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Role of the α1G T-Type Calcium Channel in Spontaneous Absence Seizures in Mutant Mice

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Alterations in thalamic T-type Ca^{2+} channels are thought to contribute to the pathogenesis of absence seizures. Here, we found that mice with a null mutation for the pore-forming α1A subunits of P/Q-type channels ($\alpha 1A^{-/-}$ mice) were prone to absence seizures characterized by typical spike-and-wave discharges (SWDs) and behavioral arrests. Isolated thalamocortical relay (TC) neurons from these mice showed increased T-type Ca^{2+} currents *in vitro*. To examine the role of increased T-currents in $\alpha 1A^{-/-}$ TC neurons, we cross-bred $\alpha 1A^{-/-}$ mice with mice harboring a null mutation for the gene encoding α1G, a major isotype of T-type Ca^{2+} channels in TC neurons. $\alpha 1A^{-/-}/\alpha 1G^{-/-}$ mice showed a complete loss of T-type Ca^{2+} currents in TC neurons and displayed no SWDs. Interestingly, $\alpha 1A^{-/-}/\alpha 1G^{+/-}$ mice had 75% of the T-type Ca^{2+} currents in TC neurons observed in $\alpha 1A^{+/+}/\alpha 1G^{+/+}$ mice and showed SWD activity that was quantitatively similar to that in $\alpha 1A^{-/-}/\alpha 1G^{+/+}$ mice. Similar results were obtained using double-mutant mice harboring the α1G mutation plus another mutation also used as a model for absence seizures, i.e., *lethargic* ($\beta 4^{lh/lh}$), *tottering* ($\alpha 1A^{tg/tg}$), or *stargazer* ($\gamma 2^{stg/stg}$). The present results reveal that α1G T-type Ca^{2+} channels play a critical role in the genesis of spontaneous absence seizures resulting from hypofunctioning P/Q-type channels, but that the augmentation of thalamic T-type Ca^{2+} currents is not an essential step in the genesis of absence seizures.

Key words: calcium; Ca; EEG; electroencephalogram; epilepsy; gene; mutant; thalamus

Introduction

Pharmacological studies suggest that low-voltage-activated T-type Ca^{2+} channels are involved in the genesis of absence seizures, which are characterized by spike-and-wave discharges (SWDs) (van Luijtelaar et al., 2000; Porcello et al., 2003). Antagonists of T-type Ca^{2+} channels suppress both slow intrathalamic rhythms *in vitro* (Porcello et al., 2003) and SWDs in human absence seizure patients and in rodent models of absence seizures (Heller et al., 1983; Hosford et al., 1992; van Luijtelaar et al., 2000). Previous genetic studies indicate that of the three α1 subunits of T-type Ca^{2+} channels (G, H, and I), the α1G subunit is critically involved in SWD genesis. Mice with a null mutation of the α1G gene lacked low-threshold burst firing in thalamocortical relay (TC) neurons *in vitro* (Kim et al., 2001) and *in vivo* (Kim et al., 2003) and were resistant to SWDs induced by $GABA_B$-receptor agonists (Kim et al., 2001).

T-type Ca^{2+} channel activity has been studied in rodent models of absence seizures to determine the role of this channel in SWD genesis. The augmentation of T-type Ca^{2+} currents in the thalamus was first reported in studies using genetic absence epilepsy rats from Strasbourg (Tsakiridou et al., 1995; Talley et al., 2000). Computational modeling studies support the concept that augmented T-type Ca^{2+} currents increase the number of burst spikes and thereby enhance thalamic synchrony (Destexhe et al., 1996, 1998; Hughes et al., 1999; Thomas and Grisar, 2000). These studies imply that functional enhancement of the T-type Ca^{2+} channel can contribute to the development of absence seizures by enhancing the probability of thalamocortical hypersynchronization. Mice with mutations in various subunits of the high-voltage-activated (HVA) Ca^{2+} channels, namely *tottering* ($\alpha 1A^{tg/tg}$), *lethargic* ($\beta 4^{lh/lh}$), and *stargazer* ($\gamma 2^{stg/stg}$) mice, display SWDs (Noebels and Sidman, 1979; Hosford et al., 1992; Qiao and Noebels, 1993). Recently, these mutant mice were shown to have higher T-type Ca^{2+} current levels in their TC neurons compared with normal mice, suggesting a possibility that the enhancement of T-currents might underlie the pathogenesis of absence seizures in those mutants (Tsakiridou et al., 1995; Zhang et al., 2002).

To address these issues, we examined absence seizures in mice with a null mutation in the gene coding for pore-forming α1A subunits ($\alpha 1A^{-/-}$ mice), which therefore lack the P/Q-type Ca^{2+} currents. We then explored the role of T-type Ca^{2+} channels in the genesis of absence seizures in these null mice and other spontaneous mutant mice. Our results provide *in vivo* evidence that baseline T-type Ca^{2+} currents but not their augmentation in TCs are necessary and sufficient to support absence seizures in various genetic mouse models.

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Materials and Methods

Animals. The α1A mice used for EEG recording and patch-clamp analyses were F2 progeny derived from intercrossing heterozygotes of the F1 (129/sv×C57BL/6J) genetic background. Mice heterozygous for the α1A null mutation (Jun et al., 1999), *lethargic* ($\beta 4^{lh/lh}$), *tottering* ($\alpha 1A^{tg/tg}$), or *stargazer* ($\gamma 2^{stg/stg}$) were mated with $\alpha 1G^{-/-}$ mice (Kim et al., 2001) to obtain $\alpha 1A^{+/-}/\alpha 1G^{+/-}$, $\alpha 1A^{tg/+}/\alpha 1G^{+/-}$, $\beta 4^{lh/+}/\alpha 1G^{+/-}$, $\gamma 2^{stg/+}/\alpha 1G^{+/-}$ offspring. Double-heterozygous mice for the two mutations were intercrossed to obtain $\alpha 1A^{-/-}$, $\beta 4^{lh/lh}$, $\alpha 1A^{tg/tg}$, $\gamma 2^{stg/stg}$ mice with different numbers of α1G gene alleles. These mice allowed examination of the effect of deleting α1G on the background of absence seizures in $\alpha 1A^{-/-}$ mice. In addition, the mice allowed investigation of the pathological role of T-current enhancement in absence seizures. The animals were housed at room temperature (22°C), fed *ad libitum*, and submitted to a 12 hr light/dark cycle. All handling of mice was in accordance with the regulations of the institute.

Electrode implantation and cortical EEG recording. Differential EEG recording was performed as described previously (Kim et al., 2001). Mice were anesthetized with avertin (tribromoethyl alcohol/tertiary amyl alcohol; Aldrich, Milwaukee, WI). Subdural tungsten electrodes (A-M Systems, Carlsborg, WA) were bilaterally or unilaterally implanted in the temporal lobe region, and a ground electrode was implanted in the occipital region of the brain (Schridde and van Luijtelaar, 2004). The head mount was secured using dental cement, and mice were allowed to recover for at least 24 hr before EEG recordings. EEG activity (sampling frequency, 200 μsec) was recorded during 0.5–1 hr samples for 1–2 hr using a pCLAMP8.0 program (Axon Instruments, Foster City, CA). Only SWDs with a minimum voltage amplitude of twice the background EEG and a minimum duration of 0.7 sec were included in analysis, and SWDs separated by $<$1 sec were regarded as a single SWD event.

To test the effects of drugs on absence seizures, EEGs of $\alpha 1A^{-/-}$ mice (3–4 weeks old) were recorded for 2 hr, starting 1 hr before drug administration. Valproic acid (Sigma, St. Louis, MO) or ethoxusimide (Sigma) was diluted in physiological saline (0.85% NaCl) and injected intraperitoneally. The selection of the drug dose was based on published data (Heller et al., 1983; Aizawa et al., 1997) and preliminary experiments.

Data analysis. EEG signals were amplified, filtered, and recorded using pCLAMP8 software (Axon Instruments). To assess the difference in EEG activity between each group of mice, we used the linear spectra of consecutive EEG data sections (duration over 1 min; range, 1–15 Hz) computed using the pCLAMP8 program using the fast Fourier transform.

Northern blot. Total RNA was isolated from the thalamic region of 3- to 4-week-old mice. RNA (20 μg per lane) was separated on 1.0% agarose gels containing 2.2 M formaldehyde and then transferred to nylon membranes by capillary blot. The hybridization solution comprised 7% SDS, 1% BSA, 0.5 M $NaHPO_4$, 1 mM EDTA, and a random-primed rat cDNA probe corresponding to nucleotides 4699–6174 bp of the α_{1G} clone. A glyceraldehyde 3-phosphate dehydrogenase probe was also hybridized to blots, and this signal was used to normalize for RNA loading. Signal detection and normalization were performed using the ImageQuant Image Analysis system (Amersham Biosciences, Arlington Heights, IL). Concentrations are expressed as "percentage of wild-type control" analyzed on the same blots.

Whole-cell voltage-clamp analysis. Patch-clamp analysis of thalamic relay neurons was performed as described previously (Kim et al., 2001). Thalamic relay neurons were acutely dissociated as described previously (Tsakiridou et al., 1995; Raman and Bean, 1999). Briefly, brains were cooled rapidly in ice-chilled slicing solution consisting of (in mM): 122 NaCl, 26 $NaHCO_3$, 1.2 NaH_2PO_4, 2 $MgCl_2$, 2 $CaCl_2$, 3 KCl and 10 glucose, after which 300 μm sections were cut in the coronal plane using a Vibratome (Ted Pella, Redding, CA). Slices containing the ventrobasal complex were dissected with a scalpel to isolate the thalamus. Thalamic slices were incubated at 35°C for 6 min with protease XXIII (3 mg/ml; Sigma) in an oxygenated HEPES-buffered solution consisting of (in mM): 82 Na_2SO_4, 30 KSO_4, 5 $MgCl_2$, 10 HEPES, 10 glucose, 0.01% phenol red, and adjusted to pH 7.4 with NaOH. The enzymatic reaction was stopped by adding BSA (1 mg/ml; Sigma) and trypsin inhibitor (1 mg/ml; Sigma). Each thalamic slice was triturated with fire-polished Pasteur pipettes and

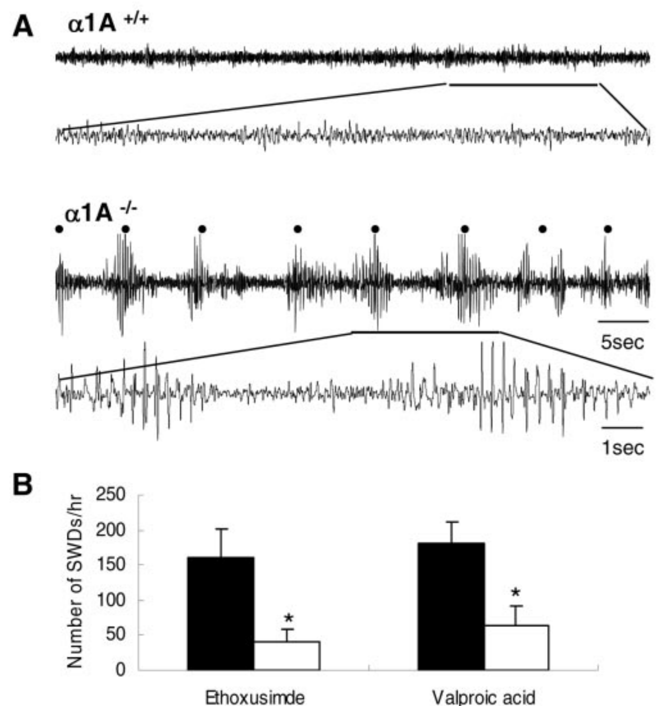

Figure 1. EEG recordings from the cortex of freely moving $\alpha 1A^{-/-}$ mice. *A*, Top, Representative EEG traces show no SWD activity from wild-type mice. Bottom, Spontaneous 3–5 Hz SWDs with high amplitude of cortical activity occurred in 3- to 4-week-old $\alpha 1A^{-/-}$ mice ($n = 10$). The $\alpha 1A^{-/-}$ mice displayed behavioral immobility and maintained a fixed posture throughout the SWDs. The black dot indicates SWDs. The thin scale bars are valid for all EEG recordings displayed. The thick-lined periods are expanded for detail. *B*, Effect of anti-epileptic drugs (white bars) and control vehicle (shaded bars) on the occurrence of absence seizures in $\alpha 1A^{-/-}$ mice. Bars represent the mean number of SWDs per hour for $\alpha 1A^{-/-}$ mice exposed to each drug. Both ethoxusimde (150 mg/kg; $n = 6$) and valproic acid (10 mg/kg; $n = 5$) were effective in reducing the occurrence of SWDs of $\alpha 1A^{-/-}$ mice (*$p < 0.025$; two-tailed *t* test). Error bars represent ±SEM.

plated onto a recording chamber. Healthy-looking neurons of triangular or multipolar shapes with processed dendrites were used for patch-clamp recordings. Recordings were performed using electrodes (3.5–6.5 MΩ) fabricated from borosilicate glass (Warner Instruments, Hamden, CT) in an extracellular solution consisting of (in mM): 55 TEA-Cl, 3 $CaCl_2$, 10 HEPES, adjusted to pH 7.4 with TEA-OH. Patch pipettes were filled with a solution containing (in mM): 110 $TrisPO_4$ dibasic, 28 Tris-base, 11 EGTA, 2 $MaCl_2$, 0.5 $CaCl_2$, 4 Na_2ATP, 0.3 GTP-Na, 0.001 TTX, pH 7.3. The series resistance compensation ($>$60%) was used routinely, and patch-recording data with access resistance ($>$20 MΩ) were discarded. The currents were leak-subtracted using a P/4 protocol. Signals were digitized using an Axopatch 200-B amplifier (Axon Instruments) and analyzed using pCLAMP8 software (Axon Instruments).

Results

Absence seizures with 3 Hz SWDs in $\alpha 1A^{-/-}$ mice

$\alpha 1A^{-/-}$ mice develop progressive neurological symptoms characterized specifically by ataxia and dystonia, before dying ~4 weeks after birth (Jun et al., 1999). We recorded cortical EEG activities in $\alpha 1A^{-/-}$ and wild-type mice at 3–4 weeks of age. We found that $\alpha 1A^{-/-}$ mice ($n = 10$) exhibited spontaneous 3–5 Hz SWDs (Fig. 1*A*) and that each episode was accompanied by behavioral arrest, often with twitching of the vibrissa. These abnormal cortical activities were not observed in wild-type littermates (Fig. 1*A*). SWDs occurred ~160 times per hour in $\alpha 1A^{-/-}$ mice (Table 1). We examined the effect of the anti-epileptic drugs ethoxusimide (150 mg/kg; $n = 6$) and valproic acid (10 mg/kg;

Table 1. Characteristic of SWDs in mutant mice

Mutant mice	Frequency (Hz)	Mean duration (sec)	Number of incidents per hour
α1A$^{-/-}$			
α1G$^{+/+}$	3–4	2.0 ± 0.2 (0.7–5)	161.7 ± 40.5
α1G$^{+/-}$	3–4	2.1 ± 0.2 (0.7–5)	156.2 ± 22.0
α1G$^{-/-}$	—	—	0**
α1A$^{tg/tg}$			
α1G$^{+/+}$	6–7	2.2 ± 0.2 (0.7–6)	98.3 ± 12.2
α1G$^{+/-}$	6–7	2.1 ± 0.2 (0.7–5)	90.2 ± 15.3
α1G$^{-/-}$	—	—	0**
β4$^{lh/lh}$			
α1G$^{+/+}$	5–6	1.5 ± 0.1 (0.7–5)	164.6 ± 25.7
α1G$^{+/-}$	5–6	1.3 ± 0.1 (0.7–5)	204.8 ± 26.0
α1G$^{-/-}$	5–6	1.6 ± 0.2 (0.7–5)	7.1 ± 4.7**
γ2$^{stg/stg}$			
α1G$^{+/+}$	5–7	1.9 ± 0.2 (0.7–10)	132.0 ± 8.7
α1G$^{+/-}$	5–7	1.8 ± 0.2 (0.7–10)	135.6 ± 20.0
α1G$^{-/-}$	5–7	1.3 ± 0.1 (0.7–2)*	9.4 ± 3.4**

Values, except for frequency, are means ± SEM. Values in parentheses are ranges. —, Not found. *p <0.05, **p <0.005 (mutants compared with α1G$^{+/+}$ within each subgroup; two-tailed t test).

n = 5) on the incidence of SWDs in α1A$^{-/-}$ mice. These drugs are documented to suppress absence seizure SWDs in humans and rodents (Heller et al., 1983; Hosford et al., 1992). Compared with vehicle-treated α1A$^{-/-}$ mice, we found that ethoxusimide decreased the incidence of SWDs from 161.7 ± 40.5 to 40.8 ± 18.6 per hour, whereas valproic acid reduced the incidence from 181.6 ± 28.9 to 64.4 ± 26.6 per hour (Fig. 1*B*). Taken together, the behavioral, electrographic, and pharmacological characters of seizures in α1A$^{-/-}$ mice are similar to those of mice absence seizures(Hosford et al., 1992; Aizawa et al., 1997).

Generation of α1A$^{-/-}$mice with differing numbers of α1G alleles

α1G is one of the T-type Ca^{2+} channels highly expressed in thalamic relay neurons and is selectively involved in SWD seizures induced by $GABA_B$ receptor agonists (Kim et al., 2001). We sought to determine whether α1G T-type Ca^{2+} channels are pathophysiologically involved in the generation and expression of absence seizures in α1A$^{-/-}$ mice. α1G$^{+/-}$ and α1A$^{+/-}$ mice were cross-bred to generate double heterozygotes, α1A$^{+/-}$/α1G$^{+/-}$ mice. A result of double heterozygote matings was α1A$^{-/-}$ mice with different numbers of α1G gene alleles, i.e., α1A$^{-/-}$/α1G$^{+/+}$, α1A$^{-/-}$/α1G$^{+/-}$, and α1A$^{-/-}$/α1G$^{-/-}$. On visual inspection, α1A$^{-/-}$/α1G$^{+/-}$ and α1A$^{-/-}$/α1G$^{-/-}$ mice exhibited severe ataxia and weakness, similar to that observed in α1A$^{-/-}$ single knock-out mice (Jun et al., 1999). In addition, α1A$^{-/-}$/α1G$^{+/-}$ and α1A$^{-/-}$/α1G$^{-/-}$ mice did not survive past weaning.

Decreased HVA Ca^{2+} currents in α1A$^{-/-}$ thalamic relay neurons

We performed whole-cell voltage-clamp experiments to examine the effect of α1A genetic deletion on HVA Ca^{2+} currents in acutely dissociated TC neurons, which are characterized by their large size and triangular or multipolar shape with truncated dendrites (Huguenard and Prince, 1992; Pape et al., 1994; Kim et al., 2001). HVA Ca^{2+} currents, supported by 3 mM Ca^{2+} as a charge carrier, were activated by step depolarization from a holding potential of −60 mV. As a result, large sustained voltage-dependent Ca^{2+} inward currents were evoked. Figure 2*A* shows a typical trace of total Ca^{2+} currents with a slowly inactivating component recorded from TC neurons. A significant difference was observed between neurons from α1A$^{-/-}$/α1G$^{+/+}$ mice compared with neurons of control (α1A$^{+/+}$/α1G$^{+/+}$) mice in terms of the amplitude of Ca^{2+} currents (Fig. 2*A*). The amplitudes of HVA Ca^{2+} currents at all command membrane potentials were smaller in α1A$^{-/-}$/α1G$^{+/+}$ (n = 12) and α1A$^{-/-}$/α1G$^{-/-}$ (n = 11) TC neurons compared with controls (n = 10). This decrease in HVA

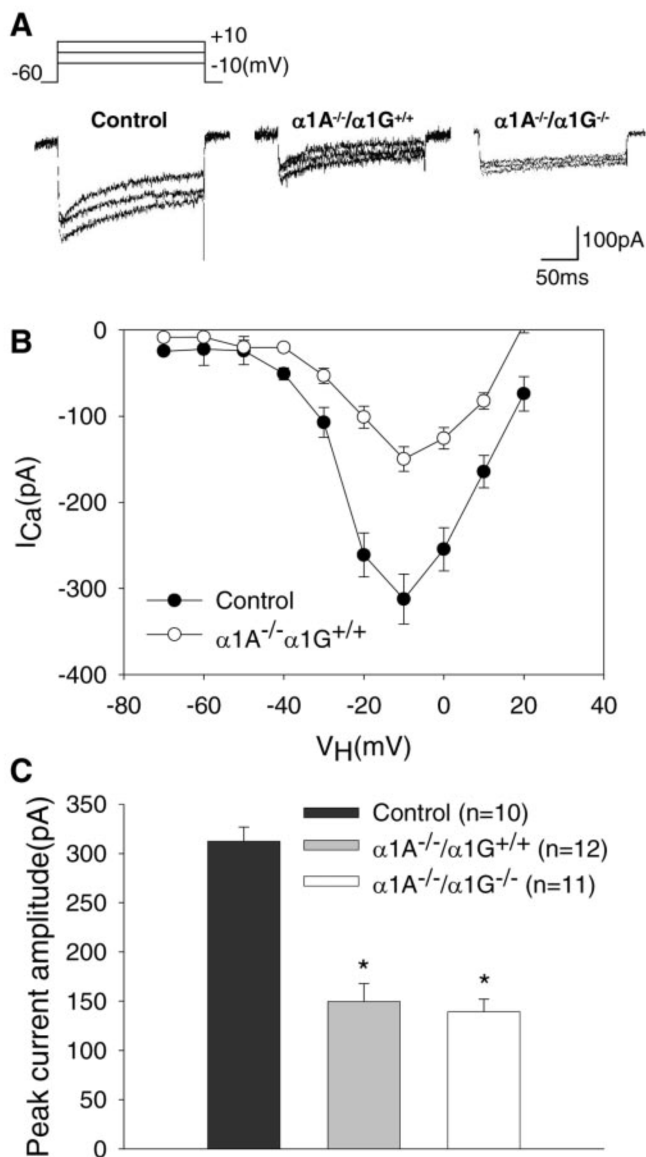

Figure 2. HVA Ca^{2+} currents in acutely isolated thalamic relay neurons. *A*, Representative traces of total Ca^{2+} currents of control, α1A$^{-/-}$/α1G$^{+/+}$, and α1A$^{-/-}$/α1G$^{-/-}$ TC neurons evoked by stepping membrane potential voltages between −10 and +10 mV in 10 mV increments from a holding potential of −60 mV. Sustained HVA Ca^{2+} currents decayed slowly during the 200 msec step commands. *B*, Mean peak *I–V* curves for total Ca^{2+} currents in TC neurons show different features between the two groups. The *I–V* relationship at this voltage protocol shows that the HVA Ca^{2+} currents in α1A$^{-/-}$/α1G$^{+/+}$ cells were dramatically reduced at all testing voltage steps above −40 mV compared with those in control. Symbols represent pooled data from α1A$^{-/-}$/α1G$^{+/+}$ (open symbols; n = 8) and control mice (filled symbols; n = 10). *C*, The HVA Ca^{2+} histogram of peak amplitude is at −10 mV in control (black bars; n = 10), α1A$^{-/-}$/α1G$^{+/+}$ (gray bars; n = 12), and α1A$^{-/-}$/α1G$^{-/-}$ (white bars; n = 11), with holding potential at −60 mV. Note that the peak amplitude of HVA Ca^{2+} currents in α1A$^{-/-}$/α1G$^{+/+}$ and α1A$^{-/-}$/α1G$^{-/-}$ mice was decreased significantly more than in α1A$^{+/+}$/α1G$^{+/+}$ mice (*p < 0.001; two-tailed t test). No statistically significant changes occurred in the HVA Ca^{2+} current of α1A$^{-/-}$/α1G$^{-/-}$ mice compared with that from TC neurons of α1A$^{-/-}$/α1G$^{+/+}$ mice (p > 0.05). Control indicates α1A$^{+/+}$/α1G$^{+/+}$.

Ca^{2+} current can be accounted for by the loss of P/Q-type Ca^{2+} currents in $\alpha 1A^{-/-}$ mice, consistent with previous results in which P/Q-type Ca^{2+} currents were shown to be a component of HVA Ca^{2+} currents in TC neurons (Pfrieger et al., 1992; Kammermeier and Jones, 1997).

To determine the current–voltage (*I–V*) relationship of total HVA Ca^{2+} currents in neurons from $\alpha 1A^{-/-}/\alpha 1G^{+/+}$ ($n = 10$) and control ($n = 8$) mice, a series of voltage steps from −70 to +20 mV with +10 mV increments was delivered from a holding potential at −70 mV. The *I–V* relationship curve in control mice revealed a prominent shoulder at negative potentials, indicating channel activation at low voltages, with the peak of the *I–V* curve occurring at −10 mV (Fig. 2*B*). In contrast to these findings using control neurons, in neurons from $\alpha 1A^{-/-}/\alpha 1G^{+/+}$ mice, the *I–V* relationships showed a significant decrease in the amplitude of the HVA Ca^{2+} current in voltage steps from −30 to +20 mV, peaking near −10 mV without a change in the shape of the *I–V* curves (Fig. 2*B*). We examined the profile of HVA Ca^{2+} currents in TC cells from neurons of $\alpha 1A^{-/-}/\alpha 1G^{-/-}$ and $\alpha 1A^{-/-}/\alpha 1G^{+/-}$ (data not shown) mice and found no difference in HVA Ca^{2+} currents between these two genotypes and $\alpha 1A^{-/-}/\alpha 1G^{+/+}$ mice (Fig. 2*A*). These findings indicate that genetic reduction of T-type Ca^{2+} currents does not strongly modulate HVA Ca^{2+} currents for compensation. We quantitatively compared the peak amplitude of inward Ca^{2+} currents evoked by depolarization from −60 to −10 mV in TC neurons from control and $\alpha 1A^{-/-}/\alpha 1G^{+/+}$ mice. Pooled data showed that the total HVA Ca^{2+} current was 312.3 ± 14.5 pA in neurons of controls ($n = 10$) (Fig. 2*C*, black bars) and 149.8 ± 18 pA in neurons of $\alpha 1A^{-/-}/\alpha 1G^{+/+}$ ($n = 12$) (Fig. 2*C*, gray bars) mice ($p < 0.001$); however, the averaged peak amplitude of the HVA Ca^{2+} current in neurons of $\alpha 1A^{-/-}/\alpha 1G^{-/-}$ mice at −10 mV (139.2 ± 12.9 pA; $n = 11$) (Fig. 2*C*, white bars) was similar to that of neurons of $\alpha 1A^{-/-}/\alpha 1G^{+/+}$ mice (149.8 ± 18 pA; $n = 12$) (Fig. 2*C*, gray bars) ($p > 0.05$). The differences in the peak amplitudes of HVA Ca^{2+} currents between neurons of $\alpha 1A^{-/-}/\alpha 1G^{+/+}$ and control mice were not caused by differences in the surface area of cells because the value of the whole-cell capacitance was not different between $\alpha 1A^{-/-}/\alpha 1G^{+/+}$ (11.5 ± 0.6 pF; $n = 12$) and control (11.5 ± 0.9 pF; $n = 10$) cells.

Increased T-type calcium currents in $\alpha 1A^{-/-}$ thalamic relay neurons

To examine T-type Ca^{2+} currents, we used a voltage protocol in which a voltage step from −110 to −45 mV activates transient T-currents (Huguenard and Prince, 1992) (Fig. 3*A*). This T-current reached peak amplitude ~10 msec after onset of the 100 msec voltage step and then inactivated rapidly. The TC neurons from $\alpha 1A^{-/-}/\alpha 1G^{-/-}$ mice displayed a near complete loss of T-type Ca^{2+} currents ($n = 8$) (Fig. 3*A*), consistent with previous data (Kim et al., 2001). In contrast, transient Ca^{2+} currents of larger amplitude were evoked from $\alpha 1A^{-/-}/\alpha 1G^{+/+}$ cells compared with control ($\alpha 1A^{+/+}/\alpha 1G^{+/+}$) cells (Fig. 3*A*). The data presented in Figure 3*B* show that the averaged peak value of T-type Ca^{2+} currents at −45 mV was significantly larger in $\alpha 1A^{-/-}/\alpha 1G^{+/+}$ neurons (264.4 ± 24.6 pA; $n = 10$) (Fig. 3*B*, gray bars) than in controls (165.2 ± 10.5 pA; $n = 13$) (black bars) ($p < 0.0005$). In contrast, the peak amplitude of T-currents from $\alpha 1A^{-/-}/\alpha 1G^{+/-}$ neurons was 125.3 ± 10.0 pA ($n = 18$) (Fig. 3*B*, dark gray bars), which is ~50% of that in $\alpha 1A^{-/-}/\alpha 1G^{+/+}$ and ~75% of that in control TC cells ($p < 0.005$). These properties of T-type Ca^{2+} currents from each genotype were consistent with the current density histogram of peak T-currents (Fig. 3*C*). These data indicate that the enhancement of T-type Ca^{2+} currents was not caused by an increase in TC neuron membrane size. No significant difference in time-to-peak was observed between groups [8.2 ± 0.4 msec for control ($n = 11$), 8.4 ± 0.6 msec for $\alpha 1A^{-/-}/\alpha 1G^{+/+}$ ($n = 11$), and 8.1 ± 0.5 msec for $\alpha 1A^{-/-}/\alpha 1G^{+/-}$ ($n = 18$) TC cells].

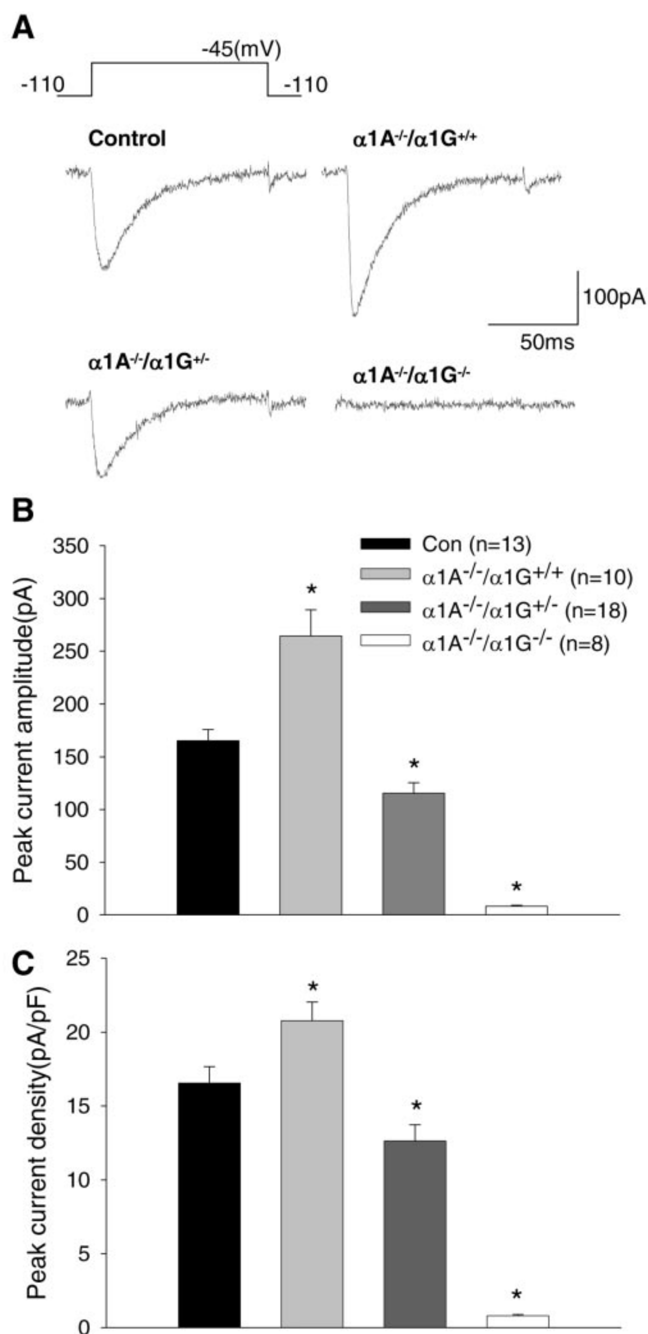

Figure 3. HVA Ca^{2+} currents in acutely isolated thalamic relay neurons. *A*, Much larger T-currents, which are rapidly inactivated, were obtained from TC neurons in $\alpha 1A^{-/-}/\alpha 1G^{+/+}$ than from control ($\alpha 1A^{+/+}/\alpha 1G^{+/+}$), whereas T-currents were nearly absent in the $\alpha 1A^{-/-}/\alpha 1G^{-/-}$ TC neurons. *B*, The histogram shows the mean peak amplitude of T-type Ca^{2+} current in acutely isolated TC neurons from control (black bars), $\alpha 1A^{-/-}/\alpha 1G^{+/+}$ (gray bars), $\alpha 1A^{-/-}/\alpha 1G^{+/-}$ (dark gray bars), and $\alpha 1A^{-/-}/\alpha 1G^{-/-}$ (white bars). *C*, The histogram indicates the mean peak T-type Ca^{2+} current densities that were from the same cells used for the histogram (*B*) in each group. The asterisks indicate the significant difference between each mutant and control (*$p < 0.05$; two-tailed *t* test).

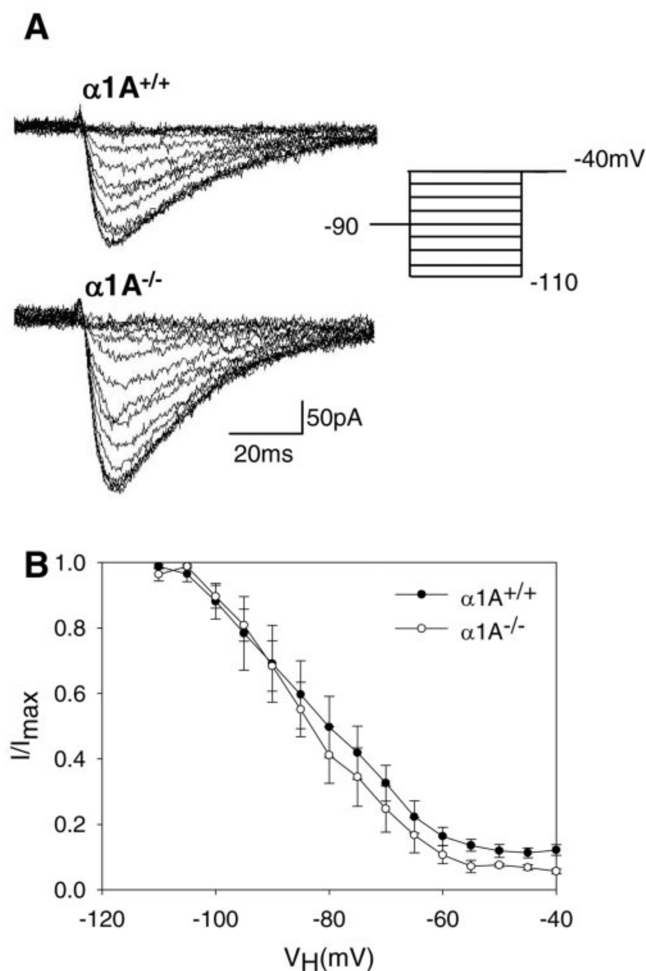

Figure 4. Steady-state inactivation of T-currents. *A*, The membrane potential was stepped to -40 mV from holding potentials ranging from -110 to -40 mV. *B*, The normalized peak amplitude of the Ca^{2+} currents elicited by the test pulse at -40 mV was plotted as a function of the holding potential. The symbols represent pooled data from $\alpha1A^{+/+}$ (filled square; $n = 6$) and $\alpha1A^{-/-}$ (open square; $n = 7$). Error bars represent $\pm$SEM.

One explanation for augmented T-type Ca^{2+} currents in $\alpha1A^{-/-}$ TC cells is a change in voltage dependence, such as steady-state inactivation to a more depolarized level. To obtain steady-state inactivation curves, we delivered a prepulse to various membrane potentials before a -40 mV test stimulus. Current traces of steady-state inactivation of T-currents are shown in Figure 4*A*. The data presented in Figure 4*B* show that despite increased T-type Ca^{2+} current amplitude, steady-state inactivation was similar for both groups when peak current values from the test pulses were normalized to the maximal current amplitude in each cell.

We examined the possibility that the alteration in T-current amplitudes was caused by an increase in the amount of T-type Ca^{2+} channels. Using rat α1G cDNA [nucleotide (nt) 4699–6174] as a probe in Northern blot analysis, we examined α1G gene expression in thalamus tissue isolated from $\alpha1A^{-/-}$ and $\alpha1A^{+/+}$ mice. Although no signal was detected in $\alpha1G^{-/-}$ thalamic tissues, visual examination of autoradiographs indicated similar α1G mRNA expression in thalamic tissues from both $\alpha1A^{-/-}$ ($n = 5$) and $\alpha1A^{+/+}$ ($n = 4$) mice (Fig. 5). Quantitative image analysis confirmed that there was no significant difference in expression (94 ± 9 for $\alpha1A^{-/-}$ and 100 ± 5% for $\alpha1A^{+/+}$; $p > 0.5$).

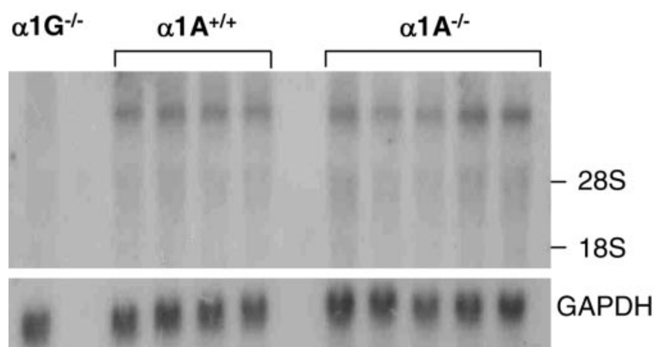

Figure 5. Northern blot analysis of α1G transcripts from thalamus. For Northern blot analysis, mouse ($\alpha1G^{-/-}$, $\alpha1A^{+/+}$, and $\alpha1A^{-/-}$) thalamic tissue was probed with α1G (nt 4699–6174 bp) and then exposed for 3 d. Internal control was performed using GADPH. Thalamic α1G transcript expression that was not detected in $\alpha1G^{-/-}$ mice did not differ between $\alpha1A^{+/+}$ and $\alpha1A^{-/-}$.

Effect of α1G allele number on SWD generation in $\alpha1A^{-/-}$ mice

We investigated whether α1G genes were necessary for spontaneous absence seizures in $\alpha1A^{-/-}$ mice, because they are functionally involved in drug-induced SWD seizures (Kim et al., 2001). Subdural EEG measurements were conducted on 3-week-old $\alpha1A^{-/-}/\alpha1G^{+/+}$ ($n = 5$) and $\alpha1A^{-/-}/\alpha1G^{-/-}$ ($n = 4$) mice. We found that $\alpha1A^{-/-}/\alpha1G^{-/-}$ mice did not exhibit the typical 3–4 Hz SWDs that were observed in $\alpha1A^{-/-}/\alpha1G^{+/+}$ littermates (Fig. 6*A*) (157.5 ± 43.3 per hour for $\alpha1A^{-/-}/\alpha1G^{+/+}$ and 0 ± 0 per hour for $\alpha1A^{-/-}/\alpha1G^{-/-}$ mice; $p < 0.005$) (Table 1). A power spectrum analysis confirmed this alteration in cortical paroxysmal activity, i.e., disappearance of the 3 Hz peak frequency in $\alpha1A^{-/-}/\alpha1G^{-/-}$ compared with $\alpha1A^{-/-}/\alpha1G^{+/+}$ mice (Fig. 7*A*). It appears that complete genetic deletion of α1G genes functionally abolishes generation of the spontaneous cortical SWD activity observed in $\alpha1A^{-/-}/\alpha1G^{+/+}$ mice.

We investigated whether augmentation of thalamic T-currents was necessary for absence seizures. We examined SWDs in α1G heterozygote mice on a $\alpha1A^{-/-}$ background. These mice were shown previously to exhibit 75% of the T-currents observed in wild-type mice (Fig. 3). Such experiments present an opportunity to investigate the functional consequence of reduced T-current on absence seizures caused by lack of α1A subunits. If increased TC neuron T-current (compared with wild type) is a causative factor in SWD genesis in $\alpha1A^{-/-}$ mice, mice with reduced or the same level of T-currents compared with non-epileptic mice should express no or altered SWD cortical activity. Contrary to our expectations, we found no significant difference between $\alpha1A^{-/-}/\alpha1G^{+/-}$ and $\alpha1A^{-/-}/\alpha1G^{+/+}$ mice in terms of SWD duration or frequency (Table 1).

Role of α1G in SWD generation in various absence seizure mice models

Having found that SWDs in α1A/G double-mutant mice differed according to α1G allele "dose," we examined the role of α1G in the genesis of SWDs in other mouse models of absence seizures. We again cross-bred heterozygote mice to produce double mutants containing $\alpha1G^{-/-}$ and $\alpha1A^{tg/tg}$ (Noebels and Sidman, 1979), $\beta4^{lh/lh}$ (Hosford et al., 1992), or $\gamma2^{stg/stg}$ (Noebels et al., 1990). Our study was on the basis of findings that compared with neurons of control mice, T-type Ca^{2+} currents are greater in

thalamic relay neurons from $\alpha 1A^{tg/tg}$, $\beta 4^{lh/lh}$, and $\gamma 2^{stg/stg}$ mice (146, 151, and 145% of control, respectively) (Zhang et al., 2002). We performed EEG recordings in freely moving young (3–4 week) $\alpha 1A^{tg/tg}/\alpha 1G^{+/+}$ ($n = 3$), $\beta 4^{lh/lh}/\alpha 1G^{+/+}$ ($n = 4$), and $\gamma 2^{stg/stg}/\alpha 1G^{+/+}$ ($n = 3$) mice on a mixed genetic background (129/sv×C57BL/6J). These mice exhibited 5–7 Hz SWDs with high amplitude, with the morphology and dominant frequency of the SWDs being slightly different among groups (Fig. 6*B–D*, Table 1). During a period of robust cortical paroxysmal activity, all mice exhibited a sudden behavioral arrest of movement and a fixed posture, indicating behavioral absence seizures. The mean seizure durations were 2.2 ± 0.2 sec (range, 0.7–6 sec) for $\alpha 1A^{tg/tg}/\alpha 1G^{+/+}$ mice, 1.5 ± 0.1 sec (0.7–5 sec) for $\beta 4^{lh/lh}/\alpha 1G^{+/+}$ mice, and 1.9 ± 0.2 sec (0.7–10 sec) for $\gamma 2^{stg/stg}/\alpha 1G^{+/+}$ mice (Table 1). The mean number of SWD events per hour were 98.3 ± 12.2 in $\alpha 1A^{tg/tg}/\alpha 1G^{+/+}$ ($n = 3$), 164 ± 25.7 in $\beta 4^{lh/lh}/\alpha 1G^{+/+}$ ($n = 4$), and 132.0 ± 8.7 in $\gamma 2^{stg/stg}/\alpha 1G^{+/+}$ ($n = 3$) mice (Table 1). The SWD patterns in these mutants were not different from those described previously (Noebels, 1984; Hosford et al., 1992; Qiao and Noebels, 1993). We took EEG measurements in double mutants completely lacking α1G genes. We found a complete suppression of SWDs in $\alpha 1A^{tg/tg}/\alpha 1G^{-/-}$ mice ($n = 5$). Indeed, we did not observe any 6–7 Hz SWDs with minimum voltage amplitude of twice the EEG background and a minimum duration of 0.7 sec (Figs. 6*B*, 7*B*; Table 1). For both $\beta 4^{lh/lh}/\alpha 1G^{-/-}$ ($n = 4$) and $\gamma 2^{stg/stg}/\alpha 1G^{-/-}$ ($n = 4$) mice, cortical SWD paroxysmal activities were strongly suppressed (Fig. 6*C*,*D*; Table 1). The 5–7 Hz SWDs with very short duration (0.7–2 sec) were rare in $\gamma 2^{stg/stg}/\alpha 1G^{-/-}$ mice (9.4 ± 3.4 per hour; $n = 4$), and very few 5–6 Hz SWDs with a duration similar to those observed in $\beta 4^{lh/lh}/\alpha 1G^{+/+}$ mice were detected on EEG recordings from $\beta 4^{lh/lh}/\alpha 1G^{-/-}$ mice (7.1 ± 4.7 per hour; $n = 4$) (Table 1). Interestingly, the results show that contrary to genetic ablation of α1G, which abolished cortical SWDs in mice harboring genetic dysfunction, in the α1A gene that encodes the main subunit of P/Q-type Ca^{2+} channel there was some paroxysmal cortical activity in $\alpha 1G^{-/-}$ mice, with a mutation in the regulatory subunit of the HVA Ca^{2+} channel (i.e., β4 and γ2). We used power spectrum analysis to simplify our data regarding changes in the dominant peak frequency. From this analysis, we conclude that there are few or no SWDs in any of these mutants as a result of homologous deletion of α1G genes (Fig. 7). Additionally, EEG analysis showed that SWDs were present in all double mutants that were heterozygous for α1G (i.e., $\alpha 1G^{+/-}$) (Fig. 6*B–D*). Indeed, there was no difference in SWDs between these mutants and those with $\alpha 1G^{+/+}$ (Table 1).

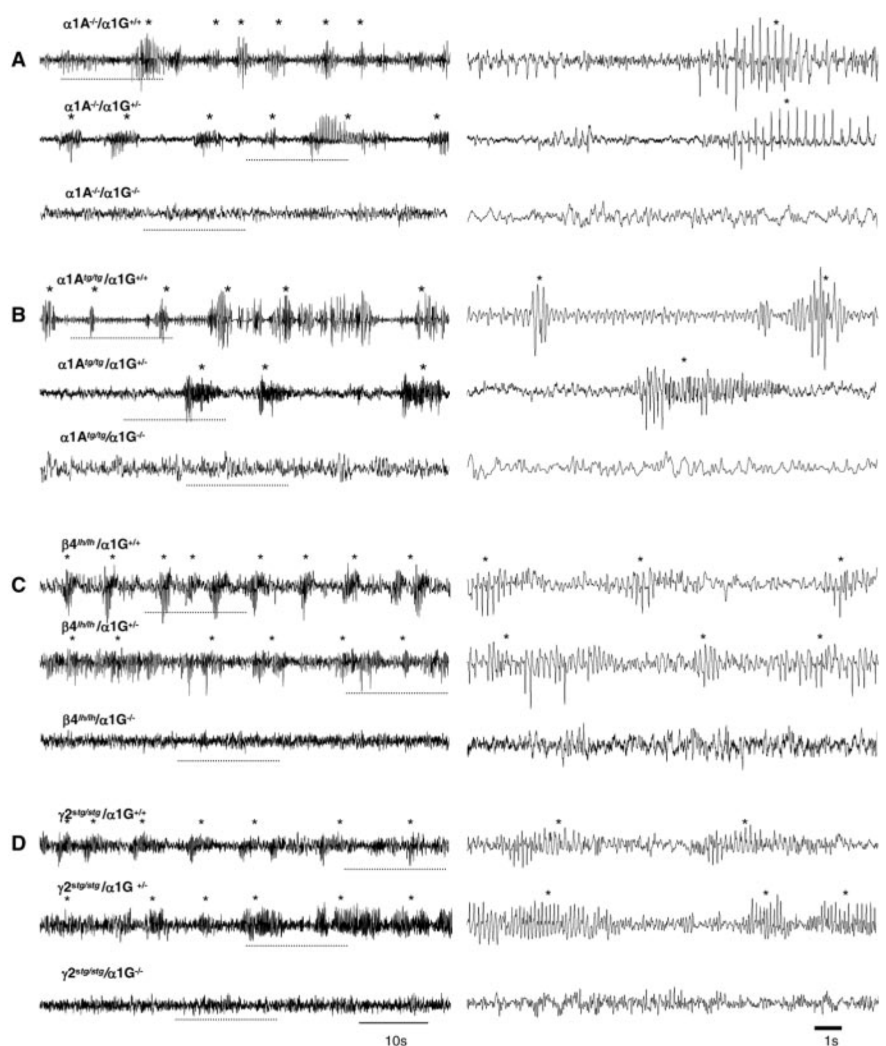

Figure 6. EEG recording of α1G genetic deletion in SWD activities of various genetic mutants. Three traces of EEG recordings from $\alpha 1A^{-/-}$ (*A*), $\alpha 1A^{tg/tg}$ (*B*), $\beta 4^{lh/lh}$ (*C*), and $\gamma 2^{stg/stg}$ (*D*) mice are illustrated according to αlG gene dosage. The dotted line shown within the 1 min trace (left) is expanded as 15 sec EEG trace (right). The asterisks indicate SWDs on the EEG traces in each genotype. *A*, Spontaneous SWD activities with high amplitude were frequently recorded in the $\alpha 1A^{-/-}/\alpha 1G^{+/+}$ (top) and $\alpha 1A^{-/-}/\alpha 1G^{+/-}$ mice (middle). SWD activities that still remained in $\alpha 1A^{-/-}/\alpha 1G^{+/-}$ could not be observed from $\alpha 1A^{-/-}/\alpha 1G^{-/-}$ mice (bottom).

Discussion

P/Q-type Ca^{2+} channels and absence seizures

HVA Ca^{2+} channels (subdivided into L, N, P/Q, and R types) play critical roles in neuron function, such as neurotransmitter release (Wheeler et al., 1994), patterning of cell excitability (Cavelier et al., 2002; Park et al., 2003), and gene expression (Sutton et al., 1999). Spontaneous absence seizures are reported in mice with Ca^{2+} channelopathy caused by various mutations in the subunits of P/Q-type Ca^{2+} channels, which show a partial reduction in P/Q-type currents with no difference in mRNA and protein levels of the channels (Fletcher et al., 1996; Mori et al., 1996; Wakamori et al., 1998; Zwingman et al., 2001). Consistent with these findings, we found that $\alpha 1A^{-/-}$ mice, which lack P/Q-type channels, had SWD activity similar to that reported in mice with other α1A point mutations (Fletcher et al., 1996; Mori et al., 1996; Wakamori et al., 1998; Zwingman et al., 2001); however, in contrast to the reports showing that mutations in $\alpha 1A^{tg/tg}$, $\beta 4^{lh/lh}$, and $\gamma 2^{stg/stg}$ mice result in increased total HVA Ca^{2+} currents in

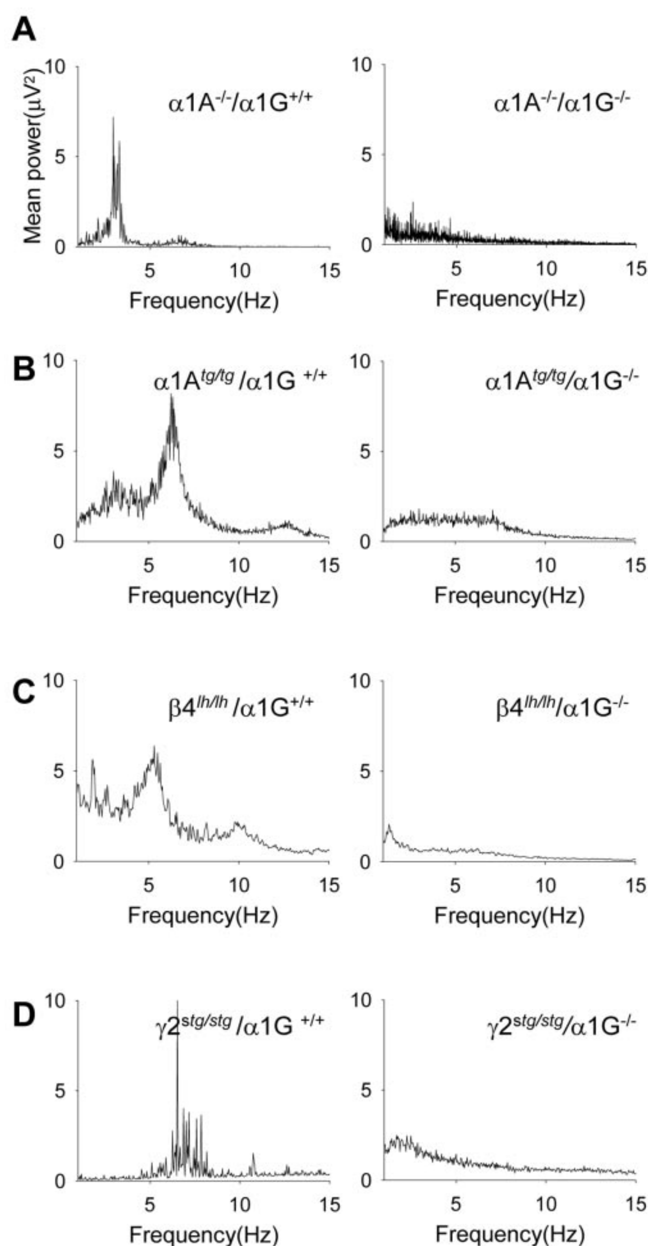

Figure 7. Power spectral analysis (1–15 Hz) of the filled potentials. *A*, Comparison of power spectra analysis between $\alpha 1A^{-/-}/\alpha 1G^{+/+}$ (left) and $\alpha 1A^{-/-}/\alpha 1G^{-/-}$ (right) mice shows disappearance of the dominant frequency (3–4 Hz) of SWDs in $\alpha 1A^{-/-}/\alpha 1G^{+/+}$. The alterations of major peak frequency in the power spectra analysis between the two groups of each spontaneous mutant line were clear as shown in α1A null mice (*B–D*). EEG traces >1 min taken from each of the double mutants were used for the power analysis ($\alpha 1A^{-/-}/\alpha 1G^{+/+}$, 6 traces from 4 mice; $\alpha 1A^{-/-}/\alpha 1G^{-/-}$, 5 traces from 3 mice; $\alpha 1A^{tg/tg}/\alpha 1G^{+/+}$, 20 traces from 3 mice; $\alpha 1A^{tg/tg}/\alpha 1G^{-/-}$, 20 traces from 5 mice; $\beta 4^{lh/lh}/\alpha 1G^{+/+}$, 10 traces from 3 mice; $\beta 4^{lh/lh}/\alpha 1G^{-/-}$, 10 traces from 3 mice; $\gamma 2^{stg/stg}/\alpha 1G^{+/+}$, 9 traces from 3 mice; $\gamma 2^{stg/stg}/\alpha 1G^{-/-}$, 9 traces from 3 mice).

TC neurons (Zhang et al., 2002), α1A null TC neurons had reduced total HVA Ca^{2+} currents, possibly because of the absence of P/Q-type Ca^{2+} currents. Thus, the present results suggest that the loss of P/Q-type current function contributes to the genesis of SWDs without any indirect changes to other HVA Ca^{2+} current types, at least in TC neurons. Consistent with this proposal, expression studies using a cloned α1A gene from a child patient with absence seizure and ataxia revealed a near-complete deletion of P/Q-type currents (Jouvenceau et al., 2001), similar to the findings obtained from $\alpha 1A^{-/-}$ mice (Kammermeier and Jones, 1997; Jun et al., 1999).

Heterogeneity of absence seizures: is α1G a common mediator for absence seizures?

Data from mouse models displaying various and nonoverlapping neurological abnormalities indicate the involvement of multiple mechanisms in the genesis of SWDs (Hosford et al., 1992; Di Pasquale et al., 1997; Zhang et al., 2002). Pharmacological studies support the complexity of SWDs. Although $GABA_B$ receptor-induced SWDs are associated with thalamocortical pathways (Caddick and Hosford, 1996; Kim et al., 2001), systemic administration of $GABA_A$ antagonists can induce SWDs in athalamic cats (Steriade and Contreras, 1998; Kim et al., 2001). Previously, we revealed that there were different mechanisms involved in $GABA_B$ receptor-mediated and $GABA_A$ antagonist-induced SWDs by showing that they are either dependent or independent of α1G T-type channels, respectively, because $\alpha 1G^{-/-}$ mice that lack thalamic burst firings are exclusively resistant to $GABA_B$ antagonist-induced SWDs (Kim et al., 2001). Thus, an aim of the present study was to characterize various mouse models of spontaneous absence seizures according to their dependence on α1G gene function. The present results show that α1G null mutation abolished SWDs of $\alpha 1A^{-/-}$ and $\alpha 1A^{tg/tg}$ mice and drastically reduced SWDs in $\beta 4^{lh/lh}$ and $\gamma 2^{stg/stg}$ mice. It is interesting to note that there were residual SWDs in double mutants with $\alpha 1G^{-/-}$ and $\gamma 2^{stg/stg}$ or $\beta 4^{lh/lh}$. Considering that both $\gamma 2$ and $\beta 4$ are auxiliary subunits of α1 subunits, which have been known to modulate voltage dependence, kinetics, and amplitude of other types of Ca^{2+} channels as well as the P/Q-type (Kang et al., 2001; Schjott et al., 2003), the residual SWDs in $\gamma 2^{stg/stg}/\alpha lG^{-/-}$ and $\beta 4^{lh/lh}/\alpha lG^{-/-}$ mice appear independent of pathological interactions between α1A and dysfunctional $\gamma 2$ and $\beta 4$ subunits. This concept is supported by pharmacological studies using these mice in which absence seizures in $\gamma 2^{stg/stg}$ mice were sensitive to MK-801, which is ineffective in the treatment of absence seizures in other mice (Heller et al., 1983; Aizawa et al., 1997); however, a common thread that weaves through the generation and propagation of absence seizures in $\alpha 1A^{-/-}$ mice, as well as other mutant mice, is critical dependence on the α1G gene.

Functional significance of T-currents in TC neurons

Many studies on Ca^{2+} channelopathy have reported altered Ca^{2+} current profiles as a result of Ca^{2+} channel mutations, indicating a strong correlation between a disease symptom and alteration in Ca^{2+} currents. For example, altered expression of the N-type Ca^{2+} channel α1B in $\beta 4^{lh/lh}$ mice is suggested as a possible mechanism underlying absence seizures (McEnery et al., 1998), and increased expression of the α1C Ca^{2+} channel in cerebellar Purkinje cells in $\alpha 1A^{tg/tg}$ mice is associated with the dystonia in these mutant mice (Campbell and Hess, 1999). Similarly, the importance of T-currents in the development of absence seizures is underlined by recent studies using animal models of absence seizures (Tsakiridou et al., 1995; Zhang et al., 2002). These findings have motivated computational modeling studies to describe how augmented T-currents in thalamic neurons contribute to either physiological or pathophysiological synchrony in thalamocortical networks (Destexhe et al., 1996, 1998; Hughes et al., 1999; Thomas and Grisar, 2000). Somewhat unexpectedly, however, the present study using double-mutant mice (epileptic mice on a $\alpha 1G^{-/-}$ background) revealed that the basal level of T-currents in TC neurons was enough to support SWD genera-

tion. There was no quantitative difference in the severity of SWDs between 75 and 150% of wild-type dosage of T-currents in TC neurons isolated from the double mutants, suggesting that an increase in thalamic T-currents might not contribute to SWD genesis *in vivo*. At this point it cannot be ruled out that the increase of T-currents in other brain regions may support absence seizure development in these mutants, considering that the expression of α1G is also detected in other regions of brain, including cortex, olfactory bulb, and cerebellum (Talley et al., 1999). The question arises as to how normal levels of T-type Ca^{2+} currents in TC neurons contribute to SWDs generation. It is interesting to note that the hyperpolarizing shift in the resting membrane potential of hyperpolarization-activated cation channel 2-deficient thalamic relay neurons removes inactivation of T-type Ca^{2+} channels and thereby promotes burst rather than tonic firing in response to depolarizing inputs resulting in increased susceptibility to oscillations (Ludwig et al., 2003). Reduced excitatory but normal inhibitory synaptic transmission in $\beta4^{lh/lh}$ and $\alpha1A^{tg/tg}$ mice thalami (Caddick et al., 1999) and enhanced $GABA_B$ receptor expression (Hosford et al., 1992) would result in relatively enhanced GABAergic input in $\beta4^{lh/lh}$ and $\alpha1A^{tg/tg}$ thalamic neurons. Thus, more effective hyperpolarization per se could increase the likelihood of a T-type Ca^{2+} channel opening in TC neurons, enough to support SWDs without an increase in T-currents.

Finally, the present results suggest that a shift in research direction is required to determine the mechanisms underlying absence seizures. Beyond the issue of augmentation of T-type Ca^{2+} channels in TC neurons, studies are required to elucidate how hyperpolarizing inputs are overloaded in these neurons. The relationship between hypofunctioning P/Q-type channels and hyperpolarization of TC neurons sheds light on a possible novel therapeutic strategy for absence seizures.

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