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Lack of $Ca_V3.1$ channels causes severe motor coordination defects and an age-dependent cerebellar atrophy in a genetic model of essential tremor

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ABSTRACT

T-type Ca^{2+} channels have been implicated in tremorogenesis and motor coordination. The α1 subunit of the $Ca_V3.1$ T-type Ca^{2+} channel is highly expressed in motor pathways in the brain, but knockout of the $Ca_V3.1$ gene ($\alpha1G^{-/-}$) *per se* causes no motor defects in mice. Thus, the role of $Ca_V3.1$ channels in motor control remains obscure *in vivo*. Here, we investigated the effect of the $Ca_V3.1$ knockout in the null genetic background of α1 $GABA_A$ receptor ($\alpha1^{-/-}$) by generating the double mutants ($\alpha1^{-/-}/\alpha1G^{-/-}$). $\alpha1^{-/-}/\alpha1G^{-/-}$ mice showed severer motor abnormalities than $\alpha1^{-/-}$ mice as measured by potentiated tremor activities at 20 Hz and impaired motor learning. Propranolol, an anti-ET drug that is known to reduce the pathologic tremor in $\alpha1^{-/-}$ mice, was not effective for suppressing the potentiated tremor in $\alpha1^{-/-}/\alpha1G^{-/-}$ mice. In addition, $\alpha1^{-/-}/\alpha1G^{-/-}$ mice showed an age-dependent loss of cerebellar Purkinje neurons. These results suggest that $\alpha1^{-/-}/\alpha1G^{-/-}$ mice are a novel mouse model for a distinct subtype of ET in human and that $Ca_V3.1$ T-type Ca^{2+} channels play a role in motor coordination under pathological conditions.

1. Introduction

Essential tremor (ET) is a common [1], complex neurologic disorder with a broad spectrum of clinical characteristics such as age of onset, primarily affected body region, and rate of progression [2,3]. Diverse neural circuits and pathophysiological mechanisms likely contribute to the complexity of ET symptoms although supporting evidences remain to be elucidated.

The search for the mechanisms underlying ET has been facilitated by the use of harmaline, a plant-derived metabolite, known to be increased in the blood of ET patients [4]. When administered to human and rodents [5,6], harmaline causes essential-like tremor. Studies revealed that harmaline induces tremor rhythms at around 10 Hz through the olivocerebellar pathway [7,8] by increasing the amplitude of subthreshold oscillations in the inferior olive neurons, which are regulated by T-type Ca^{2+} currents [9]. And among the three T-type Ca^{2+} channel isoforms ($Ca_V3.1$, $Ca_V3.2$, and $Ca_V3.3$), null mutation or shRNA-mediated knockdown of $Ca_V3.1$ ($\alpha1G^{-/-}$) in the inferior olive significantly attenuated the harmaline-induced tremor [10], suggesting that $Ca_V3.1$ is the tremor-producing isoform in the harmaline model of ET.

Recently, a novel genetic model of ET has been reported; mice lacking the $GABA_A$ receptor α1 subunit ($\alpha1^{-/-}$) show several behavioral and pharmacological characteristics of human ET [11]. The loss of GABA inhibition [12] and subsequent adaptation and reorganization of GABAergic circuits in the brain regions [13–15] may underlie the genetic tremor in $\alpha1^{-/-}$ mice, but it remains unknown whether T-type Ca^{2+} channels are involved in the tremorogenesis. To address this issue, we investigated the role of $Ca_V3.1$ T-type Ca^{2+} channels in a genetic ET model by generating double knockout mice ($\alpha1^{-/-}/\alpha1G^{-/-}$) and found that markedly increased rather than decreased tremor was observed. The pathologic tremor in $\alpha1^{-/-}/\alpha1G^{-/-}$ mice was symptomatically similar as but pharmacologically different from that of $\alpha1^{-/-}/\alpha1G^{+/+}$ mice, indicative of a mouse model for novel type of ET. Our data further support the idea that $Ca_V3.1$ T-type Ca^{2+} channels are involved in modulating motor function under pathological condition.

2. Materials and methods

2.1. Animals

$GABA_A$ receptor α1 heterozygous ($\alpha1^{+/-}$) mice with a C57BL/6J genetic background were obtained from the laboratory of Dr. Gregg E. Homanics and utilized for the production of wild-type ($\alpha1^{+/+}$) and homozygous mutants ($\alpha1^{-/-}$). Double heterozygotes were

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produced ($\alpha1^{+/-}/\alpha1G^{+/-}$) and crossed to generate double mutants ($\alpha1^{-/-}/\alpha1G^{-/-}$) and their littermate controls ($\alpha1^{-/-}/\alpha1G^{+/+}$, $\alpha1^{+/+}/\alpha1G^{+/+}$). The genotypes were confirmed by PCR analysis, as previously described [16,17]. All the experiments below were performed using 4–8 month-old mice. Animal care and handling were carried out following the institutional guidelines of the Animal Care and Use Committee of KAIST. Mice were housed in a 12-h light/dark cycle (7 AM–7 PM) with free access to water and food.

2.2. Tremor measurement and analysis

The tremor recording platform was an opaque metal cylinder (diameter 10 cm × height 15 cm) mounted with an accelerometer (DC response accelerometer, Model No. 3711D1FA3G, Piezotronics) and suspended by two elastic wires inside a soundproof chamber [10]. A video camera was placed on top of the platform and the behaviour of each mouse was monitored during the recording session.

After acclimatization to the soundproof chamber for 1 h, each mouse was placed on the recording platform and tremulous activity was measured for 10 min. Electrical signals from the accelerometer were sampled at 1 kHz and digitized with the MiniDigi 1A (Axon Instrument). Raw traces and tremor strength for individual 2-min epochs were analyzed using Clampfit 9.2 (Axon Instrument) and Fourier transformation algorithm (MATLAB 7.0; MathWorks), respectively.

2.3. Rotarod test

Mice ($n = 7$ for $\alpha1^{-/-}/\alpha1G^{-/-}$, $n = 5$ for $\alpha1^{-/-}/\alpha1G^{+/+}$) were placed on an accelerating rotating rod (3 cm in diameter) apparatus (Model No. 47600, Ugo Basile) that accelerated from 5 to 40 rpm over a period of 5 min. Each mouse was given four trials per day for four consecutive days, and each trial lasted a maximum of 5 min with a 30-min inter-trial interval. The latency for each mouse to fall off the rod was recorded.

2.4. Pharmacological analysis

Propranolol (10 and 20 mg/kg, Sigma–Aldrich) and ethanol (0.5 and 1 g/kg, OCI Company Ltd.) were dissolved in saline. The dose of each drug was determined based on previous study [11] and prepared fresh each day. Vehicles and drugs were administered intraperitoneally in a 10 ml/kg injection volume 1 h before tremor measurement. A separate group of mice was used to test each dose.

2.5. Immunohistochemical staining and counting of Purkinje cells

$\alpha1^{+/+}/\alpha1G^{+/+}$, $\alpha1^{-/-}/\alpha1G^{+/+}$, and $\alpha1^{-/-}/\alpha1G^{-/-}$ mice ($n = 3$–5 per genotype) were perfused with 4% formaldehyde in PBS at 4 and 8 month of age, and the brains were extracted and postfixed for 8 h at 4 °C. Cerebellar paraffin blocks were prepared using a Tissue Processor (Leica) and cut sagittally at 10 μm using a rotary microtome (Leica). Following standard deparaffinization procedures, the sections were antigen-retrieved by incubating at 90 °C for 50 min in 10 mM sodium citrate buffer with 0.05% Tween 20, pH 6.0. After rinsing the sections in distilled water (D.W.) for 5 min, immunoperoxidase staining was performed using the VECTASTAIN® Elite ABC kit according to the manufacturer's instructions. In brief, the sections were incubated with a 0.3% H_2O_2 solution in D.W. for 30 min, rinsed in 0.02% Tween 20 in PBS, and incubated for 30 min with 0.3% Triton®X-100, 5% horse serum, and 5% goat serum in PBS. After washing in 0.02% Tween 20 in PBS, the sections were incubated for 1 h with anti-calbindin D-28 K polyclonal antibody (1:50, Millipore) diluted in 0.3% Triton®X-100 in PBS. The sections were washed in 0.02% Tween 20 in PBS, incubated with biotinylated secondary antibody for 30 min, washed, and incubated with VECTASTAIN® Elite ABC reagent for 30 min. Sections were then washed and further incubated with peroxidase substrate solution (3,3′-diaminobenzidine tetrahydrochloride hydrate, Aldrich®) for 2 min. After washing in D.W., the sections were briefly immerged in xylene, air dried, and coverslipped with Shandon Synthetic Mountant (Thermo). All procedures were performed at room temperature. Calbindin-positive Purkinje cells were observed under a confocal microscope (LSM510, Carl Zeiss) using a 20× objective.

Nucleated Purkinje cells in the anterior lobe (lobule I-III) of the cerebellar cortex in every fifth sections were counted blind to genotypes using ImageJ software, and Purkinje cell linear density was calculated by dividing the number of cells by the linear distance of the Purkinje cell layer per section.

2.6. Statistical analysis

All data are presented as the mean ± SEM and subjected to ANOVAs with appropriate post hoc tests. This analysis was performed using SigmaStat 3.1 software package.

3. Results

$\alpha1^{-/-}/\alpha1G^{-/-}$ mice were born at the normal Mendelian ratio and showed normal growth and survival, but they were strikingly distinguishable from their wild-type ($\alpha1^{+/+}/\alpha1G^{+/+}$) and $\alpha1^{-/-}/\alpha1G^{+/+}$ littermates by outstanding tremulous activity. The double mutants shared several tremor-related features with $\alpha1^{-/-}/\alpha1G^{+/+}$ mice; they exhibited tremor when freely moved or suspended by tails, but not when relaxed, and all four limbs were equally affected. For a more quantitative analysis, the motion activity displayed by the three genotypic groups of mice was assessed and compared using an accelerometric transducer (Fig. 1). All three groups of mice showed 3–5 Hz activity reflecting basal locomotion, without quantitative differences ($p > 0.05$, Fig. 1B). $\alpha1^{-/-}/\alpha1G^{+/+}$ mice exhibited tremor at 20–25 Hz as reported previously [11], but the tremor was evident during only the first 2-min period (Fig. 1C). In $\alpha1^{-/-}/\alpha1G^{-/-}$ mice, the 20–25 Hz tremor was increased 3- to 15-fold as compared to that of $\alpha1^{-/-}/\alpha1G^{+/+}$ mice and persisted throughout the recording session (Fig. 1C).

Based on the fact that ET impairs normal motor performance in patients [18,19], rotarod testing was used to measure motor coordination (Fig. 2). The rotarod performance of $\alpha1^{+/+}/\alpha1G^{+/+}$ mice reached the maximum level (5 min) since the second day (data not shown). Compared with $\alpha1^{-/-}/\alpha1G^{+/+}$ mice, $\alpha1^{-/-}/\alpha1G^{-/-}$ mice demonstrated a significantly lower score on the third (51.8 ± 10.2 and 25.6 ± 5.2 s, respectively) and fourth (70.8 ± 2.7 and 35.4 ± 4.8 s, respectively; $^{*}p < 0.05$) day. Furthermore, $\alpha1^{-/-}/\alpha1G^{-/-}$ mice did not show motor learning at all, as measured by the increase in latency to fall from the rotating rod as the number of trials increased, while $\alpha1^{-/-}/\alpha1G^{+/+}$ mice did ($^{\#}p < 0.001$). Taken together, these results suggest that the loss of $Ca_V3.1$ channels in $\alpha1^{-/-}/\alpha1G^{+/+}$ mice leads to a more pronounced, persistent, and severe form of essential-like tremor.

Next, anti-ET drugs were used to determine whether the 20–25 Hz pathologic tremor in $\alpha1^{-/-}/\alpha1G^{-/-}$ mice had the pharmacological properties of ET. Propranolol, a first-line medication for ET, and ethanol, a more potent anti-ET drug, markedly reduced tremor amplitudes in $\alpha1^{-/-}/\alpha1G^{+/+}$ mice [11]. However, $\alpha1^{-/-}/\alpha1G^{-/-}$ mice still showed tremor even after administration of 20 mg/kg of propranolol (Fig. 3A and B), a dose two times higher than necessary to suppress the tremor in $\alpha1^{-/-}/\alpha1G^{+/+}$ mice [11]. On the contrary, ethanol effectively ameliorated the tremor by ~90% at a non-sedative dose of 1 g/kg (Fig. 3A and B). Thus, unlike the $\alpha1^{-/-}/$

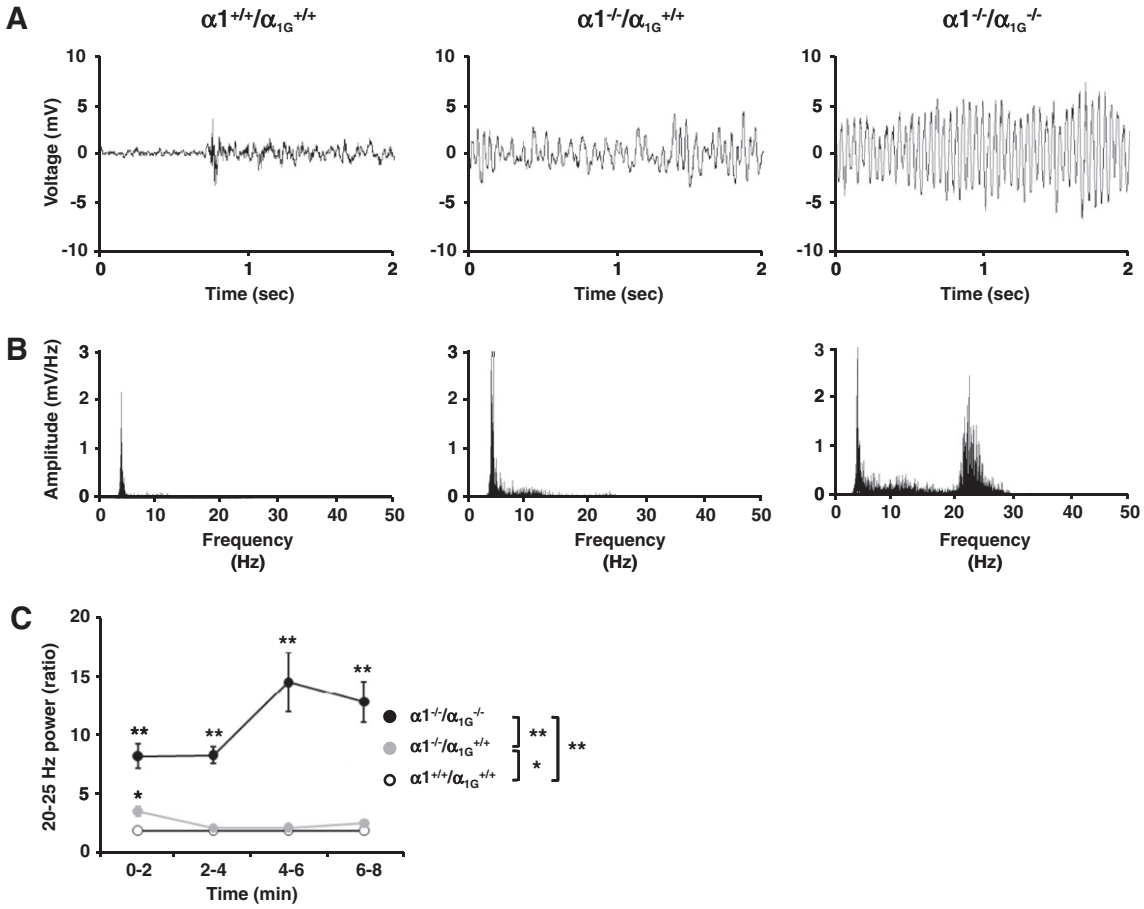


Fig. 1. Tremor measurement and analysis. Representative voltage tracings (A) and peak amplitudes (B) of tremulous activity in the three genotypes ($n = 3$ for $\alpha1^{+/+}/\alpha1G^{+/+}$ and $\alpha1^{-/-}/\alpha1G^{-/-}$, $n = 4$ for $\alpha1^{-/-}/\alpha1G^{+/+}$). (C) Relative changes in tremor strength. Data represent tremor strength (20–25 Hz; see Section 2) for each 2-min epochs in $\alpha1^{-/-}/\alpha1G^{+/+}$ (gray circle) and $\alpha1^{-/-}/\alpha1G^{-/-}$ mice (black circle), respectively, normalized by that in $\alpha1^{+/+}/\alpha1G^{+/+}$ mice (white circle; $^{*}p < 0.05$, $^{**}p < 0.001$, two-way ANOVA). Error bars indicate SEM.

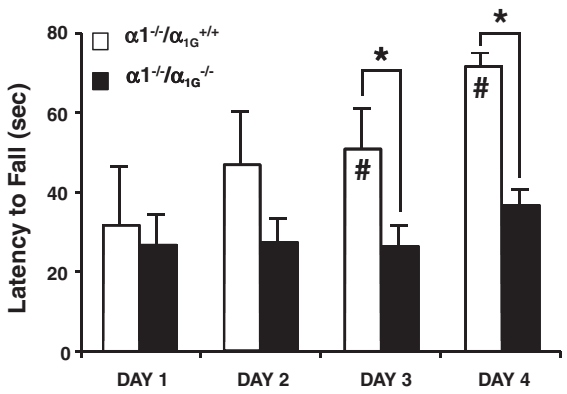


Fig. 2. Comparison of rotarod performance between the two mutants. Data represent the mean latency to fall (in seconds) ± SEM for each trial over four successive days ($^{*}p < 0.05$, $^{\#}p < 0.001$ versus day 1, two-way ANOVA with repeated measures).

$\alpha1G^{+/+}$ mice, our double knockout mice differentially respond to a subset of ET medications.

Several studies have shown that ET is accompanied by pathologic changes in the cerebellum; such as a loss of cerebellar Purkinje cells (PCs) [20–22]. In addition, PC degeneration has been shown to be involved in tremor in rodents [23,24]. However, $\alpha1^{-/-}/\alpha1G^{+/+}$ mice did not show any deficits in the number or morphology of PCs [11]. To understand the etiology of the severer essential-like tremor in $\alpha1^{-/-}/\alpha1G^{-/-}$ mice, histological analysis of the cerebellum in the three genotypic groups of mice was performed. No significant differences in the number (21.0 ± 0.2 for wild-type, 22.7 ± 0.4 for $\alpha1^{-/-}/\alpha1G^{+/+}$, and 22.3 ± 1.5 for $\alpha1^{-/-}/\alpha1G^{-/-}$), location or morphology of calbindin-stained PCs between genotypes at 4-months of age were observed (Fig. 4A). However, a mild decrease in PCs was detected in 8-month-old $\alpha1^{-/-}/\alpha1G^{-/-}$ mice (Fig. 4A and B). Given the early onset of tremulous symptoms in our mutant mice (<3-week of age), these results show that the observed PC degeneration, rather than playing a causative role, is a parallel manifestation or a by-product of the persistent, pathologic tremor in $\alpha1^{-/-}/\alpha1G^{-/-}$ mice.

4. Discussion

Our study provides the first *in vivo* evidence of the role of T-type Ca^{2+} channels in motor control. Though $\alpha1G^{-/-}$ mice showed no defects in normal motor performance [10], knockout of the $Ca_V3.1$ gene in a $GABA_A$ receptor α1 subunit-null genetic background leads to severe motor disturbances (Figs. 1 and 2) that even lead to an age-dependent loss of cerebellar Purkinje cells (Fig. 4). Thus, it is likely that the role of $Ca_V3.1$ channels in motor control is more pronounced under pathological conditions.

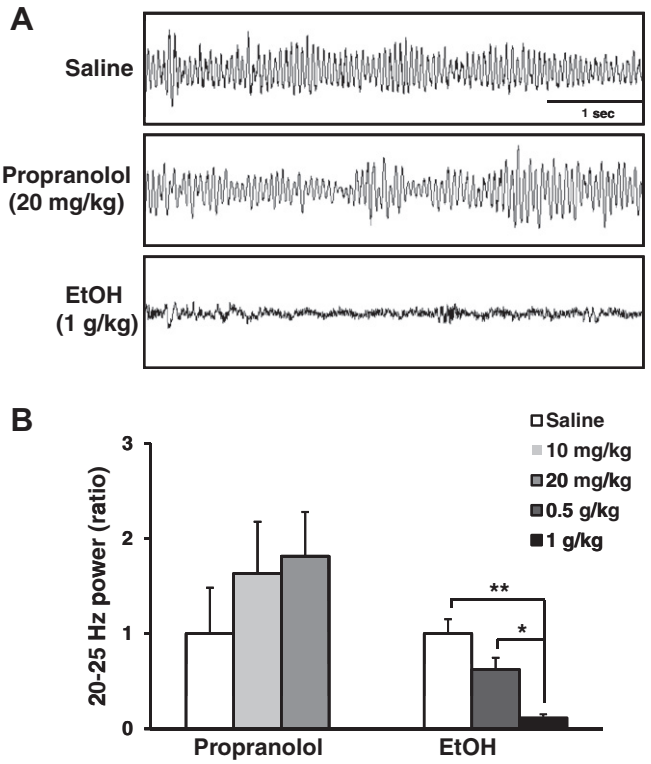
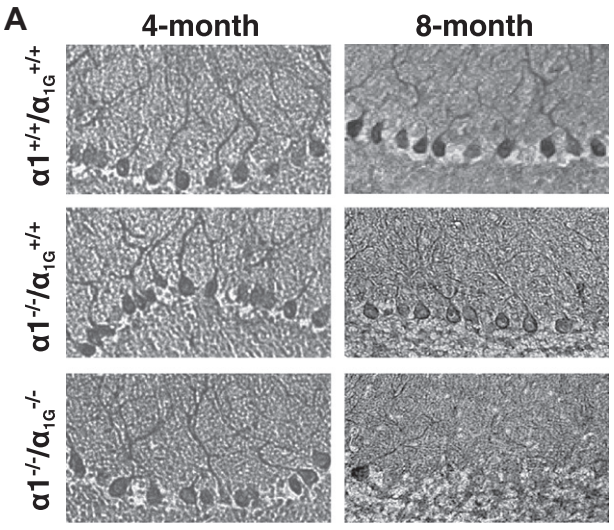


Fig. 3. Responses of $\alpha1^{-/-}/\alpha1G^{-/-}$ mice to anti-tremor agents. (A and B) Motion activity in $\alpha1^{-/-}/\alpha1G^{-/-}$ mice ($n = 3$–4 per treatment) was measured after administration of vehicle (saline), propranolol (10 and 20 mg/kg), or ethanol (0.5 and 1 g/kg). Representative voltage tracings (A) and relative tremor strength (B) from vehicle- or drug-treated $\alpha1^{-/-}/\alpha1G^{-/-}$ mice are shown. (B) Data represent the changes of mean tremor amplitudes (20–25 Hz) ± SEM for 100-s epochs in drug treatment relative to that in vehicle treatment ($^{*}p < 0.05$, $^{**}p < 0.001$, one-way ANOVA).



B

Genotypes	Purkinje cell number/mm layer
$\alpha1^{+/+}/\alpha_{1G}{}^{+/+}$	21.6±0.6
$\alpha1^{-/-}/\alpha_{1G}{}^{+/+}$	20.3±0.5
$\alpha1^{-/-}/\alpha_{1G}{}^{-/-}$	17.9±0.6*

Fig. 4. Age-dependent change of Purkinje layer integrity. (A) Calbindin-stained Purkinje cells in the cerebellum of 4- or 8-month-old mice from the three genotypic groups. Images are magnified by 400×. (B) Purkinje cell linear density in 8-month-old $\alpha1^{-/-}/\alpha1G^{-/-}$ mice and littermate controls was calculated. Data are presented as the mean cell number per mm layer ± SEM ($^{*}p < 0.05$, one-way ANOVA).

ET, once known as a monosymptomatic disorder, has nowadays been recognized as a highly heterogeneous entity. The genetic [25] and clinical [26] variability of ET thus infer diverse and complex tremorogenic mechanisms. In an attempt to better understand the etiology of ET, here we extend the scope of our previous study using harmaline model [10] to the α1-null genetic model [11], thereby generating double knockout mice ($\alpha1^{-/-}/\alpha1G^{-/-}$). Considering our previous results that $\alpha1G^{-/-}$ mice showed no overt motor defects and were resistant to the harmaline-induced tremor [10], no alterations or suppression of the genetic tremor was anticipated. Rather, however, severer tremor was produced from the double knockout mice. Compared to $\alpha1^{-/-}/\alpha1G^{+/+}$ mice, the $\alpha1^{-/-}/\alpha1G^{-/-}$ mice exhibited a pathologic tremor with increased amplitudes followed by exacerbated motor disturbances (Figs. 1 and 2).

It cannot convincingly be answered why the pathologic tremor in $\alpha1^{-/-}/\alpha1G^{+/+}$ mice was enhanced, instead of being intact or abolished, by $Ca_V3.1$ knockout. Given that the tremor frequency of $\alpha1^{-/-}/\alpha1G^{-/-}$ mice precisely overlap with that of $\alpha1^{-/-}/\alpha1G^{-/-}$ mice (20–25 Hz), present results may not be due to a non-specific increase of tremor susceptibility but rather suggest a possibility of the two ET models having different tremorogenic origins whereat the role of $Ca_V3.1$ channels differs. Harmaline tremor has been known to be induced in inferior olive neurons [10,27,28]. But the α1 subunit of the $GABA_A$ receptor is not expressed in the inferior olive [29] and the loss of $GABA_A$ receptor binding sites in $\alpha1^{-/-}$ mice was pronounced in the major motor pathways from the brainstem to the thalamus and the basal ganglia [12], suggesting a non-olivocerebellar origin of the tremor observed in $\alpha1^{-/-}/\alpha1G^{+/+}$ mice. In addition, $Ca_V3.1$ channels are highly expressed in the motor pathway [30,31], thus likely contribute to modulate the pathologic tremor. Further studies using region-specific Cre drivers and α1-floxed mice ($\alpha1^{F/F}$) [32], along with $Ca_V3.1$-specific shRNA, will be required to accurately explain present results and to support our hypothesis regarding tremor origins.

Our double mutants, as well as $\alpha1^{-/-}/\alpha1G^{+/+}$ mice, share common behavioral and pharmacological features with ET patients; $\alpha1^{-/-}/\alpha1G^{-/-}$ mice showed a persistent, pathologic tremor with postural and kinetic components and ethanol-responsiveness (Fig. 3). Furthermore, the prominent pathological changes in ET patients were also detected in $\alpha1^{-/-}/\alpha1G^{-/-}$ mice (Fig. 4). The only difference between them was that propranolol was not efficacious in reducing tremor amplitudes in $\alpha1^{-/-}/\alpha1G^{-/-}$ mice (Fig. 3). However, Deuschl et al. reported the identification of a subgroup among ET patients who showed no improvement of the tremor when given propranolol [33]. Thus, our double knockout model likely represents a distinct subtype of ET that is refractory to the β-blocker.

In conclusion, our study suggests that the $Ca_V3.1$ T-type Ca^{2+} channel plays a role in modulating motor function under pathological condition and thus, the treatment of ET by antagonizing T-type Ca^{2+} channels [34] should be considered with caution, due to possible contraindications. In addition, our $\alpha1^{-/-}/\alpha1G^{-/-}$ mice can be used as a novel genetic model for studying ET and for screening drug candidates.

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