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Modified autonomic regulation in mice mutated in the β4 subunit of the lh/lh calcium channel

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ABSTRACT

Genetic analyses have revealed an important association between P/Q-type calcium channel activities and hereditary neurological disorders. The P/Q-type channels are composed principally of heterologous multimeric subunits including CaV2.1 and CaVβ4. Of these, the β4 subunit is thought to play a significant role in channel physiology, because a mouse line mutant in that subunit (the lethargic mouse: lh) exhibits a severe ataxic phenotype. The aim of the present study was to elucidate the physiological importance of the β4 subunit.

ECG analysis showed that the T wave was high in 8-week-old lh mutants; this may be associated with hyperkalemia. Upon pharmacological ECG analysis, 2-3-week-old lh mutants exhibited reduced responses to a β-blocker and a muscarinic receptor antagonist. Analysis of heart rate variability revealed that the R–R interval was unstable in lh mutants and that both the low- and high-frequency components had increased in extent, indicating that the tonus of both the sympathetic and parasympathetic nervous systems was modified.

Thus, our present study revealed that the β4 subunit played a significant role in regulation of sympathetic and parasympathetic nerve activities.

1. Introduction

Voltage-dependent Ca^{2+} channels regulate the activities of excitable membranes, which control physiological functions including muscle contraction, and hormone and neurotransmitter release [1,2]. These channels feature a principal pore-forming α_1 subunit, encoded by a family of 10 distinct genes (CaV1.1-4, CaV2.1-3, and CaV3.1-3). Five distinct high-threshold channels (L, N, P, Q, and R) have been identified, as have low-threshold T-type voltage-dependent channels [3]. Several types of calcium channel contribute to the regulation of neuronal activity. In particular, P- and N-type channels are involved in transmitter release at the synaptic terminal [4,5]. CaV2.1 is found in two pharmacologically different P- and Q-type channels. Mutations in the CaV2.1 (α1A) gene are associated with inherited neurological diseases, including ataxia and seizures in the mouse [6], and familial hemiplegic migraine (FHM) and episodic ataxia type-2 (EA-2) in humans [7]. Notably, CaV2.1-null mutant mice develop a rapidly progressive ataxic syndrome a few weeks after birth, thus indicating the importance of CaV2.1 in the central nervous system [8].

In addition to α_1 subunits, native Ca_V channels contain two-to-three minor or auxiliary subunits, termed β, α_2/δ, and γ, that

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modulate channel expression and electrophysiological kinetics [1,2]. Although auxiliary, the calcium channel β ($Ca_v\beta$) subunits are physiologically important. The β subunits are cytoplasmically located and modulate channel properties, including current amplitude and channel properties [9]. In addition, β subunits may serve as scaffolding proteins for components of several signaling pathways located around the channel [10]; this is because the β subunits are members of the membrane-associated guanylate kinase family (MAGUK) [11]. Four different β subunit genes have been identified [12], and neurologically lethargic (lh) mice of the inbred BALB/cGn strain carry a spontaneous autosomal recessive mutation in the β4 subunit [13]; such animals exhibit sluggish behavior [14]. Homozygotes can be identified by 15 days of age, as they exhibit ataxia and lethargic behavior, followed within a few days by the onset of spontaneous focal motor seizures [14] resembling the absence seizures of human petit mal epilepsy [15]. Such animals also exhibit immunological problems including thymic involution, causing high-level mortality, suggesting that β4 subunit mutation may trigger a variety of disorders.

In the present study, we evaluated the ECG phenotype of the lethargic mouse. Upon ECG analysis, lethargic mice exhibited high-level variability in the R–R interval and reduced responses to both an adrenergic β-blocker and a parasympathetic blocker. Furthermore, we found that the elevated T-wave evident upon ECG was probably attributable to a high plasma potassium level, indicating that an electrolyte disorder may be one cause of the accelerated mortality of lethargic mice.

2. Materials and methods

2.1. Animals

"Lethargic" mice were purchased from the Jackson Laboratory (Bar Harbor, MA). The mouse mutation termed "rolling Nagoya" (Tg^{rol}) is an allelic mutation in CaV2.1, as we reported previously [16–18].

Throughout the study, animals were housed at 22 ± 0.5 °C under a constant 12-h light/dark cycle with free access to food and water. All experiments were conducted in accordance with the Guidelines for the Use of Laboratory Animals of Akita University School of Medicine.

2.2. Genotyping

The mutated sequence from the gene encoding the calcium channel β4 subunit was amplified using primers "lh-for" (5'-caactcccacaacaacaggttggg-3′) and "β4-rev" (5'-tcacctggaattgccagatgtccaaa-3′). These primers correspond to the A_{160}TPTTT$_{165}$ region, including the lh allele-specific 4-bp sequence (tggg) (lh-for) and a specific intron 5 sequence (β4-rev) of the murine calcium channel β4 subunit.

2.3. Reverse transcription–polymerase chain reaction (RT-PCR)

Poly(A)+ RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and Oligotex-dT30 (TaKaRa, Shiga, Japan). The RT reaction was performed using a first-strand cDNA synthesis kit (SuperScript II Reverse Transcriptase; Invitrogen). PCR amplification was performed using GoTaq Green Master Mix (Promega, Madison, WI, USA).

Specific calcium channel $\beta_{1\text{-}4}$, CaV1.2, and CaV1.3 sequences were amplified by PCR (34 cycles). (Detailed information is provided in the Supplementary information.)

2.4. ECG evaluation

ECG, heart rate, and RR interval were measured simultaneously (ML846 Power Lab system; AD Instruments, Dunedin, New Zealand) [19]. An M-button connector was used to connect the electrode [20]. We used HRV as a measure of cardiac autonomic nerve control [19]. The HR variability (HRV) was the standard deviation of the R–R interval (SDNN) and is considered to reflect the integrity of cardiac vagal control. HRV analysis featured both time domain analysis (based on calculation of deviations from the average R–R interval [the standard deviation of R–R intervals: SDNN]) and analysis of the HRV power spectrum. The SDNN represented the total variability. An HRV power spectrum has three components of very low frequency (VLF), low frequency (LF), and high frequency (HF). Generally, the LF component reflects sympathetic/parasympathetic tone and the HF component parasympathetic tone. We defined the ranges of the spectral components as very low frequency (VLF) < 0.15 < low frequency (LF) < 1.5 < high frequency (HF) < 5, according to the manufacturer's protocol. For pharmacological analysis, mice were given either atropine (1.0 mg/kg) to cause parasympathetic blockade or propranolol (1.0 mg/kg) to cause sympathetic blockade. (Detailed information is provided in the Supplementary information.)

2.5. Statistical analysis

All data are presented as means ± SEMs. The statistical significance of an observed difference was determined by analysis of variance (ANOVA) followed by application of Dunnett's test, and p-values less than 0.05 were considered significant.

3. Results

3.1. Gene structure and mutation

The exon–intron structure of the β4 gene is shown in Fig. 1A. The primer sequences used for genotyping and their positions in the genomic sequence are indicated. The lethargic (lh) mutation and wild-type allele were both appropriately identified (Fig. 1B). The insertion of four bases (tggg) in intron 4 of the β gene of the lh mutant is shown in Fig. 1C. The forward primer sequence for the wild-type allele is shown in red, and the insert (tggg) in the lh allele in green. The sequence of the reverse primer (B4-rev) is also indicated. The ataxic phenotype and small thymus typical of this mouse are discussed in the Supplemental data.

3.2. Modified regulation of the autonomic heart rate

As P/Q-type channel mutants (CaV2.1 mutant Tg^{rol}) are known to exhibit modified autonomic HR regulation, we next performed ECG analysis to explore HR regulation in the lh mutant. By 3 weeks of age, ECG analysis of the lh mutant revealed a regular pattern, indicating physiological pacemaking and propagation of excitation (Fig. 2A). A typical Tg^{rol} recording is also shown; Tg^{rol} ECG revealed a regular pattern.

Interestingly, after development of the typical ataxic phenotype (at 4–5 weeks of age), some mutant mice exhibited high T-waves on ECG recordings (Fig. 2A lower panel; lh mutant at 28 days); such waves are often associated with high plasma potassium levels. Most lh-mutant mice died within 1 week after the appearance of high T-waves. Statistical analysis showed that the T/R ratios of ECG recordings made at this time were elevated (Fig. 2B). Notably, the plasma potassium levels of lh mutants were significantly higher than those of wild-type controls (Fig. 2C), suggesting that one cause of early death (at ca. 5 weeks) in lh mutants might be high

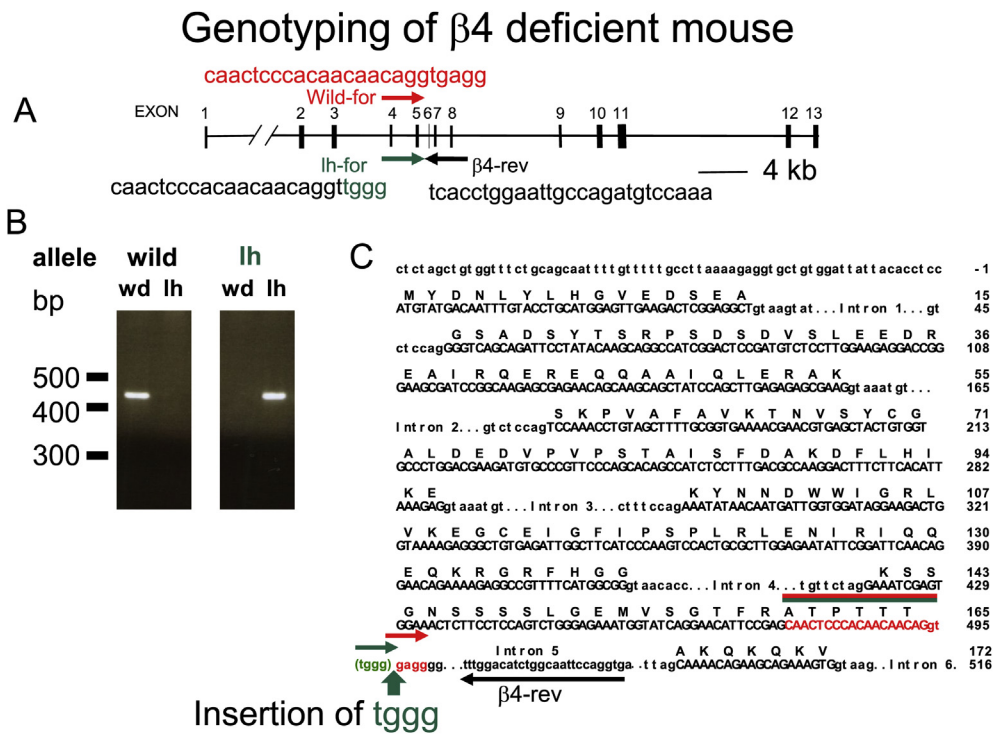

Fig. 1. A) Structure of the β4 gene and the mutational site in the lh mouse. PCR primer sequences ("wild-for," "lh-for," and "β4-rev") used for genotyping and their positions in genomic DNA. Exons (boxes) and introns (lines) are indicated. B) Typical PCR data from the wild-type (right) and lh mutant (left) alleles. The amplified sequences are indicated (wt: wild type, lh: lh mutant). C) Exon (1–6) sequences and intron sequences. The primer sequences used for genotyping are shown. The lh mutation (a tggg insertion) is shown in green. The insertion site is indicated. The red arrow corresponds to the forward primer for the wild-type allele, and the green arrow to the forward primer for the lh-mutant allele. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

potassium levels. We also performed ECG analysis on adult Tg^{rol} mice; only animals aged 12–16 weeks were available (Fig. 2A). These mice had significantly lower HR components than either wild-type animals or lh mutants (442 ± 2 bpm, $n = 6$, $p < 0.05$). Differences in HR influence ECG parameters and may compromise the analysis of pharmacological responses. Thus, we focused on differences between wild-type and lh-mutant mice.

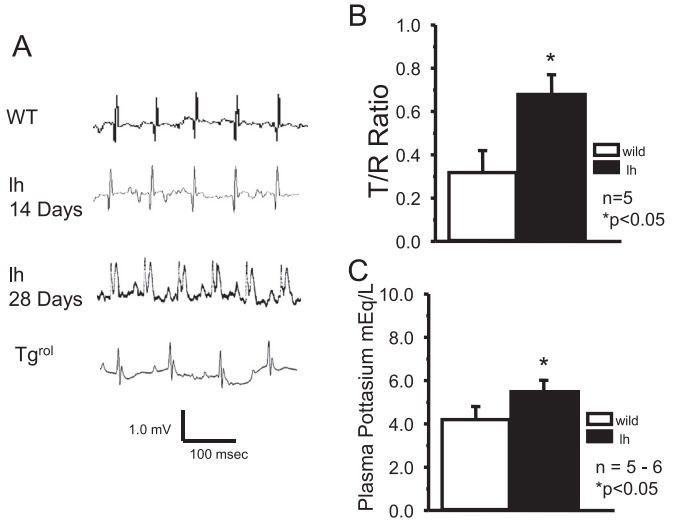

Fig. 2. A) Representative ECG traces of wild-type (WT) mice, 14-day-old (lh 14 Days) and 28-day-old lh-mutant mice (lh 28 Days), and 12-week-old rolling Nagoya mutant (Tg^{rol}) mice. Scale bars: 1.0 mV and 100 ms. B) Statistical analysis of the T:R wave ratios (T/R ratios) of wild-type (open bar) and lh-mutant mice (closed bar). *$p < 0.05$, $n = 5$. C) Plasma potassium concentrations of wild-type (open bar) and lh-mutant (closed bar) mice. *$p < 0.05$, $n = 5$.

3.3. Pharmacological analysis

To further analyze pharmacological changes in the lh mutant, we performed ECG on young animals (2–3 weeks of age) because most lh mice died by 6 weeks (Fig. 3). Over 1 h, the HRs of lh and wild-type mice were similar (688 ± 20 and 661 ± 19 bpm, respectively; $p > 0.05$, Fig. 3A).

The lh mutant was less responsive than the wild-type to intraperitoneal injection of the β-adrenergic blocker propranolol (1.0 mg/kg), suggesting a decrease in sympathetic tonus (Fig. 3A). The response to the parasympathetic blocker atropine (0.5 mg/kg, i.p.) was also reduced in the lh mutant, suggesting reduced parasympathetic tonus. The lh mutant exhibited an unstable R–R interval (Fig. 3B, lower panel), indicating a rise in HR variability and unstable pacemaking.

Together, the data show that the tonus of both sympathetic and parasympathetic nerve control were lower in the lh mutant. We have previously reported that P/Q-type channels play roles in the autonomic (both sympathetic and parasympathetic) nervous system [16], and this is consistent with our present results.

We next analyzed ECG parameters using the ML846 Power Lab system.

On average, the QT interval was shorter in the lh mutant (0.19 ± 0.08 ms) than in the wild-type (0.25 ± 0.02 ms); this may be attributable to differences in the HR component. The QT time (QTc) did not differ significantly between the wild-type and lh mutant groups (0.07 ± 0.01 vs. 0.06 ± 0.02 ms, respectively). Similarly, neither the PQ nor the QRS time differed significantly between the two strains.

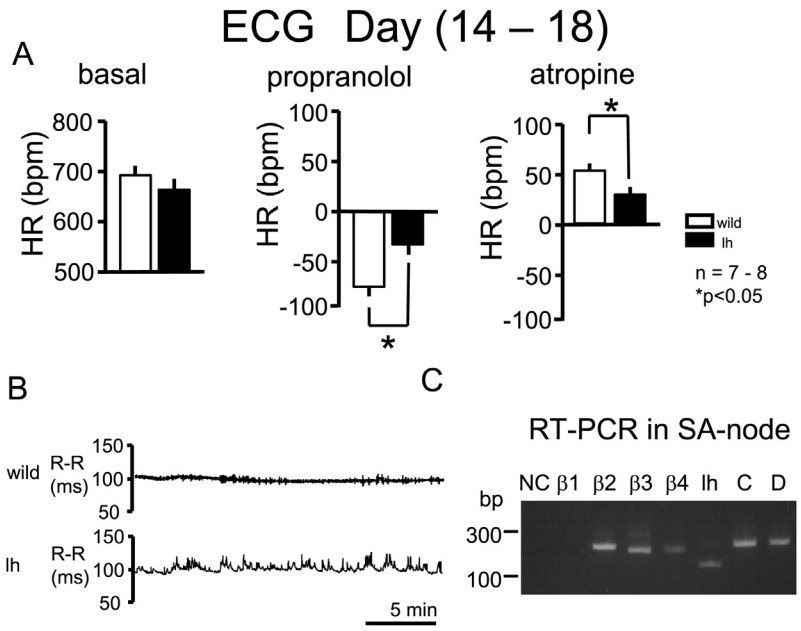

Fig. 3. A) Statistical analysis of the heart rates of 14-day-old wild-type (WT, open bar) and lh-mutant mice (lh, closed bar). The pharmacological responses to propranolol (middle panel) and atropine (right panel) are shown. The lh mutant had a reduced response to both propranolol (1.0 mg/kg, i.p.) and atropine (0.5 mg/kg, i.p.) compared with wild-type mice. *$p < 0.05$, $n = 7–8$. B) Representative time-dependent changes in the R–R intervals of wild-type (upper panel) and lh-mutant (lower panel) mice. The lh mutants had an unstable R–R interval. C) RT-PCR analysis of expression levels of β and α1 subunits in the sinus node. Identification of β2, β3, and β4 subunit-specific transcripts, and CaV1.2-specific (C) and CaV1.3-specific (D) transcripts in the murine SA node. NC, negative control (no cDNA). The PCR products amplified from the lh mutant are indicated (lh). The primer sets used in PCR amplifications are shown. β-actin expression level served as a control. No PCR amplification was detected using a β1-specific primer set.

3.4. Expression of calcium channels in the SA node (RT-PCR analysis)

Given that the lh mutants exhibited reduced responsiveness to autonomic nervous stimulation, we explored the expression levels of various calcium channel α1 and β subunits in the sinus node (Fig. 3C). Sinus (SA) nodes were visualized via dissection microscopy and were confirmed to engage in pacemaking (Dissection of an SA node is shown in Supplemental Fig. 1). Both CaV1.2 (lane C) and CaV1.3 (lane D) were expressed in SA nodes, suggesting that two L-type channels containing α1 subunits were involved in pacemaking. The β2 and β3 subunits were also expressed. Although the levels of amplified product were low, the β4 subunit was also expressed in SA nodes, suggesting that this subunit may also be involved in pacemaking. We also evaluated expression of calcium channels in the SCG and kidney, while no significant differences between wild-type and lh mutant mice were observed (Supplementary data Figs. 2 and 3). Nevertheless, expression of mutated β4 subunit was confirmed in the lh mutant.

3.5. Modified heart rate variability

As the R–R interval of the lh mutant was apparently unstable, HR parameters were further analyzed using the Power Lab HRV software. Fig. 4A shows representative Poincaré plots of wild-type (2–3 weeks of age), lh-mutant (2–3 weeks of age), and rolling-Nagoya (Tg^{rol}, 12–16 weeks of age) mice (upper panels). Wild-type mice exhibited stable changes in R–R intervals, whereas lh-mutant mice had relatively unstable R–R intervals (shown as dots). Rolling-Nagoya mice had long R–R intervals, resulting in dot scattering in the Poincaré plot, suggesting that pacemaking was unstable.

Power spectral analysis (Fig. 4; lower panels in A and B–D) showed that the LF component was greater in lh mutants (13.1 ± 2.4%, $n = 8$) compared with wild-type mice (7.1 ± 2.9 ms^2, $n = 19$). Interestingly, Tg^{rol} mice exhibited low LF (2.0 ± 0.9 ms^2, $n = 8$). The lh and Tg^{rol} mutants exhibited increased HFs (41.4 ± 5.4 ms^2, $n = 8$; 29.2 ± 7.2 ms^2, $n = 8$; lh and Tg^{rol} mutants, respectively) compared with wild-type mice (14.0 ± 2.7 ms^2, $n = 19$). Because the Tg^{rol} mutant exhibited low LF, the LF/HF ratio was also significantly lower (0.66 ± 0.02, $n = 8$) than that of wild-type mice (0.49 ± 0.08, $n = 19$). The significant increases in the LF and HF of lh-mutant mice suggest that autonomic nerve control is affected in these animals.

4. Discussion

In the present study, we explored the phenotypes of lh mutant mice.

ECG analysis showed that the T-wave was high in 8-week-old lh mutants; possibly due to hyperkalemia. Upon pharmacological ECG analysis, 2–3-week-old lh mutants exhibited reduced responses to propranolol (a β-blocker) and atropine (a muscarinic receptor antagonist). HRV analysis showed that the lh mutant had an unstable R–R interval and increased proportions of both the LF and HF components, indicating that the tonus of both the sympathetic and parasympathetic nerves was modified.

The lethargic mutation features deletion of approximately 60% of the β4 coding sequence [13]. Such a truncated β subunit would lack all three of the domains mediating wild-type interactions with the α1A and β4 subunit [21,22]. The binding affinity of β4 to the conserved α-binding domain (AID) of CaV2.1 is 10 fold higher than that of other β subunits [21]. Additionally, CaV2.1 has three β4 binding domains, but only one conserved binding site for the other β subunits [22]. The high binding affinity between Cav2.1 and the β4 subunit and the presence of several β4-binding domains in Cav2.1 suggest that the lh mutant, in which 60% of β4 is deleted, probably has significantly fewer channels on the cell surface and that the properties of these channels are altered.

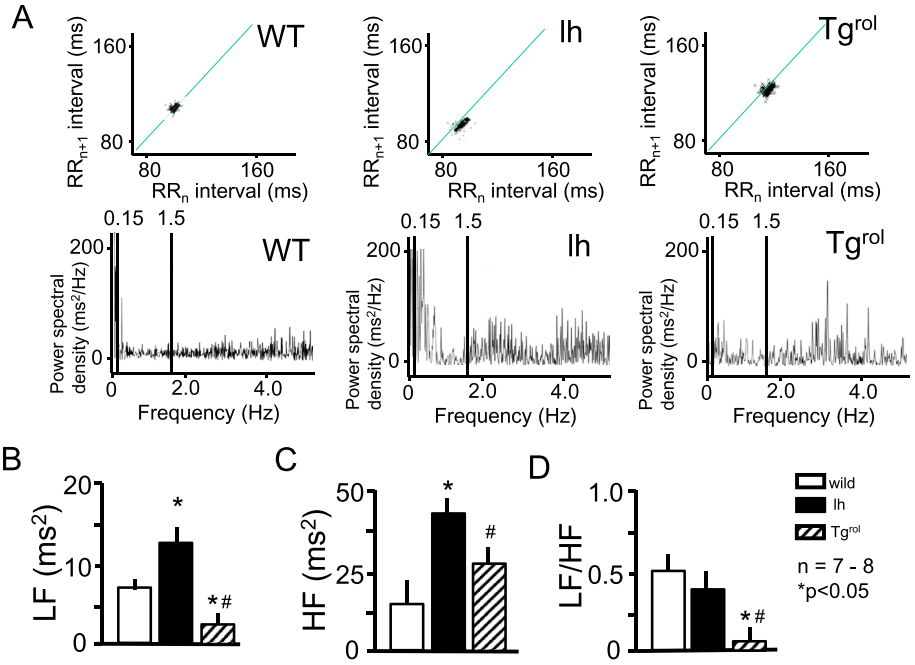

Fig. 4. A) Representative ECG Poincaré plots of 14-day-old wild-type mice (WT, left upper panel), 14-day-old lh-mutant mice (lh, middle upper panel), and 12-week-old rolling Nagoya mutant (Tg^{rol}) mice (right upper panel). Representative power spectrum analysis of 14-day-old wild-type (WT, left lower panel), 14-day-old lh-mutant (lh, middle lower panel), and 12-week-old rolling Nagoya mutant (Tg^{rol}) mice (right lower panel). Statistical analysis of the power spectra (B–D). The LF (B) and HF (C) components and the LF/HF ratios (D) of 14-day-old wild-type (WT, open bar) mice, 14-day-old lh-mutant mice (lh, closed bar), and 12-week-old rolling Nagoya mutant (Tg^{rol}, hatched bar) mice (left panel). C) Comparison of the HF components of 14-day-old wild-type (WT, open bar) mice, 14-day-old lh-mutant mice (lh, closed bar), and 12-week-old rolling Nagoya mutant (Tg^{rol}, hatched bar) mice (left panel). *p < 0.05 vs. wild-type, #p < 0.05 vs. lh-mutant, n = 7–8. Error bars indicate SEMs.

The phenotype of the lh mutant has been continuously investigated since the first description of the mutant by Dickie [14]. After identification of the mutation in the voltage-dependent calcium channel β4 subunit by Burgess et al. [13], McEnery et al. reported that expression of the β1 subunit was increased in the brains of lh mutant mice [23]. In the present study, we found no significant changes in the β−subunit expression profiles of the mouse SCG, SA node, or kidney (Supplementary data Figs. 2 and 3). However, we used RT-PCR to obtain our data, and this technique is less accurate than Western blotting or binding analysis. Thus, our negative results may conceal differences in the expression levels of various calcium channel subunits.

In addition to the role played by β subunits in channel physiology, Hibino et al. reported a direct interaction of the β4 subunit with the chromo-shadow domain of chromobox protein 2/heterochromatin protein 1γ (CHCB2/HP1 γ); this nuclear protein is involved in gene silencing and transcriptional regulation [24]. Therefore, β4 is multifunctional (a subunit of electrical channels and a regulator of gene transcription). This means that the known phenotypes of the lh mutant may be attributable to changes in calcium channel properties, modified transcriptional regulation, as yet unknown actions of β4, or secondary consequences of mutation.

Our present findings that both the β4 subunit and CaV2.1 are expressed in the kidney (Supplemental Fig. 3), that the T-wave is elevated in lh-mutant ECG recordings, and that the lh mutant has an increased plasma potassium level (Fig. 2) may suggest that calcium channels are involved in renal function, perhaps including calcium reabsorption.

The autonomic nervous system, including both the sympathetic and parasympathetic pathways, is an essential regulator of the circulatory system. Calcium influx across plasma membranes augments cytosolic free calcium levels, which facilitate neurotransmitter release at synaptic termini [25]. Several types of channel occur together in a single neuron and contribute to the regulation of neuronal activity. Hong and Chang reported that N-type channels made a major contribution, and P/Q-type channels made a minor contribution, to sympathetic neural responses [25]. Previously, we examined mice deficient in both β3 and CaV2.2, two major subunits of N-type channels, and β3-overexpressing mice [19,26]. We identified a major role for N-type calcium channels in sympathetic nerve regulation. Additionally, we demonstrated that P/Q-type channels played a significant role in regulating the parasympathetic nervous system of Tg^{rol} mice [16]. These animals exhibit significantly reduced (ca. 40%) P-type calcium channel currents [18]. Additionally, the currents are shifted in terms of voltage-dependent inactivation.

As the β4 subunit is strongly expressed in Purkinje cells and has a high affinity for CaV2.1 [21], β4 should significantly influence the P/Q-type channel properties of such cells. However, Burgess et al. reported a reshuffling of calcium channel subunit combinations and conserved P-type calcium channel currents in lh Purkinje cells [27], inconsistent with the data of our present study. However, we speculate that interpretation of the data of Burgess et al. may be difficult. Thus, lh mutants apparently exhibited cerebellar ataxia, probably associated with electrophysiological cell excitability in the cerebellum. Electrophysiological evaluation using the patch-clamp technique focuses principally on calcium channels of the soma, not those of dendrites. However, dendrites are more important in terms of neural signal transmission. Further studies are needed to explore these findings in more detail.

In conclusion, we analyzed the phenotypes of lh-mutant mice. ECG analysis showed that the lh mutant had a high T-wave, which may be associated with hyperkalemia. Upon pharmacological ECG analysis, 2–3-week-old lh mutants responded less to a β-blocker and a muscarinic receptor antagonist than did wild-type mice. HRV analysis revealed that the R–R interval of the lh mutant was

unstable, indicating that both sympathetic and parasympathetic nerve tonus was modified. Our results strongly suggest that the β4 subunit is involved in various physiological functions, including thymus development, the neural functioning of Purkinje cells, and autonomic nerve regulation within the heart.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.112.

Transparency document

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