Comparative Medicine
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Vol 54, No 6
December 2004
Pages 690-694

Effect of Genetic Background on Ca_v2 Channel α_1 and β Subunit Messenger RNA Expression in Cerebellum of N-Type Ca^{2+} Channel α_{1B} Subunit-Deficient Mice

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Although the N-type Ca^{2+} channel plays a role in a variety of neuronal functions, N-type Ca^{2+} channel α_{1B}-deficient mice exhibit normal life span without apparent behavioral or histologic abnormalities. To examine whether the reason for their normal behavior is compensation by other Ca_v2 channel α_1 or β subunit genes and to analyze whether genetic background influences the subunit expression pattern, we studied the α_{1A}, α_{1E}, β_{1b}, β_2, β_3 and β_4 subunit mRNA levels in cerebellum of α_{1B}-deficient mice with CBA × C57BL/6 or CBA/JN background. In cerebellum of the mice with a CBA × C57BL/6 background, α_{1A} mRNA was expressed at a higher level than that in wild-type or heterozygous mice, but difference in the expression levels of α_{1E}, β_{1b}, β_2, β_3, and β_4 subunits was not found among wild-type, heterozygous, and homozygous mice. In cerebellum of α_{1B}-deficient mice with CBA/JN background, β_4 mRNA was expressed at a higher level than that in wild-type or heterozygous mice, but α_{1A}, α_{1E}, β_{1b}, α_2, and β_3 transcripts were expressed at similar levels in all genotypes. Therefore, a possible explanation of the normal behavior of α_{1B}-deficient mice is that Ca_v2 channel family members compensate for the deficiency, and that the change of functional subunit expression pattern for compensation differs in animals with different genetic backgrounds.

The Ca^{2+} channel α_{1B} subunit is a pore-forming component capable of generating N-type Ca^{2+} channel activity (1, 15, 20). Although the N-type Ca^{2+} channel plays a role in a variety of neuronal functions, α_{1B}-deficient mice have drastically reduced baroreflex responses and increased arterial blood pressure and heart rate, but have normal life span without apparent behavioral abnormality (4), presumably owing to compensation by other Ca^{2+} channels. Gene families are thought to evolve from common ancestors as a means of providing more complex functions, often involving diverse gene- and tissue-specific expression mechanisms. Many gene-deficient mice have been generated by use of homologous recombination methods, and in some instances, a deficiency expected to be lethal is not, or the mice manifest normal behavior, suggesting development of compensation (i.e., another gene within the family is functional in place of the knocked-out gene). For example, in cyclooxygenase-1 (COX-1)-deficient mice, COX-2 compensation develops in the uterus during the pre-implantation period (12).

The N-type Ca^{2+} channel is one of the high voltage-sensitive Ca_v2 channels that include P/Q ($Ca_v2.1$)-, N ($Ca_v2.2$)-, and R ($Ca_v2.3$)-type channels (2). The N-type Ca^{2+} channel α_{1B}-deficient mice should be a useful model for the study of compensation by other genes within the Ca_v2 channel family. The Ca_v2 channel is composed of at least three subunits, α_1, α_2-δ, and β; the α_1 subunit is a pore-forming component, which also functions as the voltage-sensor and is capable of generating Ca^{2+} channel activity in heterogeneous expression systems (7). Results of molecular cloning studies have indicated that the α_{1A}, α_{1B}, and α_{1E} genes encode the α_1 subunits of the ω-agatoxin-IVA (ω-Åga-IVA)-sensitive P/Q-type channel, the ω-conotoxin-GVIA (ω-CgTx-GVIA)-sensitive N-type channel, and the dihydropyridine/ω-Aga-IVA/ω-CgTx-GVIA (DHP/ω-Aga-IVA/ω-CgTx-GVIA)-insensitive R-type channel, respectively (8, 9, 20). The α_2-δ subunit is encoded by a single gene, whereas four β subunit genes have been identified (1, 3). It has been reported that brain cells express β_{1b}, β_2, β_3, and β_4 subunit genes (6). Results of expression studies using the various cloned subunits indicated that each α_1 subunit reconstitutes with each β subunit to yield a functional channel (7-9). This discriminatory channel complex formation was supported by the finding that a highly conserved region of the linker of the I–II loop of all α_1 subunits interacts with each β subunit (11). To examine the hypothesis that the reason for the normal behavior of α_{1B}-deficient mice is compensation by other Ca_v2 channel α_1 or/and β subunit genes, we planned to examine the mRNA expression pattern of these genes in cerebellum of α_{1B}-deficient mice.

In the case of angiotensinogen-deficient mice, all mutants on a C57BL/6J background, but not on a C57BL/6 × CBA × ICR background, died at the neonatal stage (14). Mice deficient in homeobox11L1 (Hox11L1) had complete penetrance of intestinal pseudo-obstruction on a C57BL/6J background and low penetrance on a 129/SvJ background (10). Those reports suggest that changing the genetic background of gene-deficient mice is important for investigating the function of a specific gene product and compensatory mechanisms. For production of N-type Ca^{2+} channel α_{1B}-deficient mice, a germ line chimera was de-

Received: 6/10/04. Revision requested: 7/20/04. Accepted: 7/26/04.
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rived from TT2 embryonic stem (ES) cells with a targeted mutation of the α_{1B} subunit gene. The TT2 ES cells were established from an F1 embryo produced between CBA and C57BL/6 strains (16). In our previous study, we used a mixed genetic background (CBA × C57BL/6) to analyze α_{1B}-deficient mice (4).

In the study reported here, we developed N-type Ca^{2+} channel α_{1B}-deficient mice with a CBA/JN background, and examined the mRNA expression pattern of α_{1A}, α_{1B}, α_{1E}, β_{1b}, β_2, β_3, and β_4 subunit genes in cerebellum of α_{1B}-deficient mice with a CBA × C57BL/6 or a CBA/JN background.

Materials and Methods

Animals. All experiments conformed to the Japanese regulations for animal care and use, following the Guideline for Animal Experimentation of the Japanese Association for Laboratory of Animal Science (5), and were approved by the Animal Care and Use Committee of Eisai Co., Ltd. To generate α_{1B}-deficient mice, the targeting vector was electroporated into TT2 ES cells derived from an F1 embryo produced between CBA and C57BL/6 strains (16). Gene-targeted ES cell clones were injected into fertilized eggs at the eight-cell stage of Crj:CD-1 (ICR) mice (Charles River, Tokyo, Japan). Chimeric male mice were mated with CBA/JNCrj (CBA/JN) (Charles River) female mice. Heterozygous (+/-) α_{1B}-deficient male mice were backcrossed to CBA/JN female mice. The backcrossing was continued to the N10 generation. At the N3 generation, heterozygous (+/-) α_{1B}-deficient females were mated to CBA/JN males to introduce chromosome Y into a congenic strain. At the N10 generation, brother-sister heterozygous (+/-) α_{1B}-deficient mouse matings were conducted to obtain wild-type (+/+), heterozygous (+/-), and homozygous (-/-) α_{1B}-deficient mice with a CBA/JN background. All mice were kept in specific-pathogen-free facilities that were free of the following microorganisms: *Pseudomonas aeruginosa, Escherichia coli* O-115a*, Salmonella typhimurium, Citrobacter rodentium, Clostridium piliforme, Pasteurella pneumotropica, Corynebacterium kutscheri, Bordetella bronchiseptica, Staphylococcus pneumoniae, S. aureus, Mycoplasma pulmonis, Syphacia muris, Giardia muris, Spironucleus muris,* Sendai virus, mouse hepatitis virus, and Tyzzer's organism.

Mice were housed in groups of five male, five female, a female/male pair, or a male/female pair of pups in 31 × 23 × 15.5-cm plastic shoebox cages (M-55-TG, Okazaki Sangyo Co. Saitama, Japan) that were maintained in a laminar flow rack (LFR-A-2, Tokiwa Kagaku Kikai Co., Tokyo, Japan) in the animal facilities. In our facilities, 10 mice are selected randomly from a laminar flow rack and are monitored once a month for the aforementioned microorganisms. The environment was maintained at constant temperature (22 ± 2°C) and humidity (55 ± 5%), with a 12:12-h light:dark cycle (lights on at 6:30 a.m.) and 15 fresh air changes hourly. Commercial diet (CE-2, Nippon CLEA, Inc., Tokyo, Japan) and tap water were available ad libitum, and the bedding (Paper Clean, Japan SLC, Shizuoka, Japan) was changed once a week. Mice had ad libitum access to food and water.

Determination of genetic profile. To determine the genetic profile, the following microsatellite DNA markers were selected: *D1Mit1* (8.7), *D1Mit318* (18.5), *D1Mit5* (32.8), *D1Mit21* (32.8), *D1Mit181* (42.0), *D1Mit10* (56.6), *D1Mit194* (71.5), *D1Mit356* (95.8), *D2Mit1* (1.0), *D2Mit7* (28.0), *D2Mit43* (47.5), *D2Mit107* (75.6), *D2Mit226* (96.0), *D3Mit60* (0.0), *D3Mit239* (16.5), *D3Mit40* (39.7), *D3Mit14* (64.1), *D3Mt44* (78.5), *D3Mit19* (87.6), *D4Mit149* (0.0), D4Mit39 (10.6), *D4Mit53* (19.8), *D4Mit27* (42.5), *D4Mit76* (55.7), *D4Mit33* (79.0), *D5Mit123* (1.0), *D5Mit13* (20.0), *D5Mit18* (45.0), *D5Mit24* (60.0), *D5Mit216* (72.0), *D6Mit1* (2.8), *D6Mit268* (25.5), *D6Mit29* (36.5), *D6Mit39* (46.3), *D6Mit59* (67.0), *D6Mit15* (74.0), *D7Mit178* (0.5), *D7Mit294* (8.0), *D7Mit82* (25.0), *D7Mit148* (46.4), *D7Mit259* (72.0), *D8Mit64* (16.0), *D8Mit64* (16.0), *D8Mit178* (30.0), *D8Mit33* (45.0), *D8Mit88* (58.0), *D8Mit56* (73.0), *D9Mit2* (17.0), *D9Mit27* (29.0), *D9Mit10* (49.0), *D9Mit18* (71.0), *D10Mit75* (2.0), *D10Mit80* (4.0), *D10Mit3* (21.0), *D10Mit115* (38.4), *D10Mit10* (51.0), *D10Mit233* (62.0), *D11Mit78* (2.0), *D11Mit51* (18.0), *D11Mit70* (54.0), *D11Mit103* (76.0), *D12Mit37* (1.0), *D12Mit34* (29.0), *D12Mit5* (37.0), *D12Mit79* (53.0), *D13Mit57* (9.0), *D13Mit64* (30.0), *D13Mit26* (38.0), *D13Mit53* (62.0), *D13Mit35* (75.0), *Plau* (2.5), *D14Mit62* (18.5), *D14Mit7* (44.5), *D14Mit77* (60.0), *D15Mit8* (19.7), *D15Mit17* (32.0), *D15Mit2* (46.9), *D15Mit79* (66.2), *D16Mit129* (3.4), *D16Mit4* (27.3), *D16Mit47* (43.0), *D16Mit6* (63.2), *D17Mit23* (17.0), *D17Mit34* (18.8), *D17Mit50* (23.2), *D17Mit7* (32.3), *D17Mit93* (44.5), *D17Mit221* (56.7), *D18Mit164* (2.0), *D18Mit17* (20.0), *D18Mit40* (37.0), *D18Mit7* (50.0), *D18Mit144* (57.0), *D19Mit41* (16.0), *D19Mit19* (26.0), *D19Mit100* (27.0), *D19Mit34* (53.0), *DXMit22* (17.0), *DXMit60* (30.8), *DXMit38* (58.2), and *DXMit223* (73.3). Microsatellite DNA markers, as simple sequence length polymorphisms, were amplified by use of polymerase chain reaction (PCR) analysis of the DNA from tail biopsy specimens of 4-month-old mice, followed by agarose gel electrophoresis to determine the genotype of markers. Primer sets for microsatellite DNA markers were purchased from Research Genetics, Inc. (Invitrogen, Carlsbad, Calif.), and PCR analysis was conducted according to the manufacturer's protocol. We performed genetic monitoring of (+/-) α_{1B}-deficient mice at the N10 generation of the CBA/JN backcross line (female, male, n = 2).

Identification of genotype of α_{1B}-deficient mice. The primers and PCR program used for the screening of α_{1B} wild-type (+/+), heterozygous (+/-), or homozygous (-/-) genotype have been described (4). Genotypes were identified by use of PCR analysis of the DNA of tail biopsy specimens from 6-week-old mice.

Histologic analysis. The cerebellum from each 26- to 28-week-old mouse—(+/+), female = 4, male = 4; (+/-), female = 4, male = 4; and (-/-), female = 4, male = 4—was formalin fixed and paraffin embedded. Paraffin sections, 4 μ thick, were stained with hematoxylin and eosin as described (4).

Real-time RT-PCR analysis. Total RNA from the cerebellum of the aforementioned 26- to 28-week-old mice was isolated using the manual provided with the RNA extraction kit (TRlzol Reagent, Gibco-BRL, Md.). For quantification of α_{1A}, α_{1B}, α_{1E}, β_{1b}, β_2, β_3, and β_4 subunit gene expression levels, the real-time quantitative RT-PCR method was performed in duplicate assays using an ABI7700 Sequence Detection system (PE Applied Biosystems, Foster City, Calif.). The cerebellums from multiple animals were not pooled for each RT-PCR run. First-strand cDNA was synthesized with 1 μg of total RNA, using the TaqMan Reverse Transcription Reagent system (PE Applied Biosystems). The reaction conditions for RCR analysis were: one cycle of 94°C for 10 min, plus 40 cycles of 95°C for 15 sec, and 60°C for 1 min. The amount of α_{1A}, α_{1B}, α_{1E}, β_{1b}, β_2, β_3, and β_4 subunit genes was determined by use of Assays-on-Demand Products system (PE Applied Biosystems). To confirm equivalent loading, the amount of 18S ribosomal RNA (rRNA) in each

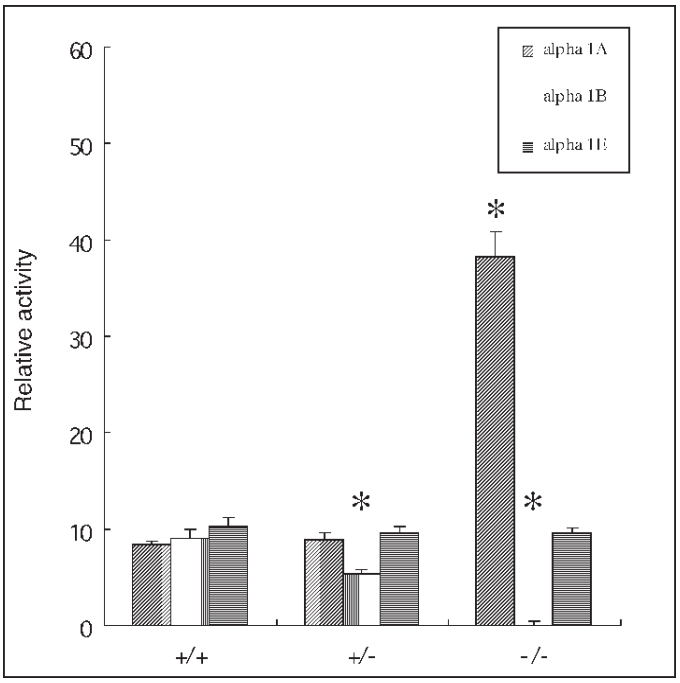

Figure 1. Real-time PCR analysis of α_{1A}, α_{1B}, and α_{1E} subunit genes in the cerebellum of wild-type (+/+), heterozygous (+/-), or homozygous (-/-) α_{1B}-deficient male mice with a CBA × C57BL/6 background; (+/+), (+/-), (-/-): n = 4. The relative amount of mRNA was expressed as the ratio to 18S ribosomal RNA. Data are reported as mean ± SD from three independent experiments. Asterisk indicates significant ($P < 0.05$) differences compared with values for (+/+) mice.

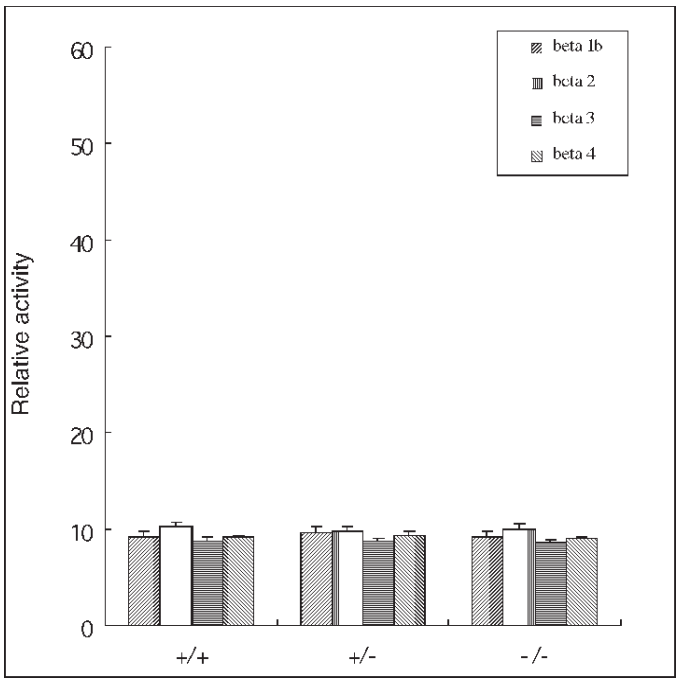

Figure 2. Real-time PCR analysis of β_{1b}, β_2, β_3, and α_4 subunit genes in the cerebellum of (+/+), (+/-), or (-/-) α_{1B}-deficient male mice with a CBA × C57BL/6 background. *See* Fig. 1 for key.

sample was determined using the standard primers and Taqman probe (PE Applied Biosystems). The mRNA level of each sample was expressed as its ratio to 18S rRNA.

Analysis of data. Data are presented as mean ± SD from three independent experiments. Statistical significance was determined by use of analysis of variance, followed by the Dunnett test; a value of $P < 0.05$ was considered significant.

Results

Genetic profile. To evaluate whether the genetic background of the offspring from the germline chimera had been converted to CBA/JN, we performed genetic monitoring using microsatellite DNA markers. Use of all markers revealed CBA/JN genetic types in heterozygous α_{1B}-deficient mice at the N10 generation of the CBA/JN backcross line (data not shown). The results indicated that we had succeeded in developing a congenic strain. All mice were free of apparent behavior defects.

Expression analysis of α_1 and β subunit mRNAs in α_{1B}-deficient mice. To examine the hypothesis that the reason for the normal behavior of N-type Ca^{2+} channel α_{1B}-deficient mice is compensation by other Ca_v2 channel α_1 or/and β subunits, and to analyze whether genetic background influences the gene expression, we studied the expression pattern of α_{1A}, α_{1B}, α_{1E}, β_{1b}, β_2, β_3, and β_4 subunit genes in the cerebellum of the mice with a CBA × C57BL/6 or a CBA/JN background. The mouse genotypes were identified by use of PCR analysis of the DNA in tail biopsy specimens (data not shown). In the cerebellum from a mouse with a CBA × C57BL/6 background, α_{1A} mRNA was expressed at a higher level than that from wild-type or heterozygous mice, but difference in the expression levels of α_{1E}, β_{1b}, β_2, β_3 and β_4 subunits was not found in wild-type, heterozygous, and homozygous mice (Fig. 1 and Fig. 2). In the cerebellum from a mouse with a CBA/JN background, β_4 mRNA was expressed at a higher level than that in wild-type or heterozygous mice, but the α_{1A}, α_{1E}, β_{1b}, β_2, and β_3 transcripts were expressed at similar levels irrespective of genotype (Fig. 3 and Fig. 4). In the cerebellum from mice with CBA × C57BL/6 and CBA/JN backgrounds, heterozygous mice expressed α_{1B} mRNA at a low level compared with that in wild-type mice, but α_{1B} mRNA could not be detected in homozygous mice (Fig. 1 and Fig. 3). Difference in the expression patterns of the transgenes was not observed between males and females in any of the mouse lines (data not shown).

Histologic analysis. Using PCR analysis of DNA in tail biopsy specimens, mouse genotypes were identified (data not shown). All α_{1B}-deficient mice were free of apparent behavior defects and anatomic-histologic changes in the cerebellum (data not shown).

Discussion

We have here documented that α_{1A} subunit gene expression is up-regulated in the cerebellum of α_{1B}-deficient mice with a CBA × C57BL/6 background, whereas α_{1E} transcripts were expressed at similar levels in (+/+), (+/-), and (-/-) mice. Thus, one reason for the normal behavior of N-type α_{1B}-deficient mice is considered to be functional compensation via increased expression of the α_{1A} subunit gene in cerebellar cells. It is reasonable that the α_{1A}, but not the α_{1E} subunit can compensate for deficiency of N-type Ca^{2+} channel function in α_{1B}-deficient mice, because it has been reported that P/Q-type α_{1A} and N-type α_{1B} subunits tend to be concentrated in nerve terminals for the release of neurotransmitters (18, 19) whereas the α_{1E} subunit is located mainly in cell bodies (17). On the other hand, in the cerebellum of α_{1B}-deficient mice with a CBA/JN background, which also exhibit normal behavior,

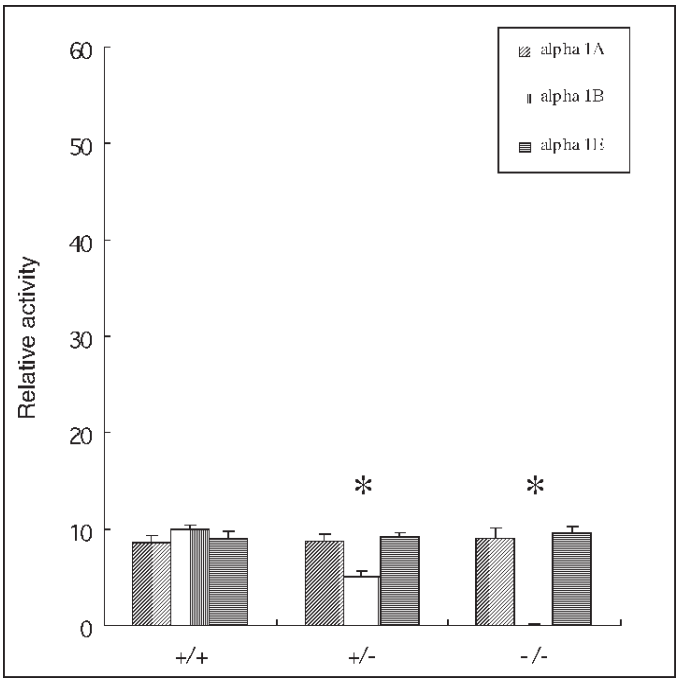

Figure 3. Real-time PCR analysis of α_{1A}, α_{1B} and α_{1E} subunit genes in the cerebellum of (+/+), (+/-), or (-/-) α_{1B}-deficient male mice with a CBA/JN background. S*ee* Fig. 1 for key.

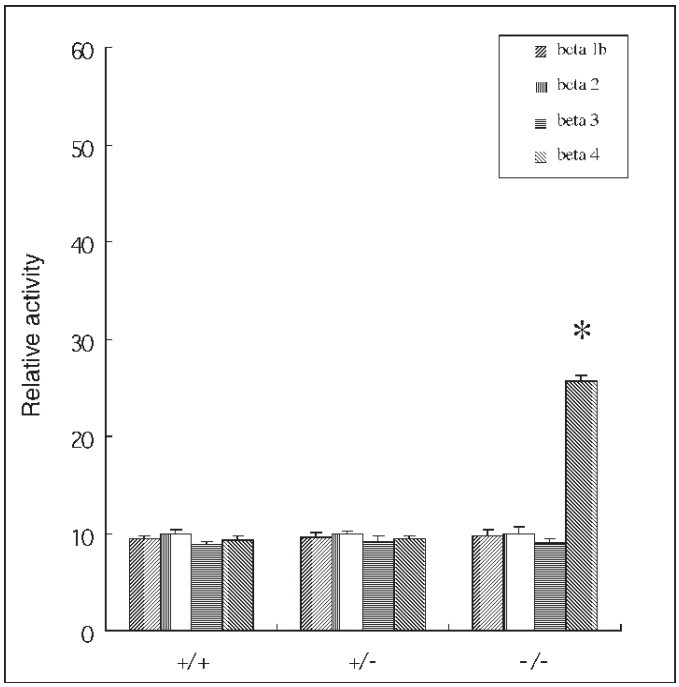

Figure 4. Real-time PCR analysis of β_{1b}, β_2, β_3, and β_4 subunit genes in the cerebellum of (+/+), (+/-), or (-/-) α_{1B}-deficient male mice with a CBA/JN background. *See* Fig. 1 for key.

the transcript of α_{1A} subunit gene was expressed at similar levels among the (+/+), (+/-), and (-/-) genotypes, whereas the β_4 subunit gene was up-regulated in (-/-) compared with (+/+) and (+/-) mice. These results suggest that genetic background influences the nature of the alteration of gene expression pattern that compensates for lack of N-type Ca^{2+} function. It has been reported that the α_{1B}/α_2-δ subunits of the N-type channel complex were immunoprecipitated together with the β_3 and β_4 subunits (13), and the α_{1A}/α_2-δ subunits of the P/Q-type channel complex were immunoprecipitated together with the $\beta_{1b,}$ $\beta_{2,}$ β_3 and β_4 subunits (6). This suggests that the β_3 or β_4 subunit is the most likely candidate molecule among β subunits to compensate for lack of N-type Ca^{2+} channel function in α_{1B}-deficient mice. Thus, in the study reported here, we detected increased expression of α_{1A} subunit in the cerebellum of α_{1B}-deficient mice with a CBA × C57BL/6 background, but increased expression of β_4 subunit in those with a CBA/JN background. These changes appear to be unrelated to each other. Nevertheless, although we should examine the Ca^{2+} current by means of an electrophysiologic study, these changes might reasonably compensate for lack of N-type Ca^{2+} channel function, because it has been reported that the P/Q-type Ca^{2+} channel α_{1A} subunit is targeted to presynaptic terminals by the β_4 subunit (21). That is, the increased expression of β_4 subunit could have the functional effect of the increasing the surface expression of the α_{1A} subunit. Our results suggest that the P/Q-type Ca^{2+} channel seems to have a compensatory role in the cerebellum of N-type Ca^{2+} channel α_{1B}-deficient mice.

It has been reported that gene expression is influenced by genetic background; for example, in Hox11L1-lacZ transgenic mice carrying putative Hox11L1 regulatory sequences fused to the *E. coli lacZ* reporter gene, lacZ expression was detected in mice with a C57BL/6 background, but not in those with a 129/SvJ background (10). Gene expression is thought to be achieved through positive regulatory mechanisms in which *trans*-acting factors enhance basal transcription activity and negative regulatory mechanisms repress transcription activity. The concerted action of positive and negative regulatory mechanisms is thought to be essential to provide complex regulation and flexibility in gene expression. In cerebellar cells of α_{1B}-deficient mice derived from a CBA × C57BL/6 background, it was suggested that the expression-regulatory region of the α_{1A} subunit gene contains enhancer *cis*-elements and that some *trans*-regulatory factors for enhancer expression is up-regulated. On the other hand, in cerebellar cells of α_{1B}-deficient mice derived from a CBA/JN background, up-regulated expression of the β_4 subunit gene, not the α_{1A} subunit gene seems to be the dominant mechanism. These results indicate that the use of gene-deficient mice with different genetic backgrounds is important for investigating the relationships among the function of a knocked-out gene product, the contribution of compensatory gene products such as *trans*-regulatory factors, and the phenotype of gene-deficient mice.

Further studies, including identification of *cis*-elements and *trans*-regulatory factors for enhancer expression of α_{1A} and β_4 subunit genes, examination of cell type-specific gene expression patterns, using transcriptome and proteome analyses, and pharmacologic analysis of neurotransmitter release, will be necessary to elucidate the molecular mechanisms of compensation that lead to normal behavior in α_{1B}-deficient mice.

Acknowledgments

We thank Norio Akaogi, Michiyo Shimada, Jiro Sonoda, and Satoru Hosokawa for assistance with histologic analysis, and Norimasa Miyamoto for generation of the congenic α_{1B}-deficient mouse strain.

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