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Enhanced Expression of Ca^{2+} Channel α_{1A} and β_4 Subunits and Phosphorylated Tyrosine Hydroxylase in the Adrenal Gland of N-type Ca^{2+} Channel α_{1B} Subunit-deficient Mice with a CBA/JN Genetic Background

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Electrophysiologic studies have demonstrated that adrenal medulla chromaffin cells express voltage-dependent P/Q-, N-, L-, and R-type Ca^{2+} channels and that these channels regulate release of norepinephrine and epinephrine. However, N-type Ca^{2+} channel α_{1B}-deficient mice with a CBA/JN background show normal plasma norepinephrine and epinephrine levels, presumably owing to compensation by other gene(s). To examine the expression patterns of the P/Q-type α_{1A}, L-type α_{1C}/α_{1D}, and R-type α_{1E}, β_1, β_2, β_3, and β_4 subunits, as well as of tyrosine hydroxylase (Th), dopamine β hydroxylase (Dbh), and phenylethanolamine-N-methyltransferase (Pnmt) in the adrenal gland of α_{1B}-deficient mice, we used real-time quantitative reverse transcription–polymerase chain reaction and Western blot analyses. The expression levels of α_{1A}, β_4, Th, and Th phosphorylated at serine 40 were higher in homozygous mice than in wild-type and heterozygous mice, but the expression levels of α_{1C}, α_{1D}, α_{1E}, β_1, β_2, β_3, Dbh, and Pnmt did not differ among wild-type, heterozygous, and homozygous mice. These results suggest that the compensatory mechanisms to maintain normal levels of epinephrine and norepinephrine in the adrenal gland of N-type Ca^{2+} channel α_{1B}-deficient mice include increased expression of α_{1A} and β_4 subunits and increased catecholamine biosynthetic activity.

Abbreviations: Dbh, dopamine β hydroxylase; PCR, polymerase chain reaction; Pnmt, phenylethanolamine-N-methyltransferase; pTh, typroxine hydroxylase phosphorylated at serine 40; RT-PCR, reverse transcription–polymerase chain reaction; SD, standard deviation; SDS, sodium dodecyl sulfate; Th, tyrosine hydroxylase

The voltage-dependent Ca^{2+} channel α_{1B} subunit generates N-type Ca^{2+} channel activity.[31] Although the N-type Ca^{2+} channel is localized in the plasma membrane and is essential for neurotransmitter release,[3] N-type Ca^{2+} channel α_{1B}-deficient mice live for more than a year without apparently abnormal behavior.[6] To date, many gene-deficient mice have been generated by using homologous recombination methods and, in some cases, a deficiency expected to be lethal is not, or the mice show normal behavior. For example, in low-density lipoprotein receptor-related protein 1b (LRP1b)-deficient mice, LRP1 compensation occurs.[11] In such compensation, another gene within the same family may be functional in place of the knocked-out gene, because gene families are thought to have evolved from common ancestors as a means of providing more complex functions, often involving diverse gene- and tissue-specific expression mechanisms.

N-type Ca^{2+} channel α_{1B}-deficient mice likely will be a useful model for the study of compensation by other Ca^{2+} channel family members. Structurally, the Ca^{2+} channels are composed of at least 3 subunits, the α_1, α_2-δ, and β subunits.[27] The α_1 subunit is a pore-forming component, functions as a voltage-sensor, and is capable of generating Ca^{2+} channel activity in heterogeneous expression systems.[3] Molecular cloning studies have revealed that the α_{1C} ($Ca_v1.2$), α_{1D} ($Ca_v1.3$), α_{1A} ($Ca_v2.1$), α_{1B} ($Ca_v2.2$) and α_{1E} ($Ca_v2.3$) genes encode the α_1 subunits of the dihydropyridine-sensitive L-type channel, ω-agatoxin- IVA-sensitive P/Q-type channel, ω-conotoxin-GVIA -sensitive N-type channel, and dihydropyridine/ω-agatoxin-IVA /ω-conotoxin-GVIA-insensitive R-type channel, respectively.[4,12,13,16,31] Expression studies with the various cloned subunits showed that each α_1 subunit reconstitutes with each β subunit to yield a functional channel.[17] This result of discriminatory channel complex formation is 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.[17]

Electrophysiologic studies have demonstrated that adrenal medulla chromaffin cells express L-, P/Q-, N-, and R-type channels[1] and that these channels regulate release of catecholamine with exocytotic machinery.[19,28] However, N-type Ca^{2+} channel α_{1B}-deficient mice with a CBA × C57BL/6 genetic background

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showed normal plasma norepinephrine levels,[6] suggesting that compensation by other Ca^{2+} channel α_1 or β subunit gene(s) or both might occur in adrenal gland to maintain normal circulating levels of norepinephrine. Indeed, in our previous study, we showed that P/Q-type Ca^{2+} channel α_{1A} subunit gene expression is upregulated in the adrenal gland of N-type α_{1B}-deficient mice with a CBA × C57BL/6 genetic background.[25] In the case of angiotensinogen-deficient mice, all mutants died at the neonatal stage if they had a C57BL/6J background but not if they had a C57BL/6 × CBA × ICR background.[22] This report suggests that changing the genetic background of gene-deficient mice is important for investigating the function of a specific gene product and compensatory mechanisms. Catecholamine neurotransmitters are synthesized in adrenal medulla chromaffin cells from tyrosine—via dopa, dopamine, and norepinephrine—to epinephrine.[14] To control the levels of norepinephrine and epinephrine in the absence of N-type Ca^{2+} channel function, changes might occur in the gene expression pattern of catecholamine-biosynthesizing enzymes, including tyrosine hydroxylase (Th), dopamine beta-hydroxylase (Dbh), and phenylethanolamine N-methyltransferase (Pnmt).

In this study, to investigate the influence of genetic background on specific gene expression and of lack of N-type Ca^{2+} channel function on the catecholamine biosynthesis pathway, we examined the circulating levels of norepinephrine and epinephrine and studied the expression patterns of α_{1A}, α_{1C}, α_{1D}, α_{1E}, β_1, β_2, β_3, and β_4 subunits; Th; Dbh; and Pnmt in the adrenal gland of N-type Ca^{2+} channel α_{1B}-deficient mice with a CBA/JN background.

Materials and Methods

Animals. All animal procedures conformed to Japanese regulations on animal care and use, following the Guideline for Animal Experimentation of the Japanese Association for Laboratory Animal Science,[7] and were approved by the Animal Care and Use Committee of Eisai. Mice with a nonfunctional α_{1B} subunit of the N-type Ca^{2+} channel had previously been generated by gene-targeting methods by using a TT2 embryonic stem cell line derived from an F1 embryo of CBA and C57BL/6.[6] In a previous study, we examined plasma levels of norepinephrine[6] and expression patterns of α_{1A}, α_{1C}, α_{1D}, α_{1E}, β_1, β_2, β_3 and β_4 subunits on a CBA × C57BL/6 genetic background.[25] For the present experiments, α_{1B}-deficient mice were backcrossed onto a CBA/JNCrj (CBA/JN; Charles River Japan, Tokyo, Japan) genetic background.[24] The backcrossing was continued to the N15 generation. At the N3 generation, heterozygous (+/–) α_{1B}-deficient female mice were mated to CBA/JN males to introduce chromosome Y into a congenic strain. At the N15 generation, brother–sister matings of heterozygous (+/–) α_{1B}-deficient mice were established to obtain wild-type (+/+), heterozygous (+/–) and homozygous (–/–) α_{1B}-deficient mice with a CBA/JN background.[24] All mice were kept in specific pathogen-free (SPF) facilities that were free of the following microorganisms: *Pseudomonas aeruginosa*, *Escherichia coli o-115a*, *Salmonella typhimurium*, *Citrobacter rodentium*, *Clostridium piliforme*, *Pasteurella pneumotropica*, *Corynebacterium kutsheri*, *Bordetella bronchiseptica*, *Staphylococcus pneumoniae*, *Staphylococcus aureus*, *Mycoplasma pulmonis*, *Syphacia muris*, *Giardia muris*, *Spironucleus muris*, Sendai virus, mouse hepatitis virus, and *Bacillus piliformis*. The mice were housed in groups of 5 males; 5 females; 1 female–male pair; or sire, dam, and pups in 31 × 23 × 15.5-cm plastic shoebox cages (M-55-TG, Okazaki Sangyo, Saitama, Japan), which were maintained in laminar-flow racks (LFR-A-2, Tokiwa Kagaku Kikai, Tokyo, Japan) in the animal facilities. In our facilities, 10 mice are selected randomly once monthly from a laminar-flow rack and are monitored for the microorganisms described earlier. The animal facilities were maintained at constant temperature (22 ± 2 °C) and humidity (55% ± 5%), with a 12:12-h light:dark cycle (lights on, 0630) and 15 fresh-air changes hourly. Commercial diet (CE-2, Nippon CLEA, Tokyo, Japan) and tap water were available ad libitum, and bedding (Paper Clean, Japan SLC, Shizuoka, Japan) was changed once weekly.

Identification of genotype of α_{1B}-deficient mice. The primers and PCR program used for the screening of α_{1B} wild-type (+/+), heterozygous (+/–), and homozygous (–/–) genotypes were described previously.[6] Genotypes were identified by PCR from the DNA of tail biopsies of 6-wk-old mice.

Histologic analysis. Adrenal glands of 10-wk-old wild-type, heterozygous, and homozygous mice (4 females and 4 males of each genotype) were formalin-fixed and paraffin-embedded. Each paraffin section, 4 µm thick, was stained with hematoxylin and eosin as described previously.[6]

Determination of plasma catecholamine level. For assay of plasma catecholamine levels, blood samples were collected from 10-wk-old wild-type, heterozygous, and homozygous mice (6 females and 6 males of each genotype) anesthetized with intraperitoneal injection of 1.5 g/kg urethane (Nacalai Tesque, Kyoto, Japan). At 30 min after induction of anesthesia, 800 µl blood was drawn from the left atrium, and norepinephrine and epinephrine levels were measured using CatCombi ELISA kit (Immuno-Biological Labs, Gunnma, Japan) according to the manufacturer's instructions.

Real-time reverse transcription–polymerase chain reaction (RT-PCR). Total RNA from the adrenal gland of 10-wk-old wild-type, heterozygous, and homozygous mice (6 females and 6 males of each genotype) was isolated according to manufacturer's instructions (Isogen Solution, Nippon Gene, Tokyo, Japan). Real-time RT-PCR for quantification of gene expression was performed using an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA).[9] The reaction mixtures contained the primers and Taqman probes for the genes for the α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E}, β_1, β_2, β_3, and β_4 subunits; Th, Dbh, and Pnmt (Assays-on-Demand Products, PE Applied Biosystems), together with 10 ng total RNA, PCR buffer, 5.5 mM $MgCl_2$, 0.3 mM of each dNTP, 5 U RNAse inhibitor, 12.5 U murine leukemia virus reverse transcriptase, and 1.5 U Ampli*Taq* Gold DNA polymerase (PE Applied Biosystems). The conditions for PCR amplification were: 48 °C for 30 min for RT, and then 1 cycle of 94 °C for 10 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min, according to the manufacturer's protocol. To confirm correct amplification of each subunit gene, PCR products were sequenced. The amount of 18S rRNA in each sample was used for an internal standard to confirm equivalent loading, by using standard primers and Taqman probe (PE Applied Biosystems). The mRNA level of each sample was expressed relative to that of 18S rRNA. Data are presented as mean ± standard deviation (SD) of 3 independent experiments. Statistical significance was determined by analysis of variance followed by Dunnett tests, and P values of less than 0.05 were considered to be significant. Statistical analysis was conducted using the software package SAS 8.1 (SAS Institute Japan, Tokyo, Japan).

Western blot analysis. Western blot analysis was performed to measure the protein levels of the α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E}, β_1, β_2, β_3 and β_4 subunits; Dbh; Pnmt; Th; and Th phosphorylated at serine 40 (pTh) in the adrenal gland of 10-wk-old wild-type, heterozygous, and homozygous (8 females and 8 males of each genotype)

Table 1. Plasma levels of norepinephrine and epinephrine of α1B-deficient mice

Genotype	Norepinephrine (pg/ml)	Epinephrine (pg/ml)
Wild-type (+/+)	780 ± 169	330 ± 80
Heterozygous (+/–)	756 ± 145	325 ± 73
Homozygous (–/–)	695 ± 170	290 ± 85

mice. Adrenal glands were dissected on ice and homogenized in ice-cold buffer containing 10 mM Tris-HCl-buffered 0.32 M sucrose (pH 7.4), 1 mM potassium ethylene glycol bis (2-aminoethyl ether)-N,N,N',N'-tetraacetic acid, and protease inhibitors (1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 µg/ml aprotinin, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml benzamidine, and 8 µg/ml each of calpain inhibitors I and II; Wako Pure Chemical Industries, Osaka, Japan).

For the analysis of the α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E}, β_1, β_2, β_3, and β_4 subunits, protein samples (50 µg/lane) were electrophoresed in a 6% sodium dodecyl sulfate (SDS)–polyacrylamide gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane (BA83, Schleicher and Schuell, Keene, NH) in a buffer containing 12.5 mM Tris base, 96 mM glycine, 0.1% SDS, and 15% (v/v) methanol. The membrane was blocked with 5% skim milk in 50 mM Tris-HCl (pH 7.2) containing 200 mM NaCl (TBS) for 1 h at room temperature. The blot was incubated with anti-α_{1A} (1:200; Alomone Labs, Jerusalem, Israel), anti-α_{1B} (1:100; Alomone Labs), anti-α_{1C} (1:100; Alomone Labs), anti-α_{1D} (1:200; Alomone Labs), anti-α_{1E} (1:200; Alomone Labs), anti-β_1 (1:100; Chemicon International, Temecula, CA), anti-β_2 (1:200; Chemicon International), anti-β_3 (1:100; Chemicon International), or anti-β_4 (1:200; Chemicon International) antibody diluted in TBS containing 0.05% Tween 20 and 3% skim milk at room temperature for 3 h. For detection of Dbh, Pnmt, Th, and pTh, protein samples (40 µg/lane) were electrophoresed in a 6% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane as described; the blot then was incubated with anti-Dbh (1:100; Abcam, Cambridge, MA), anti-Pnmt (1:100; Abcam), anti-Th (1:200; Abcam), or anti-pTh (1:500; Abcam) antibody diluted in the previously described buffer at room temperature for 2 h.

After washing the membranes at room temperature for 30 minutes with 3 changes of TBST buffer containing 25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.1% Tween 20, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (1:1000; Medical and Biological Laboratories, Tokyo, Japan) for 1 h at room temperature. Signals were detected with ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ). Prior to reprobing with actin antibody (1:5000; Abcam), antibodies were stripped from membranes by using the protocol included with the ECL reagents. The densities of specific bands were determined by densitometry (Alpha Innotech, San Leandro, CA) and normalized against that of actin. Each point represents the mean ± 1 SD of triplicate determinations. Statistical significance was determined by analysis of variance followed by Dunnett tests.

Results

Assay of norepinephrine and epinephrine levels. The mouse genotypes were identified by PCR of DNA from tail biopsy samples (data not shown). The numbers of wild-type, heterozygous, and homozygous mice derived from matings between heterozygous mice were consistent with Mendelian segregation, and the ratio of females and males was the same in all groups (data not shown). None of the genotypes of α_{1B}-deficient mice showed any apparent behavioral abnormality, and all survived for more than a year (data not shown). Plasma levels of norepinephrine and epinephrine tended to be lower in homozygous mice than in wild-type and heterozygous mice (Table 1), but this difference was not significant.

Histologic analysis of α_{1B}-deficient mice. All genotypes of

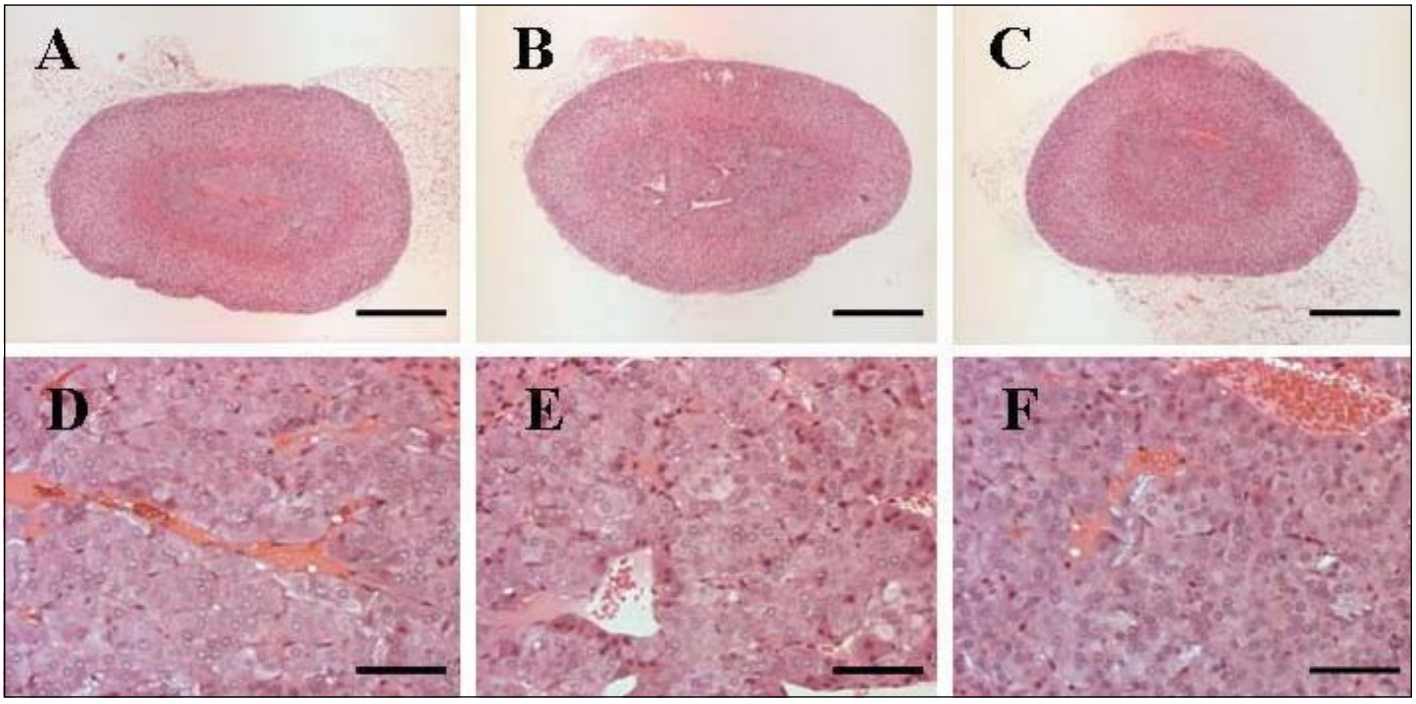

Figure 1. Histologic analysis of the adrenal gland (A through C) and adrenal medulla (D through F) of wild-type (+/+, A and D), heterozygous (+/–, B and E), and homozygous (–/–, C and F) α_{1B}-deficient mice. Hematoxylin and eosin stain. Bar = 200 µm (A through C), 100 µm (D through F).

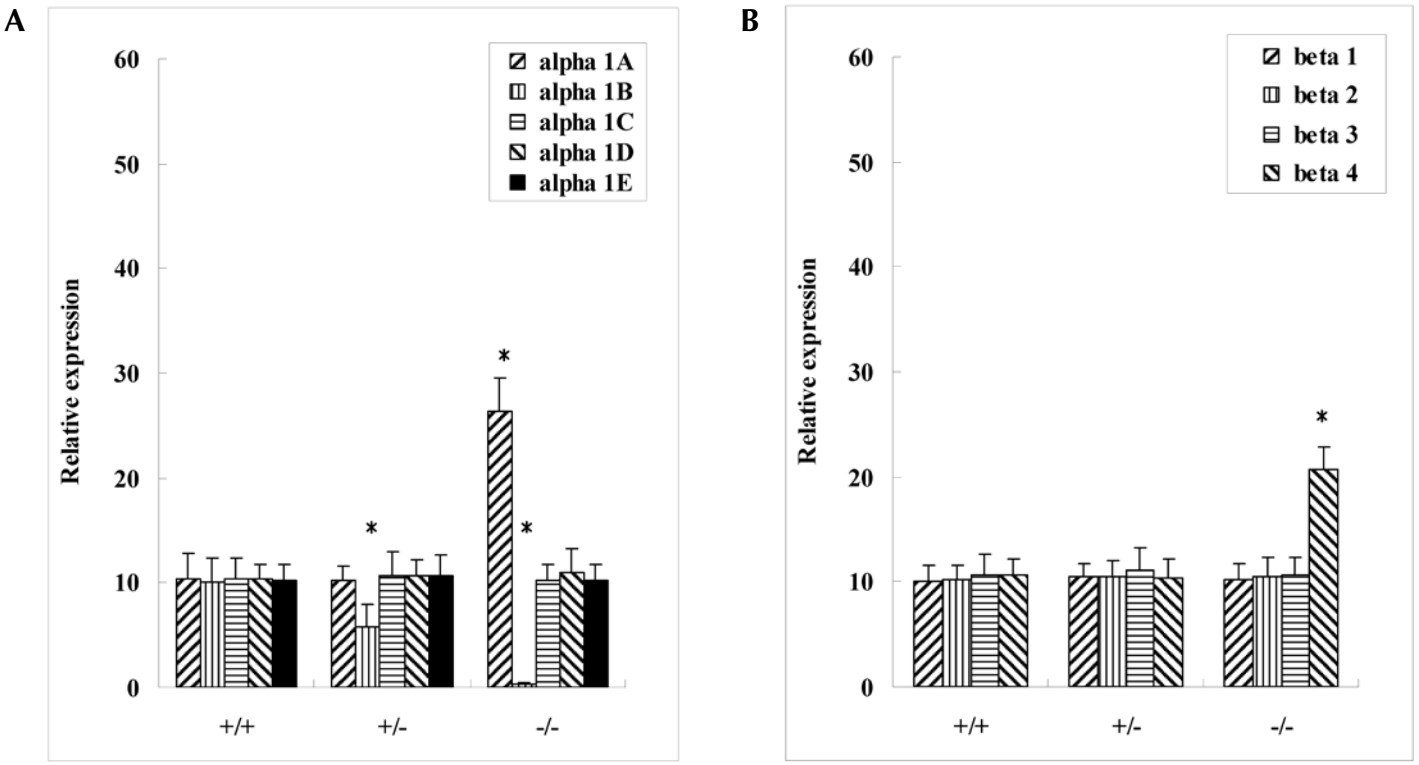

Figure 2. Real-time PCR analysis of α_1 (A) and β (B) subunit genes in the adrenal gland of wild-type (+/+), heterozygous (+/–), and homozygous (–/–) α_{1B}-deficient mice. The amount of mRNA is expressed relative to that of 18S ribosomal RNA. Data are reported as means ± standard deviation of 3 independent experiments. *, $P < 0.05$ versus value for wild-type mice.

α_{1B}-deficient mice were free from apparent anatomic histologic changes in the adrenal gland (Figure 1).

Expression of α_1 and β subunit mRNAs in the adrenal gland of α_{1B}-deficient mice. Real-time RT-PCR was performed to examine the mRNA transcription levels of α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E}, β_1, β_2, β_3, and β_4 subunit genes in the adrenal gland. The α_{1A} mRNA level was similar in wild-type and heterozygous mice, whereas homozygous mice showed more than 2-fold greater expression (Figure 2 A). The mRNA transcription levels of the α_{1C}, α_{1D}, and α_{1E} subunits were similar in wild-type, heterozygous, and homozygous mice (Figure 2 A), as were those of the β_1, β_2, and β_3 subunits (Figure 2 B). Homozygous mice showed more than 2-fold greater transcription of β_4 mRNA than did wild-type and heterozygous mice (Figure 2 B). In the adrenal gland of mice with CBA/JN backgrounds, heterozygous mice expressed less α_{1B} mRNA than did wild-type mice, but no α_{1B} mRNA could be detected in homozygous mice (Figure 2 A). The transcription patterns of the transgenes did not differ between males and females of any of the mouse genotypes (data not shown).

Expression of Th, Dbh, and Pnmt mRNAs in the adrenal gland of α_{1B}-deficient mice. We used real-time RT-PCR to examine the mRNA expression levels of Th, Dbh, and Pnmt. The Th mRNA level was similar in wild-type and heterozygous mice, whereas homozygous mice showed more than 4-fold greater expression (Figure 3). In contrast, the Dbh and Pnmt mRNA levels were similar among all genotypes (Figure 3). None of the mRNA expression patterns of these transgenes differed between males and females in any of the mouse genotypes (data not shown).

Protein levels of the α_{1A} and β_4 subunits, Th, and pTh in the adrenal gland of α_{1B}-deficient mice. Western blot analysis was performed to examine the protein levels of the α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E}, β_1, β_2, β_3, and β_4 subunits; Dbh; Pnmt; Th; and pTh in the adrenal gland of α_{1B}-deficient mice. Overall the protein levels were consistent with the mRNA expression patterns (Figure 4). The α_{1A} (Figure 4 A, B) and β_4 (Figure 4 C, D) protein levels were similar in wild-type and heterozygous mice, whereas homozygous mice showed more than 2-fold greater expression. The expression level of α_{1B} protein was lower in heterozygous mice than in wild-type mice, but no α_{1B} protein could be detected in homozygous mice (Figure 4 B). The protein expression levels of the α_{1C}, α_{1D}, and α_{1E} subunits were similar among all genotypes (Figure 4 B), as were those of the β_1, β_2, and β_3 subunits (Figure 4 D); Dbh; and Pnmt (Figure 4 G). Th (Figure 4 E, G) and pTh (Figure 4 F, G) protein levels were similar in wild-type and heterozygous mice, whereas homozygous mice showed more than 4-fold greater expression. No difference was observed in the protein expression patterns of the transgenes between male and female mice in any of the transgenic lines (data not shown).

Discussion

Although mouse adrenal medullary chromaffin cells have been shown to express L-type, N-type-, P/Q-type, and R-type Ca^{2+} channels,[1] N-type Ca^{2+} channel α_{1B}-deficient mice with a CBA × C57BL/6 genetic background showed normal behavior and norepinephrine levels and survived for more than a year.[6] In a previous study, we showed that P/Q-type Ca^{2+} channel α_{1A} subunit expression was upregulated, but no change was observed in the expression patterns of the α_{1C}, α_{1D}, α_{1E}, β_1, β_2, β_3, and β_4 subunit genes in the adrenal gland of α_{1B}-deficient mice with a CBA × C57BL/6 genetic background.[25] This suggested the occurrence of compensation by another Ca^{2+} channel α_1 subunit

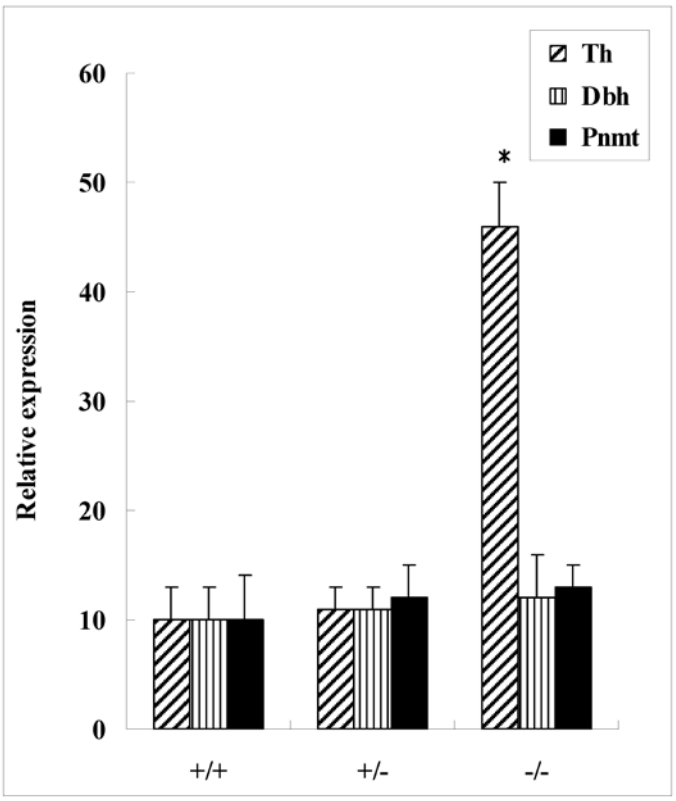

Figure 3. Real-time PCR analysis of tyrosine hydroxylase (Th), dopamine β hydroxylase (Dbh), and phenylethanolamine-N-methyltransferase (Pnmt) transcription in the adrenal gland of wild-type (+/+), heterozygous (+/−), and homozygous (−/−) α_{1B}-deficient mice. The amount of mRNA is expressed relative to that of 18S ribosomal RNA. Data are reported as means ± standard deviation of 3 independent experiments. *, $P < 0.05$ versus value for wild-type mice.

gene. In the present study, α_{1B}-deficient mice with a CBA/JN genetic background showed normal behavior, had normal norepinephrine and epinephrine levels, and survived for more than a year; the α_{1A} subunit gene also was upregulated in the adrenal gland of these mice. It is reasonable that the α_{1A} subunit can compensate for deficiency of N-type Ca^{2+} channel function in the adrenal gland of α_{1B}-deficient mice, because the α_{1A} and α_{1B} subunits show the greatest similarity of localization in the nervous system[26,29–31] and have the greatest similarity of sequence among α_1 subunits.[12,13,16,31] Because the expression pattern of the α_{1A} subunit gene in adrenal gland was the same in α_{1B}-deficient mice derived from CBA × C57BL/6 background as that in mice with a CBA/JN background, the compensation appears to be independent of genetic background. In our previous study, we showed that the 6.3-kb fragment of the 5′-upstream region of the α_{1A} gene contains a *cis* enhancer element(s) associated with compensation in the adrenal gland of α_{1B}-deficient mice derived from CBA × C57BL/6 mice.[25] Our current results suggest that a similar enhancer is present in α_{1B}-deficient mice with a CBA/JN genetic background.

In contrast, transcription and expression of the β_4 subunit in the adrenal gland of α_{1B}-deficient mice with a CBA/JN background was increased in addition to those of the α_{1A} subunit. It seems that this particular occurrence of compensation via enhanced Ca^{2+} channel subunit gene expression is dependent on genetic background. The β subunit is reported to regulate Ca^{2+} entry into the cells by increasing the peak Ca^{2+} current,[21,32] by shifting the voltage dependence of activation and inactivation[15] and by modulating G protein inhibition of the α_1 subunit.[2,18] The α_{1B}/α_2-δ subunits of the N-type channel complex immunoprecipitated with the β_3 and β_4 subunits,[20] whereas the α_{1A}/α_2-δ subunits of the P/Q-type channel complex immunoprecipitated with the β_1, β_2, β_3, and β_4 subunits.[10] This pattern suggests that the β_3 or β_4 subunit is the most likely candidate molecule among the β subunits to compensate for the lack of N-type Ca^{2+} channel function in α_{1B}-deficient mice. Although we should examine the Ca^{2+} current by means of an electrophysiologic study, upregulated expression of the β_4 subunit might reasonably compensate for lack of N-type Ca^{2+} channel function, because the P/Q-type Ca^{2+} channel α_{1A} subunit is targeted to presynaptic terminals by the β_4 subunit.[33] That is, increased expression of β_4 subunit could have the functional effect of increasing the surface expression of the α_{1A} subunit.

In our previous study, cerebellar expression of the α_{1A} subunit in α_{1B}-deficient mice with a CBA × C57BL/6 background was higher than that in wild-type or heterozygous mice,[24] whereas in cerebellum of α_{1B}-deficient mice with a CBA/JN background, expression of the β_4 subunit was higher, but that of the α_{1A} subunit was similar among genotypes.[24] These results suggest that the functional subunit expression pattern for compensation differs not only among genetic backgrounds but also among tissues.

Gene expression is thought to be achieved through positive regulatory mechanisms, in which *trans*-acting factors enhance basal transcriptional activity, as well as negative regulatory mechanisms, which repress transcriptional activity. The concerted action of positive and negative regulatory mechanisms is thought to be essential to provide complex regulation and flexibility in gene expression. Our results suggest that a *trans* regulatory factor for compensatory α_{1A} subunit enhanced expression is functional in the adrenal gland of α_{1B}-deficient mice with a CBA × C57BL/6 background, whereas expression of both the α_{1A} and β_4 subunits is enhanced in mice with a CBA/JN background. So far no information is available regarding the regulatory elements and promoter region of the β_4 subunit gene. Identification of the *cis* element(s) and *trans* regulatory factor(s) for expression of the β_4 subunit gene is needed to elucidate the molecular mechanisms of compensation. The difference in the β_4 subunit gene expression pattern between α_{1B}-deficient mice on CBA × C57BL/6 and CBA/JN backgrounds might be due to an influence of the genetic background derived from C57BL/6. We are now generating a congenic α_{1B}-deficient mouse strain with a C57BL/6N background in order to examine the expression pattern of the α_{1A} and β_4 subunit genes. Analysis of differences in neural or cardiovascular function of congenic strains of CBA/JN and C57BL/6N backgrounds would be useful in understanding the biologic importance of β_4 subunit expression in the adrenal gland of α_{1B}-deficient mice of CBA/JN versus CBA × C57BL/6 background. Although the transcriptional mechanisms are dependent upon genetic background, our results suggest that the P/Q-type Ca^{2+} channel seems to have a compensatory role in the adrenal gland of N-type Ca^{2+} channel α_{1B}-deficient mice. Although we found that the α_{1A} and β_4 subunit expression levels are enhanced, it is important to examine whether the increased protein expression levels of α_{1A} and β_4 subunits translate into changes of function. Other mechanisms, such as alternative splicing or post-translational processing, might actually generate the Ca^{2+} channel activity that substitutes for N-type

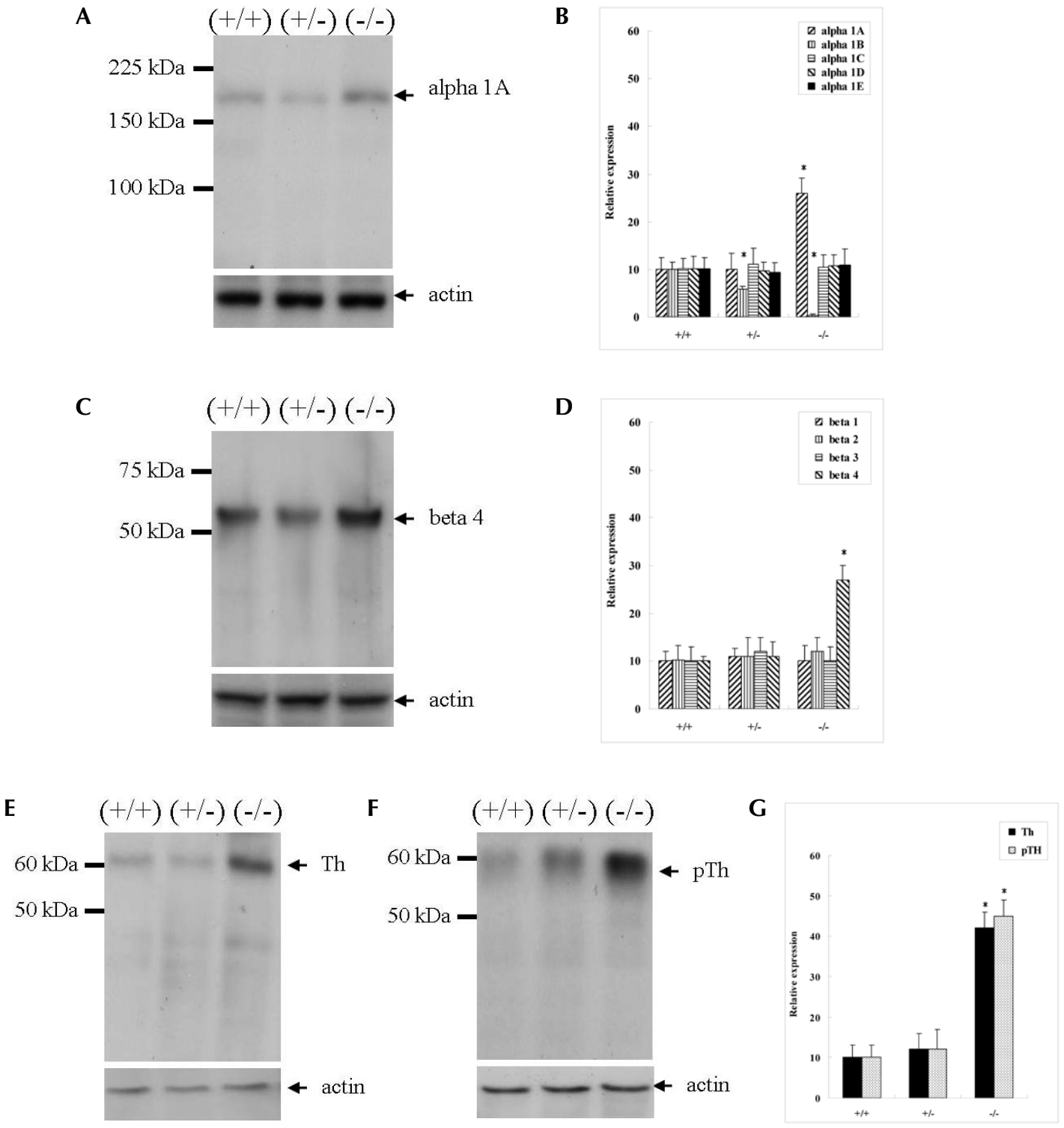

Figure 4. Western blot analysis of protein levels of α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E}, β_1, β_2, β_3, and β_4 subunits; dopamine β hydroxylase (Dbh), phenylethanolamine-N-methyltransferase (Pnmt), tyrosine hydroxylase (Th), and Th phosphorylated at serine 40 (pTh) in the adrenal gland of wild-type (+/+), heterozygous (+/–), and homozygous (–/–) α_{1B}-deficient mice. The bottom panel shows the expression of actin as a loading control (A, C, E, and F). Data are from 1 representative experiment of 3 that gave qualitatively identical results. The density of bands was measured by densitometry and normalized against that of actin (B, D, and G). Each point represents the mean ± standard deviation of triplicate determinations. *, $P < 0.05$ versus value for wild-type mice.

Ca^{2+} channel function in α_{1B}-deficient mice.

The catecholamines, including dopamine, norepinephrine, and epinephrine, are synthesized from dietary tyrosine in the adrenal medulla by the sequential action of several enzymes.[14] Th, the 1st and rate-limiting enzyme in catecholamine synthesis, catalyzes the conversion of tyrosine to L-dopa, a substrate for aromatic L-amino acid decarboxylase, which converts L-dopa to dopamine. In noradrenergic neurons and adrenal chromaffin cells, dopamine is converted to norepinephrine by Dbh, whose expression is primarily restricted to these cells. Norepinephrine is converted to epinephrine by Pnmt in adrenergic cells in the adrenal medulla. To extend our analysis of compensation mechanisms, we examined the expression levels of the Th, Dbh, and Pnmt genes in the adrenal gland of α_{1B}-deficient mice with a CBA/JN background. We found that Th mRNA was expressed at a higher level in homozygous α_{1B}-deficient mice than in wild-type or heterozygous mice, but no difference in the mRNA expression levels of Dbh and Pnmt was found among wild-type, heterozygous, and homozygous mice. The 5′-upstream region of the Th gene contains the cAMP response element, and experiments involving deletion of this element indicate that it is an inducible enhancer for Th transcription.[8] In addition, α_{1A} subunit-transfected cells showed that the level of phosphorylated cAMP response element binding protein is upregulated in HEK293 cells expressing functional P/Q-type Ca^{2+} channel and that this phosphorylation is dependent on Ca^{2+} influx through functional P/Q-type Ca^{2+} channel, because it returns to basal levels in the presence of ω-agatoxin-IVA.[23] Therefore, upregulated expression of the Th gene may be mediated by enhanced activity of the P/Q-type Ca^{2+} channel.

Serine-40 phosphorylation of Th by cAMP-dependent protein kinase plays an important role in the regulation of Th activity and thereby in the biosynthesis of catecholamine.[5] The level of phosphorylated Th in homozygous α_{1B}-deficient mice was higher than that in wild-type and heterozygous mice. Although we did not examine the level of dopamine in adrenal gland, in view of the normal expression levels of Dbh and Pnmt genes and the normal plasma levels of norepinephrine and epinephrine, the dopamine level is also likely to be normal owing to enhanced biosynthesis. The mechanism of activation of cAMP-dependent protein kinase is not clear in this study, but these results indicate that although enhanced activity of the P/Q-type Ca^{2+} channel seems to be functional in the release of catecholamines to compensate for the deficiency of N-type Ca^{2+} channel, this alone is not enough. An enhanced catecholamine biosynthetic pathway also seems to be needed to maintain normal circulating norepinephrine and epinephrine levels in α_{1B}-deficient mice. Catecholamine biosynthesis could be enhanced by upregulation of Th and its phosphorylation, and it is not certain whether overexpression of α_{1A} and β_4 would fully mimic the catecholamine secretion mediated by N-type Ca^{2+} channels, indicating that the turnover rate of catecholamine in adrenal gland also is accelerated. The expression levels of Th and pTh in the adrenal gland of homozygous α_{1B}-deficient mice were also higher than those in wild-type and heterozygous mice with a CBA × C57BL/6 background (data not shown). Our results suggest that the compensation mechanisms in the catecholamine biosynthetic pathway and turnover rate of catecholamine are independent of genetic background.

In this study, our results indicate that changing the genetic background and studying the gene products responsible for functional compensation are important for understanding apparently normal phenotype in gene-deficient mice. Further studies, including identification of *cis* element(s) and *trans* regulatory factor(s) for enhanced expression of the α_{1A} and β_4 subunit and Th genes, examination of cell-type-specific gene expression patterns using transcriptome and proteome analyses, and electrophysiologic and pharmacologic analysis of catecholamine release, will be necessary to elucidate the molecular mechanisms of compensation that lead to normal behavior and normal plasma catecholamine levels in N-type Ca^{2+} channel α_{1B}-deficient mice.

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