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Generation of RGS8 Null Mutant Mice by Cre/loxP System

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Regulators of G-protein signaling (RGS) proteins are negative regulators of heterotrimeric guanine-nucleotide-binding proteins (G-proteins) by activating intrinsic GTPase activity of Gα and thereby terminating G-protein coupled receptor-associated signal transduction. RGS8 belongs to B/R4 subfamily of RGS proteins and is expressed in the central nervous system, especially dense in cerebellar Purkinje cells. RGS8 binds specifically to Gαo and Gαi3 *in vitro* and activates their intrinsic GTPase activities. To investigate the role of RGS8 *in vivo*, we generated mice lacking RGS8 by gene targeting in embryonic stem (ES) cells using Cre/loxP system. RGS8 null mutant mice were viable, fertile and showed apparently normal development. Histological analysis showed no apparent abnormalities in morphology of cerebellar layer or Purkinje cells in RGS8 null mutant mice.

RGS proteins are negative regulators of heterotrimeric guanine-nucleotide-binding protein (G-protein) cycle. The G-protein cycle is initiated by binding of an agonist to its target G-protein coupled receptor (GPCR), which associates with a heterotrimeric G-protein on the cytoplasmic side of cell membrane. In the inactive state, G-protein localizes at the cell membrane as a complex of GDP-bound form of Gα (Gα-GDP) and Gβγ. Upon activation, Gα changes from Gα-GDP to Gα-GTP and dissociates from Gβγ. Both Gα and Gβγ can activate their distinct effectors including enzymes (e.g., adenylyl cyclases and phospholipases) and ion channels [e.g., G-protein coupled inwardly rectifying potassium (GIRK) channels]. Then GTP hydrolysis takes place and Gα-GDP can reassociate with Gβγ thus terminating the signal. It is well established that Gα has an intrinsic GTPase activity but the activity is very weak without GTPase activating proteins (GAPs). RGS proteins were first identified as GAPs and cloning studies have identified over 30 distinct RGS genes in mammals (1, 6, 8, 13).

Of these RGS proteins, RGS8 belongs to B/R4 subfamily. *In situ* hybridization analysis revealed that RGS8 mRNAs are expressed widely but differentially in the central nervous system (CNS), especially dense in cerebellar Purkinje cells and relatively dense in brainstem (5). RGS8 has conserved RGS domain and SYP motif allowing for 14-3-3 binding (2). In addition, RGS8 can bind specifically to the Gαo and Gαi3 and regulate GIRK current *in vitro* (10), although physiological roles of RGS8 *in vivo* remain largely unknown. These results indicate that RGS8 has a potential drug target for CNS diseases (9, 13). In this study, to elucidate physiological roles of RGS8 in the CNS, we generated mice deficient in RGS8

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by gene targeting in embryonic stem (ES) cells. RGS8 null mutant mice were viable. They were fertile and showed no apparent developmental defects. Furthermore, the loss of RGS8 did not affect morphology of cerebellar layer or Purkinje cells.

MATERIALS AND METHODS

Targeting strategy. A 14.5-kb genomic DNA fragment containing exons 2 to 5 of the RGS8 gene (GenBank Acc. No. NM_026380) was subcloned from a bacterial artificial chromosome (BAC) clone obtained by screening an RPCI-22 129 mouse BAC genomic library (CHORI BACPAC Resources, Oakland, CA). To generate RGS8 gene-targeting vector, subcloned fragments were inserted into neoFRT/loxP cassette (a gift of Dr. Sakimura) containing two loxP sequences and neomycin resistance gene (neo) flanked by two Flp recognition target (frt) sites (Fig. 1). The RGS8 targeting vector contained neoFRT, exons 4 and 5 of RGS8 gene flanked by loxP sequences, the 7.2 kb upstream and 2.8 kb downstream genomic sequences, and a negative selection marker, the diphtheria toxin A-fragment (DT-A) expression cassette (12). Cre recombination resulted in the deletion of exons 4 and 5 as well as neoFRT (Fig. 1).

Generation of RGS8 (-/-) mice. The targeting vector was introduced into 129/Ola-derived EB3 ES cells (a gift of Dr. Niwa) by electroporation. G418 selection (200 μg/ml) was applied 24 h after transfection and G418-resistent colonies were isolated on day 7 of selection. To screen homologous recombinants, genomic DNA from these clones was digested with EcoRI or EcoRV and hybridized with 5' or 3' probes, respectively (not shown). Chimeric mice were generated by injection of the homologous recombinants into C57BL/6 blastocysts. The chimeric mice were mated with C57BL/6 mice to yield RGS8 (+/flox-neo) mice (P generation). By mating RGS8 (+/flox-neo) mice with EIIa-cre transgenic mice (7), RGS8 (+/-) mice (F_1) were obtained. Southern blotting analysis of tail DNA confirmed the removal of exons 4 and 5 and neo. Genotypes of RGS8 (+/flox-neo) mice and RGS8 (+/-) mice were determined by Southern blotting using an internal probe (Fig. 1) and polymerase chain reaction (PCR) analysis with primers (R14, 5'-GGAGTCTTTCGATGTGCTTC-3'; R17, 5'-CTCCCAAGCTAATGACCTTC-3'; R19, 5'-TAATATCCGAGAACCTGGGG-3') (Fig. 1). Mice were housed in a temperature- and humidity- controlled room under a 12-h light/dark cycle with food and water ad libitum. This study was performed in accordance with the Guidelines of Animal Experimentation at Kobe University School of Medicine (Permission number: P-060904R).

Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR). To detect RGS8 mRNA, RT-PCR was used with primers corresponding to the exons 4 and 5 (Fig. 2C). Total RNAs were isolated from cerebellum and the first strand cDNA was synthesized as a template of PCR analysis with a set of primers for RGS8 (R1, 5'-GAACAAAGGCATGAG GACTCGACTGG -3' and R2, 5'-ACTTATGAGAGAAGCACATCGAAAG -3') and a set of primers for β-actin (5'-CAACGTCACACTTCATGATGG-3' and 5'-CTACAATGAG CTGCGTGTGG-3'; GenBank Acc. No. NM_007393).

Immunoblot analysis. P2-fractions were prepared from cerebella as described previously (11). Proteins were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore, Billerica, MA). The membrane was probed with chick polyclonal antibody against RGS8 (ab14267, abcam, Cambridge, UK) or mouse monoclonal antibody against Rac1 (R56220, BD bioscience, San Diego, CA), followed by HRP-conjugated secondary antibodies against chick IgY (703-035-155, Jackson Immuno Research, West Grove, PA) or mouse IgG (715-035-151, Jackson Immuno Research), respectively. Bound

antibodies were visualized with ECL detection reagents (GE Healthcare Bio-Sciences Corp, Piscataway, NJ).

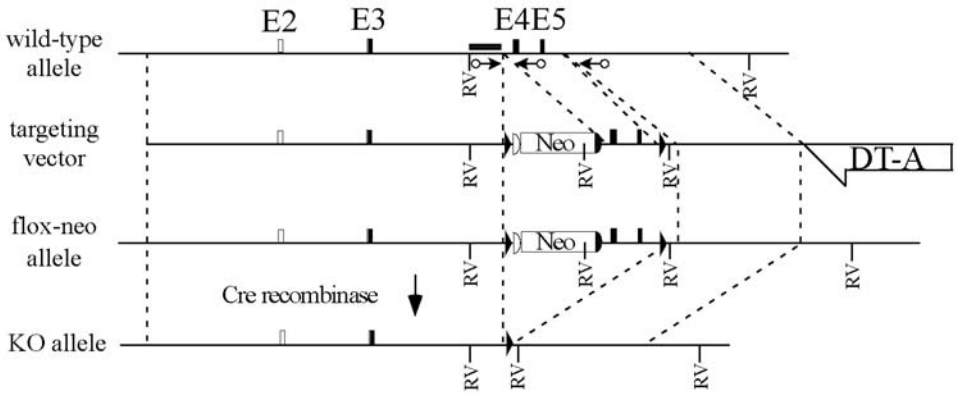

FIG. 1 Targeting strategy for generation of RGS8 null mutant mice.
Wild-type RGS8 allele with exons 2 to 5 are shown (closed boxes, coding region; open box, non coding region). Exons 4 and 5 encode a part of the RGS domain. Restriction sites for EcoRV (RV), PCR primers (black arrow) for PCR genotyping and an internal probe (bold line) for Southern blotting analysis are indicated. In the targeting vector, two loxP sites (black triangles) flank a 1.8 kb neoFRT fragment and a 3.2 kb genomic DNA fragment including exons 4 (102 bp) and 5 (65 bp). When Cre enzyme is provided, neoFRT and the exons 4 and 5 in flox-neo allele are excised to result in KO allele.

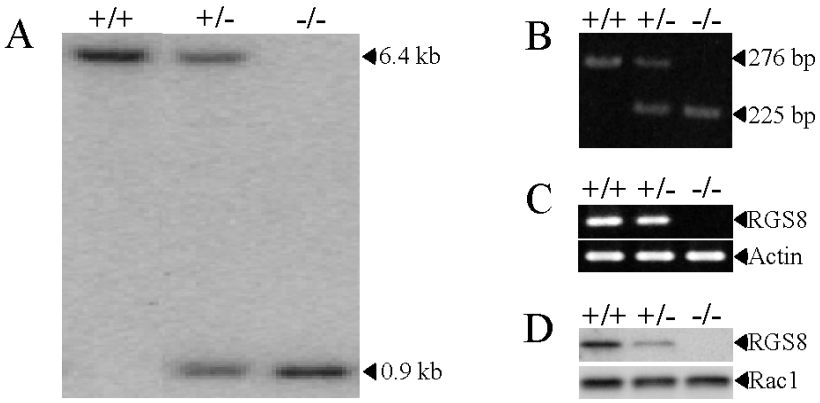

FIG. 2 Generation of RGS8 (-/-) mice.
(A) Southern blotting analysis of tail DNA from wild-type (+/+), RGS8 (+/-) and RGS8 (-/-) mice. EcoRV-digested DNA was hybridized with the internal probe (Fig. 1). Wild-type and KO alleles are indicated by the presence of a 6.4 kb fragment and a 0.9 kb fragment, respectively. (B) PCR analysis of tail DNA from wild-type (+/+), RGS8 (+/-) and RGS8 (-/-) mice. Wild-type and KO alleles are indicated by the presence of a 276 bp band and a 225 bp band, respectively. (C) RT-PCR analysis of total RNA from wild-type (+/+), RGS8 (+/-) and RGS8 (-/-) cerebellum (8-week-old male). PCR with RGS8-forward and RGS-reverse primers amplified a 167 bp fragment corresponding to exons 4 and 5. No amplified band from RGS8 (-/-) RNA was detected. A 260 bp β-actin fragment was amplified as a positive control (Actin). (D) Immunoblot analysis of proteins extracted from wild-type (+/+), RGS8 (+/-) and RGS8 (-/-) cerebellum (8-week-old male) using anti-RGS8 and anti-Rac1 antibodies. RGS8 protein was detected in wild-type (+/+) and RGS8 (+/-) mice but not in the RGS8 (-/-) mice.

Immunofluorescence staining of the tissue sections. To analyze morphology of cerebellum and Purkinje cells, immunofluorescence staining was carried out. Adult mice were deeply anesthetized with pentobarbital and were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion, removed brains were exposed to 4% paraformaldehyde for 2 h, washed with phosphate buffered saline (PBS), embedded in 2% agarose in PBS, and cut on a microslicer (VT 1000S-V1.0E, Leica Microsystems, Nussloch, Germany) into 50 μm-thick parasagittal sections. The free-floating sections were incubated with antibodies to calbindin (Chemicon International, Temecula, CA) overnight at 4°C. These sections were then washed with PBS and incubated with Cy3-conjugated antibody to Alexa488-conjugated antibody to rabbit IgG (Jackson Immuno Research) for 1 h at room temperature. Neurons were subsequently detected with NeuroTrace (Invitrogen, Carlsbad, CA) fluorescent Nissl stain. Photographs were taken with a fluorescence microscope (BZ-8000, Keyence, Osaka, Japan) or confocal microscope (LSM510, Carl Zeiss, Jena, Germany).

RESULTS

Generation of RGS8 mutant ES cells. The RGS8 targeting vector was constructed from a genomic BAC DNA clone that contains exons 2 to 5 of the mouse RGS8 gene. In this vector two loxP sites were inserted to flank exons 4 and 5 of RGS8 and neoFRT. Since neo is flanked by two frt sites, Flp-mediated recombination could induce deletion of neo, resulting in flox allele of RGS8 gene. When Cre enzyme is provided, neoFRT and exons 4 and 5 of RGS8 gene in flox-neo allele are excised (KO allele; Fig. 1). Deletion of the exons 4 and 5 introduces a frame shift mutation, creating a stop codon in exon 7. The targeting vector was introduced into EB3 ES cells by electroporation and we verified the correct recombination event by Southern blotting analysis using 5' and 3'probes (data not shown).

Generation of RGS8 (-/-) mice. Injection of three homologous recombinant clones into C57BL/6 blastocysts produced germline male chimeras. The chimeric mice were mated with inbred C57BL/6 mice and yielded RGS8 (+/flox-neo) mice (P generation). By mating RGS8 (+/flox-neo) mice with EIIa-cre transgenic mice which express Cre in preimplantation embryos (7), we obtained RGS8 (+/-) mice (F_1). Southern blotting analysis of tail DNA confirmed the removal of exons 4 and 5 as well as neo (Fig. 1 and 2A). We obtained F_4 hybrids of RGS8 (+/-) mice by backcrossing RGS8 (+/-) mice (F_1) to inbred C57BL/6 mice three times. Interbreeding of RGS8 (+/-) mice (F_4) produced RGS8 null mutant [RGS8 (-/-)] mice. Southern blotting and PCR analyses confirmed the deletion of exons 4 and 5 encoding part of RGS domain in RGS8 (-/-) mice (Fig. 2A and 2B). RGS8 (-/-) offspring from intercrossing of RGS8 (+/-) mice was obtained in roughly Menderian ratios. The loss of RGS8 mRNA in RGS (-/-) mice was confirmed by RT-PCR analysis of total RNA from mouse cerebellum (Fig. 2C). Western blot analysis of P2 fraction of cerebellum using antibodies against RGS8 confirmed that the RGS8 (-/-) mice did not express RGS8 protein (Fig. 2D). Therefore we concluded that the RGS8 null mutant mice were established.

General appearance of RGS8 (-/-) mice. At 8 weeks of age, gross appearance of RGS8 (-/-) mice was indistinguishable from wild-type littermates. RGS8 (-/-) mice appeared healthy, well groomed, and were capable of caring for themselves. In addition, both female and male RGS8 (-/-) mice were fertile.

Morphology of Purkinje cells in RGS8 (-/-) mice. Because RGS8 is expressed prominently in cerebellar Purkinje cells (5), we focused our attention on Purkinje cells. The gross anatomy of the cerebellum in RGS8 (-/-) mice appeared normal as revealed by Nissl staining (Fig. 3A). The size and density of the Purkinje cells in RGS8 (-/-) cerebellum

seemed comparable to controls (Fig. 3B). Staining with anti-calbindin antibodies showed that morphology of Purkinje cell dendrites in RGS8 (-/-) mutant mice appeared normal as compared with wild-type controls (Fig. 3C).

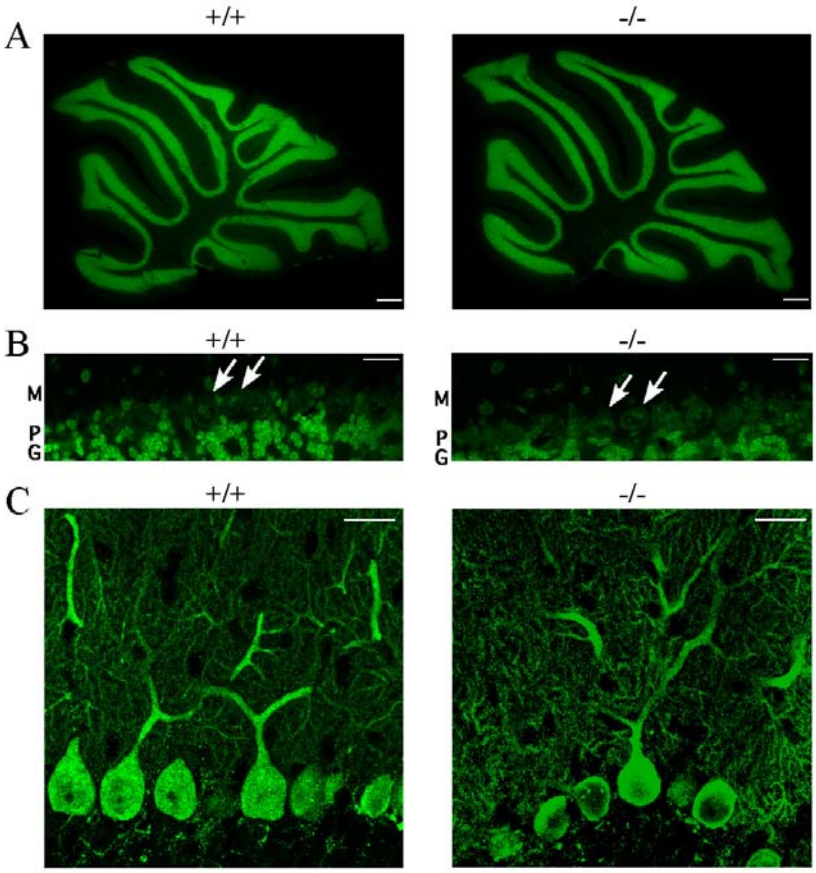

FIG. 3 Anatomy of RGS8 (-/-) cerebellum.
(A, B) Parasagittal sections of wild-type (+/+) and RGS8 (-/-) cerebella were stained with NeuroTrace. Arrows indicate Purkinje cells. M, molecular layer; P, Purkinje cell layer; G, granule cell layer. (C) Parasagittal sections of wild-type (+/+) and RGS8 (-/-) cerebella were stained with antibodies against calbindin. Scale bars in (A), 300 μm; in (B) and (C), 20 μm.

DISCUSSION

In this study, we generated RGS8 (-/-) mice using a Cre/loxP system. Development of RGS8 (-/-) mice appeared normal. In spite of expression of RGS8 in testis (10), RGS8 (-/-) mice are fertile. Since RGS8 is expressed strongly in Purkinje cells and binds to Gαo and Gαi, RGS8 (-/-) mice were expected to exhibit phenotypes related to dysfunction of signal transduction via Gi-coupled $GABA_B$ receptors in Purkinje cells. It was reported that the loss of $GABA_{B(2)}$ receptor or GABA transporter subtype 1 (GAT1) resulted in spontaneous seizures or ataxia, respectively (3, 4). However, our RGS8 (-/-) mice did not show apparent seizure and ataxia. Currently we are performing more quantitative and intensive analyses of Purkinje cell-related motor behavior and learning of RGS8 (-/-) mice using such behavioral

tasks as rota-rod. Histological analysis of RGS8 (-/-) cerebellum showed that loss of RGS8 did not affect the cerebellum structure or morphology of Purkinje cell dendrites. When co-expressed in Xenopus oocytes with a GPCR and a GIRK, RGS8 accelerated not only the turning off but also the turning on of the GIRK current upon receptor stimulation, without affecting the dose-response relationship (10). Thus, it would be interesting to examine whether the signal transduction via Gi-coupled $GABA_B$ receptors in Purkinje cells would be affected in RGS8 (-/-) mice in the future.

We obtained RGS8 (+/flox-neo) mice during the course of generating RGS8 (-/-) mice. By breeding the RGS8 (+/flox-neo) mice to transgenic mice expressing Flp in the early mouse embryo, we could generate progeny in which neoFRT is deleted in RGS8 allele [RGS8 (+/flox) mice]. If we would find any significant phenotypes in RGS (-/-) mice in the future, we could take advantage of tissue specific RGS8 knockout using the RGS (flox/flox) mice and tissue specific Cre transgenic lines to determine the role of RGS8 in the specific tissue.

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