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ORIGINAL ARTICLE

Simultaneous deletion of floxed genes mediated by CaMKIIα-Cre in the brain and in male germ cells: application to conditional and conventional disruption of Goα

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The Cre/LoxP system is a well-established approach to spatially and temporally control genetic inactivation. The calcium/calmodulin-dependent protein kinase II alpha subunit (CaMKIIα) promoter limits expression to specific regions of the forebrain and thus has been utilized for the brain-specific inactivation of the genes. Here, we show that CaMKIIα-Cre can be utilized for simultaneous inactivation of genes in the adult brain and in male germ cells. Double transgenic Rosa26$^{+/stop\text{-}lacZ}$::CaMKIIα-Cre$^{+/Cre}$ mice generated by crossing CaMKIIα-Cre$^{+/Cre}$ mice with floxed ROSA26 lacZ reporter (Rosa26$^{+/stop\text{-}lacZ}$) mice exhibited lacZ expression in the brain and testis. When these mice were mated to wild-type females, about 27% of the offspring were whole body blue by X-gal staining without inheriting the Cre transgene. These results indicate that recombination can occur in the germ cells of male Rosa26$^{+/stop\text{-}lacZ}$::CaMKIIα-Cre$^{+/Cre}$ mice. Similarly, when double transgenic Gnao$^{+/f}$::CaMKIIα-Cre$^{+/Cre}$ mice carrying a floxed *Go-alpha* gene (Gnao$^{f/f}$) were backcrossed to wild-type females, approximately 22% of the offspring carried the disrupted allele (Gnao$^{\Delta}$) without inheriting the Cre transgene. The Gnao$^{\Delta/\Delta}$ mice closely resembled conventional Go-alpha knockout mice (Gnao$^{-/-}$) with respect to impairment of their behavior. Thus, we conclude that CaMKIIα-Cre mice afford recombination for both tissue- and time-controlled inactivation of floxed target genes in the brain and for their permanent disruption. This work also emphasizes that extra caution should be exercised in utilizing CaMKIIα-Cre mice as breeding pairs.
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INTRODUCTION

Heterotrimeric G proteins transduce numerous extracellular signals from receptors to intracellular signaling pathways. The Gi and Go proteins are activated by the same neurotransmitter receptors including D2 and D4, type 1 serotonin, M2 and M4 ACh, GABA-B and group 2 metabotropic glutamate receptors.[1] However, despite 70–85% identity, targeted inactivation of each of the *Giα* and *Goα* genes has markedly different consequences. For example, Giα2-knockout mice show severe immunological deficits, including inflammatory bowel disease, and other immune abnormalities that precede an ulcerative colitis syndrome.[2] In contrast, deletion of Goα, which is abundantly expressed in the central nervous system, causes severe neurological deficits, such as hyperlocomotion, occasional seizure, hyperalgesia and loss of light response.[2] These distinctive behaviors point to a unique role of Goα in the brain.

Gene targeting is a powerful technique to study the physiological functions of a gene and its product(s).[3] However, studies with conventional Goα-knockout mice have been hampered, because loss of Goα leads to extremely low birth rates, and the survival rate of occasionally born pups

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decreases markedly with ages.[4] The rare survival of Goα knockout to adulthood precludes analysis of Goα functions in the adult brain. To circumvent these problems, we used a Cre/loxP system[5] in which the Cre recombinase expression is driven by the promoter of calcium/calmodulin-dependent protein kinase II alpha subunit (CaMKIIα). Among several CaMK isozymes, CaMKIIα is predominantly expressed in the cerebral cortex and hippocampus but very low in the striatum, cerebellum and brainstem of adult mice[6] where it mediates diverse physiological reactions in response to intracellular Ca^{2+} signals.[7] Genetic ablation of CaMKIIα impairs learning and memory in mice.[8,9] Similarly, the Cre recombinase activity driven by the CaMKIIα promoter (CaMKIIα-Cre) is detected in the cortex, striatum, hippocampus, but very little in the cerebellum.[10,11] When crossed with a strain containing *loxP* sites flanking sequences of interest, Cre-mediated recombination occurs in these regions.

Previous reports have demonstrated conventional knockout mice can be generated by a single cross of *loxP* containing mice to Cre transgenic mice in which Cre expression is driven by germline-specific promoters such as zona pellucid glycoprotein 3 (Zp3) that is expressed in growing oocytes[12] and protamine 1 that is expressed in haploid round spermatids.[13] Interestingly, CaMKIIα-Cre is expressed in the testis like the natural CaMKIIα.[10] The natural CaMKIIα is mildly expressed in the testis,[14] where it regulates acrosomal reaction of spermatozoa.[15] Although the CaMKIIα-Cre could induce germ line recombination in the testis, the recombined allele, however, was never transmitted to the progeny in some transgenic mouse lines.[16] Thus, it is worthwhile to investigate systematically the CaMKIIα-Cre activity during spermatogenesis and the efficiency of germ line transmission to the next generation.

In this study, we show that CaMKIIα-Cre mice can be used to induce brain-specific disruption of a floxed gene in one generation as well as to obtain global disruption in the next generation through germline recombination. We show that when CaMKIIα-$Cre^{+/Cre}$ mice were crossed with floxed ROSA26 lacZ reporter ($Rosa26^{+/stop\text{-}lacZ}$) mice, the expression of lacZ was simultaneously induced by CaMKIIa-Cre in the brain and testis. Mating of such mice expressing lacZ in the testis yielded a progeny that expressed lacZ in the entire body. These results suggest that recombination events can occur in the germline of male $Rosa26^{+/stop\text{-}lacZ}$::CaMKIIα-$Cre^{+/Cre}$ mice. Similarly, when CaMKIIα-$Cre^{+/Cre}$ mice were crossed with mice whose *Goα* subunit gene (Gnao) had two loxP sites flanking exons 5 and 6,[17] the Gnao was deleted in the adult brain and testis. The same mice were utilized to generate Goα-null mice ($Gnao^{\Delta/\Delta}$) that showed the same neurological phenotypes as conventional Gnao-knockout mice ($Gnao^{-/-}$).[4] These results indicate that the use of CaMKIIα-Cre mice affords an efficient way to generate both conditional knockout mice with brain-specific disruption of the gene and to simultaneously obtain conventional knockout mice that carry the null allele disrupted in fertilized eggs. The results also imply that caution should be exercised when using CaMKIIα-Cre mice for breeding.

MATERIALS AND METHODS

Animals

CaMKIIα-$Cre^{+/Cre}$ mice were a kind gift of Kong YY (Seoul National University), which were originally obtained from Artemis Pharmaceuticals (Cologne, Germany). $Rosa26^{+/stop\text{-}lacZ}$ (B6;129S4-Gt(ROSA)26Sor^{tm1Sor}/J) mice originally developed by Soriano[18] were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The Goα-floxed mice ($Gnao^{f/f}$) containing loxP sites flanking exon 5 and 6 of Goα were previously reported.[17] Male $Rosa26^{+/stop\text{-}lacZ}$ mice (homozygous for the transgene) or $Gnao^{f/f}$ mice were crossed to female CaMKIIα-$Cre^{+/Cre}$ mice (heterozygous for the transgene) to generate mice with brain-specific deletion of the floxed gene. The F1 male progeny ($Rosa26^{+/stop\text{-}lacZ}$::CaMKIIα-$Cre^{+/Cre}$ or $Gnao^{+/f}$::CaMKIIα-$Cre^{+/Cre}$) were crossed to wild-type female mice to confirm germline recombination in the F2 progeny. Food and water were provided *ad libitum*, and all experimental procedures were reviewed and approved by the Institutional Animal Research Ethics Committee at the Ajou University Medical Center (Suwon, South Korea).

Genotyping

Genotypes were verified by polymerase chain reaction (PCR) using genomic DNA isolated from mouse tail biopsies. In brief, tail pieces were placed in 250 μl lysis solution (50 mM NaOH) and boiled at 95 °C for 30 min. PCR was carried out with 2 μl of crude tail lysate for 30 cycles at the indicated temperature with each pair of specific primers for Gnao, Cre and lacZ (Table 1). PCR products were separated by electrophoresis in 2% agarose gels and visualized using ethidium bromide.

X-gal staining

Adult animals or pregnant female mice with embryos of gestational age 13.5 days were transcardially perfused with 0.9% saline and then with 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) under

Table 1 The primer sequences for PCR reaction

Target	*Sequence*	*Product*
Gnao		
Forward	ACCTGGCCTCCCTTGGGAATACAG	+: 224 bp
Reverse	CAGCGATCTGAACGCAAGAAGTGG	f: 303 bp
Gnao (Δ)		
Forward	AAGAATAGAACCTAGGACTGGAGG	445 bp
Reverse	GCAGACAAGTGAACAAGTGAAACCC	
lacZ		
Forward	GTTGCAGTGCACGGCAGATACACTTGCTGA	89 bp
Reverse	GCCACTGGTGTGGGCCATAATTCAATTCGC	
Cre		
Forward	GCGGTCTGGCAGTAAAAACTATC	100 bp
Reverse	GTGAAACAGCATTGCTGTCACTT	
Gapdh		
Forward	GTTGCTGTTGAAGTCACAGGAGAC	395 bp
Reverse	TCCATGACAACTTTGGCATCG TGG	

deep anesthesia. The brains, livers, hearts, lungs, kidneys, spleens and testes were trimmed, post-fixed in 2% paraformaldehyde for 12 h and incubated at 37 °C overnight in X-gal staining solution (1 mg ml^{-1} X-gal [5-bromo-4-chloro-3-indolyl-D-galactopyranoside], 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$, 0.02% Nonidet P-40, 0.1 M phosphate buffer, pH 7.4). To confirm regional expression patterns, the brain was sectioned with 2 mm thickness using a brain mold.

RT–PCR

Gnao$^{+/f}$::CaMKIIα-Cre$^{+/Cre}$ mice of 10 weeks of age were used for analyses. Age-matched littermates, without the Cre transgene, were used as controls. For RNA analyses, the brain, liver, heart, lungs, spleen, kidneys and testis were dissected from lethally anesthetized mice and snap frozen. Total RNA was extracted from homogenized frozen tissues using RNAzol B (Tel-Test, Friendswood, TX, USA), and cDNA was synthesized in a 20 μl reaction volume containing 1 μg total RNA using the First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN, USA), according to the manufacturer's recommendations. The RT–PCR reactions were carried out for 30 cycles with primers specific for Cre and for 26 cycles with primers specific for the mouse *glyceraldehyde 3-phosphate dehydrogenase* gene (Gapdh).

Western blot analysis

Approximately 100 mg of brain tissue was homogenized in 1 ml RIPA buffer (50 mM Tris–Cl, pH 8.0, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl). The supernatant was obtained after centrifugation in a microcentrifuge at 13 000 g for 10 min at 4 °C. The protein was quantitated with a Bradford assay. Fifty micrograms of protein from each genotype was used for electrophoresis on 10% SDS-PAGE and transferred to a PVDF membrane. The membranes were blocked in 5% skim milk in PBS-T (phosphate-buffered saline with 0.1% Tween 20) and then incubated overnight at 4 °C with an anti-Goα rabbit antibody (1:1000, Santa Cruz, CA, USA). After washing with PBS-T three times, the specific immunoreactivity was probed with a horseradish peroxidase-conjugated anti-rabbit antibody (1:5000, Zymed, San Francisco, CA, USA) for 1 h at room temperature. An ECL kit (Pierce, Rockford, IL, USA) was used to visualize the immunoreactivity following the manufacturer's protocol.

Assessment of locomotor activity

Animals were introduced to the test room and habituated to the novel test environment for 1 h. Then animals were placed in a transparent activity cage (opaque plastic, $30 \times 30 \times 30\,cm^3$) under subdued illumination, and video tracking was conducted for 1 h to record locomotor activity.

RESULTS

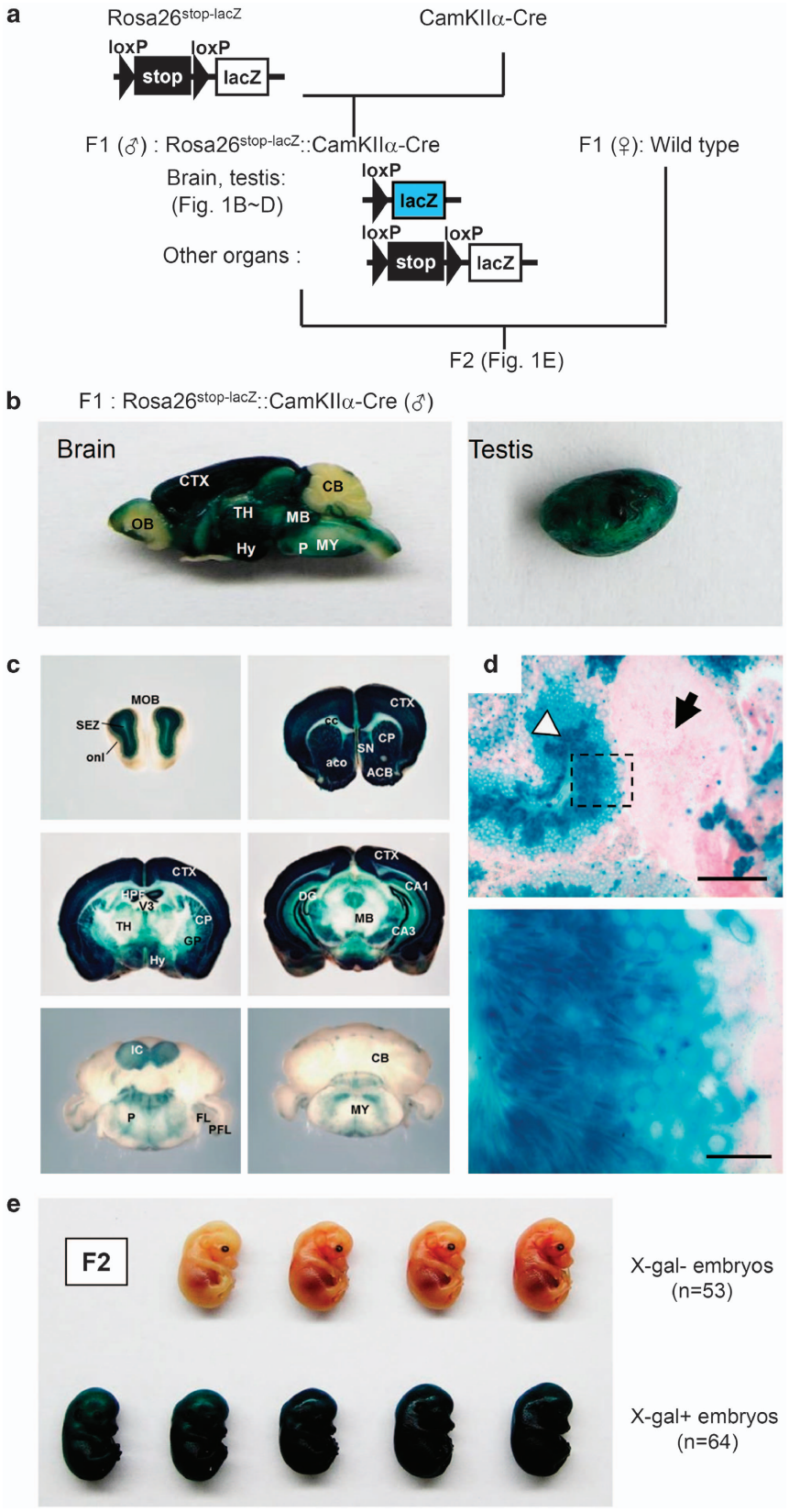
Cre recombinase activity in CaMKIIα-Cre transgenic mice

The CaMKIIα promoter was proven to drive a robust expression of Cre recombinase in the brain, however, its subregional expression varies in different CaMKIIα-Cre founder lines.[10,11,16,19] To confirm the pattern of Cre expression in our CaMKIIα-Cre mice, heterozygous CaMKIIα-Cre$^{+/Cre}$ mice were bred with Rosa26$^{+/stop\text{-}lacZ}$ mice that harbored a floxed stop cassette upstream of the *β-galactosidase* (lacZ) gene at the ubiquitously expressed ROSA locus.[18] The F1 male offspring (Rosa26$^{+/stop\text{-}lacZ}$::CaMKIIα-Cre$^{+/Cre}$) were killed at 10 weeks of age (Figure 1a), and the activity of Cre recombinase was determined by X-gal staining of diverse organs including the brain, heart, kidney, liver, lung, spleen and testis. In agreement with the previous report, Rosa26$^{+/stop\text{-}lacZ}$::CaMKIIα-Cre$^{+/Cre}$ mice exhibited intense X-gal staining only in the brain and testis (Figure 1b). Other organs were essentially negative (data not shown). A series of coronal images reconstructed along the anterior-posterior axis of the whole mouse brain revealed X-gal-positive signals in the main olfactory bulb, cerebral cortex, striatum, septal nucleus, hippocampus, dentate gyrus, hypothalamus, ventral midbrain and few dorsal nuclei of medulla oblongata. By comparison, X-gal signals were not detected or very low in the thalamus, pons and cerebellum (Figure 1c).

Interestingly, the cross section of the testis showed a mosaic pattern of lacZ staining (Figure 1d). Negative (arrows) and positive (arrowhead) seminiferous tubules were intermingled in the testis, suggesting that the Cre recombinase was activated and promoted mosaic deletion of floxed allele in subpopulations of the seminiferous tubules. Therefore, when these F1 male Rosa26$^{+/stop\text{-}lacZ}$::CaMKIIα-Cre$^{+/Cre}$ mice were crossed with wild-type female mice, 64 out of 117 offspring were stained with X-gal in their entire body (Figure 1e). The segregation pattern for both the Cre transgene and the target gene was analyzed by PCR with genomic DNA obtained from tail biopsies (Table 2). Out of 117 F2 offspring, 54 (46%) carried the Cre transgene indicating that the Cre allele is equally segregated in F1 germ cells or F2 offspring independently of Rosa26$^{stop\text{-}lacZ}$ allele. Interestingly, 31 out of 64 X-gal positive offspring with a genotype of Rosa26$^{+/lacZ}$ (recombined allele) did not inherit the Cre transgene. These results suggest that Cre-mediated recombination occurs before the first meiotic division during spermatogenesis and the disrupted allele is inheritable to the progeny.

Application of CaMKIIα-Cre mediated germline recombination to floxed gnao mice

We tested whether CaMKIIα-Cre could be universally applicable to deletion of other floxed genes through germline recombination. Mice whose Go alpha subunit exons 5 and 6 were flanked with loxP (Gnao$^{f/f}$) were bred to CaMKIIα-Cre$^{+/Cre}$ mice. F1 male offspring with a genotype of Gnao$^{+/f}$::CaMKIIα-Cre$^{+/Cre}$ were killed at 10 weeks of age (Figure 2a), and genomic DNA (gDNA) from the tail and mRNA from diverse organs was isolated for PCR and RT–PCR analyses, respectively. The Cre mRNA was expressed only in the brain and testis, where the Cre recombinase deleted the floxed exon 5 and 6 segment of the *Gnao* gene yielding the disrupted Gnao allele (Gnao$^{\Delta}$) (Figure 2b, lanes 2, 8). Cre mRNA expression was not detectable in other organs. The F1 male Gnao$^{+/f}$::CaMKIIα-Cre$^{+/Cre}$ mice were mated to the wild-type female mice. Out of 43 F2 offspring that once had carried floxed Gnao allele,


a
Rosa26stop-lacZ
loxP
stop
loxP
lacZ
CamKIIα-Cre
F1 (♂) : Rosa26stop-lacZ::CamKIIα-Cre
F1 (♀): Wild type
Brain, testis:
(Fig. 1B~D)
Other organs :
loxP
lacZ
loxP
stop
loxP
lacZ
F2 (Fig. 1E)
b
F1 : Rosa26stop-lacZ::CamKIIα-Cre (♂)
Brain
CTX
CB
OB
TH
MB
Hy
P
MY
Testis
c
MOB
SEZ
onl
CTX
cc
CP
SN
aco
ACB
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HPF
V3
TH
CP
GP
Hy
CTX
DG
CA1
MB
CA3
IC
P
FL
PFL
CB
MY
d
e
F2
X-gal- embryos
(n=53)
X-gal+ embryos
(n=64)

31 inherited the recombined Gnao$^{\Delta}$ allele, whereas 12 still retained the intact Gnao^{f} allele, giving a recombination efficiency of 72.1% efficiency (Table 3). Again, 19 Gnao$^{\Delta}$ offspring did not inherit the Cre transgene (Figure 2c, lane 3). By comparison, the recombination efficiency for floxed Rosa26$^{stop\text{-}lacZ}$ by CaMKIIα-Cre was 98.5% and only one F2 offspring retained the original stop-lacZ sequence (Table 2). These results indicated that, although the CaMKIIα-Cre-mediated germline recombination is universally applicable to floxed genes, the recombination efficiency may vary depending on the floxed target genes.

Generation and characterization of gnao$^{\Delta/\Delta}$ mice

To generate homozygous Gnao$^{\Delta/\Delta}$ mice, we intercrossed heterozygous mice carrying the disrupted Gnao allele (Gnao$^{+/\Delta}$) without the Cre as shown in Figure 2c (lane 3), and genotyped each of F3 progeny by PCR analysis (Figure 3a). Western analysis indicated that the Goα protein was not detected at all in the brain from F3 homozygous Gnao$^{\Delta/\Delta}$ mice, whereas it was decreased to approximately one-half in the heterozygous Gnao$^{+/\Delta}$ mice (Figure 3b). Finally, we compared the locomotion activity of Gnao$^{\Delta/\Delta}$ mice with that of the Goα$^{-/-}$ null mice obtained through the conventional knockout process.[4] As reported earlier, conventional Gnao$^{-/-}$ mice exhibited lower body weight (<45% of the wild-type littermates body weight) before 3 weeks of age but gained weight to the similar level of their wild-type littermates at 8 weeks of age.[4] In addition, they showed behavioral defects such as low survival rates, generalized tremor, hyperactive locomotion and turning behavior.[4] Similarly, Gnao$^{\Delta/\Delta}$ mice exhibited perinatal death and lower body weight than the wild-type littermates at 2 weeks of age. When they survived, the weight difference gradually diminished toward adulthood (data not shown). Similar to the previously reported Gnao$^{-/-}$ null mice, the locomotor activity of Gnao$^{\Delta/\Delta}$ mice was significantly increased and the duration of the mice in the center of an open field was markedly increased compared with that of the wild-type litter mates in an open field test (Figure 3c). The results clearly demonstrated that it is possible to generate a conventional knockout line from a conditional line by crossing with CaMKIIα-Cre mice.

DISCUSSION

Goα is one of the most abundant membrane proteins in the brain, but its functions are poorly understood. To understand the Goα functions in the adult brain, targeted disruption of Gnao is a method of choice. However, the deletion of Goα by the conventional knockout technology is associated with low survival rates of neonates.[4] In addition, continuous intercross of the original heterozygous Goα-null mice has progressively further lowered the birth rates of homozygous pups, probably due to unknown functions of Goα in the prenatal period. Fortunately, the development of conditional knockout mice has allowed us to disrupt the gene in a tissue selective manner. The conditional gene knockout technique is based on phage-derived Cre/loxP or yeast-derived Flp/FRT systems.[20] Both recombinases are functional and act with similar efficiency, but the most widely used method is the Cre/loxP system. The 38 kDa Cre recombinase catalyzes DNA recombination between specific 34-bp sequences called loxP,[21] thus Cre-mediated recombination can be applied to

Table 2 Summary of the F2 offspring from mating shown in Figure1a

Genotypes of breeding pairs	*F2 offspring*		
	Floxed allele	*Cre*	*No. offspring (%)*
F1 male: Rosa26$^{+/stop\text{-}lacZ}$:: CaMKIIα-Cre$^{+/Cre}$	+/stop-lacZ	+	1 (0.9)
	+/stop-lacZ	Cre	0 (0.0)
	+/lacZ	+	31 (26.5)
	+/lacZ	Cre	33 (28.2)
Female: wild type	+/+	+	31 (26.5)
	+/+	Cre	21 (17.9)
	Total		117 (100)

The lacZ and stop-lacZ alleles were determined by X-gal staining. The CaMKIIα-driven Cre recombinase deleted the stop sequence and the resulting lacZ embryos were X-gal positive in the entire body (Figure 1e).

Figure 1 CaMKIIα-Cre activity in brain and testis. (**a**) Breeding scheme for generation of the F1 Rosa26$^{+/stop\text{-}lacZ}$::CaMKIIα-Cre$^{+/Cre}$ and the F2 offspring. The Rosa26$^{stop\text{-}lacZ}$ transgene carries the stop sequences flanked by loxP sites and lacZ is not expressed (X-gal negative, white box). After Cre deletes the stop sequence in front of the lacZ gene, lacZ is expressed (X-gal positive, blue box). (**b**) The brain and testis from male Rosa26$^{+/stop\text{-}lacZ}$::CaMKIIα-Cre$^{+/Cre}$ mice were stained for β-galactosidase activity using X-gal. The only X-gal-positive tissues were brain and testis among major organs. (**c**) Serial coronal sections of the brain with a 2 mm thickness show X-gal-positive signals in ACB, nucleus accumbens; CA1,3, hippocampus CA1,3; CP, caudaoputamen; CTX, cortex; GP, globus pallidus; HPF, hippocampal formation; Hy, hypothalamus; MOB, main olfactory bulb; SN, septal nucleus, but the absence of X-gal reactivity in CB, cerebellum; cc, corpus callosum; FL, flocculus; IC, inferior colliculus; MB, midbrain; MY, medullar; onl, olfactory nerve layer; P, pons; PFL, paraflocculus; SEZ, subependymal zone; TH, thalamus; V3, third ventricle. (**d**) The top panel shows coexistence of seminiferous tubules with Cre recombinase activity (X-gal positive, arrow head) and without Cre recombinase activity (X-gal negative, arrow) in the testis of Rosa26$^{+/stop\text{-}lacZ}$::CaMKIIα-Cre$^{+/Cre}$ mice. The section is lightly counterstained with nuclear fast red. Scale bar = 100 μm. The bottom panel is a high magnification of the boxed area. Scale bar, 20 μm. (**e**) Male Rosa26$^{+/stop\text{-}lacZ}$::CaMKIIα-Cre$^{+/Cre}$ mice were crossbred with wild-type female mice. Offspring of 13.5 embryonic days carrying the recombined allele were whole-body blue by X-gal staining.

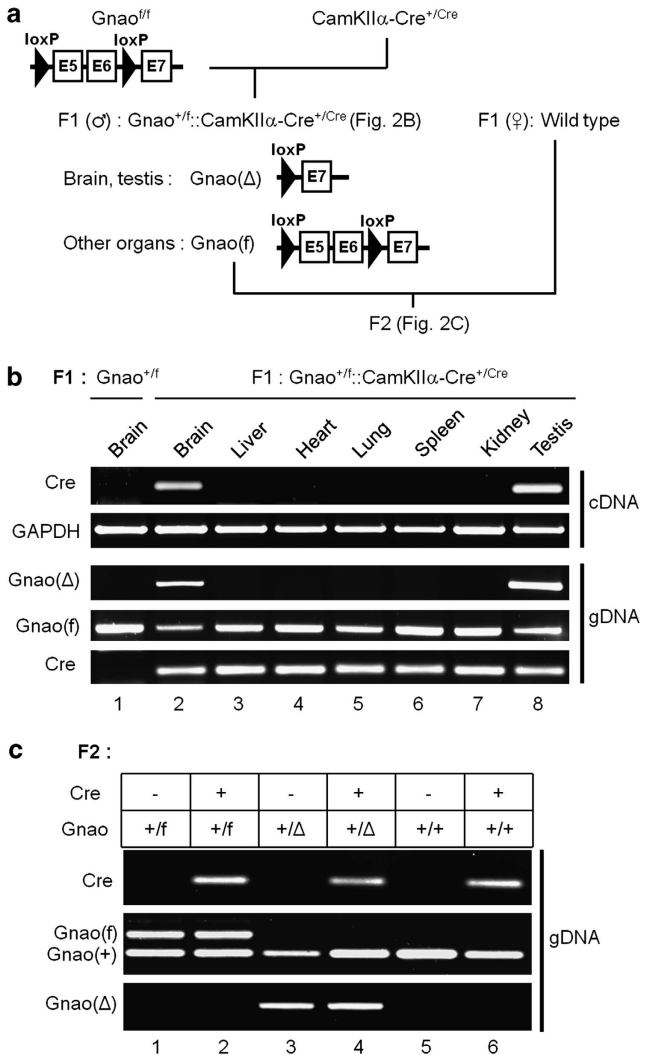

Figure 2 CaMKIIα-Cre mediated disruption of the Gnao allele in the brain and testis. (**a**) Breeding scheme for generation of the F1 Gnao$^{+/f}$::CamKIIα-Cre$^{+/Cre}$ mice and the F2 generation. Cre-mediated recombination leads to the deletion of exons 5 and 6 of the Gnao allele (Gnao$^{\Delta}$) in the brain and testis. (**b**) Genomic DNA (gDNA) from major organs were examined by PCR for recombination of floxed alleles in male Gnao$^{+/f}$::CamKIIα-Cre$^{+/Cre}$ mice. Recombination was observed in the brain and testis, but not in other organs. When examined for cDNA by RT–PCR, expression of the Cre transgene transcript was detected in both the brain and testis but not in other organs. Gnao$^{+/f}$ from parallel breeding was used as a control. (**c**) The male Gnao$^{+/f}$::CamKIIα-Cre$^{+/Cre}$ mice were mated to the wild-type females. Six different genotypes were identified in the F2 offspring.

Table 3 Summary of the F2 offspring from mating shown in Figure 2a

Genotypes of breeding pairs	*F2 offspring*		
	Floxed allele	*Cre*	*No. offspring (%)*
F1 male:	+/f	+	6 (6.9)
Gnao$^{+/f}$::	+/f	Cre	6 (6.9)
CaMKIIα-Cre$^{+/Cre}$	+/Δ	+	19 (21.8)
Female: wild type	+/Δ	Cre	12 (13.8)
	+/+	+	18 (20.7)
	+/+	Cre	26 (29.9)
	Total		87 (100)

The CaMKIIα-driven Cre recombinase deleted the exon 5 and 6 of Gnao^{f} and yielded Gnao$^{\Delta}$ alleles (Figure 2a). The Gnao^{f} and Gnao$^{\Delta}$ alleles were distinguished by PCR using each set of Gnao or Gnao$^{\Delta}$ primers (Table 1, Figure 2c).

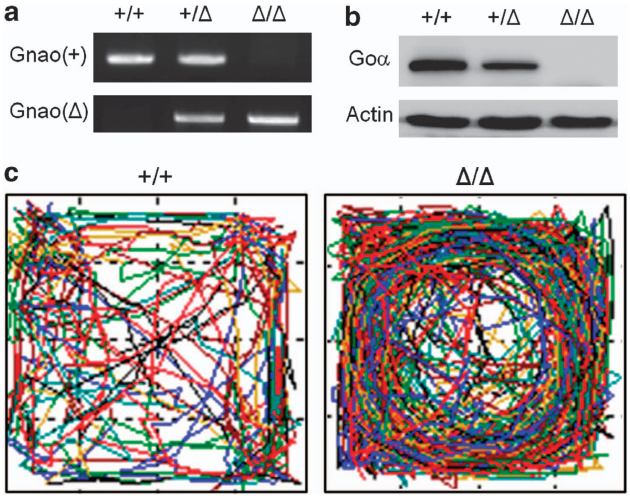

Figure 3 Hyperactive locomotion activity of Gnao$^{\Delta/\Delta}$ mice. Heterozygous Gnao$^{+/\Delta}$ mice were intercrossed to obtain F3 Gnao$^{\Delta/\Delta}$ mice. (**a**) Genotypes of the F3 were analyzed by PCR analysis of genomic DNA isolated from the tail. (**b**) Deletion of Gnao in Gnao$^{\Delta/\Delta}$ mice was verified by western analysis of brain extracts. (**c**) Representative data recorded for 1 h showed hyper locomotor activity of Gnao$^{\Delta/\Delta}$ mice in an open field test.

various types of gene manipulation. In addition, the use of specific promoters to regulate the Cre recombinase has allowed gene deletion in a tissue- or time-specific manner.

In the CaMKIIα-Cre mice, the Cre recombinase is under the control of the mouse CaMKIIα promoter and was first used to attempt gene inactivation in limited areas of the brain.[22] This transgene drives the expression of Cre recombinase in the cerebral cortex, striatum and hippocampus causing inactivation of the target genes in these regions.[11] Using CaMKIIα-Cre mice, we were able to selectively delete the target genes in the brain, thus the F1 males carrying Rosa26$^{+/stop\text{-}lacZ}$ and CaMKIIα-Cre$^{+/Cre}$ genotype showed lacZ expression in the expected areas of the brain (Figures 1b and c). We also found the Cre activity in the testis, which caused recombination in the male gametes. Our results clarify the germline recombination of CaMKIIα-

Cre, which had been anecdotally known, by presenting the quantitative analysis of the progeny in two floxed genes. However, the efficiency may differ depending on the genes. For Rosa26$^{stop\text{-}lacZ}$, 98% of the floxed gene was recombined (64 out of 65), whereas 72.1% for Gnao^{f} (31 out of 43) (Tables 2 and 3). One possible explanation for this difference between Rosa26$^{stop\text{-}lacZ}$ and Gnao^{f} is the accessibility of loxP sites integrated in target genes. It has been reported that DNA methylation, one of the primary mechanisms of DNA modification, can influence the accessibility of the loxP sites for Cre-mediated recombination.[23] Therefore, the recombination efficiency may also vary due to the locus into which the Cre transgene is integrated.

Interestingly, germline recombination has been previously reported with Synapsin1 (Syn1) promoter-driven Cre, which is also utilized for brain-specific recombination.[24–26] However, in contrast to CaMKIIα that is naturally active and induces production of the native CaMKIIα protein in the testis,[14,15] the native Syn1 protein is not found in the testis. Nevertheless, the Syn1 promoter is aberrantly active and induces the Cre expression.[27] Similarly, cornea-specific Keratocan-Cre,[28] skin-specific Keratin-Cre,[29] endothelium-specific Tie2-Cre and smooth muscle-specific Smmhc-Cre mice[30] also show unexpected Cre expression in the testis. Recently, genome-wide studies have revealed that transcriptomes are highly complex in the brain and testis compared with the other organs.[31] The high complexity of the testis arises from 'leaky' transcription of functional and nonfunctional portions of the genome from transcriptionally permissive chromatin[32] as a result of continuous repackaging of DNA into a high degree of chromatin compaction during spermatogenesis.[33] Thus, unexpected expression of the Cre transgene is probably due to the functionally irrelevant consequence of chromatin remodeling and aberrant activation of the transgene promoter in the testis.

In the present work, we have conclusively demonstrated CaMKIIα-Cre-mediated germline recombination and important implications in Cre/loxP-mediated conditional gene ablation with a tissue-specific promoter that only allows Cre production in limited tissues. A set of PCR primers that can distinguish between the floxed and recombined allele will be helpful to detect unexpected recombination of the floxed allele. Our method of using CaMKIIα-Cre mice is effective for simultaneously disrupting the gene in the brain for the study of neurological functions and in male germ cells to generate constitutive knockout mice.

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