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Generation of L7-tTA Knock-in Mice

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We generated a versatile mouse line, L7-tTA knock-in mouse, in which tetracycline-responsive transcriptional activator (*tTA*) gene was introduced into exon 2 of *L7* locus. Since *L7* is specifically expressed in cerebellar Purkinje cells, we expected Purkinje cell-restricted expression of *tTA* gene in the knock-in mice. *In situ* hybridization analysis exhibited that *tTA* mRNAs in those mice were expressed only in Purkinje cells. Introduction of transgene consisting of tetracycline-responsive element that is a binding site for tTA and ideal cDNAs into L7-tTA knock-in mouse would result in specific expression of cDNA encoding proteins in Purkinje cells and its expression could be controlled by doxycycline administration.

L7-tTA knock-in mice would provide us with opportunity to elucidate the role of specific genes in cerebellar Purkinje cells.

Tetracycline-responsive transcriptional activator (tTA) is a transcriptional factor which binds tetracycline-responsive element (TRE), and then activates transcription of a gene downstream of TRE. tTA is inactivated by antibiotic tetracycline or its analogue doxycycline (DOX), and activated by withdraw of DOX. This tetracycline-regulated system is very useful because a gene expression downstream of TRE can be controlled by DOX administration at specific developmental stage (2, 5,10).

The Purkinje cell (PC) is an only output neuron in cerebellar cortex. Proximal dendrites of each PC are innervated by a single climbing fiber (CF) in adult brain and form glutamatergic synapses. PC is innervated by multiple CFs during early postnatal development, but establishes mono innervation by the end of the third postnatal week in mice (9, 16). Distal dendrites of PCs are innervated by parallel fibers (PFs), another glutamatergic input. To explore the molecular mechanism of motor learning, synaptic plasticity and neural circuit refinement that require PC function, we need the mice expressing the specific genes exclusively in PCs. *L7* gene is expressed in PCs and retinal bipolar cells (3, 4). So we planed to insert *tTA* gene into *L7* locus to accomplish *tTA* expression in PCs.

The postsynapses of CF-PC and PF-PC synapses contain metabotropic glutamate receptor 1 (mGluR1) and α-amino-3 hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor (6, 13, 15). PF-PC synapses, but not CF-PC synapses, have glutamate receptor delta 2 (GluRδ2) (11). These glutamate receptors are involved in cerebellar motor learning (6, 7,

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8). For example, mGluR1 knock-out mice exhibit deficient LTD, motor discoordination, and multiple innervations of CF in adulthood (1, 7, 9). These glutamate receptors are good candidates to be analyzed using mice expressing tTA in PCs.

In this study, we generated L7-tTA knock-in mice which possess *tTA* gene downstream of *L7* promoter and confirmed that *tTA* mRNA was exclusively expressed in PCs in L7-tTA knock-in brain.

MATERIALS AND METHODS

This study was conducted in accordance with the guidelines of Kobe University and Hokkaido University. Mice were housed in a temperature- and humidity- controlled room under a 12-hr light and dark cycle with food and water ad libitum. All restriction enzymes and pKF3 vectors were purchased from Takara. Biodyne PLUS membrane was purchased from Pall corporation. RPCI-22 129 mouse BAC genomic library was purchased from CHORI BACPAC Resources.

Targeting strategy. A 10.9 kb genomic DNA fragment containing *L7* gene was obtained from a bacterial artificial chromosome (BAC) clone obtained by screening an RPCI-22 129 mouse BAC genomic library. *tTA* gene was cut out from pTet-Off vector (CLONTECH) and was inserted into PvuI site of exon 2 of *L7* gene. *loxP*-*neo*^{r} cassette containing neomycin resistant gene driven by the HSV-*tk* promoter flanked by two *loxP* sequences was inserted into the ApaI site in the intron between exons 1 and 2 of *L7* gene as a positive selection marker. The diphtheria toxin A fragment (*DT-A*) gene driven by the MC1 promoter was inserted into BalI site at the 3′ end of the targeting vector as a negative selection marker.

Generation of L7-tTA knock-in mice. 129/Ola-derived EB3 ES cells (3.2×10^7 cells) were transfected with 100 µg of the linearized targeting vector by electroporation (Bio-Rad Gene Pulser, 0.8 kV/3 µF). Followed by selection with G418 (175 or 200 µg/ml) 24hr after the transfection, 168 G418-resistant colonies were picked up on day 7 and 8 of the selection, and the genomic DNAs from the ES cells were subjected to Southern blot and PCR analysis for confirmation of homologous recombination. For 5’ probe, the genomic DNAs were digested with KpnI and XhoI, separated on a 0.8% agarose gel and transferred to Biodyne PLUS membrane. For 3’ probe, the genomic DNAs were digested with EcoRV, separated on a 0.8% agarose gel and transferred to Biodyne PLUS membrane. A 630 bp DNA fragment positioning upstream of *L7* gene coding region and a 250 bp fragment positioning downstream of *L7* gene coding region were used as hybridization probes. Hybridization probes were visualized by using Bio-Imaging Analyzer BAS-2500 (Fuji Photo Film). The targeted ES clones isolated were microinjected into C57BL/6 blastocysts to generate chimeric mice. The chimeric males were mated with C57BL/6 females. Germ line transmission of the targeted allele was confirmed by Southern blot analysis of the genomic DNA prepared from these litters. After heterozygous mutant animals were obtained, the *neo*^{r} gene was deleted by mating with adult EIIa-Cre transgenic mice. To examine excision of the *neo*^{r} gene, genomic DNAs isolated from the mice tail were subjected to PCR analysis.

RT-PCR analysis. To detect *tTA* mRNA, RT-PCR analysis was conducted. Total RNAs were isolated from cerebella, cerebral cortices and whole brains. The first strand cDNA was synthesized as a template of PCR analysis with a set of primers for tTA (5’-AAGTGATTAACAGCGCATTAG-3’ and 5’-TAGCGCTACTGAATCATTTCG-3’).

***In situ* hybridization.** To detect *tTA* mRNAs, *in situ* hybridization analysis was carried out using an antisense oligonucleotide probe. The probe sequence was 5’-GGGGGCGTCGTCGTCCGGGAGATCGAGCAGGCCCTCGATGGTAGACCCGT-3’. Under deep pentobarbital anesthesia, whole brains were taken out from adult mice. Fresh

frozen brain sections (20 μm) in the parasagittal plane were mounted on grass slides coated with 3-aminopropyltriethoxysilane. The sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min, incubated 2 mg/ml glycine in phosphate-buffered saline for 10 min, acetylation with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min. Then the sections were prehybridized for 1 hr in a buffer containing 50% formamide, 50 mM Tris-HCl (pH 7.5), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.25% SDS, 0.6 M NaCl, 1 mM EDTA and 200 μg/ml tRNA. All incubations mentioned above were conducted at room temperature. Hybridization was performed at 42℃ for 10 h in the prehybridization buffer mixed with 10% dextran sulfate and 10000 c.p.m./μl of ^{33}P-labeled probe. The sections were washed twice at 55℃ for 40 min in 0.1×SSC containing 0.1% sarcosyl. The hybridized signals were visualized using BioMax film (Kodak) for 4 weeks.

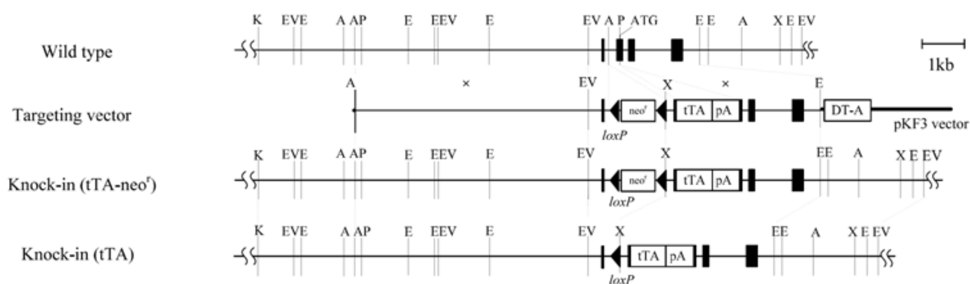

Figure 1 Targeting strategy of L7-tTA knock-in mouse. An initiation codon of *L7* gene exists in the exon 2. *tTA* gene was inserted into PvuI site upstream of the initiation codon in exon 2. Neomycin resistant gene (*neo^r*) was flanked by *loxP* sequences and inserted into ApaI site. The *neo^r* can be deleted by Cre-*loxP* recombination. DT-A gene was inserted downstream of the exon 4. Restriction enzymes sites; A, ApaI; E, EcoRI; EV, EcoRV; K, KpnI; P, PvuI; X, XhoI.

RESULT

Construction of a targeting vector. We generated L7-tTA knock-in mouse. A targeting vector was constructed from an RPCI-22 129 mouse BAC genomic DNA clone that contains exons 1 to 4 of the mouse *L7* gene. This vector has a *tTA* gene upstream of an initiation codon of *L7* gene in exon 2, and a *loxP-neo^r* cassette between exons 1 and 2. *neo^r* gene was flanked by two *loxP* sequences. When Cre enzyme acts on it, *neo^r* gene is excised. *DT-A* gene was inserted into BalI site of the vector as a negative selection marker (Figure 1).

Generation of L7-tTA knock-in mice. The targeting vector was linearized by restriction enzyme KpnI. The linearized vector was electroporated into 129/Ola-derived EB3 ES cells. After 24 hr from the transfection, 168 G418 resistant colonies were picked up. To screen correct homologous recombinant clones, Southern blot analysis was conducted along Figure 2 (data not shown). We obtained 13 homologous recombinant clones from 168 G418 resistant clones. Percentage of homologous recombinant clones which we obtained was 7.7%. Five homologous recombinant clones were injected into C57BL/6 blastocysts to obtain male chimeric mice. The male chimeric mice were mated with female C57BL/6 mice to obtained L7 (+/tTA-neo^r) mice (F1 generation). Genotypes of these mice were determined by Southern blot analysis of the tail DNAs (Figure 2). In Southern blot analysis using 5’ probe,

we detected the 12.4 kb fragment in wild-type mouse and the 9.6 kb and 12.4 kb fragments were detected in L7 (+/tTA-neo^r) mouse. In Southern blot analysis using 3' probe, we detected the 5.1 kb fragment in wild-type mouse and the 8.0 kb and 5.1 kb fragments were detected in L7 (+/tTA-neo^r) mouse. These results showed that homologous recombination between wild-type allele and targeting vector occurred correctly. Interbreeding of L7 (+/tTA-neo^r) mice produced L7 (tTA-neo^r/tTA-neo^r) mice. Gross appearance of L7 (tTA-neo^r/tTA-neo^r) mice was indistinguishable from wild-type littermates. L7 (tTA-neo^r/tTA-neo^r) mice showed no apparent abnormalities in their gait or motor coordination.

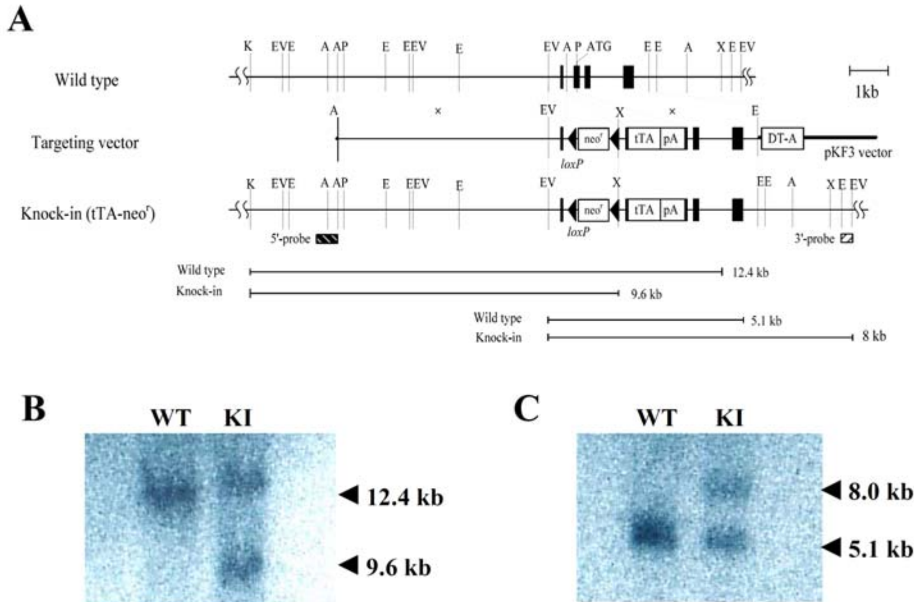

Figure 2 Southern blot analysis of tail DNAs from F1 mice. (A) Strategy of Southern blot analysis. (B) Southern blot analysis using 5' probe. Genomic DNAs were digested by KpnI and XhoI. The 12.4 kb fragment was detected in wild-type mouse (WT). The 9.6 kb fragment was detected in L7 (+/tTA-neo^r) mouse (KI). (C) Southern blot analysis using 3' probe. F1 tail DNAs were digested by EcoRV. The 5.1 kb fragment was detected in WT. The 8.0 kb fragment was detected in KI.
Restriction enzyme sites: A, ApaI; E, EcoRI; EV, EcoRV; K, KpnI; P, PvuI; X, XhoI.

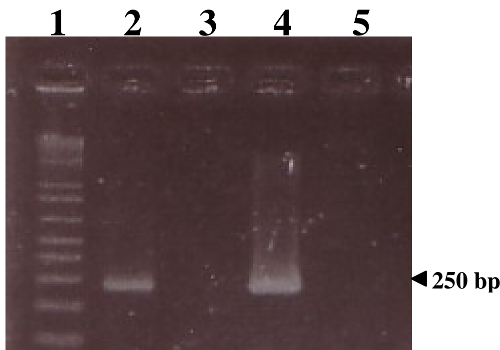

Figure 3 RT-PCR analysis of RNA from L7-tTA knock-in mouse. RT-PCR analysis showed 250 bp band for *tTA* mRNA. Lane 1, size marker; lane 2, cerebellum of L7 (+/tTA-neo^r) mouse; lane 3, cerebral cortex of L7 (+/tTA-neo^r) mouse; lane 4, whole brain of CaMKII-tTA transgenic mouse; lane 5, whole brain of wild-type mouse.

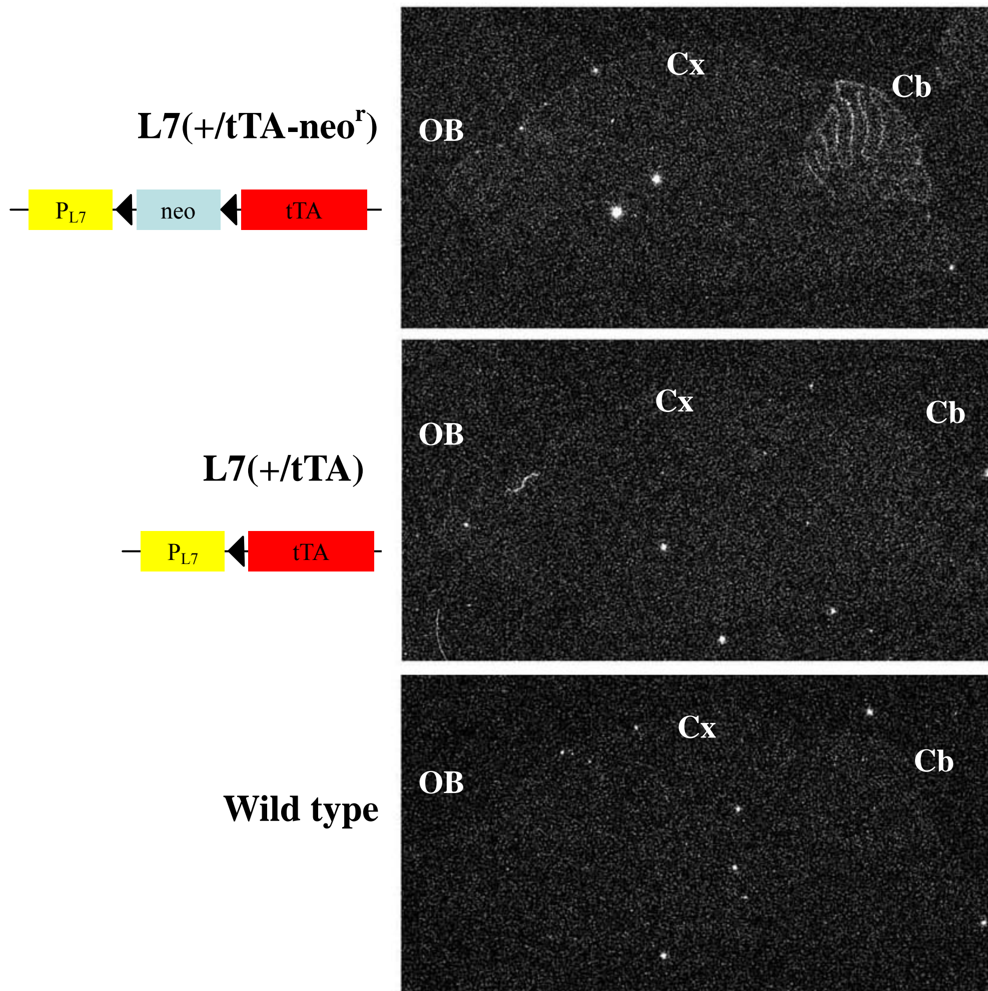

Figure 4 *In situ* hybridization analysis for *tTA* mRNA in parasagittal sections of the brains. ^{33}P-labelled antisense probes for *tTA* mRNA were hybridized. Top, L7 (+/tTA-neo^r) brain. Middle, L7 (+/tTA) brain. Bottom, wild-type brain. Specific signals for *tTA* mRNA were observed in the cerebellum of L7 (+/tTA-neo^r) brain. These signals disappeared in L7 (+/tTA) brain in which neomycin resistant gene (*neo^r*) was deleted by Cre-*loxP* recombination. OB, olfactory bulb; Cx, cerebral cortex; Cb, cerebellum.

Expression of *tTA* mRNA in L7-tTA knock-in mice. The expression of *tTA* mRNA was confirmed by RT-PCR of total RNA from cerebellum of L7 (+/tTA-neo^r) mouse and whole brain of CaMKII-tTA transgenic mouse as positive control. *tTA* mRNA was not detected in cerebral cortex of L7 (+/tTA-neo^r) mouse or whole brain of wild-type mouse (Figure 3).

***In situ* hybridization analysis of L7-tTA knock-in mice.** We obtained L7 (+/tTA) mice by mating L7 (+/tTA-neo^r) mice (F1 generation) with EIIa-Cre transgenic mice which express Cre in preimplantation embryos. To confirm *tTA* was expressed in PCs, *in situ* hybridization analysis using ^{33}P-labelled probe was conducted. L7 (+/tTA-neo^r) mouse showed PC-specific expression of *tTA* gene (Figure 4). *tTA* mRNA signals were not detected

in the cerebral cortex or olfactory bulb. These results are consistent with the results obtained by RT-PCR analysis. However *tTA* mRNA signals were not detected in any regions in the brain of L7 (+/tTA) mouse (Figure 4).

DISCUSSION

In this study, we generated L7-tTA knock-in mice in which *tTA* was introduced into *L7* locus. L7 is known as a Purkinje cell protein-2 (Pcp2) and is expected to play important roles in PC function (14). L7 protein interacts with heterotrimeric G protein $G_{\alpha o}$ *in vitro* and they were co-immunoprecipitated from the lysate of the COS cells which were co-transfected with *L7* and $G_{\alpha o}$ genes (12). Since L7 stimulates GDP release from $G_{\alpha o}$, L7 functions as a nucleotide exchange factor for $G_{\alpha o}$. Because $G_{\alpha o}$ is expressed in central nervous system including PCs and heart (12), knockout of *L7* could result in dysfunction of PC. *L7* gene was expected to be disrupted by introducing *tTA* gene into exon 2 in our L7-tTA knock-in mice. However, we could not find ataxia or other cerebellum-related abnormalities in L7 (tTA-neo^r /tTA-neo^r) mice. This result is consistent with a previous report that conventional L7 knock-out mice exhibited no abnormality in their behavior (14).

We established a mouse line which expresses *tTA* mRNAs specifically in PCs. The L7-tTA knock-in mice would be mated with transgenic mice which possess TRE and cDNA of the genes which could play roles in PCs function downstream of TRE. mGluR1, AMPA receptor and GluRδ2 are good candidates, because they play important roles in PCs (1, 6, 7, 8, 11, 13). For example, when the transgenic mice possessing TRE upstream of mGluR1 cDNA and L7-tTA knock-in mice are crossed with mGluR1 knock-out mice, we will obtain the mice expressing mGluR1 in PCs. The expression of mGluR1 in this mouse could be stopped by DOX administration in specific developmental stage.

As discussed above, tetracycline-regulated system used in this study is useful to study specific gene function in a specific region at a certain developmental stage. Conventional knock-out mouse technology has limitations when gene function at a certain developmental stage must be elucidated or when conventional knock-out mouse results in a lethal phenotype (2). These problems can be solved by tetracycline-regulated system (2, 5, 10). We used knock-in strategy to express *tTA* with PC specific manner. Transgenic approach could also be achieved by using same *L7* promoter. However, the region-specificity and strength of the *tTA* expression could be affected by the chromosomal locus where the transgene has been inserted. Our knock-in strategy has an advantage of ensuring that the *tTA* gene is regulated in a PC specific manner. On the other hand, we did not detect *tTA* mRNA in cerebellum of L7 (+/tTA) mouse in spite of expression of *tTA* mRNAs in L7 (+/tTA-neo^r) mouse. These results suggest that HSV-*tk* promoter or *neo^r* gene by itself in *loxP-neo^r* cassette affect the transcription of *tTA* gene in L7 (+/tTA-neo^r) mice.

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