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# Motor Deficits and Hyperactivity in Cerebral Cortex-specific *Dyt1* Conditional Knockout Mice

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**DYT1 dystonia is a primary generalized early-onset torsion dystonia caused by mutations in *DYT1* that codes for torsinA and has an autosomal dominant inheritance pattern with ~30% penetrance. Abnormal activity in the pallidal–thalamic–cortical circuit, especially in the globus pallidus internus, is the proposed cause of dystonic symptoms. However, recent neuroimaging studies suggest significant contribution of the cerebral cortex. To understand the contribution of the cerebral cortex to dystonia, we produced cerebral cortex-specific *Dyt1* conditional knockout mice and analysed their behaviour. The conditional knockout mice exhibited motor deficits and hyperactivity that mimic the reported behavioural deficits in *Dyt1* ΔGAG knockin heterozygous and *Dyt1* knockdown mice. Although the latter two mice exhibit lower levels of dopamine metabolites in the striatum, the conditional knockout mice did not show significant alterations in the striatal dopamine and its metabolites levels. The conditional knockout mice had well-developed whisker-related patterns in somatosensory cortex, suggesting formations of synapses and neural circuits were largely unaffected. The results suggest that the loss of torsinA function in the cerebral cortex alone is sufficient to induce behavioural deficits associated with *Dyt1* ΔGAG knockin mutation. Developing drugs targeting the cerebral cortex may produce novel medical treatments for DYT1 dystonia patients.**

**Key words: cerebral cortex, conditional knockout mouse, DYT1 dystonia, early-onset dystonia, torsinA.**

Abbreviations: DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; *DYT1* (*TOR1A*), TorsinA gene in human; *Dyt1* (*Tor1a*), TorsinA gene in mouse; HVA, homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid); KO mouse, knockout mouse.

Dystonia is a movement disorder defined by patterned, directional and often sustained muscle contractions that causes twisting and repetitive movements or abnormal postures. Dystonia is topographically classified into five groups, *i.e.* focal, segmental, multifocal, generalized and hemi-dystonia (*1*). DYT1 dystonia is a primary generalized early-onset torsion dystonia (*2*). Symptoms usually appear in childhood or adolescence, first affecting a limb and eventually traveling to other limbs (*3*). DYT1 dystonia is an autosomal dominant genetic disease with ~30% penetrance (*4*). Mutational analysis identified a 3-bp deletion of GAG codon in *DYT1* (*TOR1A*) corresponding to a glutamic acid residue (torsinA$^{\Delta E}$) in the C-terminal region of the encoded torsinA (*5*). An 18 bp deletion in *DYT1* was also reported in dystonia patients (*6*, *7*). TorsinA is a member of the AAA$^{+}$ superfamily of ATPase that belongs to a class of molecular chaperones. TorsinA is widely expressed throughout the central nervous system in human and rodents (*8–12*). In rodents, torsinA is highly expressed during prenatal and early postnatal development.

Several mouse lines have been produced to model DYT1 dystonia. Transgenic mice over-expressing the human torsinA$^{\Delta E}$ showed circling and hyperactivity and impaired motor learning (*13*, *14*). *Dyt1* ΔGAG knockin heterozygous mouse exhibited deficits in fine motor coordination and balance (*15*). Similar motor deficits were also observed in *Dyt1* knockdown (KD) mice suggesting that a partial loss of torsinA function contributes to the pathology (*16*). Furthermore, both *Dyt1* ΔGAG knockin homozygous and *Dyt1* knockout mice lacking exons 2–4 died during early neonatal development, suggesting torsinA$^{\Delta E}$ protein is not fully functional (*15*, *17*).

Pathological studies of patient brains suggest that dystonia has a functional rather than degenerative aetiology (*18*). Abnormal activities in the pallidal–thalamic–cortical circuitry are thought to be involved in primary dystonia (*19*, *20*). Surgical interventions in this circuit have proven effective in treating dystonia (*21*). For example, destructive pallidotomy has been used for drug-resistant dystonia (*22*) and deep brain stimulation

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(DBS) of the globus pallidus interna (GPi) yields beneficial results in generalized dystonia (*23*, *24*). As a result of the success in treating dystonia by pallidotomy and bilateral pallidal DBS, the GPi has been thought to be a major contributor to the pathogenesis of this disease, while the cortex, thalamus, brainstem and spinal cord are believed to not be the source of dysfunction in primary dystonia (*20*).

However, abnormal activity in the cerebral cortex has been reported in several types of dystonia (*25*). Occupational focal dystonia is thought to be associated with organizational changes in the cortical somatosensory presentation of the hand (*26*). A reduction in regional cerebral blood flow was reported in severe idiopathic generalized dystonia patients (*27*). Furthermore, abnormal activities in the cerebral cortex were observed in DYT1 dystonia patients and mutation carriers in studies using positron emission tomography (*28*, *29*). Therefore, abnormal activities in the cerebral cortex seem to be a common phenomenon in these dystonia. The cerebral cortex is well-known to play an important role in the motor system (*30*). However, the abnormal activities in DYT1 dystonia patients and the carriers are often observed together with abnormal activities in other brain regions. Therefore, it is not clear whether the cerebral cortex is a source of dysfunction or the abnormal activity in the cerebral cortex is caused by pathological changes in other brain region. To analyse the effect of the loss of torsinA function in cerebral cortex, we employed a genetic approach using conditional mutagenesis to inactivate *Dyt1* in the developing mouse cerebral cortex. The conditional knockout mice exhibited motor deficits that mimic *Dyt1* ΔGAG knockin heterozygous and *Dyt1* KD mice.

## MATERIALS AND METHODS

*Making of* Dyt1 loxPneo *Mice—Dyt1* of 129/Sv origin was subcloned as described previously (*15*). A targeting construct was made with two *loxP* sites flanking exons 3 and 4 with a *PGKneo* cassette flanked by two *FRT* sites (*31*, *32*). The construct was transfected into D3 129/Sv ES cells and the clones that had appropriately undergone homologous recombination were identified by Southern blot analysis. DNA fragments for the 5′ external probe was used to identify the homologous recombinants as described previously (*15*). The DNA fragment for 5′ internal probe was obtained from a subclone containing a *Hind*III/*Bsp*EI digest of *Dyt1* encoding exons 3 and 4. The internal probe was used to exclude false positive clones with undesirable recombination between two *loxP* sites. The transfectants were microinjected into C57BL/6 blastocysts (*33*). Chimeric male mice were generated and crossed with C57BL/6 females to transmit the germline of the targeted mutation. The progenies with the mutation were named *Dyt1 loxPneo* mice. The genotyping of the *loxP* locus was performed by PCR using tail DNA with a combination of F (5′-ATTCAAAAATGTT GTCATAGCCAGG-3′) and T (5′-CTACAGTGACCTGAA TCATGTGGC -3′) primers.

*Making of* Dyt1 *Knockout Mice—Dyt1 loxPneo* mice were crossed with *CMV-cre* BALB/c mice (Jackson Laboratory, stock no. 003465) to delete *Dyt1* exons 3 and 4 (*34*). Deletion of the exons was confirmed by PCR-based genotyping method (*35*). The deletion of exons 3 and 4 with neomycin cassette (*PGKneo*) were genotyped using F and pgkp (5′-TCCATCTGCACGAGACTAGTG AG-3′) primers. The progenies carrying the deleted locus with the neomycin cassette were mated with *FLP* mice (Jackson Laboratory, Stock no. 003946) to remove the cassette (*36*). Mice heterozygous for the deleted locus (Δ) were intercrossed to produce *Dyt1* knockout homozygous (Δ/Δ) mice. The deletion of exons 3 and 4 without the cassette was confirmed by PCR using F and Tcko2 (5′-CCATAGCTGGACCTGCAATTAAG-3′) primers.

*Making of Cerebral Cortex-specific* Dyt1 *Conditional Knockout Mice—Dyt1 loxPneo* mice were mated with *FLP* mice to remove the cassette. The progenies without the cassette were named *Dyt1 loxP* mice. *Dyt1 loxP* mice were crossed with *Emx1-cre* knockin mice (*37–39*) to obtain double heterozygous mice. The double heterozygous mice were crossed with *Dyt1 loxP* homozygous mice to derive cerebral cortex-specific *Dyt1* conditional knockout mice (cKO mice) and their control littermates. Genotyping for cKO and control littermates was performed by multiplex PCR using T and F primer sets for *Dyt1 loxP*, and creA and cre6 primer sets for *cre* (*40*). Tissue-specific deletions of exons 3 and 4 were confirmed by 30 cycles of PCR using 10 ng of DNA isolated from the cerebral cortex, striatum, thalamus, mesencephalic tectum, cerebellum, medulla and spinal cord from a cKO mouse with F and Tcko2 primer sets.

*Immunohistochemistry*—The cKO and control littermate mice at 24-days old were deeply anaesthetized and perfused with 0.1 M phosphate buffer (pH 7.4; PB) followed by 4% paraformaldehyde in 0.1 M PB. Brains were dissected and fixed in the paraformaldehyde solution overnight at 4°C followed by 30% sucrose in 0.1 M PB overnight. The brains were frozen and sectioned sagittally at a thickness of 50 μm using a Histoslide 2000 sliding microtome (Reichert-Jung). The floating sections were treated with 0.3% hydrogen peroxide and blocked in PB containing Vectastain rabbit normal serum. The sections were incubated with torsinA (S-20) goat polyclonal antibody (Santa Cruz, sc-19483) at 1 : 200 dilution in the blocking solution at 4°C overnight and stained with Peroxidase goat IgG ABC kit (Vectastain, PK-4005) and peroxidase substrate DAB kit (Vector, SK-4100). They were mounted on slide, dehydrated with xylene and then coverslipped with DPX mountant (Fluka).

*Histochemical Procedures*—For staining of the whisker-related patterns in cerebral cortex of cKO and the control mice, the cortices were flattened between two glass slides for 24 h and tangential 50 μm-thick slices were cut as described previously (*41*). The slices were then washed three times with PB over a period of 1 h and incubated with the cytochrome oxidase staining solution (*41*) or with β-GAL staining solution (*42*). The reactions were stopped between 2 and 15 h, when individual whisker-related patterns were clearly discernible from the background. They were then rinsed in PB and mounted on gelatin-subbed slides, and coverslipped with an aqueous gelatin medium.

*Motor Behaviour Tests*—To compare the behaviour test results with those of previously reported *Dyt1* ΔGAG knockin heterozygous and *Dyt1* KD mice, the same mixed genetic background mice of C57BL/6, 129/Sv and BALB/c were used for motor behaviour tests. To minimize the contribution of genetic background to the behaviour, control mice were randomly selected from the littermates of each cKO mouse. Mice were housed under a 12 h-light and 12 h-dark cycle condition. Behaviour tests were performed within the last 4 h of the light period after acclimation to a sound-attenuated testing room for 1 h as described previously (*15*, *16*, *43*). A group of 17 cKO (nine males and eight females) and 18 control littermates (nine males and nine females) from 116 to 197-days old was used for the open-field test. Spontaneous activities under light condition of each mouse in an open-field apparatus (AccuScan Instruments) was automatically recorded for 15 min using DigiPro software as described (*43*). The mice were allowed to rest for 1 week and rotarod test was performed as previously published (*15*). The same group of mice from 164 to 245-days old was used for pawprint gait analysis as described previously (*15*). To compare the results with those of *Dyt1* KD mice, the pawprint gait analysis was also performed for 21 *Dyt1* KD mice (8 males and 13 females) and 20 wild-type littermates (8 males and 12 females) prepared as previously described (*16*). The second group of 16 cKO (eight males and eight females) and 17 control littermates (7 males and 10 females) from 115 to 192-days old was used for beam-walking test. Mice were trained to transverse a medium square beam (14 mm wide) in three consecutive trials each day for 2 days. The trained mice were tested twice on the medium square beam and medium round beam (17 mm diameter) on the third day and small round beam (10 mm diameter) and small square beam (7 mm wide) on the fourth day. The hindpaw slips on each side were recorded. All behavioural tests were performed by investigators blind to the genotypes.

*HPLC Analysis*—The striata were dissected from 28 mice, 11 cKO (six males and five females) and 17 control littermates (eight males and nine females) from 264 to 344-days old, for HPLC analysis at the same period of the day as described previously (*15*, *43*). The tissues were homogenized in an ice-cold 0.2 N perchloric acid (5 μl/mg tissue) solution for 1 min. The homogenates were centrifuged for 15 min at 15,000*g* at 4°C to remove debris. Ten microlitre of each supernatant was applied to a C18 reverse phase column (Varian) equilibrated with 50 mM potassium phosphate buffer with 0.5 mM octyl sulfate (Sigma) and 8% acetonitrile connected to an ESA model 5200A electrochemical detector. Dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid) (HVA) were separated at 0.8 ml/min and quantified by comparing to the standard reagents (Sigma). HPLC analysis was performed by investigators blind to the genotypes.

*Statistical Analysis*—Statistics were performed using SAS/STAT Analyst software (Version 9.1.3; SAS institute Inc. NC) as described previously (*15*, *16*, *43*). Significance was assigned at the $P < 0.05$ level. When the genotype and sex interaction had significant difference or trend ($P \leq 0.1$), the data were stratified by sex and analysed further in each group separately. Most of the data of open-field test, pawprint gait analysis and HPLC analysis were normally distributed and was analysed by ANOVA mixed model with repeated measurements as appropriate. The data of slip numbers in the beam-walking test for all four beams were analysed together by logistic regression (GENMOD) with negative binominal distribution using GEE model. This is possible, because of no significant interaction between genotype and beam. Control mice were normalized to zero.

## RESULTS

*Generation of Targeted* Dyt1 *Conditional Locus*—To investigate the contribution of torsinA in the pathogenesis of DYT1 dystonia, we made *Dyt1* knockout mice using embryonic stem cell-based gene targeting. A construct was made with two *loxP* sites flanking exons 3 and 4 (Fig. 1A). Two targeted mouse ES cell clones were isolated from 130 clones (Fig. 1B). Chimerical mice were obtained by the microinjection methods and then *Dyt1 loxPneo* mice were obtained by the breeding. The neomycin cassette and exons 3 and 4 were deleted by crossing with *FLP* mice and *CMV-cre* mice, respectively (Fig. 2A). The *Dyt1* heterozygous knockout mice (+/Δ) were crossed to produce *Dyt1* knockout (Δ/Δ) newborn pups and the genotypes were confirmed by PCR (Fig. 2B). Deletion of exons 3 and 4 caused a frame shift and created a premature stop codon in exon 5, when splicing occurred between exons 2 and 5. *Dyt1* knockout homozygous mice were easily identified as newborn pups without milk in their stomach (Fig. 2C). The neonatal lethality was similar to *Dyt1* ΔGAG knockin homozygous mice (*15*) and *Dyt1* knockout mice lacking exons 2 through 4 (*17*). The results suggested that the lack of exons 3 and 4 also reproduced the phenotype caused by the loss of torsinA function. We used *Dyt1 loxP* mice to bypass the lethality in *Dyt1* knockout mice and analyse the effect of loss of torsinA function in the cerebral cortex.

*Anatomical Characterization of the Cerebral Cortex-specific* Dyt1 *Knockout Mice*—Cerebral cortex-specific *Dyt1* conditional knockout (cKO) mice were produced using *Dyt1 loxP* and *Emx1-cre* knockin mice (Fig. 3A and B). In *Emx1-cre* knockin mice, *cre* expression is driven by an *Emx1* endogenous promoter that has an expression pattern mainly restricted to the cerebral cortex (*37*, *38*). Therefore, cKO mice could delete *Dyt1* gene specifically in developing cerebral cortex. Tissue-specific deletion of exons 3 and 4 of cKO mice was confirmed by PCR using DNA isolated from each brain region. The deletion in cerebral cortex was confirmed as predicted (Fig. 3C). It was not detected in spinal cord and other brain regions (striatum, thalamus, mesencephalic tectum, cerebellum and medulla). The reduction of torsinA expression in the cerebral cortex of cKO mouse brain was further conformed by immunohistochemistry (Fig. 4A and B).

Although the complete *Dyt1* knockout mice died at neonatal stage, cKO mice could survive to adult. There are recent reports implicating torsinA in synaptogenesis

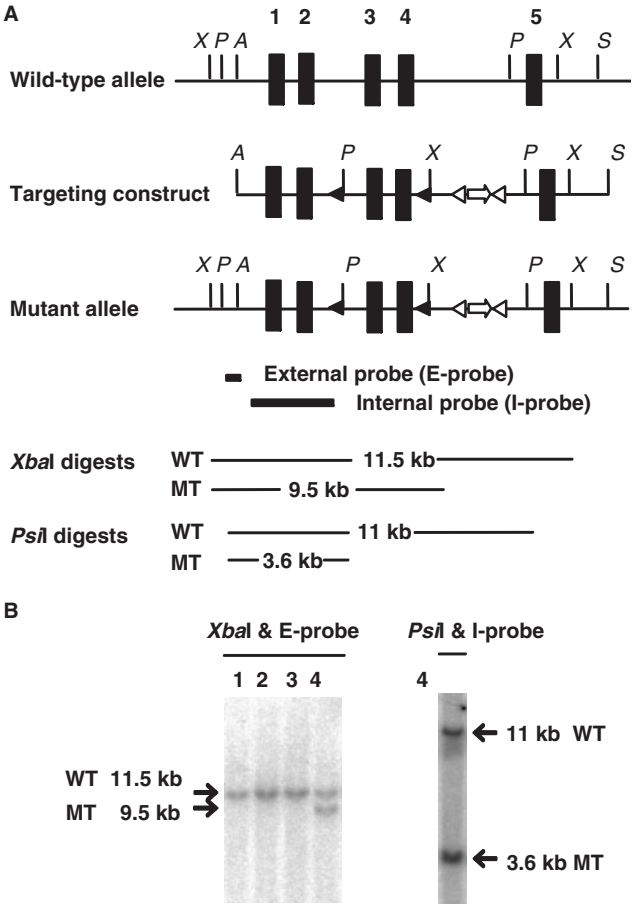


Fig. 1. **Making of *Dyt1 loxPneo* mice.** (A) Map of *Dyt1* wild-type allele, targeting construct and mutant alleles. Filled boxes indicate exons. The targeting construct was made with two *loxP* sites (filled triangles) flanking exons 3 and 4 with a *PGKneo* cassette without a polyA sequence (open right arrow) flanked by two *FRT* sites (open triangles). The locations of 5′external and internal probes are indicated under the map and predicted restriction fragments are depicted below the map. *X*, *Xba*I; *P, Psi*I; *A*, *Afl*II; *S*, *Sma*I. (B) Representative Southern blot analysis of the transfectants. The genomic DNA digested by *Xba*I or *Psi*I were hybridized with 5′external or internal probes, respectively. Targeted clones were selected first using the external probe (left figure, clone 4), followed by a second screening using 5′ internal probe (right figure, clone 4).

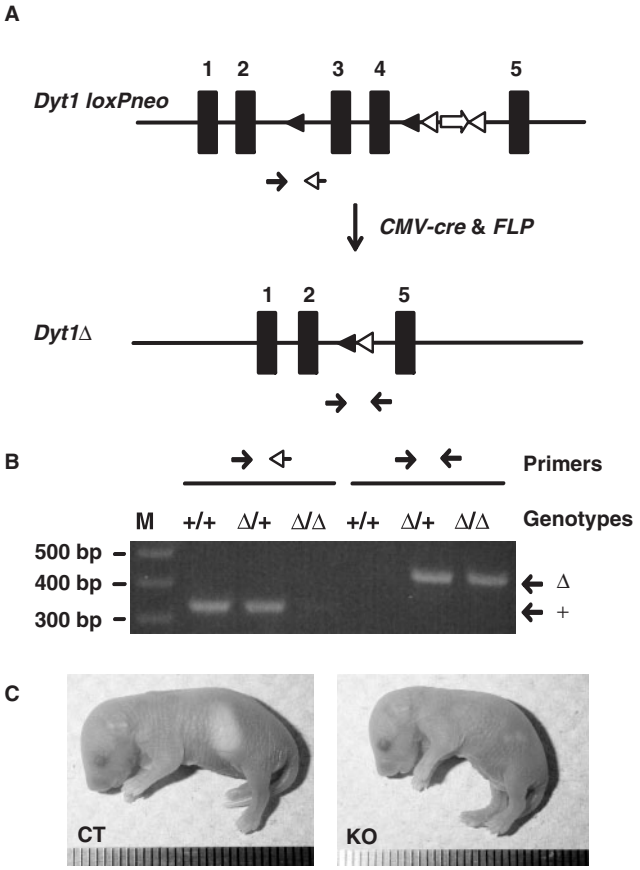


Fig. 2. **Making of *Dyt1* knockout mice and phenotype.** (A) *Dyt1 loxPneo* mice was obtained as shown above. Filled triangles indicate *loxP* sites inserted. Open triangles indicate the *FRT* sites that were incorporated to remove the *PGKneo* cassette (open right arrow). *PGKneo* and exons 3 and 4 were removed by crossing with *CMV-cre* and *FLP* mice. The primer sites to amplify the exons 3 and 4-containing locus (+) and exon 3 and 4-deleted locus (Δ) were shown by combination of arrow and open arrow pairs, and arrow pairs, respectively. (B) Detection of wild-type and exons 3 and 4-deleted loci by PCR-based genotyping. In wild-type (+/+) mice, only wild-type loci were detected. In *Dyt1* heterozygous knockout (Δ/+) mice, both wild-type and the deleted loci were detected. In the *Dyt1* homozygous knockout (Δ/Δ) mice, only the deleted loci were detected. (**C**) Phenotype of *Dyt1* knockout mice. Control mice drank milk (CT) and grew up to adults. However, *Dyt1* knockout homozygous mice (KO) had no milk and died within one or two days after birth. One unit of the scales under the mice is 1 mm.

and neurite outgrowth (*44*, *45*). We analysed the formation of whisker-related patterns (barrels) in somatosensory cortex (*46*) to address this. Cytochrome oxidase staining of the tangential sections through the somatosensory cortex clearly showed rows of well-developed barrels that were indistinguishable between control and cKO mice (Fig. 5A). *Emx1-cre* knockin mice contain a *lacZ* driven by the endogenous *Emx1* promoter that encodes β-galactosidase with a nuclear localization signal (*37*). We used β-galactosidase staining as a variation of Nissl staining to detect the cytoarchitectonic barrels. The advantage of the β-galactosidase staining over the traditional Nissl staining is the ability to examine only cells affected by *cre*-mediated recombination. Staining by β-galactosidase in both control *Emx1-cre* and cKO mice also revealed well-developed barrels (Fig. 5B). Both staining results demonstrated that the whisker-related patterns formed normally in the absence of torsinA in the developing cerebral cortex, suggesting formations of synapses and neural circuits were largely unaffected.

*Deficits in Motor Coordination and Balance*—Motor deficits in beam-walking tests have been reported in several dystonia mouse models (*15*, *16*, *43*, *47*). We used this test to evaluate the motor performance of cKO mice. Analysis of slips numbers revealed that cKO mice showed 71% more slips than control littermates (Fig. 6A; $Z = 2.10$, $P = 0.0361$). The deficits of cKO mice in beam-walking test suggest that the cKO mice had poor

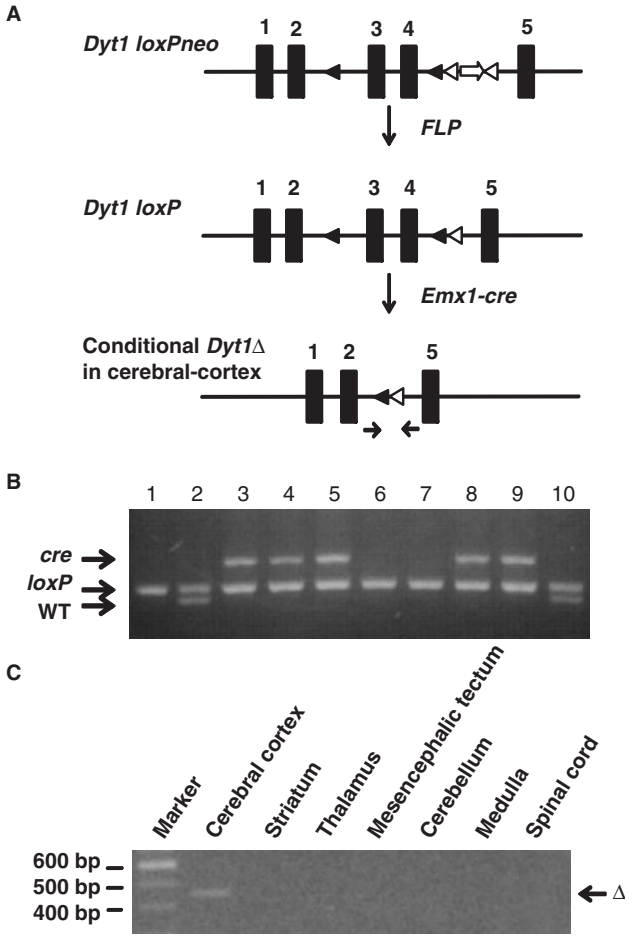


Fig. 3. **Making of cKO mice.** (A) Strategies to generate conditional KO mice. *Dyt1 loxPneo* mice were crossed with *FLP* mice to remove *PGKneo* cassette. *Dyt1 loxP* mice were then crossed with *Emx-cre* mice to obtain double heterozygotes. The double heterozygotes were crossed with *Dyt1 loxP* homozygotes to obtain conditional knockout mice. The primer sites to amplify the exons 3 and 4-deleted locus (Δ) were shown by the arrow pair under the map. (B) A representative of PCR-based genotyping of cKO mice is shown. Top band is PCR product from *cre*. Middle band is for *Dyt1 loxP* locus and the bottom band is *Dyt1* wild-type locus. Lanes 1, 6, 7: *Dyt1 loxP* homozygous mice. Lanes 2, 10: *Dyt1 loxP* heterozygous mice. Lanes 3, 4, 5, 8, 9: cKO mice. (C) Tissue-specific deletion of *Dyt1* exons 3 and 4 in cKO mice was confirmed by PCR using DNA isolated from each brain region. The deletion was detected only in cerebral cortex as predicted (Δ).

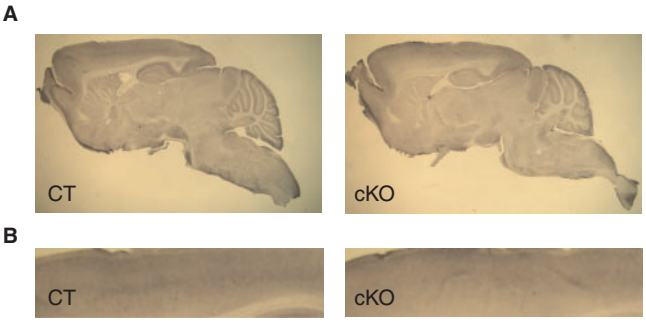


Fig. 4. **Immuohistochemistry with torsinA antibody.** (A) Sagittal sections of the whole brains from cKO and control littermate (CT) were treated with torsinA antibody and stained. Reduction of torsinA expression in the cerebral cortex was confirmed in cKO mouse. The expression of torsinA in the striatum and thalamus was unchanged. (B) Enlarged images of the cerebral cortex in cKO and control littermate (CT). The reduction of torsinA in the cortical layers was evident in cKO mouse.

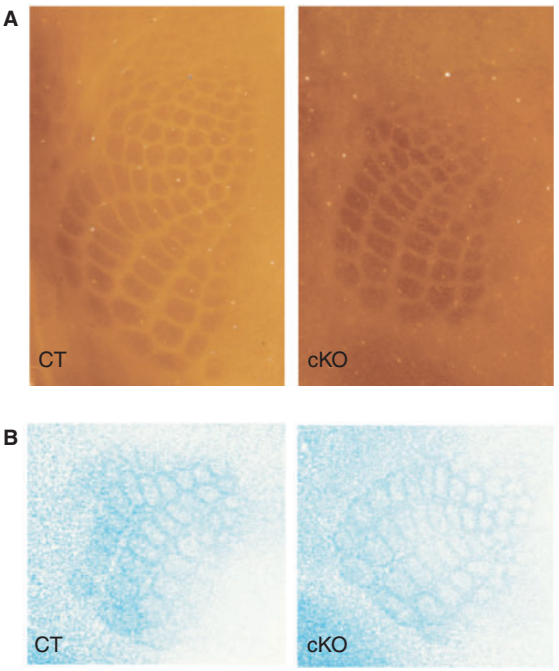


Fig. 5. **Anatomical characterization in somatosensory cortex.** Well-developed whisker-related patterns (barrels) in somatosensory cortex were detected in both control and cKO mice by cytochrome oxidase staining (A) and β-galactosidase staining (B). CT, control mouse; cKO, cerebral cortex-specific *Dyt1* conditional knockout mouse.

motor coordination and balance. Similar to *Dyt1* ΔGAG knockin and *Dyt1* KD mice (*15*, *16*), the cKO mice showed normal performance in rotarod test ($P = 0.7$, data not shown).

To investigate the motor performance further, we tested the cKO mice using pawprint gait analysis. The cKO mice showed no significant difference in the stride length and overlap of the placement of paws (data not shown). However, the hind base of male cKO mice was significantly smaller than those of control males, suggesting an abnormality in gait (Fig. 6B; $P = 0.0156$). To compare the results with those of *Dyt1* KD mice, the pawprint gait analysis was also performed for *Dyt1* KD mice. The hind base of male KD mice was significantly smaller than those of control males, suggesting an abnormality in gait similar to cKO mice (Fig. 6C; $P = 0.008$).

*Hyperactivity of Locomotion*—Since hyperactivity of locomotion is commonly observed in transgenic mouse

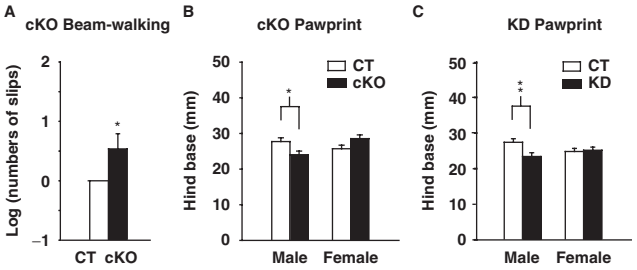


Fig. 6. **Deficits in motor coordination and balance in cKO mice.** (A) Beam-walking test showed significantly more slips in cKO mice. (B) Pawprint gait analysis of cKO mice showed that male cKO mice exhibited significantly smaller hind base compared to the control mice. (C) Pawprint gait analysis of *Dyt1* KD mice also showed that male cKO mice exhibited significantly smaller hind base compared to the control mice. CT, control littermate mice; cKO, cerebral cortex-specific *Dyt1* conditional knockout mice; KD, *Dyt1* knockdown mice. $^{*}P<0.05$, $^{**}P<0.01$. Vertical bars represent mean ± standard errors (SE).

models of dystonia (*13*, *15*, *16*, *43*, *47*), locomotion was assessed in the open-field test. The cKO mice also showed hyperactivity in the open-field test. The cKO exhibited increased activity, total distance travelled, number of movements and the time spent on the movement (Fig. 7). Although the cKO mice showed higher stereotypic activity, it should be noted that stereotypic activity has been conventionally defined as repetitive behavioural, such as grooming and head bobbing. Several labs, however, have noted that an open-field apparatus such as the one used in this study does not give accurate measurements of these types of activities (*48*). We make note of that possibility too, but present this observation here with the speculation that the stereotypy count could be measurements of other stereotypic activity such as gait hesitation, which has been documented to contribute to the stereotypy count by an open-field apparatus (*49*). Similar to transgenic mice over-expressing torsinA$^{\Delta E}$ and *Dyt1* ΔGAG knockin heterozygous mice, the cKO mice also showed

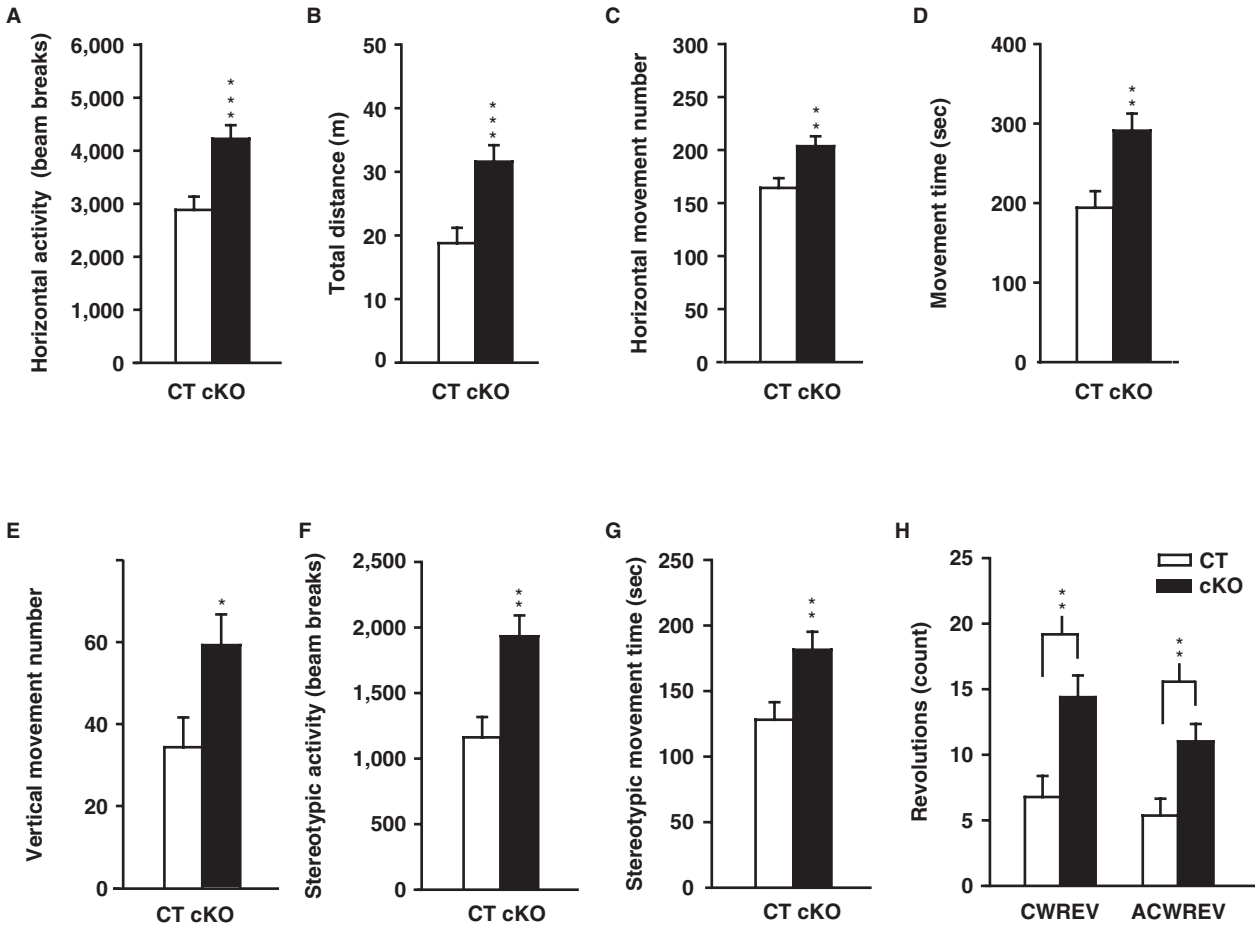


Fig. 7. **Hyperactivity of cKO mice in open-field test.** The cKO mice exhibited significantly increased horizontal activity (A, $P=0.0008$), total distance travelled (B, $P=0.0009$), horizontal movement number (C, $P=0.0056$) and time spent on horizontal movement (D, $P=0.0032$). The cKO mice showed increased vertical movement number (E, $P=0.0243$). The cKO mice had increased stereotypic activity (F, $P=0.0017$) and stereotypic movement time (G, $P=0.0094$). The cKO mice showed increased clockwise (H, CWREV, $P=0.0027$) and counter-clockwise (H, ACWREV, $P=0.0049$) circling in the open field. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Vertical bars represent mean ± SE.

Table 1. **DA and their metabolites in the striatum.**

| Content or ratio | CT | cKO | *P* |
|---|---|---|---|
| DA | 20.71 ± 2.03 | 21.67 ± 2.64 | 0.80 |
| DOPAC | 1.11 ± 0.09 | 1.22 ± 0.12 | 0.55 |
| HVA | 1.50 ± 0.12 | 1.73 ± 0.15 | 0.29 |
| DOPAC/DA | 0.057 ± 0.005 | 0.061 ± 0.006 | 0.64 |
| HVA/DA | 0.080 ± 0.007 | 0.090 ± 0.010 | 0.44 |

The values of neurochemical are shown as mean ± standard errors (in ng/mg of wet tissue). The turnover of metabolites is shown as the ratio of the neurochemicals.

significantly more clockwise and counterclockwise revolutions or circling behaviours.

*Neurochemicals in the Striatum*—To determine whether the motor deficits and hyperactivity accompany any alterations of striatal DA metabolism, we measured concentrations of DA and their metabolites in the striatum. Unlike most of the *Dyt1* mouse models reported so far (*13*, *15*, *16*), the levels of DA, DOPAC and HVA, and the ratios of DOPAC or HVA to DA in the cKO mice were not significantly different from those in the control mice (Table 1).

## DISCUSSION

Although torsinA is widely expressed throughout the brain, the fact that mutant *DYT1* causes a predominantly motor phenotype suggests that motor control regions of the brain are severely affected by the mutation. Identification of the specific motor control regions of the brain that is the source of the dysfunction in motor coordination is important for understanding the mechanism of the disease. Knowing the mechanism could potentially be useful for the development of effective medical treatments for patients. It has been proposed that the GPi is the major site of pathogenesis and that the cortex, thalamus, brainstem and spinal cord are not the source of dysfunction in primary dystonia (*20*). However, recent neuroimaging studies suggested contributions of the cerebral cortex. In this study, we produced cKO mice and analysed the effect of loss of torsinA function in cerebral cortex. Beam-walking test and pawprint gait analysis revealed that cKO mice exhibited motor deficits that mimic the behavioural deficits in *Dyt1* ΔGAG knockin heterozygous and *Dyt1* KD mice. This is the first report to suggest that the loss of torsinA function in the cerebral cortex plays an important role in the pathogenesis of this disease. Furthermore, we found that there was no significant difference in striatal DA and its metabolites levels between the cKO and control mice. The results suggest that the loss of torsinA function in cerebral cortex causes hyperactivity and motor deficits without significant change of DA metabolism in the striatum.

TorsinA has been implicated in neurodevelopment. Manipulation of torsinA expression in neuroblastoma cells revealed that torsinA may control neurite outgrowth by interaction with microtubule associated proteins (*44*). In a *Drosophila* model of DYT1 dystonia, over-expression of human mutant torsinA, but not normal human torsinA, leads to morphological deficits at the neuromuscular junction in larvae, suggesting torsinA might be involved in regulating synapse formation (*45*). To determine the possible role of torsinA in synapse and circuit formation, we examined the development of whisker-related patterns in somatosensory cortex. The whisker-related patterns developed normally in the absence of functional torsinA during development, suggesting formations of synapses and neural circuits were unaffected by the mutant protein.

Dystonia has been recognized as a neuronal circuit disorder. One model proposes that abnormal activity in the pallidal–thalamic–cortical circuit causes primary dystonia (*20*). According to this model, the cerebral cortex receives enhanced excitatory inputs from the motor thalamus and releases enhanced excitatory outputs to the brainstem, striatum and motor thalamus in dystonia patients. Since the *Dyt1* deletion in our mouse model is restricted to the cerebral cortex, the motor deficits may be caused by the abnormal output signals originating from the cerebral cortex. The abnormal outputs from the cerebral cortex may affect the brainstem, striatum and motor thalamus according to this model. Of particular interest, the cerebral cortex may alter striatal function through the corticostriatal pathway.

The results suggest that functional changes in the cerebral cortex could lead to motor deficits. However, this finding does not exclude the possibility of contribution of other brain regions because torsinA is expressed throughout the central nervous system. The striatum and motor thalamus are the candidates among them because those brain regions function downstream of the cerebral cortex. We demonstrated in this study that the generation of tissue-specific conditional knockout mice followed by behaviour tests is a powerful approach to identify a brain region, which contributes to the pathogenesis of this disease. The same strategy can be applied to identify other brain regions. Future study using *Dyt1* conditional knockout mice that restrict torsinA inactivation to the striatum (*50*) or motor thalamus would address specific neuronal circuit involved in the deficits. The results also suggest that loss of torsinA function in cerebral cortex does not affect DA metabolism in the striatum. The significant differences in striatal DA metabolites found in *Dyt1* ΔGAG knockin heterozygous mice (*15*) and *Dyt1* KD mice (*16*) are likely caused by loss of torsinA function in dopaminergic neurons derived from substantia nigra. Generating of substantia nigra-specific conditional knockout mice may further allow us to determine the origin of the DA metabolism deficits observed in *Dyt1* ΔGAG knockin heterozygous mice and *Dyt1* KD mice. The future study using these conditional knockout mice will uncover the neuronal circuits, origin, sequence and neuropathological processes underlying DYT1 dystonia.

Although there are many medical treatments for dystonia, there is no definitive cure specific for DYT1 dystonia. Levodopa administration to patients with segmental or generalized dystonia beginning in childhood or adolescnce was proposed as potential therapy (*51*). However, this treatment was not effective for some DYT1 dystonia patients (*52*, *53*). Anti-cholinergic treatments,

such as trihexyphenidyl, are useful if the optimal dose is carefully managed depending on the progress of disease while considering the side effects. Pallidotomy (*22*) and GPi DBS (*54*) are effective surgical therapies for generalized dystonia, although it has not been established yet whether they are effective throughout the whole life. These therapies are used sequentially in clinical practice until each therapy becomes ineffective (*51*). Most of the available treatments are symptomatic treatments instead of pathogenesis-targeted ones. The results presented in this study suggest that the cerebral cortex can be a novel target for medical treatments. Motor cortex stimulation has in fact been used in a limited number of cases with patients who have focal dystonia (*55*). Developing or surveying drugs targeting the cerebral cortex that compensate for the loss of torsinA function may produce effective medical treatments for DYT1 dystonia.

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