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Earlier onset of motor deficits in mice with double mutations in *Dyt1* and *Sgce*

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DYT1 early-onset generalized torsion dystonia is an inherited movement disorder caused by mutations in *DYT1* coding for torsinA with ~30% penetrance. Most of the DYT1 dystonia patients exhibit symptoms during childhood and adolescence. On the other hand, *DYT1* mutation carriers without symptoms during these periods mostly do not exhibit symptoms later in their life. Little is known about what controls the timing of the onset, a critical issue for *DYT1* mutation carriers. DYT11 myoclonus-dystonia is caused by mutations in *SGCE* coding for ε-sarcoglycan. Two dystonia patients from a single family with double mutations in *DYT1* and *SGCE* exhibited more severe symptoms. A recent study suggested that torsinA contributes to the quality control of ε-sarcoglycan. Here, we derived mice carrying mutations in both *Dyt1* and *Sgce* and found that these double mutant mice showed earlier onset of motor deficits in beam-walking test. A novel monoclonal antibody against mouse ε-sarcoglycan was developed by using *Sgce* knock-out mice to avoid the immune tolerance. Western blot analysis suggested that functional deficits of torsinA and ε-sarcoglycan may independently cause motor deficits. Examining additional mutations in other dystonia genes may be beneficial to predict the onset in *DYT1* mutation carriers.

Keywords: antibody/dystonia/ε-sarcoglycan/myoclonus-dystonia/torsinA.

Abbreviations: *DYT1* (*TOR1A*), TorsinA gene in human; *Dyt1* (*Tor1a*), TorsinA gene in mouse; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; KI mouse, knock-in mouse; KO mouse, knockout mouse; *SGCE*, ε-sarcoglycan gene in human; *Sgce*, ε-sarcoglycan gene in mouse; WT mouse, wild-type mouse.

DYT1 early-onset generalized torsion dystonia is an inherited movement disorder caused by mutations in *DYT1 (TOR1A)*, which codes for torsinA (*1*). TorsinA is a member of AAA+ family of ATPases and may work in trafficking of polytopic membrane proteins and protein processing in the secretory pathway (*2*, *3*). ATPase activity (*4*, *5*) and molecular chaperon activity of torsinA (*6*) have also been reported *in vitro*. TorsinA is localized in the endoplasmic reticulum (*7*, *8*) and nuclear envelope (*9*, *10*). Most of the patients have a 3 bp deletion, ΔGAG, in *DYT1* corresponding to a loss of a glutamic acid residue in the C-terminal region of torsinA. The ΔGAG mutation causes abnormal activation of the brain in humans and mice (*11*, *12*). An 18 bp deletion in *DYT1* was also reported in a family (*13*). The 18-bp mutation reduces ATPase activity *in vitro* (*5*) and induces locomotor disability and anatomical changes in fruit flies (*14*). A third mutation that causes an Arg288Gln exchange was also reported (*15*). Genetic studies using *Dyt1* ΔGAG knock-in (KI), *Dyt1* knockout (KO), *Dyt1* knock-down and the cerebral cortex-specific *Dyt1* conditional KO mice suggested that a loss of torsinA function contributes to the pathology of the disease (*16*–*18*). Moreover, we reported that chemical enhancement of torsinA rescued *Dyt1* ΔGAG KI mice from their motor deficits (*19*). Recent studies also suggested that the mutant forms of torsinA are quickly degraded by both the proteasome and macroautophagy–lysosome pathways in transfected cells while wild-type (WT) torsinA is stable and degraded primarily through the macroautophagy–lysosome pathway (*20*, *21*).

DYT11 myoclonus-dystonia is another inherited movement disorder caused by mutations in *SGCE*, which codes for ε-sarcoglycan (*22*). Epsilon-sarcoglycan is a membrane protein and widely expressed in the body (*23*–*25*). The protein sequence homology analysis and 3D structure modeling suggest that ε-sarcoglycans has a cadherin-like domain at the N-terminal extracellular region, suggesting it may function in intercellular adhesion (*26*). Mouse ε-sarcoglycan has alternative splicing variants and the brain-specific isoforms have PDZ-binding motifs (*27*). Mouse ε-sarcoglycan is enriched in pre- and post-synaptic membrane fractions, suggesting a role in synaptic transmission (*28*). *Sgce* is maternally imprinted and paternally expressed in humans and rodents (*27*, *29*, *30*). We previously reported the making of *Sgce* KO mice lacking exon 4 and

demonstrated that paternally inherited *Sgce* heterozygous KO mice did not express maternally inherited WT *Sgce* in the brain (*27*). The *Sgce* KO mice exhibited myoclonus, motor deficits, alterations in emotional responses and monoamine metabolism (*31*).

Since the penetrance of DYT1 dystonia is ~30% (*32*), mutation in other genes, environmental factors or both have been considered as risk factors that may contribute to the incidence of this disease. Finding risk factors in *DYT1* mutation carriers may help to predict the onset and prognosis of this disease. It may also elucidate the mechanism of the penetrance and help to find potential approaches to prevent the onset of this disease. Previous studies reported two myoclonus-dystonia patients in a family with double mutations in *DYT1* and *SGCE* (*33*, *34*). They inherited an 18 bp in-frame deletion in *DYT1* from their mother and a 587T>G missense mutation (Leu196Arg) in *SGCE* from their father. Both patients exhibited more severe symptoms than their parents. However, it is not clear whether this additional mutation truly affects the onset of dystonia because of the limited number of the patients. Genetic animal models provide an attractive alternative to address this question.

A recent study suggested that torsinA participates in the quality control of ε-sarcoglycan (*35*). TorsinA forms a stable complex with missense-mutant forms of ε-sarcoglycan and facilitates their degradation in transfected cells. Although torsinA does not make a stable complex with WT ε-sarcoglycan, the transfected torsinA facilitates the reduction of both WT and mutant forms of ε-sarcoglycan in the co-transfected cells. Therefore, loss or reduction of the torsinA function may affect the quality control of ε-sarcoglycan and alter the amount of ε-sarcoglycan *in vitro*. Whether similar interaction occurs *in vivo* is not known.

In previous studies, we reported the making of *Dyt1* ΔGAG heterozygous KI mice as a genetic model of DYT1 dystonia that exhibited motor deficits in males at about 6.5 months of age in the beam-walking test (*36*). We also reported the making of paternally inherited *Sgce* heterozygous KO mice, which also showed similar motor deficits at 6.5–7.5 months of age (*31*). In this study, we produced the single and double mutant mice and littermate control mice to evaluate their motor performance at 5.5 months of age and to determine whether the two mutations affect the age of onset of the motor deficits. Furthermore, it was reported that torsinA shows a trend of reduction in the whole brain of perinatal *Dyt1* ΔGAG heterozygous KI mice (*18*). Here we quantified this reduction and analysed the effect of the *Dyt1* ΔGAG mutation on the level of ε-sarcoglycan and the effect of *Sgce* KO on the level of torsinA in the striatum that plays an important role in motor coordination, balance and learning (*37*).

Materials and Methods

Mice

All experiments were carried out in compliance with the USPHS Guide for Care and Use of Laboratory Animals and approved by IACUC of University of Illinois at Urbana-Champaign (UIUC) and University of Alabama at Birmingham (UAB). *Dyt1* homozygous KO mice, *Dyt1* ΔGAG heterozygous KI mice, paternally inherited *Sgce* heterozygous KO mice and *Sgce* homozygous KO mice were prepared and genotyped by PCR as described earlier (*12*, *17*, *27*, *36*). *Sgce* heterozygous KO male mice were crossed with *Dyt1* ΔGAG heterozygous KI female mice to produce the single and double mutant mice, and their control littermates. Mice were housed under a 12 h light and 12 h dark cycle.

Open-field test

A group that had 17 *Dyt1* ΔGAG heterozygous KI mice (9 males and 8 females), 16 paternally inherited *Sgce* heterozygous KO mice (5 males and 11 females), 16 double-mutated mice (11 males and 5 females) and 13 control littermates (8 males and 5 females) from 133 to 161-days old (average: 151 days) was used for open-field test. Mice were moved into a sound-attenuated testing room and acclimated for >1 h. Open-field test was performed as described earlier (*38*). Spontaneous activities of individual mice were recorded by infrared light beam sensors in a 41 × 41 × 31 cm acryl case for 15 min at 1-min intervals using DigiPro software (AccuScan Instruments) under a 60 W light condition. Data in the open-field test were analysed by the ANOVA-mixed model with SAS program as described earlier (*36*).

Beam-walking test

Two groups of the mice at 2- and 5.5-months old were prepared for beam-walking tests. The first group consisted of 11 *Dyt1* ΔGAG heterozygous KI mice (7 males and 4 females), 16 paternally inherited *Sgce* heterozygous KO mice (11 males and 5 females), 13 double-mutated mice (9 males and 4 females) and 13 control littermates (6 males and 7 females) at average 2-months old. The second group had 17 *Dyt1* ΔGAG heterozygous KI mice (9 males and 8 females), 16 paternally inherited *Sgce* heterozygous KO mice (5 males and 11 females), 16 double-mutated mice (11 males and 5 females) and 13 control littermates (8 males and 5 females) from 150 to 175-days old (average: 167 days). Their motor performance was evaluated by the beam-walking test as described earlier (*36*). Briefly, the beam-walking test was performed within the last 8 h of the light period after acclimation to a sound-attenuated testing room for 1 h. The mice were trained to transverse a medium square beam in three consecutive trials each day for 2 days and tested twice each on a medium square beam and a medium round beam on the third day. The mice were then tested twice each on a small round beam and a small square beam on the fourth day. Their hindpaw slips on each side were counted. The beam-walking test was performed by investigators blind to the genotypes. The beam-walking data were analysed by logistic regression (GENMOD) with negative binominal distribution using GEE model in SAS/STAT Analyst software (Version 9.1.3; SAS institute Inc., NC, USA), using sex, age and body weight as variables (*17*). The data were analysed after log transformation to obtain a normal distribution. Control WT mice were normalized to zero. The beam-walking data at 2-months old were further analysed by Student's *t*-test. Statistical significance was assigned at the $P < 0.05$.

Western blot for torsinA

To check the quality of a commercial torsinA antibody, we used a *Dyt1* homozygous KO mouse brain as a negative control and a WT mouse brain as a positive control in the western blot analysis. Since *Dyt1* homozygous KO mutation is neonatal lethal, the brain was obtained right after birth. The whole brain was homogenized in SDS–PAGE loading buffer and sonicated. The homogenate was boiled for 5 min, incubated on ice for 1 min, and then centrifuged for 5 min to obtain the supernatant. The proteins were separated by SDS–PAGE and transferred to a PROTRAN nitrocellulose transfer membrane (Whatman). The membrane was blocked in 5% milk (Bio-Rad) in a wash buffer [20 mM Tris–Cl (pH 7.6), 137 mM NaCl, 0.1% (v/v) Tween20] and incubated overnight at 4°C with rabbit polyclonal torsinA antibody (Abcam; ab34540) in the blocking buffer. The membrane was washed in the wash buffer and incubated with bovine anti-rabbit IgG-HRP (Santa Cruz; sc-2370) in the blocking buffer at room temperature for 1h, and then washed. The band was detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). The signal was captured by Alpha

Innotech FluorChem FC2. The molecular mass was estimated with Precision Plus Protein Standards All Blue (Bio-Rad).

For quantitative analysis, protein extracts were prepared from the striatum in a group of mice at ~5.5-months old that comprised *Dyt1* ΔGAG heterozygous KI mice ($n=4$), paternally inherited *Sgce* heterozygous KO mice ($n=4$), the double mutant mice ($n=4$) and their WT littermates ($n=4$). The striata were dissected from the mouse brains and quickly frozen in liquid nitrogen. The striata were homogenized in 200 µl of ice-cold lysis buffer [50 mM Tris–Cl (pH 7.4), 175 mM NaCl, 5 mM EDTA, Complete Mini protease inhibitor cocktail (Roche)] and sonicated for 10 s. One-ninth volume of 10% TritonX-100 in the lysis buffer was added to the homogenates. The homogenates were incubated for 30 min on ice and then the supernatants were obtained by centrifuging at 10,000*g* for 15 min at 4°C. The protein concentration was measured by the Bradford assay with bovine serum albumin as standards. The homogenates were mixed with the SDS–PAGE loading buffer and boiled for 5 min, incubated on ice for 1 min, and then centrifuged for 5 min to obtain the supernatant. Of the samples, 40 µg was loaded on SDS–PAGE and the separated proteins were transferred to the nitrocellulose membrane. To compare the striatal torsinA levels in *Dyt1* ΔGAG heterozygous KI mice, paternally inherited *Sgce* heterozygous KO mice and the double mutant mice with that in WT littermates, we used β-actin as a loading control detected with HRP-conjugated β-actin antibody (Santa Cruz; sc-47778 HRP). The bands were detected as described earlier and the density of the bands was quantified with UN-SCAN-IT gel (Silk Scientific) software. The density of torsinA bands was normalized to that of β-actin bands, and analysed by Student's *t*-test. Control WT mice were normalized to 100%.

Making of monoclonal antibodies and western blot for ε-sarcoglycan

A full-length mouse ε-sarcoglycan cDNA (DDBJ/EMBL/GenBank accession No. AB200281) was used as a template to amplify a partial-length cDNA (1,158 bp) to produce the protein in *Escherichia coli* (*27*). The cDNA was amplified by using Pfx polymerase (Invitrogen) with a primer set of Msgce21F (5′-CACCGAATTCATGGGCCGGAACGTGTACC-3′) and Msgce1R (5′-GAATTCTCATGTAGTCTGCGGTTGAGGG-3′), and subcloned into pET100/D-Topo vector (Invitrogen). This cDNA codes for most of ε-sarcoglycan, but does not code for the predicted signal peptide at the N-terminal region (*23*) or the alternative variant sequences at the C-terminal region (*27*). The partial ε-sarcoglycan was expressed as a His-tag fusion protein in *E. coli* (BL21), and then purified by using Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen).

Monoclonal antibodies against this partial ε-sarcoglycan were produced by using *Sgce* homozygous KO mice to avoid the immune tolerance. The mice were supplied with Tylenol in the drinking water for 48 h prior to the first injection of the antigen and for 72 h after the injection. Mice were anaesthetized and injected in one footpad with 0.1 ml of the antigen protein (200 µg) in the emulsion with Freund's complete adjuvant. In the second week, the mice were injected with 0.1 ml of the antigen protein in the emulsion with Freund's incomplete adjuvant. This booster injection was repeated once a week for three weeks. The local lymph nodes were dissected from the mice and the isolated lymphocytes were fused with NS-1 myeloma cells.

The full-length ε-sarcoglycan cDNA (1,263 bp) was amplified by PCR using a primer set of MsgceF1F (5′-GAGGATCCGCCACCATGAGCCCCGCGACC-3′) and MsgceF1R (5′-GCCTCGAGTTAAATTCCCGAGAGCAGTAAT-3′), and subcloned into pcDNA3.1 (Invitrogen) to produce the full-length ε-sarcoglycan in CHO cells, which was used for ELISA, FACS and the western blot for screening of the positive hybridomas. The positive hybridomas were used for the second screening by western blot for the striatal protein extracts from WT and paternally inherited *Sgce* heterozygous KO mice. Goat anti-mouse Ig, Human ads-HRP (SouthernBiotech), was used as the secondary antibody. The band was detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Clones were selected that reacted to the striatal protein extract from a WT mouse, but not to that from a paternally inherited *Sgce* heterozygous KO mouse.

The striatal samples for western blot were also prepared as described earlier from adult *Dyt1* ΔGAG heterozygous KI mice ($n=4$) and their WT littermates ($n=4$). The western blot for ε-sarcoglycan was performed as described earlier with mSE 3A9 as the primary antibody and bovine anti-mouse IgG-HRP (Santa Cruz; sc-2371) as the secondary antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected with HRP-conjugated GAPDH antibody (Santa Cruz; sc-25778 HRP) as a loading control. The bands were detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). The western blot was performed in triplicate. The standard curves for molecular mass were calculated by the least-squares means from the migration distance of the marker bands after log transformation of the molecular mass. The density of ε-sarcoglycan was normalized to that of GAPDH and analysed by Student's *t*-test. Control WT mice were normalized to 100%.

Preparation of synaptosomal fraction and western blot for ε-sarcoglycan

Synaptosomal fraction was prepared from the cerebral cortex based on a standard protocol (*39*). Briefly, the cerebral cortexes were dissected from four *Dyt1* ΔGAG heterozygous KI mice and four WT littermates and quickly frozen in liquid nitrogen. The cortexes were defrosted in 5 ml of ice-cold TEVP buffer [10 mM Tris–Cl (pH 7.4 at 4°C), 5 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA] containing 320 mM sucrose for 5 min and homogenized with Dounce homogenizer. The homogenates were centrifuged for 10 min at 800*g*, 4°C and the supernatants were collected (S1 supernatants). S1 supernatants were centrifuged for 15 min at 9,200*g* in Beckman L8-60M ultracentrifuge with Type 70.1 Ti rotor and the pellets were obtained (P2 pellets). After briefly rinsing with 1 ml TEVP buffer containing 35.6 mM sucrose, P2 pellets were re-suspended in 2 ml TEVP buffer containing 35.6 mM sucrose and put on ice for 30 min. P2 samples were vortexed and centrifuged for 20 min at 25,000*g*, 4°C. Pellets were briefly rinsed with 1 ml of TEVP buffer and re-suspended in 1 ml TEVP buffer (LP1 fraction; synaptosomal fraction). LP1 fraction was sonicated for 10 s and the protein concentration was measured by the Bradford assay with bovine serum albumin as standards. Sonicated LP1 fraction was stored at −80°C. Sonicated LP1 fraction was diluted with water containing Complete Mini protease inhibitor cocktail (Roche) and mixed with equal volume of 2 × SDS–PAGE loading buffer to the final protein concentration of 0.5 µg/µl. The solution was then boiled for 5 min, incubated on ice for 1 min, and centrifuged for 5 min to obtain the supernatant for loading at 10 µg in each lane. The separated proteins were transferred to the nitrocellulose membrane. The membrane was blocked with 5% milk in the wash buffer and treated with the monoclonal ε-sarcoglycan antibody to detect ε-sarcoglycan. The membrane was also treated with rabbit polyclonal anti-synaptophysin antibody (Thermo Scientific, PA1-1043) to detect synaptophysin as a loading control. The secondary antibodies and detecting reagents were the same as described earlier. The experiments were performed in duplicate. The quantified ε-sarcoglycan level was normalized to the synaptophysin level, and analysed by Student's *t*-test. The levels in *Dyt1* ΔGAG heterozygous KI mice were then normalized to WT mice.

Results

Decreased locomotion in the double mutant mice compared to Dyt1 *ΔGAG heterozygous KI mice*

We produced the single and double mutant mice of *Dyt1* ΔGAG KI and *Sgce* KO, and their littermate controls. Spontaneous activities in the mutant mice were assessed in the open-field apparatus and compared to those in control littermates. The double mutant mice did not exhibit significant differences in comparison to control littermates in horizontal (horizontal beam breaks, $P=0.59$; total distance travelled, $P=0.35$; movement number, $P=0.07$; movement time, $P=0.55$) or vertical locomotion (vertical beam breaks, $P=0.85$; vertical movement number, $P=0.77$; vertical time, $P=0.85$). There was no significant difference in clockwise or anti-clockwise circulation between the double mice and the control littermates ($P=0.07$ and $P=0.45$, respectively).

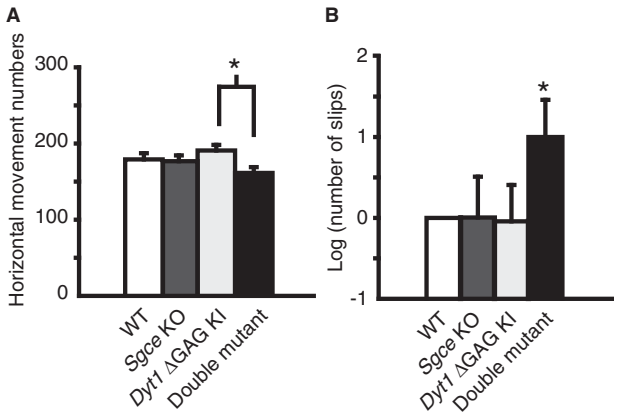

Fig. 1 Motor behaviour tests in the mutant mice. (A) Horizontal movement numbers in the open-field test. The double mutant mice exhibited significant decreased horizontal movement numbers in comparison to *Dyt1* ΔGAG heterozygous KI mice. (B) The beam-walking test for the mutant mice and their control littermates at 5.5 months of age. *Dyt1* ΔGAG heterozygous KI mice and paternally inherited *Sgce* heterozygous KO mice did not show a significant difference in the number of slips when compared to control littermates. However, the double mutant mice showed a significantly increased number of slips compared to the control littermates. The vertical bars represent means ± standard errors. $*P<0.05$.

However, the double mutant mice exhibited significant decreased horizontal movement numbers ($P=0.04$; Fig. 1A) in comparison to *Dyt1* ΔGAG heterozygous KI mice. The results suggest that locomotion in double mutant mice were mostly normal at this age while additional mutation in *Sgce* caused hypoactivity in comparison to *Dyt1* ΔGAG heterozygous KI mice.

Earlier onset of motor deficits in double mutant mice in the beam-walking test

To determine whether the two mutations affect the age of onset of the motor deficits, motor performance was evaluated at 2 months and 5.5 months of age using the beam-walking test, which tests motor coordination and balance (*40*). Five and half months of age was chosen because it is about 1 month before the onset of the motor deficits seen in the *Dyt1* ΔGAG heterozygous KI mice and the paternally inherited *Sgce* heterozygous KO mice. At 2 months of age, 51 mice did not show any slips on any beams while a double mutant female showed a left slip on the small round beam and a *Sgce* KO male showed two slips on both sides on the small square beam. Since most of the mice had no slips, the SAS statistical program could not generate any reliable estimate. We further analysed these data with the *t*-test by simply comparing slip numbers of the mutant mice to those of the wild-type mice. There was no significant difference between the mutant and WT mice at 2 months of age (*Sgce* KO mice, $P=0.20$; Double mutant mice, $P=0.32$). However, the double mutant mice exhibited significant motor deficits at 5.5 months of age ($P=0.03$; Fig. 1B) while neither the *Dyt1* ΔGAG KI heterozygous nor the paternally inherited *Sgce* KO heterozygous mice showed significant motor deficits ($P=0.92$ and $P=0.99$, respectively; Fig. 1B). These results suggest that the double mutations cause earlier onset of motor deficits in mice.

Loss of ε-sarcoglycan did not significantly affect torsinA levels in the striatum

To analyse the effect of loss of ε-sarcoglycan on the expression level of torsinA, we performed the western blot using striatal protein extracts from paternally inherited *Sgce* heterozygous KO mice and their WT littermates. We first assessed the quality of a commercial torsinA antibody (Abcam; ab34540) by western blot using protein extracts from a *Dyt1* homozygous KO mouse brain and a WT brain. Although the torsinA antibody showed a strong band at 36 kDa for the WT mouse brain, it did not detect any band around this molecular mass in *Dyt1* homozygous KO mouse brain (Fig. 2A). Therefore, this antibody is suitable for the western blot analysis of mouse torsinA.

We analysed the striatal torsinA level in WT littermates, paternally inherited *Sgce* heterozygous KO mice, *Dyt1* ΔGAG heterozygous KI mice and the double mutant mice using this antibody through western blot analysis. The torsinA level in the striatum was not altered by the loss of ε-sarcoglycan ($P=0.70$; Fig. 2B). On the other hand, torsinA was significantly reduced both in *Dyt1* ΔGAG heterozygous KI mice ($P=0.02$; Fig. 2B) and the double mutant mice with *Dyt1* ΔGAG heterozygous KI and paternally inherited *Sgce* heterozygous KO ($P=0.007$; Fig. 2B) relative to their WT littermates. There was no significant difference between *Dyt1* ΔGAG heterozygous KI mice and the double mutant mice ($P=0.52$; Fig. 2B).

Dyt1 *ΔGAG mutation did not significantly affect ε-sarcoglycan levels in the striatum*

To analyse the effect of *Dyt1* ΔGAG mutation on the expression level of ε-sarcoglycan, we performed western blot using striatal protein extracts from *Dyt1* ΔGAG heterozygous KI mice and their WT littermates. Since none of the tested commercial antibodies were suitable to detect and quantify mouse ε-sarcoglycan in western blot analysis (Supplementary Fig. S1), a novel mouse monoclonal ε-sarcoglycan antibody (mSE 3A9) was developed using *Sgce* KO mice to avoid the immune tolerance. Although a single strong band at 52 kDa was detected by the monoclonal antibody in the WT striatal sample, it did not detect any bands around this molecular mass in the striatal protein extract from the paternally inherited *Sgce* heterozygous KO mice (Fig. 3A). Therefore, this antibody is suitable for western blot analysis of mouse ε-sarcoglycan. The lack of ε-sarcoglycan detection also confirmed the exclusive paternal expression of ε-sarcoglycan in the mouse striatum, which was suggested by our previous study using RNA from the mouse brains (*27*). Western blot analysis revealed that the ε-sarcoglycan level in the striatum was not altered in *Dyt1* ΔGAG heterozygous KI mice ($P=0.92$; Fig. 3B).

Dyt1 *ΔGAG mutation did not significantly affect the trafficking of ε-sarcoglycan*

Mouse ε-sarcoglycan is a membrane protein (*23*) and enriched in pre- and post-synaptic membrane fractions (*28*). Since torsinA may work in trafficking of

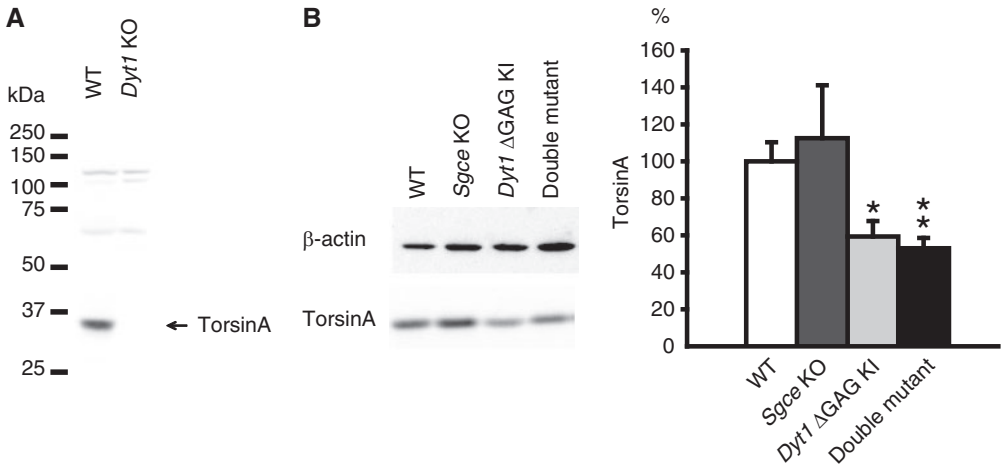

Fig. 2 Western blot for torsinA. (A) Specificity of a commercial torsinA antibody in western blot analysis demonstrated by using whole brain extracts from a WT mouse and that from a *Dyt1* homozygous KO mouse. Arrow indicates a strong major band at 36 kDa corresponding to torsinA. (B) Striatal torsinA levels in WT littermates, paternally inherited *Sgce* heterozygous KO mice, *Dyt1* ΔGAG heterozygous KI mice and the double mutant mice. The torsinA level in the striatum was not altered by the loss of ε-sarcoglycan. On the other hand, torsinA was significantly reduced both in *Dyt1* ΔGAG heterozygous KI mice and the double mutant mice with *Dyt1* ΔGAG heterozygous KI and paternally inherited *Sgce* heterozygous KO mice relative to their WT littermates. There was no significant difference between *Dyt1* ΔGAG heterozygous KI mice and the double mutant mice. The representative bands are shown on the left and their quantification is shown on the right. The vertical bars represent means ± standard errors. $*P<0.05$, $**P<0.01$.

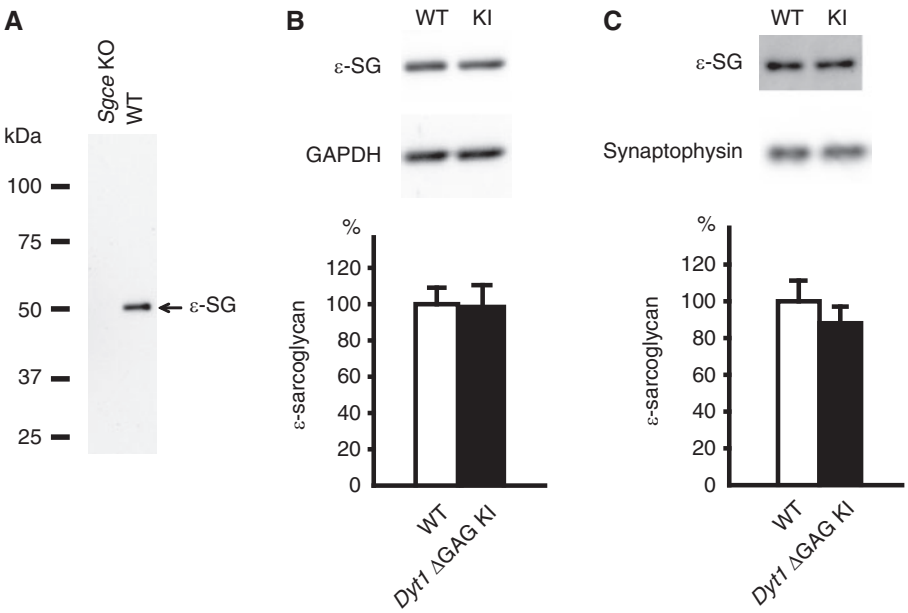

Fig. 3 Western blot for ε-sarcoglycan. (A) Specificity of a novel monoclonal antibody in western blot analysis by using the striatal protein extracts from a paternally inherited *Sgce* heterozygous KO mouse and a WT littermate. Arrow indicates a strong major band at 52 kDa corresponding to ε-sarcoglycan (ε-SG) that was detected only in WT mouse. (B) The amounts of ε-sarcoglycan in the striatal protein extracts from *Dyt1* ΔGAG heterozygous KI mice and WT littermates. The representative bands of ε-sarcoglycan and GAPDH are shown on the top and the quantified ε-sarcoglycan is shown on the bottom. There was no significant difference in ε-sarcoglycan levels in the striatum between WT control mice and *Dyt1* ΔGAG heterozygous KI mice. (C) The amounts of ε-sarcoglycan in the synaptosomal fractions from *Dyt1* ΔGAG heterozygous KI mice and WT littermates. The representative bands of ε-sarcoglycan and synaptophysin are shown on the top and the quantified ε-sarcoglycan is shown on the bottom. There was no significant difference in ε-sarcoglycan levels in synaptosomal fractions between WT control mice and *Dyt1* ΔGAG heterozygous KI mice. The vertical bars represent means ± standard errors.

membrane proteins (*2*), we examined whether *Dyt1* ΔGAG mutation affects the trafficking of ε-sarcoglycan in mouse brains. Synaptosomal fraction was prepared from the cerebral cortexes in *Dyt1* ΔGAG heterozygous KI mice and WT littermates and ε-sarcoglycan levels were analysed by western blot. The ε-sarcoglycan levels were standardized with synaptophysin levels as a loading control. There was no significant difference in ε-sarcoglycan levels in the synaptosomal fractions between WT control mice and *Dyt1* ΔGAG heterozygous KI mice ($P=0.44$; Fig. 3C). The results suggest that *Dyt1* ΔGAG

mutation does not significantly affect the trafficking of ε-sarcoglycan *in vivo*.

Discussion

To determine the effect of the additional *Sgce* mutation to the onset of the motor deficits in *Dyt1* ΔGAG KI mice, we produced the single mutant mice, the double mutant mice and the control WT littermates, and evaluated their motor performance by the beam-walking test. The double mutant mice exhibited significant motor deficits at 1 month before the onset of motor deficits in single mutant mice (Fig. 1B), suggesting that the double mutations cause earlier onset of motor deficits in mice. This is the first report that simultaneous mutations in two dystonia genes facilitate the onset of motor deficits in mice, suggesting that the additional multiple mutations are a risk factor of early onset in this disease. Detection of additional mutations in other dystonia genes may be beneficial to predict the onset and penetrance in *DYT1* mutation carriers.

Although a previous paper suggested that torsinA contributes to the quality control of ε-sarcoglycan and facilitates the degradation of both WT and missense-mutant forms of ε-sarcoglycan in transfected cells (*35*), the present results showed a significant reduction in the striatal torsinA level caused by *Dyt1* ΔGAG mutation (Fig. 2B) did not alter the levels of ε-sarcoglycan (Fig. 3B). It was reported that the loss of a member in the sarcoglycan complex reduces the amount of other sarcoglycan members in the complex (*41*). If ε-sarcoglycan makes a stable complex with torsinA *in vivo*, the loss of ε-sarcoglycan should have affected the amount of torsinA. However, our data showed that loss of ε-sarcoglycan did not alter the torsinA levels (Fig. 2B). Moreover, the loss of ε-sarcoglycan did not produce a greater reduction of the torsinA level in the double mutant mice compared to *Dyt1* ΔGAG heterozygous KI mice (Fig. 2B). These results suggest that ε-sarcoglycan does not make a stable complex with torsinA. Furthermore, western blot results using synaptosomal fractions suggest that *Dyt1* ΔGAG mutation does not significantly affect the trafficking of ε-sarcoglycan *in vivo* (Fig. 3C). The present results are consistent with a previous report that WT ε-sarcoglycan was not co-precipitated with torsinA in co-transfected cells (*35*), suggesting that the interaction between torsinA and ε-sarcoglycan may be quite limited *in vivo*. TorsinA may only work for degradation of misfolded proteins such as mutant forms of ε-sarcoglycan and the reduction of ε-sarcoglycan may cause DYT11 myoclonus-dystonia in these missense mutation carriers. Finally, the present results do not exclude a possibility that the reduced level of torsinA in *Dyt1* ΔGAG heterozygous KI mice is still enough to maintain the normal level of ε-sarcoglycan and normal trafficking of it to synaptosomes. However, the earlier onset of motor deficits in the double mutant mice should be caused by the independent pathway rather than the maintenance or trafficking of ε-sarcoglycan because ε-sarcoglycan is not expressed in the double mutant mice.

Since it has been reported that both *Dyt1* ΔGAG homozygous KI and *Dyt1* KO mice showed nuclear envelope abnormality in the cerebral cortical neurons (*18*), we examined the nuclear envelope in the primary motor cortex to determine indirectly whether there is any functional alteration of torsinA in *Sgce* KO mice and the double mutant mice. However, we could not find any blebbing or other nuclear envelope abnormalities in the cortical neurons in all four genotypes (Supplementary Fig. S2). The results suggest that loss of ε-sarcoglycan did not produce significant loss of torsinA function in the cerebral cortex. Therefore, the results presented in this study suggest that the functional deficits of torsinA and ε-sarcoglycan may cause the motor deficits by independent pathways and the combination of these deficits caused earlier onset of motor deficits in double mutant mice. This conclusion is further supported by our previous studies. Although alterations of striatal monoamine contents were present in both *Dyt1* ΔGAG KI mice and *Sgce* KO mice, 3-methoxy-4-hydroxyphenylacetic acid (HVA) was significantly decreased in *Dyt1* ΔGAG KI male mice (*36*) while dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and HVA were significantly increased in *Sgce* KO mice (*31*). Taken together, our results suggest different pathways are affected between DYT1 generalized torsion dystonia and DYT11 myoclonus-dystonia. Identification of these pathways will elucidate the pathophysiological mechanism of common and different symptoms of these dystonias.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest

None declared.

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