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Myoclonus, Motor Deficits, Alterations in Emotional Responses and Monoamine Metabolism in ε-Sarcoglycan Deficient Mice

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Mutations of ε-sarcoglycan gene (*SGCE*) have been implicated in myoclonus-dystonia (M-D), a movement disorder. To determine the pathophysiology of M-D, we produced *Sgce* knockout mice and found that the knockout mice exhibited myoclonus, motor impairments, hyperactivity, anxiety, depression, significantly higher levels of striatal dopamine and its metabolites, and an inverse correlation between the dopamine and serotonin metabolites. The results suggest that the diverse symptoms associated with M-D are indeed resulted from a single *SGCE* gene mutation that leads to alterations of dopaminergic and serotonergic systems. Therefore, antipsychotic agents and serotonin reuptake inhibitors may offer potential benefits for M-D patients.

Key words: dopamine, dystonia, myoclonus, sarcoglycan, serotonin.

Abbreviations: DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin; HVA, 3-methoxy-4-hydroxyphenylacetic acid; KO mouse, knockout mouse; 3-MT, 3-methoxytyramine; M-D, myoclonus-dystonia; *SGCE* (for human) or *Sgce* (for mouse), ε-sarcoglycan gene.

Sarcoglycans are transmembrane glycoproteins with six different isoforms, α, β, γ, δ, ε, and ξ (*1*). They are components of sarcoglycan complexes, which associate with sarcospan and dystroglycan complex to make dystrophin-glycoprotein complexes in muscles (*2*). Epsilon-sarcoglycan is also detected in diverse brain regions with high expression levels in the olfactory mitral cells, the cerebellar Purkinje cells, and the midbrain monoaminergic neurons (*3*). Mutations in the *SGCE*, the human gene coding for ε-sarcoglycan, have been linked to DYT11 dystonia, a subtype of M-D (*4*). M-D is a movement disorder characterized by myoclonic jerks and rapid muscle contraction combined with dystonic postures of sustained twisting and repetitive movements (*5*). DYT11 dystonia is often accompanied by diverse psychiatric symptoms, such as anxiety disorder, depression, panic attacks, and obsessive-compulsive disorder (*4*, *6–8*).

Although human linkage studies associated mutant *SGCE* with the diverse neurological and psychiatric symptoms, conflicting findings about the association have been reported. Some M-D patients had *SGCE* mutation along with other mutated genes, such as *DRD2* for dopamine receptor 2 and *TOR1A* (*DYT1*) for torsinA (*9*, *10*). Furthermore, a significant number of M-D patients appear to have no detectable *SGCE* mutations (*11–13*) while a new locus for M-D has been reported in other patients (*14*), suggesting M-D is genetically heterogeneous. The genetic and phenotypic heterogeneity of M-D patients makes it difficult to determine if these various symptoms are truly caused by the *SGCE* mutant allele. An animal model with targeted ε-sarcoglycan mutation is needed to determine the contribution of the *SGCE* mutation to the pathogenesis of the disease.

We previously reported the making of *Sgce* knockout mice lacking exon 4 and demonstrated that paternally-inherited *Sgce* heterozygous knockout (KO) mice do not express maternally-inherited wild-type *Sgce* in the brain (*15*). To determine the effects of the loss of ε-sarcoglycan on the animal's motor and emotional behaviors and to understand the pathophysiology of the DYT11 dystonia, we performed a series of behavioral tests and analyzed the levels of dopamine (DA), serotonin (5-HT), and their metabolites in the striatum.

MATERIALS AND METHODS

Sgce *Knockout Mice*—We chose paternally-inherited *Sgce* heterozygous knockout mice (+/Δ) as a genetic disease model of DYT11 dystonia over homozygous knockout mice (*Sgce* Δ/Δ) because the majority of DYT11 patients has paternally-inherited mutant *SGCE* (*3*). Paternally-inherited *Sgce* +/Δ mice have previously been shown to not express maternally-inherited *Sgce* mRNA in the brain (*15*). Paternally-inherited *Sgce* +/Δ mice lacking exon 4 (+/Δ) without a neomycin cassette were produced as previously reported (*15*). *Sgce* +/Δ males (mixed genetic background of C57BL/6, 129/Sv and BALB/c) were crossed with C57BL/6 females to obtain paternally-inherited heterozygous *Sgce* knockout mice and their wild-type littermates. To minimize the contributions of the mixed genetic background to the analysis of phenotypes, we used littermates as controls for all of the experiments in this study. Mice were housed under a 12-h light and dark cycle condition. Two groups of mice were used. The first group of 17 KO (9 males and 8 females) and 22 wild-type

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(WT) littermates (11 males and 11 females) was used for scoring the abnormality of body form, accelerated rotarod test, pawprint analysis, beam-walking test, grip strength test, and spontaneous myoclonus test in this sequence. The second group of 22 KO (11 males and 11 females) and 17 WT littermates (9 males and 8 females) was used for the open-field test, light-dark box test, tail suspension test, and neurochemical analysis. Mice were allowed to rest for one to two weeks in between tests. Behavior tests were performed within the last 4 hr of the light period after acclimation to a sound-attenuated testing room for 1 hr. Dissection of striata for HPLC analysis was also performed within the same light period of a day. All the experiments were performed by investigators blind to the genotypes.

Motor Behavior Tests—Scoring the abnormality of body form, accelerated rotarod test, pawprint analysis, beam walking test, and open-field test were performed as described previously (*16*). Briefly, beam walking was performed using mice that were from 197 to 228 days old and trained to transverse a medium square beam (14 mm wide) in three consecutive trials each day for two days. The mice were then 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 diameter) on the fourth day. Their hindpaw slips on each side were recorded. Grip strength test was performed using a commercially available apparatus (San Diego Instruments, CA).

Spontaneous Myoclonus Test—Mice (210 to 241 days old) were placed in a transparent flat bottom rodent restrainer (model 541-RR; Plas-labs, Inc. MI) to minimize voluntary movements and videotaped for an hour. The mice were habituated for approximately 30 min in the restrainer and the numbers of spontaneous myoclonus jerks of the whole body were counted during the subsequent 30 min (*17*).

Light-Dark Box Test—Each mouse from 223 to 249 days old was tested as described previously (*18*). The latency in the light box before the first entry to the dark box (entry time) was videotaped and counted.

Tail Suspension Test—Each mouse (from 237 to 263 days old) was suspended by its tail with adhesive tape attached to a rope and videotaped. The total immobility time during 6 min was evaluated (*19*). Mice that climbed up the rope were excluded from the statistical analysis (3 KO and 2 WT mice).

HPLC Analysis—Striata were dissected from the brains of the KO and WT littermates from 329 to 355 days old. DA, DOPAC and HVA were analyzed as described (*16*) except using 50 mM potassium phosphate buffer with 0.5 mM octyl sulfate (Sigma) and 8% acetonitrile as running buffer. 5-HT, 5-HIAA, 3-methoxytyramine (3-MT) and adrenaline were measured by the Neurochemistry Core Lab., Vanderbilt University Medical Center, Nashville, TN (*20*).

Statistical Analysis—Statistics were performed using SAS/STAT Analyst software as described (*16*). Significance was assigned at the $p < 0.05$ level. When the genotype and sex interaction had significant difference or trend ($p \leqslant 0.1$), the data were stratified by sex and analyzed further in each group separately (*21*). Myoclonus was analyzed by logistic regression (GENMOD) with negative binominal distribution. WT mice were normalized to zero. Correlations were calculated with the CORR procedure program. The data of slip numbers in the beam-walking test for all four beams were analyzed together by logistic regression (GENMOD) with negative binominal distribution using GEE model. Control mice were normalized to zero. To compare the effect of motor learning during the beam-walking test, a dummy variable that combined genotype and trial number was used and contrasted to derive the p-value between trials 1 and 2 for medium round, small round and small square beams.

RESULTS

Spontaneous Myoclonus and Motor Deficits—The spontaneous myoclonus numbers of individual mice were counted and plotted as the accumulated frequency in each genotype (Fig. 1A). About 30% of KO mice showed more than 40 myoclonus jerks in 30 min while none of the WT mice did. The KO mice on average exhibited 28 times more myoclonus than WT littermate mice (Fig. 1B; $p \leqslant 0.0001$).

In the beam-walking test, which measures fine motor coordination and balance, KO mice showed 122% more number of slips compared with WT mice (Fig. 2A, Total; $p = 0.0472$). Detailed analysis revealed that KO mice had a significantly higher number of the right side slips (215%

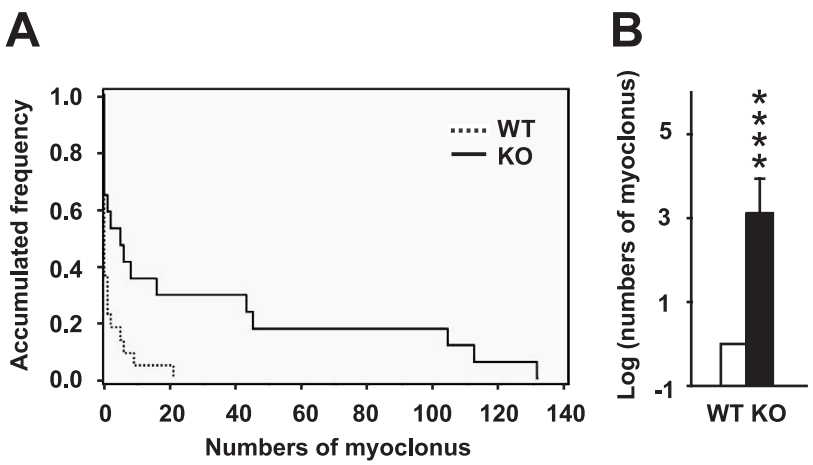

Fig. 1. **Spontaneous myoclonus test.** (A) The spontaneous myoclonus numbers in 30 min of individual mouse are plotted to show the accumulated frequency of the whole WT and KO group. (B) The total spontaneous myoclonus numbers of KO and WT mice. Data were normalized to WT mice. ****$p \leqslant 0.0001$. Vertical bars represent mean ± SE.

more slips, $p = 0.0032$; genotype and side interaction, $p = 0.0747$) while their left side slips were not significantly different from those of WT mice ($p = 0.6869$).

Recently, deficits in motor learning have been reported in carriers with *TOR1A* (*DYT1*) mutation that is responsible for DYT1 dystonia (*22*) and in transgenic mice carrying the same mutation (*23*). To compare the motor learning between KO and WT mice, we analyzed the total slip numbers of the first and second trials using medium round, small round and small square beams. The slip numbers in medium square beam were excluded because it was used for training. WT mice showed less slips and improved significantly in trial 2 over trial 1 (Fig. 2B; $p = 0.0398$). However, in KO mice, there was no significant difference between trials 1 and 2 ($p = 0.0790$). The results suggest that KO mice exhibited deficits that were consistent with impairment of motor learning as reported in DYT1 patients and transgenic mice.

To determine whether the motor deficits in KO mice were complicated with myoclonus, we analyzed the correlation between the numbers of myoclonus and total slips of each individual mouse. Significant correlation between myoclonus and slips numbers were detected neither in KO (Pearson correlation coefficients = –0.07559, $p = 0.7731$) nor in WT (0.20253, 0.3660, respectively) mice. Thus, the increased slips were not accompanied with myoclonus but reflect intrinsic deficits of motor coordination and balance. The independent appearance of myoclonus and dystonia symptoms was also reported in a study involving 24 DYT11 dystonia patients: 98% patients exhibited myoclonus while only 54% exhibited dystonia symptoms (*7*).

Scoring of body form abnormality, accelerated rotarod, grip strength test and pawprint gait analysis did not reveal significant difference between KO and WT mice (data not shown). The appearance of significant differences in the beam walking test and not in the rotarod test is also reported in the mouse models of DYT1 dystonia (*16*). These two tests could have different sensitivities for detecting motor impairment.

Motor Hyperactivity—We assessed locomotive behavior in the open-field test. There was a significant interaction between genotype and sex in horizontal locomotion ($p = 0.0338$). Female KO mice exhibited increased horizontal activity (Fig. 3A; $p = 0.0329$) while KO males did not exhibit significant differences in comparison to WT mice. Furthermore, the KO mice regardless of sex showed significant increases in vertical movement number (Fig. 3B; $p = 0.0016$), vertical activity ($p = 0.0058$), and vertical movement time ($p = 0.0016$).

Anxiety-like Behaviors—Anxiety corresponds to less time spent in the center region of the open-field test area and decreased ratio of central versus total distance the mice traveled (*24*). Therefore, we evaluated the amount of the time and the ratio of the distance. There was a significant interaction between genotype and sex for the amount of time spent ($p = 0.0077$) and for the ratio of the distance traveled ($p = 0.0157$) in the central region. Male KO mice spent significantly less central time (Fig. 4A; $p = 0.0032$) and showed significantly decreased central distance ratio (Fig. 4B; $p = 0.0127$) than their WT littermates. No such differences were present between female KO and WT mice. Taken together, compared to WT male mice, KO males showed a significantly higher level of anxiety.

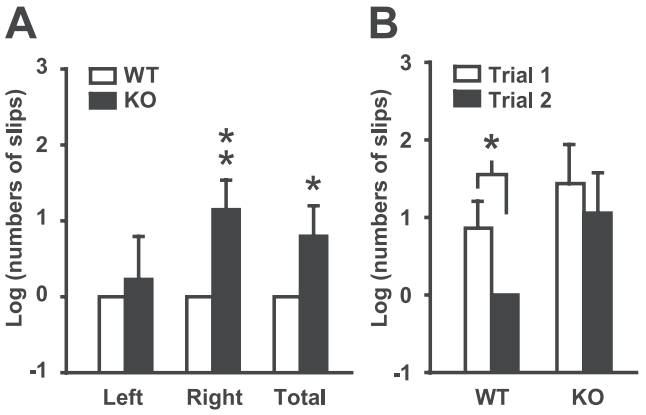

Fig. 2. **Beam-walking test.** (A) Slip deficits and affected side in KO mice. Both male and female KO mice exhibited more slips. Numbers of slips on each side (Left and Right) and the combined (Total) are plotted. (B) Impaired motor learning in KO mice. WT mice showed significantly less slips and improved significantly in trial 2 over trial 1. However, in KO mice, there was no significant difference between trials 1 and 2. Mice were tested from 197 to 228 days old. Data were normalized to WT mice (A) or trial 2 of WT mice (B). $*p < 0.05$, $**p < 0.01$. Vertical bars represent mean ± SE.

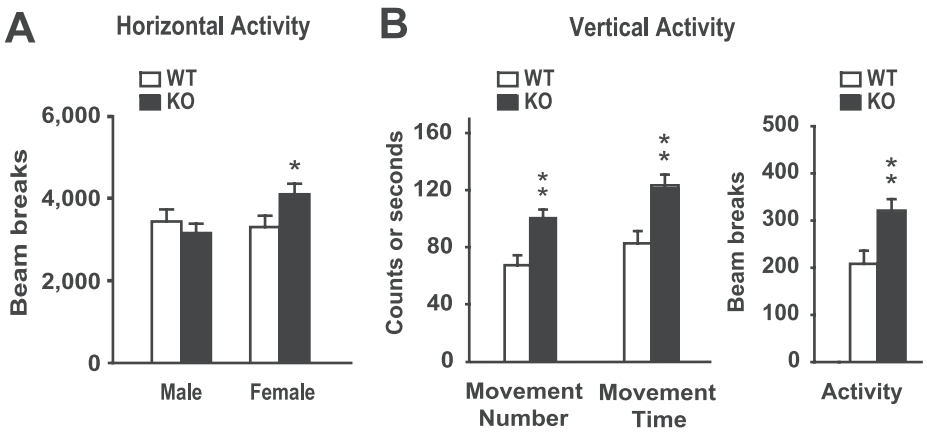

Fig. 3. **Locomotion in open-field test.** (A) The female KO mice showed significantly more horizontal activity than WT mice. (B) Vertical movement number, vertical movement time (s), and vertical activity in 15 min. $*p < 0.05$. $**p < 0.01$. Vertical bars represent mean ± SE.

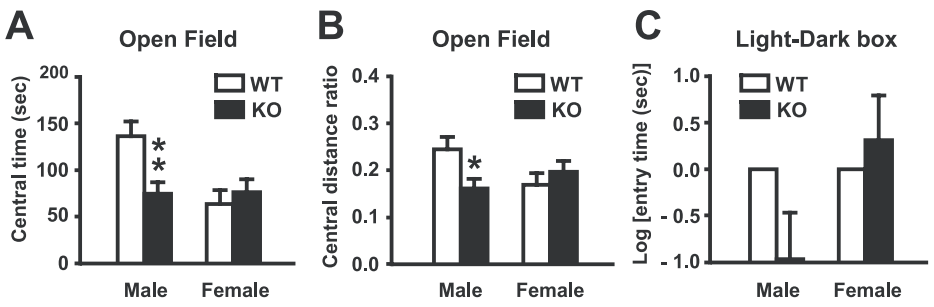

Fig. 4. **Anxiety-like behavior of male KO mice in open-field and light-dark box tests.** (A) Total central time (s) in 15 min. (B) Central distance ratio. (C) The first entry time into the dark box in light-dark box test. Data were analyzed by log transformation with WT normalized to zero. $^{*}p < 0.05$, $^{**}p < 0.01$. Vertical bars represent mean ± SE.

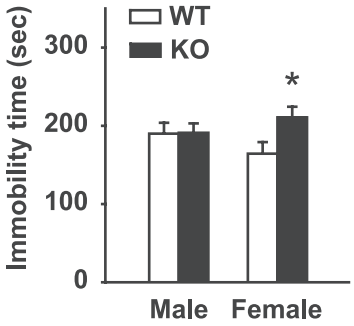

Fig. 5. **Depression-like behaviors in female KO mice.** Total immobility time (s) in 6 min tail suspension test. Female KO mice showed significantly longer immobility time. $^{*}p < 0.05$. Vertical bars represent mean ± SE.

Table 1. **DA, 5-HT, and their metabolites in the striatum.**

Content or ratio	WT	KO	p
DA	18.42 ± 2.24	25.37 ± 1.89	0.0248*
DOPAC	1.11 ± 0.09	1.36 ± 0.07	0.0352*
HVA	1.74 ± 0.13	2.12 ± 0.11	0.0302*
3-MT	1.47 ± 0.17	1.58 ± 0.14	0.6228
Adrenaline	0.27 ± 0.07	0.39 ± 0.06	0.1606
5-HT	2.12 ± 0.17	2.09 ± 0.15	0.9085
5-HIAA	0.46 ± 0.04	0.55 ± 0.04	0.1037
DOPAC/DA	0.068 ± 0.006	0.064 ± 0.005	0.5883
HVA/DA	0.106 ± 0.010	0.100 ± 0.008	0.6255
5-HIAA/5-HT	0.227 ± 0.015	0.264 ± 0.013	0.0652

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. $^{*}p < 0.05$.

Table 2. **Correlations among DA, 5-HT, the metabolites, and vertical activities.**

Compared subjects	WT PCC	WT p	KO PCC	KO p
DOPAC:VMOVNO	–0.22474	0.4027	0.42488	0.0487*
DOPAC:VACTV	–0.20069	0.4561	0.42076	0.0512
DOPAC:VTIME	–0.25345	0.3436	0.45050	0.0354*
5-HIAA:VMOVNO	–0.12567	0.6428	–0.49268	0.0198*
5-HIAA:VACTV	–0.03901	0.8859	–0.45170	0.0348*
5-HIAA:VTIME	0.12466	0.6455	–0.49361	0.0196*
5-HT:DA	–0.04219	0.8767	–0.56566	0.0061**
5-HT:DOPAC	–0.20349	0.4497	–0.57940	0.0047**
5-HT:HVA	–0.23508	0.3808	–0.56269	0.0064**
5-HIAA:DA	–0.07832	0.7731	–0.63970	0.0013**
5-HIAA:DOPAC	–0.03430	0.8996	–0.72820	0.0001****
5-HIAA:HVA	–0.06804	0.8023	–0.63787	0.0014**

PCC, Pearson Correlation Coefficients; VMOVNO, vertical movement number; VACTV, vertical activity; VTIME, vertical movement time; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p \leqslant 0.0001$.

The anxiety-like behavior was also assessed using a light-dark box test. Decreased number of transition between the two compartments and/or time and activity in the light side are indicative of increased anxiety. KO mice were hyperactive which could have prevented the accurate measurement and meaningful interpretation of total transitions and time in the dark box. As a result, we used a less sensitive measure of entry time instead. There was a trend for KO male to enter the dark box quicker than WT males (Fig. 4C; genotype, $p = 0.0531$; genotype and sex interaction, $p = 0.0065$).

Depression-Like Behaviors—Depression in mice was evaluated by the tail suspension test (*19*). When suspended by the tail, KO female exhibited significantly more time in immobility than WT females (Fig. 5; genotype, $p = 0.0250$; genotype and sex interaction, $p = 0.0784$), suggesting that the knockout females exhibited more depression-like behaviors. Male KO and WT mice had the same level of immobility in this test ($p = 0.9511$).

Neurochemicals in the Striatum—To determine the neurochemical basis of the observed behavioral impairment, we measured concentrations of neurochemicals involved in movement and emotional control. The levels of DA, DOPAC and 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid; HVA) in the striatum of KO mice were significantly higher than those of WT mice (Table 1). 5-HT and its metabolites were also analyzed. There was no significant difference in the 5-HT levels between KO and WT mice. However, the level of 5-hydroxyindoleacetic acid (5-HIAA) and the ratio of 5-HIAA to 5-HT had a tendency to be higher in KO than WT mice, although the difference did not reach significance.

We then analyzed the correlations between the levels of the neurochemicals and behavioral results of the individual mouse. In KO mice, DOPAC levels directly correlated and 5-HIAA levels inversely correlated with vertical movement number, vertical activity, and vertical movement time (Table 2). However, such correlations were absent in WT mice, suggesting that the voluntary vertical activities in WT mice were independent of overall DA and 5-HT metabolisms. Furthermore, a significant inverse correlation between DA and 5-HT metabolism was present in KO mice while such relationship was absent in WT mice.

DISCUSSION

DYT11 dystonia is a complex disease with myoclonus, dystonia and diverse psychiatric symptoms. In this

study, we evaluated the behaviors of *Sgce* KO mice and the neurochemicals in the striatum. The KO mice showed myoclonus, deficits in motor coordination, balance, and learning, and psychiatric alterations that were consistent with anxiety and depression. The KO mice showed significantly increased vertical hyperactivity that is correlated to DOPAC and inversely correlated to 5-HIAA. The vertical hyperactivity in KO mice likely represents the compulsive-checking behavior (*25*) and functional changes of dopaminergic and serotonergic neurons have been implicated in patients with obsessive compulsive disorder (*26–28*). Our results suggested that the diverse symptoms associated with DYT11 dystonia are indeed the effects of a single gene mutation involving *SGCE*. The trend of high serotonin turnover suggests that serotonin reuptake inhibitors may offer potential benefits for M-D patients.

Our neurochemical study showed inverse correlation of DA and 5-HT metabolites in the KO mice. This is consistent with the high level of expression reported for ε-sarcoglycan protein and mRNA in dopaminergic and serotonergic neurons (*3*). Based on the DA measurements, we propose to categorize DYT11 dystonia as a hyperdopaminergic dystonia. The mechanism through which the loss of ε-sarcoglycan causes hyperdopaminergic striatum remains to be investigated. Several forms of dystonia have been associated with either an increase or decrease of DA in the striatum. The most well-known is dopa-responsive dystonia caused by mutations in genes for GTP-cyclohydrolase I (*29*) or tyrosine hydroxylase (*30*). Early-onset DYT1 dystonia patients also have either lower DA levels or an increase in DA turnover in the brain, although they do not respond to L-dopa treatment (*31*, *32*). While a decrease in DA availability can lead to dystonia, an increase of it has also been shown to cause dystonia. L-dopa–induced dystonia is a major side effect challenging patients who are on long term treatments of L-dopa (*33*). In animal models, elevated levels of DA also have been shown to correlate with the dystonic symptoms. For example, injections of GDNF into the striatum, which causes an increase in DA production, has been shown to cause dystonia in rats (*34*). Furthermore, in a mutant hamster model of paroxysmal dyskinesia, striatal levels of DA and DOPAC are significantly elevated during periods of stress-induced dystonic attacks (*35*). Of the five DYT11 dystonia patients treated with L-dopa that were reported in a recent study, only one showed an improvement of symptoms and four experienced no improvement, with substantial side effect occurring in one case (*36*). Our neurochemical results suggest that L-dopa therapy may not be effective in relieving the symptoms of DTY11 dystonia; instead it may aggravate the symptoms. Hyperdopaminergia in the striatum of the KO mice suggests that antipsychotic agents to block dopaminergic pathway may be explored as a medical treatment for DYT11 dystonia.

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