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An anticholinergic reverses motor control and corticostriatal LTD deficits in *Dyt1* ΔGAG knock-in mice

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Abstract

DYT1 early-onset generalized torsion dystonia is an inherited movement disorder associated with mutations in *DYT1* that codes for torsinA protein. The most common mutation seen in this gene is a trinucleotide deletion of GAG. We previously reported a motor control deficit on a beam-walking task in our *Dyt1* ΔGAG knock-in heterozygous mice. In this report we show the reversal of this motor deficit with the anticholinergic trihexyphenidyl (THP), a drug commonly used to treat movement problems in dystonia patients. THP also restored the reduced corticostriatal long-term depression (LTD) observed in these mice. Corticostriatal LTD has long been known to be dependent on D2 receptor activation. In this mouse model, striatal D2 receptors were expressed at lower quantities in comparison to wild-type mice. Furthermore, the mice were also partially resistant to FPL64176, an agonist of L-type calcium channels that have been previously reported to cause severe dystonic-like symptoms in wild-type mice. Our findings collectively suggest that altered communication between cholinergic interneurons and medium spiny neurons is responsible for the LTD deficit and that this synaptic plasticity modification may be involved in the striatal motor control abnormalities in our mouse model of DYT1 dystonia.

Keywords

dopamine receptor; dystonia; long-term depression; torsinA; trihexyphenidyl

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1. Introduction

Dystonia is a movement disorder of disrupted muscle control caused by excessive activity of opposing muscle groups causing patients to experience sustained muscle contractions and twisting postures. Dystonia is a disorder of the central nervous system that is at least in part due to a disruption of normal basal ganglian activity. Neurodegeneration has not been found to be involved in primary dystonia, setting it apart from other major movement disorders involving the basal ganglia, such as Parkinson's and Huntington's diseases [1]. DYT1 early-onset generalized dystonia is a subtype of primary dystonia that is an inherited form of the movement disorder with onset during childhood or adolescent [2]. Most DYT1 dystonia patients have a trinucleotide deletion of a GAG (Δ GAG) sequence in the *DYT1* gene that corresponds to a deletion of a glutamic acid residue (Δ E) [3]. Two additional mutations, an 18-bp deletion and an Arg288Gln exchange have also been reported [4].

DYT1 encodes the protein torsinA. Its function is still unknown; however gene sequence comparisons identify it as an AAA+ ATPase, a family of protein with a variety of functions including serving as a chaperone molecule for the unfolding of other proteins and DNA structures [5]. Localization studies show that mRNA levels of *DYT1* are concentrated in dopaminergic nigrostriatal neurons [6], making the dopaminergic system an appropriate focus of dystonia research. Analyses of post-mortem brain tissues of human patients and brain tissues of transgenic and knock-in heterozygous mouse models of DYT1 dystonia, have identified abnormalities in multiple aspects of the dopaminergic system. Altered dopamine metabolism, determined by measuring the metabolites of dopamine, and a reduction in dopamine release by evoked potentials or by amphetamine exposure have been reported in several mouse models [7–9]. In non-manifesting human carriers of the *DYT1* mutation, dopamine receptor type 2 (D2R) ligand-binding in the striatum measured by positron emission tomography has been shown to be decreased; this reduction in D2R was first observed in focal dystonia patients [10, 11]. Collectively, these studies identified the dopaminergic system as an integral part of the pathophysiology of dystonia, but how these neurochemical changes can lead to the abnormal movements seen in dystonia is still unclear. Presently, we are left to hypothesize broadly that the changes to the dopaminergic system affects motor function by providing inappropriate neurochemical signals within the motor control system.

The function of torsinA also has been approached by studying its localization in cellular structures. In human patients and mouse models carrying the mutation, ubiquitin- and torsinA-containing aggregates in neurons of the pontine nuclei have been reported [7, 12]. Neurons from mice that either do not express torsinA or that are homozygous for the mutation have disrupted nuclear membrane [13]. In addition, multiple studies showed that torsinA is essential to normal protein processing and that the Δ E in torsinA may disrupt that role [14, 15]. These findings indicate that torsinA is important in normal cellular development and neurochemical processing; however, it remains to be seen how many of these reported observations are directly involved in the pathophysiology of dystonia. Also, we continue to struggle with the fact that we still cannot look at these molecular and cellular abnormalities and lay out a clear mechanism for dystonia's physical debilitation from improper motor control.

An avenue of research that in recent years has gained much attention, which may begin to address this problem, is the role of abnormal synaptic plasticity in the loss of motor control. Using a variety of artificial paradigms of plasticity, including high frequency transcranial magnetic stimulation, paired association stimulation, and theta burst stimulation, multiple research groups showed that primary dystonia patients have enhanced response to the external stimuli and loss of spatial specificity in the sensorimotor cortex [16–19]. These

findings provide significant support for the theory that the increase in muscle activation of dystonia may be caused by both excessive signaling and lack of inhibition in the motor corticostriatal pathway [20, 21]. However, the nature of the relationship, causal or correlative, between the synaptic plasticity changes and the manifesting symptoms remain unknown. For example, we are still unclear about whether the abnormalities in the somatosensory cortex seen in primary dystonia is the cause or the result of the abnormal movements [22].

In this study, we further explore the relationship between synaptic plasticity and motor control in dystonia by employing our *Dyt1* Δ GAG heterozygous knock-in (KI) mice that carries one *Dyt1* allele with the trinucleotide deletion, similar to that seen in DYT1 patients. We previously reported that these mice have a reproducible fine motor coordination and balance deficit on the beam-walking task. In this study, we show that our dystonia mouse model have a deficit in synaptic plasticity, specifically a loss of corticostriatal long-term depression (LTD) that we predict may play a role in the motor abnormality. The LTD deficit was restored by the anticholinergic, trihexyphenidyl (THP). We also show that THP can reverse the motor discoordination and imbalance seen in the KI mice. In addition, we further characterize the role of striatal cholinergic neurons in LTD and the involvement of D2R in the communication between cholinergic neurons and striatal medium spiny neurons.

2. Materials and methods

2.1. Animals and housing

All experiments were carried out in compliance with the USPHS Guide for Care and Use of Laboratory Animals and approved by the IACUC of University of Illinois at Urbana-Champaign (UIUC) and the University of Alabama at Birmingham (UAB). *Dyt1* Δ GAG KI mice and their littermate control wild-type WT mice were prepared and genotyped by PCR as described earlier [7, 23]. Since male *Dyt1* Δ GAG KI mice exhibited significant motor deficits in the previous studies, only male mice were used for every experiment in this study. Mice were housed under a 12 hr light, 12 hr dark cycle with *ad libitum* access to food and water. All of the behavior tests were performed by investigators blind to the genotypes and treatments.

2.2. Field potential recording

Brain slices containing both striatum and cortex were prepared from male mice between 3 and 4 weeks old. Animals were sacrificed by decapitation, and the brains were transferred rapidly to ice-cold modified artificial cerebrospinal fluid (ACSF) containing: 194 mM sucrose, 30 mM NaCl, 4.5 mM KCl, 1 mM MgCl₂, 26 mM NaHCO₃, 1.2 mM NaH₂PO₄, and 10 mM D-glucose. Modified ACSF was brought to pH 7.4 by aeration with 95% O₂/5% CO₂. Coronal sections (300 μ m thick) were cut in ice-cold modified ACSF using a vibrotome 1000. Slices were transferred immediately to a nylon net submerged in normal ACSF (124 mM NaCl, 4.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 26 mM NaHCO₃, 1.2 mM NaH₂PO₄, and 10 mM D-glucose). Normal ACSF was maintained at pH 7.4 by aeration with 95% O₂/5% CO₂ at room temperature (19–22°C). Following at least 1 hr of incubation at room temperature, hemislices were transferred to a recording chamber, submerged in normal ACSF and maintained at a temperature between 28 and 30°C stable within $\pm 1^\circ\text{C}$ during any given experiment.

Pipettes with resistances ranging from 2–4 M Ω were pulled from borosilicate glass capillaries on a Flaming-Brown micropipette puller (Sutter Instruments, Novato, CA), and filled with 1 M NaCl. Stimulation, given at 0.05 Hz, was delivered by a Master-8 stimulator and optical stimulus isolation unit (A.M.P.I., Jerusalem, Israel) through a bipolar twisted

tungsten wire that was placed in the white matter just dorsal to the dorsomedial or dorsolateral striatum. The recording electrode was placed in the dorsomedial for long-term potentiation (LTP) or dorsolateral striatum for LTD, as previously described [24, 25]. Stimulation intensity was adjusted to yield an evoked population spike amplitude approximately one-half the amplitude of the maximal evoked response. Stimulus pulse duration ranged from 0.02 to 0.05 m sec, and stimulation intensity ranged from 0.5 to 0.9 mA. Data were filtered (high pass 0.1 Hz, low pass 3 kHz), then amplified and digitized using an Axoclamp 1D amplifier and Digidata 1322 interface (Axon Instruments/Molecular Devices, Sunnyvale, CA). To induce LTP or LTD, 4 trains of 100 Hz stimulation were delivered 10 sec apart, at an intensity that evoked the maximal PS. The induction protocol for striatal LTD is the same as that for striatal LTP; the only difference is that LTD is recorded from the dorsolateral, or sensorimotor, striatum.

In experiments examining the effect of muscarinic receptor blockade on LTD, THP (5 μ M) was applied to the bath perfusion at the start of the recording and was present throughout the experiment. THP was made from a 500 $\times$ concentrated stock solution in distilled H₂O. The population spike amplitude was measured using Clampfit 9.2, as previously described [26].

2.3. THP injection and Beam-walking test

The beam-walking test was performed as described previously [7]. Animals were trained to traverse a medium square beam (14 mm wide) in three consecutive trials each day for 2 days. After training was completed, the experiment commenced with recordings of beam transversal time and number of hindpaw slips for each of the two trials per beam. On the first test day, animals were made to cross the medium square and round beam (17 mm diameter) and on the second day, a small round beam (10 mm diameter) and an additional small square beam (7 mm wide). A total of 20 WT and 20 *Dyt1* Δ GAG KI male mice about 5 to 6 months of age were used. Either WT or *Dyt1* Δ GAG KI mice were further randomly assigned to two groups with 10 mice each. THP solution in saline was injected intraperitoneally once on each test day about 2 hr before the beginning of the beam-walking test. THP in 0.9% NaCl was used at 0.8 mg/kg. One group was injected with THP and the other injected with a vehicle solution of 0.9% NaCl.

2.4. Golgi staining

Brains from three male mice per genotype of approximately 8 months old were processed with the Rapid Golgi staining kit (FD NeuroTechnologies, Ellicott City, MD), based on the manufacturer's protocol. Stained slices were sectioned at a thickness of 200 μ m. Two to three medium spiny neurons of the striatum of each mouse were selected based on guidelines established previously [27]. Neurons were traced using Neurolucida software (MicroBrightFields, Colchester, VT) at 40 $\times$ magnification in oil. Analysis of neuronal tracings was performed with NeuroExplorer (MicroBrightFields). Branching numbers per order and spine densities of mice from each genotype were compared.

2.5. D2R measurements

2.5.1. Brain tissue binding autoradiography—Brains from three pairs of WT and *Dyt1* Δ GAG KI male mice of six months old were frozen in isopentane (Sigma-Aldrich) cooled by dry ice. Coronal sections were cut in a cryostat and thaw-mounted onto gelatin-coated glass slides. Sections that were used corresponded to plates 20 to 25 of Franklin and Paxinos mouse brain slices collection [28]. Binding was performed according to a published protocol [29]. Briefly, sections were incubated in 180 pM [³H] YM-09151 in incubation buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM MgCl₂, 1 μ M pargyline, 100 mg/L ascorbate) for 3 h at room temperature. After incubation, sections were washed for 10 min in cold incubation buffer followed by a dip in cold H₂O. When sections were dry, they were

exposed to BAS-TR 2025 imaging plate for 72 h and scanned on an FLA-7000 bio-imager analyzer (Fuji Film).

2.5.2. Radioligand binding in membrane preparations—For D2R binding assay, striata were removed from three male mouse brains of each genotype of at least six months of age and homogenized in nine volumes of ice-cold 50 mM Tris-HCl, 8 mM MgCl₂, 5 mM EDTA disodium (pH 7.1). The homogenate was centrifuged at 18,000 × g for 20 minutes at 4°C and the pellet was suspended in the same buffer and stored at -80°C. The protein concentration of the membrane preparation was determined by Bradford assay using bovine serum albumin as a standard. [³H]YM-09151 binding was determined using previously described methods [30]. Briefly, an aliquot of the homogenate was suspended in 200 µl of binding buffer [50 mM Tris-HCl, 120 mM NaCl, 5mM KCl, 5 mM MgCl₂, 1.5 mM CaCl₂, 1 mM EDTA disodium, 10 µM pargyline hydrochloride (Sigma-Aldrich) and 0.1% ascorbic acid, pH 7.4] with 0.03 to 1.92 nM [³H]YM-09151 (2.64TBq/mmol, Perkin Elmer). The reaction mixture was incubated at 25°C for 40 minutes, and then rapidly filtered under vacuum through glass microfiber GF/B filter (Whatman). The filter was then washed four times with 4 ml of the ice-cold binding buffer without the isotope and then dried. The radioactivity of the filters was measured in 6 ml of ScintiSafe Econo1 (Fisher Scientific) by a Beckman liquid scintillation counter. Non-specific binding of [³H] YM-09151 was measured in the presence of 30 µM (S)-(-)-sulpiride (Sigma-Aldrich). The experiments were done in duplicates.

2.5.3. qRT-PCR of mRNA for striatal D2L and D2S—Striata were dissected from four *Dyt1* ΔGAG KI and four WT male mouse brains of 6 months old and quickly frozen in liquid nitrogen. The RNA was isolated by RNeasy mini kit (Qiagen) and the cDNA was synthesized by SuperScript III First-Strand SuperMix for qRT-PCR kit (Invitrogen). The real-time (RT) PCR was performed by using iQ5 real-time systems (Bio-rad) with SYBR Green ER qPCR SuperMix for iCycler instrument (Invitrogen) and the following primer sets. The primer sets of D2E7 (5'-GGAGTTTCCCAGTGAACAGGCGG-3') and D2E8 (5'-TTGCTATGTAGACCGTGGTGGGATG-3') for dopamine receptor D2L, D2E8 and D2S (5'-CACCACTCAAGGATGCTGCCCG-3') [31] for dopamine receptor D2S, and E1 (5'-CACCCGCGAGCACAGCTTCTTTG-3') and E2 (5'-AATACAGCCCCGGGAGCATCGTC-3') for β-actin, were used for the real-time PCR. The iQ5 real-time systems was programmed at 50°C for 2 min, 95°C for 8 min 30 sec, 40 cycles of 95°C for 30 sec and 66°C for 1 min 30 sec, followed by melting curve analysis. The relative quantities of cDNA for receptor D2L and D2S were calculated by ΔC_T methods normalized to β-actin cDNA as a reference [32].

2.5.4. Western blot analysis—Striata were dissected from three *Dyt1* ΔGAG KI and three WT male mouse brains of six months old and homogenized in 200 µl of ice-cold lysis buffer [50mM Tris-Cl (pH 7.4), 175mM NaCl, 5mM EDTA with a protease inhibitor cocktail tablet (Roche)]. The homogenate was sonicated for 10 sec. Triton X-100 was added to 1% w/v final concentration and the mixture was incubated on ice for 30 min and centrifuged at 10,000 × g for 15 min at 4°C. The protein concentration of the supernatant was measured by BCA protein assay (Bio-Rad). An aliquot of the supernatant corresponding to 25 µg of protein was mixed with 2 × loading buffer containing 2-mercaptoethanol and boiled for 5 min and spun down. The proteins were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. After blocking with 5% non-fat milk (Bio-Rad) in washing buffer [20mM Tris-Cl (pH 7.6), 137 mM NaCl, 0.1% Tween 20], the membrane was incubated overnight at 4°C with rabbit anti-human dopamine receptor D2 (Chemicon, AB5084P) at 1:1,000 dilution in 5% non-fat milk in the washing buffer. Membrane was washed with the washing buffer and then incubated with peroxidase AffiniPure donkey anti-

rabbit IgG (H+L) antibody (Jackson ImmunoResearch) at room temperature for 1 h. The signals were detected with ECL kit (GE Healthcare) and X-ray film. The antibodies on the membrane was stripped with Pierce restore western blot stripping buffer and treated with mouse anti- β -actin antibody (Sigma, A5441) at 1:10,000 dilution and peroxidase AffiniPure donkey anti-mouse IgG (H+L) antibody (Jackson ImmunoResearch) as described above. The density of the bands was quantified by UN-SCAN-IT gel (Silk Scientific).

2.6. FPL64176 injection for induction of dystonia-like symptoms

Induction of dystonic-like behaviors after subcutaneous injections of FPL64176 (Sigma-Aldrich) and quantification of dystonia were performed according to published methods [33]. Briefly, ten WT and six *Dyt1* Δ GAG KI male littermates approximately 8–12 months old were subcutaneously injected with escalating doses of FPL64176 (2, 4, 8, and 12 mg/kg) on four consecutive days. Mice were placed in a clear plastic box 18 $\times$ 30 cm similar to the home cage. The dystonic-like movements and postures were assessed on a four-point scale for one minute at 10 min intervals for 1 hr. The four-point scale consisted of the following: 0 point for normal motor behavior, 1 point for no impairment, slightly slowed or abnormal motor behavior, 2 points for mild impairment, limited ambulation unless disturbed, transient abnormal postures, infrequent falls, 3 points for moderate impairment, limited ambulation, even when disturbed, frequent abnormal postures, frequent falls but upright for majority of time, 4 points for severe impairment, almost no ambulation, sustained abnormal postures, not upright for majority of time [33].

2.7. Statistics

The beam-walking data were analyzed by using SAS/STAT Analyst software (Version 9.1.3; SAS institute Inc. NC) as described [34]. In brief, slip numbers for all four beams were analyzed together by logistic regression (GENMOD) with negative binominal distribution using GEE model. The beam-walking slips were natural log transformed to obtain normal distribution to fit the statistical model, stratified by genotype, and normalized to WT or *Dyt1* Δ GAG KI mice with vehicle injection. Dendritic branches and spine numbers in Golgi staining, D2 receptor binding, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), Western blot data were analyzed by Student's t-test. Dystonia scores after FPL64176 injection were analyzed by using logistic regression (GENMOD) with GEE model and normalized to base levels and WT mice, respectively. Significance was assigned at $P < 0.05$.

3. Results

3.1. Striatal HFS-LTD, but not HFS-LTP is impaired in striatal slices from KI mice

We looked at long-term plasticity in the striatum using field potential recording. Striatal field potentials were readily evoked in dorsomedial and dorsolateral striatum subregions of brain slices from both WT and *Dyt1* Δ GAG KI mice. The stimulus intensities needed to evoke half-maximal amplitude and maximal amplitude population spikes (PS) were comparable in slices from both sets of animals, as were the PS amplitudes themselves.

Delivery of high frequency stimulation (HFS, 4 trains of 1 sec duration, 100 Hz stimulation, 1 train per 10 sec), in the dorsolateral striatum generally evokes LTD in field potential or whole-cell recordings [27, 35, 36]. Indeed, LTD was consistently observed in slices from WT mice as evidenced by a post-HFS decrease in PS amplitude of $29 \pm 10\%$ percent (Fig. 1A; $n = 6$ slices). No LTD was observed in WT mouse slices ($n=4$ slices) in the presence of the D2R antagonist sulpiride (10 μ M), indicating that LTD in these slices shows dopamine/D2R dependence similar to that observed in previous studies of rat and mouse striatum [35, 37, 38]. In contrast to findings in WT mice, LTD was not observed following HFS in a large

majority of the *Dyt1* ΔGAG KI slices examined. Only 2 of 6 KI slices examined (from 6 mice) exhibited a >15% decrease in PS amplitude following HFS, and the average decrease in this group was $3 \pm 6\%$ at the end of the experiment.

In the dorsomedial striatum, LTP was consistently induced by HFS in slices from both WT and *Dyt1* ΔGAG KI mice. The time course of LTP onset and the magnitude of the increase in PS amplitude (Fig. 1B; ~125% of baseline) were similar in both groups (n=5 slices). Thus, of the two predominant forms of long-term synaptic plasticity expressed in striatum, only LTD is disrupted in the mutant mice.

3.2. THP reverses the HFS-LTD deficit in KI mice

Activity of M1-like muscarinic acetylcholine receptors (mAChRs) suppresses LTD induction and limits the magnitude of LTD in the striatum [35, 38, 39]. Application of mAChR antagonists enhances the magnitude of HFS-LTD and can rescue LTD in the presence of D2 receptor antagonist. WT littermate mice showed consistent LTD (Fig. 1C; $18 \pm 5\%$ decrease in PS amplitude, 14 slices from 8 mice). *Dyt1* ΔGAG KI mice showed no consistent LTD (1 of 7 slices from 6 mice showing >15% decrease, $1 \pm 7\%$ average decrease, Fig. 1C). When we applied 5 μM THP to striatal slices of the *Dyt1* ΔGAG KI mice, we observed consistent LTD (Fig. 1C; PS amplitude decreased by >15% in 7 of 10 slices from 6 mice, $22 \pm 4\%$ average decrease). The stimulus parameters used to evoke maximal and half-maximal PS responses, as well as the PS amplitudes themselves did not differ between WT and *Dyt1* ΔGAG KI mice or in the presence of THP.

3.3. No overt structural changes in striatal neurons of KI mice

To rule out structural changes to the striatal neurons that may interfere with synaptic plasticity, we analyzed the branching patterns of the neurons and dendrite numbers of mutant mice. Golgi staining and tracing of neurons with subsequent analysis of branching patterns and spine density counts were performed. *Dyt1* ΔGAG KI mice had unaltered branching patterns in comparison to WT littermates. They had a similar degree of branching with an average degree of branching to the fifth order in both *Dyt1* ΔGAG KI mice and WT groups ($P = 1$, n=8 or 9 of each genotype). The number of dendrites per branch order was also similar between the two groups (Fig. 1D; WT: 2 to 9.8 ± 0 to 1.2 dendrites, KI: 2 to 9.3 ± 0 to 1.4 dendrites, $P = 0.42$ to 1.0). Spine densities per 50 μm segment of second order dendrites were unchanged in *Dyt1* ΔGAG KI mice (Fig. 1E; WT: 36 ± 3 , n = 8; KI: 34 ± 2 , n = 8; $P = 0.58$) in comparison to WT littermates.

3.4. THP rescued motor control deficit in KI mice

Dyt1 ΔGAG KI mice were previously shown to have significantly greater number of hindpaw slips on a beam-walking test [7]. We looked at the effects of THP on slips numbers in the beam-walking test. Slips numbers are normalized to saline-injected group of each genotype. Reduced slips numbers is an indicator of rescue of motor deficit. Although THP injection at 0.8 mg/kg did not affect slip numbers in WT mice (Fig. 2; Log-transformed slip numbers normalized to WT with saline: 0 ± 0 ; WT with THP: 0.0334 ± 0.3884 ; n=10 mice, $P = 0.9315$, in each group), slips numbers were significantly reduced in *Dyt1* ΔGAG KI mice, suggesting reversal of motor control deficit in *Dyt1* ΔGAG KI mice (Fig. 2; Log-transformed slips numbers normalized to KI with saline: 0 ± 0 ; KI with THP: -0.8553 ± 0.4167 ; n=10 mice in each group, $P = 0.0401$).

3.5. Striatal D2R binding, D2R levels, but not D2R mRNA levels, are decreased in KI mice

Cortico-striatal LTD dependence on D2R and the lack of LTD in *Dyt1* ΔGAG KI mice led to an examination of the striatal D2R levels in *Dyt1* ΔGAG KI mice. D2R levels of *Dyt1*

Δ GAG KI mice were measured by three different methods: autoradiographic binding, radioligand binding in a membrane fraction, and Western blot analysis. Autoradiographic binding with a radioligand for D2R, [3 H] YM-09151, performed on coronal brain sections showed that *Dyt1* Δ GAG KI mice had a reduction in striatal D2R binding (Fig. 3A; WT: 250 ± 14 pixels, $n = 3$; KI: 211 ± 14 pixels, $n = 3$; $P = 0.048$). To further confirm receptor binding levels and to determine receptor binding affinities, we assessed radioligand binding in striatal membrane fractions from WT and *Dyt1* Δ GAG KI mice also using [3 H] YM-09151. The B_{max} value for [3 H] YM-09151 binding to D2R was significantly lower in *Dyt1* Δ GAG KI mice in comparison to WT mice (Fig. 3B; WT: 633 ± 40 fmol/mg, $n = 3$; KI: 429 ± 40 fmol/mg, $n = 3$; $P = 0.02$). Receptor affinity for the ligand as indicated by K_d , however, was comparable between mice of both genotypes (WT: 64.0 ± 11 pM, $n = 3$; KI: 91 ± 11 pM, $n = 3$; $P = 0.16$).

To determine if the decreased receptor binding is the result of a decrease in mRNA or protein levels, mRNA quantity of both forms of D2R, long (D2RL) and short (D2RS) were measured by qRT-PCR. No differences between WT and *Dyt1* Δ GAG KI mice were observed in the mRNA levels of both forms of D2R tested (Fig. 3C; D2RL – WT: 0.0730 ± 0.0112 ratio of receptor cDNA to β -actin cDNA, $n = 4$, KI: 0.071 ± 0.003 , $n = 4$, $P = 0.89$; D2RS – WT: 0.014 ± 0.001 , $n = 4$, KI: 0.013 ± 0.001 , $n = 4$, $P = 0.67$). Relative D2R protein levels standardized to levels of β -actin, on the other hand, were significantly reduced in *Dyt1* Δ GAG KI mice (Fig. 3D; D2R: WT 0.83 ± 0.08 ratio of D2R to β -actin pixels, $n=3$; KI 0.51 ± 0.11 ratio of D2R to β -actin pixels, $n=3$; $P=0.04$).

3.6. KI mice are partially resistant to FPL64176-induced dystonic-like symptoms

Corticostriatal HFS-LTD has been shown to require D2R-induced inhibition of acetylcholine release from interneurons which normally act on muscarinic acetylcholine receptors (mAChRs) of medium spiny neurons [38]. This inhibition of mAChR activation removes the inhibition of calcium influx through the Cav1.3 calcium channels in response to synaptic depolarization. We hypothesized that because of the decrease in D2R in our *Dyt1* Δ GAG KI mice, acetylcholine release may not be properly suppressed during HFS, causing an over-activation of mAChRs. This overactivation would cause our mutant mice to have a greater inhibition of Cav1.3 calcium channels, which alters their behavioral response to calcium channel activation.

To explore this possibility, we examined the ability of the L-type calcium channel agonist FPL64176 to produce dystonic-like postures and behaviors in KI mice. This calcium channel activating drug is known to produce dystonic-like behavior in wild-type mice [33]. Ten WT and six *Dyt1* Δ GAG KI mice were injected subcutaneously on four consecutive days with escalating doses of FPL64176 (2, 4, 8, 12 mg/kg). Both *Dyt1* Δ GAG KI and WT mice developed FPL64176-induced dystonic-like postures and behaviors. However, on a four-point scale that semi-quantitatively represented the severity of dystonic-like postures, *Dyt1* Δ GAG KI mice overall displayed significantly less severe dystonic-like postures and behaviors across all FPL64176 doses (Fig. 4; normalized dystonia score to WT; WT 0 ± 0 , KI -0.41 ± 0.2 , $P < 0.02$). On average, the *Dyt1* Δ GAG KI mice responded to the drug treatment with behavior that was semi-quantitatively measured as a half point less severe in motor disruption when compared to WT littermates.

4. Discussion

In this report we show that an anticholinergic can reverse both corticostriatal LTD and motor control deficits in our *Dyt1* Δ GAG KI mice. The reduction in striatal D2R protein expression of *Dyt1* Δ GAG KI mice, deficit of HFS-LTD and its rescue by an anticholinergic THP, collectively suggest the importance of cholinergic interneurons modulation of synaptic

plasticity potential of medium spiny neurons. We propose that our data lend support to the theory that aberrant D2R activity causes cholinergic interneurons to be disinhibited from secreting acetylcholine. The increased amount of acetylcholine acts on medium spiny neuron mAChRs to inhibit the activation of Ca_v1.3 channels (Fig. 5A). The inhibition of Ca_v1.3 channels reduces the release of endocannabinoids, which is thought to be responsible for the downregulation of glutamate release that in wild-type mice leads to HFS-LTD [35, 38]. The restoration of LTD by THP, an anticholinergic, bypasses the D2R deficiency and acts directly on the post-synaptic mAChRs to rescue the HFS-LTD (Fig. 5B, C).

Our *Dyt1* ΔGAG KI mice are partially resistant to the behavioral changes induced by FPL64176, L-type calcium channel agonist. FPL64176, when applied to brain tissue, has been demonstrated to induce LTD which is sufficiently strong that no further HFS-LTD induction could be achieved [37]. Given the partial resistance to FPL64176 demonstrated by our mutant mice, we speculate that the reverse of this occlusion phenomenon may be occurring in our mouse model, whereby the suppression of calcium channel opening achieved by modulation from the interneurons may be strong enough to attenuate the opening of channels by a direct agonist.

We confirmed in our study with FPL64176 that activation of L-type calcium channels by FPL64176 leads to dystonic-like symptoms in wild-type mice. In this report, we also show data that *Dyt1* ΔGAG KI are deficient in LTD, most likely because of an attenuation of calcium channel activation. If these two findings are compared, clearly we see that they contradict; however, we propose that a comparison between the findings cannot be strictly made because the differences in the two experimental mouse systems are significant. In our genetically modified mice, the LTD deficit may have occurred since birth causing currently unknown developmental changes to occur, while FPL64176 administration leads to very acute and transient changes in mice with normal development.

The loss of corticostriatal HFS-LTD, its reversal by THP, and the reduction of striatal D2 receptors in our *Dyt1* ΔGAG KI mice are consistent with previously published data obtained from a transgenic mouse model of DYT1 dystonia called hMT mice [40, 41]. Unlike our *Dyt1* ΔGAG KI mice that expresses wild-type and ΔE mouse torsinA at physiologic level, the hMT mouse overexpresses ΔE human torsinA ectopically [42]. Prior published reports show that hMT mice appear to behave similarly to our *Dyt1* ΔGAG KI mice. hMT mice have also been found to have motor coordination deficits on the beam-walking task and have a disruption in dopamine metabolism as compared to wild-type littermates [43]; however, in most behavioral performance tasks, the performance of hMT mice was not significantly different from that of mice overexpressing the wild-type human torsinA, which leads one to question the general effects of overexpressing torsinA, whether it be mutant or wild-type. In addition, data exists to support that the ΔGAG mutation may not necessarily be a gain-of-function mutation, but instead could have a component of loss-of-function, a genetic mutation consequence that a transgenic mouse line would be unable to model [44]. For these reasons, confirmatory evidence of synaptic plasticity alteration in a heterozygous knockin mouse model such as ours is informative and necessary.

While corticostriatal HFS-LTD was deficient in our *Dyt1* ΔGAG KI mice, LTP was unchanged from that of WT mice. This observation is different from the enhanced LTP detected in the transgenic mouse model [40]. In that model, the excess in what was described as a heightened cholinergic tone, was thought to be responsible for the augmented LTP [45]. Comparisons between our data and that used on hMT mice must be made cautiously since the protocols for induction of LTD and LTP were different in each study. Ours relied on anatomical-specificity of dorsolateral versus dorsomedial for differential production of LTD versus LTP respectively while the protocol used on hMT mice involved

recording from the same anatomical region with removal of voltage-dependent magnesium block of NMDA receptor channels for induction of LTP. We view corticostriatal HFS-LTD and LTP as experimental events that represent long-lasting changes in the striatal synapses caused by input from the cerebral cortex, and changes to them represent a change in synaptic plasticity in general. Regardless of the difference in the methods, our *Dyt1* Δ GAG KI mice and hMT mice both have altered corticostriatal synaptic plasticity. These experimental synaptic plasticity events have been strongly associated with motor control and motor learning [27, 46, 47]. Specific to dystonia, synaptic plasticity alterations have also been thought about in context of the current hypothesis that dystonia is a disorder resulting from a lack of “surround inhibition” of motor patterns that along with the expansion of center facilitation results in excessive co-contraction of muscle groups [20, 21, 40]. How these changes in surround inhibition and center facilitation directly specific correlate with synaptic plasticity abnormalities seen as changes to LTD or LTP continues to require further elucidation.

We acknowledge that our data still does not provide direct causative evidence for synaptic plasticity changes resulting in the motor abnormality. Our LTD data was obtained in young mice while the behavioral deficit was seen in older mice. The inability to test both events within mice of the same age group stems from the specific protocol and previous findings within these mice. We have previously tested our mice at 3 months of age and did not see a significant motor coordination and balance abnormality at that time. Aging appears to be necessary for a measureable phenotype, which may be related to the variable penetrance of the *Dyt1* gene, with only 30% of carriers exhibiting a visibly significant motor abnormality. The protocol we used of HFS-LTD and LTP induction recording is more consistent with young mice before they reach adulthood [25]. For this reason, we were not able to perform LTD induction recordings in mice age 3 months and 6 months to more directly correlate the motor behavioral deficit with loss of LTD. Confirmatory studies using methods that are less sensitive to age is a warranted future avenue to explore.

In addition, we understand that the explanation we propose to correlate THP to the improvement of motor deficits via correction of LTD does not take into account the fact that acetylcholine is important in other neurochemical pathways. For example, within the striatum activation of M1 muscarinic receptors also have been shown to induce striatal dopamine release [48]. The inhibition of this release may play a role in dystonia, although no evidence exists to date of increased striatal dopamine release in DYT1 dystonia. In fact, amphetamine-induced dopamine release has been shown to be decreased in the hMT mouse [8].

The implications of our findings are limited first, by the possibility that the reduction in D2R and its significance in the development of *Dyt1* Δ GAG KI mice may not be restricted to interneurons and that the D2R population on the medium spiny neurons may also be essential in controlling the neuron’s capacity for synaptic potentiation. In the transgenic DYT1 dystonia mouse tissues, a reversal of the LTD deficit was also seen in the mice when given SCH 58261, a specific A2AR antagonist of a receptor that inhibits D2R on medium spiny neurons, thus implicating the importance of post-synaptic D2R [41]. Secondly, our data does not address a specific mechanism for how torsinA can affect D2R. The reduction in D2R binding corresponded with a reduction in protein but not with mRNA levels, suggesting that mutant torsinA most likely does not regulate transcription, but is involved in protein translation or post-translational processes such as the receptors’ assembly, stabilization, or trafficking pattern. Wild-type and not mutant torsinA in cell cultures was shown to affect the assembly of polytopic membrane proteins such as D2R receptors and the dopamine transporter molecule [49]. Also, a known binding partner of torsinA is calnexin which is involved in dopamine receptor trafficking [50, 51]. Thirdly, we show changes in a

highly specific region of the brain, while torsinA is widely expressed in the brain and may be a disorder of a network of abnormalities involving multiple regions of the brain [7, 12, 52]. Tissue-specific expression of the mutation *Dyt1* at physiological level would be an important tool for the further evaluation of the brain regions and neurodevelopmental consequences of the mutation.

In conclusion, we present behavioral, electrophysiology, and biochemical findings that, collectively suggest that synaptic plasticity alterations in the corticostriatal pathway may be associated with the motor control deficit seen in dystonia. We show evidence for how altered communication between striatal cholinergic interneurons and medium spiny neurons may be responsible for the observed corticostriatal LTD deficit seen in the *Dyt1* Δ GAG KI mice. The reversal of the LTD deficit and motor control abnormalities by the anticholinergic provide further support that the two events may share a causative relationship.

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Abbreviations

ACSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
D2R	dopamine receptor type 2
D2RL	dopamine receptor type 2 long form
D2RS	dopamine receptor type 2 short form
<i>DYT1</i> (<i>TORIA</i>)	torsinA gene in human
<i>Dyt1</i> (<i>Tor1a</i>)	torsinA gene in mouse
HFS	high frequency stimulation
KI	knock-in
LTD	long-term depression
LTP	long-term potentiation
mAChR	muscarinic acetylcholine receptor
PS	population spikes
SE	standard errors
THP	trihexyphenidyl
WT	wild-type

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Research highlights

- Anticholinergic trihexyphenidyl restored long-term depression in *Dyt1* Δ GAG mice.
- Trihexylphenidyl also reversed motor control deficit in dystonia mouse model.
- Striatal D2 receptors were expressed at lower quantities in *Dyt1* Δ GAG mice.
- Ca^{2+} channel agonist FPL64176 only partially induced dystonic signs in mutant mice.
- Cholinergic tone is important in synaptic plasticity and motor control in dystonia.

Fig. 1.

Corticostriatal LTD is deficient in *Dyt1* Δ GAG KI mice and is restored by THP. (A) LTD in the dorsolateral striatum was induced by the same stimulus protocol in slices from WT and *Dyt1* Δ GAG KI mice. In contrast to WT mice, LTD was not observed following the same HFS protocol in *Dyt1* Δ GAG KI mouse slices. LTD also was not observed in WT mouse slices when D2Rs were blocked by the antagonist sulpiride (10 μ M). (B) LTP in the dorsomedial striatum was consistently induced by 4 trains of 100 Hz stimulation to the cortex in slices from both *Dyt1* Δ GAG KI and WT mice. (C) WT animals from the same breeding litters showed consistent LTD. (D) Application of mAChR antagonist, THP (5 μ M), rescued LTD in *Dyt1* Δ GAG KI mouse slices to levels similar to those seen in WT mice. (E) *Dyt1* Δ GAG KI mice had similar dendritic branching patterns in comparison to WT mice. (F) The number of spines per branch was also similar between the two groups. Vertical bars represent mean $\pm$ SE.

Fig. 2. THP rescued motor control deficit in KI mice. The motor control performance in beam-walking was not affected in WT mice by THP injection. The slips numbers in beam-walking was improved in the *Dyt1* Δ GAG KI mice after administration of THP. Vertical bars represent mean $\pm$ SE. * $P < 0.05$.

Fig. 3.

D2 receptors are significantly reduced in the striatum of *Dyt1* Δ GAG KI mice. (A) Autoradiographic binding with radioligands for D2R show *Dyt1* Δ GAG KI mice had a reduction in striatal D2R binding. (B) D2R binding in a radioligand binding assay of striatal membranes shows significantly lower Bmax values in *Dyt1* Δ GAG KI mice in comparison to WT mice. Receptor affinity for the ligand, however, was comparable between mice of both genotypes. (C) No differences between WT and *Dyt1* Δ GAG KI mice were observed in the mRNA levels of both the long and short forms of D2R. (D) Relative D2R protein levels standardized to levels of β -actin, however, were significantly reduced in *Dyt1* Δ GAG KI mice.

Fig. 4.

Dyt1 Δ GAG KI mice are partially resistant to FPL64176-induced dystonic-like symptoms. Calcium channel agonist FPL64176 causes dystonia-like movements in WT mice, an effect that is attenuated in *Dyt1* Δ GAG KI mice. Inset: ten minutes after the injection of FPL64176, the representative *Dyt1* Δ GAG KI mouse (top) demonstrated slowness of movement, but remained upright and able to respond to tactile stimulation. The WT mouse (bottom) had sustained abnormal postures and was not able to remain upright during the majority of the 10 min. Vertical bars represent mean $\pm$ SE. * $P < 0.05$.

Fig. 5.

Hypothesis of functional alteration of medium spiny neurons in *Dyt1*ΔGAG KI mice. (A) In WT mouse during HFS, cholinergic interneurons do not release acetylcholine to act on muscarinic receptors of medium spiny neurons. This allows for the opening of Ca_v1.3 calcium channels that initiates a series of events leading to the inhibition of glutamate release from cortical cells to allow for HFS-stimulated LTD. (B) In *Dyt1* ΔGAG KI mice, the low level of D2R disinhibits the release of acetylcholine, allowing for activation of post-synaptic muscarinic receptors, which inhibits the opening of Ca_v1.3 channels. (C) Anticholinergic THP inhibits the activation of muscarinic receptors to restore the opening of Ca_v1.3 channels in *Dyt1* ΔGAG KI mice.