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Cholinergic dysregulation produced by selective inactivation of the dystonia-associated protein TorsinA

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Abstract

DYT1 dystonia, a common and severe primary dystonia, is caused by a 3-bp deletion in *TOR1A* which encodes torsinA, a protein found in the endoplasmic reticulum. Several cellular functions are altered by the mutant protein, but at a systems level the link between these and the symptoms of the disease is unclear. The most effective known therapy for DYT1 dystonia is use of anticholinergic drugs. Previous studies have revealed that in mice, transgenic expression of human mutant torsinA under a non-selective promoter leads to abnormal function of striatal cholinergic neurons. To investigate what pathological role torsinA plays in cholinergic neurons, we created a mouse model in which the *Dyt1* gene, the mouse homolog of *TOR1A*, is selectively deleted in cholinergic neurons (ChKO animals). These animals do not have overt dystonia, but do have subtle motor abnormalities. There is no change in the number or size of striatal cholinergic cells or striatal acetylcholine content, uptake, synthesis, or release in ChKO mice. There are, however, striking functional abnormalities of striatal cholinergic cells, with paradoxical excitation in response to D2 receptor activation and loss of muscarinic M2/M4 receptor inhibitory function. These effects are specific for cholinergic interneurons, as recordings from nigral dopaminergic neurons revealed normal responses. Amphetamine stimulated dopamine release was also unaltered. These results demonstrate a cell-autonomous effect of *Dyt1* deletion on striatal cholinergic function. Therapies directed at modifying the function of cholinergic neurons may prove useful in the treatment of the human disorder.

Introduction

Dystonias are characterized by repetitive involuntary muscle contractions causing twisting movements (Fahn and Eldridge, 1976). Early onset torsion dystonia (DYT1, or Oppenheim dystonia) is a genetically determined form of “primary” dystonia (Albanese et al., 2011)

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with autosomal dominant inheritance, reduced penetrance, and onset most often before young adulthood (Breakefield et al., 2008). The mutation is a 3-bp deletion in the gene *TOR1A*, encoding torsinA (Ozelius et al., 1997), a protein expressed throughout the brain and found in the endoplasmic reticulum and nuclear envelope (Augood et al., 2003; Gonzalez-Alegre and Paulson, 2004). TorsinA is a member of the AAA+ (ATPases Associated with a variety of cellular Activities) (Ozelius et al., 1997), and has a number of potential functions including interactions with the cytoskeleton, binding in the nuclear envelope, chaperone activity, and vesicle transportation and secretion (Granata et al., 2009; Warner et al., 2010).

The pathophysiology of DYT1 dystonia is thought to involve abnormalities in neural processing and plasticity. In carriers of the DYT1 mutation, there is abnormal metabolic activity in areas involved in sensorimotor processing and motor planning (Carbon and Eidelberg, 2009), abnormal synaptic plasticity following repetitive transcranial magnetic stimulation (rTMS) (Edwards et al., 2006), and disparities in motor sequence learning (Ghilardi et al., 2003). Structural studies suggest altered connectivity between cortex, thalamus, and cerebellum (Carbon et al., 2008). Pathological study of DYT1 brains, however, has revealed no evidence of neurodegeneration (Rostasy et al., 2003; Standaert, 2011), and the connection between the cellular functions of torsinA and the systems-level abnormalities is unclear (Breakefield et al., 2008).

The most effective treatment currently available for DYT1 dystonia is high-dose anticholinergic medications (Burke et al., 1986), suggesting that dystonia may be related to cholinergic function. In a mouse model produced by expression of the mutant torsinA under a CMV promoter (hMT1 mice), we have observed altered cholinergic function with a paradoxical excitatory, rather than inhibitory, response to D2 agonists (Pisani et al., 2006; Sciamanna et al., 2011) and alterations in synaptic plasticity which can be normalized by reducing cholinergic transmission (Martella et al., 2009). Together, these observations point to abnormal cholinergic function and a hypercholinergic state as the source of abnormal plasticity in human DYT1 dystonia as well as in the hMT1 model.

Cholinergic transmission in the basal ganglia is mediated primarily by large, aspiny striatal interneurons (Pisani et al., 2006). An important question is whether the cholinergic abnormalities associated with mutant torsinA arises from actions of the protein within cholinergic neurons, or rather as a consequence of upstream changes; the hMT1 mice do have other neurochemical abnormalities, including altered dopamine turnover (Balcioglu et al., 2007). To determine whether there are cell-autonomous consequences of torsinA dysfunction, we have created mice in which the *Dyt1* gene, the mouse homolog of *TOR1A*, is selectively inactivated within cholinergic neurons. We find that this reproduces features observed in the hMT1 mice, including impaired motor behavior and paradoxical activation of striatal cholinergic interneurons by dopamine D2 receptor agonists. These findings suggest that mutant torsinA protein has a cell-autonomous effect on cholinergic neurons, and that these neurons are an important target for potential therapies for the human disease.

Methods

Animals

Chat-cre mice (B6;129S6-*Chat^{tm1(cre)}Low*/J; Jackson laboratory, stock no. 006410) were imported and crossed with homozygous *Dyt1 loxP* mice created previously (Yokoi et al., 2008) to produce a colony of cholinergic knock-out mice (ChKO). Genotyping was performed by PCR on tail DNA using primer pairs for cre (GTTTGCAGAAGCGGTGGG forward primer and CCTTCTATCGCCTTCTTGACG reverse primer) and for the loxP locus (ATTCAAAAATGTTGTCATAGCCAGG forward primer and

CTACAGTGACCTGAATCATGTGGC reverse primer). PCR products were run using a 2% agarose gel. Mice were housed on a 12 hour light/dark cycle with ad libum access to water and food.

LacZ staining

ROSA lacZ reporter mice (B6.129S4-*Gt(ROSA)26Sor^{tm1Sor}/J*; Jackson Laboratories, stock no. 003474) (Soriano, 1999) were imported and crossed with *Chat-cre* mice to produce *Chat-cre/ROSA* mice. Mice were anesthetized with pentobarbital and transcardially perfused with 0.1M PBS followed by 4% paraformaldehyde. Brains were dissected, fixed in 4% paraformaldehyde at 4C for 6 hrs, and then soaked in 30% sucrose for 72 hrs at 4C. 50 μ m brain sections were cut using the microtome. Sections were washed for 10 min $\times$ 3 in 0.1M phosphate buffer and 10 min $\times$ 3 in 0.1M phosphate buffer, 2mM MgCl₂, 0.01% sodium deoxycholate, and 0.02% NP-40. Sections were stained with x-gal solution overnight at 37°C before being mounted and coverslipped with Kaiser's glycerol jelly. Slides were viewed using a Nikon Eclipse microscope (E800M).

Laser capture microdissection

Brains were dissected and quick frozen on dry ice. 8 μ m striatal coronal sections were cut on a cryostat and mounted on plain glass slides. Sections were stained either with 1% methylene blue or with primary antibody, 1:10 goat anti-choline acetyltransferase (AB144P; Millipore, Billerica, MA), followed by secondary antibody, 1:50 Cy3-conjugated AffiniPure donkey anti-goat IgG (705-265-003; Jackson Immuno Research Laboratories, West Grove, PA), using methods previously described (Zucker et al., 2005). Striatal neurons were captured using a Veritas Microdissection System (Arcturus, Mountain View, CA) using CapSure HS LCM caps with a laser spot diameter of approximately 30 μ m/neuron. RNA from the LCM caps was extracted and purified using the PicoPure RNA Isolation components kit (Arcturus). RNA was reverse transcribed to cDNA using Superscript III reverse transcriptase (18080-093; Invitrogen, Carlsbad, CA). Q-PCR was run using a Bio-Rad iCycler and iQ SYBR Green Supermix (Biorad; Hercules, CA) to measure levels of torsinA, choline acetyl-transferase (ChAT), and β -Actin for normalization. Duplicate samples were run through 50 PCR cycles (95°C for 30s, 57°C for 30s, 72°C for 45s) and starting quantity was measured using a standard curve. Specificity of primer PCR products was verified by melt-curve analysis and gel electrophoresis. Primer sequences were as follows: torsinA exons 3–4 (GenBank: BC017683.1) GGGAAGCAGAGGGAAGAAAT forward primer and ATGAGGTTCCGGTCAATGAG reverse primer, ChAT (GenBank: BC119322.1) GTGAGACCCTGCAGGAAAAG forward primer and GCCAGGCGGTTGTTTAGATA reverse primer, and β -Actin (GenBank: BC138614.1) AGATCTGGCACCACACCTTC forward primer and CTTTTCACGGTTGGCCTTAG reverse primer.

Motor behavior

All behavior experiments were performed with the investigator blind to genotype and with equivalent number of male and female subjects. Rotarod and beam walking experiments were performed as described previously (Dang et al., 2005) using mice aged 8 to 11 months. Briefly, for the rotarod mice were tested for two consecutive days with three trials each day separated by one hour. Each rotarod trial started with at an initial 4 rpm and accelerated at 0.2 rpm/s up to a maximum cutoff of 28 rpm at 2 min. Latency for the mouse to fall off was measured. Rotarod testing was performed on a 47600 Mouse RotaRod (Ugo Basile, Collegeville, PA). For beam walking, mice were trained for two consecutive days with three sessions per day to cross a 14 mm wide square beam. After training, animals were tested for two consecutive days. On the first day a 17 mm wide square beam and a 17 mm wide round beam were used with two trials on each beam. The second day animals were tested on a

smaller 10 mm wide round beam and on a 7 mm wide square beam with two trials on each beam. The number of hind limb slips was recorded for each trial. For testing in the openfield, elevated plus maze, and Barnes maze, mice between the ages of 3 to 8 months were used. Mice were tested in the openfield (Columbus instruments) for one trial lasting 4 min and total distance traveled was measured. Mice were tested in the elevated plus maze (San Diego Instruments) for one trial lasting 4 min and number of entries, time spent, and distance traveled in the open and closed arms was measured. Barnes maze paradigm was performed as previously described (Dawood et al., 2004). The Barnes maze apparatus (Columbus instruments) contained 40 holes surrounding the perimeter with an escape chamber underlying one hole containing sunflower seeds and bedding. The escape chamber remained in the same position throughout the experiment. The day prior to testing, mice were pre-trained using four consecutive trials where mice were allowed to explore the maze before being guided to the escape chamber. Mice were subsequently tested on the following four days by placing each mouse in the center of the maze and measuring distance traveled and time to find the escape chamber. On each test day mice were tested for 4 trials separated by approximately 30 min. If mice did not find escape hole by 4 min the trial was ended and the mouse was guided to escape chamber. Measurements for openfield, elevated plus maze, and the Barnes maze were made using Ethovision XT software (Noldus).

Analysis of striatal acetylcholine

Mouse brains were fixed using a Microwave Fixation System (Muromachi Kikai Co., Tokyo, Japan). Striatal tissue was dissected and sent to Vanderbilt Neurochemistry Core for analysis by HPLC. Tissue was homogenized in 250 μ l acetonitrile followed by centrifuge at 13000g $\times$ 30 min to remove debris. The supernatant was collected, washed with heptane, and the acetonitrile was evaporated using a stream of nitrogen. The sample was re-suspended in 75 μ l of mobile phase (37.5 mM phosphoric acid, pH 8.5). To measure acetylcholine (ACh) content, 50 μ l of sample was loaded on an Acetylcholine/Choline Column (Bioanalytical Systems) and detected with an Antec Decade II electrochemical detector. Total protein measurements were made using BCA Protein Assay kit (Pierce Chemical Company, Rockford, IL).

Stereology

For stereology fixed sections were washed, quenched in 3% H₂O₂ and 50% methanol, and blocked in normal horse serum. Sections were incubated in primary antibody, 1:1000 goat anti-choline acetyltransferase over night at 4°C, and secondary antibody, 1:500 biotinylated horse anti-goat IgG (Vector, BA-9500) for 1 hr. For immunoperoxidase staining, ABC reagent (Vector, PK-7100) was applied for 30 min followed by DAB substrate (Vector, SK-4100) for 7 min. Sections were rinsed in graded alcohols and cover-slipped with permount mounting medium.

Unbiased cell population estimates were performed using an Olympus BX51 microscope equipped with StereoInvestigator 9 software (MicroBrightfield, Williston, VT). The entire volume of the striatum was sampled, starting at the rostral-most section containing the corpus callosum and extending to the caudal-most section containing the fully crossed anterior commissure. Every fourth section was used for population measurements. The optical fractionator method was employed to estimate the population of cholinergic interneurons in the striatum. The grid size used to count cells was 100 $\times$ 100 μ m, and an average of 30 sites was sampled per section.

Neuron morphology

For studies of neuron morphology, fixed sections were stained with an immunofluorescent method and examined using confocal microscopy. Striatal sections were blocked for 30 min

with 3% normal donkey serum and 0.3% triton-x100 in 0.1M PBS. Sections were stained with primary antibody, 1:400 goat anti-choline acetyltransferase, overnight at 4°C and secondary antibody, 1:500 Cy3-donkey anti-goat IgG, for 2 hrs at room temperature. Several striatal sections were double stained for ChAT and dopamine and cyclic AMP-regulated phosphoprotein (DARPP-32). These sections were blocked with 5% normal donkey serum and 0.3% Triton-x100 in 0.1M PBS for 1 hr. Primary antibodies used were rabbit polyclonal antibody to DARPP32 (1:800; Cell Signaling, Danvers, MA) overnight at 4°C and goat anti-ChAT antibody (1:500) overnight at 4°C the next day. The sections were then incubated with secondary antibodies, Cy3- donkey anti-goat IgG (1:300) and donkey anti-rabbit IgG conjugated to FITC (1:150; Jackson ImmunoResearch, West Grove, PA), sequentially for 2 hrs at room temperature. Sections were mounted to slides and coverslipped with Vectashield mounting medium (Vector Labs, Burlingame, CA).

Fluorescently labeled cholinergic interneurons were imaged with a confocal microscope (Leica SP5). Six single neuron image stacks were taken at random from each animal using a 40× oil immersion lens and a z axis spacing of 0.5µm between each image. The image stacks were uploaded into Neurolucida (MicroBrightfield, Williston, VT) for cell volume and total area analysis. Each cell was outlined at each focal plane to reconstruct the cell in 3D. For each reconstructed cell body, Neuroexplorer (MicroBrightfield, Williston, VT) was used to measure enclosed volume (µm³) and surface area (µm²).

[³H]-Acetylcholine Uptake and Release

Mice were euthanized with CO₂ and brains were cut into 300 µm striatal sections using a vibratome. Slices were soaked in aCSF with 95% O₂ and 5% CO₂ at 34°C for 30 min before incubating with ³H-[choline chloride] for 1 hour to allow uptake. Slices were transferred to a superfusion chamber and washed with oxygenated aCSF containing 10mM hemicholinium-3 for 1 hour. Superfusate samples were then collected every 5 min to obtain baseline levels before switching to stimulation buffer containing 20 mM KCl in aCSF. After superfusion, tissue sections were removed from the chamber and placed in 1M HCl and 0.1% Triton X-100 before being homogenized to allow quantification of total uptake. 200ul from each sample was mixed with 15 ml EconoSafe Scintillation fluid before measurement on a Beckman LS 6000IC scintillation counter.

Electrophysiology

Experiments were carried out in accord with both the EC and Italian guidelines (86/609/EEC; D.Lvo 116/1992) and approved by the University of Rome “Tor Vergata” (n. 153/2001A). All efforts were made to minimize the number of animals utilized and to reduce their suffering. Transgenic mice (8–10 weeks old) were sacrificed by cervical dislocation under ether anaesthesia. Coronal striatal slices (250–300 µm thick) and horizontal slices of the ventral midbrain (300 µm thick) were cut with a vibratome in Krebs' solution (in mM: 126 NaCl, 2.5 KCl, 1.3 MgCl₂, 1.2 NaH₂PO₄, 2.4 CaCl₂, 10 glucose, 18 NaHCO₃), bubbled with 95% O₂/ and 5% CO₂ (Mercuri et al., 1995; Pisani et al., 2006). After 30–60 min recovery, individual slices were transferred into a recording chamber (0.5–1 ml volume), continuously superfused with oxygenated Krebs' medium, at 2.5–3 ml/min and maintained constantly at 32–33°C.

Whole-cell recordings were performed from individual interneurons, visualized by means of IR-DIC system (Bonsi et al., 2007) by using a Multiclamp 700b amplifier (Axon Instruments), and borosilicate glass pipettes (1.5 mm outer diameter, 0.86 inner diameter) pulled on a P-97 Puller (Sutter Instruments). For whole-cell recordings, electrodes were filled with a solution containing (in mM): 120 CsMeSO₃, 15 CsCl, 8 NaCl, 10 tetraethylammonium-Cl, 10 HEPES, 2–5 QX-314 [N-(2,6-

dimethylphenylcarbamoylmethyl)triethylammonium bromide], 0.2 EGTA, 2 Mg-ATP, and 0.3 Na-GTP, pH 7.3 adjusted with CsOH, and were performed at room temperature. Whole-cell solution was also used for perforated-patch recordings. Gramicidin-A-B-C (Sigma) was dissolved in dimethylsulfoxide (10 mg ml⁻¹) and then diluted in the intrapipette solution at a final concentration of 50–100 µg/ml. The tip of the pipette was filled with gramicidin-free intracellular solution. The perforation process was considered complete when the amplitude of the action potentials was steady and > 60 mV, and electrode resistance was steady and < 50 MΩ. To calculate the variations in membrane potential in spontaneously active neurons we considered the value of membrane potential measured 100 ms after the peak of the afterhyperpolarizing potential (AHP) following each single spike. At this time-point the phase of repolarization is rather complete, reaching a “plateau” that allows to extrapolate the membrane potential. Reported values were obtained by averaging the measures at “plateau” recorded in the 10 seconds pre-drug application.

Data were acquired with pClamp software and analyzed offline (Clampfit 10.2, Molecular Devices, USA; MiniAnalysis 6.0, Synaptosoft, USA and Prism 3.02, GraphPad software Inc., USA). Membrane currents were continuously monitored and access resistance measured in voltage-clamp was in the range of 5–30 MΩ prior to electronic compensation (60–80% routinely used).

Current-clamp recordings from nigral neurons were performed blindly using sharp microelectrodes filled with 2M KCl (40–60 MΩ). Slices were transferred into a recording chamber maintained constantly at 34–35°C. Signal acquisition and off-line analysis were performed with an Axoclamp 2B amplifier and pClamp9 software (Molecular Devices, USA). Nigral neurons were identified according to their specific electrophysiological characteristics, as described previously (Lacey et al., 1987; Mercuri et al., 1995; Napolitano et al., 2010).

In vivo microdialysis

Mice approximately seven months-old were used in microdialysis studies. Microdialysis procedures were performed in alert, freely moving mice as previously described (Zhang et al., 2007). Briefly, mice were anesthetized with ketamine/xylezine (100/10 mg/kg) before the stereotaxic implantation of a probe (Eicom; P-I-6-02) into the striatum at coordinates (+0.0 mm anteroposterior, +2.5 mm mediolateral from the bregma, and –4.4 mm dorsoventral with respect to dura). Probes were secured onto the skull using stainless-steel screws and dental acrylic. Twenty-four hrs after surgery, *in vivo* microdialysis was performed on conscious mice. Probes were perfused continuously with artificial CSF (147 mM NaCl, 4 mM 227 KCl, and 2.3 mM CaCl₂) at a flow rate of 1.5 µl/min. The dialysate was collected in 20-min fractions. Baseline levels of extracellular dopamine were established prior to the administration of amphetamine. Amphetamine (5mg/kg; Sigma-Aldrich Corporation, St Louis, MO) was dissolved in physiological saline and was injected subcutaneously in a volume of 10 ml/kg, as previously described (Zhang et al., 2006). Dopamine levels in microdialysis samples were measured by high performance liquid chromatography (HPLC) using a reversed phase column (Dionex ESA MD-150×3.2 150 mm Dionex, Chelmsford, MA, USA), as previously described (Balcioglu et al., 2007). Probe locations were verified in all mice at the end of the microdialysis experiment. The animal was anaesthetized with ketamine/xylezine (100/10 mg/kg) and perfused transcardially with 4% paraformaldehyde in saline. Brains were removed and placed in fixative overnight at 4 °C. After equilibrating in 30% sucrose in 0.1 M phosphate buffer, brains were cut into 50µm coronal sections using a sliding microtome (Histoslide 2000, Reichert-Jung) and then Nissl stained.

Statistical analysis

Students' unpaired *t*-test was used for statistical analysis of qPCR, stereology, neuron morphology, openfield, and 3H-choline uptake and release experiments. Elevated plus maze and microdialysis data were analyzed by 2-way analysis of variance (ANOVA) and Barnes maze data was analyzed by repeated measures 2-way ANOVA. Ach HPLC data was analyzed by one-way ANOVA. Rotarod and beam-walking data were log transformed to fit a normal distribution and were analyzed by logistical regression (GENMOD) with negative binominal distribution using GEE model as previously described (Deandrade et al., 2011). Electrophysiological data were analyzed using ANOVA test with a post-hoc Tukey test between groups (WT and ChKO mice) or Student *t*-test ($p < 0.05$; $\alpha = 0.01$). For all analyses, p value < 0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism software, SAS/STAT 9.1 (SAS Institute), and IBM SPSS Statistics 19.

RESULTS

Generation and characterization of ChKO mice

In order to selectively target cholinergic neurons, ChAT-cre mice were utilized in which the expression of an internal ribosome entry site-linked cre recombinase gene (IRES-Cre) is driven by the endogenous promoter for ChAT (Ivanova et al., 2010; Madisen et al., 2010; Pecho-Vrieseling et al., 2009; Wang et al., 2009). The *Dyt1* gene was deleted in cholinergic neurons by crossing ChAT-Cre mice with *Dyt1 loxP* homozygous mice to produce ChKO mice (Fig. 1). In comparison to other mouse models of DYT1 dystonia in which either homozygous *Dyt1* Δ GAG knock-in (Dang et al., 2005) or homozygous *Dyt1* knock-out leads to lethality (Goodchild et al., 2005), ChKO mice show no signs of lethality at birth.

Since torsinA is thought to play a role in the developing nervous system and is highly expressed during mouse development (Vasudevan et al., 2006), we assessed the timing of Cre recombinase expression under the ChAT promoter by crossing with Rosa reporter mice which drive expression of LacZ. Sections from these animals obtained at postnatal days 7, 21 and 90 were stained for beta-galactosidase expression. We found that LacZ staining indicated that Cre recombinase activity was robust as early as postnatal day 7, and was detectable in striatal cholinergic neurons as well as other cell groups known to express ChAT, such as the basal forebrain and the motor nuclei of cranial nerves (Fig. 2).

To verify that expression of Cre recombinase did indeed lead to selective inactivation of the *Dyt1* gene, laser capture microdissection (LCM) was used to isolate cholinergic and non-cholinergic neurons from the striatum of ChKO animals (Figure 3). Using LCM we obtained enriched population of cholinergic neurons as indicated by the increased amount of ChAT mRNA in striatal neurons stained positive for ChAT (Fig. 3C; $p < 0.05$; $n = 5$ for ChKO, and $n = 5$ for *loxP*). As hypothesized, in ChKO mice we found that torsinA mRNA expression was decreased in cholinergic neurons compared to control (*loxP*) mice (Fig. 3D; $p < 0.05$; $n = 5$ for ChKO, and $n = 5$ for *loxP*) and, furthermore, we found that torsinA expression in non-cholinergic neurons was unchanged in ChKO mice compared to control mice (Fig. 3D). These data also illustrate that the baseline level of torsinA mRNA, relative to that of beta-actin mRNA, is higher in cholinergic than non-cholinergic neurons in the striatum; a similar preferential expression of torsinA mRNA in cholinergic cells has been observed in human striatum (Augood et al., 1999).

Selective inactivation of Dyt1 in cholinergic neurons leads to subtle motor deficits

Other DYT1 mouse models show no gross signs of dystonia or abnormal motor behavior, but they do show impairments in fine motor coordination and balance as indicated by increased slips on beam-walking and decreased latency on the rotarod (Dang et al., 2005;

Dang et al., 2006; Jinnah et al., 2005; Sharma et al., 2005). Similarly, ChKO mice show no gross signs of dystonic behavior. However, when ChKO mice were tested on the rotarod they showed significantly lower latencies to falling off compared to *loxP* mice (Fig. 4A; $p = 0.0410$; $n = 20$ for ChKO; CT mice consisted of *Dyt1 loxP* mice, $n = 20$, and heterozygous *loxP/Chat-cre* mice, $n = 4$). On the beam-walking task, ChKO mice showed no significant difference in number of hind limb slips compared to *loxP* mice (Fig. 4B). Additional behavioral paradigms tested included the openfield for spontaneous locomotor activity, the elevated plus maze for anxiety, and the Barnes maze for spatial learning, all of which showed no difference between ChKO mice and *loxP* control mice (data not shown).

Selective inactivation of *Dyt1* does not alter striatal cholinergic neuron numbers or size

Since the non-selective homozygous deletion of *Dyt1* gene can lead to lethality, we evaluated the possibility that inactivation of the *Dyt1* gene in cholinergic neurons might lead to neuronal loss or alterations in cellular morphology. Histological examination of brains from ChKO animals did not disclose any obvious abnormalities of brain structure. To test for changes in cholinergic population number, striatal sections from ChKO mice were stained for ChAT and unbiased stereology was used to determine the number of ChAT-positive neurons present in the striatum (Fig. 5A). We found that the selective inactivation of *Dyt1* in cholinergic cells did not result in any reduction in the number of striatal ChAT-positive neurons; indeed, there was a trend towards an increased number of ChAT neurons in the ChKO animals. (Fig. 5C; $n = 3$ for ChKO and $n = 3$ for *loxP*). To test for changes in cell volume, confocal imaging z-stacks were used to reconstruct striatal cholinergic neuron cell bodies and make volumetric measurements (Fig. 5B). We found no significant difference in the cell body enclosed volume of striatal cholinergic neurons from ChKO and *loxP* mice (Fig. 5D; $n = 3$ for ChKO and $n = 3$ for *loxP*).

Selective inactivation of *Dyt1* in cholinergic neurons does not alter striatal Ach content, uptake, or release

Since previous studies had suggested abnormalities of cholinergic release in hMT1 mice, we directly examined the content of Ach in the striatum of ChKO mice, as well as the ability of tissue from these mice to synthesize and release acetylcholine. For determination of striatal Ach content, rapid *in vivo* fixation with microwave irradiation was used to prevent postmortem degradation of Ach. Using this approach, we found no significant difference in striatal Ach content in ChKO mice compared to *loxP* mice (Figure 6; $n = 8$ for ChKO, and $n = 8$ for *loxP*). In slices from ChKO and control (*loxP*) animals, we estimated Ach synthesis and release by incubation with [3 H]-choline. After loading with [3 H]-choline, release was evoked by high K^+ in the presence of hemicholineum to prevent reuptake. ChKO mice showed no significant difference in the capacity to take up [3 H]-choline compared to *loxP* mice (Figure 7A; $n = 8$ for ChKO, and $n = 15$ for *loxP*). We observed Ach release induced by high potassium, but the amount released did not differ between ChKO and controls (Fig. 7B; $n = 4$ for ChKO, and control group consisted of *loxP*, $n = 7$, and heterozygous *loxP/Chat-cre* mice, $n = 3$).

Selective inactivation of *Dyt1* in cholinergic neurons leads to loss of M2/M4 receptor inhibitory function, and paradoxical activation by D2 agonists

For the electrophysiological studies, cholinergic interneurons (ChIs) were identified by their size and shape, by means of IR-DIC video microscopy, and according to their peculiar electrophysiological properties which include a depolarized resting membrane potential (RMP), a long-lasting afterhyperpolarization (AHP) and a prominent I_h current activated by hyperpolarizing current pulses. All these properties have been unequivocally attributed to ChIs (Bennett et al., 2000). Spontaneous firing activity requires maintenance of an ionic equilibrium and signaling molecules. Thus, to preserve these properties, we performed

perforated-patch recordings. In such configuration, spontaneous, ongoing spiking activity was observed in nearly all of the recorded cells. Interneurons recorded from ChKO mice did not exhibit significant differences from WT mice in their basic membrane properties, such as RMP (WT -48 ± 1.8 mV; ChKO -52 ± 2.3 mV), input resistance (WT 168 ± 4.2 M Ω ; ChKO 154 ± 2.9 M Ω), AHP duration (WT 298 ± 27 ms; ChKO 309 ± 33 ms), I_h (WT 289.73 ± 44 pA; ChKO 301 ± 68 pA) ($n=16$; $p>0.05$; not shown).

Activation of M2/M4 receptors in ChIs, generates a membrane hyperpolarization with a reversible cessation of firing activity which often serves an autoreceptor function by creating a negative feedback effect that is important for homeostasis of striatal Ach levels (Calabresi et al., 1998; Pisani et al., 1999). In WT mice, bath-application of either muscarine (10 μ M, 90 s) or the selective M2/M4 receptor agonist oxotremorine (300 nM, 2 min) caused a membrane hyperpolarization and abolished firing activity (respectively 7.2 ± 1.3 mV and 6.98 ± 2.3 mV, $n=4$ for each group; Fig 8A;). Notably, this self-inhibitory response was absent in ChIs from ChKO animals. In fact, activation of M2/M4 receptors did not produce any significant membrane hyperpolarization or firing rate reduction ($p>0.05$, Fig. 8B).

To control for non-specific effects of the inactivation of *Dyt1* in cholinergic cells, we studied the effects of application of baclofen, a GABA_B receptor agonist, which exerts an inhibitory effect on ChI's similar to that of M2/M4 agonists (Pisani et al., 1999). Baclofen (10 μ M, 1 min) caused a comparable membrane hyperpolarization along with full blockade of firing activity both in WT (Fig. 9A; 5.3 ± 0.8 mV, $n=4$, $p>0.05$) and ChKO animals (Fig. 9A1; 4.7 ± 1.0 mV, $n=4$, $p>0.05$).

We have previously observed that in transgenic mice overexpressing mutant torsinA throughout the brain (hMT1 mice), application of the D2 agonist quinpirole produces a paradoxical excitatory effect on ChI spontaneous firing (Pisani et al., 2006). A remarkably similar response was observed in recordings from ChIs from ChKO mice. Bath-application of quinpirole (10 μ M, 2 min) in WT mice did not change the intrinsic membrane properties of the recorded cells, and slightly reduced the spontaneous firing rate of these neurons (Fig. 10A; from 0.23 ± 0.01 Hz to 0.18 ± 0.02 ; $n=6$; $p>0.05$). Conversely, in ChKO mice, quinpirole induced a membrane depolarization coupled to an increase in firing rate (Fig. 10B; from 0.18 ± 0.03 Hz to 0.36 ± 0.05 ; $n=6$ $p<0.05$). To verify that the paradoxical effect was directly mediated by D2 receptor activation, quinpirole was applied in the presence of the D2 receptor antagonist sulpiride (10 μ M). Sulpiride fully prevented the quinpirole-dependent effect on ChKO mice (not shown). To verify the specificity of the depolarizing response, we examined the effect of dihydroxyphenylglycine (3,5-DHPG), a group 1 mGluR non-selective agonist, which has a robust excitatory effect on this cell type (Bonsi et al., 2005). Bath-applied 3,5-DHPG (30 μ M, 30 s) induced a rapid and reversible membrane depolarization and an increase in firing rate that was identical in WT and ChKO animals (WT: from 0.24 ± 0.01 Hz to 0.56 ± 0.1 ; ChKO from 0.19 ± 0.01 Hz to 0.68 ± 0.05 ; $n=3$ for each group; Fig. 9B–B1).

Selective inactivation of *Dyt1* in cholinergic neurons does not alter the properties of dopaminergic neurons, their response to D2 agonists, or amphetamine stimulated dopamine release

Since abnormalities of dopaminergic function have been observed in some mouse models of dystonia, we also examined the effects of selective inactivation of *Dyt1* in cholinergic neurons on the activity and pharmacological responsiveness of dopaminergic neurons in the substantia nigra.

Spontaneous, rhythmic firing activity (mean rate of ~ 2 – 4 Hz) is a peculiar feature of nigral neurons. Hyperpolarizing current pulses evoke a prominent sag, mediated by an

hyperpolarization-activated cation current (I_h) (Mercuri et al., 1995). Both maximal firing rate and sag amplitude are considered distinguishing features of dopaminergic neurons (Liss et al., 2005), and we used these features to identify neurons for study. Spontaneous firing activity of dopaminergic nigral neurons in slices from ChKO mice was comparable to that recorded from control mice (Fig. 11; WT: 3.5 ± 1.2 Hz; ChKO: 3.56 ± 1.32 ; $p > 0.05$ $n = 15$ for each group). Pacemaking activity of nigral neurons is regulated by somatodendritic D2 autoreceptors (Mercuri et al., 1997; Napolitano et al., 2010). Accordingly, dopamine (DA) application ($100 \mu\text{M}$, 30 s) abolished cell firing and hyperpolarized the cell membrane to a similar extent in slices from control (Fig. 11; 13.9 ± 1.75 mV; $n = 12$), and ChKO mice (17.6 ± 3.6 mV $n = 11$; $p > 0.05$). Similarly, the D2 receptor agonist quinpirole ($10 \mu\text{M}$, 2 min) caused a membrane hyperpolarization and blockade of firing discharge (Fig. 12; WT: 15.5 ± 1.6 mV; ChKO: 16.02 ± 1.4 mV; $n = 11$; $p > 0.05$), an effect that was prevented by the D2 antagonist sulpiride ($3 \mu\text{M}$), confirming the requirement for activation of the D2 receptors (Fig. 12).

In a final set of experiments, we tested the effects of amphetamine, which causes increases of DA in the synaptic cleft. In fact bath-application of amphetamine ($100 \mu\text{M}$, 2 min) hyperpolarized nigral neurons, and abolished their firing activity to a similar extent in midbrain slices from both control and ChKO mice (Fig. 13; WT: 16.5 ± 3 mV; $n = 13$; ChKO: 16.4 ± 3.2 ; $n = 13$; $p > 0.05$). Since a decrease in amphetamine-stimulated DA release has been shown in the hMT1 mouse model (Balcioglu et al., 2007), we performed *in vivo* microdialysis to measure baseline and amphetamine stimulated levels of extracellular striatal DA. We found similar levels of striatal extracellular dopamine at baseline (Fig. 14; WT: 24.70 ± 0.82 nM; ChKO: 26.37 ± 0.88 ; $p > 0.05$; $n = 12$ for each group) and, contrary to findings from the hMT1 mice, no difference following amphetamine stimulation in ChKO and control mice (Fig. 14; WT: 228.22 ± 60.10 nM; ChKO: 219.83 ± 70.61 ; $p > 0.05$; $n = 12$ for each group).

DISCUSSION

In this study we used a cholinergic specific conditional knock out of *Dyt1* in a mouse model to determine whether deletion of this protein would produce cell-autonomous effects on cholinergic neuron function or behavior of the animals. We found that this produces pathologic effects with altered electrophysiological properties of striatal cholinergic interneurons, including a paradoxical excitatory response to D2 receptor agonists and impaired muscarinic receptor function, as well as subtle defects in movement behavior. These features are very similar to those observed in transgenic animals with expression of mutant human torsinA throughout the brain (hMT1 mice; Pisani et al., 2006; Sharma et al., 2005; Zhao et al., 2011). The deletion of *Dyt1* from cholinergic cells does not, however, alter the number or size of cholinergic neurons present in the striatum, or basic properties of striatal choline uptake and acetylcholine release in response to depolarization. Together this data supports the concept that mutant torsinA has a dominant negative effect, inhibiting the normal actions of torsinA (Breakefield et al., 2001; Granata et al., 2009; Torres et al., 2004; Warner et al., 2010), and that cholinergic interneurons are a critically important site for the effects of the mutant protein.

The ChKO mouse model employs *Cre-loxP* recombination, with ChAT as the promoter for Cre recombinase, to target *Dyt1* deletion in cholinergic neurons. We tested the specificity using a Rosa reporter mouse and found that Cre expression was limited to known cholinergic neuron populations. These studies also revealed that by postnatal day seven, robust expression of Cre enzyme activity is evident, indicating that inactivation of the target *Dyt1* gene should occur early in the postnatal period. We tested the efficacy of Cre recombinase activity by using LCM. In ChKO mice we found that torsinA mRNA

expression was significantly decreased in striatal cholinergic neurons, while torsinA expression in non-cholinergic striatal neurons was unchanged. Because our LCM samples were obtained using a circular capture area of fixed size, they are enriched samples of the ChAT labeled neurons, but do likely include some contribution from adjacent non-cholinergic cells. Thus, complete elimination of TorsinA mRNA was not expected in our LCM samples from cholinergic neurons in ChKO mice. We also observed that the baseline level of torsinA in cholinergic neurons, relative to beta-actin, was higher in cholinergic neurons than in non-cholinergic neurons. A similar preferential expression of torsinA mRNA in cholinergic vs. non-cholinergic striatal neurons has been observed in human brain (Augood et al., 1999). In concordance with the lack of neurodegeneration in DYT1 dystonia, we observed no difference in number or size of striatal cholinergic neurons in ChKO mice (Rostasy et al., 2003; Standaert, 2011).

Similar to most of the previously described mouse models of genetic forms of dystonia, ChKO mice do not exhibit any behavior which is obviously “dystonic” in nature (Breakefield et al., 2008; Jinnah et al., 2005). They do, however, show subtle motor deficits, in this case best revealed by decreased latencies on the rotarod, a defect also observed in other mouse models of DYT1 (Grundmann et al., 2007; Sharma et al., 2005). The ChKO mice exhibited normal behavioral on a number of other tests, including open field activity, tests for anxiety and spatial learning. ChKO mice showed no impairment in beam walking, a task which requires fine motor balance and coordination. This is in contrast to several other mouse models which do show beam walking impairment including the hMT1 mouse, DYT1 knock in mice, *Dyt1* knock down mice, cerebral cortex specific *Dyt1* knock out mice, striatal specific knock out mice, and a mouse model expressing human mutant torsinA selectively in dopaminergic cells (Dang et al., 2005; Dang et al., 2006; Page et al., 2010; Yokoi et al., 2008; Yokoi et al., 2011; Zhao et al., 2008). The subtle nature of all of these defects makes it difficult to assign causality to the different behavioral features, but these data at least suggest that the deletion of *Dyt1* from cholinergic neurons alone in the ChKO animals may reproduce only part of the phenotype seen with more global deletions or expression of the mutant torsinA. Interestingly, recent data shows that the anticholinergic trihexyphenidyl can reverse beam walking deficits in a DYT1 knock in mouse model (Dang et al., 2012). Collectively, all of these mouse models of DYT1 dystonia are probably more reflective of the non-manifesting carrier state of human DYT1, as opposed to the state of symptomatic DYT1 dystonia (Breakefield et al., 2008; Ghilardi et al., 2003; Sharma et al., 2005).

Here we examined Ach content, uptake, synthesis, and release in response to high potassium, in ChKO mice and found no significant differences between the ChKO animals and controls. These data, together with anatomical evidence that the number and morphology of ChAT neurons are preserved in these animals, demonstrates that deletion of *Dyt1* does not alter these basic properties of striatal cholinergic neurons. These static measurements of Ach content, uptake, and synthesis do not, however, exclude the possibility that there are dynamic alterations in Ach homeostasis in the ChKO mice. Ach concentration and distribution within the synapse cannot be interpreted from these measurements. Further, alterations in Ach modulation by dopaminergic or cholinergic receptors may depend on patterns of activation by ligands for these sites. There have been no previous studies which have directly examined cholinergic function in human DYT1 dystonia. The hMT1 mice do show increased acetylcholinesterase activity, which may be a reflection of the underlying cholinergic abnormalities in these animals (Martella et al., 2009).

Despite the lack of neurochemical differences in ChKO mice, recordings from striatal cholinergic interneurons in these mice showed striking abnormalities of cholinergic function. We found that selective deletion of *Dyt1* from cholinergic neurons reproduced the

same paradoxical excitatory response to D2 receptor agonists that was previously seen in hMT1 mice (Pisani et al., 2006). This defect was selective for D2 receptors, in that deletion of *Dyt1* from cholinergic neurons did not alter their response to baclofen, a GABA_B receptor agonist, or 3,4,-DHPG, a group 1 mGluR agonist. These data are consistent with a recent study in the hMT1 model, in which a similar deficit in D2 signaling in striatal non-cholinergic cells was observed, with inability of a D2R agonist to re-establish normal corticostriatal synaptic plasticity and reduced sensitivity to haloperidol-induced catalepsy, along with reduced abundance of D2R protein and impaired ability of D2Rs to activate their cognate Go/i proteins (Napolitano et al., 2010).

We also observed that cholinergic neurons in the ChKO animals lacked the normal muscarinic receptor response. Since these receptors are known to function as an autoreceptors, this deregulation would further contribute to a hypercholinergic state. While the electrophysiological defect is clear, the mechanism of this effect is not certain. One possibility is that selective inactivation of torsinA induces compensatory adaptations in ion channel activity, which offset the effect of muscarinic activation without altering basic membrane properties. Alternatively, loss of torsinA activity disrupts post-receptor signaling mechanisms linked to muscarinic receptor activation.

Although the normal functions of torsinA are not well understood, the data presented here support the view that the *TOR1A* mutation in torsinA produces pathological effects through a dominant negative action. In our model system, the selective deletion of *Dyt1* in cholinergic neurons produces a physiological phenotype in these cells which is similar to that observed with global transgenic expression of human mutant torsinA in the hMT1 mice (Pisani et al., 2006). The structural basis for this dominant negative effect is likely to be the ability of mutant torsinA to form homohexameric ring structures which are characteristic of AAA+ proteins (Breakefield et al., 2001; Granata et al., 2009; Torres et al., 2004; Warner et al., 2010).

The data presented here support the view that DYT1 dystonia is associated with abnormalities in cholinergic transmission. This view is consistent with the clinical observations of the efficacy of anticholinergic drugs, as well as our observations in both the ChKO and hMT1 mouse models (Martella et al., 2009; Pisani et al., 2006). Our data show that these cholinergic abnormalities are at least in part a cell-autonomous effect, related directly to the actions of torsinA within cholinergic neurons. The fact that ChKO mice did not recapitulate findings of reduced amphetamine stimulated dopamine release seen in hMT1 mice indicates some pathologic role TorsinA outside of cholinergic neurons. Thus, it will be important to examine the effects of *Dyt1* mutation and deletion in other models systems including other components of basal ganglia circuitry. Nevertheless, our data do suggest that approaches targeting the function of cholinergic neurons may be a fruitful approach to developing novel therapies for DYT1 dystonia, and possibly other forms of human dystonia.

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ABBREVIATIONS

ChKO cholinergic-specific conditional knock-out

ChAT	choline acetyl-transferase
Ach	acetylcholine
LCM	laser capture microdissection
ChI	Cholinergic interneuron
D2	dopaminergic receptor
DA	dopamine
WT	wild type
M2/M4	muscarinic acetylcholine receptor
mGluR	metabotropic glutamate receptor

References

- Albanese A, et al. EFNS guidelines on diagnosis and treatment of primary dystonias. *Eur J Neurol*. 2011; 18:5–18. [PubMed: 20482602]
- Augood S, et al. Distribution and ultrastructural localization of torsinA immunoreactivity in the human brain. *Brain Res*. 2003; 986:12–21. [PubMed: 12965225]
- Augood SJ, et al. Distribution of the mRNAs encoding torsinA and torsinB in the normal adult human brain. *Ann Neurol*. 1999; 46:761–9. [PubMed: 10553994]
- Balcioglu A, et al. Dopamine release is impaired in a mouse model of DYT1 dystonia. *J Neurochem*. 2007; 102:783–8. [PubMed: 17550429]
- Bennett BD, et al. Intrinsic membrane properties underlying spontaneous tonic firing in neostriatal cholinergic interneurons. *J Neurosci*. 2000; 20:8493–503. [PubMed: 11069957]
- Bonsi P, et al. Modulatory action of metabotropic glutamate receptor (mGluR) 5 on mGluR1 function in striatal cholinergic interneurons. *Neuropharmacology*. 2005; 49(Suppl 1):104–13. [PubMed: 16005029]
- Bonsi P, et al. Endogenous serotonin excites striatal cholinergic interneurons via the activation of 5-HT 2C, 5-HT6, and 5-HT7 serotonin receptors: implications for extrapyramidal side effects of serotonin reuptake inhibitors. *Neuropsychopharmacology*. 2007; 32:1840–54. [PubMed: 17203014]
- Breakefield X, et al. The pathophysiological basis of dystonias. *Nat Rev Neurosci*. 2008; 9:222–34. [PubMed: 18285800]
- Breakefield X, et al. TorsinA: movement at many levels. *Neuron*. 2001; 31:9–12. [PubMed: 11498045]
- Burke R, et al. Torsion dystonia: a double-blind, prospective trial of high-dosage trihexyphenidyl. *Neurology*. 1986; 36:160–4. [PubMed: 3511401]
- Calabresi P, et al. Muscarinic IPSPs in rat striatal cholinergic interneurons. *J Physiol*. 1998; 510(Pt 2):421–7. [PubMed: 9705993]
- Carbon M, Eidelberg D. Abnormal structure-function relationships in hereditary dystonia. *Neuroscience*. 2009; 164:220–9. [PubMed: 19162138]
- Carbon M, et al. Microstructural white matter changes in primary torsion dystonia. *Mov Disord*. 2008; 23:234–9. [PubMed: 17999428]
- Dang M, et al. Generation and characterization of Dyt1 DeltaGAG knock-in mouse as a model for early-onset dystonia. *Exp Neurol*. 2005; 196:452–63. [PubMed: 16242683]
- Dang M, et al. Motor deficits and hyperactivity in Dyt1 knockdown mice. *Neurosci Res*. 2006; 56:470–4. [PubMed: 17046090]
- Dang MT, et al. An anticholinergic reverses motor control and corticostriatal LTD deficits in Dyt1 DeltaGAG knock-in mice. *Behav Brain Res*. 2012; 226:465–72. [PubMed: 21995941]
- Dawood MY, et al. Accelerated Barnes maze test in mice for assessment of stress effects on memory. *Ann N Y Acad Sci*. 2004; 1032:304–7. [PubMed: 15677437]

- Deandrade MP, et al. Characterization of Atp1a3 mutant mice as a model of rapid-onset dystonia with parkinsonism. *Behav Brain Res.* 2011; 216:659–65. [PubMed: 20850480]
- Edwards M, et al. Abnormalities in motor cortical plasticity differentiate manifesting and nonmanifesting DYT1 carriers. *Mov Disord.* 2006; 21:2181–6. [PubMed: 17078060]
- Fahn S, Eldridge R. Definition of dystonia and classification of the dystonic states. *Adv Neurol.* 1976; 14:1–5. [PubMed: 941763]
- Ghilardi MF, et al. Impaired sequence learning in carriers of the DYT1 dystonia mutation. *Ann Neurol.* 2003; 54:102–9. [PubMed: 12838525]
- Gonzalez-Alegre P, Paulson H. Aberrant cellular behavior of mutant torsinA implicates nuclear envelope dysfunction in DYT1 dystonia. *J Neurosci.* 2004; 24:2593–601. [PubMed: 15028751]
- Goodchild RE, et al. Loss of the dystonia-associated protein torsinA selectively disrupts the neuronal nuclear envelope. *Neuron.* 2005; 48:923–32. [PubMed: 16364897]
- Granata A, et al. TorsinA and dystonia: from nuclear envelope to synapse. *J Neurochem.* 2009; 109:1596–609. [PubMed: 19457118]
- Grundmann K, et al. Overexpression of human wildtype torsinA and human DeltaGAG torsinA in a transgenic mouse model causes phenotypic abnormalities. *Neurobiol Dis.* 2007; 27:190–206. [PubMed: 17601741]
- Ivanova E, et al. Characterization of transgenic mouse lines expressing Cre recombinase in the retina. *Neuroscience.* 2010; 165:233–43. [PubMed: 19837136]
- Jinnah HA, et al. Rodent models for dystonia research: characteristics, evaluation, and utility. *Mov Disord.* 2005; 20:283–92. [PubMed: 15641011]
- Lacey MG, et al. Dopamine acts on D2 receptors to increase potassium conductance in neurones of the rat substantia nigra zona compacta. *J Physiol.* 1987; 392:397–416. [PubMed: 2451725]
- Liss B, et al. K-ATP channels promote the differential degeneration of dopaminergic midbrain neurons. *Nat Neurosci.* 2005; 8:1742–51. [PubMed: 16299504]
- Madisen L, et al. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat Neurosci.* 2010; 13:133–40. [PubMed: 20023653]
- Martella G, et al. Impairment of bidirectional synaptic plasticity in the striatum of a mouse model of DYT1 dystonia: role of endogenous acetylcholine. *Brain.* 2009; 132:2336–49. [PubMed: 19641103]
- Mercuri NB, et al. Properties of the hyperpolarization-activated cation current *I_h* in rat midbrain dopaminergic neurons. *Eur J Neurosci.* 1995; 7:462–9. [PubMed: 7773443]
- Mercuri NB, et al. Loss of autoreceptor function in dopaminergic neurons from dopamine D2 receptor deficient mice. *Neuroscience.* 1997; 79:323–7. [PubMed: 9200717]
- Napolitano F, et al. Dopamine D2 receptor dysfunction is rescued by adenosine A2A receptor antagonism in a model of DYT1 dystonia. *Neurobiol Dis.* 2010; 38:434–45. [PubMed: 20227500]
- Ozelius LJ, et al. The early-onset torsion dystonia gene (DYT1) encodes an ATP-binding protein. *Nat Genet.* 1997; 17:40–8. [PubMed: 9288096]
- Page ME, et al. Cell-autonomous alteration of dopaminergic transmission by wild type and mutant (DeltaE) TorsinA in transgenic mice. *Neurobiol Dis.* 2010; 39:318–26. [PubMed: 20460154]
- Pecho-Vrieseling E, et al. Specificity of sensory-motor connections encoded by Sema3e-Plxnd1 recognition. *Nature.* 2009; 459:842–6. [PubMed: 19421194]
- Pisani A, et al. Electrophysiological recordings and calcium measurements in striatal large aspiny interneurons in response to combined O2/glucose deprivation. *J Neurophysiol.* 1999; 81:2508–16. [PubMed: 10322086]
- Pisani A, et al. Altered responses to dopaminergic D2 receptor activation and N-type calcium currents in striatal cholinergic interneurons in a mouse model of DYT1 dystonia. *Neurobiol Dis.* 2006; 24:318–25. [PubMed: 16934985]
- Rostasy K, et al. TorsinA protein and neuropathology in early onset generalized dystonia with GAG deletion. *Neurobiol Dis.* 2003; 12:11–24. [PubMed: 12609485]
- Sciamanna G, et al. Developmental profile of the aberrant dopamine D2 receptor response in striatal cholinergic interneurons in DYT1 dystonia. *PLoS One.* 2011; 6:e24261. [PubMed: 21912682]

- Sharma N, et al. Impaired motor learning in mice expressing torsinA with the DYT1 dystonia mutation. *J Neurosci*. 2005; 25:5351–5. [PubMed: 15930383]
- Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet*. 1999; 21:70–1. [PubMed: 9916792]
- Standaert DG. Update on the pathology of dystonia. *Neurobiol Dis*. 2011; 42:148–51. [PubMed: 21220015]
- Torres G, et al. Effect of torsinA on membrane proteins reveals a loss of function and a dominant-negative phenotype of the dystonia-associated DeltaE-torsinA mutant. *Proc Natl Acad Sci U S A*. 2004; 101:15650–5. [PubMed: 15505207]
- Vasudevan A, et al. Developmental patterns of torsinA and torsinB expression. *Brain Res*. 2006;1073–1074. 139–45.
- Wang Z, et al. Presynaptic and postsynaptic interaction of the amyloid precursor protein promotes peripheral and central synaptogenesis. *J Neurosci*. 2009; 29:10788–801. [PubMed: 19726636]
- Warner T, et al. TorsinA and DYT1 dystonia: a synaptopathy? *Biochem Soc Trans*. 2010; 38:452–6. [PubMed: 20298201]
- Yokoi F, et al. Motor deficits and hyperactivity in cerebral cortex-specific Dyt1 conditional knockout mice. *J Biochem*. 2008; 143:39–47. [PubMed: 17956903]
- Yokoi F, et al. Motor deficits and decreased striatal dopamine receptor 2 binding activity in the striatum-specific Dyt1 conditional knockout mice. *PLoS One*. 2011; 6:e24539. [PubMed: 21931745]
- Zhang L, et al. Protective effects of minocycline on behavioral changes and neurotoxicity in mice after administration of methamphetamine. *Prog Neuropsychopharmacol Biol Psychiatry*. 2006; 30:1381–93. [PubMed: 16839653]
- Zhang L, et al. Minocycline attenuates hyperlocomotion and prepulse inhibition deficits in mice after administration of the NMDA receptor antagonist dizocilpine. *Neuropsychopharmacology*. 2007; 32:2004–10. [PubMed: 17228338]
- Zhao Y, et al. Abnormal motor function and dopamine neurotransmission in DYT1 DeltaGAG transgenic mice. *Exp Neurol*. 2008; 210:719–30. [PubMed: 18299128]
- Zhao Y, et al. The DYT1 carrier state increases energy demand in the olivocerebellar network. *Neuroscience*. 2011; 177:183–94. [PubMed: 21241782]
- Zucker B, et al. Transcriptional dysregulation in striatal projection- and interneurons in a mouse model of Huntington's disease: neuronal selectivity and potential neuroprotective role of HAP1. *Hum Mol Genet*. 2005; 14:179–89. [PubMed: 15548548]

HIGHLIGHTS

- Cholinergic-specific conditional knock out mouse model of DYT1 dystonia was created
- ChKO mice show subtle impairment in motor behavior
- Cholinergic neurons in ChKO mice show abnormal responses to D2 receptor agonists
- Cholinergic neurons in ChKO mice show loss of muscarinic receptor inhibitory function
- These data imply a cell-autonomous effect of torsinA on cholinergic neurons

Figure 1. Genotyping of *Chat-cre*, *Dyt1 loxP*, and ChKO mice

Top band shows PCR product for *Cre*, middle band represents *Dyt1 loxP* locus, and bottom band shows *Dyt1* wild type locus. Lanes 1, 2, and 9 represent ChKO mice. Lanes 3, 7, 8 represent heterozygous *Dyt1 loxP* mice. Lanes 4 and 5 represent mice expressing ChAT-cre and heterozygous for *Dyt1 loxP*. Lane 6 is representative of a *Chat-cre* mouse.

Figure 2. β -galactosidase staining in *Chat-cre/ROSA* reporter mice

Striatal (Str) and brainstem (Bs) sections shown at postnatal day 7 (A,D), 21 (B,E), 90 (C,F). Expression of Cre-recombinase is shown early as postnatal day 7 in striatum and brainstem sections. Inset boxes illustrate higher magnification of staining in cells from each section.

Figure 3. Validation of selective inactivation of *Dyt1* in ChKO mice using laser capture microdissection

(A) Example of fluorescent staining for ChAT positive striatal neurons prior to LCM capture and (B) after LCM capture. (C) ChAT stain positive neurons (cholinergic) show 29.3 fold higher expression of ChAT mRNA (normalized to beta-actin) compared to non-cholinergic neurons. (D) TorsinA mRNA expression normalized to beta-actin is significantly decreased in cholinergic neurons from ChKO mice compared to *loxP* mice, and is unchanged in non-cholinergic neurons from ChKO compared to *loxP* mice. * $P < 0.05$.

Figure 4. ChKO mice show impaired motor behavior

(A) ChKO mice show a decrease in latency to falling off the rotarod compared to control mice (CT). (B) ChKO mice showed no significant difference in number of hind limb slips on beam walking task compared to *loxP* mice. Bars represent mean $\pm$ SEM. * $P < 0.05$.

Figure 5. Striatal cholinergic population number and volumetric size is unchanged in ChKO mice

(A) Example of staining for choline acetyltransferase (ChAT) to identify striatal cholinergic neurons. (B) Wire diagrams outlining cell body parameters for volumetric size measurements in striatal cholinergic neurons from *loxP* and ChKO mice. (C) Estimated striatal cholinergic neuron populations from *loxP* and ChKO mice show no significant difference. (D) Enclosed volumes of striatal cholinergic neuron cell bodies in ChKO and *loxP* mice show no significant difference. Bars represent mean $\pm$ standard error of the (SEM).

Figure 6. HPLC analysis shows no significant difference in Ach content between ChKO and loxP mice

Choline content is shown as a control. Bars represent mean $\pm$ SEM.

Figure 7. [3H]-choline uptake and release

(A) Total [^3H]-choline uptake in ChKO mice is not significantly different compared to *loxP* mice. (B) Release induced by high potassium was not different between ChKO mice and the control group (*loxP/het*).

Figure 8. Recordings from striatal cholinergic neurons in ChKO mice show altered response to muscarinic receptor activation

(A) Bath-application of the non-selective M2/M4 receptor agonist muscarine (10 μ M, 90 s) caused a membrane hyperpolarization and cessation of spontaneous firing activity in a cholinergic interneuron recorded from a WT mouse. This inhibitory response was reversible upon drug washout. Insets show the firing rate at higher speed. (B) In ChKO mice, the inhibitory response to muscarine was absent.

Figure 9. Cholinergic interneurons exhibit normal response to GABA_B receptor and group I mGlu receptor activation

A. The GABA_B receptor agonist baclofen (10 μM, 1 min) blocked the firing activity of interneurons recorded from both WT (A) and ChKO (A1) mice to a similar extent. B. Similarly, the group I mGlu receptor agonist 3,5-DHPG (30 μM, 30 s) induced a membrane depolarization coupled to increase in firing activity in interneurons recorded from both WT (B) and ChKO (B1) mice.

Figure 10. Abnormal responses to D2 dopamine receptor activation

Quinpirole (10 μ M, 2 min) did not modify the RMP of an interneuron recorded from a WT mouse (A). The inset (below) shows at higher sweep speed the firing rate of the cell.

Analysis of the inter-spike interval (ISI, inset on the right) did not reveal changes between pre- and post-quinpirole. Conversely, in interneurons from ChKO mice (B), quinpirole (10 μ M, 2 min) caused a paradoxical excitatory effect, with a net increase in firing frequency, as measured by the leftward shift of the ISI after quinpirole application (b).

Figure 11. Intrinsic properties of dopaminergic neurons and normal response to dopamine in ChKO mice

Nigral dopaminergic neurons recorded from WT mice did not exhibit significant differences compared to ChKO mice. Current steps in both depolarizing and hyperpolarizing directions revealed no significant differences in spike threshold and I_h current of the recorded neurons (A,A1). Similarly, the basal firing frequency of the cells did not differ in the two strains (B). Bath-application of dopamine (DA, 100 μ M, 30 s) inhibited cell firing and hyperpolarized the cell membrane to a similar extent in slices from control and KO mice (C). The extent of the hyperpolarization and the recovery to resting levels upon drug washout did not differ in WT and ChKO mice (D,E).

Figure 12. Normal responses of dopaminergic neurons from ChKO mice to quinpirole
 Bath-application of the D2 receptor agonist quinpirole (10 μ M, 2 min) caused a membrane hyperpolarization and blockade of firing discharge both in WT and ChKO mice, an effect that was prevented by pretreatment of the slice with the selective antagonist sulpiride (3 μ M) (A). The inset shows the lack of any significant change in firing rate in the presence of sulpiride. Summary plots of the hyperpolarizing response to quinpirole, and time to recovery to resting levels in the two strains (B,C).

Figure 13. Recordings from dopaminergic neurons in ChKO mice show no alteration in response to amphetamine

(A) Amphetamine (100 μ M, 2 min) hyperpolarized nigral neurons and blocked their firing activity to a similar extent in midbrain slices from both control and ChKO mice. (B) Summary plots of the amphetamine effects in both strains of mice.

Figure 14. Microdialysis measurements show no difference in baseline or amphetamine stimulated striatal extracellular dopamine levels
Bars represent mean $\pm$ SEM.