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Trihexyphenidyl rescues the deficit in dopamine neurotransmission in a mouse model of DYT1 dystonia

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ABSTRACT

Trihexyphenidyl, a nonselective muscarinic receptor antagonist, is the small molecule drug of choice for the treatment of DYT1 dystonia, but it is poorly tolerated due to significant side effects. A better understanding of the mechanism of action of trihexyphenidyl is needed for the development of improved treatments. Because DTY1 dystonia is associated with both abnormal cholinergic neurotransmission and abnormal dopamine regulation, we tested the hypothesis that trihexyphenidyl normalizes striatal dopamine release in a mouse model of DYT1 dystonia using *ex vivo* fast scan cyclic voltammetry and *in vivo* microdialysis. Trihexyphenidyl increased striatal dopamine release and efflux as assessed by *ex vivo* voltammetry and *in vivo* microdialysis respectively. In contrast, L-DOPA, which is not usually effective for the treatment of DYT1 dystonia, did not increase dopamine release in either Dyt1 or control mice. Trihexyphenidyl was less effective at enhancing dopamine release in Dyt1 mice relative to controls *ex vivo* (mean increase WT: 65% *vs* Dyt1: 35%). Trihexyphenidyl required nicotinic receptors but not glutamate receptors to increase dopamine release. Dyt1 mice were more sensitive to the dopamine release decreasing effects of nicotinic acetylcholine receptor antagonism (IC_{50}: WT = 29.46 nM, Dyt1 = 12.26 nM) and less sensitive to acetylcholinesterase inhibitors suggesting that nicotinic acetylcholine receptor neurotransmission is altered in Dyt1 mice, that nicotinic receptors indirectly mediate the differential effects of trihexyphenidyl in Dyt1 mice, and that nicotinic receptors may be suitable therapeutic targets for DYT1 dystonia.

1. Introduction

Dystonia is characterized by abnormal muscle contractions that cause debilitating abnormal postures and/or twisting movements (Jankovic and F, 1998; Albanese et al., 2013). DYT1 dystonia is one of the most prevalent forms of inherited dystonia. Most DYT1 patients carry a 3 base-pair in-frame deletion (ΔGAG) in the *TOR1A* gene resulting in a single glutamic acid deletion in the TorsinA protein (Ozelius et al., 1997; Jankovic and F, 1998; Ozelius et al., 1998). Despite the identification of a causal mutation, pharmacological interventions for DYT1 dystonia are limited. The preferred oral medication is the nonselective muscarinic acetylcholine receptor (mAChR) antagonist, trihexyphenidyl (THP) (Schwarz and Bressman, 2009; Thenganatt and Jankovic, 2014), which binds to all 5 mAChR subtypes at low nanomolar affinity (M_1 = 1.6 nM, M_2 = 7 nM, M_3 = 6.4 nM, M_4 = 2.6 nM, M_5 = 15.9 nM). (Dorje et al., 1991; Bolden et al., 1992) Although THP can be an effective treatment for DYT1 dystonia, it is often poorly tolerated due to dose-limiting side effects associated with its broad anti-muscarinic receptor activity (Burke et al., 1986; Jabbari et al., 1989; Guthrie et al., 2000; Lumsden et al., 2016). Further, these side-effects are exacerbated by the high doses required to treat dystonia. A significant obstacle to the development of improved therapeutics is that the mechanism of action of THP is poorly understood. A better understanding of the therapeutic actions of THP could improve treatment options.

One possible mechanism of action of THP may involve the regulation of striatal dopamine (DA) neurotransmission, which is abnormal in DYT1 dystonia. Previous studies have demonstrated reductions in

Abbreviations: mAChR, muscarinic acetylcholine receptor; THP, trihexyphenidyl; DA, dopamine; ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; ChI, cholinergic interneuron; NAcc, nucleus accumbens; FSCV, fast scan cyclic voltammetry; AChE, acetylcholinesterase; DHβE, dihydro-β-erythroidine

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striatal D2 receptor density and abnormalities in DA metabolites in DYT1 patients (Augood et al., 2002; Asanuma et al., 2005). Additionally, a significant reduction in striatal DA release is observed in several mouse models of DYT1 dystonia (Balcioglu et al., 2007; Page et al., 2010; Song et al., 2012). Striatal DA release is regulated by acetylcholine (ACh) neurotransmission *via* both mAChR and nicotinic acetylcholine receptors (nAChRs) (Zhang et al., 2002a,b; Exley and Cragg, 2008; Zhang et al., 2009a,b; Threlfell and Cragg, 2011). Previous studies have implicated striatal ACh in the pathophysiology of dystonia (Bonsi et al., 2008; Bonsi et al., 2011; Maltese et al., 2014; Scarduzio et al., 2017; Bonsi et al., 2018). Cholinergic innervation in the striatum is provided by both cholinergic interneurons (ChIs)(Bolam et al., 1984) and cholinergic projections from brainstem nuclei(Dautan et al., 2016) although striatal DA release is apparently mediated by ChIs (Brimblecombe et al., 2018). ACh released by ChIs activates nAChRs on DA terminals to enhance DA release (Rice and Cragg, 2004; Exley and Cragg, 2008; Threlfell and Cragg, 2011). Activation of presynaptic M_2 and M_4 mAChR on ChIs inhibits ACh release (Zhang et al., 2002a,b), thereby attenuating DA release (Threlfell et al., 2010). Therefore, we hypothesized that THP increases DA release in the striatum by inhibiting this negative feedback mechanism. Indeed, we found that DA release was significantly reduced in Dyt1 knockin mice. THP restored DA release in Dyt1 mice through a nAChR-dependent mechanism, suggesting a mechanism of action for THP in the treatment of dystonia and identifying nAChRs as a therapeutic target.

2. Materials and methods

2.1. Animals

Dyt1 knockin mice heterozygous for the ΔE-torsinA mutation (*Tor1a*$^{+/\Delta E}$)(Goodchild et al., 2005) and normal littermates (*Tor1a*$^{+/+}$) on a C57BL/6J background were bred at Emory University. Animals were maintained on a 12 h light/dark cycle, with *ad libitum* access to food and water. Mice were group housed with nestlets for environmental enrichment. Male and female mice between 12 and 14 weeks old were used in all experiments. Mice were genotyped using PCR (forward primer, GCTATGGAAGCTCTAGTTGG; reverse primer CAGCCAGGGCT AAACAGAG). All studies were approved by the Institutional Animal Care and Use Committee at Emory University.

2.2. Brain slice preparation

Mice were euthanized by cervical dislocation, and the brain was sectioned at 300 μm in ice-cold, oxygenated sucrose artificial cerebral spinal fluid (aCSF) containing [in mM]: sucrose [194], NaCl [20], KCl [4.4], $CaCl_2$ [1.2], $MgCl_2$ [1.2], NaH_2PO_4 [1.2], $NaHCO_3$ [25], D-glucose [11] at pH 7.4. Slices containing the dorsolateral striatum were selected by using the presence of a fused anterior commissure (Bregma +0.26 mm) or, for the ventral striatum (nucleus accumbens; NAcc), the presence of the unfused anterior commissure (Bregma +0.98 mm) as landmarks. Brain slices were collected in a holding chamber containing oxygenated, bicarbonate-buffered aCSF containing [in mM]: NaCl [126], KCl [2.45], $CaCl_2$ [2.4], $MgCl_2$ [1.2], NaH_2PO_4 [1.2], $NaHCO_3$ [25], D-glucose [11] and maintained at room temperature for 45–60 min before experiments began.

2.3. Fast scan cyclic voltammetry

A slice was transferred to the recording chamber and perfused with oxygenated aCSF at 32 °C. After 30 min, a carbon fiber electrode was inserted approximately 50 μm into the surface of the slice and a bipolar tungsten stimulating electrode was placed approximately 200 μm away. Recordings were conducted in the dorsolateral striatum or in the core of the NAcc. Dorsolateral striatum was selected because this region receives innervation from the motor cortex (Hintiryan et al., 2016). DA release was elicited by either 1-pulse (600 μA, 4 ms pulse width) or 5-pulse 100 Hz electrical stimulation at 5 min inter-stimulus intervals to avoid rundown. The scan rate for voltammetry was 400 V/s from −0.4 V to 1.3 V to −0.4 V verses Ag/AgCl with a sampling rate of 10 Hz using a Chem-Clamp voltammeter-amperometer (Dagan Corporation, Minneapolis, MN, USA). All experiments were conducted and analyzed using Demon voltammetry software (Wake Forest University) (Yorgason et al., 2011). All drugs were diluted in aCSF and bath applied. Drugs were equilibrated in the bath for 10–20 min before recordings commenced. For the nicotine experiment, baseline DA release was recorded, then nicotine was added to the slice for 5 mins, and then washed out for 40 mins while DA release was recorded. For the L-DOPA administration experiment, mice were injected with L-DOPA plus benserazide (10 mg/kg and 5 mg/kg respectively, subcutaneous) and then euthanized 30 mins later. FSCV was then performed as described above. All electrodes were calibrated to known DA standards in aCSF using a custom-made flow cell.

2.4. In vivo microdialysis

Microdialysis was performed as previously described (Song et al., 2012). Briefly, a concentric microdialysis probe that was manufactured in-house was calibrated with 100 ng/mL DA in aCSF containing [in mM]: NaCl [147], KCl [3.5], $CaCl_2$ [1.2], $MgCl_2$ [1.2], NaH_2PO_4 [1] at pH 7.0–7.4. After anesthesia with isoflurane, the probe was implanted in the dorsal striatum (anterior 0.6 mm, lateral 1.7 mm, and ventral 4.5 mm from bregma). The probe was perfused with aCSF at a flow rate of 0.6 μL/min while the mice habituated to the experimental chamber overnight. On the day of the experiment, 4 baseline samples were collected every 20 mins to establish basal monoamine concentrations. All collection tubes contained 1 μL of 6.25 mM ascorbic acid to preserve monoamines. Mice were either injected i.p. with a single 20 mg/kg dose of THP or 300 nM THP was reverse dialyzed into the striatum. Samples were collected every 20 mins after injection for 2 h and stored at −80 °C until high-performance liquid chromatography (HPLC) analysis. After sample collection, the probe location was verified by reverse dialysis of 3% bromophenol blue, and only mice with a probe correctly located in the dorsal striatum were included in the analysis.

2.5. Monoamine detection

DA was detected in microdialysis samples using HPLC with electrochemical detection as previously described (Jinnah et al., 1994). The system consisted of an ESA MD-150 × 3.2 mm column, an ESA 5020 guard cell, and an ESA 5600A Coularray detector with an ESA 6210 detector cell (ESA, Bedford MA). The guard cell potential was 475 mV; and the analytic cell potentials were set at −175, 100, 350 and 425 mV. Samples were eluted at a flow rate of 0.4 mL/min with a mobile phase composed of [in mM], [1.7] 1-octanesulfonic acid sodium, [75] NaH_2PO_4, 0.25% triethylamine, and 8% acetonitrile at pH 2.9. Monoamines were identified by retention time and electrochemical profile in comparison with known standards.

2.6. Acetylcholinesterase activity assay

Acetylcholinesterase (AChE) activity was assessed in striatal homogenates using a commercially available colorimetric AChE assay (Abcam, Cambridge, UK), according to the manufacturer's instructions. Briefly, whole striatum was homogenized in lysis buffer by sonicating on ice. Striatal homogenates were incubated with acetylthiocholine and production of thiocholine was identified by development of DTNB-thiol adduct. The reaction was read at 410 nm using a spectrofluorometer (Molecular Devices, San Jose, CA, USA) and absorbance was calibrated to known standards of AChE (mU/mL). Activity was normalized to total protein and data were expressed as mU/mg protein.

2.7. Quantitative real-time PCR

Total RNA was extracted from mouse midbrain using Pure Link RNA mini kit (Life Technologies, Carlsbad, CA, USA). First-strand cDNA synthesis was performed using 1 μg of total RNA and SuperScript II Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). qRT-PCR was performed using 20 ng cDNA, 450 nM primers, and 2× SYBR® Select Master Mix (Applied Biosystems, Foster City, CA, USA). PCR conditions were: 95 °C for 2 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Melting curves were used to validate a single PCR product. Data was analyzed using the comparative C_T method using β-actin as a control (Livak and Schmittgen, 2001). Primers used included: β-actin, forward 5′-AAGGCCAACCGTGAAAAGAT-3′ and reverse 5′-GTGGTACGACCAGAGGCATAC-3′; α4 nAChR, forward 5′-AGCGGC TCCTGAAGAGACTC-3′ and reverse 5′-ACACGTTGGTCGTCATCATC-3′; α6 nAChR, forward 5′-CGGTTTATGTCTGTGGCTATG-3′ and reverse 5′-CCCAACGCAATCTGTAGTCC-3′; α7 nAChR, forward 5′-GCAGATCA TGGATGTGGATG-3′ and reverse 5′-CAAGACGTTGGTGTGGAATG-3′; β2 nAChR, forward 5′-AGATCTACAGGAGCATTAGAG-3′ and reverse 5′-CTTGGAGGGTGCGTGGATCT-3′.

2.8. Compounds

Trihexyphenidyl, atropine, nicotine, neostigmine, L-DOPA, and dopamine were obtained from Sigma-Aldrich (St. Louis, MO). Dihydro-β-erythroidine (DHβE), ambenonium, and CNQX were obtained from Tocris (Minneapolis, MN).

2.9. Statistical analysis

All data are represented as means with standard error. Dose response data were analyzed with non-linear regression to determine IC_{50} or EC_{50}. All other experiments were analyzed with either two-tailed Student's *t*-test or two-way ANOVA with *post hoc* Sidak's multiple comparison test. Statistical analyses were performed using Graphpad Prism 7 (https://www.graphpad.com). Statistical significance is defined as $*p < .05$, $**p < .01$, $***p < .001$, $****p < .0001$.

3. Results

3.1. DA release is reduced in Dyt1 knockin mice

FSCV was used to measure evoked DA release in the dorsolateral striatum. DA release was stimulated with a single electrical stimulation using a perimaximal stimulus current. DA was identified by characteristic oxidation and reduction peaks at +600 mV and -200 mV respectively (Fig. 1A). There was a significant reduction in DA release in Dyt1 mice compared to control mice ($Tor1a^{+/+}$, 1.85 ± 0.12 μM, $Tor1a^{+/\Delta E}$,0.91 ± 0.08 μM, Student's *t*-test, p < .0001) (Fig. 1B). Because it is not known if the DA release deficit is mediated by region-specific microcircuits within the dorsal striatum, which is integrally involved in motor control, or if the decrement in release is a more general phenomenon, we examined DA release in the core of the NAcc. DA release was significantly reduced in the NAcc in Dyt1 mice compared to control mice ($Tor1a^{+/+}$, 1.07 ± 0.09 μM, $Tor1a^{+/\Delta E}$, 0.44 ± 0.06 μM, Student's *t*-test, $p < .01$) (Fig. 1C). Despite the decrement in DA release in Dyt1 knockin mice, the clearance of dopamine (tau), an indirect measure of DA uptake (Yorgason et al., 2011), was not significantly different between Dyt1 and control mice ($Tor1a^{+/+}$, 0.647 ± 0.052 s, $Tor1a^{+/\Delta E}$, 0.707 ± 0.040 s, Student's *t*-test, $p > .05$)(Fig. 1D).

3.2. Trihexyphenidyl enhances DA release

Because previous studies have shown that mAChRs regulate striatal DA release (Zhang et al., a,b; Zhang et al., 2002a,b; Threlfell et al., 2010; Threlfell and Cragg, 2011), we tested the hypothesis that THP enhances DA release by performing FSCV in dorsolateral striatum while bath applying 3–300 nM THP. This dose range was selected based on the affinities of THP for mAChRs (2–20 nM depending on the mAChR subtype(Bolden et al., 1992)), and on known THP receptor occupancy in humans (Shinotoh et al., 1994), which suggests that THP concentrations in brain are near-saturating for all mAChRs subtypes at the high doses used for the treatment of dystonia. A concentration of 300 nM THP achieves ~97–99% mAChR occupancy. THP dose-dependently increased DA release in both control and Dyt1 mice (Fig. 2A; two-way repeated measures ANOVA drug effect, $F_{1,10} = 14.44$, $p < .01$). There was also a significant genotype x dose interaction effect (two-way repeated measures ANOVA, $F_{4,20} = 5.26$, $p < .01$), whereby Dyt1 mice exhibited a significantly reduced response to THP than control mice at 100 nM and 300 nM ($p < .05$ and $p < .01$, respectively, Sidak multiple comparison test). There was no significant difference in the EC_{50} of THP between the genotypes (Student's *t*-test, $p > .05$). To determine if the potentiation of DA release was an extraneous off-target effect of THP, we conducted a FSCV dose-response experiment using the prototypical mAChR antagonist atropine (Fig. 2B). Atropine dose-dependently increased DA release in both genotypes (two-way repeated measures ANOVA effect of dose, $F_{5,20} = 59.78$, $p < .0001$), but was significantly less effective in Dyt1 mice than control mice (two-way repeated measures ANOVA genotype x dose interaction effect $F_{5,20} = 6.912$, $p < .001$) at 10 nM and 30 nM ($p < .05$ and $p < .01$, respectively, Sidak multiple comparison test), in agreement with the THP data. There was no significant difference in the EC_{50} of atropine between the genotypes (Student's *t*-test, $p > .05$). While THP is an effective treatment in DYT1 dystonia, L-DOPA and other dopaminergic agonists are seldom used (Schwarz and Bressman, 2009; Thenganatt and Jankovic, 2014). We predicted that, in contrast to THP, L-DOPA would not rescue the deficit in DA release given that DA synthesis and content are normal in DYT1 dystonia (Furukawa et al., 2000). To test this, mice were pretreated with 10 mg/kg L-DOPA

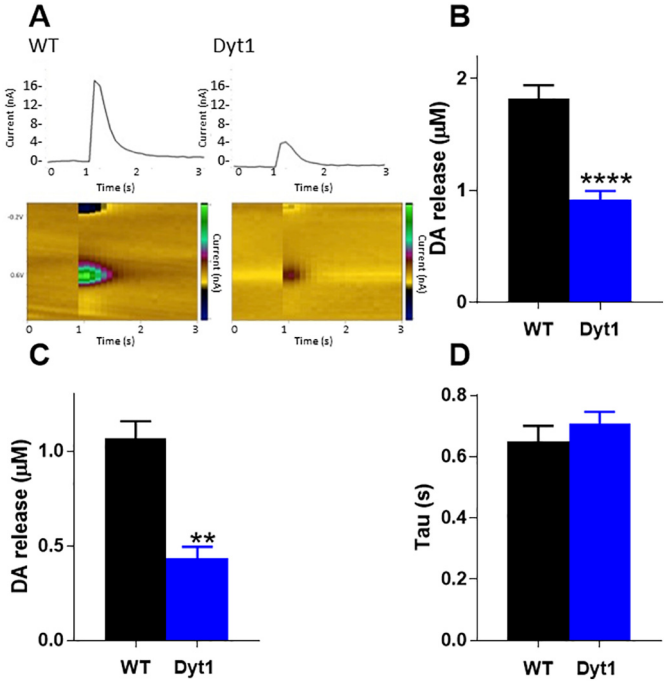

Fig. 1. DA release in Dyt1 mice. (A)Representative voltammograms and current traces of WT and Dyt1 DA release. 1-pulse electrical stimulation occurred at $t = 1$ s. (B) Striatal DA release is significantly reduced in Dyt1 mice as assessed by FSCV (WT $n = 15$; Dyt1 = 17). (C) DA release is significantly reduced in the nucleus accumbens core ($n = 3$). (D) There is no change in DA uptake (tau) between Dyt1 and control animals ($n = 5$). Values represent mean ± SEM.

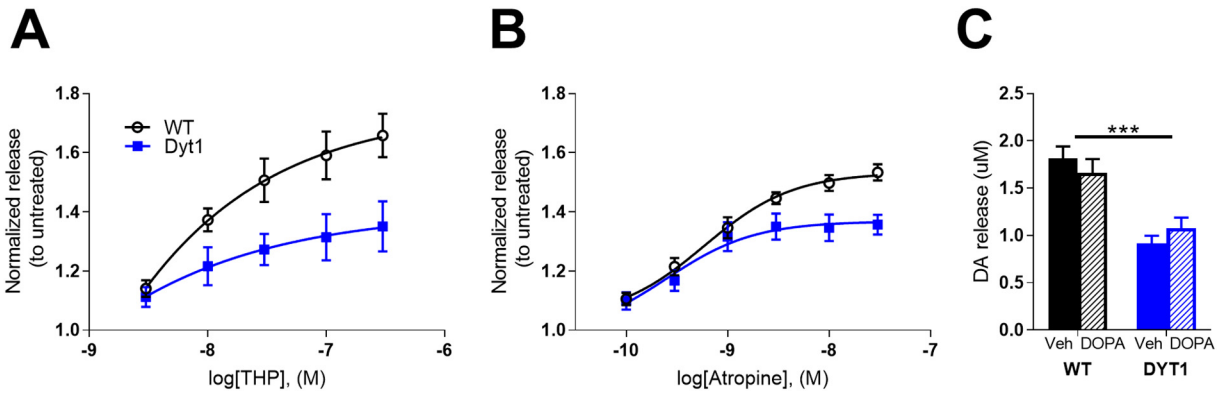

Fig. 2. Muscarinic antagonists increase DA release (A) Dose response curves of the effect of THP on DA release. THP dose-dependently increased DA release in WT and Dyt1 mice, but was less effective in Dyt1 (WT n = 6; Dyt1 n = 6). (B). Dose response curves of the effect of atropine, a prototypical mAChR antagonist, on DA release. Atropine significantly increased DA release, but was significantly less effective in Dyt1 mice (WT n = 5; Dyt1 n = 5). (C) Effect of L-DOPA on DA release. L-DOPA administration had no effect on DA release in Dyt1 or control mice (WT = 4; Dyt1 = 4). Values are normalized to untreated for each genotype and represent mean ± SEM.

with 5 mg/kg benserazide (s.c.) and then *ex vivo* FSCV was performed to assess DA release (Fig. 2C). 10 mg/kg L-DOPA was chosen because this dose is sufficient to induce L-DOPA induced dyskinesia in mice (Fieblinger et al., 2014; Bordia et al., 2015; Lim et al., 2015; Solis et al., 2017). Consistent with this hypothesis, L-DOPA had no significant effect on DA release in control or Dyt1 mice (two-way ANOVA treatment effect $F_{1,36} = 0.0012$, $p > .05$).

3.3. THP normalizes extracellular DA in Dyt1 mice

To test the DA-enhancing effects of THP *in vivo*, microdialysis was conducted in awake, behaving mice. THP was reverse dialyzed and extracellular DA was measured both pre- and post- treatment (Fig. 3A). Extracellular DA was significantly reduced at baseline in Dyt1 mice relative to controls (*Tor1a*$^{+/+}$, 3.07 ± 0.434 ng/mL, *Tor1a*$^{+/\Delta E}$, 1.88 ± 0.205 ng/mL; Student's *t*-test, $p < .05$), consistent with previous results (Song et al., 2012). THP significantly increased extracellular DA in both control and Dyt1 mice (two-way repeated measures ANOVA, main effect of treatment, $F_{1,14} = 38.02$, $p < .0001$). In fact, THP increased extracellular DA in Dyt1 mice to normal WT concentrations (Tukey's post-hoc, $p > .05$). Additionally, extracellular DA was measured using microdialysis after a single peripheral administration of THP (20 mg/kg i.p.). Previous studies demonstrated that this dose reduces the severity of the dystonia in a mouse model of DOPA-responsive dystonia, but does not reduce locomotor activity (Rose et al., 2015). Peripherally administered THP significantly increased extracellular DA (Fig. 3B); (two-way repeated measures ANOVA, main effect of treatment, $F_{1,16} = 7.003$, $p < .05$).

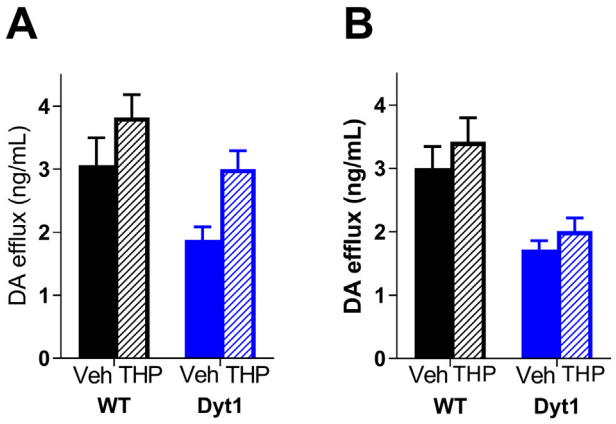

Fig. 3. THP enhances DA release and extracellular DA. (A) DA efflux after reverse dialysis of 300 nM THP. THP significantly increased extracellular DA in both WT and Dyt1 mice (WT n = 9; Dyt1 n = 7). (B) DA efflux after i.p. injection of 20 mg/kg THP. THP significantly increased DA release in both genotypes (WT n = 8; Dyt1 n = 10). Values represent mean ± SEM.

3.4. Functional nAChRs are required for the THP-induced increase in DA release

Current evidence suggests that mAChR agonists primarily influence DA release indirectly through ChIs. Activation of mAChR autoreceptors on ChIs reduces the release of ACh, thereby reducing the activation of nAChRs on DA terminals to decrease DA release (Rice and Cragg, 2004; Exley and Cragg, 2008; Threlfell et al., 2010; Threlfell and Cragg, 2011; Threlfell et al., 2012). Conversely, the mAChR antagonist THP may increase DA release indirectly through nAChRs. To test the hypothesis that THP requires functional nAChRs to enhance DA release, *ex vivo* FSCV was performed in the presence of THP (300 nM) while blocking nAChRs with the selective β2 subunit-containing nAChR antagonist DHβE (1 μM) (Harvey et al., 1996). β2 nAChR subunits are present in all nAChR on DA terminals in the dorsal striatum but absent from glutamatergic terminals in the striatum (Wonnacott et al., 2000; Exley et al., 2008; Zhang et al., 2009a,b). Further, DHβE is sufficient to completely block DA release induced by optogenetic stimulation of ChIs (Fieblinger et al., 2014). DHβE alone significantly decreased DA release in both control and Dyt1 mice (Fig. 4A) (two-way repeated measures ANOVA effect of treatment, $F_{3,30} = 77.29$, $p < .0001$; Sidak's post-hoc test, $p < .001$). DA release was significantly reduced in Dyt1 relative to control mice when nAChRs were blocked by DHβE ($p < .01$, Student's *t*-test). The THP-induced increase in DA release was abolished in the presence of DHβE (Sidak's post-hoc test, $p > .05$). However, THP significantly increased DA release after DHβE washout (Sidak's post-hoc test, $p < .001$). These results suggest that THP requires functional nAChR to exert its DA-enhancing effects.

3.5. The THP-induced increase in DA release is not dependent on glutamatergic signaling

Previous studies have shown that corticostriatal glutamatergic afferents drive DA release indirectly through excitation of ChIs (Kosillo et al., 2016). To determine if glutamate signaling is required for the THP-induced increase in DA release, we co-applied the non-specific glutamate receptor antagonist CNQX (10 μM) with THP (300 nM) (Fig. 4B). CNQX alone had no effect on DA release (Sidak's post-hoc test, $p > .05$). In the presence of CNQX, THP significantly increased DA release compared to baseline (two-way repeated measures ANOVA,

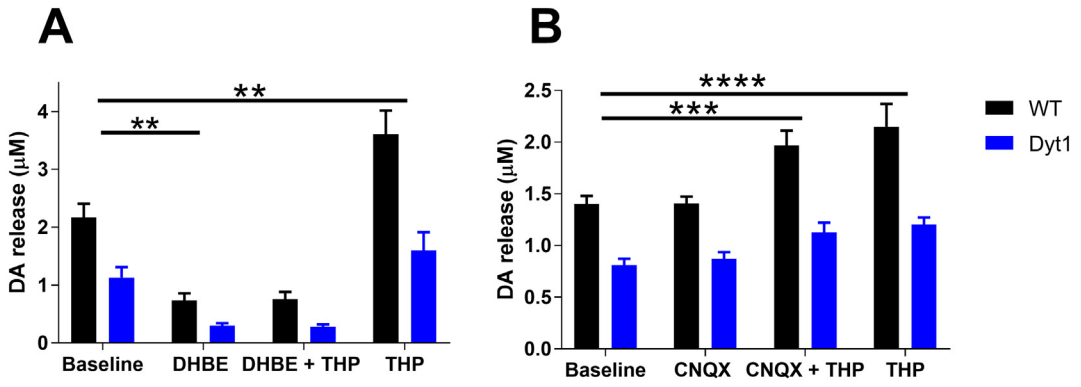

Fig. 4. Glutamate and ACh involvement in THP mechanism of action. (A) THP depends on nAChR to increase DA release. Blockade of nAChR with DHβE abolishes THP's DA enhancing effects (WT n = 6; Dyt1 n = 6). (B) THP does not depend on glutamate signaling to increase DA release. Blockade of glutamate receptors does not alter THP's DA-enhancing effect (WT *n* = 4; Dyt1 n = 4). Values represent mean [DA] ± SEM.

effect of treatment, $F_{3,18} = 29.91$, $p < .001$; Sidak's post-hoc test, $p < .001$). Application of THP alone after CNQX washout significantly increased DA release compared to baseline (Sidak's post-hoc test, $p < .0001$) and this increase was not significantly different from that observed with the combined CNQX plus THP treatment (Sidak's post-hoc test, $p > .05$). These results suggest that the increase in DA release induced by THP is not dependent on glutamatergic signaling.

3.6. Dyt1 mice are more sensitive to nAChR antagonists

Because nAChRs are necessary for the DA release-enhancing effect of THP and this effect is attenuated in Dyt1 knockin mice, we hypothesized that nAChR neurotransmission is abnormal in Dyt1 knockin mice. Therefore, a dose-response experiment using DHβE (1–100 nM) was performed. Dyt1 mice exhibited a leftward shift in the dose-response (Fig. 5A) as demonstrated by a significant reduction in the IC_{50} in Dyt1 mice compared to control mice (*Tor1a*$^{+/+}$, 29.5 ± 6.5 nM, *Tor1a*$^{+/\Delta E}$, 12.3 ± 4.3 nM, Student's *t*-test, $p < .05$) (Fig. 5A inset). The leftward shift in the antagonist DHβE dose-response curve implies that neurotransmission *via* nAChRs is functionally attenuated in Dyt1 mice. To test this hypothesis, dose-response experiments were conducted with two different AChE inhibitors, neostigmine and ambenonium. The increased extracellular ACh induced by these compounds results in the rapid desensitization of nAChRs, thereby reducing DA release (Zhang et al., 2004). A rightward shift in the dose-response curve for both neostigmine and ambenonium treatment was observed for Dyt1 mice compared to controls (Fig. 5B & C) with a significant increase in the IC_{50}s for both neostigmine (*Tor1a*$^{+/+}$, 308.9 ± 50.6 nM; *Tor1a*$^{+/\Delta E}$, 764.3 ± 42.7 nM; Student's *t*-test, $p < .001$) and ambenonium (*Tor1a*$^{+/+}$ 59.24 ± 8.11 nM; *Tor1a*$^{+/\Delta E}$, 142.6 ± 19.9 nM; Student's *t*-test, $p < .0001$) compared to controls. Because the attenuated response to the AChE inhibitors in Dyt1 mice could be due to differences in AChE activity in the striata of control *vs* Dyt1 mice, we measured AChE activity. AChE activity was not significantly different between Dyt1 and control animals (*Tor1a*$^{+/+}$, 50.05 ± 7.32 mU/mg protein, *Tor1a*$^{+/\Delta E}$, 36.99 ± 4.86 mU/mg protein, Student's *t*-test, $p > .05$) (Fig. 5D). These results are consistent with the hypothesis that nAChR neurotransmission is attenuated in Dyt1 mice.

Presynaptic nAChR act as a frequency filter on DA terminals such that, when nAChR are active, tonic (1-pulse stimulation) and burst (5-pulse, 100 Hz stimulation) firing yields similar levels of DA release. However, when nAChR are blocked or desensitized, DA release is significantly reduced in response to tonic stimulation compared to burst stimulation (Zhang et al., 2009a,b; Threlfell and Cragg, 2011). Therefore, if nAChR function itself is attenuated in Dyt1 mice, the response to tonic and burst firing should resemble the blocked nAChR state. In both Dyt1 and control mice, 1-pulse stimulations evoked levels of DA release that were comparable to 5-pulse stimulations (two-way repeated measures ANOVA effect of genotype, $F_{1,8} = 1.138$, $p > .05$). Blocking nAChRs with DHβE diminished DA release evoked by 1p stimulation, and the ratio between 5p and 1p evoked-release was not significantly different between Dyt1 and control mice (Student's *t*-test, $p > .05$), suggesting that nAChR function is intact in the context of this challenge (Fig. 5E). Because the attenuated nAChR function could be due to chronically desensitized receptors, DA release was assessed after exposure to a high dose of nicotine (500 nM), which causes rapid desensitization of nAChRs and a subsequent decrease in DA release (Rice and Cragg, 2004). Acute nicotine exposure caused a rapid decrease in DA release followed by a return to baseline DA release after ~20 mins (Fig. 5F) (two-way repeated measures drug effect $F_{9,81} = 105.6$, $p < .0001$). There was no significant difference in response between Dyt1 and control mice (two-way repeated measures ANOVA genotype effect $F_{1,9} = 3.908$, $p > .05$). Further, we examined midbrain nAChR mRNA expression using RT-qpCR (Fig. S1). We found no significant difference in the expression of α4, α6, α7, and β2 nAChR subunits between Dyt1 knockin and control mice (two-way ANOVA genotype effect $F_{1,40} = 3.961$, $p > .05$).

4. Discussion

Here, we demonstrate that THP, one of the few small molecule drugs available for the treatment of dystonia, rescued the deficit in striatal dopamine release in Dyt1 mice. Furthermore, we demonstrate that the DA-enhancing effect of THP is dependent on nAChR neurotransmission, which is attenuated in Dyt1 mice.

Reductions in DA neurotransmission are consistently observed in a variety of Dyt1 mouse models, including transgenic Dyt1 mice (Shashidharan et al., 2005; Zhao et al., 2008; Hewett et al., 2010) and knockin Dyt1 mice (Balcioglu et al., 2007; Song et al., 2012). Indeed, a similar diminution in striatal DA release was observed using FSCV in transgenic mice that overexpress torsinA (ΔE) specifically in TH+ neurons (Page et al., 2010). Our results demonstrate that the deficiency in DA release is not attributable to abnormal DA reuptake or abnormal firing rates because the deficit is observed with evoked release in coronal slices, where DA neuron cell bodies are absent. Further, DA release is reduced in the NAcc, suggesting that the dysfunction is not specific to striatal microcircuits or specific to motor control regions. Interestingly, this finding may explain why DYT1 patients experience higher incidences of risk-taking behaviors than healthy controls (Arkadir et al., 2016). Future studies are needed to determine if torsinA (ΔE) causes a global decrease in neurotransmitter release, or if abnormalities are confined to DA neurons. Indeed, a previous study demonstrated that hippocampal glutamatergic neurotransmission is reduced in Dyt1

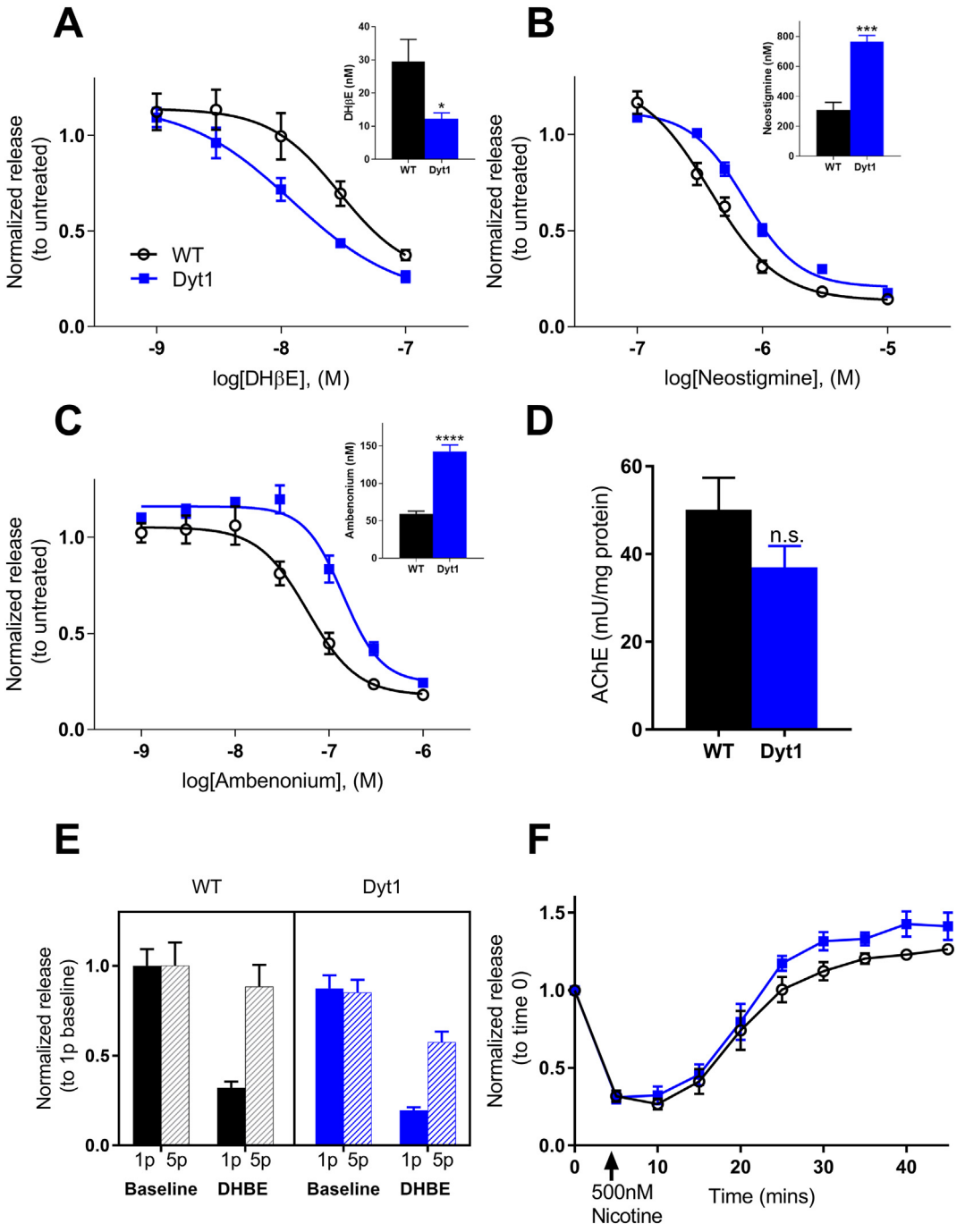

Fig. 5. Nicotinic receptor function and ACh tone is altered in Dyt1 mice. (A) The nAChR antagonist DHβE dose-dependently decreases DA release. The dose-response curve has a significant leftward shift for Dyt1 mice and the IC_{50} is significantly reduced for Dyt1 (A inset) (WT n = 6; Dyt1 n = 6). (B) Response to the AChE inhibitor neostigmine is altered in Dyt1 mice. There is a significant rightward shift in the dose-response curve for Dyt1 mice and the IC_{50} is significantly increased in Dyt1 mice (B inset) (WT n = 5; Dyt1 n = 5). (C) Response to the AChE inhibitor ambenonium is also altered in Dyt1 mice. There is a significant rightward shift in the dose-response curve for Dyt1 mice and the IC_{50} is significantly increased (C inset) (WT n = 5; Dyt1 n = 5). (D) AChE activity is unaltered between Dyt1 and control animals (WT n = 6; Dyt1 n = 6). (E) DA release in response to 1-pulse and 5-pulse electrical stimulation at baseline and after DHβE treatment. There is no significant difference in responses between genotypes (WT n = 5; Dyt1 n = 5). (F) Response to high concentration nicotine treatment is similar between WT and Dyt1 mice (WT n = 5; Dyt1 n = 5). Values are normalized to untreated for each genotype and represent mean ± SEM.

knockin mice (Yokoi et al., 2013).

Both THP and atropine dose-dependently increased *ex vivo* DA release suggesting that the DA-enhancing mechanism of action is likely at mAChRs rather than off-target effects. Importantly, direct infusion of THP into the striatum increased extracellular DA in Dyt1 mice to normal levels *in vivo* demonstrating that the site of action is the striatum. Further, the THP-induced increase in DA release was dependent on nAChRs suggesting THP increases DA release indirectly by modifying nAChR neurotransmission. In striatum, it is known that ACh release from ChIs is regulated by M_2 and M_4 mAChRs that inhibit ACh release (Zhang et al., 2002a,b; Threlfell et al., 2010). It is possible that THP acts through one or both of these receptor subtypes. However, we cannot exclude potential effects of other mAChR subtypes or cell types. For example, THP may act directly at M1 and/or M4 mAChRs on

SPNs to enhance DA release *via* known retrograde endocannabinoid signaling between spiny projection neurons and DA neurons or *via* axon collaterals onto cholinergic interneurons (Alcantara et al., 2001; Tepper et al., 2008; Gonzales et al., 2013; Foster et al., 2016), which could indirectly modify DA release. Additional studies using mAChR subtype-selective and cell-type selective manipulations of cholinergic neurotransmission are needed to elucidate the precise mechanism.

Despite the strong association between DA and dystonia, dopaminergic drugs are not generally used for the treatment of the dystonias. The exception is DOPA-responsive dystonia (DRD), which is caused by a reduction in DA due to mutations in genes critical for DA synthesis. In DRD patients, L-DOPA, alleviates dystonia by restoring presynaptic DA concentrations and therefore activity-dependent DA release (Ichinose and Nagatsu, 1999). However, in the context of abnormal DA release despite normal striatal DA concentrations, as observed in Dyt1 knockin mice, L-DOPA did not overcome the release deficit to provide significant benefit whereas THP rescued DA release. Further, unlike direct-acting DA receptor drugs that obscure presynaptic DA neuron firing patterns, THP acts indirectly on activity-dependent DA release. However, it is likely that the therapeutic effects of THP are not confined to rescuing striatal DA. Previous studies have shown that Dyt1 mice have abnormal corticostriatal LTP (Martella et al., 2009; Martella et al., 2014; Maltese et al., 2018), and THP and the M_1 mAChR selective antagonist VU0255035 restored normal plasticity (Maltese et al., 2014), suggesting that a multipronged approach may be necessary for the treatment of dystonia.

While THP potentiated DA release, it was less effective in Dyt1 than normal mice. Because THP acts indirectly through nAChRs to increase DA release, abnormal nAChR or ChI function may underlie the reduced efficacy in Dyt1 mice. While nAChR regulation appeared normal in Dyt1 mice, as demonstrated by desensitization/resensitization to nicotine, response to tonic and phasic stimulation, and nAChR expression, Dyt1 mice exhibited an increased sensitivity to the nAChR antagonist DHβE. The simplest explanation for a leftward shift in an antagonist dose-response curve is a reduction in the competing ligand, in this case extracellular ACh. This hypothesis that extracellular ACh is reduced in Dyt1 mice is also supported by the rightward shift observed for the AChE inhibitors. In contrast, a previous study using microdialysis suggests extracellular ACh is increased in the striatum of Dyt1 mice using *in vivo* microdialysis. (Scarduzio et al., 2017) It is likely that the fundamental differences in the techniques particularly temporal resolution, stimulation parameters, and *in vivo vs ex vivo* approaches account for the discrepancies. It is also important to note that we did not directly assess surface expression of nAChR or changes in nAChR subunit composition, which are difficult to measure due to the dynamic moment-to-moment regulation of nAChRs, so we cannot exclude those factors as alternative explanations. Regardless, the reduction in DA release in Dyt1 knockin mice is not solely attributable to abnormal ACh tone because DA release in Dyt1 knockin mice was attenuated even after ACh neurotransmission was abolished by antagonists.

5. Conclusions

Taken together, our data suggest a potential mechanism of action for THP through the regulation of striatal DA and suggest nAChRs as a possible target for therapeutics. Indeed, two case reports found that transdermal nicotine patches or nicotine lozenges improved spastic dystonia, supporting the utility of either nAChR agonists or, more likely, nAChR positive allosteric modulators in dystonia (Lees, 1984; Vaughan et al., 1997). Future studies are needed to assess the contribution of specific mAChR subtypes to the therapeutic effects of THP to facilitate the discovery of more efficacious therapeutics with fewer side effects.

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Author contribution

A.M.D., E.J.H, & H.A.J conceived of project. A.M.D. & E.J.H designed experiments. A.M.D., X.F., & Y.D performed experiments. A.M.D. wrote the first draft. All authors reviewed the manuscript.

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