BLOCKADE OF M4 MUSCARINIC RECEPTORS ON STRIATAL CHOLINERGIC INTERNEURONS NORMALIZES STRIATAL DOPAMINE RELEASE IN A MOUSE MODEL OF *DYT1-TOR1A* DYSTONIA

Abbreviated title: M4 muscarinic antagonists in *DYT1-TOR1A* dystonia

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ABSTRACT

Trihexyphenidyl (THP), a non-selective muscarinic receptor (mAChR) antagonist, is the preferred oral pharmaceutical for the treatment of *DYT1-TOR1A* dystonia. A better understanding of the mechanism of action of THP is a critical step in the development of better therapeutics with fewer side effects. Using a mouse model of *DYT1-TOR1A* dystonia (*Tor1a*$^{+/\Delta E}$ KI mice), we recently found that THP normalized striatal DA release, revealing a plausible mechanism of action for this compound. However, the exact mAChR subtypes that mediate this effect remain unclear. In this study we used a combination of a newly developed M4 subtype-selective mAChR antagonist and cell-type specific mAChR KO mice to determine which mAChR subtypes mediate the DA enhancing effects of THP. We determined that THP and the M4 subtype-selective mAChR antagonist enhance striatal DA release by blocking M4 mAChR on striatal cholinergic interneurons in *Tor1a*$^{+/\Delta E}$ KI mice. However, in *Tor1a*$^{+/+}$ mice THP increases striatal DA release through a combination of M1 and M4 mAChR, which reveals an alteration in M1 mAChR function in *Tor1a*$^{+/\Delta E}$ KI mice. Taken together these data implicate a principal role for M4 mAChR located on striatal cholinergic interneurons in the mechanism of action of THP and suggest that M4-subtype selective mAChR antagonists may be more efficacious therapeutics for *DYT1-TOR1A* dystonia.

SIGNIFICANCE STATEMENT

Trihexyphenidyl, a non-selective muscarinic receptor antagonist, is the preferred oral therapeutic for *DYT1-TOR1A* 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 therapeutics. We recently found that trihexyphenidyl rescues the deficit in both striatal dopamine release and steady-state extracellular striatal dopamine concentrations in a mouse model of *DYT1-TOR1A* dystonia. However, the precise muscarinic receptor subtype(s) that mediate these effects are unknown. We used a newly developed M4 muscarinic receptor subtype-selective antagonist along with M1 and M4 muscarinic receptor knockout mice to determine the precise muscarinic receptor subtypes that mediate the dopamine-enhancing effects of trihexyphenidyl.

INTRODUCTION

DYT1-TOR1A dystonia is an inherited form of dystonia caused by a three base pair deletion (Δgag) in the *TOR1A* gene (Ozelius et al., 1992; Ozelius et al., 1997). Because the precise mechanism(s) that lead to the abnormal movements are unknown, treatments for *DYT1-TOR1A* dystonia are inadequate. The non-selective muscarinic acetylcholine receptor (mAChR) antagonist trihexyphenidyl (THP) is the preferred oral pharmaceutical for *DYT1-TOR1A*, but its clinical use is limited due to significant dose-limiting side effects associated with its nonselective action at all five mAChRs subtypes (Schwarz and Bressman, 2009; Thenganatt and Jankovic, 2014). These side effects include cognitive impairment, constipation, dry mouth, urinary retention, and others (Burke et al., 1986; Jabbari et al., 1989; Guthrie et al., 2000; Lumsden et al., 2016). A better understanding of the mAChR subtype(s) responsible for the therapeutic effect of THP is critical for the development of targeted treatments with fewer side effects.

One potential mechanism of action of THP is the regulation of striatal dopamine (DA) neurotransmission. Abnormal striatal DA neurotransmission is consistently observed in both *DYT1-TOR1A* patients and mouse models of *DYT1-TOR1A* dystonia. In *DYT1-TOR1A* patients, brain DA metabolites are abnormal and D2 DA receptor availability is reduced (Augood et al., 2004; Asanuma et al., 2005). In mouse models of *DYT1-TOR1A,* striatal DA release is reduced despite normal total tissue DA concentrations (Balcioglu et al., 2007; Page et al., 2010; Song et al., 2012; Downs et al., 2019). Striatal DA release is regulated by acetylcholine (ACh) (Zhang et al., 2002b; Zhang et al., 2002a; Exley and Cragg, 2008; Zhang et al., 2009a; Zhang et al., 2009b; Threlfell et al., 2010) and we recently discovered that THP rescues the deficit in both evoked DA release and steady-state extracellular DA concentrations in the striatum of a mouse knockin (KI) model of *DYT1-TOR1A* (*Tor1a*$^{+/\Delta E}$ KI mice) (Downs et al., 2019). However, the mAChR subtype(s) and striatal cell type that mediate the effects of THP are unknown.

THP binds to all 5 mAChRs at nanomolar affinity (Dorje et al., 1991; Bolden et al., 1992), and all 5 mAChRs are expressed in the striatum (Buckley et al., 1988; Levey et al., 1991; Harrison et al., 1996; Alcantara et al., 2001; Yamada et al., 2003; Hernandez-Flores et al., 2015). M1 and M4 mAChRs are the most highly expressed subtypes in the striatum (Levey, 1993). M1 mAChRs are expressed on both direct and indirect striatal projection neurons (dSPNs and iSPNs) where they mediate corticostriatal plasticity (Harrison et al., 1996; Hernandez-Flores et al., 2015). M1 mAChRs also mediate extracellular DA concentrations, although the underlying mechanisms are unknown (Gerber et al., 2001). M4 mAChRs are expressed on dSPNs where they regulate corticostriatal plasticity (Nair et al., 2015; Nair et al., 2019) and mediate striatal DA release via endocannabinoid signaling (Foster et al., 2016). Additionally, M4 mAChRs expressed on cholinergic interneurons (ChIs) act as inhibitory autoreceptors to modulate extracellular ACh concentrations, which play a central role in mediating DA release (Threlfell et al., 2010; Pancani et al., 2014). Thus, THP may act through M1 or M4 mAChRs or both to enhance DA release. In this study, we used a combination of a newly developed subtype-selective M4 mAChR antagonist and cell-type specific mAChR knockout (KO) mice to determine the role of M1 and M4 mAChRs in the potentiation of DA release by THP in a KI mouse model of *DYT1-TOR1A* (*Tor1a*$^{+/\Delta E}$). We found that M4 mAChRs on ChIs mediate the THP-induced increase in striatal DA release in both control (*Tor1a*$^{+/+}$) and *Tor1a*$^{+/\Delta E}$ mice. M1 mAChRs also contribute to the DA-enhancing effects of THP in *Tor1a*$^{+/+}$ mice, but not in *Tor1a*$^{+/\Delta E}$ mice. Our results pinpoint a specific cell type and mAChR subtype underlying the effects of THP and also reveal a novel abnormal cholinergic signaling mechanism in *Tor1a*$^{+/\Delta E}$ mice.

MATERIALS AND METHODS

Animals

Male and female mice (8-14 weeks of age) inbred on C57BL/6J were used for all studies. All mice were bred at Emory University. Animals were maintained on a 12h light/dark cycle and

allowed *ad libitum* access to food and water. All experimental procedures were approved by the Emory Animal Care and Use Committee and followed guidelines set forth in the *Guide for the Care and Use of Laboratory Animals.* Heterozygous knockin mice carrying the *Tor1a(Δgag)* mutation (*Tor1a*$^{+/\Delta E}$) (Goodchild et al., 2005) and normal littermates (*Tor1a*$^{+/+}$) were genotyped using PCR (forward primer 5'-GCTATGGAAGCTCTAGTTGG-3'; reverse primer 5'-CAGCCAGGGCTAAACAGAG-3'). The following strains were used for the conditional mAChR KO mice experiments: M4 mAChR flox (M4$^{flox/flox}$) (Jeon et al., 2010), M1 mAChR flox (M1$^{flox/flox}$) (Kamsler et al., 2010), *ChAT-cre* (ChAT$^{tm1(cre)/Lowl}$/MwarJ; JAX #031661), *D1-cre* (Tg(Drd1-cre)^{EY262Gsat}/Mmucd; MMRRC #030989-UCD), and *A2A-cre* (Tg(Adora2a-cre)^{KG139Gsat}/Mmucd; MMRRC #036158-UCD). These strains were used to generate both *Tor1a*$^{+/+}$ and *Tor1a*$^{+/\Delta E}$ mice with conditional KO of M4 mAChR from cholinergic neurons (ChAT-cre; M4$^{flox/flox}$), KO of M4 mAChR from dSPNs (D1-cre; M4$^{flox/flox}$), KO of M1 mAChR from dSPNs (D1-cre; M1$^{flox/flox}$), and KO of M1 from iSPNs (A2A-cre; M1$^{flox/flox}$). Mice were genotyped using PCR with the following primers *D1-cre* (forward primer 5'- GCTATGGAGATGCTCCTGATGGAA-3'; reverse primer 5'-CGGCAAACGGACAGAAGCATT -3'); *A2A-cre* (forward primer 5'-CGTGAGAAAGCCTTTGGGAAGCT-3'; reverse primer 5'- CGGCAAACGGACAGAAGCATT-3'); *ChAT-cre* (WT forward primer, 5'-GCAAAGAGACCTCATCTGTGGA-3'; cre forward primer, 5'-TTCACTGCATTCTAGTTGTGGT-3'; common reverse primer, 5'-GATAGGGGAGCAGCACACAG-3'); *M1 flox* (WT forward primer 5'-GAGCCTCAGTTTTCTCATTGG-3'; mutant forward primer 5'- AACACTACTTACACGTGGTGC-3'; common reverse primer 5'- TCAACCTGTACTGGTGATACG-3'); *M4 flox* (forward primer 5'-TGCAGATGTAGCTCAGCTCAGCGGTAC-3'; reverse primer 5'-TGAAGGTTGTAGACAAAGCTATACACATGGC-3').

Fluorescent in situ hybridization

Brains were rapidly dissected, frozen and kept at -80°C until sectioning (10 µm). RNAscope fluorescent *in situ* hybridization was performed according to manufacturer's instructions (Advanced Cell Diagnostics, Newark, CA, USA). Briefly, slides were fixed in ice-cold 4% paraformaldehyde in 0.1M PBS for 15 mins. Slides were then dehydrated in graded ethanols and allowed to air dry for 5 minutes. Slides were treated with Protease-IV solution for 30 mins at room temperature and then incubated with the appropriate probes for 2 hours at 40 °C. The following probes were used: Mm-*Chat*-C1 (cat # 408731), Mm-*Chrm4*-C2 (cat # 410581-C2), Mm-*Drd1*-C3 (cat # 461901-C3), Mm-*Chrm1*-C1 (cat # 495291), Mm-*Adora2a*-C2 (cat # 409431-C2). A series of four amplification steps were then performed according to manufacturer's instructions with 2 X 2 min washes in wash buffer between each amplification step. Slides were counterstained with DAPI and then coverslipped using Prolong Glass Antifade Mounting media (Thermo Fisher Scientific, Waltham, MA, USA). Slides were stored at 4°C until imaging.

Slides were imaged using a Leica SP8 confocal microscope using Leica Acquisition Suite (LAX S) (Leica Microsystems, Buffalo Grove, IL, USA). A total of 6 regions of interest (ROIs) from the dorsolateral quadrant of the striatum were taken from each section using a 40X or 60X objective and an optical thickness of ~5 µm. The same optical settings were used for image acquisition from each mouse with the settings titrated for each specific probe. Quantification of labeled cells were performed using the Cell Counter function in ImageJ (NIH). Cells were considered positive for a given probe if there were >5 particles clustered around the nucleus.

Fast scan cyclic voltammetry

Fast scan cyclic voltammetry (FSCV) to measure DA release was performed according to previously published methods (Downs et al., 2019). Mice were euthanized using cervical dislocation and 300 µm sections were prepared using a vibratome in ice-cold oxygenated sucrose-supplemented 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. After sectioning, brain slices were maintained 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.

All FSCV experiments were performed in the dorsolateral striatum at ~ Bregma +0.26 mm because this region receives dense innervation from the motor cortex (Hintiryan et al., 2016). A slice was transferred to the recording chamber and perfused with oxygenated aCSF at 32°C for 30 min to equilibrate to the chamber. A carbon fiber electrode constructed in-house was inserted approximately 50 µm into the surface of the slice and a bipolar tungsten stimulating electrode was placed approximately 200 µm away. DA release was evoked by a 1-pulse (600 µA, 4 ms pulse width) electrical stimulation at 5 min inter-stimulus intervals to prevent 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). FSCV experiments were conducted and analyzed using Demon voltammetry software (Wake Forest University) (Yorgason et al., 2011) to determine peak DA release. All compounds were diluted in aCSF at the time of recording and bath applied for 10-20 mins before recordings commenced to allow for equilibration. All electrodes were calibrated to known DA standards in aCSF using a custom-made flow cell.

Compounds

THP was purchased from Sigma-Aldrich (St. Louis, MO). Dihydro-β-erythroidine (DHβE) was obtained from Tocris (Minneapolis, MN). VU6021625 was generously provided by Dr. Jeffrey Conn (Vanderbilt University, TN).

Statistical analysis

All data are presented as means with standard error. Dose response experiments were analyzed with non-linear regression to determine EC_{50}. EC_{50} was analyzed using a two-tailed Student's *t*-test. Fluorescent *in situ* hybridization data was analyzed using one-way ANOVA with *post hoc* Dunnett's multiple comparison test. Experiments assessing DA release were analyzed using 2-way repeated measures ANOVA (genotype x treatment) with *post hoc* Sidak's multiple comparison test. Analyses were performed separately for *Tor1a*$^{+/+}$ and *Tor1a*$^{+/\Delta E}$ in mAChR KO experiments. All analyses were performed using Graphpad Prism 9 (https://www.graphpad.com/). Statistical significance is defined as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

RESULTS

M4 mAChRs in striatal cholinergic cells are necessary for the effects of THP

We have previously demonstrated that the non-selective mAChR antagonist THP enhances DA release in both *Tor1a*$^{+/+}$ and *Tor1a*$^{+/\Delta E}$ KI mice and rescues the deficiency in DA release in *Tor1a*$^{+/\Delta E}$ KI mice (Downs et al., 2019). However, the mAChR subtype(s) and cell types that mediate this effect are unknown. M4 mAChRs expressed on dSPNs, ChIs, and cortical glutamatergic terminals directly or indirectly regulate striatal DA release (Harrison et al., 1996; Threlfell et al., 2010; Threlfell and Cragg, 2011; Pancani et al., 2014). Therefore, to determine if THP depends on M4 mAChRs on any of these cell types, we assessed striatal DA release in response to challenge with THP in *Tor1a*$^{+/+}$ and *Tor1a*$^{+/\Delta E}$ KI mice lacking M4 mAChRs on ChIs (ChI-M4$^{-/-}$) or M4 mAChRs on dSPNs (dSPN-M4$^{-/-}$). Deletion of M4 mAChRs from cortical glutamatergic inputs was not investigated because we have previously demonstrated that blocking ionotropic glutamate receptors does not affect the ability of THP to enhance DA release (Downs et al., 2019).

Previous studies have demonstrated that M4 mAChRs on dSPNs regulate striatal DA release by modulating endocannabinoid production (Foster et al., 2016). To test the hypothesis that THP enhances DA release by acting on dSPNs, we measured electrically evoked DA release

in striatal slices from mice lacking M4 mAChRs in direct SPNs (dSPN-M4$^{-/-}$) in the presence of THP (300 nM). The dSPN-M4 KO had no effect on baseline DA release in either *Tor1a*$^{+/+}$ mice (Sidak's multiple comparison test, p = 0.98) or *Tor1a*$^{+/\Delta E}$ (Sidak's multiple comparison test, p = 0.92). Deleting M4 mAChR expression from dSPNs had no effect on the THP-induced increase in DA release in either *Tor1a*$^{+/+}$ mice (two-way repeated measures ANOVA main effect of treatment, $F_{1,9}$ = 208.2, p < 0.0001; treatment x genotype interaction effect, $F_{1,9}$= 2.92, p = 0.12) or *Tor1a*$^{+/\Delta E}$ mice (**Fig 1A**, two-way repeated measures ANOVA main effect of treatment, $F_{1,9}$ = 17.52, p = 0.0024; treatment x genotype interaction effect, $F_{1,9}$ = 0.038, p = 0.848).

To verify that the M4 mAChR conditional knockout in dSPNs was both effective and cell-type specific, we used fluorescent *in situ* hybridization with probes to *Chrm4*, *Drd1* and *ChAT* to assess striatal mRNA expression. *Chrm4* encodes the M4 mAChR. *Drd1* encodes the D1 DA receptor which is a marker for dSPNs and *ChAT* encodes choline acetyltransferase, a marker for ChIs. In control mice, *Chrm4* mRNA was expressed in all *ChAT*-positive cells and almost all *Drd1*-positive cells. In dSPN-M4$^{-/-}$ mice, there was a significant reduction in the percent of cells expressing both *Chrm4* and *Drd1* mRNA to ~13% of control mice while there was a corresponding increase in the percent of cells that expressed only *Drd1* without *Chrm4* (**Fig 1C vs D & Table 1,** one-way ANVOA $F_{2,6}$ = 554.7, p < 0.0001). There was no change in *Chrm4* mRNA expression in *Chat*-positive striatal ChIs in dSPN-M4$^{-/-}$ compared to control mice. These results validate the cell-type specific conditional knockout of M4 mAChRs in dSPNs but not ChIs.

M4 mAChRs are expressed on ChIs where they act as inhibitory autoreceptors. It is known that activation of these autoreceptors results in a reduction in DA release via a decrease in nAChR activation on DA terminals (Threlfell et al., 2010). To test the hypothesis that THP exerts its effect by blocking M4 mAChRs expressed on ChIs, we assessed DA release in striatal slices from mice lacking M4 mAChRs in ChIs (ChI-M4$^{-/-}$). KO of the M4 mAChR from ChIs had no effect on baseline DA release in either *Tor1a*$^{+/+}$ (Sidak's multiple comparison test, p = 0.81) or *Tor1a*$^{+/\Delta E}$ mice

(Sidak's multiple comparison test, p = 0.65). While THP increased DA release in both *Tor1a*$^{+/+}$ and *Tor1a*$^{+/+}$; ChI-M4$^{-/-}$ mice (**Fig 1B,** two-way repeated measures ANOVA main effect of treatment $F_{1,10}$ = 119.7, p < 0.0001), the effect of THP was significantly attenuated in *Tor1a*$^{+/+}$; ChI-M4$^{-/-}$ mice to 65% of *Tor1a*$^{+/+}$ mice (two-way repeated measures ANOVA treatment x genotype interaction effect, $F_{1,10}$ = 24.31, p = 0.0006, Sidak's multiple comparison test, p = 0.35). In *Tor1a*$^{+/\Delta E}$ mice, THP significantly increased DA release (two-way repeated measures ANOVA, main effect of treatment, $F_{1,10}$ = 10.58, p = 0.0087; treatment x genotype interaction effect $F_{1,10}$ = 2.89, p = 0.12). However, THP did not significantly enhance DA release in *Tor1a*$^{+/\Delta E}$; ChI-M4$^{-/-}$ mice (Sidak's multiple comparisons test, p = 0.51). These results suggest that M4 mAChRs on ChIs mediate the effects of THP in *Tor1a*$^{+/\Delta E}$ KI mice. However, in *Tor1a*$^{+/+}$ mice, the effects of THP are mediated only in part by M4 mAChR on ChIs.

Fluorescent *in situ* hybridization with probes to *Chrm4*, *Drd1* and *ChAT* was used to verify that the M4 mAChR conditional knockout in ChIs was accomplished. In control mice, *Chrm4* mRNA was detected in all *ChAT*-positive cells. However, in ChI-M4$^{-/-}$ mice, *Chrm4* mRNA expression was not detected in any *ChAT*-positive cells (**Fig 1C vs E & Table 1,** one-way ANVOA $F_{2,6}$ = 18.68, p = 0.0002). There was no change in *Chrm4* mRNA expression in *Drd1*-positive cells in ChI-M4$^{-/-}$ compared to control mice. Thus, the conditional *Chrm4* deletion targeted to ChIs was cell-type specific and highly effective.

A selective M4 mAChR antagonist is sufficient to recapitulate the effects of THP

The cell-type selective KO experiments suggest that M4 mAChRs are necessary for the DA release-enhancing effects of THP in *Tor1a*$^{+/\Delta E}$ KI mice. However, because THP acts at all five mAChRs, it is not clear if selective blockade of M4 mAChRs would rescue DA release in *Tor1a*$^{+/\Delta E}$ KI mice similar to the effects of THP. Therefore, we assessed DA release in the presence of the subtype-selective M4 mAChR antagonist VU6021625, which was shown to be effective in ameliorating the dystonia exhibited by a mouse model of DOPA-response dystonia (Moehle et

al., 2020). VU6021625 dose-dependently enhanced DA release in both *Tor1a*$^{+/+}$ and *Tor1a*$^{+/\Delta E}$ KI mice (**Fig 2A**). There was no significant difference in EC_{50} between genotypes (Student's *t*-test, p = 0.87). DA release was significantly increased in both *Tor1a*$^{+/+}$ and *Tor1a*$^{+/\Delta E}$ KI mice at the maximal concentration of 300nM VU6021625 (**Fig 2B**, two-way repeated measures ANOVA, main effect of treatment, $F_{1,8}$ = 30.19, p < 0.0006). VU6021625 was similarly effective in both *Tor1a*$^{+/+}$ and *Tor1a*$^{+/\Delta E}$ KI mice (two-way repeated measures ANOVA genotype x treatment interaction effect, $F_{1,8}$ = 1.376, p = 0.27). Importantly, VU6021625 normalized DA release in *Tor1a*$^{+/\Delta E}$ KI mice to *Tor1a*$^{+/+}$ levels (Sidak's multiple comparison's test, p = 0.58).

Blockade of M4 mAChRs in ChIs but not dSPNs recapitulates the effects of THP

To determine the specific cell type(s) that mediate the effects of the M4 mAChR subtype-selective antagonist, we assessed DA release in response to VU6021625 (300 nM) in dSPN-M4$^{-/-}$ mice. Deleting M4 mAChR expression from dSPNs had no effect on the VU6021625-induced increase in DA release in *Tor1a*$^{+/+}$ (two-way repeated measures ANOVA main effect of treatment $F_{1,8}$ = 36.15, p = 0.0003; treatment x genotype interaction effect, $F_{1,8}$= 0.00024, p = 0.99) or *Tor1a*$^{+/\Delta E}$ dSPN-M4$^{-/-}$ mice **(Fig 3A**, two-way repeated measures ANOVA main effect of treatment F1,8 = 242.2, p < 0.0001; treatment x genotype interaction effect, $F_{1,8}$ = 0.578, p = 0.47).

Next, we determined the role of M4 mAChR expressed on ChIs in mediating the increase in DA release in response to VU6021625 treatment. KO of M4 mAChR from ChIs eliminated the DA enhancing effect of VU6021625 in *Tor1a*$^{+/+}$ mice (**Fig 3B**, two-way repeated measures ANOVA, treatment x genotype interaction effect, $F_{1,9}$ = 9.81, p = 0.012). KO of M4 mAChR from cholinergic neurons also eliminated the DA enhancing effect of VU6021625 in *Tor1a*$^{+/\Delta E}$ KI mice (two-way repeated measures ANOVA, treatment x genotype interaction effect, $F_{1,9}$ = 51.46, p < 0.0001). These data suggest that the M4 subtype selective mAChR antagonist enhances DA release by blocking M4 mAChR on ChIs in both *Tor1a*$^{+/+}$ and *Tor1a*$^{+/\Delta E}$ KI mice.

M1 mAChRs on dSPNs and iSPNs are required for the effects of THP in Tor1a$^{+/+}$ but not Tor1a$^{+/\Delta E}$ KI mice

M1 mAChRs are expressed on both direct and indirect SPNs (Harrison et al., 1996; Hernandez-Flores et al., 2015). Previous studies have demonstrated that M1 mAChR regulates extracellular DA in mice (Gerber et al., 2001). Although the mechanism mediating this effect is largely unknown, it is known that SPNs provide GABAergic input to ChIs (Tepper et al., 2008). It is possible that THP mediates DA release indirectly by blocking excitatory M1 mAChRs on SPNs to disinhibit ChI activity thereby increasing ACh release and nAChR activity on DA terminals. To determine if M1 mAChRs on either direct or indirect SPNs are required for the DA release-enhancing effects of THP, we assessed striatal DA release in response to challenge with THP in *Tor1a*$^{+/+}$ and *Tor1a*$^{+/\Delta E}$ KI mice lacking M1 mAChR on dSPNs (dSPN-M1$^{-/-}$) or iSPNs (iSPN-M1$^{-/-}$).

In *Tor1a*$^{+/+}$ mice lacking M1 mAChR in dSPNs, the increase in DA release in response to THP was diminished compared to control animals (**Fig 4A,** two-way repeated measures ANOVA treatment x genotype interaction effect, $F_{1,10} = 8.226$, $p = 0.016$). However, THP still significantly increased DA release in *Tor1a*$^{+/+}$ dSPN-M1$^{-/-}$ mice (two-way repeated measures ANOVA treatment effect, $F_{1,10} = 109.1$, $p < 0.0001$, Sidak multiple comparisons test, $p = 0.0006$). In contrast, KO of M1 mAChR from dSPNs did not affect the response to THP in *Tor1a*$^{+/\Delta E}$ KI mice (two-way repeated measures ANOVA treatment x genotype interaction effect $F_{1,10} = 0.128$, $p = 0.73$). There were no differences observed in baseline DA release in either *Tor1a*$^{+/+}$ (Sidak's multiple comparison test, $p = 0.90$) or *Tor1a*$^{+/\Delta E}$ (Sidak's multiple comparison test, $p = 0.83$) due to dSPN-M1 KO.

In *Tor1a*$^{+/+}$ mice lacking M1 mAChR in iSPNs, THP significantly increased DA release, but the effect was significantly diminished compared to *Tor1a*$^{+/+}$, similar to *Tor1a*$^{+/+}$ dSPN-M1$^{-/-}$ mice (**Fig 4B,** two-way repeated measures ANOVA treatment x genotype interaction effect, $F_{1,10} = 9.455$, $p = 0.012$, Sidak multiple comparisons test, $p = 0.0003$). In contrast, the effect of THP in

Tor1a$^{+/\Delta E}$ iSPN-M1$^{-/-}$ mice did not differ from *Tor1a*$^{+/\Delta E}$ mice (two-way repeated measures ANOVA treatment x genotype interaction effect $F_{1,10} = 0.6647$, $p = 0.43$). Deletion of M1 mAChRs from iSPNs had no effect on baseline release in either *Tor1a*$^{+/+}$ (Sidak's multiple comparison test, $p = 0.89$) or *Tor1a*$^{+/\Delta E}$ (Sidak's multiple comparison test, $p = 0.92$). These results demonstrate that M1 mAChRs contribute to the effects of THP on DA release in *Tor1a*$^{+/+}$ but not *Tor1a*$^{+/\Delta E}$ KI mice.

To confirm that the SPN cell-type specific KO of M1 mAChRs were effective, we used fluorescent *in situ* hybridization with probes specific to *Chrm1*, *Adora2a*, and *Drd1*. *Chrm1* encodes the M1 mAChR receptor. *Adora2a* encodes the A2A adenosine receptor and is a specific marker for iSPNs. *Drd1* is a specific marker for dSPNs. In control mice, *Chrm1* was expressed in almost all *Adora2a*-positive and *Drd1*-positive cells. However, in dSPN-M1$^{-/-}$ mice, the percentage of cells expressing both *Chrm1* and *Drd1* mRNA was significantly reduced to ~2% of control levels (**Fig 4C vs D & Table 2,** one-way ANVOA $F_{2,6} = 90.44$, $p < 0.0001$) while the percentage of cells expressing both *Chrm1* and *Adora2a* was unchanged. In iSPN-M1$^{-/-}$mice, the percentage of cells expressing both *Chrm1* and *Adora2a* mRNA was significantly reduced to ~5% of control levels (**Fig 4C vs E & Table 2,** one-way ANOVA $F_{2,6} = 57.37$, $p < 0.0001$), while the percentage of cells expressing both *Chrm1* and *Drd1* mRNA was unchanged. These results demonstrate that the M1 mAChR knockouts were specific and effective.

DISCUSSION

THP is the only oral medication to be proven effective in a double-blind placebo-controlled trial for dystonia (Fahn, 1983; Burke et al., 1986). Unfortunately, most patients cannot use THP due to intolerable side-effects at the high doses necessary for efficacy in dystonia (Schwarz and Bressman, 2009; Thenganatt and Jankovic, 2014). Here we used THP as a platform to further understand the mechanism of action of mAChR antagonists and identify specific mAChR subtypes that may serve as targets for more efficacious therapeutics with fewer side effects. We demonstrated that M4 mAChR located on striatal ChIs mediate the THP-induced increase in striatal DA release. Furthermore, we described a role for M1 mAChR in the increase in striatal DA release caused by THP in *Tor1a*$^{+/+}$ mice, but not in *Tor1a*$^{+/\Delta E}$ KI mice.

Our results demonstrate that M4 mAChRs expressed on ChIs are both necessary and sufficient for the THP-induced rescue of DA release in *Tor1a*$^{+/\Delta E}$ KI mice. KO of M4 mAChR from ChIs, but not dSPNs, abolished the increase in DA release caused by THP in *Tor1a*$^{+/\Delta E}$ KI mice, while a selective M4 mAChR antagonist recapitulated the effect of THP. KO of M4 mAChR from ChIs abolished the ability of the M4 subtype selective mAChR antagonist VU6021625 to enhance DA release in both *Tor1a*$^{+/+}$ and *Tor1a*$^{+/\Delta E}$ KI mice, which demonstrates the receptor specificity of the compound. The results from the antagonist experiments are also notable because gene deletion experiments can be confounded by compensatory developmental changes in response to the knockout. The consistency of our results across both the M4 mAChR antagonist challenge in genetically unaltered adult mice and the conditional knockout experiments suggests that developmental confounds do not likely account for the results.

While our results implicate M4 mAChRs on ChIs in the action of THP, the conditional knockout used here was not specific to ChIs and it is likely that M4 mAChR expression was deleted from other cholinergic projections to the striatum, including projections from the pedunculopontine nucleus and laterodorsal tegmentum (Dautan et al., 2016). However, a recent

study found these brainstem cholinergic projections do not play a role in mediating striatal DA release in this *ex vivo* slice preparation (Brimblecombe et al., 2018). Further, both M2 and M4 mAChRs act as autoreceptors on ChIs to mediate DA release (Smiley et al., 1999; Threlfell et al., 2010). While we did not specifically address the contribution of M2 mAChRs, the near total abolition of DA release enhancement by THP after M4 mAChR KO from ChIs in *Tor1a*$^{+/\Delta E}$ KI mice suggests that M2 mAChRs play a minor role, if any. Thus, while other cell types and receptors may play a role in the mechanism of action of THP in DA release, our results suggest that M4 mAChRs on ChIs play a dominant role.

The role of ChIs in the mechanism of action of THP is consistent with the considerable evidence implicating ChI dysfunction in *DYT1-TOR1A* dystonia. Normally, D2R activation inhibits ChI firing rates, causing a reduction in extracellular ACh. In contrast, in *Tor1a(ΔE)* rodent models, the D2R agonist quinpirole induces an increase in the ChI firing rate (Pisani et al., 2006; Martella et al., 2009; Grundmann et al., 2012; Sciamanna et al., 2012). Both THP and a more selective M2/M4 mAChR antagonist restore D2R-mediated ChI responses in *ex vivo* brain slices of *Tor1a*$^{+/\Delta E}$ KI mice (Scarduzio et al., 2017). Thus, THP and other more selective mAChR antagonists normalize both DA release and striatal ChI responses in *Tor1a*$^{+/\Delta E}$ KI mice suggesting a central role for ChI in the development of novel therapeutics. However, it is not yet known if a selective M4 mAChR antagonist alone would similarly normalize ChI responses.

Our studies suggest that M1 mAChR function is abnormal in *Tor1a*$^{+/\Delta E}$ KI mice. Consistent with previous work (Gerber et al., 2001), we demonstrate that M1 mAChRs mediate DA release in normal mice, as KO of M1 mAChR from either dSPNs or iSPNs attenuated the DA release-enhancing effects of THP. While the precise mechanisms that mediate this effect are unclear, it is likely indirect perhaps via SPN axon collateral synapses onto ChIs (Tepper et al., 2008). In contrast to normal mice, the response to THP in *Tor1a*$^{+/\Delta E}$ KI mice was not affected by deletion of M1 mAChRs suggesting either hypofunctional M1 mAChR signaling on dSPNs and iSPNs or a

defect in the local striatal microcircuit in *Tor1a*$^{+/\Delta E}$ KI mice. As previous studies have not identified changes in M1 mAChR expression levels in *Tor1a*$^{+/\Delta E}$ KI mice (Richter et al., 2019) and M1 mAChR function appears to be normal in *Tor1a*$^{+/\Delta E}$ KI mice (Martella et al., 2014), a local microcircuit defect seems to be the most likely explanation and future experiments will be required to address this question. Although M1 mAChRs do not contribute to the THP-induced normalization of DA release in *Tor1a*$^{+/\Delta E}$ KI mice, the action of THP via M1 mAChRs rescues the abnormal corticostriatal plasticity, including diminished long-term depression and loss of synaptic depotentiation in SPNs, observed in *Tor1a*$^{+/\Delta E}$ KI mice (Maltese et al., 2014; Martella et al., 2014). Thus, a combination of M1 and M4 antagonists may be required to simultaneously enhance striatal DA while also restoring normal corticostriatal plasticity to effectively treat dystonia.

Taken together, our data demonstrate that THP normalizes DA release in *Tor1a*$^{+/\Delta E}$ KI mice by blocking M4 mAChR on ChIs. This suggests that M4 subtype-selective mAChR antagonists, alone or in combination with other compounds, may be promising therapeutics for *DYT1-TOR1A* dystonia. Further, because many of the dose-limiting peripheral side effects associated with THP, including constipation, dry mouth, and urinary retention, are mediated by M2 and M3 mAChR (Bymaster et al., 2003), M4 subtype-selective mAChR antagonists may improve dystonia symptoms while avoiding many of the side effects of the non-selective mAChR antagonists that are currently available to patients. Indeed a recent study demonstrating that the M4 mAChR subtype-selective antagonist VU6021625 reduces dystonic movements in a knockin mouse model of DOPA-responsive dystonia provides support for this approach (Moehle et al., 2020). That VU6021625 ameliorates the abnormal phenotypes in two different mouse models of dystonia demonstrates the promise of more selective mAChR antagonists as therapeutics for dystonia.

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FIGURE LEGENDS

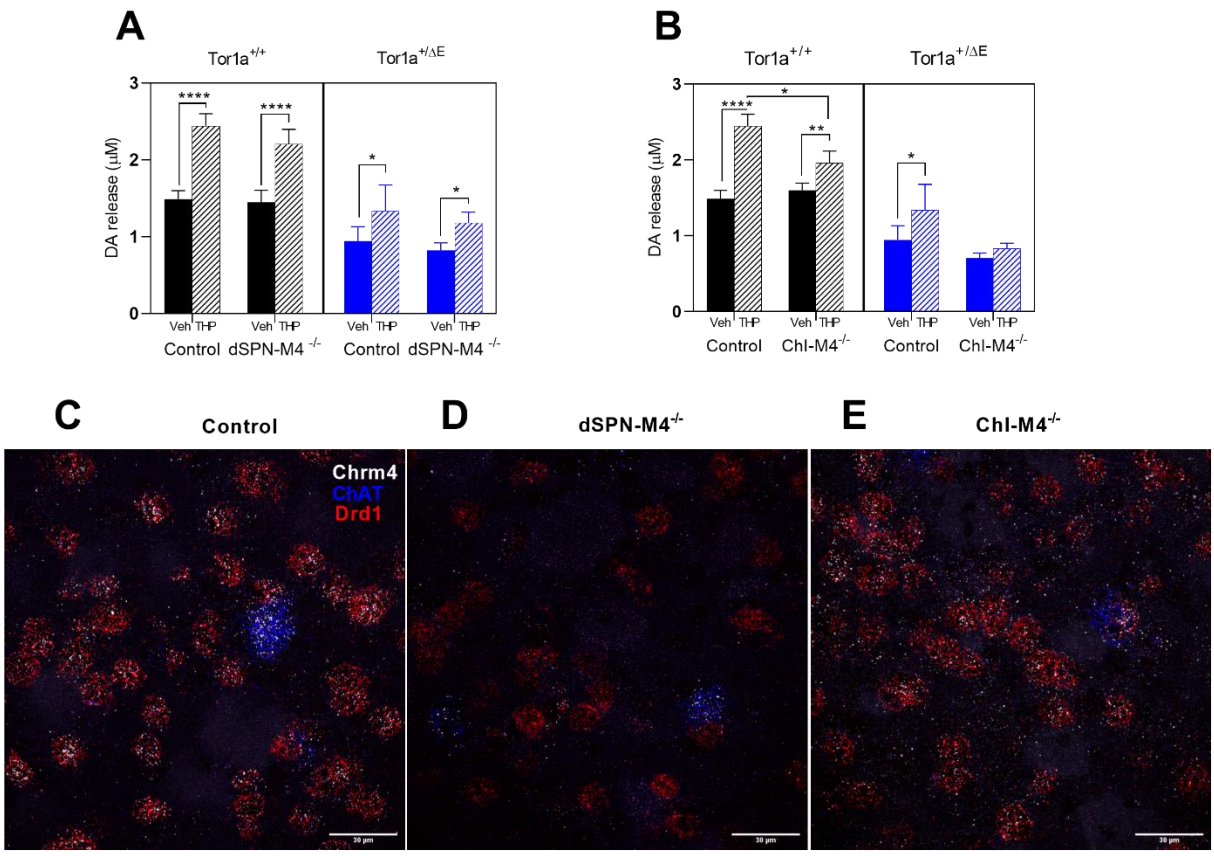

Fig 1. **Effect of cell-type specific deletion of M4 mAChRs on striatal DA release in *Tor1a*$^{+/+}$ and *Tor1a*$^{+/\Delta E}$ KI mice** (**A**) KO of M4 mAChR from direct SPNs did not significantly change the DA-release enhancing effects of 300nM THP in either genotype. (**B**) In *Tor1a*$^{+/+}$ mice, deletion of M4 mAChR from ChIs reduced the effect of THP relative to controls. KO of M4 mAChR from ChIs abolished the effects of THP in *Tor1a*$^{+/\Delta E}$. Data are expressed as mean peak concentration of DA released (μM) ± SEM (all groups n=6); *p<0.05, **p<0.01, ****p<0.0001. Representative images of fluorescent *in situ* hybridization in control (**C**), dSPN-M4$^{-/-}$ (**D**), and ChI-M4$^{-/-}$ (**E**) mice using probes to ChAT, Chrm4, and Drd1 mRNA.

Table 1. M4 mAChR KO mouse validation

	Cell markers			
Genotype	*ChAT+, Chrm4+*	*ChAT+*	*Drd1+, Chrm4+*	*Drd1+*
WT	6.2±0.4% (21)	0.0±0.0% (0)	92.9±1.2% (314)	0.9±0.8% (3)
dSPN-M4$^{-/-}$	7.3±1.8% (27)	0.0±0.0% (0)	12.1±1.9%**** (45)	80.7±2.9%**** (290)
ChI-M4$^{-/-}$	0.0±0.0%** (0)	6.6±0.9%*** (25)	92.9±1.1% (344)	0.5±0.3% (2)

Table 1. **M4 mAChR KO mouse validation**. Quantification of *Chat*- *Drd1*- and *Chrm4*-positive cells in M4 mAChR cell-type specific knockout mice. Data represent mean percentage ± SEM of positive cells with the number of cells in parentheses. Data within each column were analyzed with one-way ANOVA with *post hoc* Dunnett's multiple comparisons test. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ compared to control.

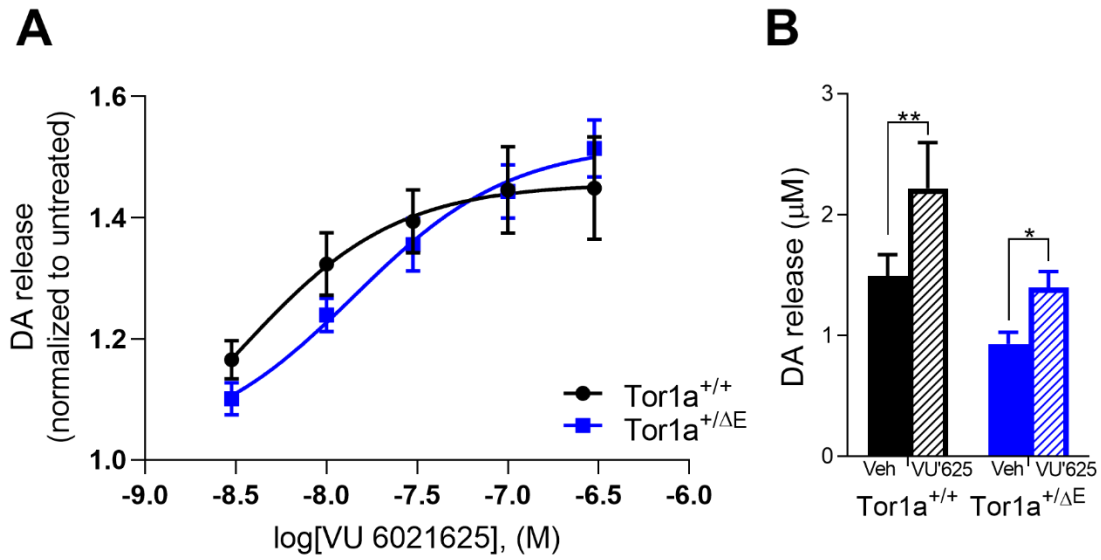

Fig 2. **Effect of VU6021625 on striatal DA release in *Tor1a*$^{+/+}$ and *Tor1a*$^{+/\Delta E}$ KI mice.** (**A**) Dose response curve of the effect of the subtype-selective M4 mAChR antagonist VU6021625 on DA release. VU6021625 dose-dependently enhanced DA release in both *Tor1a*$^{+/+}$ and *Tor1a*$^{+/\Delta E}$ KI mice. Values are normalized to untreated for each genotype. (**B**) Effect of maximal concentration of VU6021625 on DA release. 300nM VU6021625 significantly enhanced striatal DA release in both *Tor1a*$^{+/+}$ and *Tor1a*$^{+/\Delta E}$ KI mice. Values represent mean ± SEM (n=5); *p<0.05, **p<0.01.

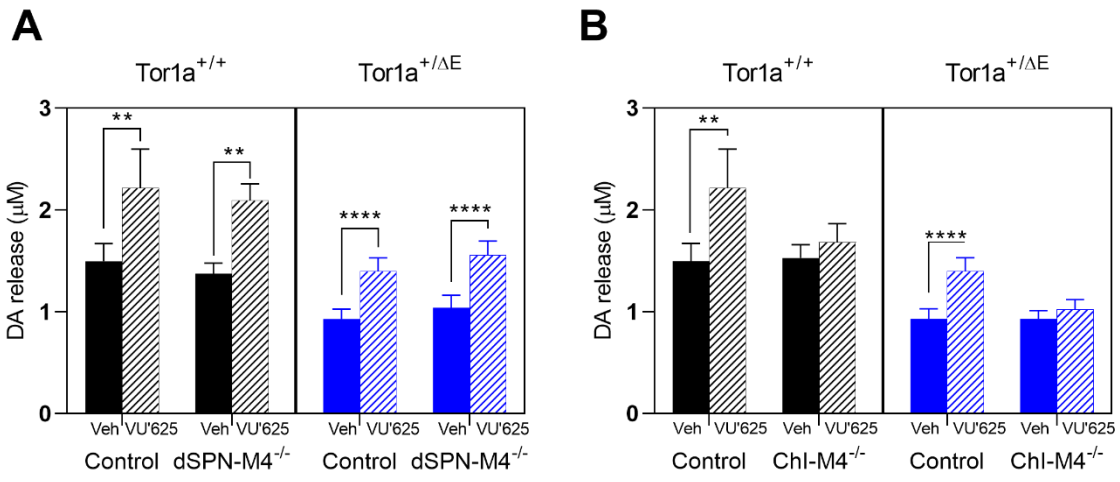

Fig 3. **Effect of M4 mAChR KO in dSPNs or ChIs on the VU6021625-induced increase in DA release** (**A**) KO of M4 mAChR from dSPNs did not significantly change the DA release-enhancing effects of 300nM VU6021625 in either genotype. (**B**) KO of M4 mAChR from ChIs reduced the DA release enhancing effects of VU6021625 in both *Tor1a*$^{+/+}$ and *Tor1a*$^{+/\Delta E}$ mice. Data are expressed as concentration of DA released (μM) ± SEM (all groups n=6); **p<0.01, ****p<0.0001.

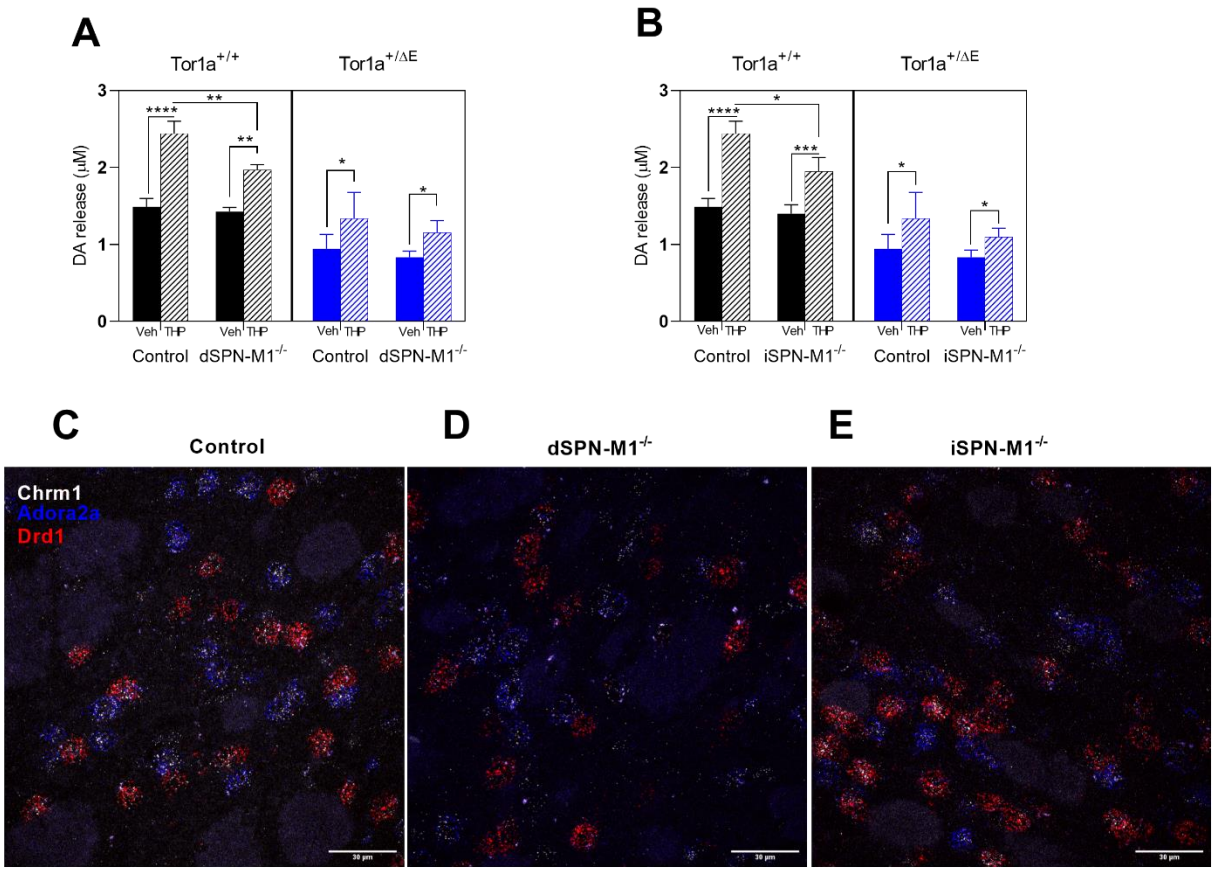

Fig 4. **Effect of cell-type specific deletion of M1 mAChRs on striatal DA release in *Tor1a*$^{+/+}$ and *Tor1a*$^{+/\Delta E}$ KI mice** (**A**) KO of M1 mAChR from dSPNs reduced but did not eliminate the effects of THP in *Tor1a*$^{+/+}$ mice. However, KO of M1 mAChR from direct SPNs did not alter the effects of THP in *Tor1a*$^{+/\Delta E}$ KI mice. (**B**) KO of M1 mAChR from indirect SPNs in *Tor1a*$^{+/+}$ mice reduced but did not eliminate the effects of THP. KO of M1 mAChR from indirect SPNs in *Tor1a*$^{+/\Delta E}$ KI mice did not alter the effects of THP. Data are expressed as concentration of DA released (μM) ± SEM. (all groups n=6); *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Representative images of control (**C**), dSPN-M4$^{-/-}$ (**D**), and iSPN-M4$^{-/-}$ (**E**) mice labeled for ChAT, Chrm4, and Drd1 mRNA.

Table 2 M1 mAChR KO mouse validation

	Cell Markers			
Genotype	*Adora2a+, Chrm1+*	*Adora2+*	*Drd1+, Chrm1+*	*Drd1+*
Control	47.4±4.0% (213)	3.2±0.6% (16)	49,1±3.1% (226)	0.2±0.2% (1)
dSPN-M1$^{-/-}$	46.5±3.4% (220)	4.0±0.3% (20)	2.3±0.6%**** (11)	46.9±3.3%**** (223)
iSPN-M1$^{-/-}$	4.4±1.0%*** (23)	42.3±3.2%**** (224)	52.8±3.7% (279)	0.4±0.4% (2)

Table 2. **M1 mAChR KO mouse validation.** Quantification of *Chrm1*- *Adora2a*- and *Drd1*-positive cells in M1 mAChR cell-type specific knockout mice. Data represent mean percentage ± SEM of positive cells (number of cells). Data within each column were analyzed with one-way ANOVA with *post hoc* Dunnett's multiple comparisons test. ***$p < 0.001$, **** $p < 0.0001$ compared to Control.