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Anticholinergic drugs rescue synaptic plasticity in DYT1 dystonia: role of M_1 muscarinic receptors

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Abstract

Background—Broad spectrum muscarinic receptor antagonists have represented the first available treatment for different movement disorders such as dystonia. However, the specificity of these drugs and their mechanism of action is not entirely clear.

Methods—We performed a systematic analysis of the effects of anticholinergic drugs on short- and long-term plasticity recorded from striatal medium spiny neurons from DYT1 dystonia knock-in ($Tor1a^{+/\Delta gag}$) mice heterozygous for ΔE-torsinA and their controls ($Tor1a^{+/+}$ mice).

Results—Antagonists were chosen that had previously been proposed to be selective for muscarinic receptor subtypes and included pirenzepine, trihexyphenydil, biperiden, orphenadrine, and a novel selective M_1 antagonist, VU0255035. $Tor1a^{+/\Delta gag}$ mice exhibited a significant impairment of corticostriatal synaptic plasticity. Anticholinergics had no significant effects on intrinsic membrane properties and on short-term plasticity of striatal neurons. However, they exhibited a differential ability to restore the corticostriatal plasticity deficits. A complete rescue of both long-term depression (LTD) and synaptic depotentiation (SD) was obtained by applying the M_1-preferring antagonists pirenzepine and trihexyphenidyl as well as VU0255035. Conversely, the non-selective antagonists orphenadrine produced only a partial rescue of synaptic plasticity, whereas biperiden and ethopropazine failed to restore plasticity. The selectivity for M_1 receptors

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Conflict of interest: nothing to report.

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was further demonstrated by their ability to counteract the M_1-dependent potentiation of NMDA current recorded from striatal neurons.

Conclusions—Our study demonstrate that selective M_1 muscarinic receptor antagonism offsets synaptic plasticity deficits in the striatum of mice with the DYT1 dystonia mutation, providing a potential mechanistic rationale for the development of improved antimuscarinic therapies for this movement disorder.

Keywords

dystonia; striatum; muscarinic receptor antagonists; synaptic plasticity

Introduction

DYT1 dystonia is a severe form of inherited dystonia caused by a deletion in the gene encoding the protein torsinA. Currently the medical therapy for this disorder is still largely unsatisfactory. Synaptic plasticity abnormalities have been demonstrated both in patients[1–3] as well as at corticostriatal synapses of multiple rodent models, either over-expressing the human mutant torsinA[4,5] or in knock-in mice heterozygous for mutant torsinA[6]. These synaptic alterations were normalized by antimuscarinic agents, indicating a fundamental involvement of striatal acetylcholine in the pathogenesis of this disorder. Accordingly, broad spectrum muscarinic receptor (mAChR) antagonists have long been used to treat dystonia, but their side effects have significantly reduced their use[7]. The exact mechanism of action of anticholinergic drugs in the relief of dystonic symptoms remains undetermined, although it is believed that their effect is centrally mediated and that restores the imbalance between striatal dopamine and acetylcholine.

The mAChRs are G-protein-coupled receptors subdivided into two major classes according to their pharmacological and signalling properties[8]. The M_1, M_3, and M_5 mAChRs couple to the Gq/G11-type G-proteins, leading to IP3 and DAG formation, whereas the M_2 and M_4 mAChRs activate Gi/Go-type G-proteins, inhibiting adenylyl cyclase. M_1 mAChRs are expressed on dendrites and spines of medium spiny neurons (MSNs), and are therefore strategically positioned to influence motor control and synaptic plasticity[8–11]. The lack of subtype-selective ligands for the mAChRs has prevented a more comprehensive understanding of the role of mAChR subtypes in distinct brain regions.

More recently, novel compounds have been developed, displaying higher selectivity for single mAChRs[15].

We performed a systematic characterization of the effects of antimuscarinic agents on short- and long-term synaptic plasticity of MSNs recorded from mice with the DYT1 dystonia mutation. We characterized the effects of antimuscarinic drugs such as trihexyphenydil, orphenadrine, biperiden and ethopropazine, and compared these results with those obtained with the novel, selective M_1 mAChR antagonist *N*-[3-oxo-3-[4-(4-pyridinyl)-1-piperazinyl]propyl]-2,1,3-benzothiadiazole-4-sulfonamide (VU0255035)[16]. Clarifying the functions of mAChRs in striatum could indicate the direction for a modern and selective strategy for pharmacological intervention in dystonia.

Methods

Tissue preparation and physiology

The Animal Care and Use Committee of "Tor Vergata" University approved all experiments, in accord with EC, Internal Institutional Review Committee, EU directive and Italian rules (86/609/EEC; D.Lvo 116/1992, 63/2100 EU, 153/2001A–IHM and 5/2010UV). All efforts were made to reduce the number of animals used. Colonies of both knock-in Tor1a$^{+/\Delta gag}$ mice heterozygous for ΔE-torsinA and their controls (Tor1a$^{+/+}$ mice)[17] were bred at our animal house. Genotyping was performed as indicated[17]. Parahorizontal corticostriatal slices (180–200 μm) were cut in Krebs' solution[4] (in mM: 126 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 10 glucose, 18 $NaHCO_3$, 95% O_2, 5% CO_2). Slices were transferred into a recording chamber, superfused with oxygenated Krebs' medium (32–33°C). In these slices a knife-cut was made between the striatum and the thalamus, to prevent contamination from thalamostriatal inputs[4]. Sharp-microelectrode recordings were performed blindly with 2M KCl (40–60 MΩ). Signal acquisition and off-line analysis were performed with an Axoclamp 2B amplifier and pClamp9 software (Molecular Devices). MSNs were identified according to their electrophysiological characteristics[4,6]. Glutamatergic excitatory postsynaptic potentials (EPSPs) were evoked with a bipolar electrode in the cortex (V–VI layer), in picrotoxin (50 μM) to block $GABA_A$ receptors[6]. Synaptic stimuli were delivered at 0.1 Hz, and 6 events were averaged. Paired-pulse facilitation was assessed by presenting two stimuli (ISI 50 ms) and measuring the ratio (EPSP2/EPSP1). One neuron per slice was used for plasticity experiments. For high-frequency stimulation (HFS, 3 trains: 3 sec, 100 Hz, 20 s interval), stimulus intensity was raised to spike threshold. EPSP amplitude was averaged and plotted as % of control before HFS. Magnesium was removed to optimize LTP induction[18]. Synaptic depotentiation (SD) was induced by a low-frequency stimulation (LFS) protocol (2 Hz, 10 min), ~30 min after LTP stabilization. Whole-cell recordings were performed as described[19], with a Multiclamp 700b amplifier, using borosilicate glass pipettes (resistance range: 2.5–5 MΩ). Membrane currents were continuously monitored and access resistance measured was between 5–30 MΩ prior to electronic compensation (60–80% routinely used). For NMDA-mediated currents, pipettes contained (mM): K^+-gluconate (125), NaCl (10), $CaCl_2$ (1.0), $MgCl_2$ (2.0), 1,2-bis (2-aminophenoxy) ethane-N,N,N,N-tetra-acetic acid (BAPTA) (1), Hepes (10), GTP (0.3) M_g-ATP (2.0); pH adjusted to 7.3 with KOH. Cells were clamped at an holding potential (HP) of −80 mV. Data were analyzed offline (Clampfit 10.2; MiniAnalysis 6.0, Synaptosoft; Prism 3.02, GraphPad).

Drug source and handling

Biperiden, ethopropazine, orphenadrine were from Sigma (Italy), pirenzepine from TOCRIS Bioscience (UK), VU0255035 was provided by Dr. Conn. Drugs were applied by bath perfusion.

Statistical analysis

Electrophysiological results are means ± SEM. Student's *t*-test and non-parametric Mann-Whitney test were used to compare means pre- and post-HFS/drug. Analysis of variance

(ANOVA) test with a *post-hoc* Tukey-test were performed among groups ($p<0.05$; $\alpha=0.01$). P value <0.05 was considered statistically significant.

Results

Membrane and synaptic responses to antimuscarinic agents in striatal medium spiny neurons

MSNs from both Tor1a$^{+/+}$ and Tor1a$^{+/\Delta gag}$ mice had similar resting membrane potential, were silent at rest and, upon depolarizing current pulses showed membrane rectification and tonic action potential discharge[6] (Fig. 1AC). Each of the tested drugs failed to modify intrinsic properties of MSNs (Suppl. Table 1). Then, we measured PPR as an indicator of presynaptic activity[20]. No significant differences in the PPR were found between Tor1a$^{+/+}$ (Fig. 1C; n=8, 1.03±0.01%) and Tor1a$^{+/\Delta gag}$ neurons (Fig. 1C; n=9, 1.04±0.02%, $p>0.05$). The selective M_1 mAChR antagonist, VU0255035 (0.05–1 μM), preserved the physiological I/V curve recorded in MSNs from both Tor1a$^{+/+}$ (n=11) and Tor1a$^{+/\Delta gag}$ (n=15) mice (Fig 1B, $p>0.05$). Additionally, no difference in PPR was measured with VU0255035 (100–300 nM) in Tor1a$^{+/+}$ (n=10, 1.02 ±0.01 %) and Tor1a$^{+/\Delta gag}$ (n=11, 1**.03±0.02%)** slices (Fig 1D, Suppl. Table 1; ANOVA $p>0.05$), indicating that M_1 mAChR antagonism does not affect basal striatal glutamatergic transmission at the doses utilized.

M_1 mAChR antagonism rescues striatal synaptic plasticity in knock-in mice

A bidirectional impairment of synaptic plasticity has been described in Tor1a$^{+/\Delta gag}$ mice[6]. As expected, HFS of corticostriatal afferents led invariably to the induction of long-term depression (LTD) in MSNs recorded from Tor1a$^{+/+}$ mice (data not shown; n=12, 52.8 ±6.4% of control, measured 20 min post-HFS; *t*-test $p<0.05$), whereas HFS failed to elicit LTD in Tor1a$^{+/\Delta gag}$ MSNs (Fig. 2; n=10, 98.04 ±5.8% of control, 20 min post-HFS; *t*-test $p>0.05$). Endogenous acetylcholine modulates corticostriatal synaptic plasticity through M_1 mAChRs[11,21]. Each of the mAChR antagonists tested did not affect physiological LTD in Tor1a$^{+/+}$ mice (data not shown; VU0255035: n=6, 50.6 ±4.3%; *t*-test $p<0.05$; pirenzepine: n=6, 49.1 ±5.9%; *t*-test $p<0.05$; tryhexyphenidyl: n=5, 54.7 ±7.7%; Mann-Whitney: $p<0.05$; orphenadrine: n=6, 51.9 ±6.5%; *t*-test $p<0.05$; biperiden: n=5, 50.6 ±4.3%; Mann-Whitney: $p<0.05$; ethopropazine: n=6, 51.1 ±6.2%; *t*-test $p<0.05$).

Conversely, in Tor1a$^{+/\Delta gag}$ mice, anticholinergic agents displayed a distinct profile, according to their ability to antagonize M_1 mAChRs, without affecting basal EPSP amplitude. The novel selective M_1 mAChRs antagonist, VU0255035 (100 nM, 20 min) fully restored LTD (Fig. 2A; n=16, 47.9 ±6.2%; *t*-test $p<0.05$). Similarly, both M_1-preferring antagonists pirenzepine (100 nM, 20 min) and trihexyphenidyl (3 μM, 20 min) were able to rescue a physiological LTD in Tor1a$^{+/\Delta gag}$ MSNs (Fig. 2B,C; pirenzepine: n=12, 48.2 ±6.6%; *t*-test $p<0.05$; tryhexyphenidyl: n=13, 53.87 ±5.4%; Mann-Whitney: $p<0.05$). Conversely, the non-selective mAChR antagonist orphenadrine (100 nM, 20 min) produced only a partial rescue of LTD in MSNs from Tor1a$^{+/\Delta gag}$ mice (Fig. 2D; n=12, 69.57 ±2.5%; *t*-test $p<0.05$). Additionally, both biperiden (10–50 μM, 20 min) and ethopropazine (100 μM, 20 min) failed to restore LTD in Tor1a$^{+/\Delta gag}$ mice (Fig 2E,F; biperiden: n=10, 105.8 ±4.7%; ethopropazine: n=10, 102.2 ±6.9%; Mann-Whitney: $p>0.05$ for both).

A physiological LTP was measured in Tor1a$^{+/+}$ mice (data not shown, n=6, 170.9 ±8.3% of control, measured 20 min post-HFS; *t*-test p<0.05), whereas an LTP of significantly increased amplitude was recorded in MSNs from Tor1a$^{+/\Delta gag}$ mice (Fig. 3; n=10, 202.6 ±6.2% of control, 20 min post-HFS; Mann-Whitney: p<0.05). Once LTP is stabilized, a LFS protocol reverts synaptic activity to resting levels, a phenomenon termed synaptic depotentiation (SD)[22]. LFS caused a normal SD in Tor1a$^{+/+}$ mice (data not shown, n=6, 105.5 ±7.9% of control, 10 min post-LFS; *t*-test p<0.05) but was unable to depotentiate corticostriatal synapses in Tor1a$^{+/\Delta gag}$ MSNs (Fig. 3; n=10, 242.4 ±7.8% of control, 10 min post-LFS; Mann-Whitney: p<0.05).

In Tor1a$^{+/+}$ mice, a physiological SD was measured, without significant effects by the tested drugs (data not shown; VU0255035: n=5, 98.8 ±6.2%; Mann-Whitney: p<0.05; pirenzepine: n=6, 99.8 ±9.1%; *t*-test p<0.05. tryhexyphenidyl: n=5, 100.3 ±4.9%; Mann-Whitney: p<0.05; biperiden: n=5, 101.1 ±5.5%; Mann-Whitney: p<0.05; ethopropazine: n=6, 102.9 ±8.2%; *t*-test p<0.05). However, in knock-in mice, VU0255035 (100 nM, 20 min) was able to completely rescue SD in Tor1a$^{+/\Delta gag}$ mice (Fig. 3A; n=10, 95.1 ±6.8%; Mann-Whitney: p<0.05) as well as both pirenzepine (100 nM, 20 min) and trihexyphenidyl (3 μM, 20 min) (Fig 3B,C; pirenzepine: n=10, 96.4 ±8.9%; *t*-test p<0.05; tryhexyphenidyl: n=8, 102.87 ±9.36%; Mann-Whitney: p<0.05).

Orphenadrine has been shown to inhibit NMDA responses[23]. Indeed, when bath-applied in low-magnesium solution, which relieves the Mg^{2+}-dependent NMDA receptor blockade[18], orphenadrine (n=6), prior to LFS protocol, reduced the amplitude of the recorded EPSP (Fig. 3D, blue arrow; 30% of control). Under these conditions, LFS caused a partial SD, although this could well be related to the NMDA antagonism; therefore, although a significant difference emerges compared to the pre-LFS values (Fig 3D; n=8, 153.19 ±5.9%; *t*-test p<0.05), the efficacy of orphenadrine in rescuing SD cannot be ascribed solely to muscarinic antagonism.

SD deficit was not normalized by treatment with both biperiden (20 μM, 20 min) and ethopropazine (100 μM, 20 min) in Tor1a$^{+/\Delta gag}$ mice (Fig 3E,F; biperiden: n=6, 231.5 ±5.02%; *t*-test p>0.05; ethopropazine: n=6, 228.1 ±9.3%; *t*-test p>0.05).

Muscarinic antagonists preferentially target M_1 mAChR-dependent responses in striatal MSNs

Selective activation of M_1 mAChRs results in the potentiation of NMDA receptor-mediated currents in MSNs[24]. To demonstrate the selectivity of VU0255035, we recorded NMDAR-mediated currents in MSNs from both Tor1a$^{+/+}$ and Tor1a$^{+/\Delta gag}$ mice (Fig. 4). Whole-cell recordings were performed in picrotoxin (50 μM) plus TTX (1 μM). Bath-application of NMDA (20 s, 30 μM) produced a transient and reversible inward current in MSNs recorded from both Tor1a$^{+/+}$ (n=7) and Tor1a$^{+/\Delta gag}$ (n=10) mice (Fig. 4A,B). Preincubation with the selective M_1 mAChR agonist McN-A-343 (3 μM), potentiated the NMDA-induced current in all the recorded MSNs from both Tor1a$^{+/+}$ (n=7) and Tor1a$^{+/\Delta gag}$ (n=10) mice (Fig. 4A–C; 175.2 ±6.8%; 174.5 ±6.3%, respectively; p<0.05). In the presence of 100 nM VU0255035 (15 min), the McN-A-343-induced potentiation of NMDA currents was fully prevented in MSNs from both Tor1a$^{+/+}$ and Tor1a$^{+/\Delta gag}$ mice (Fig. 4A–C; 83.7 ±3.9% and

86.6 ±3.9%, respectively; p>0.05). Similarly, in both genotypes, pirenzepine completely prevented the potentiation of NMDA-mediated currents caused by McN-A-343 (3 μM; 86 ±3.4%, n=5; p>0.05; not shown).

In keeping with the hypothesis that selective M_1 antagonism normalizes synaptic plasticity deficits, we performed similar whole-cell recordings with biperiden, ethopropazine and orphenadrine. In Tor1a$^{+/\Delta gag}$ MSNs, both biperiden and ethopropazine failed to prevent the enhancement of NMDA-mediated currents caused by McN-A-343 (Fig. 4D; biperiden: 163.3 ±4.6%; n=6; p<0.05; ethopropazine: 185.1 ±7.5%; n=6; p<0.05). A final set of recordings was performed with orphenadrine, which acts as non-competitive NMDA antagonist in cultured neurons[23]. Orphenadrine (100 nM, 10–15 min) per sè reduced significantly NMDA currents in MSNs (not shown, 44.7 ±3.1% inhibition of NMDA-induced current; n=7; p<0.05). Such inhibitory effect did not allow to test further its potential efficacy on M_1-induced potentiation.

Anticholinergics do not affect the M_2/M_4-mediated responses in striatal neurons

M_2/M_4 mAChRs mediate two responses in distinct striatal neuron subtypes. In cholinergic interneurons, M_2/M_4 autoreceptor activation generates a membrane hyperpolarization/outward current, by closing CaV2 Ca^{2+} channels, and by increasing opening of Kir3 potassium channels, which hyperpolarize terminals and further reduce Ca^{2+} channel opening[25]. In addition, the cortical glutamatergic drive to MSNs is reduced by presynaptic M_2/M_4 mAChRs located at corticostriatal terminals[14,26]. This latter effect was excluded since no change in EPSP amplitude was measured, as described above for plasticity experiments. Then, we tested the effects of antimuscarinic agents on cholinergic interneurons, identified by means of their electrophysiological properties[27,28]. Intrinsic membrane properties of these interneurons were not significantly different between genotypes, as reported[6]. In cells from both strains, muscarine (10 μM, 90s) induced a membrane hyperpolarization and interrupted their firing activity, an effect that was blocked by the M_2/M_4 receptor-preferring antagonist methoctramine (300 nM, 10 min; not shown). No significant difference was measured between genotypes, showing that the muscarinic autoreceptor function is preserved in Tor1a$^{+/\Delta gag}$ mice. To exclude a non-specific involvement of M_2/M_4 mAChRs, we tested pirenzepine, trihexyphenydil and VU0255035 on the response to muscarine. VU0255035 affected neither the intrinsic properties nor the inhibitory response to muscarine, ruling out a recruitment of M_2/M_4 mAChRs (Fig. 5; n=6, p>0.05). Similar results were obtained with pirenzepine and tryhexyphenydil (n=4 for each drug, p>0.05; not shown).

Discussion

Anticholinergic treatments are used in different forms of dystonia, although the existing drugs are non-selective muscarinic antagonists, associated with a broad array of undesirable central and peripheral side effects[7,29]. Therefore, it is important to develop a detailed understanding of the roles of individual mAChR subtypes in basal ganglia function. Surprisingly, few studies analyzed the mechanism of action of this class of drugs, despite the evidence that it still represents one of the few medical options for the treatment of

dystonia[7,29]. Our study demonstrates that M_1 mAChR antagonism offsets striatal synaptic plasticity deficits in mice with the DYT1 dystonia mutation, providing a potential mechanistic rationale for the development of improved antimuscarinic therapies for this movement disorder.

Striatal acetylcholine and dystonia

Acetylcholine plays key roles in the striatal regulation of normal voluntary movement, as well as the motor dysfunction that occurs in different movement disorders, such as Parkinson's disease and dystonia[30]. The striatum contains a small percentage of interneurons which provide this area with one of the highest levels of acetylcholine in the brain[31]. Besides the local striatal innervation, a number of cholinergic projections arise from nuclei of the basal forebrain, as well as from the pedunculopontine-lateral dorsal tegmental nuclei[32], therefore caution is required in assuming that striatal mAChR antagonism might fully explain the therapeutic efficacy of antimuscarinic agents. However, emerging common themes in DYT1 dystonia indicate that disruption of synaptic plasticity processes represents a reproducible feature in multiple models, and might underlie the motor learning abnormalities observed in patients[1–3]. In different rodent models of DYT1 dystonia, striatal MSNs exhibit a loss of LTD, whereas LTP is enhanced in magnitude. Low-frequency stimulation (LFS) can normally revert potentiated synapses to resting levels, a phenomenon termed synaptic depotentiation (SD)[22]. However, LFS fails to induce SD both in mice and rats overexpressing mutant torsinA, as well as in DYT knock-in mice[4–6]. Collectively, a loss of inhibitory plasticity phenomena (LTD and SD) and concomitant increase in LTP amplitude emerges from these models. Consistently, the current pathophysiological hypothesis for dystonia suggests that dystonia may result from a deficient "surround inhibition" of competing motor patterns, coupled to an enhanced plasticity in motor areas[33,34]. In such context, cholinergic transmission is profoundly involved, as it plays a key role in the impairment of corticostriatal synaptic plasticity[4–6,35].

Muscarinic receptor antagonism in dystonia

MAChR subtypes M_1, M_2 and M_4, appear to be dominant in the striatum[12,13]. MSNs express primarily M_1, variable levels of M_4, but very low levels of M_2, M_3 and M_5 mAChRs[10]. Cholinergic interneurons by contrast have dominant expression and function of M_2 and M_4 mAChRs[11,36]. Some of the adverse effects of mAChR inhibitors are mediated by peripheral M_2 and M_3 mAChRs, whereas M_1 is responsible for the effects on cognition and motor function[8,9,11,37]. M_1 receptor activation increases MSN excitability by reducing KCNQ and Kir2 currents[38–40], and exert a central role in striatal long-term plasticity[11,21].

Our results indicate that M_1 mAChR antagonism is specifically required to offset plasticity deficits in mice with mutant torsinA. Although a morphological characterization was not performed between MSNs of the direct and indirect pathways, MSNs express homogeneously M_1 mAChRs and no specific difference was measured in their responses to muscarinic agents. Moreover, we had previously shown that the vast majority of morphologically-identified MSNs had a similar response to muscarinic modulation[4]. At the doses utilized, trihexyphenydil, pirenzepine, VU0255035 restored a normal LTD, as well as SD. Conversely, biperiden and ethopropazine failed to rescue LTD and SD. Orphenadrine

should not be considered in this experimental setting, since its antagonistic action on NMDA receptor function prevents a reliable evaluation[23]. We further demonstrated the specificity of effect on M_1 mAChR by testing their ability to prevent the M_1–dependent potentiation of NMDA currents. Only VU0255035, pirenzepine and tryhexyphenydil blocked the enhancement induced by M_1 agonists, whereas biperiden, ethopropazine were ineffective. Accordingly, VU0255035 antagonizes potentiating NMDA receptor currents induced by muscarinic agonists in hippocampal CA1 cells[16].

Additionally, we also ruled out a potential involvement of M_2/M_4 mAChRs, by testing antimuscarinic agents on the autoreceptor response that was unaffected by all the drugs tested. Although firing activity of other striatal interneurons[41,42] may partially resemble that of cholinergic interneurons, we are confident that the electrophysiological and pharmacological profile allows to unequivocally identify cholinergic interneurons. Moreover, these drugs failed to affect the presynaptic inhibition by M_2/M_4 mAChRs[14,26,43], indicating the specificity for M_1 mAChRs. Dystonic reactions can also appear as a consequence of treatment with neuroleptic drugs[44]. Recently, in a model of haloperidol-induced catalepsy, anticholinergic drugs displaying a higher affinity for M_1 mAChRs were more potent in counteracting catalepsy[45]. Collectively, our data suggest that irrespective of the specific form of dystonia, M_1 mAChRs may represent a preferred target for relieving dystonic symptoms.

A note of caution is required in translating these effects into their therapeutic efficacy. Indeed, the observation that biperiden, orphenadrine and ethopropazine were unable to rescue synaptic plasticity is in disagreement with their clinical efficacy. Our work does not address this issue, but demonstrates that their mechanism of action cannot be attributed to a M_1 mAChR antagonism, rather it may rely on their ability to act at different transmitter receptors simultaneously. This is consistent with previous observations reporting their anti-NMDA, anti-histamine and anti-nicotinic receptor properties[7,46,47]. Further work is required to address these issues.

In conclusion, our results indicate that subtype selectivity will be crucial to achieving clinical efficacy without adverse effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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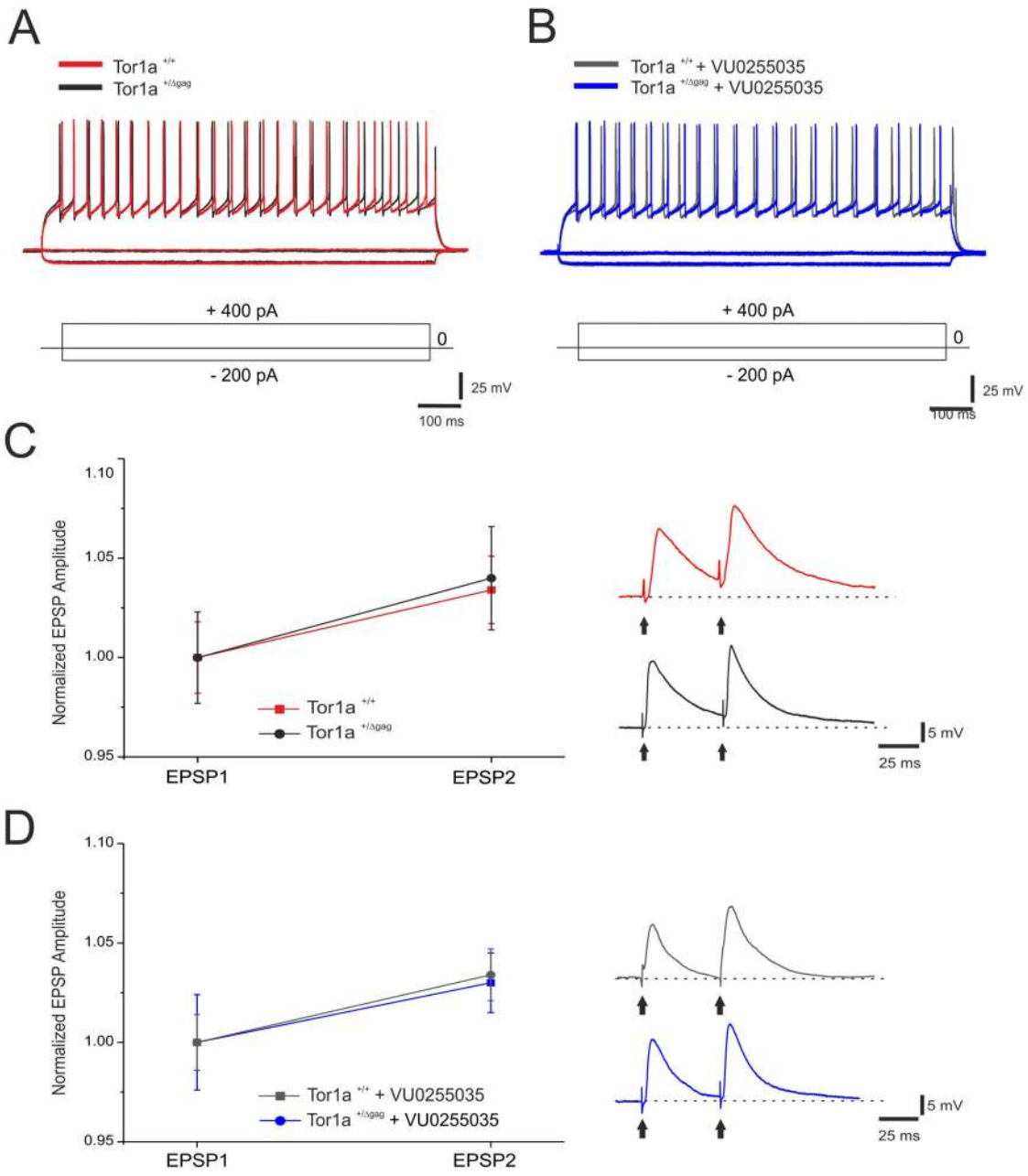

Figure 1. Selective M_1 mAChR antagonism does not modify intrinsic and synaptic properties in Tor1a$^{+/+}$ and Tor1a$^{+/\Delta gag}$, mice

(**A**) Superimposed traces showing voltage responses to current steps in both depolarizing and hyperpolarizing direction from Tor1a$^{+/+}$ (red, RMP=−89 mV) and Tor1a$^{+/\Delta gag}$ (black, RMP=−90 mV) MSNs. (**B**) Superimposed voltage responses to both depolarizing and hyperpolarizing current steps in MSN recorded from either Tor1a$^{+/+}$ (grey, RMP=−89 mV) or Tor1a$^{+/\Delta gag}$ (blue, RMP =−89 mV) mice, in the presence of the selective M_1 mAChR antagonist, VU0255035 (100 nM). (**C**) Paired-pulse facilitation (50 ms interstimulus interval) does not show significant differences between Tor1a$^{+/+}$ and Tor1a$^{+/\Delta gag}$ mice. (***Right***) Representative paired recordings of EPSPs from both Tor1a$^{+/+}$ and Tor1a$^{+/\Delta gag}$

MSNs (red and black, respectively). (**D**) Paired-pulse facilitation measured from both Tor1a$^{+/+}$ and Tor1a$^{+/\Delta gag}$ MSNs is unaffected by VU0255035 (100 nM). (***Right***) Representative paired recordings of EPSPs from both genotypes MSNs (grey and blue, respectively). Each data point in the plot is the mean ± SEM of >8 independent recordings.

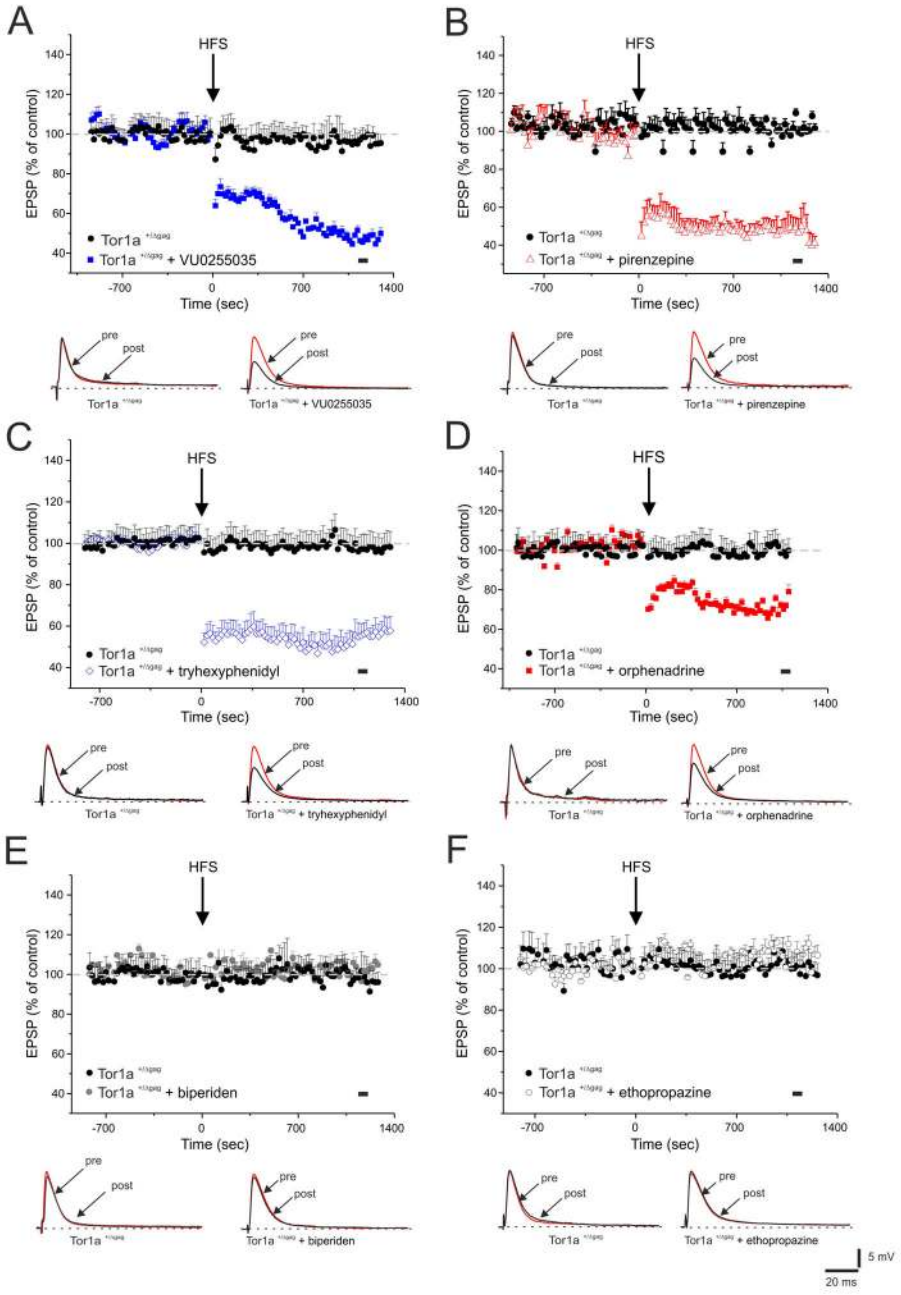

Figure 2. Corticostriatal LTD is restored by M_1-preferring mAChRs antagonists in Tor1a$^{+/\Delta gag}$ MSNs

(**A**) HFS (arrow) fails to induce LTD in Tor1a$^{+/\Delta gag}$ mice (black circles), whereas preincubation with the selective M_1 mAChR antagonist, VU0255035 (100 nM, 20 min, blue squares), fully restores LTD in Tor1a$^{+/\Delta gag}$ MSNs. (**B, C**) Similarly, M_1-preferring antagonists, pirenzepine (100 nm, 20 min, red triangles) and tryhexyphenidyl (3 μM, 15 min, blue diamonds) rescue LTD in Tor1a$^{+/\Delta gag}$ mice. (**D**) Conversely, slice preincubation with orphenadrine (100 nM, 15 min, red squares), only partially offsets LTD deficits in Tor1a$^{+/\Delta gag}$ mice. (**E, F**) In slices treated with biperiden (20 μM, 20 min, grey filled circles)

and ethopropazine (100 μM, 20 min, grey open circles), HFS fails to induce LTD. Below each plot, superimposed traces of EPSPs recorded before (pre) and 20 min after HFS (post). The black spot indicates at which time point samples were measured. Each data point represents the mean ± SEM of ≥10 independent observations.

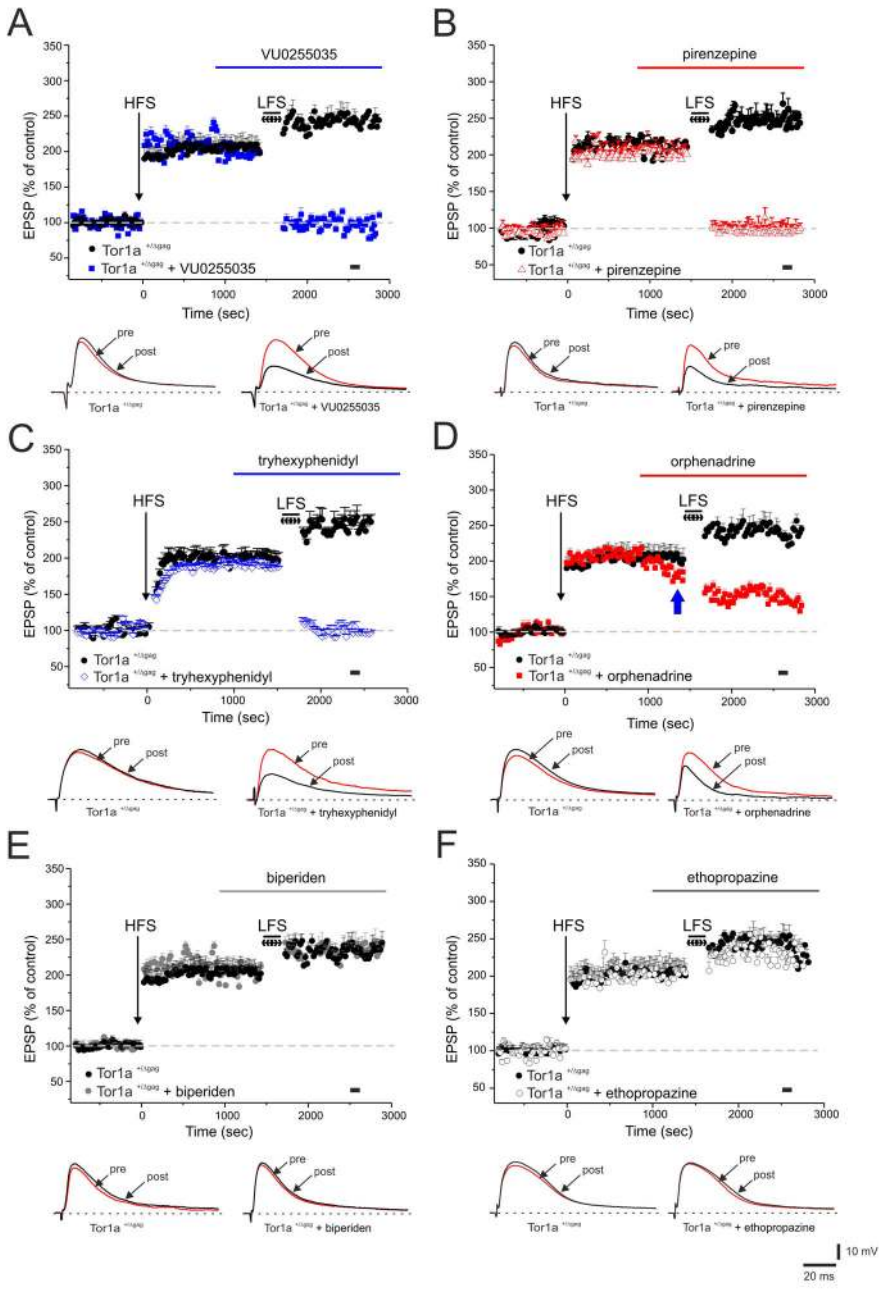

Figure 3. Antagonists of mAChRs restore synaptic depotentiation (SD) in Tor1a$^{+/\Delta gag}$ MSNs

(**A**) After LTP induction, a low-frequency stimulation, LFS (2 Hz, 10 min) protocol fails to induce SD in Tor1a$^{+/\Delta gag}$ MSNs (black circles). Before LFS, bath-application of VU0255035 (100 nM, blue bar) restores SD in Tor1a$^{+/\Delta gag}$ mice. (**B, C**) Similarly, both pirenzepine (100 nM, red triangles) and tryhexyphenidyl (3 μM, blue diamonds), restore SD. Conversely, bath-applied orphenadrine (100 nM, red bar) causes only a partial rescue of SD in Tor1a$^{+/\Delta gag}$ MSNs **(D)** (blue arrow indicates the decrease in EPSP amplitude by orphenadrine). Biperiden (20 μM, grey filled circles) and ethopropazine (100 μM, grey open circles), fail to restore the LFS-induced SD (**E, F**). ***Below*** each plot representative EPSPs

recorded before (pre) and 15 min after (post) LFS protocol. The black spot indicates at which time point samples were measured. Each data point represents the mean ± SEM of ≥8 independent observations.

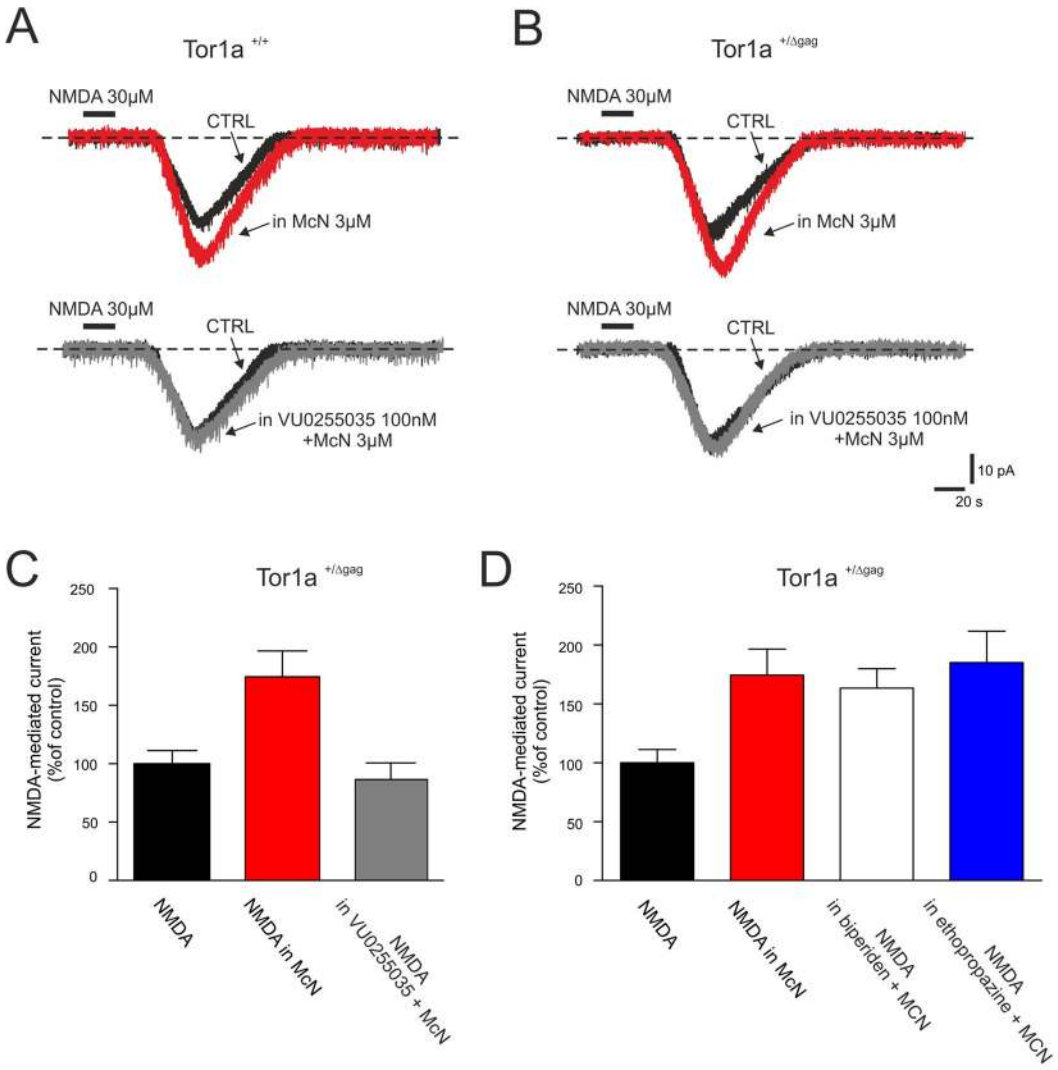

Figure 4. VU0255035 prevents the M_1 mAChR-mediated potentiation of NMDA-responses in MSNs

(**A, B**) Whole-cell recordings in the voltage-clamp mode, showing that bath-applied NMDA (30 μM, 20 s) induces an inward current in the recorded MSN from both Tor1a$^{+/+}$ and Tor1a$^{+/\Delta gag}$ mice. In both genotypes, pretreatment with the selective M_1 mAChR agonist McN-A-343 (3 μM, red traces) significantly potentiates the NMDA-induced current, which returns to control levels in the presence of VU0255035 (100 nM, grey traces). H.P.: −80 mV. (**C, D**). Summary plots of the effects (expressed as % of the NMDA-induced control current) of anticholinergic agents on the McN-A-343-induced potentiation of NMDA currents in Tor1a$^{+/\Delta gag}$ mice.

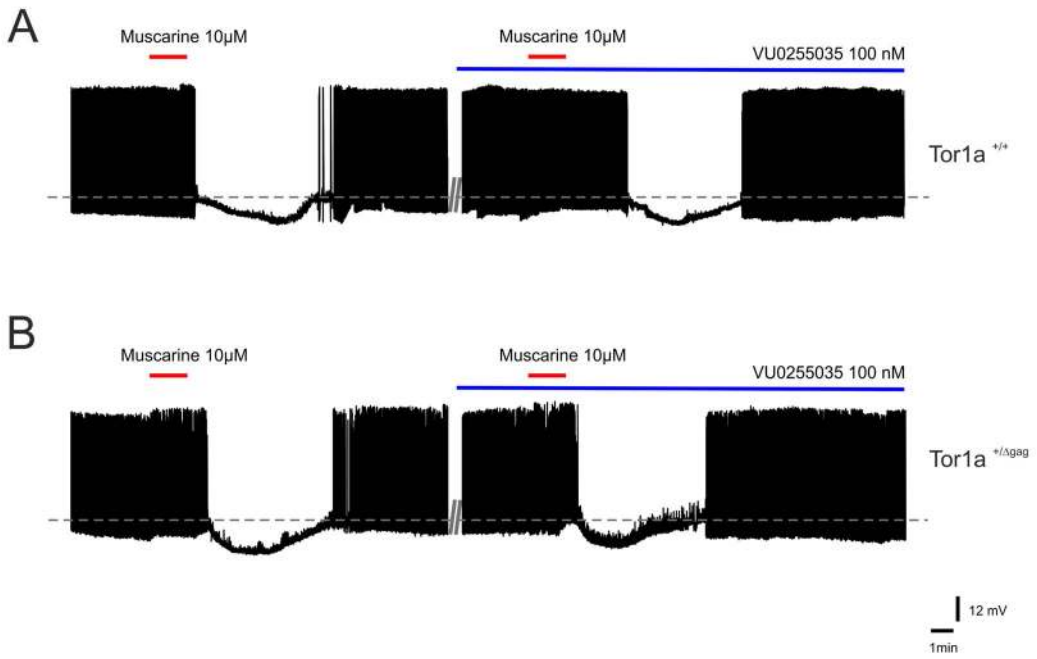

Figure 5. VU0255035 does not affect M_2/M_4 mAChR-mediated responses

(**A, B**) Sample traces of striatal cholinergic interneurons recorded from both genotypes, showing a typical spontaneous, pacemaking firing activity. Bath-application of muscarine (10 μM, 90 s) causes a transient membrane hyperpolarization and cessation of firing activity in the recorded interneurons, recorded from both Tor1a$^{+/+}$ and Tor1a$^{+/\Delta gag}$ mice. This inhibitory response, mediated by M_2/M_4 mAChRs, is not prevented by pretreatment with VU0255035 (100 nM) in both strains of mice.