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RESEARCH ARTICLE

Activation of M_4 muscarinic receptors in the striatum reduces tic-like behaviours in two distinct murine models of Tourette syndrome

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Background and Purpose: Current pharmacotherapies for Tourette syndrome (TS) are often unsatisfactory and poorly tolerated, underscoring the need for novel treatments. Insufficient striatal acetylcholine has been suggested to contribute to tic ontogeny. Thus, we tested whether activating M_1 and/or M_4 receptors—the two most abundant muscarinic receptors in the striatum—reduced tic-related behaviours in mouse models of TS.

Experimental Approach: Studies were conducted using CIN-d and D1CT-7 mice, two TS models characterized by early-life depletion of striatal cholinergic interneurons and cortical neuropotentiation, respectively. First, we tested the effects of systemic and intrastriatal xanomeline, a selective M_1/M_4 receptor agonist, on tic-like and other TS-related responses. Then, we examined whether xanomeline effects were reduced by either M_1 or M_4 antagonists or mimicked by the M_1/M_3 agonist cevimeline or the M_4 positive allosteric modulator (PAM) VU0467154. Finally, we measured striatal levels of M_1 and M_4 receptors and assessed the impact of VU0461754 on the striatal expression of the neural marker activity c-Fos.

Key Results: Systemic and intrastriatal xanomeline reduced TS-related behaviours in CIN-d and D1CT-7 mice. Most effects were blocked by M_4, but not M_1, receptor antagonists. VU0467154, but not cevimeline, elicited xanomeline-like ameliorative effects in both models. M_4, but not M_1, receptors were down-regulated in the striatum of CIN-d mice. Additionally, VU0467154 reduced striatal c-Fos levels in these animals.

Conclusion and Implications: Activation of striatal M_4, but not M_1, receptors reduced tic-like manifestations in mouse models, pointing to xanomeline and M_4 PAMs as novel putative therapeutic strategies for TS.

KEYWORDS
animal models, muscarinic receptor, striatum, tic disorders, xanomeline

Abbreviations: CIN, cholinergic interneuron; CIN-d, cholinergic interneuron depletion; PPI, prepulse inhibition; SPN, striatal projection neuron; TS, Tourette syndrome.

1 | INTRODUCTION

Tics are sudden, non-rhythmic movements or vocalizations characterized by variable intensity and complexity. Tic disorders, such as Tourette syndrome (TS), have detrimental impacts on psychosocial functioning, educational attainment and overall quality of life (Evans et al., 2016; Houghton et al., 2017; Pérez-Vigil et al., 2018). These effects are often compounded by comorbid conditions, such as obsessive-compulsive disorder (OCD), attention-deficit hyperactivity disorder (ADHD), and anxiety (Eapen et al., 2016). While dopaminergic blockers represent the most effective pharmacological approach to managing tic disorders (Weisman et al., 2013), their use is impeded by extrapyramidal, cognitive and metabolic adverse effects. This background highlights the critical and urgent need for novel therapeutic interventions.

Tics are posited to arise from dysregulated activity within the cortico-striatal-thalamic-cortical (CSTC) circuitry (Stern et al., 2000), and specifically from the activation of aberrant striatal foci (Mink, 2001). These formations are characterized by an imbalance between excess stimulation of striatal projection neurons (SPNs) and insufficient inhibition from local interneurons. Several studies have reported a decrease in cholinergic interneurons (CINs) within the dorsal striatum of individuals with severe TS (Kalanithi et al., 2005; Kataoka et al., 2010; Lennington et al., 2016). To understand the functional significance of these findings, we subjected mice to partial CIN depletion in the striatum and found that this manipulation leads to tic-like behaviours and other TS-related responses after exposure to acute stressors (Cadeddu et al., 2023; Xu et al., 2015). Specifically, mice subjected to early-life CIN depletion (CIN-d) exhibit tic-like jerks, grooming and digging stereotypies, and sensorimotor gating deficits in response to a brief, mildly stressful spatial confinement session (Cadeddu et al., 2023).

The two main **muscarinic acetylcholine receptors** expressed in the striatum are **M_1** and **M_4** (Bonsi et al., 2011; Zhou et al., 2003), primarily signalling through the $G_{q/11}$ and $G_{i/o}$ proteins, respectively (Santiago & Abrol, 2019). M_1 and M_4 receptors are expressed in **D_1** and **D_2receptor** expressing SPNs (Hersch et al., 1994; Ince et al., 1997) and have been shown to modulate their intrinsic excitability (Girasole & Nelson, 2015). Activation of postsynaptic dendritic and somatic M_1 receptors is postulated to enhance SPN excitability (Figueroa et al., 2002; Galarraga et al., 1999; Moehle et al., 2017; Shen et al., 2007). Conversely, M_4 receptors predominate in D_1-SPNs, where they inhibit the downstream effects of D_1 receptor activation (Brady et al., 2008; Jeon et al., 2010; Mamaligas & Ford, 2016; Nair et al., 2019).

Drawing from this background, we proposed that activating M_1 and/or M_4 receptors could offset the deficient **acetylcholine** neurotransmission observed in the striatum of CIN-d mice and reduce TS-related behavioural abnormalities. To investigate this hypothesis, we assessed the effects of the M_1/M_4 muscarinic receptor agonist **xanomeline** (XAN) in CIN-d mice. Additionally, we evaluated its impact on D1CT-7 mice, a mutant line with a neuropotentiating transgene expressed in D_1-expressing neurons. D1CT-7 mice exhibit sudden tic-like jerks, which are exacerbated by spatial confinement (Godar et al., 2016; Nordstrom & Burton, 2002). Despite the distinct mechanisms underlying tic development in CIN-d and D1CT-7 mice, both models demonstrate high face and predictive validity (Bortolato & Cadeddu, 2022).

Our interest in XAN stems from its well-established clinical efficacy as an antipsychotic (Shekhar et al., 2008). By itself, XAN is associated with peripheral adverse effects such as nausea, vomiting, and gastrointestinal distress (Shekhar et al., 2008), leading to high discontinuation rates. However, a novel formulation combining XAN with **trospium**, a non-selective muscarinic antagonist with limited brain penetrance, has been shown to mitigate these adverse effects (Correll et al., 2022) while maintaining XAN therapeutic efficacy (Sauder et al., 2022; Weiden et al., 2022). Building on these premises, we studied the potential efficacy of XAN as a treatment for tic disorders.

What is already known?

- Tics are thought to be contributed by striatal cholinergic deficits.
- The two main muscarinic receptors in the striatum are M_1 and M_4.

What does this study add?

- The muscarinic agonist xanomeline reduced tic-related behaviours in two distinct mouse models.
- The ameliorative effects of xanomeline are mediated by M_4, but not by M_1, receptors.

What is the clinical significance?

- Xanomeline and M_4 receptor activators may be effective in reducing tic severity.

2 | METHODS

2.1 | Animals

CIN-d mice were generated from offspring of homozygous **ChAT**-cre female mice (B6; 129S6- $ChAT^{tm2[cre]Lowl/J}$ obtained from The Jackson Laboratory, 006410) crossed with C57BL/6J male mice (The Jackson Laboratory, 000664; Bar, ME), as previously described (Cadeddu et al., 2023). ChAT-cre pups were cryoanesthesised and underwent bilateral stereotaxic infusion within the dorsal striatum (coordinates: AP − 0.5 mm; ML ± 1.6 mm; DV − 2.8 mm) of either a viral construct expressing the simian diphtheria toxin receptor (DTR) exclusively in

cre-positive cells or an inactive control virus (Cadeddu et al., 2023; Xu et al., 2015). Following surgery, mice were left undisturbed in their home cage until postnatal day 18, when they received a single injection of diphtheria toxin (1 μg·kg^{-1}, IP); this treatment led to the depletion of the DTR-expressing CINs. D1CT-7 mice (C.Cg-Tg [DRD1-ctxA] 7Burt/J from Jackson Laboratory, 008367, maintained in a 25% C57BL/6 and 75% Balb/c background) and their wild-type (WT) littermates were bred in the husbandry facilities of the University of Utah and genotyped as previously described (Godar et al., 2016). Mice from both backgrounds were weaned at postnatal day 21 and group-housed (three per cage) with food and water ad libitum. Housing facilities were maintained at 22°C with a 12-h light/dark cycle (lights on from 6:00 AM to 6:00 PM). Handling and experimental procedures were performed in compliance with the National Institute of Health guidelines and approved by the Institutional Animal Care and Use Committee at the University of Utah (Protocols N. 0001425 and 2106002). Animal studies are reported in compliance with the ARRIVE guidelines (Percie Du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). Every effort was made to minimize the number of animals and their suffering. Thus, animal numbers for each experiment were determined through power analyses conducted on preliminary results. Based on these calculations, we designed each experiment with eight mice per group for systemic treatments and six mice per group for intracerebral infusions. No animals were excluded from the study.

2.2 | Materials

XAN oxalate and the potent M_1/M_3 agonist **cevimeline** hydrochloride (CEV) (Bio-Techne, Minneapolis, MN) were dissolved in 0.9% NaCl with < 0.5% DMSO, administered IP and/or infused into the dorsolateral striatum (DLS). The M_4 positive allosteric modulator **VU0467154** (donated by Lundbeck Pharma, Denmark) was suspended in 10% Tween80, 90% water, v/v; 10kg^{-1} (Gould et al., 2018). These drugs were administered systemically at 10 ml·kg^{-1} body weight. For intracerebral infusion, drugs were dissolved in a Ringer solution. The highly selective, competitive muscarinic M_1 receptor antagonist **VU0255035** (Abcam, Boston, MA), and the potent selective M_4 receptor antagonist VU6028418 (Bio-Techne) were suspended in 0.1% Tween80, 0.5% MC in water, and administered PO at 10 ml·kg^{-1} as in Spock et al. (2021) or infused into the DLS. All dose ranges were informed by the available literature on rodent models (Cikowski et al., 2022; Montani et al., 2021; Ono et al., 2012; Sheffler et al., 2009) and established based on pilot dose-curve investigations on CIN-d and control mice.

2.3 | Experimental procedures

A total of 722 male mice (273 CIN-d, 145 controls, 216 D1CT-7, and 88 WT) were used in the present study. Only males were used since our previous results in our models showed that the intensity of behavioural deficits was significantly greater in males than females (Cadeddu et al., 2023; Godar et al., 2016), in keeping with the marked male preponderance of TS (Freeman et al., 2000). Other behavioural domains associated with frequently comorbid disorders were tested only in D1CT-7 mice, because no spontaneous behavioural alterations were observed in CIN-d mice (Cadeddu et al., 2023). Experimental manipulations were carried out between 9:00 AM and 4:00 PM. All assessments were performed by trained personnel, blind to treatment and experimental groups. The study included five sets of experiments (Figure 1; for all methodological details, see below):

Study 1: Impact of systemic XAN on behavioural responses related to TS and comorbid disorders. Initially, we examined the effects of XAN on TS-related behaviours in both CIN-d and D1CT-7 mice. Twenty min post-treatment with either XAN (0.5,5 mg·kg^{-1}, IP) or its vehicle, mice were exposed to spatial confinement, because this condition elicits overt TS-related manifestations in both models (Cadeddu et al., 2023; Godar et al., 2016). Following a 10-min acclimation period (30 min post-treatment), sequential assessments of grooming, tic-like jerks, and acoustic prepulse inhibition (PPI) of the startle reflex were conducted. To determine if the effects of XAN were associated with changes in locomotion or other behavioural abnormalities related to TS comorbidities, we assessed the effects of XAN (5 mg·kg^{-1}) on various behavioural tasks, including open field, rotarod, marble-burying task, elevated-plus maze, tail suspension and novel object exploration.

Study 2: Involvement of M_4 receptors in the effects of XAN. In the initial experiment, CIN-d and D1CT-7 mice were treated with either the M_4-selective antagonist VU6028418 (1–3 mg·kg^{-1}, PO) or its vehicle. Fifteen min later, mice received an injection of either XAN (5 mg·kg^{-1}, IP) or its vehicle. Twenty min after XAN treatment, animals were exposed to spatial confinement and underwent the same behavioural assessments as in the first study. In the subsequent experiment, separate groups of mice were administered the M_4-selective positive allosteric modulator (PAM) VU0467154 (0.3–3 mg·kg^{-1}, IP). Animals were exposed to spatial confinement 5 min later, and behavioural evaluations commenced after a 10-min acclimation period.

Study 3: Role of M_1 receptors in the effects of XAN. This series of experiments followed a protocol similar to that of the second set. Initially, CIN-d and D1CT-7 mice were treated with either vehicle or the M_1-selective antagonist VU0255035 (3–30 mg·kg^{-1}, PO). After 15 min, XAN (5 mg·kg^{-1}, IP) or vehicle was administered. Twenty min after XAN treatment, animals were exposed to spatial confinement, and behavioural assessments began 10 min later. In another experiment, different groups of mice were administered vehicle or cevimeline (CEV; 5–10 mg·kg^{-1}, IP), a muscarinic agonist with high affinity and potency on M_1 and M_3 but not M_4 receptors. Twenty min post-treatment, animals were exposed to spatial confinement, and behavioural evaluations commenced after a 10-min acclimation period.

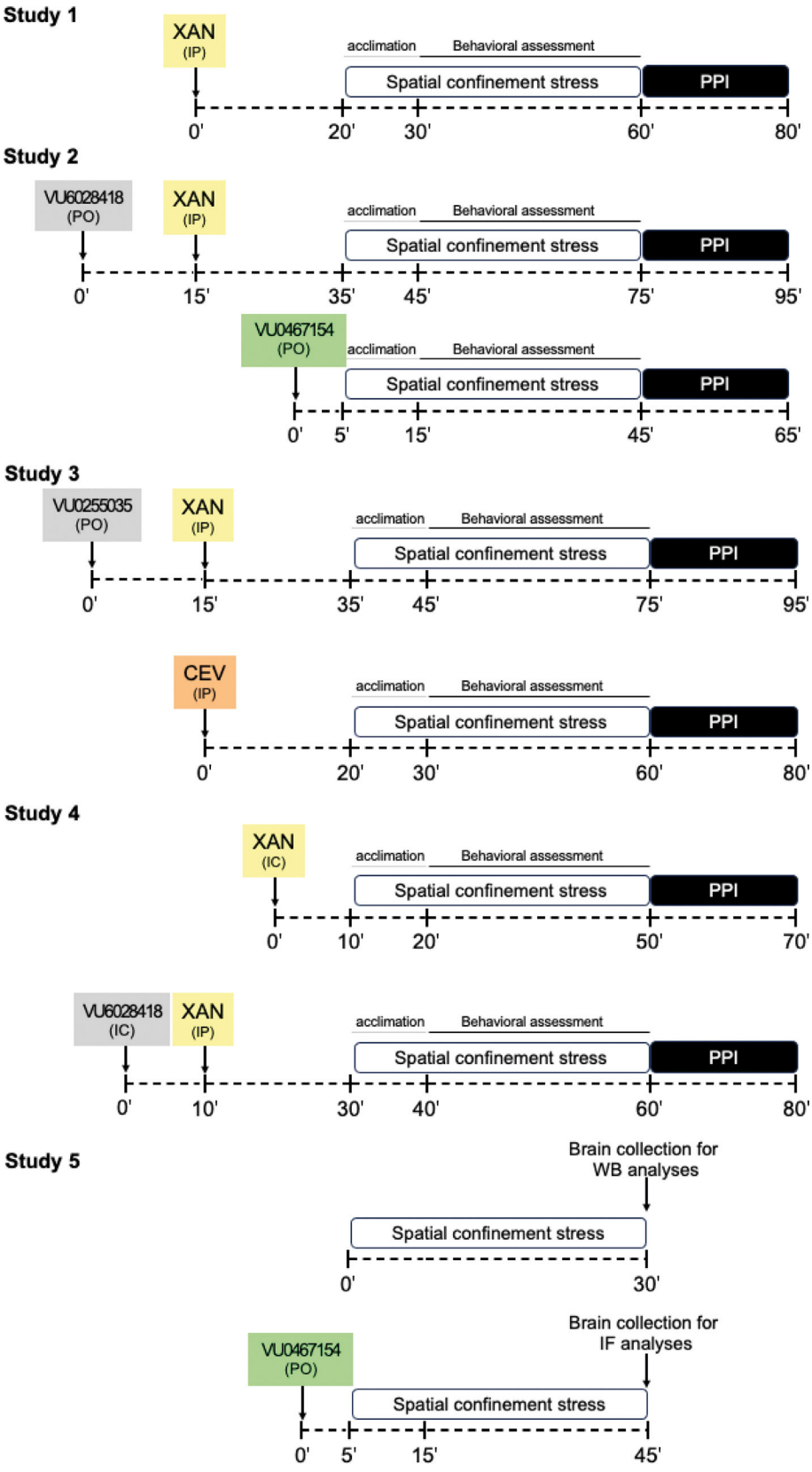

FIGURE 1 Outline of the experimental design. Study 1 evaluated the impact of systemic xanomeline (XAN) on behavioural responses related to TS and comorbid disorders. Study 2 was designed to evaluate the involvement of M_4 receptors in the effects of XAN, leveraging the selective M_4 antagonist, VU06028418 and the M_4 positive allosteric modulator (PAM), VU0467154. Study 3 was designed to evaluate the involvement of M_1 receptors in the effects of XAN, using the selective M_1 antagonist, VU0255035 and cevimeline (CEV), a muscarinic agonist with high affinity and potency on M_1 and M_3 but not M_4 receptors. Study 4 evaluated the involvement of the dorsal striatum in the behavioural effects of XAN. Study 5 was used for the neurochemical investigations. The timelines for treatments and procedures are outlined in each panel. Abbreviations: PPI, prepulse inhibition of the acoustic startle. WB, western blotting. IF, immunofluorescence. For further details, see text.

Study 4: Involvement of the dorsal striatum in the behavioural effects of XAN. Initially, CIN-d and D1CT-7 mice underwent bilateral cannulation in the dorsal striatum. Ten days later, local administration of XAN or vehicle (0.25 μg per side per mouse) was performed 10 min before subjecting the animals to spatial confinement. After a 10-min acclimation period, the TS-like

phenotype of the experimental animals was evaluated. In the subsequent experiment, different groups of animals were subjected to intrastriatal administration of the M_4-selective antagonist VU6028418 (0.25 μg per side per mouse) 10 min prior to administration of XAN (5 mg·kg^{-1}, IP) or its vehicle. Twenty min post-XAN treatment, animals were exposed to spatial confinement, and behaviour was assessed 10 min later.

Study 5: Neurochemical investigations. Initially, we measured the levels of M_1 and M_4 proteins in the striatum of CIN-d and control mice. Animals were euthanized by isoflurane anesthesia (5%) followed by rapid decapitation and their striata were harvested and processed for western blotting of M_1 and M_4 receptors (see methodological details). In a subsequent experiment aimed at understanding the neural correlates of the effects of the M_4 receptor PAM VU0467154 (3 mg·kg^{-1}, IP), animals were exposed to spatial confinement for 30 min after treatment. Subsequently, animals were anaesthetised and intracardially perfused with 60 ml of PBS. Brains were collected and fixed in 4% paraformaldehyde for 48 hours before being utilized for immunofluorescent staining (see detailed description below).

2.4 | Assessment of grooming and tic-like jerks

Both CIN-d and D1CT-7 mice were tested using a battery of behavioural tasks capturing TS-related responses. Grooming and tic-like jerks were measured under conditions of spatial confinement as previously described (Cadeddu et al., 2023; Godar et al., 2016). Briefly, animals were confined within a clear, bottomless Plexiglas cylinder (10 cm in diameter × 30 cm in height), placed in a familiar cage, and deeply sunk into the bedding to ensure stability. The procedure lasted 40 min, and behaviours were video recorded for the last 30 min to avoid potential behavioural alterations caused by neophobia induced by exposure to the unfamiliar enclosure. Grooming behaviour (including complete and incomplete sequences of licking, scratching, and washing the paws, head, body and tail), eye blinking, and axial head and body jerks were scored by trained observers blinded to experimental groups and treatments. Each animal was used only once in this procedure to avoid potential carry-over effects of stress.

2.5 | Acoustic PPI of the startle reflex

Startle testing was conducted in sound-attenuating ventilated startle chambers (SR-LAB, San Diego Instruments, San Diego, CA), as previously detailed (Bortolato et al., 2004). The acoustic PPI protocol featured a 70-dB background broadband noise (5 min acclimation period), followed by three consecutive blocks of 'pulse', 'prepulse + pulse' and 'no stimulus' trials. During the first and third blocks, mice received five 'pulse alone' trials of 115 dB. During the second block, animals underwent a pseudo-random sequence of 50 trials, consisting of 'pulse-alone' trials (12 events), pulses preceded by 73 (PP3), 76 (PP6), or 82 (PP12) dB 'prepulses' (10 events for each level of prepulse) and 'no stimulus' trials (eight events, consisting of background white noise delivery). Intertrial intervals (ITI) in both protocols were selected randomly between 10 and 15 s. A dynamic calibration system was used to ensure comparable sensitivities across chambers. Percent PPI (%PPI) was calculated using the formula (Mean Startle Amplitude, MSA, calculated as the mean peak voltage of the startle response to pulse-alone trials):

$$100 - MSA \times \text{`}pre-pulse\ pulse\text{'}\ \text{trials} / MSA \times \text{`}pulse\ alone\text{'}\ \text{trials} \times 100$$

Since our initial characterization of CIN-d mice showed a robust main effect of prepulse loudness levels (see Results) but no interactions between this factor and others (Cadeddu et al., 2023), values were averaged across the three prepulse loudness levels for all studies. When PPI and startle amplitude were measured in conjunction with confinement stress, PPI was tested after confinement.

2.6 | Open field

The open field was a Plexiglas square grey arena (40 × 40 cm) surrounded by four black walls (40 cm high). Two concentric zones of equivalent areas were defined on the floor: a central square quadrant and a peripheral frame directly adjacent to the walls. Thirty min after XAN (5 mg·kg^{-1}, IP) or vehicle treatment, mice were placed in the centre, and their behaviour was monitored for 5 min. Analysis of locomotor activity was performed using Ethovision (Noldus Instruments, The Netherlands). Behavioural measures included the distance travelled and duration in the central zone.

2.7 | Rotarod

Motor function and balance were measured using a rotarod apparatus (MED Associates Inc, St Albans, VT; ENV-574 M). The treadmill consists of a 3.6-cm cylindrical treadmill connected to a computer-controlled stepper motor-driven drum that can be programmed to operate at a constant speed or in a defined acceleration mode. When the mice fall off the rotating drum, individual sensors at the bottom of each separate compartment automatically record the latency to fall. Mice were trained at a 4-rpm constant speed for over 1 min. The test was based on a 5-min session in acceleration mode (4–40 rpm) 30 min after XAN (5 mg·kg^{-1}, IP) or vehicle treatment.

2.8 | Marble-burying test

Testing was performed as previously described (Godar et al., 2011). Each mouse was transferred individually into a clean home cage (29 × 17 × 13 cm) containing sawdust (5 cm thick). After 15 min of habituation, mice (n = 8 per group) were removed from the cage and treated with XAN (5 mg·kg^{-1}, IP) or vehicle. Thirty min later, 16 coloured marbles were homogeneously distributed on the surface

of the cage and mice were reintroduced to the cage and kept for 15 min under dim-light conditions (100 lx). At the end of the session, buried marbles were counted by personnel blind to condition and treatment. A marble was considered buried when at least 2/3 of its surface was covered by sawdust.

2.9 | Elevated plus maze

Anxiety-like behaviour was assessed using an elevated plus-maze as previously described (Wyatt et al., 2013). The maze consisted of two open arms (35 × 6 cm) and two closed arms (35 × 6 × 21 cm) extending from a central platform (6 × 6 cm) elevated at 74 cm from the ground. Environmental light was kept at 100 lx to promote exploration in a novel maze. Thirty min after XAN (5 mg·kg^{-1}, IP) or vehicle treatment, mice were placed in the centre area of the maze with their heads directed towards an open arm. The time spent in the open arms (with all four paws in the arm) and the overall number of head dips were measured by personnel blind to experimental groups.

2.10 | Tail suspension test

The tail suspension test was performed as described elsewhere (Bortolato et al., 2013). Thirty min after XAN (5 mg·kg^{-1}, IP) or vehicle treatment, mice were individually suspended by the tail using medical tape affixed to a hook 30 cm from the floor. Environmental light was kept at 300 lx. Animals were video recorded for 5 min and the duration of immobility was measured.

2.11 | Novel object recognition

We used a modified version of the protocol described in Bortolato et al. (2013) to evaluate how XAN may modify information encoding in D1CT-7 and WT mice. Animals were individually acclimatized to black Plexiglas cages (20 × 20 × 30 cm) for 15 min each. The day after, animals were treated with XAN (5 mg·kg^{-1}) or its vehicle. Thirty min later, they were placed into the same cages with two novel black plastic cylinders (8 cm tall × 3.5 cm in diameter), affixed to the floor and symmetrically placed at 6 cm from the two nearest walls. Mice were placed in a corner, facing the centre and at equal distance from the two objects. Their start position was rotated and counterbalanced for each treatment throughout the test. Twenty-four hours later, mice were placed in the same cage for long-term memory testing. One of the cylinders was replaced by a novel plastic rectangular block (6 cm tall × 3 × 3 cm), which was placed in a counterbalanced fashion to avoid bias. Behaviours for both sessions were videotaped for 15 min. Exploration was defined as sniffing or touching either of the two objects with the snout; sitting on the object was not considered exploration. In the second exploration trial, a novel exploration index (%NEI) was calculated as the ratio of the duration of the exploratory approaches targeting the novel objects over the time of exploration of both objects.

2.12 | Stereotaxic surgery and microinjections

Stereotaxic surgery was performed as previously described (Cadeddu et al., 2021). Briefly, mice were anaesthetised with **xylazine** / **ketamine** (20/80 mg·kg^{-1}, IP) and then placed onto a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Stainless steel 26-G bilateral guide cannulae (P1 Technologies Inc., Roanoke, VA, USA) were implanted in the dorsal striatum (AP + 1.2 mm, ML ± 1.6 mm, DV − 3.8 mm from the skull surface) according to the stereotaxic brain atlas of Paxinos and Franklin (2019). The lengths of the cannulae were selected to end 0.5 mm above the targeted area, and the injector projected 1 mm beyond the guide cannula. After implantation, cannulae were plugged with wire stylets to avoid clogging during the post-operative recovery. Antibiotic therapy was administered for 2 days (enrofloxacin, Bayer HealthCare, Shawnee Mission, KS, USA). Mice were allowed to recover in their home cages (single-housed) with food and water ad libitum. Four to 5 days after cannulation, mice received bilateral microinjections in the targeted area (as detailed below for each experiment). Microinjections were performed by gently restraining the animal, removing the stylet and replacing it with the injector (P1 Technologies Inc., Roanoke, VA, USA) connected to 250 µl Hamilton syringes via PE tubing. A microinfusion pump (KD Scientific, Holliston, MA, USA) delivered 0.5 µl per side of drug solution (or its vehicle solution) at a constant flow per rate of 0.25 µl·min^{-1}. After infusion, the injector was left in place for two min to allow fluid diffusion. Behavioural experiments were carried out immediately after infusion. After behavioural tests, animals were killed, and the histological verification of the cannula location was confirmed. Animals with errant locations of either device or damage to the targeted area(s) were excluded from the analysis.

2.13 | Western blotting

Mice were killed after induction of anaesthesia by **isoflurane** (5%) inhalation, and rapid decapitation via guillotine was performed. The dorsal striatum was dissected and flash-frozen for western blot analysis. Samples were stored at −80°C until assayed. To analyse the expression levels of M_1 and M_4 receptors, tissues were weighted and diluted (50 µg per 10 µl) in RIPA buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 µg·ml leupeptin and protease inhibitor cocktail. Small homogenate aliquots were used for protein determination using a modified Lowry protein assay method (DC protein assay, Bio-Rad Laboratories, Hercules, CA, USA). Samples containing 50 µg of total proteins were run in duplicate onto 4%–15% Criterion™ TGX Stain-free™ precast gels (Bio-Rad) and transferred into nitrocellulose membranes (Bio-Rad). Stain-free™ gel formulation includes a trihalo compound that, when exposed to ultraviolet (UV) irradiation, generates a covalent reaction with tryptophan residues of proteins and allows them to be visualized within the gel or after transfer to a blotting membrane. Following protein transfer, the

membrane was detected by UV, and a blot image was collected for total protein. Primary antibodies against M_1 (Cat# NBP1–87466, RRID: AB_11021120, dilution 1:750; Novus Biological, Littleton, CO), M_4 (Cat# NBP3-03052, RRID:AB_2943415, dilution 1:1000; Novus Biological) were incubated in TBS-T containing 3% (w/v) BSA buffer overnight at 4°C. Next, blots were washed in TBS-T and then incubated in TBS-T containing goat anti-rabbit HRP-conjugated (Cat# 31462, RRID: AB_228338, dilution 1:10000; ThermoFisher Scientific, Waltham, MA) secondary antibodies, for 90 min at room temperature. Chemiluminescence was detected with the ChemiDocTM XRS + Imaging System using the Clarity Western ECL substrate (Bio-Rad). Bands were quantified in arbitrary units and normalized using Image Lab (Bio-Rad) software. Samples containing the same amounts of total proteins in each experimental group were run on the same immunoblots and then analysed together. Membranes were normalized against total lane protein to control for equal loading.

2.14 | Immunofluorescence

Fixed brains were sliced to obtain 50 µm-coronal sections by using a vibratome (PELCO 1000, Redding, CA) and collected in 48-well plates filled with PBS + 0.02%NaN_3. Selected sections (containing the striatum) were used for the immunofluorescence protocol for c-Fos (monoclonal antibody, cat. #MA5-15055, RRID: AB_10984728, ThermoFisher). Briefly, after 3 washes in TBS (150 mM NaCl and 50 mM Tris–HCl pH = 7.4), sections were permeabilized in TBS-A (TBS + 0.1% TritonX) for 15 min and then blocked in TBS-B (TBS-A + 2% BSA). Primary antibody was diluted 1:200 in TBS-B and incubated overnight at 4°C. To remove the excess of unbound primary antibody, sections were washed sequentially in TBS-A and TBS-B. The secondary antibody (cat. #A-21244, RRID: AB_2535812, ThermoFisher) was diluted 1:200 in TBS-B and incubated for 1 h in the dark. Sections were washed in TBS, mounted on slides, and coverslipped with DAPI mountant (cat. # P36935, ThermoFisher). Five image fields within the x-y plane of the tissue section were randomly selected inside the dorsal striatum for each sample (n = 3 per group). They were acquired with a 40 × water immersion objective using the SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany) For each image, channels were split to allow separate pixel quantification using the following automated macro with the ImageJ software (available at www.imagej.net) in the 405-nm and 647-nm channels:

```
run('8-bi'");
run('Auto Threshold...,' 'method = MaxEntropy white');
makeRectangle(3, 3, 508, 504);
run('Measure')
```

2.15 | Statistical analyses

Normality and homoscedasticity of data distribution were tested using Kolmogorov–Smirnov and Bartlett's tests. Statistical analyses of behavioural and immunofluorescence data were performed using two-way ANOVA (with treatment and genotype per group as fixed factors). Multiple comparisons were performed using Tukey's test. Western-blotting data were analysed via a two-tailed *t*-test. All data are expressed as mean ± SEM, and analyses were performed using GraphPad Prism 10 statistical package (GraphPad, San Diego, CA, USA). Significance was set at $P < 0.05$. Data reporting and analyses were performed in compliance with the editorial guidelines on experimental design and analysis in pharmacology (Curtis et al., 2022).

2.16 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in https://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2022/23 (Alexander et al., 2023).

3 | RESULTS

3.1 | XAN reduces TS-related responses in CIN-d and D1CT-7 mice

We initially examined the impact of XAN (0.5–5 $mg{\cdot}kg^{-1}$, IP) on various behaviours relevant to TS in both CIN-d and D1CT-7 mouse models. Beginning with the CIN-d mice, we tested the effects of spatial confinement on grooming response in a two-way ANOVA design (Figure 2a). As previously reported (Cadeddu et al., 2023), CIN-d mice treated with saline exhibited an increased number of jerks during spatial confinement, but this effect was reversed by XAN (5 $mg{\cdot}kg^{-1}$, IP) (group × treatment interaction: $F[2,42] = 7.08$, $P = 0.002$). Similar effects were found with respect to stress-induced grooming (group × treatment interaction: $F[2,42] = 5.84$, $P = 0.006$) (Figure 2b). CIN-d mice had no alteration in startle amplitude (Figure 2c) but did exhibit PPI deficits, which were reduced by XAN (group × treatment interaction: $F[2,42] = 4.26$, $P = 0.02$) (Figure 2d). D1CT-7 mice exhibited a markedly greater number of body and head jerks under confinement compared to their WT counterparts (genotype × treatment interaction: $F[2,42] = 6.06$, $P = 0.005$). This alteration was mitigated by XAN administration (Figure 2e). Although a statistically significant interaction effect was found between XAN treatment and genotype ($F[2,42] = 4.51$, $P = 0.017$), Tukey's test did not identify significant differences (Figure 2f). While no significant differences were observed in startle amplitude (Figure 2g), a significant genotype × treatment interaction ($F[2,42] = 6.61$, $P = 0.003$) revealed that XAN reversed the pronounced PPI deficit exhibited by D1CT-7 mutants (Figure 2h). We next explored the effects of XAN (5 $mg{\cdot}kg^{-1}$, IP) in D1CT-7 mice on other behavioural phenotypes (Figure S1). No significant effects of XAN were found in open-field locomotor activity, rotarod assay, tail-suspension paradigm, and encoding in the novel object recognition task. Notably, XAN treatment resulted in a substantial reduction in marble-burying behaviour in both genotypes (main effect of XAN: F

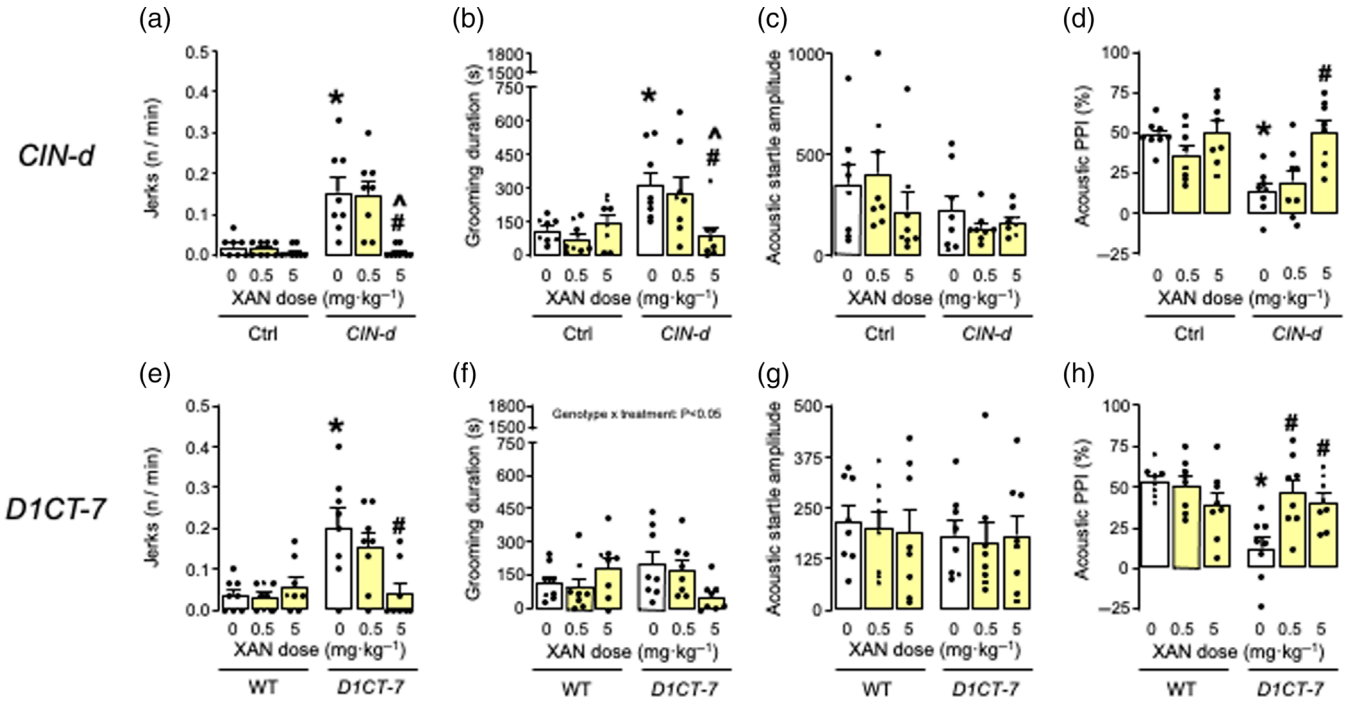

FIGURE 2 Xanomeline (XAN; 0.5–5 mg·kg^{-1}, IP) reduced TS-related behaviours. Panels A-D show the effects of XAN on (a) frequency of body and head jerks, (b) overall duration of grooming stereotypies, (c) acoustic startle amplitude and (d) percent of acoustic prepulse inhibition (PPI) in CIN-d and control (Ctrl) mice. Panels (e)–(h) represent the effects of XAN on the same behavioural paradigms in D1CT-7 and wild-type (WT) male littermates. Data were analysed with two-way ANOVAs. *, $P < 0.05$ for comparisons of vehicle-treated D1CT-7 versus WT or CIN-d versus Ctrl mice; #, $P < 0.05$ for comparison between XAN and its vehicle in D1CT-7 or CIN-d mice; ^, $P < 0.05$ for comparison between 0.5 and 5 mg·kg^{-1} XAN in CIN-d mice. All data are shown as means ± SEM. n = 8 per group. For further details, see text.

[1,28] = 7.98, $P = 0.009$) (Figure S1d). Finally, there was no reduction in open-arm time in the elevated plus maze (Figure S1e); however, XAN reduced the number of head dips in this paradigm in both genotypes (main effect of XAN: F[1,28] = 9.54, $P = 0.005$) (Figure S1f).

3.2 | The M_4-selective antagonist VU6028418 reverses the effects of XAN in TS-related responses in CIN-d and D1CT-7 mice

Building on the finding of ameliorative properties of XAN in both mouse models of TS, we tested the involvement of M_4 receptors in these effects. To this end, we investigated whether the M_4 receptor antagonist VU6028418 (1–3 mg·kg^{-1}, PO) could counteract the effects of XAN in CIN-d and D1CT-7 mice. The analysis of jerks in CIN-d mice disclosed a significant interaction between XAN and VU6028418 (F[2,42] = 3.31, $P = 0.046$), which reflected the ability of VU6028418 to reverse the ameliorative effects of XAN (Figure 3a). Similarly, VU6028418 (3 mg·kg^{-1}) reversed the effects of XAN on grooming (treatment × treatment interaction: F[2,42] = 11.26, $P = 0.0001$) (Figure 3b). While no significant differences were observed in startle analysis (Figure 3c), we found a significant interaction between treatments in PPI (F[2,42] = 18.40, $P < 0.00001$), which was due to the reversal of XAN's effects by both doses of VU6028418 (Figure 3d). The analysis of body and head jerks in D1CT-7 mice revealed a significant interaction between treatments (F[2,41] = 5.34, $P = 0.009$), indicating that VU6028418 (1 mg·kg^{-1}) reversed the effects of XAN (Figure 3e). The same type of effect was found in grooming (XAN × VU6028418 interaction: F[2,42] = 12.42, $P = 0.00006$) (Figure 3). The analysis of acoustic startle amplitude did not yield any significant differences (Figure 3g). Conversely, PPI analyses disclosed that both doses of VU6028418 fully reversed the ameliorative effects of systemic XAN in this index (XAN × VU6028418 interaction: F[2,42] = 6.51, $P = 0.003$) (Figure 3h).

3.3 | The M_4-selective positive allosteric modulator VU0467154 reduces TS-related responses in CIN-d and D1CT-7 mice

To verify whether M_4 activation is not only necessary, but also sufficient to elicit therapeutic effects in TS, we examined the effects of the positive allosteric modulator (PAM) VU0467154 (0.3–3 mg·kg^{-1}, IP) on TS-related behavioural responses. Both doses of VU0467154 significantly opposed tic-like jerks in CIN-d mice (genotype × treatment interaction: F[2,42] = 9.584, $P = 0.0004$) (Figure 4a). Conversely, only the highest dose of VU0467154 mitigated the increase in grooming behaviour exhibited by CIN-d mice under confinement (VU0467154 × genotype interaction: F[2,42] = 10.35, $P = 0.0002$) (Figure 4b). CIN-d mice exhibited a significant elevation in startle

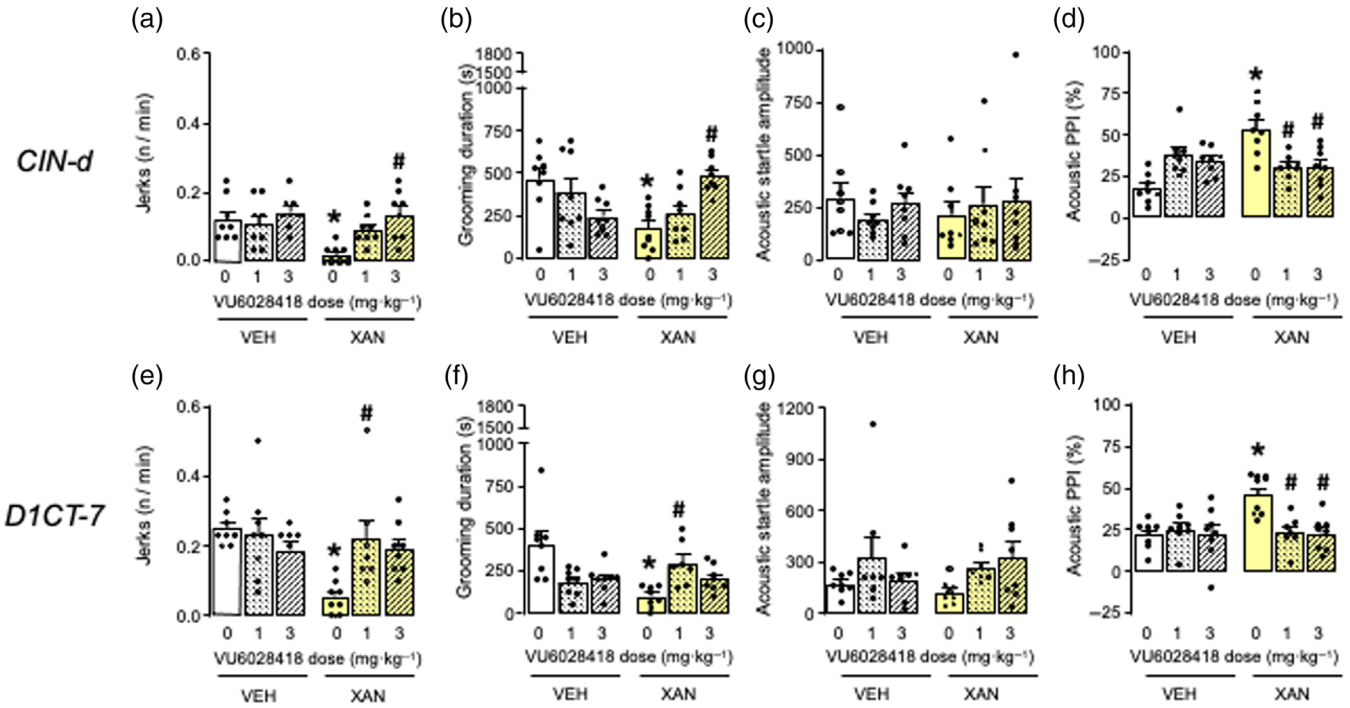

FIGURE 3 The M_4-selective antagonist VU6028418 (1–3 mg·kg^{-1}, PO) reverses the ameliorative effects of xanomeline (XAN; 5 mg·kg^{-1}, IP) in TS-related behaviours. Panels A-D show the combined effects of VU6028418 and XAN on (a) frequency of body and head jerks, (b) overall duration of grooming stereotypies, (c) acoustic startle amplitude and (d) acoustic prepulse inhibition (PPI) in CIN-d and control (Ctrl) mice. Panels (e)–(h) represent the effects of VU6028418 and XAN on the same behavioural paradigms in D1CT-7 and wild-type (WT) male littermates. Data were analysed with two-way ANOVAs. *, $P < 0.05$ for comparisons of vehicle-treated D1CT-7 versus WT or CIN-d versus Ctrl mice; #, $P < 0.05$ for comparison between XAN and its vehicle in D1CT-7 or CIN-d mice. All data are shown as means ± SEM. n = 8 per group. For further details, see text.

amplitude in this cohort (main effect of group: $F[1,42] = 5.39$, $P = 0.025$) (Figure 4c); however, this effect was not impacted by VU0467154 treatment. Finally, the highest dose of the M_4 PAM reversed the PPI deficits in CIN-d mice (group × treatment interaction: $F[2,42] = 3.776$, $P = 0.059$) (Figure 4d).

In D1CT-7 mice, VU0467154 led to similar improvements in body and head jerks (group × treatment interaction: [Figure 6g]. $F[2,42] = 3.68$, $P = 0.03$) (Figure 4e) and stress-induced grooming (group × treatment interaction: $F[2,42] = 12.99$, $P = 0.00004$) (Figure 4f). No differences in startle amplitude were observed (Figure 4g); however, PPI analysis revealed a significant genotype × treatment interaction ($F[2,42] = 3.92$, $P = 0.03$), indicating that VU0467154 (3 mg·kg^{-1}) opposed a marked deficit in this index in spatially confined D1CT-7 mice (Figure 4h).

3.4 | The M_1-selective antagonist VU0255035 does not reverse the effects of XAN in TS-related responses in CIN-d and D1CT-7 mice

We next tested the contribution of the M_1 receptors to the ameliorative effects of XAN (5 mg·kg^{-1}, IP). To this end, we evaluated the effects of the M_1-selective antagonist VU0255035 (3–30 mg·kg^{-1}, PO) in XAN-treated CIN-d and D1CT-7. In CIN-d mice, VU0255035 failed to significantly counteract the effect of XAN on tic-like jerks (main effect of XAN: $F[1,42] = 43.67$, $P < 0.00001$; XAN × VU0255035 interaction: $F[2,42] = 2.13$, $P = 0.11$) (Figure 5a) and grooming stereotypies (main effect of XAN: $F[1,42] = 49.53$, $P < 0.00001$; XAN × VU0255035 interaction: $F[2,42] = 2.127$, $P = 0.12$) (Figure 5b). While the analysis of startle amplitude did not yield any significant findings (Figure 5c), a significant XAN × VU0255035 interaction was observed in the analysis of PPI values (Figure 5d) ($F[2,42] = 4.27$, $P = 0.02$), reflecting the fact that XAN increased PPI irrespective of VU0255035 co-treatment.

In D1CT-7 mice, XAN reduced confinement-associated jerks, but this was not reversed by the M_1 receptor antagonist (main effect of XAN: $F[1,42] = 36.51$, $P < 0.0001$; XAN × VU0255035 interaction: $F[2,42] = 0.15$, $P = 0.86$) (Figure 5e); furthermore, XAN decreased grooming responses, and this effect was not countered by VU0255035 (main effect of XAN: $F[1,42] = 19.86$, $P = 0.00006$; XAN × VU0255035 interaction: $F[2,42] = 1.37$, $P = 0.26$) (Figure 5f). Neither XAN nor VU0255035 exerted any influence on startle amplitude (Figure 5g). XAN again enhanced PPI values in D1CT-7 mice (main effect of XAN: $F[1,42] = 51.45$, $P < 0.00001$); in this case, VU0255035 partially reversed the effects of XAN (XAN × VU0255035 interaction: $F[2,42] = 3.27$, $P = 0.048$); although Tukey's test identified no significant effects of VU0255035 in combination of XAN, no significant difference was found between the

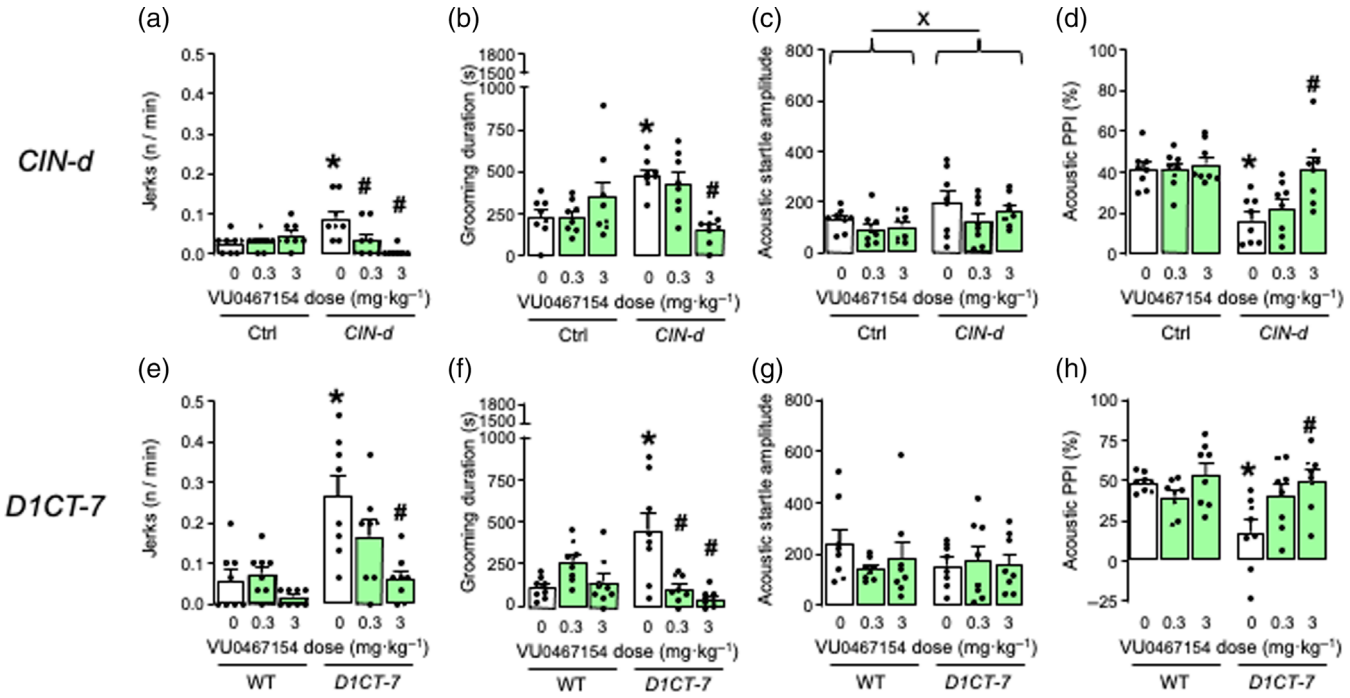

FIGURE 4 The M_4-selective positive allosteric modulator VU0467154 (0.3–3 mg·kg^{-1}, IP) reduced TS-related behaviours. Panels (a)–(d) show the effects of VU0467154 on (a) frequency of body and head jerks, (b) overall duration of grooming stereotypies, (c) acoustic startle amplitude and (e) percent of acoustic prepulse inhibition (PPI) in CIN-d and control (Ctrl) mice. Panels (e)–(h) represent the effects of VU0467154 on the same behavioural paradigms in D1CT-7 and wild-type (WT) male littermates. Data were analysed with two-way ANOVAs. ^{x}, $P < 0.05$ for main-effect comparison of group (CIN-d vs. Ctrl); *, $P < 0.05$ for comparisons of vehicle-treated D1CT-7 versus WT or CIN-d versus Ctrl mice; $^{\#}$, $P < 0.05$ for comparison between XAN and its vehicle in D1CT-7 or CIN-d mice. All data are shown as means ± SEM. n = 8 per group. For further details, see text.

highest dose of VU0255035 and XAN and the combination of the M_1 antagonist with the vehicle of XAN, suggesting a partial involvement of M_1 receptors in the effects of XAN on sensorimotor gating (Figure 5h).

3.5 | CEV does not reduce TS-related responses in CIN-d and D1CT-7 mice

In a converse experiment, we assessed the effects of CEV (5–10 mg·kg^{-1}, IP). This agent was selected because it is (i) approved for clinical use and (ii) a potent agonist of M_1, M_3 and to a lesser extent M_5, receptors, with low affinity in the micromolar range for M_4 receptors (Heinrich et al., 2009). CEV is therefore optimally suited to compare and contrast its effects with those of XAN and identify the relative influence of M_1 and M_4 receptors on TS-related behaviours. In CIN-d mice, CEV did not attenuate jerks (main effect of group: $F[1,42] = 19.14$, $P = 0.00008$; genotype × treatment interaction: $F[2,42] = 1.54$, $P = 0.23$) (Figure 6a) or grooming stereotypies under confinement (main effect of group: $F[1,42] = 8.596$, $P = 0.005$; genotype × treatment interaction: $F[2,42] = 1.48$, $P = 0.24$) (Figure 6b). Furthermore, CEV did not affect startle amplitude (Figure 6c) as well as PPI deficits observed in CIN-d mice (main effect of group: $F[1,42] = 24.11$, $P = 0.00001$; genotype × treatment interaction: $F[2,42] = 0.98$, $P = 0.38$) (Figure 6d). As before, D1CT-7 mice exhibited a greater frequency of tic-like jerks relative to wild-type controls; CEV did not significantly modulate neither tic-like jerks (main effect of genotype: $F[1,42] = 38.18$, $P < 0.00001$; genotype × treatment interaction: $F[2,42] = 2.182$, $P = 0.13$) (Figure 6e) nor grooming (Figure 6f). Furthermore, CEV did not influence startle amplitude (Figure 6g) and failed to ameliorate the PPI impairments that were again observed in D1CT-7 mice (main effect of genotype: $F[1,42] = 38.18$, $P < 0.00001$; genotype × treatment interaction: $F[2,42] = 0.49$, $P = 0.62$) (Figure 6h).

3.6 | Intrastriatal XAN reduces TS-related responses in CIN-d and D1CT-7 mice

To ascertain whether the effects of XAN were mediated through the activation of muscarinic receptors within the striatum, we tested the impact of intrastriatal XAN (0.25 µg per side per mouse) on TS-related behaviours in CIN-d and D1CT-7 mouse models. Intrastriatal XAN reduced motor jerks (group × treatment interaction: $F[1,20] = 20.86$, $P = 0.0002$) (Figure 7a) and grooming behaviour (group × treatment interaction: $F[1,20] = 11.29$, $P = 0.003$) (Figure 7b) of CIN-d mice. Startle amplitude analysis revealed a significant main effect of XAN ($F[1,20] = 15.00$, $P = 0.0009$), but no group differences

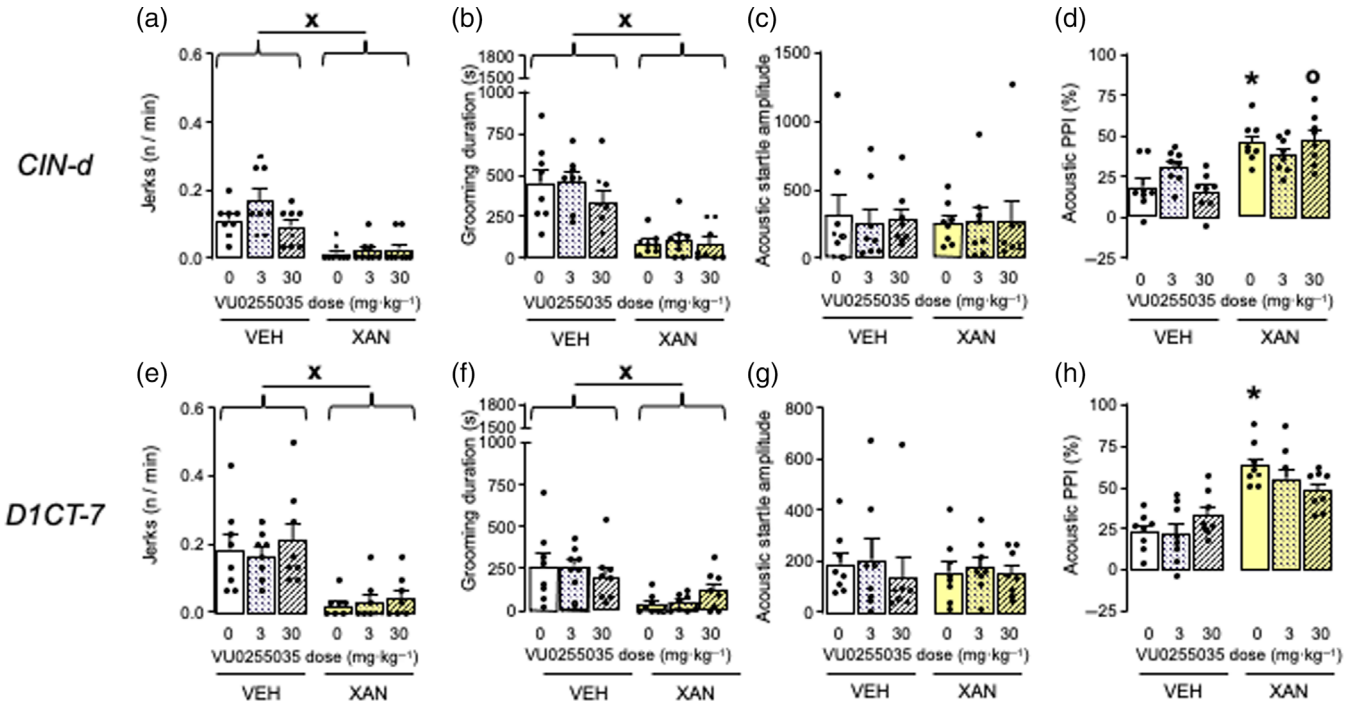

FIGURE 5 The M_1-selective antagonist VU0255035 (3–30 mg·kg^{-1}, PO) does not reverse the ameliorative effects of xanomeline (XAN; 5 mg·kg^{-1}, IP) in TS-related behaviours. Panels A-D show the combined effects of VU0255035 and XAN on (a) frequency of body and head jerks, (b) overall duration of grooming stereotypies, (c) acoustic startle amplitude and (d) acoustic prepulse inhibition (PPI) in CIN-d and control (Ctrl) mice. Panels (e)–(h) represent the effects of VU0255035 and XAN on the same behavioural paradigms in D1CT-7 and wild type (WT) male littermates. Data were analysed with two-way ANOVAs. ^{X}, $P < 0.05$ for main effect comparison of vehicle (VEH) with XAN; *, $P < 0.05$ for comparisons of vehicle-treated D1CT- 7 versus WT or CIN-d versus Ctrl mice; ^{O}, $P < 0.05$ for comparison of XAN and VU0244035 (30 mg·kg^{-1}) versus VEH and VU0244035 (30 mg·kg^{-1}). All data are shown as means ± SEM. n = 8 per group. For further details, see text.

were detected (group × XAN interaction: $F[1,20] = 1.36$, $P = 0.26$) (Figure 7c). XAN was found to reverse the PPI deficits observed in CIN-d mice (group × treatment interaction: $F[1,20] = 8.83$, $P = 0.008$) (Figure 7d). In the D1CT-7 mouse model, XAN opposed the increase in body and head jerks (genotype × treatment: $F[1,20] = 13.75$, $P = 0.001$) (Figure 7e), even though it failed to mitigate the increase in stress-induced grooming behaviour (main effect for genotype: $F[1,20] = 10.21$, $P = 0.0045$; treatment × genotype interaction: $F[1,20] = 0.61$, $P = 0.44$) (Figure 7f). While no significant differences were observed in startle amplitude (Figure 7g), PPI analysis revealed a significant genotype × treatment interaction ($F[1,20] = 5.908$, $P = 0.02$), indicating a significant impairment in D1CT-7 mice ($P < 0.01$ for comparison between WT and D1CT-7 with vehicle), which was reversed by intrastriatal XAN ($P < 0.05$ between D1CT-7 treated with vehicle and XAN) (Figure 7h).

3.7 | Intrastriatal infusion of VU6028418 reverses the effects of systemic XAN in TS-related responses in CIN-d and D1CT-7 mice

To elucidate whether the effects elicited by XAN were contingent upon the activation of striatal M_4 receptors, we assessed how the effects of systemic XAN were modified by intrastriatal infusions of the M_4 receptor antagonist VU6028418 in CIN-d and D1CT-7 mouse models. A significant interaction emerged between the two treatments in confinement-induced jerking behaviour in CIN-d mice ($F[1,19] = 12.91$, $P = 0.002$) (Figure 8a). A similar interaction was found for grooming behaviour (interaction: $F[1,20] = 4.70$, $P = 0.04$) (Figure 8b). A main effect of XAN was found on startle amplitude ($F[1,20] = 6.19$, $P = 0.02$), but this was unaffected by VU6028418 treatment (interaction: $F[1,20] = 1.125$, $P = 0.30$) (Figure 8c). Lastly, PPI evaluations yielded a significant interaction between treatments ($F[1,20] = 12.21$, $P = 0.002$), which was found to reflect the positive effect of XAN in this index. Nevertheless, intrastriatal VU6028418 administration failed to negate the effects of systemic XAN (Figure 8d).

In D1CT-7 mice, the M_4 receptor antagonist significantly blocked the ameliorative effects of XAN on motoric jerks (XAN × VU6028418 interaction: $F[1,20] = 4.65$, $P = 0.043$) (Figure 8e) and grooming (XAN × VU6028418 interaction: $F[1,20] = 17.29$) (Figure 8f). In evaluating startle amplitude, we detected a significant XAN × VU interaction ($F[1,20] = 4.69$; $P = 0.043$), which was found to reflect the ability of XAN to reduce this parameter in co-treatment with VU6028418 (Figure 8g). A main effect of XAN was found on PPI ($F[1,20] = 31.29$, $P = 0.00002$), but this effect was not modified by intrastriatal VU6028418 (interaction: $F[1,20] = 0.86$, $P = 0.37$) (Figure 8h).

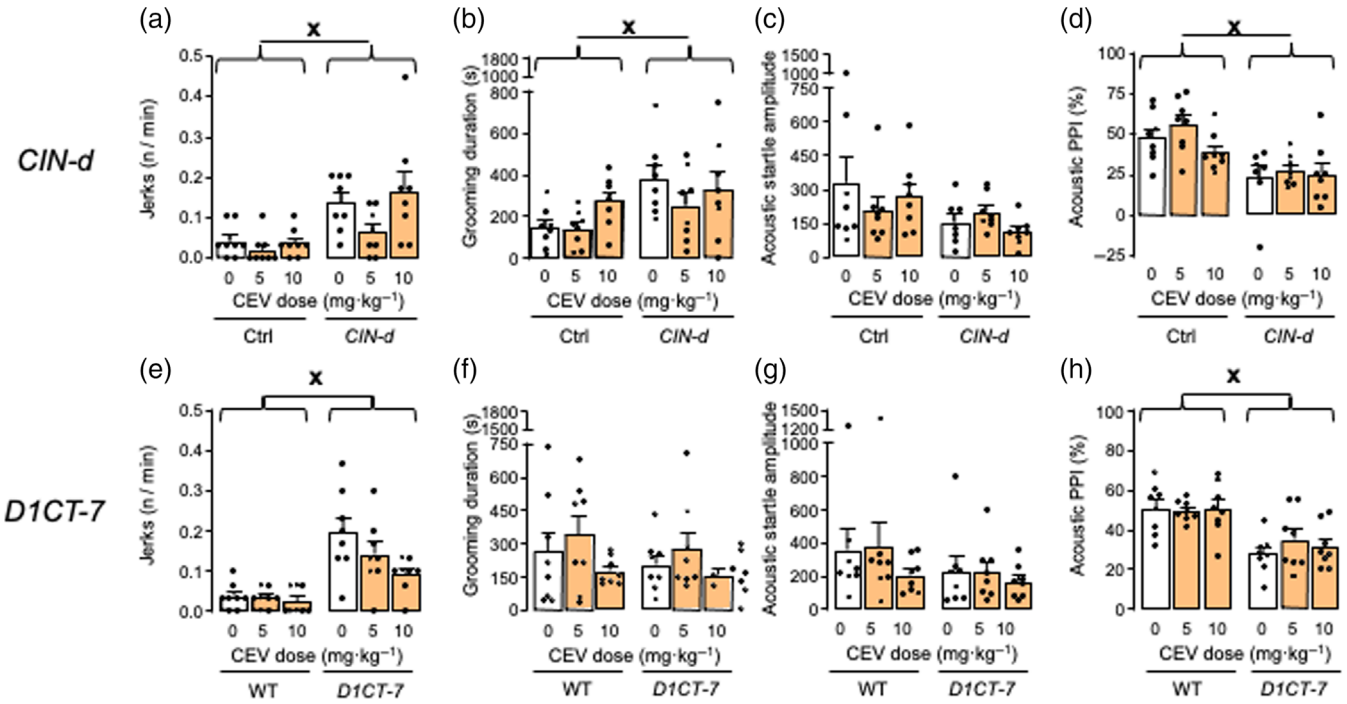

FIGURE 6 Cevimeline (CEV; 5–10 mg·kg^{-1}, IP) failed to reduce TS-related behaviours. Panels A-D show the effects of CEV on (a) frequency of body and head jerks, (b) overall duration of grooming stereotypies, (c) acoustic startle amplitude and (d) percent of acoustic prepulse inhibition (PPI) in CIN-d and control (Ctrl) mice. Panels (e)–(h) represent the effects of CEV on the same behavioural paradigms in D1CT-7 and wild type (WT) male littermates. Data were analysed with two-way ANOVAs. ^{X}, $P < 0.05$ for main effect comparison of genotype (D1CT-7 vs. WT) or group (CIN-d vs. Ctrl). All data are shown as means ± SEM. n = 8 per group. For further details, see text.

3.8 | CIN-d mice show a significant reduction in M_4, but not M_1, receptor levels in the dorsal striatum

To investigate whether the depletion of cholinergic interneurons in CPu lead to a different expression of M_1 and M_4 receptors, we analysed total lysates of CIN-d and Ctrl mice via Western blot (Figure 9a, n = 6 per group). *t*-test analyses revealed no significant difference in M_1 expression between Ctrl and CIN-d mice ($t[11] = 0.87$, NS) (Figure 9b). Conversely, M_4 receptor levels were significantly reduced in the striatum of CIN-d mice ($t[11] = 2.49$; $P = 0.03$) (Figure 9c).

3.9 | VU0467154 reduced c-Fos levels in the dorsal striatum of CIN-d mice after exposure to spatial confinement

Building on these data, we hypothesized that the behavioural effects of the M_4 PAM, VU0467154 reflected a selective change in the neuronal activity in the striatum of animal models of tic pathophysiology. To test this hypothesis, we measured levels of c-Fos, a well-established marker of neural activity, in CIN-d and control mice treated either with vehicle or VU0467154 before exposure to spatial confinement (Figure 10a). We detected a significant interaction between genotype and treatment (interaction: $F[1,49] = 43.71$, $P < 0.00001$) (Figure 10b). Notably, stressed CIN-d mice exhibited significantly higher c-Fos levels compared to stressed control mice. Treatment with VU0467154 reduced c-Fos expression levels in CIN-d mice. In striking contrast with these results, the M_4 PAM markedly increased c-Fos levels in control mice.

4 | DISCUSSION

The main results of this study showed that systemic and intrastriatal administration of XAN reduced tic-related motoric behaviours and PPI deficits in two distinct mouse models capturing complementary mechanisms of tic pathophysiology. Many of XAN's effects were blocked by systemic or intrastriatal administration of the M_4 receptor antagonist VU6028418, while the M_1 antagonist VU0255035 had little effect. In line with these findings, the M_4-selective PAM VU0467154 elicited a robust amelioration of tic-related responses and PPI deficits. This effect was not observed following treatment with CEV, a potent agonist for M_1 and M_3, but not M_4 receptors. These data indicate that striatal M_4 receptor activation is required to reduce TS-related behavioural abnormalities in relation to different models capturing complementary aspects of tic pathophysiology.

The effects of XAN and other muscarinic receptor activators were first tested in CIN-d mice (Cadeddu et al., 2023). These animal models are designed to reproduce the loss of CINs observed in postmortem striatal specimens from TS-affected subjects (Kalanithi et al., 2005; Kataoka et al., 2010). When confronted with spatial confinement stress, CIN-d mice engage in grooming stereotypies and tic-like

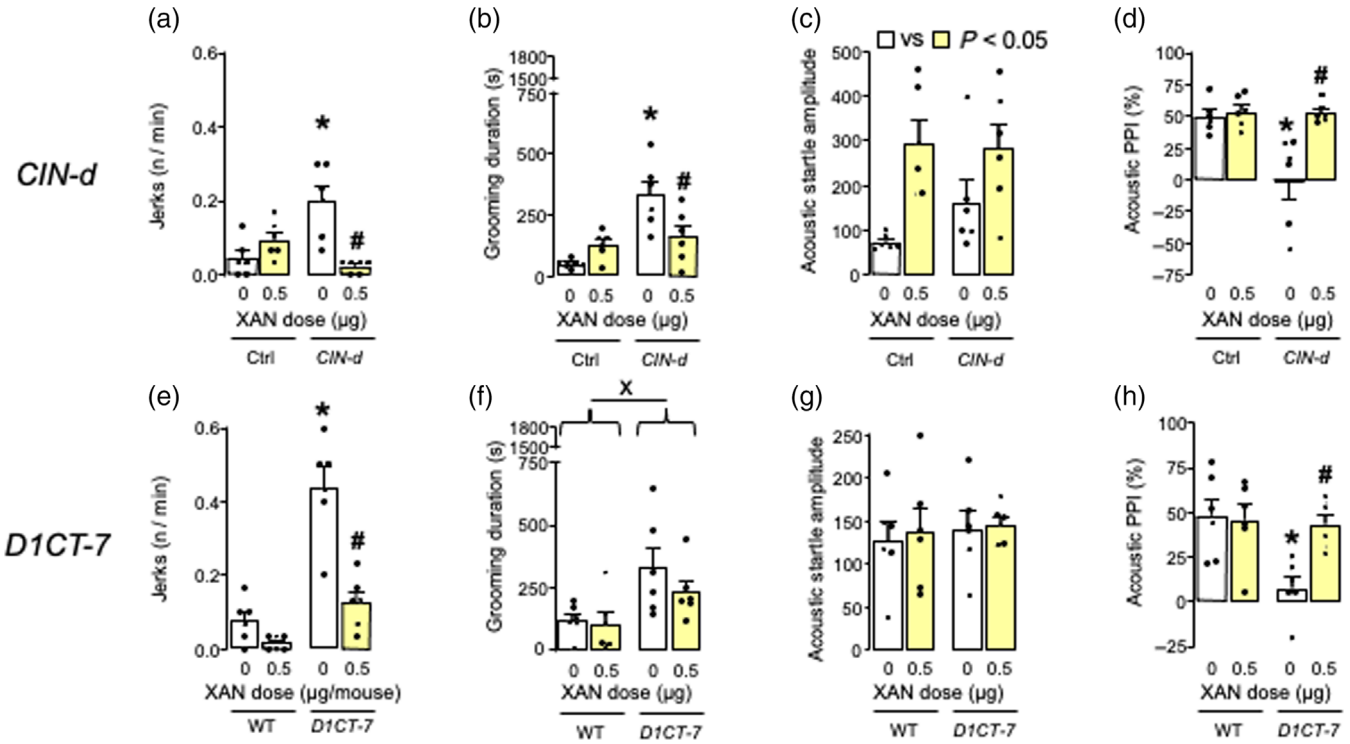

FIGURE 7 Intrastriatal xanomeline (XAN; 0.25 µg per side per mouse) reduced TS-related behaviours. Panels (a)–(d) show the effects of XAN on (a) frequency of body and head jerks, (b) overall duration of grooming stereotypies, (c) acoustic startle amplitude and (d) percent of acoustic prepulse inhibition (PPI) in CIN-d and control (Ctrl) mice. Panels (e)–(h) represent the effects of XAN on the same behavioural paradigms in D1CT-7 and wild type (WT) male littermates. Data were analysed with two-way ANOVAs. ^{X}, $P < 0.05$ for main effect of genotype (D1CT-7 vs. WT); *, $P < 0.05$ for comparisons of vehicle-treated D1CT-7 versus WT or CIN-d versus Ctrl mice; $^{\#}$, $P < 0.05$ for comparison between XAN and its vehicle in D1CT-7 or CIN-d mice. All data are shown as means ± SEM. n = 8 per group. For further details, see text.

sporadic motor jerks (Cadeddu et al., 2023; Xu et al., 2015). These abnormal responses are likely underpinned by reduced striatal cholinergic neurotransmission, resulting in excess activation of D_1- and D_2-SPNs. Accordingly, the antagonism of both D_1 and D_2 receptors reversed their abnormal phenotypes (Cadeddu et al., 2023). In this study, we documented a decrease in M_4 receptors, but not M_1 receptors, within the dorsal striatum of CIN-d mice. This reduction in M_4 receptors likely contributes to the atypical behaviours exhibited by CIN-d mice. This inference is supported by the finding that activation of M_4 receptors reduces tic-like behaviours and other TS-related abnormal responses to levels comparable with those of control animals. In further support of this idea, the administration of the M_4-selective PAM VU0467154 normalized the levels of c-Fos within the striatum of stress-exposed CIN-d mice. This transcription factor is a well-established indicator of neural activity (Herrera & Robertson, 1996). Conversely, VU0467154 increased c-Fos in control mice. This dichotomous effect may signify that, in CIN-d mice, due to the reduction of M_4 autoreceptors in CINs, M_4 activators may primarily impact postsynaptic receptors on D_1-SPNs and heteroreceptors on glutamatergic and dopaminergic afferents. As mentioned earlier, the activation of these receptors counteracts the effects of D_1 receptor stimulation in SPNs (Jeon et al., 2010; Mamaligas & Ford, 2016; Nair et al., 2019), resulting in an overall reduction of neural activity within this brain region of CIN-d mice. Conversely, in control animals, where there is no depletion of CINs, it is expected that VU0467154 would exert a greater action on M_4 autoreceptors, leading to a decrease in local acetylcholine release (Bymaster et al., 2003) and a reduction in the modulatory function of CINs on SPNs. An alternative explanation for the observed effects may come from the finding that M_4 receptors induce intracellular calcium currents in direct pathway SPNs (Hernández-Flores et al., 2015). Notably, both the activation and blockade of striatal M_4 receptors have been linked to **levodopa**-induced dyskinesias, which also are dependent on the excessive activation of D_1-SPNs, underscoring the diverse effects of different M_4 receptor subpopulations in the striatum (Brugnoli et al., 2020). Further research is needed to elucidate the cellular mechanisms underlying M_4-dependent effects in distinct striatal cell populations.

The effects of XAN and other muscarinic receptor activators were tested in D1CT-7 mice. These mice harbour a transgene expressing the enzymatic subunit A_1 under the control of the dopamine D_1 receptor promoter (Campbell et al., 1999; Nordstrom & Burton, 2002). D1CT-7 mice exhibit sudden axial jerks and other hyperkinetic manifestations (Campbell et al., 1999; Nordstrom & Burton, 2002). In the presence of acute stress, these responses are dramatically exacerbated (Godar et al., 2016), and several other TS-relevant deficits emerge, including PPI deficits (Godar et al., 2016) akin to those observed in TS patients (Swerdlow et al., 2001). Tic-like behaviours in D1CT-7 are currently interpreted as resulting from the

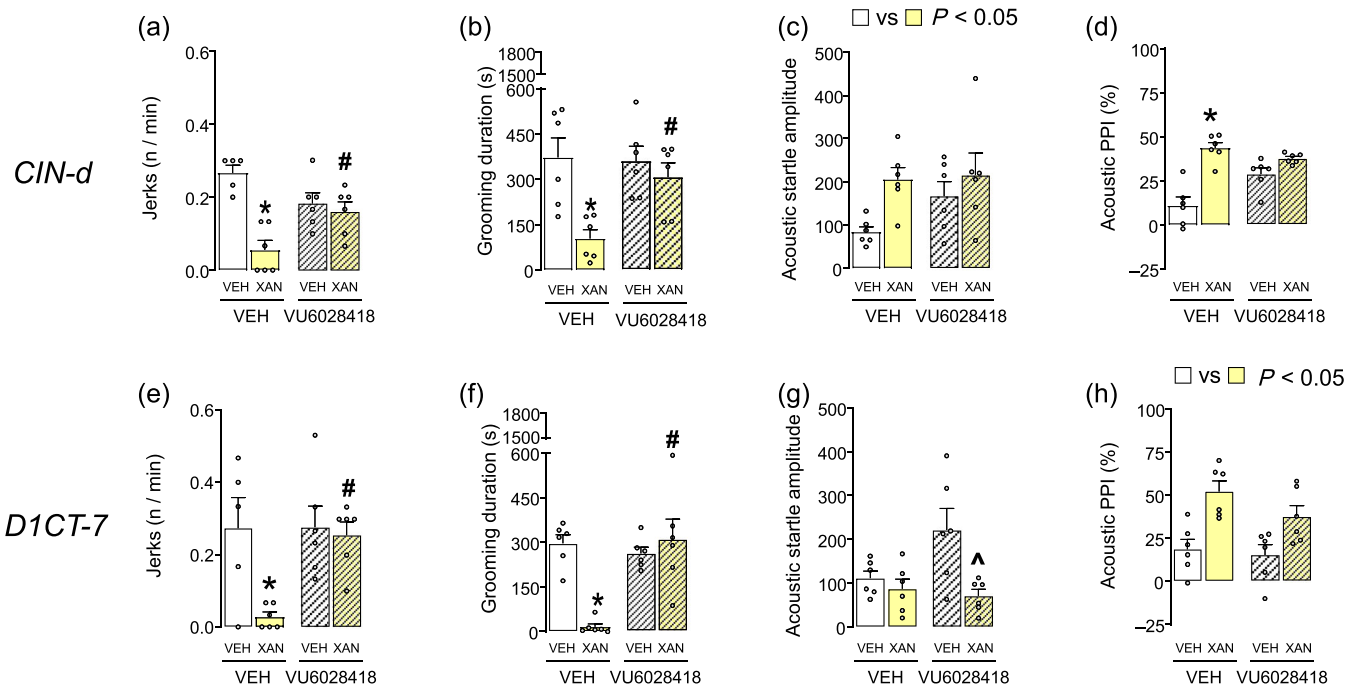

FIGURE 8 Intrastriatal infusions of the M_4-selective antagonist VU6028418 (0.25 μg per side per mouse) reverses the ameliorative effects of xanomeline (XAN; 5 mg·kg^{-1}, IP) in TS-related behaviours. Panels (a)–(d) show the combined effects of VU6028418 and XAN on (a) frequency of body and head jerks, (b) overall duration of grooming stereotypies, (c) acoustic startle amplitude and (d) acoustic prepulse inhibition (PPI) in CIN-d and control (Ctrl) mice. Panels (e)–(h) represent the effects of VU6028418 and XAN on the same behavioural paradigms in D1CT-7 and wild type (WT) male littermates. Data were analysed with two-way ANOVAs. *, $P < 0.05$ for comparisons of vehicle (VEH) + vehicle (VEH) versus XAN + VEH; #, $P < 0.05$ for comparison between XAN and VU6028418 and XAN and VEH. All data are shown as means ± SEM. n = 8 per group. For further details, see text.

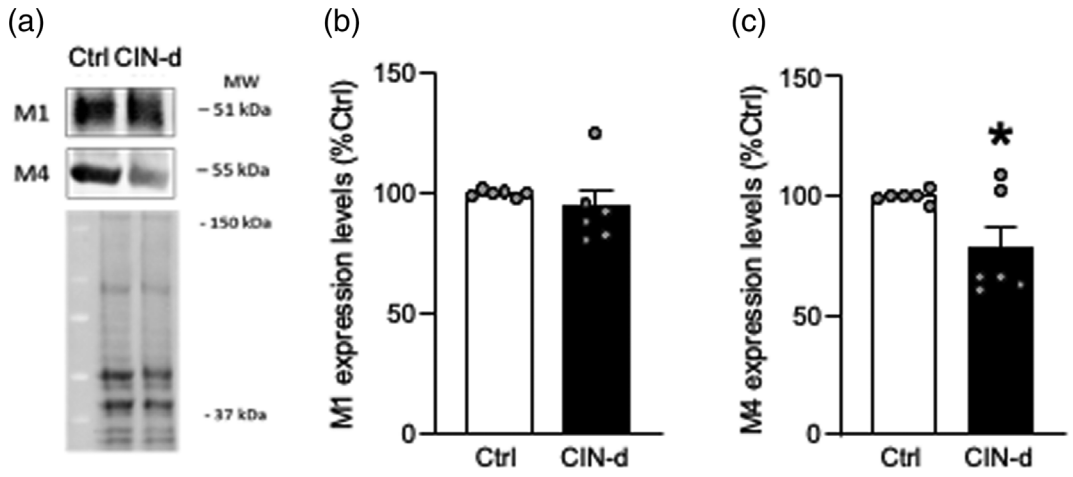

FIGURE 9 CIN-d mice show a significant reduction in M_4, but not M_1, receptor levels in the dorsal striatum. Representative immunoblot (a) and quantification of M_1 (b) and M_4 (c) expression levels in dorsal striatum expressed as a percentage of the control group (Ctrl). Values are expressed as mean ± SEM, n = 6.

neuropotentiation of a subset of pyramidal neurons in layer 2/3 of the somatosensory, insular and piriform cortex, which would lead to tics via increased **glutamate** release to the dorsal striatum. From this perspective, the ameliorative effects of systemic and intrastriatal XAN and VU0467154 may depend on reducing striatal glutamate release from cortical glutamatergic projections. In keeping with this interpretation, Pancani et al. (2014) showed that M_4 presynaptic heteroreceptors form axospinous asymmetric synapses on glutamatergic terminals in the striatum of rodents, where they regulate glutamate release at the projections of cortical pyramidal cells onto SPNs.

In addition to the effects on tic-like jerks and stereotypies, systemic and intrastriatal XAN opposed the deficits in sensorimotor

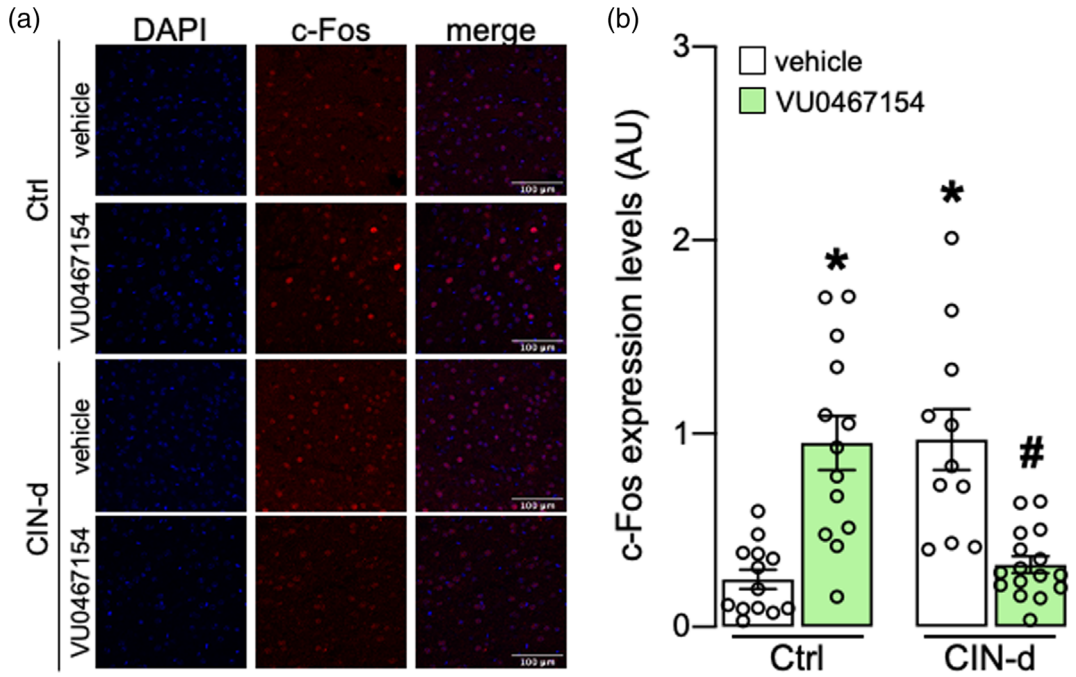

FIGURE 10 c-Fos levels in the dorsal striatum of CIN-d mice after exposure to spatial confinement were reduced by systemic VU0467154 treatment (3 mg·kg^{-1}, IP). (a) Representative immunofluorescent images. (b) Data are presented as mean ± SEM of the ratio between the absolute intensity signal of c-Fos and DAPI. Data were analysed with two-way ANOVA. * $P < 0.05$ for comparisons versus control (Ctrl) vehicle. # $P < 0.05$ for comparisons versus CIN-d vehicle.

gating observed in CIN-d and D1CT-7 mice; however, intrastriatal infusions of the M_4 receptor antagonist did not reverse the effects of XAN on this index in CIN-d mice. This discrepancy may partially reflect that PPI is primarily controlled by the ventral, rather than the dorsal, striatum. Accordingly, we showed that the nucleus accumbens is relevant to the PPI deficits of other mouse models related to TS (Cadeddu et al., 2021). Alternatively, a role of M_1 receptors could be inferred based on the finding that infusion of the antagonist of this receptor partially countered the effects of XAN in D1CT-7 mice. Further studies will be needed to test the specific muscarinic mechanisms underlying PPI regulation.

Several limitations need to be acknowledged. First, our assessments of M_1 and M_4 receptors relied exclusively on pharmacological tools rather than genetic constructs, which restricted our ability to utilize entirely selective methods. Second, we failed to measure the extracellular levels of each compound at the target site following systemic administration, thus limiting our capacity to draw comprehensive conclusions regarding receptor specificity. Third, the absence of electrophysiological and microdialysis studies in our research hampers clear discrimination of the underlying mechanisms driving the observed effects. Finally, as the study was only limited to male mice, we cannot draw any meaningful inference on the therapeutic potential of XAN and M_4 activators in TS-affected females.

These limitations notwithstanding, the observation that XAN and VU0467154 produced beneficial effects in two distinct, yet complementary models of tic pathophysiology and phenomenology suggests that stimulating M_4 receptors might effectively diminish the development of tics and other TS-relevant phenotypes independent of the specific pathophysiological mechanisms at play. Furthermore, the divergent effects of VU0467154 in CIN-d and control mice underscore that the influence of M_4 activation is probably directed specifically towards tic disorders. This suggests a potential for more targeted effects with fewer adverse consequences than existing treatments. Current interest in muscarinic receptors as prospective therapeutic targets emerged following the discovery of the antipsychotic properties exerted by XAN (Breier, 2005; Shekhar et al., 2008). Nonetheless, the advancement of therapeutics focused on these receptors was temporarily hindered due to adverse events such as excessive salivation, sweating, urination, excess gastric secretion and motility (Bodick et al., 1997). Such side effects result from activating peripheral muscarinic receptors in the parasympathetic system. To overcome this problem, XAN has been recently co-formulated with trospium, a muscarinic receptor antagonist with restricted peripheral activity. The combination of these two drugs has been found to reduce schizophrenia symptoms in Phase 2 and Phase 3 studies (Sauder et al., 2022; Weiden et al., 2022); at the same time, the safety and tolerability of this co-formulation are high, thanks to the mitigation of peripheral side effects (Correll et al., 2022). At the time of this writing (March 2024), the US Food and Drug Administration (FDA) is evaluating the potential of the XAN and trospium combination as a novel therapeutic intervention for schizophrenia.

M_4-positive allosteric modulators also are under development, given that these compounds should elicit fewer adverse events than orthostatic agonists. Accordingly, the recently developed M_4 PAM

emraclidine is tolerated well in patients (Krystal et al., 2022). These premises highlight the high potential of M_4-activating therapies as a novel, upcoming strategy for treating schizophrenia. Based on our results, we predict that such treatments may be repurposed for managing TS or other comorbid disorders, such as OCD.

AUTHOR CONTRIBUTIONS

M. B. and M. S. T. conceived and designed the study; R. C, G. B, C.B. and E. v. L. performed experiments; C. P. provided the construct for the development of CIN-d mice; R. C., C. B. and M. B. conducted data analysis; R. C. and M. B. wrote the manuscript. G. B., C. B., C. P., M. S. T. and M. B. reviewed and edited the manuscript.

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CONFLICT OF INTEREST STATEMENT

C. P. has consulted in the past 12 months for Biohaven Pharmaceuticals, Ceruvia Lifesciences, Transcend Therapeutics, Freedom Biosciences, UCB BioPharma, Nobilis Therapeutics and F-Prime Capital Partners; receives research funding from Biohaven, Transcend and Freedom; receives royalties from Oxford University Press and Up To Date; and has equity in Alco Therapeutics. M. S. T. works as a full-time employee of the pharmaceutical company H. Lundbeck A/S. MB consults for Asarina Pharmaceuticals and receives research funding from Asarina and Lundbeck Pharmaceuticals. The other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Natural Products Research, Design and Analysis, Immunoblotting and Immunochemistry and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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SUPPORTING INFORMATION

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