Characterization of Selective M₅ Acetylcholine Muscarinic Receptor Modulators on Dopamine Signaling in the Striatum

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Abstract

The type-5 muscarinic acetylcholine receptor (mAChR, M5) is almost exclusively expressed in dopamine (DA) neurons of the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc); therefore, ideally located to modulate DA signaling and underlying behaviors. However, the role of M5 in shaping DA release is still poorly characterized. In this study, we first quantitatively mapped the expression of M5 in different neurons of the mouse midbrain, then used voltammetry in mouse striatum to evaluate the effect of M5-selective modulators on DA release. The M5 negative allosteric modulator, ML375, significantly decreased electrically-evoked DA release and blocked the effect of Oxtremorine-M (Oxo-M, non-selective mAChR agonist) on DA release in presence of an acetylcholine nicotinic receptor blocker. Conversely, the M5 positive allosteric modulator, VU0365114, significantly increased electrically-evoked DA release and the Oxo-M effect on DA release. We then assessed M5 impact on mesolimbic circuit function in vivo. Although psychostimulant-induced locomotor activity models in knockout mice have previously been used to characterize the role of M5 in DA transmission, the results of these studies conflict, leading us to select a different in vivo model, namely a cocaine self-administration paradigm. In contrast to a previous study which also used this model, however, in the current study administration of ML375 did not decrease cocaine self-administration in rats (using fixed and progressive ratio). These conflicting results illustrate the complexity of M5 modulation and the need to further characterize its involvement in the regulation of dopamine signaling, central to multiple neuropsychiatric diseases.
Significance statement: This work describes the type-5 muscarinic receptor (M₅) pattern of expression within the midbrain as well as its physiological modulation by selective compounds at the axon terminal level in the striatum, where M₅ directly shapes dopamine transmission. It offers the first direct readout of mesolimbic dopamine release modulation by M₅, highlighting its role in regulating neurocircuits implicated in the pathophysiology of neuropsychiatric disorders such as substance use disorders, major depressive disorder, and schizophrenia.
Introduction

Alterations in dopamine (DA) and acetylcholine (Ach) transmission within the mesocorticolimbic circuits are thought to play central roles in the pathophysiology of multiple neuropsychiatric disorders bipolar disorder, major depressive disorder and schizophrenia (Kapur, 2003; Bender et al., 2019; Nunes et al., 2020, 2022). Although excitability of DA cell bodies was believed to be the main driver of DA release, afferent neuronal projections from the striatum also modulates DA release at the axon terminal level (Rice and Cragg, 2004; Threlfell et al., 2010; Cachope et al., 2012; Kramer et al., 2022). In the striatum, cholinergic interneurons (CINs) provide the main source of ACh and exert a robust modulation of DA release through multiple types of presynaptic and postsynaptic receptors. For instance, activation of nicotinic ACh receptors (nAChRs) on DA terminals is sufficient to trigger DA release (Sulzer et al., 2016; Yorgason et al., 2017).

Muscarinic ACh receptors (mAChRs) comprise a family of 5 subtypes of GPCRs (M1-5), some of which also are known to modulate DA transmission. Thus, they may afford relevant therapeutic targets for neuropsychiatric diseases as illustrated by the antidepressant effects of the non-selective antagonist scopolamine (Drevets et al., 2020) or the antipsychotic effects of M1/M4 agonists (Foster et al., 2021). G_{i/o}-coupled M2 and M4 receptors are expressed by CINs (Zhang et al., 2002) and can directly modulate DA release (Shin et al., 2015), while the G_{q/11}-coupled M5 receptor is highly expressed in midbrain DA neurons (Weiner et al., 1990) and their terminals in the striatum (Razidlo et al., 2022; Shin et al., 2015). Unlike other mAChRs subtypes, the function of M5 remains elusive and conflicting experimental results across studies confound the existing literature.

Both midbrain dopaminergic nuclei, namely the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNC), contain glutamate and GABA neurons as well as neurons expressing multiple classical neurotransmitter specific markers. It has been shown that some DA neurons also express the type-2 vesicular glutamate transporter (VGLUT2), a specific
marker of glutamate neurons and co-release both glutamate and DA (Hnasko et al., 2010; Stuber et al., 2010; Trudeau et al., 2014; Root et al., 2016; Zell et al., 2020). Despite GABA neurons usually opposing the function of DA neurons in the VTA (Tan et al., 2012; Matsui et al., 2014; Bariselli et al., 2016), a small fraction express VGLUT2, co-release glutamate, and can drive reinforcement (Hnasko et al., 2010; Root et al., 2014; Yoo et al., 2016). Comprehensive mapping of M₅ in midbrain cell types and pharmacological characterization of its function are needed to elucidate its role in modulating mesolimbic dopaminergic transmission.

Recent pharmacological developments have led to the generation of the selective M₅ negative and positive allosteric modulators: ML375 (M₅ NAM): M₅ IC₅₀ = 3.49 µM, M₁₋₄ EC₅₀ > 30 µM and VU 0365114 (M₅ PAM): M₅ EC₅₀ = 2.7 µM, M₁₋₄ EC₅₀ > 30 µM; ( Bridges et al., 2010; Gentry et al., 2013; Kurata et al., 2015). In vivo studies showed intracranial co-delivery of VU 6000181 (M₅ NAM) with physostigmine caused sex-dependent differences in effort-choice behavior (Nunes et al., 2022), and ML375 decreased cocaine (Gunter et al., 2018), ethanol (Berizzi et al., 2018) and opioid agonist (Gould et al., 2019) self-administration. The impact of these compounds on DA release ex vivo remains unexplored. Although these results corroborate previous studies showing that M₅ KO mice display reduced response to cocaine reinforcement (Fink-Jensen et al., 2003; Thomsen et al., 2005), conflicting locomotor activity data in response to amphetamine (Wang et al., 2004; Schmidt et al., 2010) emphasizes the need for further investigation.

To refine our knowledge of midbrain M₅ expression pattern and its modulatory role on DA release, we first used fluorescent in situ hybridization to confirm and supplement previous findings showing prominent expression of M₅ not only in DA neurons (Gould et al., 2019; Razidlo et al., 2022), but also in glutamate and dual DA/glutamate neurons. Then, utilizing ex vivo fast-scan cyclic voltammetry (FSCV), we demonstrated that enhancement of M₅ activity can increase DA release while reduction of M₅ activity can diminish DA release in the striatum. Finally, we determined the in vivo effect of ML375 on cocaine self-administration in rats, to
assess the modulatory role of M5 on mesolimbic circuit function; but, unexpectedly, did not reproduce the results of a previous study which reported an attenuation of reward-seeking behavior (Gunter et al., 2018). Collectively, these data widen our knowledge on the expression and modulatory role that M5 exerts on DA release and underline the intricacy of M5 function in the mesolimbic circuitry.
Materials and Methods

Animals.

Male mice on a C57BL/6 background (n=5, Jackson Labs, USA), aged 4 to 20 weeks, and housed on a 12h light/dark cycle with food and water ad libitum were used for multiplex RNA in situ hybridization or fast-scan cyclic voltammetry. For ex vivo experiments, M5 knockout mice (Chrm5<sup>−/−</sup>, Deltagen Research Laboratories LLC, USA) were referred to as M5 KO mice, and control were littermate heterozygote mice (Chrm5<sup>+/−</sup>) and referred as littermate controls (n=24 mice total). The cocaine self-administration study was performed at Aptuit (an Evotec company, Verona Srl, Via Fleming 4, 37135 Verona, Italy) a GLP Test Facility that is part of the Italian Health Authorities Compliance program. For this study, adult male Sprague Dawley rats (n=25) were used. After surgery, animals were maintained single-housed to preserve the integrity of the implanted catheters and were then group-housed in plastic cages with access to food and water ad libitum before the experiment. All experiments were conducted in accordance with protocols approved by the Janssen Institutional Animal Care and Use Committee. General procedures for animal care and housing were in accordance with the current Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) recommendations.

M5 KO Animal genotyping

2-mm tail tip samples from each animal were collected and lysed overnight at 55°C in the lysis buffer (10 mM Tris, pH 8.3; 5 mM EDTA; 300 mM Na Acetate, 1% Triton X-100; 500 µg/ml Proteinase K). The genotype of the animals was then established using PCR and agarose gel electrophoresis. In brief, three reaction types used to genotype mice. For both F1 and F2 generations a “multiplex” reaction is performed which is designed to detect both wild-type and targeted alleles. F1 mice are screened using a second PCR reaction that is designed to detect only the targeted allele. This step ensures that only heterozygous mice proceed to the breeding programs. For F2 mice, in addition to the multiplex PCR reaction, a second reaction is
performed that only detects the wild-type or endogenous allele. This quality-control step ensures that homozygous mutant mice are properly genotyped (Supplemental Table 2).

**Multiplex RNA In Situ hybridization**

Mice (n=5) were euthanized by CO\(_2\) induction at 10-30% of the chamber volume per min and intracardially perfused with 10% Neutral Buffered Formalin (VWR International, cat no. 89370-094). Brains were then extracted and placed in 10% formalin for 24 hours, and then slabbed into 4 different pieces. The pieces of mouse brain were placed into cassettes and processed using the Tissue-Tek VIP 6 (Sakura Finetek USA; Torrence, CA). During processing, brain specimens underwent a dehydration step with increasing grades of alcohol; the alcohol was then removed with xylene before being fully infused with paraffin. After processing, the samples were embedded into formalin-fixed, paraffin-embedded (FFPE) blocks before sectioning. For the multiplex in situ hybridization (ISH) staining, the FFPE blocks were sectioned at 4 µm (n=2-3 sections per mouse for each brain region) with and mounted on SuperFrost plus glass slides (VWR International, cat no. 48311-703).

The multiplex ISH assay was developed on the Leica Bond Rx autostainer (Leica Biosystems; Wetzlar, Germany) using the following probes and staining kits: mRNA detection probes for mouse specific M\(_5\) RNA in channel 1 (RNAscope 2.5 LS Probe – Mm Chrm5; ACDBio, cat no. 495308), mouse specific VGLUT2 RNA in channel 2 (RNAscope 2.5 LS Probe – Mm-Slc17a6; ACDBio, cat no. 319178-C2), mouse specific GAD1 RNA in channel 3 (RNAscope 2.5 LS Probe – Mm-Gad1-C3; ACDBio, cat no. 400958-C3) and mouse specific DAT RNA in channel 4 (RNAscope 2.5 LS Probe – Mm-Slc6a3-C4; ACDBio, cat no. 315448-C4). The probes were hybridized using the RNAscope 2.5 Multiplex Fluorescent Reagent Kit (ACDBio, cat no. 322800) and RNAscope LS 4-Plex Ancillary Kit for LS Multiplex Fluorescent (ACDBio, cat no. 322830). The hybridized probes were visualized using the Opal-Fluophore dyes: Opal 520 (Akoya Biosciences, cat no. FP1487001KT), Opal 570 (Akoya Biosciences, cat
no. FP1488001KT), Opal 620 (Akoya Biosciences, cat no. FP1495001KT) and Opal 690 (Akoya Biosciences, cat no. FP1497001KT). Each of the Opal dyes were reconstituted in 75 µL of DMSO included in each reagent kit.

Glass slides with mouse brain FFPE tissue were loaded onto the Leica Bond RX where slides were first baked at 60 °C for 30 mins and then deparaffinized with the generic Bond RX deparaffinization protocol. Heat-induced epitope retrieval with an EDTA-based solution (pH ~9.0) was then performed for 15 min at 95 °C. The probes were then cocktailed together by diluting the 50x concentrated VGLUT2, GAD1 and DAT probes at 1:50 into the 1x M₅ probe solution and hybridized for 120 minutes at 42 °C. After undergoing amplification steps, Opal dyes at various concentrations react with HRP to create a fluorescent signal. M₅ staining was detected using Opal 520 at 1:500 concentration. VGLUT2 staining was detected using Opal 570 at 1:1500 concentration. GAD1 staining was detected using Opal 620 at 1:3000 concentration. DAT staining was detected using Opal 690 at 1:3000 concentration. Stained slides were then coverslipped using ProLong Gold Antifade Mountant (ThermoFisher Scientific, cat no. P36934). After leaving the slides to dry overnight at RT in a fume hood, the slides were imaged on the PhenolImager HT (Akoya Biosciences, Marlborough, MA).

Stained images were analyzed using HALO AI v3.5.3577 (Indica Labs, Albuquerque, NM); using the Nuclei Seg (Plugin) – FL v1.0.0, a nuclear segmentation classifier was trained and used in conjunction with a FISH-IF algorithm to quantify multiple phenotypes. Regions of analysis were manually annotated to analyze the lateral and medial parts of the ventral tegmental area and the substantia nigra pars compacta. Both regions were delineated by the expression pattern of the dopamine transporter.

Fast-scan cyclic voltammetry

Adult mice (7–12 weeks, n=24 total) were deeply anaesthetized with isoflurane (3-4 %, Piramal) and transcardially perfused with ~10 mL ice-cold sucrose-artificial cerebrospinal fluid
(ACSF) containing (in mM): 150 sucrose, 50 NaCl, 2.5 KCl, 7 MgSO4, 0.5 CaCl2, 1.25 NaH2PO4, 25 NaHCO3 and continuously bubbled with carbogen (95% O2 + 5% CO2). Brains were extracted then cut into 300-µm coronal slices in ice-cold sucrose-ACSF using a Leica Vibratome (VT 1200 S, Leica). Slices were transferred to a perfusion chamber containing ACSF at 33°C (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO4, 2.5 CaCl2, 1.0 NaH2PO4, 26.2 NaHCO3, 11 glucose, continuously bubbled with carbogen. After 30-45 min recovery, slices were maintained at room temperature and transferred to a recording chamber continuously perfused with ACSF (2–3 ml·min⁻¹) maintained at 29–31 °C using an in-line heater (Harvard Apparatus).

For recording electrodes, cylindrical carbon-fiber microelectrodes were prepared with 7 µm diameter fibers (GoodFellow) inserted into a borosilicate capillary (1.0 mm, A-M systems) and pulled to seal the pipette around the carbon-fiber with a pipette puller (Narishige). The carbon tip was trimmed to ~100 µm of exposed fiber using micro-scissors under a microscope. The electrode was held at −0.4 V versus Ag/AgCl reference, and a triangular voltage ramp (−0.4 to +1.3 V at 400 V·s⁻¹) was delivered at 10 Hz. Before recordings, pipettes were backfilled with 3M KCl and cycled for 10 minutes at 60 Hz to reach signal stability. DA transients were electrically evoked every 2 min by electrical single-pulse stimulations in either the lateral shell of the nucleus accumbens or the dorsal striatum, where larger DA transients could be recorded in presence of dihydro-β-erythroidine application (DHβE,1 µM). For electrical stimulation, a plate concentric stimulation electrode was placed near the tip of the carbon fiber (~100–200 µm), and a rectangular pulse (100–500 µA, 0.2-0.5 ms) was applied every 2 min (pre-stim event trigger time set at 5s for a total time of 20s per sweep). Stimulation intensity was chosen to be sub-saturating to avoid rundown of DA current amplitude. Data were collected and analyzed using Tarheel CV software. Oxo-M (10 µM, Tocris), ML375 (10 µM, Sigma) and VU 0365114 (10 µM, Tocris) were diluted 1000-fold in ACSF from stock solutions daily and before bath application. DHβE (1 µM, Tocris) was used in ACSF constantly in selected experiments. DA currents were measured at the oxidation peak region and the amplitude was calculated by using peak
amplitude from baseline in Tarheel CV software. Recordings were excluded if greater than 20% dopamine current variations (80-120%) were observed during baseline measurements (10 min).

**Cocaine operant self-administration.**

Male Sprague Dawley rats \( (n = 26) \) were cannulated in the femoral vein and were trained to self-administer cocaine \((0.5 \text{ mg·kg}^{-1})\) intravenously \((\text{iv})\). In this procedure, animals performed an operant daily task \( \text{(i.e., pressing a lever)} \) under fixed ratio \((\text{FR})\) and progressive ratio \((\text{PR})\) schedules of reinforcement to receive an intravenous dose of cocaine \((\text{O’Connor et al.}, 2011)\). Self-administration sessions were performed in operant chambers \((\text{Med Associates, USA})\), each placed within a sound- and light-attenuating box and equipped with two retractable levers. A house-light was located on the opposite wall and cue lights placed above each lever together with a Sonalert device \((2.9 \text{ kHz}, 70 \text{ dB})\). Outside each chamber, an infusion pump \((\text{Model PHM-100VS or other equivalent, Med Associates, USA})\) was connected to the implanted catheter through a single channel liquid swivel mounted on the top of the chamber.

Each session was performed once a day. At the start of each session, the house light was illuminated and both levers \( \text{(one active, one inactive, randomized across animals)} \) were exposed. When the rat pressed the active lever, the infusion pump delivered the cocaine solution \((1.5 \text{ mg·mL}^{-1})\), the house light was switched off for 20 sec \((\text{time out})\) and both conditioned stimuli \((\text{CSs})\), were activated for 6.3 seconds: the light located above the active lever as well as the Sonalert device went on. Presses on both levers during the time out had no programmed consequences but were recorded. After the time out, the house light was switched on. Each session lasted 2 hours or were ended when animals had achieved 25 reinforcers, whichever occurred first. Once animals had achieved stable responding under an FR1, the FR was increased initially to FR2, FR5 and FR10 \((\text{ten active lever presses were required to obtain a cocaine infusion})\). The criteria to define stability of responding were i) \( >10 \text{ infusions/day for 3 consecutive days} \) and ii) the number of infusions over the last three sessions of cocaine self-
administration acquisition with a percent coefficient of variation (% CV) ≤20 % of the mean of infusions of the last 2 sessions. All behavioral testing was conducted during the first half of the light portion of the light/dark cycle, between 08:00 am-02:00 pm. At the completion of training sessions, rats received both a p.o. and an i.p. administration of water or saline, respectively, given 60 and 15 min before the start of the 0.5 mg·kg⁻¹ cocaine self-administration session (defined as maintenance cocaine session) to accustom animals to the p.o. and i.p. procedures.

Animals engaging in the FR10 sessions earned 0.5 mg·kg⁻¹ cocaine infusions. The session ended after 2 hours, or when animals had achieved 40 reinforcers. For the PR study, animals earned 0.5 mg·kg⁻¹ cocaine under a progressive ratio schedule of reinforcement in which the response requirement for delivery of each reinforcer increased as determined by the exponential equation used by Richardson and Roberts (Richardson and Roberts, 1996), ratio = [5 × e^(R × 0.2)] – 5. Break point was defined as the last final ratio completed; sessions were terminated after 20 min without completion of the current ratio requirement. The dose 30 mg·kg⁻¹ of ML375 was given intraperitoneally (i.p.; 2 mL·kg⁻¹ as administration volume) 15 min before the beginning of the session, and was selected based on previous studies showing oral CNS distribution study in rat (Gentry et al., 2013) as well as previously published results showing efficacy in reduction of cocaine self-administration (Gunter et al., 2018). Our internal data suggest that i.p. and p.o. dosing routes yield similar plasma exposures as defined by Cmax and AUC₀-2₄h even as oral ML375 is absorbed slower upon oral exposure (Supplemental Figure 3, Supplemental Table 2) Cocoaine was presented at single (0.5 mg·kg⁻¹) or ascending doses (0.15 or 0.25 or 0.50 or 0.75 mg·kg⁻¹), and at least 2 maintenance cocaine sessions (with cocaine presented at 0.50 mg·kg⁻¹) were interposed between testing sessions.

Rodent Pharmacokinetic Studies.

Rat pharmacokinetic studies were both conducted by Pharmaron. ML375 was prepared as a solution in 50% PEG-400 in water to a concentration of 0.5 mg·mL⁻¹ and pH of
7.0. Separate cohorts (n=3 / cohort, n=12 total rats) of non-fasted male Sprague Dawley rats were administered ML375 at a dose of 0.5 mg·kg⁻¹ i.v. or 2.5 mg·kg⁻¹ i.p., p.o., or s.c. Blood samples were collected at 0.033 (i.v. only), 0.083 (i.v. only), 0.25, 0.5, 1, 2, 4, 8, and 24 hours post dose via the jugular vein catheter. Blood samples were collected into tubes containing K₂EDTA and placed on wet ice. The plasma fraction was separated by centrifugation and kept frozen at -20 °C. Concentrations of ML375 in plasma were determined using a qualified liquid chromatography-triple quadrupole mass spectrometry (LC-MS/MS) method with a limit of quantification (LOQ) of 1 ng·mL⁻¹. Pharmacokinetic parameter values were derived from noncompartmental analysis of the plasma concentration versus time data using Phoenix WinNonlin software (Certara, Princeton, NJ). Area under the curve to infinity (AUCₙᵢₙfinity) and half-life were not reported if the terminal phase was poorly defined or if extrapolation exceeded 20%.

**Rodent Blood Brain Barrier Studies.**

ML375 was either prepared as a solution or suspension to support rat blood brain barrier studies. As a solution, ML375 was prepared to 3 mg·mL⁻¹ in 100% PEG-400 followed by dilution in an equal volume of water to achieve a final concentration of 1.5 mg·mL⁻¹ and pH of 7.0. As a suspension, ML375 was prepared in 0.5% HPMC. The material was vortexed, sonicated, and stirred overnight to achieve a homogenous suspension with a final measured pH of 7.47.

Rat blood brain barrier studies were conducted by Pharmaron. Separate cohorts (n=3 / timepoint) of non-fasted male Sprague Dawley rats were orally administered ML375 at a dose of 15 mg·kg⁻¹ using the 50% PEG-500 solution or 30 mg·kg⁻¹ using the 0.5% HPMC homogenous suspension with terminal collections made at 1, 2 (suspension only), 4, 7, and 24 h. At each timepoint, animals were euthanized by increasing CO₂ concentration. Blood was removed by
cardiac puncture and transferred to tubes containing K$_2$EDTA while on ice, while the brain was excised and washed with saline before transfer into pre-weighted test tubes and storage at -75 °C. The plasma fraction was separated from blood samples by centrifugation and kept frozen at -20 °C. The brain samples were mixed with deionized water to a ratio of 1 gram tissue per 3 mL water and homogenized. Concentrations of ML375 were determined using a qualified liquid chromatography-triple quadrupole mass spectrometry (LC-MS/MS) method with a LOQ of 1 ng·mL$^{-1}$ and 5 ng·g$^{-1}$ for plasma and brain homogenate, respectively.

**Statistical analysis.**

Statistical analysis was performed with Prism 8 (GraphPad). ANOVA followed by Tukey-Kramer multiple comparison test was used to analyze the RNA Scope ® and the FSCV study. A linear mixed effects model for repeated measures has been used for FSCV. ANOVA and ANCOVA were followed by Dunnett’s or Tukey’s post hoc test for the in vivo study. All data are expressed as mean ± SEM unless stated otherwise.
Results

1. M₅ expression in midbrain neuronal subtypes

To assess the expression of M₅ we multiplexed Chrm5 RNA with probes targeted against the RNA of dopamine transporter (DAT), the type-2 glutamate vesicular transporter (VGLUT2) and glutamate decarboxylase 1 (GAD1) using RNA Scope ® in the mouse midbrain (Figure 1A). Leveraging an automated multiplex quantification we found that M₅ is expressed by the majority of dopamine neurons in the SNc and lateral VTA (79.14 ± 1.14 % and 64.15 ± 4.39 %, respectively, n=5, Figure 1B-C), corroborating previous observations (Gould et al., 2019; Razidlo et al., 2022). In the medial VTA, the colocalization is significantly less prominent (41.76 ± 5.06 %, n=5, p<0.0001, Figure 1B). Accordingly, out of all neurons that express M₅, a large majority also express DAT in the SNc and lateral VTA (80.29 ± 1.73 % and 66.77 ± 5.61 %, respectively) but again, significantly less in the medial VTA (47.25 ±5.90 %, p=0.0014, Figure 1E-F). The same M₅ population colocalizes with VGLUT2 mostly in the medial VTA (61.52 ± 13.87 %, n=5) and significantly less in the lateral VTA and SNc (26.30 ± 5.75 and 14.56 ± 1.94 %, respectively, n=5, p=0.0067). In all three subregions, the M₅ population shares the least amount of overlap with GAD1 RNA. In the midbrain, it is known that glutamate, GABA and DA neuron populations overlap to a significant extent (Root et al., 2016; Yoo et al., 2016; Morales and Margolis, 2017). We thus analyzed the expression of M₅ in overlapping glutamate / GABA as well as DA / glutamate dual neurons. We observed that M₅ is expressed in DAT-only, VGLUT2-only, and dual DAT/VGLUT2 populations in the SNc and lateral VTA (Supplemental Figure 1). In the medial VTA, M₅ is mostly expressed in dual DA / glutamate and glutamate-only neurons. Interestingly, M₅ expression in GAD1-only neurons is the lowest in all 3 regions and virtually absent in dual glutamate / GABA neurons (Supplemental Figure 1).

2. Specific modulation of M₅ modulates dopamine release in the striatum
We used ex vivo FSCV to measure the M₅ functional modulation of DA release in mouse brain slices. Dopamine currents were recorded in the lateral shell of the nucleus accumbens (NAc) and were evoked by local electrical stimulation. Bath application of the M₅-selective PAM, VU 0365114 (10 µM) significantly increased DA transients from 5.76 ± 0.96 nA at baseline to 6.38 ± 0.92 nA (100.00 ± 16.61% to 110.86 ± 15.90%, n=6, p=0.0016, Figure 2A). In another subset of experiments, application of the M₅-selective NAM, ML375 (10 µM) significantly decreased DA transients from 6.38 ± 0.39 nA to 4.91 ± 0.66 nA (100.00 ± 6.12% to 76.98 ± 10.30%, n=7, p < 0.0001, Figure 2B).

Next, we evaluated the effect of both M₅-specific PAM and NAM on Oxtremorine-M (Oxo-M) induced increase in DA release. Oxo-M is a non-selective mAChR agonist, and has been shown to increase DA release in the striatum when applied at 10 µM due to activation of M₅, as that effect is absent in M₅ KO mice (Yan, 2017; Razidlo et al., 2022). Our characterization of M₅ KO mice shows an absence of detectable levels of M₅ RNA in the midbrain, and, importantly, the basal DA release is similar between WT and M5 KO mice (Supplemental Figure 2). In this subset of experiment, we bath-applied both M₅ modulators or vehicle for 20 minutes before application of Oxo-M (10 µM). These recordings were performed in presence of the nicotinic acetylcholine receptor (nAChR) antagonist dihydro-β-erythroidine hydrobromide (DHβE, 1 µM) to block nicotinic-driven presynaptic depression (Shin et al., 2017; Yan, 2017). Under these experimental conditions, Oxo-M produced a significant increase in electrically evoked DA current amplitude (vehicle, +26.88%, n=5, p=0.0054, Figure 2C-D), corroborating previous findings (Yan, 2017; Razidlo et al., 2022). In slices from M₅ KO mice, Oxo-M (10 µM) did not increase DA amplitude; a slight decrease in DA amplitude was observed vs. vehicle ( -12.40%, n=5, p=0.033). When ML375 (10 µM) was perfused in the recording chamber, Oxo-M (10 µM) displayed similar effect to the recordings in the M₅ KO, specifically no increase in DA current amplitude was observed (-9.79%, n=5, p=0.061). Bath application of VU 0365114 significantly potentiated DA transient amplitude (+16.88%, n =4, p=0.049). Taken
together, our ex vivo results demonstrate that activation of M₅ can increase DA transmission in the striatum.

3. ML375 does not reduce cocaine self-administration in rats

In an aim to reproduce literature findings showing ML375-mediated reduction of cocaine self-administration in rodents, we used similar fixed ratio (FR) and progressive ratio (PR) reinforcing protocols in rats as described by Gunter and collaborators (Gunter et al., 2018). During the 2-h sessions, animals received a cocaine infusion following 10 active lever presses (FR10) and were split into vehicle (n=13) or ML375 (30 mg·kg⁻¹, i.p., n=13) pretreatments to assess the impact of M₅ modulation on reinforcement (Figure 3A-C). We found that despite a strong reinforcing effect of cocaine administration (rats made significantly more presses on the active lever when compared to the inactive lever, triggering more cocaine infusions), ML375 pretreatment did not produce any change in the number of active lever presses and no significant difference was found between vehicle and ML375-treated groups (see Supplemental Table 1). Consequently, ML375 did not impact the number of infusions and no difference was found in the number of inactive lever presses.

When we used the same FR10 delivery schedule paired with an ascending dose of cocaine (0.15, 0.25, 0.50 and 0.75 mg·kg⁻¹, Figure 3D-F), cocaine had a strong reinforcing effect on rats that displayed significantly more active vs. inactive lever presses, self-administering more cocaine doses over sessions. The number of active lever press and administered doses proportionally decreased with higher cocaine concentration, corroborating previous observations (Gunter et al., 2018). However, no statistically significant effect of ML375 was observed on either the number of active lever presses or infusions (Supplemental Table 1).

Lastly, a PR schedule was introduced where animals had to lever press incrementally more to get the same dose of cocaine. Under this schedule animals displayed a significantly higher
number of lever presses to sustain the same number of injections (Figure 3G-I). ML375 did not produce any significant change in the number of active or inactive lever presses or infusions (Supplemental Table 1).

Discussion

In this study, we described the expression pattern of M5 in different cell types of the VTA and SNC. We demonstrated that M5 is predominantly expressed by DA, glutamate, and dual DA/glutamate neurons. Functionally, we showed that M5 modulation directly shapes DA release in the striatum, and finally, in contrast to data previously reported in the literature, we did not detect the previously reported decrease in cocaine self-administration following ML375 administration.

M5 is differentially expressed in SNC and VTA neuron subpopulations.

Multiple reports have shown that M5 expression is enriched in midbrain DA neurons (Gould et al., 2019; Razidlo et al., 2022; Shin et al., 2015). Still, to the best of our knowledge, no study until now investigated M5 expression in the context of the full complexity of overlapping neurotransmitter-defined cell types in VTA and SNC (Trudeau et al., 2014). Here, we leveraged a multiplexed fluorescent ISH to perform a co-detection of specific markers for DA, GABA, and glutamate neurons together with M5 to offer a more comprehensive overview of M5 expression.

In this study, we used DA transporter mRNA (DAT) as a specific marker for DA neurons, together with VGLUT2 and GAD1 mRNA to quantify M5 expression in glutamate and GABA neurons, respectively, as well as overlapping subpopulations. Indeed, some midbrain neurons express both dopaminergic and glutamatergic markers, co-release DA and glutamate, and have been shown to promote reinforcement (Wang et al., 2017; Zell et al., 2020). To add to this existing complexity, a subset of midbrain GABA neurons express VGLUT2, can co-release
glutamate, and their activation has also been shown promote reinforcement (Root et al., 2014; Yoo et al., 2016). However contradictory observations have also been reported (Root et al., 2018, 2020; Barbano et al., 2020). A more thorough characterization of M₅ expression in all of the above neuron subtypes may better elucidate the functional capacities of M₅ modulation.

First, we corroborated previous reports showing that M₅ is predominantly expressed in DA neurons (Figure 1, Supplemental Figure 1). We expanded these results further by establishing that M₅ is highly expressed in VGLUT2 and dual glutamate/DA neurons (DAT⁺/VGLUT2⁺) in the midbrain (Supplemental Figure 1). Expression of M₅ by these subpopulations suggests that M₅ can synergistically influence reward processing through either DA and/or glutamate striatal release. In line with our anatomical findings, Shin and collaborators previously demonstrated that striatal glutamate transmission is also modulated by M₅ (Shin et al., 2015). The VTA and SNc contain not only glutamate and DA neurons, but also GABA neurons known to oppose DA function either locally (Tan et al., 2012), or via projections to the nucleus accumbens (NAc) (Al-Hasani et al., 2021). Our data reveals that M₅ is expressed in a very small fraction of GAD1-only expressing (GABA) neurons, as reported previously (Gould et al., 2019), and is virtually absent from dual glutamate/GABA neurons. Although most M₅-expressing neurons in the midbrain have been shown to promote reward, its expression, although low, in neurons that oppose this function remains unclear, and additional experimental work is needed to fully resolve this apparent contradiction. Since striatal DA release is instrumental for assessing the mesolimbic circuit function, we utilized ex vivo voltammetry to directly measure its modulation by M₅ in acute mouse brain slices.

Increase and reduction of M₅ activity by selective pharmacological tools modulates dopamine release in the striatum.

Only recently, researchers began to characterize the modulatory role of M₅ on striatal DA release and few reports have been published that indicate direct modulation. Using knockout
mice instead of selective ligand-based modulation, previous studies indirectly demonstrated M₅ activation increases striatal DA transmission (Razidlo et al., 2022; Shin et al., 2015). More recently, Nunes and collaborators injected a M₅ NAM directly in the VTA which increased evoked striatal DA release of male adult Sprague Dawley rats, in the presence of physostigmine, a choline acetyltransferase blocker (Nunes et al., 2023).

Here, we measured the direct effect of a positive and a negative allosteric M₅ modulator on striatal DA release and showed that ML375 (NAM) significantly decreased DA release while VU 0365114 (PAM) increased electrically-evoked DA release in the striatum in the absence of a nicotinic receptor blocker. These observations are supported by our expression data (Figure 1) and by previous work showing that activation of M₅ facilitates DA release in the striatum (Shin et al., 2015). This is to our knowledge the first demonstration that allosteric M₅ modulators alone (i.e., without blocking nicotinic ACh receptors) can influence electrically-evoked DA release in striatum. This relatively large effect of allosteric M₅ modulators in quasi-physiological conditions could be due to the fact that concentration of acetylcholine at M₅ receptors is lower (spillover Ach acting on more distal M5 receptor). One might expect that allosteric ligands would have a larger effect on slow synapses because of the lower concentration of neurotransmitter.

We also tested the effect of the same M₅ modulators on Oxo-M induced DA release, i.e. muscarinic mediated signaling. In the presence of DHβE, Oxo-M significantly increased DA release in slices from WT but not M₅ KO mice, as shown previously (Shin et al., 2015), demonstrating the M₅-specific effect on DA release. In M₅ KO, Oxo-M produced a significant decrease in evoked DA release (Figure 2C-D). This effect suggests that Oxo-M may recruit non-cholinergic local neurons or axon terminals expressing mAChRs that, in turn, modify striatal DA release. One possible actor might be cortical glutamate afferences expressing M₄ that once inhibited decrease their excitatory tone onto DA terminals to decrease DA release (Pancani et al., 2014). CINs themselves express M₂ and M₄ in the striatum (Yan & Surmeier, 1996); their activation would reduce CINs excitability and could lead to a reduction of DA signaling.
opposing the effect of M₅ (Shin et al., 2015; Weiner et al., 1990). Further, ML375 completely blocked the Oxo-M effect on DA release, in line with the first set of experiments, and demonstrating that ML375 is sufficient to block M₅ modulation of DA release. VU 0365114 significantly potentiated Oxo-M-induced DA release, indicating basal M₅ activity in slice preparations is not saturating.

Direct measurement of extracellular DA release allowed us to show that M₅ participates in shaping DA signaling in the striatum. To the best of our knowledge, this study is the first to demonstrate a direct ex vivo modulation of DA release by M₅ using specific modulators in native tissue. Despite some clear results, the literature also contains discrepancies in the characterization of the role of M₅ in behavior relevant of neuropsychiatric illnesses. We chose to conclude our current investigations with an in vivo model of reward-seeking behavior shown to be reduced by the administration of ML375 in rats.

**Blockade of M₅ by ML375 does not decrease cocaine-induced reinforcing behavior.**

The development of brain penetrant M₅ modulators appeared only recently (Gentry et al., 2013). Despite the apparent literature consensus about the effect of ML375 producing a reduction of self-administration of ethanol, cocaine and opiates (Berizzi et al., 2018; Gould et al., 2019; Gunter et al., 2018), other conflicting studies indicate that amphetamine administration either increased or decreased locomotor activity in M₅ knockout mice (Schmidt et al., 2010; Wang et al., 2014). We thus selected a cocaine self-administration paradigm that has been shown to be disrupted by ML375 (Gunter et al., 2018).

In our study, rats responded robustly to cocaine infusion, but treatment with ML375 did not significantly reduce any reinforcing parameters (Figure 3). Despite the fact that ML375 is brain penetrant and reaches high concentration in the brain, it is strongly protein bound, leaving only a very low brain free fraction (f_{u,br}; rat, 0.003) with total and unbound brain-plasma partition coefficients of K_p=1.8 and K_p,uu=0.2 (Gentry et al., 2013) (see also pharmacokinetic profile of...
ML375 in Supplemental Figure 3 and Supplemental Table 2). Therefore, the lack of effect in our in vivo experiments might be due to the very low free brain concentration. Still, these possible explanations don’t reconcile the discrepancy between our lack of effect and the reported reduction in self-administration in previous studies (Gunter et al., 2018). It is reasonable to postulate that reproducibility of data in self-administration studies is cofounded by the moderate potency of ML375 combined with its strong protein binding, and/or the discrete modulatory role of M₅. This is not the only in vivo experiment where a change of M₅ signaling was reported to have variable effect. For instance, the various reported effect of challenging M₅ knockout mice with amphetamine remains highly unclear and debated: while some authors showed a decreased locomotor activity (Wang et al., 2004), other showed an increase (Schmidt et al., 2010). These unexplained differences could result from a potential role of DAT inhibition following M₅ activation (Garcia-Olivares et al., 2013), where amphetamine mechanism of action is more complex than cocaine, leading to a wider range of effects depending on experimental conditions. Lastly, recent efforts to better characterize effect of M₅ show a sex difference in its modulation in vivo, where male had an elevated striatal DA release paired with an increase in effort-base behavior following midbrain infusion of the NAM, VU 1000161 (Nunes et al., 2023). Since our study used only males, we are unable to characterize such a distinction, but another possible explanation could be disparate M₅ expression between sexes.

Taken together, we corroborated and extended the characterization of the pattern of expression of M₅ in midbrain neuronal populations. Using M₅-specific modulators in mouse brain slices, we refined existing data on our understanding of striatal DA transmission modulation by M₅. Finally, in contrast to data reported in the literature, systemic administration of ML375 did not decrease cocaine self-administration in rats. This disparity highlights the complexity of M₅ modulation and its measurable impact on the mesocorticolimbic dopamine circuit. Further studies will be necessary to better understand the role of M₅ on complex circuits involved in adaptive reward processing and in neuropsychiatric disorders.
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8129, National Academy of Sciences.


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e) Conflict of interest: All the authors are current or former employees of Johnson & Johnson and may hold the stock of the company.

Data Availability Statement

All the data will be made available upon request.
Figure Legends

Figure 1. M5 is mostly expressed by dopamine and glutamate neurons in the midbrain. A. Coronal midbrain section of a mouse brain labeled with fluorescent probes targeted against dopamine transporter transcripts (DAT, red) in the SNc and the VTA. B. Most of DAT+ neurons also express M5 in the SNc (79.13 ± 1.14 %), medial (41.76 ± 5.06%) and lateral (64.15 ± 4.39%) VTA. The level of M5 expression differs significantly between the 3 midbrain subregions (One-way ANOVA, F(2, 12)=22.98, p<0.0001, Tukey Kramer multiple comparisons: SNc vs. medial VTA p<0.0001, SNc vs. lateral VTA p=0.0472, medial vs. lateral VTA p=0.0044). C. Most of DAT+ neurons also express M5 (full arrows) in the medial VTA, and few DAT- also express M5 (empty arrows). D. The vast majority of GAD1+ neurons don’t express M5 in the medial VTA. E. Both VGLUT2-only and dual VGLUT2+ / DAT+ neurons co-express M5. F. In the SNc, M5 is expressed significantly more in DAT-expressing neurons when compared to VGLUT2+ and GAD1+ neurons (One-way ANOVA, F(2, 12)=52.06, p<0.0001). In the medial VTA, M5 is expressed predominantly by VGLUT2- and DAT-expressing neurons (One-way ANOVA, F(2, 12)=14.87, p=0.006), while in the lateral VTA, M5 is significantly more expressed on DAT+ neurons (One-way ANOVA, F(2, 12)=27.26, p<0.0001). Scales: 25 µm. All values expressed as mean ± SD (n=5 mice, 2-3 sections per region per mouse). See also Supplemental Figure 1. Significance keys: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 2. Modulation of dopamine release by VU 0365114 (M5 PAM) and ML375 (M5 NAM) in the striatum. A. Bath application of VU 0365114 produced a significant increase of electrically evoked DA release in the striatum (6.7 ± 0.4 nA to 7.2 ± 0.4 nA, n=6 slices from 3 mice, p=0.0016). Heat map insets show example DA peak amplitude before and after compound application. Voltammogram scale: 2.5 nA; 0.25 V. Example traces scale: 5 nA, 5 s. Data expressed as mean ± SD. B. Bath application of ML375 produced a significant decrease of
electrically evoked DA release in the striatum (6.2 ± 0.7 nA to 5 ± 0.7 nA, n=7 slices from 5 mice, p<0.0001). Example traces scale: 2.5 nA, 5 s. Voltammogram scales: 2.5 nA; 0.25 V. Data expressed as mean ± SD. C. In presence of the acetylcholine nicotinic receptor antagonist DHβE (1 µM), Oxo-M (10 µM) induced an increase of DA release in the striatum of wild type mouse but not in M₅ KO mice. Pre-incubation with ML375 (10 µM) completely abolished Oxo-M induced increase in DA release. Pre-incubation with VU 0365114 (10 µM) produced an increase in Oxo-M induced increase in DA release. Right insets show example DA peak amplitude traces during each condition. Scale: 5 nA, 5 s. Data expressed as mean ± SEM. D. Oxo-M (O-M) significantly increased DA release vs control (Ctl) in wild type mice and after VU 0365114 preincubation, but neither after ML375 pre-incubation nor in M₅ KO (n=5, p=0.0333), Vehicle (n=5, p=0.0054), ML375 (n=5, p=0.0612), VU 0365114 (n=4, p=0.0487, n= 16 mice). Data expressed as mean ± SD. Significance keys: *p<0.05, **p<0.01, ***p<0.001.

Figure 3. Effect of systemic ML375 on cocaine self-administration. A. On a cocaine self-administration FR10 schedule, treatment with ML375 (30 mg·kg⁻¹ i.p.) did not significantly impact the number of active lever presses and was not different from vehicle treatment (b: baseline; t: treatment). B. The number of infusions or C. number of inactive lever presses were not impacted by ML375 treatment. D. In a similar FR10 schedule with an ascending dose of cocaine infusion, there was no significant impact of ML375 treatment on the number of active lever presses (b: baseline; ML: ML375 treatment), E. infusions or F. inactive lever presses. G. On a PR cocaine self-administration schedule, treatment with ML375 (30 mg·kg⁻¹ i.p.) did not significantly impact the number of active lever presses and was not different from vehicle treatment (b: baseline; t: treatment). H. The number of infusions or I. number of inactive lever presses were not impacted by ML375 treatment. J. In a PR schedule with ascending doses of cocaine, ML375 did not produce any significant change in the number of active lever presses,
K. infusions, or L. inactive lever presses. All data expressed as mean ± SD (n=4-13 rats per group). See also Supplemental Figure 3 and Supplemental Table 1 and 2.
Figure 2

A

VU 0365114

DA amplitude (% control)

0 20 40 60 80 100 120 140 160

0 10 20 30

time (min)

(p=0.0016, n=6)

**

B

ML375

DA amplitude (% control)

0 20 40 60 80 100 120 140

0 10 20 30

time (min)

(p<0.0001, n=6)

***

C

compound

Oxo-M

vehicle

VU 0365114

in DHB

DA amplitude (% control)

0 50 100 150

0 10 20 30 40 50

time (min)

VU 0365114

ML375

M5 KO

D

DA amplitude (fold control)

0 1 2 3

Ctl O-M

M5 KO

Vehicle ML375

VU 0365114

* ** n.s.

*
Figure 3

A  Fixed ratio  D  Fixed ratio  G  Progressive ratio  J  Progressive ratio

B  

C  

E  

F  

H  

I  

L  

# of active lever presses

# of infusions

# of inactive lever presses

Vehicle ML375 (30 mg·kg⁻¹)

[coke] mg·kg⁻¹

0.15 0.25 0.50 0.75

Vehicle ML375 (30 mg·kg⁻¹)

[coke] mg·kg⁻¹

0.15 0.25 0.50 0.75

Vehicle ML375 (30 mg·kg⁻¹)

[coke] mg·kg⁻¹

0.15 0.25 0.50 0.75

Vehicle ML375 (30 mg·kg⁻¹)

[coke] mg·kg⁻¹

0.15 0.25 0.50 0.75

Vehicle ML375 (30 mg·kg⁻¹)

[coke] mg·kg⁻¹

0.15 0.25 0.50 0.75

Vehicle ML375 (30 mg·kg⁻¹)