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Effects of muscarinic receptor antagonists on cocaine discrimination in wild-type mice and in muscarinic receptor M_1, M_2, and M_4 receptor knockout mice

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Abstract

Muscarinic M_1/M_4 stimulation can reduce abuse-related effects of cocaine and may represent avenues for treating cocaine addiction. Muscarinic antagonists can mimic and enhance effects of cocaine, including discriminative stimulus (S^D) effects, but the receptor subtypes mediating those effects are not known. A better understanding of the complex cocaine/muscarinic interactions is needed to evaluate and develop potential muscarinic-based medications. Here, knockout mice lacking M_1, M_2, or M_4 receptors ($M_1^{-/-}$, $M_2^{-/-}$, $M_4^{-/-}$), as well as control wild-type mice and outbred Swiss-Webster mice, were trained to discriminate 10 mg/kg cocaine from saline. Muscarinic receptor antagonists with no subtype selectivity (scopolamine), or preferential affinity at the M_1, M_2, or M_4 subtype (telenzepine, trihexyphenidyl; methoctramine, AQ-RA 741; tropicamide) were tested alone and in combination with cocaine. In intact animals, antagonists with high affinity at M_1/M_4 receptors partially substituted for cocaine and increased the S^D effect of cocaine, while M_2-preferring antagonists did not substitute, and reduced the S^D effect of cocaine. The cocaine-like effects of scopolamine were absent in $M_1^{-/-}$ mice. The cocaine S^D attenuating effects of methoctramine were absent in $M_2^{-/-}$ mice and almost absent in $M_1^{-/-}$ mice. The findings indicate that the cocaine-like S^D effects of muscarinic antagonists are primarily mediated through M_1 receptors, with a minor contribution of M_4 receptors. The data also support our previous findings that stimulation of M_1 receptors and M_4 receptors can each attenuate the S^D effect of cocaine, and show that this can also be achieved by blocking M_2 autoreceptors, likely via increased acetylcholine release.

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Keywords

Muscarinic receptor; cholinergic; cocaine; knockout mouse; drug discrimination; M_1; M_2; M_4

1. Introduction

There are currently no approved or widely effective medications to curb addiction to psychostimulant drugs like cocaine. Manipulations targeting muscarinic cholinergic systems can modulate abuse-related effects of cocaine, and muscarinic receptors are thus emerging as potential targets for medications development in cocaine addiction [1]. We have previously shown that agonists or positive allosteric modulators that selectively stimulate either the M_1 or the M_4 muscarinic receptor subtypes attenuate the discriminative stimulus (S^D) [1]effects and reinforcing effects of cocaine in rats and mice ([2-5]; [6]). Studies in knockout mice that lack M_1 receptors ($M_1^{-/-}$), M_4 receptors ($M_4^{-/-}$), or both receptors, suggested that both receptor subtypes mediate the attenuation of cocaine's S^D effects, while other receptors, likely M_3, mediate the rate-suppressing side effects that were observed with less selective ligands [2, 5]. Conversely, muscarinic receptor antagonists increased the S^D effect of cocaine, and can produce cocaine-appropriate responding when substituted for cocaine [2, 7-9].

Based on rat studies that used intracranial infusions or lesions, striatal areas appear to be central to mediating both the S^D effects of cocaine [10, 11], and the modulation of the cocaine S^D effect by muscarinic receptor ligands [12-15]. Striatal tissues express predominantly the M_1 and M_4 muscarinic receptor subtypes, and lower densities of the M_2 subtype: M_1 receptors mostly postsynaptically, M_4 receptors both pre-and postsynaptically, and M_2 receptors mostly as presynaptic inhibitory autoreceptors [16-20]. M_2 and M_4 receptors modulate the tonic acetylcholine release by striatal cholinergic interneurons, which in turn modulates striatal dopamine release via a nicotinic receptor-dependent mechanism [21-25]. Postsynaptically, M_1 and M_4 receptors modulate the excitability and activity patterns of GABAergic medium spiny neurons, the striatum's main output neurons. M_1 and M_4 receptor activation has long been known to produce functional dopamine antagonism, but more recent studies are showing the reciprocal modulation of striatal dopamine and acetylcholine release to be quite complex (for review, see [26-29]).

Here, we used subtype-preferring muscarinic receptor antagonists and knockout mice lacking M_1, M_2, or M_4 receptors to investigate the contributions of specific muscarinic receptor subtypes in the muscarinic/cocaine interaction in the drug discrimination assay. Scopolamine is a non-selective muscarinic antagonist with comparable affinities at the M_1 and M_3-M5 subtypes and a marginally lower affinity at the M_2 subtype [30-32]. Telenzepine is a moderately M_1-preferring antagonist, and trihexyphenidyl has about equal affinity at M_1 and M_4 receptors, with modest selectivity over M_2, M_3, and M5 subtypes [30, 33, 34]. Tropicamide was reported to be a modestly M_4-preferring antagonist with comparable affinities across M_1-M_3 subtypes [35]. Methoctramine and AQ-RA 741 are M_2-preferring

[1]Abbreviations: S^D discriminative stimulus, DAR: drug-appropriate responding

antagonists, with methoctramine generally showing a more selective profile than AQ-RA 741 [33, 36-39]. Methoctramine binds at both the orthosteric site and an allosteric site on M_2 receptors, but the latter only at high concentrations less likely to be relevant in vivo [40, 41]. Each antagonist was either shown to be fully brain penetrant, or has been inferred to penetrate the central nervous system based on producing effects known to be centrally mediated, following systemic administration [32, 42-44].

2. Materials and Methods

2.1 Animals

Male Swiss-Webster, C57BL/6NTac, $M_1^{-/-}$, $M_2^{-/-}$ and $M_4^{-/-}$ mice were acquired from Taconic Farms (Germantown, NY) at 4-8 weeks of age, the knockout mice with the permission of Dr. Jürgen Wess. $M_1^{-/-}$, $M_2^{-/-}$ and $M_4^{-/-}$ mice were generated as described previously [45-47] and backcrossed 11 generations to C57BL/6NTac females. Age- and sex-matched wild-type C57BL/6NTac (Taconic Farms) mice served as controls. Mice were acclimated to the housing facilities for at least 7 days before training began, at no earlier than 7 weeks of age. Mice were kept in a 12-h light/dark cycle, group housed up to four per cage. Experiments were conducted during the light phase of the circadian cycle. Water was accessible ad libitum and food (rodent diet 5001; PMI Feeds, Inc., St. Louis, MO) was provided daily after training/testing sessions, 4 g/mouse/day. Rodent “treats”, nesting material, and exercise/nesting devices were provided for enrichment. Some mice had been tested previously with muscarinic receptor ligands (M_1 agonist and/or M_4 positive allosteric modulator) before the tests reported here. All procedures were approved by the McLean Hospital Institutional Animal Care and Use Committee and were carried out in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research and US laws.

2.2 Training and evaluation in cocaine discrimination

Operant-conditioning chambers and the experimental procedure were as previously described [2]. In brief, each chamber contained two nose-poke holes each equipped with a photocell and a cue light, and a cup into which liquid food was delivered from a syringe pump. Mice were trained to discriminate 10 mg/kg cocaine from saline (i.p.), reinforced with Vanilla-flavored Ensure nutrition drink. 30 reinforcers were available per 20-min session. Mice were trained initially under a FR 1 schedule, then, the ratio was gradually increased to FR 10, with increasing pretreatment time spent in the chamber rather than home cage. Eventually, sessions were preceded by the 10-min pretreatment period in the chamber, during which all lights were off, and responding had no scheduled consequences. Cocaine and saline were presented in pseudorandom order across daily training sessions, typically five days/week, and mice were counterbalanced with cocaine trained on the left or right nose-poke. Stable discrimination was defined as at least 7 of 8 consecutive sessions satisfying the following criteria: 1) ≥10 reinforcers earned per session, 2) ≥80% correct responses for the first reinforcer, and 3) ≥90% correct total responses.

Once criteria were met, mice were tested with saline, 0.32, 1.0, 3.2, 10, and 18 mg/kg cocaine to generate dose-effect functions. The non subtype-selective antagonist scopolamine

(0.032–10 mg/kg i.p.), the M_1/M_4-preferring antagonists telenzepine (3.2-56 mg/kg s.c.) and trihexyphenidyl (0.032– 32 mg/kg i.p.), the M_4-preferring antagonist tropicamide (0.1–10 mg/kg s.c.), and the M_2-preferring antagonists AQ-RA 741 and methoctramine (0.1–3.2 mg/kg s.c.) were each tested alone (i.e., as "substitution" for cocaine stimulus). In addition, pretreatment/combination tests were conducted, in which cocaine doses were tested in combination with scopolamine (0.32 mg/kg i.p., administered with cocaine as a single injection), tropicamide (0.1 mg/kg s.c.), AQ-RA 741 (0.1–1 mg/kg s.c.), and methoctramine (0.0032–1.0 mg/kg s.c.). Pretreatment times before cocaine injection (when tested in combination) or before session start (when tested alone as substitution) were: telenzepine, 10 min; methoctramine, 15 min; all other drugs, immediately before. Doses were tested within-subjects in a counterbalanced sequence. At least one training session was interspersed between each test session, and tests were only performed when mice satisfied discrimination criteria. If responding was suppressed to less than 10 responses in a session, the quantity of behavior was considered insufficient to evaluate response selection and the percentage of drug-appropriate responding (DAR) was not included in the data set.

2.3 Drugs

Cocaine hydrochloride was supplied by the National Institute on Drug Abuse (National Institutes of Health, Bethesda, MD), Scopolamine hydrobromide, telenzepine dihydrochloride hydrate, trihexyphenidyl hydrochloride, tropicamide, and methoctramine hydrate were purchased from Sigma-Aldrich (St. Louis, MO). AQ-RA 741 was purchased from R&D Systems, Inc (formerly Tocris, Ellisville, MO). Cocaine, scopolamine, telenzepine, and AQ-RA 741 were dissolved in 0.9% saline, methoctramine, in sterile water. Trihexyphenidyl was dissolved by gentle heating in sterile water. Tropicamide was dissolved in ethanol and diluted to ≤1% ethanol in sterile water. All drug doses refer to the weights of the respective salts. Vehicles, route of administration, pretreatment times, and initial dose ranges were selected based on published reports, and adjusted empirically in initial studies [2, 44, 48-51].

2.4 Data analysis

The %DAR for the whole session and total rates of responding (i.e., in both holes) are presented. Comparable effects were observed in %DAR for the first reinforcer, unless stated otherwise. Repeated measures ANOVA were performed with dose of pretreatment drug and/or cocaine or dose of substitution drug, as variables, on %DAR and response rate. For knockout strain studies, ANOVA were performed with genotype as between-subjects variable and drug doses/pretreatments as repeated-measures variables. Occasionally, responding was eliminated or suppressed to the point that no reinforcers were earned during the session; in those cases, no %DAR was calculated for that mouse (i.e., missing value). Data are reported as group means with standard error of the mean. Significance level was set at $P<0.05$; statistical software was Stata/SE for Mac.

3. Results

3.1 "Cocaine-like" muscarinic receptor antagonists in Swiss-Webster mice

In outbred Swiss-Webster mice, the M_1/M_4-preferring antagonists telenzepine and trihexyphenidyl, and the M_4-preferring antagonist tropicamide, each produced some cocaine-appropriate responding, with peak averages between 38% and 61% (Fig. 1). DAR was related to antagonist dose for telenzepine [$F(3,21)=4.07$, $P<0.05$], trihexyphenidyl [$F(3,39)=3.68$, $P<0.05$], and tropicamide [$F(5,39)=3.82$, $P<0.01$]. Trihexyphenidyl decreased rates of responding at the highest dose tested (main effect [$F(3,39)=14.3$, $P<0.0001$]; 32mg/kg vs. vehicle $P<0.001$). Tropicamide and telenzepine did not affect rates of responding significantly.

When telenzepine (3.2 mg/kg) and tropicamide (0.1 mg/kg) were tested in combination with cocaine, each antagonist produced a small shift of the cocaine dose-effect function to the left (Fig. 2), but only the effect of tropicamide reached statistical significance by ANOVA [$F(1,63)=10.6$, $P<0.01$]. The effect of cocaine dose on response allocation was always highly significant ($P<0.0001$). In two mice, telenzepine profoundly suppressed responding at the highest doses (despite no to minimal effects on rates of responding in other mice), which resulted in missing values and consequently reduced statistical power. As an alternative analysis method less affected by missing values, potencies of cocaine were calculated by interpolation in each mouse with and without telenzepine, which confirmed a leftward shift (1.34 mg/kg, 95% confidence interval 0.68 – 2.65 vs. 0.39 [0.23 – 0.65] mg/kg, $P<0.01$ by paired-sample t-test). Telenzepine produced a small decrease in rates of responding regardless of the cocaine dose [$F(1,62)=6.90$, $P<0.05$], while tropicamide did not affect rates.

3.2 "Cocaine-like" muscarinic receptor antagonists in muscarinic receptor knockout mice

Wild-type mice and knockout mice lacking M_1, M_2, or M_4 receptors acquired cocaine discrimination after on average 89.3±5.4, 85.5±5.2, 96.3±5.8, and 129.9±10.5 sessions, respectively. The $M_4^{-/-}$ mice required significantly longer training to meet criteria relative to wild-type mice ($\chi^2=13.1$, $P<0.001$). The genotypes did not differ in their cocaine dose-effect functions (Supplemental Fig. 1).

To test the hypothesis that M_1 and/or M_4 receptors mediated the cocaine-like discriminative stimulus effect of the non subtype-selective muscarinic antagonist scopolamine, scopolamine was tested as substitution in each knockout strain. Tests were aborted in the $M_2^{-/-}$ strain due to unexpected toxicity, as the first few $M_2^{-/-}$ mice tested with doses of scopolamine above 0.32 mg/kg died within a day. Thus, dose-effect functions for scopolamine substitution were obtained in wild-type mice, $M_1^{-/-}$ mice, and $M_4^{-/-}$ mice (Fig. 3). DAR was related to both genotype [$F(2,71)=3.54$, $P<0.05$] and scopolamine dose [$F(4,71)=8.47$, $P<0.0001$]. Scopolamine produced partial substitution, and significant effects on DAR, in the wild-type mice [$F(4,25)=7.28$, $P<0.001$] and in the $M_4^{-/-}$ mice [$F(4,24)=3.41$, $P<0.05$], but not in the $M_1^{-/-}$ mice. Rates of responding were also affected differentially (genotype effect [$F(2,74)=6.41$, $P<0.01$], scopolamine effect [$F(4,74)=6.08$, $P<0.001$].

A low dose of scopolamine can shift the cocaine discrimination dose-effect function to the left in intact mice [2]. To test the hypothesis that M_1 receptors also mediated the potentiation of the cocaine discriminative stimulus effect by scopolamine, we determined cocaine dose-effect functions with and without 0.32 mg/kg scopolamine in wild-type mice, $M_1^{-/-}$ mice, $M_2^{-/-}$ mice, and $M_4^{-/-}$ mice. We observed a qualitative difference between strains, in that scopolamine produced the expected shifts to the left in the wild-type mice, $M_2^{-/-}$ mice, and $M_4^{-/-}$ mice, but produced a shift to the *right* in the $M_1^{-/-}$ mice (Fig. 4). A three-way ANOVA with the factors genotype, cocaine, and scopolamine confirmed a significant genotype by scopolamine interaction [$F(3,237)=6.11$, $P<0.0001$], and follow-up simple effects confirmed a significant effect of scopolamine in each knockout line ($P<0.05$ to $P<0.001$). No evidence of toxicity was observed in the $M_2^{-/-}$ mice with this dosing. The effect of cocaine dose on DAR was significant in all pretreatment/combinations tests ($P<0.0001$).

3.3 “Cocaine-attenuating” muscarinic receptor antagonists in Swiss-Webster mice

The M_2-preferring antagonist methoctramine produced little cocaine-appropriate responding per se, with a maximal DAR of 35% at the highest dose (main effect [$F(4,26)=3.43$, $P<0.05$]; 3.2mg/kg vs. vehicle $P<0.05$), (Fig. 5A). Methoctramine was tested over a range of doses, up to doses that produced rate-decreasing effects [$F(4,28)=7.21$, $P<0.001$].

We then tested methoctramine in combination with cocaine, testing for a shift in the cocaine dose-effect function at two doses of methoctramine, and testing a range of methoctramine doses in combination with 3.2 mg/kg cocaine. As opposed to scopolamine and the M_1/M_4 receptor-preferring antagonists telenzepine, trihexyphenidyl, and tropicamide, methoctramine moderately *attenuated* the cocaine discriminative stimulus effect in Swiss-Webster mice: administration of 0.01 mg/kg methoctramine produced a small shift of the dose-effect function to the right (Fig. 5B; methoctramine effect [$F(1,53)=4.34$, $P<0.05$], cocaine by methoctramine interaction [$F(4,53)=2.67$, $P<0.05$]. At 1.0 mg/kg, methoctramine had no signnificant effect on DAR (in fact showed a trend to shift the cocaine curve to the left), and moderately decreased rates of responding ([$F(1,63)=17.6$, $P=0.0001$; Fig. 5C). This “biphasic” effect was also apparent when testing a range of methoctramine doses with 3.2 mg/kg cocaine, in which doses from 0.01 to 0.32 mg/kg methoctramine decreased DAR (main effect of methoctramine dose [$F(6,30)=3.28$, $P<0.05$]), but 1 mg/kg had no effect (Fig. 5D).

In an effort to replicate those findings, the putative M_2-preferring antagonist AQ-RA 741 was also tested. AQ-RA 741 alone produced no cocaine-appropriate responding in a range of doses previously shown to be active in vivo (no effect of dose on DAR or rate; see Supplemental Fig. 2A). However, the selectivity of AQ-RA 741 is modest, and the usefulness of those data is limited by the fact that higher, rate-suppressing doses were not tested, out of concerns for potential toxicity. Administration of 1.0 mg/kg AQ-RA 741 produced a small shift of the cocaine dose-effect function to the right (Supplemental Fig. 2B), although the effect only reached statistical significance for the first reinforcer (AQ-RA 741 dose [$F(1,62)=4.45$, $P<0.05$], AQ-RA 741 by cocaine interaction [$F(4,62)=2.98$, $P<0.05$]), but not for total-session responding. A range of AQ-RA 741 doses were tested with 3.2 mg/kg cocaine (Supplemental Fig. 2C). Again, AQ-RA 741 produced moderate

decreases in DAR that was significant for first-reinforcer responding [$F(3,15)=4.43$, $P<0.05$] (total session analysis: $P=0.06$). Rates of responding were not significantly affected.

3.4 "Cocaine-attenuating" muscarinic receptor antagonists in muscarinic receptor knockout mice

Because methoctramine has relatively low selectivity for the M_2 subtype, we wanted to test the hypothesis that effects on cocaine discrimination were mediated through M_2 receptors. To this end, we tested 0.032 mg/kg methoctramine as pretreatment to cocaine in wild-type mice, $M_1^{-/-}$ mice, $M_2^{-/-}$ mice, and $M_4^{-/-}$ mice (Fig. 6). A three-way ANOVA with the factors genotype, cocaine dose and methoctramine dose confirmed a significant genotype by methoctramine interaction [$F(3,205)=2.97$, $P<0.05$]. As hypothesized, methoctramine had no effect in the $M_2^{-/-}$ mice (Fig. 6C), while the moderate rightward shift observed in the Swiss-Webster mice was confirmed in the wild-type mice ($P=0.001$; Fig. 6A). The effect appeared intact in the $M_4^{-/-}$ mice ($P<0.01$; Fig. 6D), but was diminished in the $M_1^{-/-}$ mice, in which the shift was not significant (Fig. 6B).

4. Discussion

Muscarinic antagonists can partially mimic and increase the S^D effects of cocaine, but the muscarinic receptor subtypes involved in these effects are not known. We tested muscarinic receptor antagonists with no subtype selectivity, or moderately preferential affinity at the M_1, M_2 or M_4 subtype, in wild-type mice, $M_1^{-/-}$ mice, $M_2^{-/-}$ mice and $M_4^{-/-}$ mice trained to discriminate 10 mg/kg cocaine from saline. We found that the non-selective antagonist scopolamine or antagonists with relatively higher affinity at M_1/M_4 receptor subtypes produced some cocaine-appropriate responding per se, and produced leftward shifts in the cocaine S^D effect curve in intact animals. Experiments in the knockout mice further indicated that M_1 receptors, rather than M_4 receptors, mediate the cocaine-like S^D effects of scopolamine. In contrast, antagonists with relatively higher affinity at the M_2 receptor subtype attenuated the cocaine S^D effect. Experiments in knockout mice also supported the notion that M_2 receptors mediate the cocaine S^D attenuating effects of methoctramine.

4.1 Cocaine-like effects of non-selective and M_1/M_4-preferring antagonists in intact mice

We previously showed that scopolamine produced cocaine-appropriate responding in Swiss-Webster mice (partial substitution with an inverted U-shaped curved), and that a sub-threshold dose of scopolamine shifted the cocaine dose-effect curve to the left [2]. Methylscopolamine, which has poor brain penetration, was less potent than scopolamine in producing these effects, indicating a centrally mediated effect [2]. This is in agreement with earlier studies in rats, in which the non subtype-selective muscarinic antagonists atropine and scopolamine produced leftward shifts in cocaine's S^D effects [7, 8]. Potentiation of psychostimulant effects by muscarinic receptor blockade has been observed consistently in mice, rats, and non-human primates across a range of endpoints, including locomotor activity, stereotypies, and intravenous self-administration [8, 52-55]. Isobolographic analyses confirmed that the effects of cocaine-scopolamine combinations were more than additive both in drug discrimination in mice, and in locomotor activity in rats, implying that muscarinic receptor antagonists and cocaine produce their effects through different brain

pathways [2, 56]. The interaction likely involves dopamine release, as systemic administration of muscarinic antagonists induces striatal dopamine release in humans and rats, and potentiates cocaine-induced dopamine increases [34, 57, 58].

Here, M_1 and/or M_4-preferring antagonists produced some cocaine-appropriate responding, comparable to results obtained previously with scopolamine [2]. Previous investigations similarly found that the moderately M_1-preferring antagonists telenzepine and trihexyphenidyl potentiated the locomotor stimulant and/or S^D effects of cocaine in rats [9, 34]. Tropicamide was described as somewhat M_4-preferring, although recent data suggest that is a relatively nonspecific muscarinic antagonist [35, 59]. Effects of M_4 receptor blockade on cocaine's S^D effects have not been reported previously, but our findings are consistent with a report that tropicamide increased or prolonged cocaine-induced stereotypies in rats [52]. The notion that blocking either M_1 receptors or M_4 receptors increases effects of cocaine is also consistent with our findings that, conversely, pharmacological stimulation of M_1 and/or M_4 receptors attenuated the S^D effects and reinforcing effects of cocaine in rats and mice ([2-5]; [6]). Taken together, this first data set suggests that M_1 and/or M_4 receptor blockade at least partly mediate the effects of scopolamine in the cocaine discrimination assay.

4.2 Cocaine-like effects of muscarinic antagonists in muscarinic receptor knockout mice

Brain-penetrant muscarinic receptor antagonists with a high degree of selectivity for each of the five receptor subtypes are still being developed, and we therefore used a combination of subtype-preferring ligands and receptor knockout mice to determine the contributions of individual receptor subtypes to the above effects. Cocaine produced comparable S^D dose-effect functions in all four strains – wild-type, $M_1^{-/-}$, $M_2^{-/-}$, and $M_4^{-/-}$. In contrast, substitution of scopolamine for cocaine revealed striking differences: scopolamine produced over 80% cocaine-appropriate responding in the wild-type mice, but no appreciable cocaine-appropriate responding in the $M_1^{-/-}$ mice in the same dose range. Partial substitution was observed in the $M_4^{-/-}$ mice. Although we cannot exclude that higher doses of scopolamine could have produced some cocaine-appropriate responding in the knockout mice, these findings indicate that M_1 receptors are necessary for scopolamine to produce cocaine-S^D like effects, while M_4 receptors may contribute partially to the effect.

When we tested scopolamine in combination with cocaine, the $M_1^{-/-}$ mice again showed a qualitatively different effect: whereas scopolamine produced leftward shifts in the cocaine dose-effect function in wild-type mice, $M_2^{-/-}$ mice, and $M_4^{-/-}$ mice, $M_1^{-/-}$ mice showed a *rightward* shift. Thus, the cocaine-potentiating effect of scopolamine appears to be dependent upon blockade of M_1 receptors.

These findings illustrate the opposing modulatory effects of different muscarinic receptor subtypes on striatal dopaminergic transmission (for review, see [28]; Thomsen et al. 2017 under review). In the absence of M_1 receptors, scopolamine would block M_2-M5 receptors, and the fact that scopolamine produced a rightward shift, not leftward shift, in $M_1^{-/-}$ mice, suggests that blocking M_4 receptors alone is not sufficient to potentiate the effects of cocaine. Rather, antagonism of M_2 receptors and the resulting increase in acetylcholine tone appears to produce sufficient stimulation of M_4 receptors to attenuate cocaine's effects,

consistent with the effect of an M_4 positive allosteric modulator in the same assay [6]. It is possible that effects at M5 receptors play a role as well [66, 67]. $M_1^{-/-}$ mice (but not $M_2^{-/-}$ mice or $M_4^{-/-}$ mice) showed plasma levels of scopolamine roughly twice as high as the wild-type controls 30 min after an intraperitoneal injection of 1 mg/kg, as well as a trend for higher brain levels [32]. However, given that scopolamine produced cocaine-like effects up to at least 10 mg/kg in wild-type mice, it seems unlikely that this slightly higher blood level would account for the complete reversal of effect observed here between the wild-type mice and the $M_1^{-/-}$ mice.

The scopolamine-induced leftward shift in cocaine S^D was preserved or if anything larger in the $M_2^{-/-}$ mice and $M_4^{-/-}$ mice relative to wild-type mice. Loss of inhibitory autoreceptors may be expected to lead to increased extracellular levels of acetylcholine, and/or reduced effect of scopolamine on acetylcholine release. Indeed, these effects were observed in $M_2^{-/-}$ mice, $M_4^{-/-}$ mice, and $M_2^{-/-}M_4^{-/-}$ double knockout mice [25, 68]. Thus, loss of scopolamine-increased acetylcholine tone (which would tend to attenuate the cocaine S^D effect) would unmask the M_1 receptor-mediated potentiation of the cocaine S^D effect. These data suggest that M_1 receptors, not M_4 receptors, play the major role in scopolamine-potentiated cocaine S^D effects. Studies using $M_1^{-/-}$ mice, $M_4^{-/-}$ mice, and double $M_1^{-/-}M_4^{-/-}$mice similarly indicated that M_1 receptors, not M_4 receptors, mediated the disruption of prepulse inhibition of the startle response by scopolamine, another effect that is shared between muscarinic receptor antagonists and psychomotor stimulant drugs [69].

4.3 Cocaine-attenuating effects of M_2-preferring antagonists

In contrast to the M_1/M_4 receptors, we did not anticipate that blockade of the (primarily presynaptic) M_2 receptors would produce cocaine-like effects in the drug discrimination assay. Indeed, neither methoctramine nor AQ-RA 741 substituted for cocaine in intact mice, with the caveat that neither ligand is highly selective for the M_2 receptor, and that neither ligand was tested at doses that produced strong behavioral suppression, due to expected risks of toxicity. To evaluate the effects of methoctramine in combination with cocaine, we therefore tested a wide range of doses (two and a half log units), including doses that may bind M_2 receptors preferentially, and doses that may have off-target effects. This produced a biphasic dose-effect function, from an ineffective, sub-threshold dose, over a range of doses that decreased cocaine-appropriate responding, to a complete reversal of effect this at the highest dose. A biphasic dose-effect function of methoctramine has been observed previously, similarly attributed to recruitment of non-M_2 receptors [60]. M_2 receptor antagonists reduced the S^D effects of 3.2 mg/kg cocaine by up to 50% in Swiss-Webster mice, an effect similar to that produced by M_1/M_4 agonists [2, 5]. When tested against a range of cocaine doses, a small rightward shift was obtained.

Brain M_2 receptors are mainly inhibitory autoreceptors [61, 62]. M_2 receptors serve as autoreceptors throughout the brain with the probable exception of the nucleus accumbens, while M_4 receptors appear to serve this function only in the striatum [18, 21, 23, 25]. Consistent with this function, in vitro and in vivo studies have shown that M_2-preferring antagonists increase acetylcholine in dorsal striatum, hippocampus, and cortex [39, 60, 63-65]. We therefore hypothesize that the M_2 antagonists attenuated the cocaine S^D effect

indirectly, through stimulation of brain M_1 and/or M_4 receptors by endogenous acetylcholine. Nicotinic receptors, which also modulate dopamine release in the dorsal and ventral striatum, could also be involved [23]. A complete lack of effect of methoctramine in the $M_2^{-/-}$ mice supports the interpretation that methoctramine attenuated the cocaine S^D effect in intact mice via antagonism of M_2 receptors. Although rat studies indicated that the nucleus accumbens is important in mediating the S^D effects of cocaine [10, 11], the above results suggest that the effect of methoctramine is likely mediated at least partially in dorsal striatum (and/or other brain regions) rather than the nucleus accumbens, because M_4 receptors, not M_2 receptors, serve as autoreceptors in the accumbens [23]. While methoctramine produced a rightward shift of the cocaine curve in wild-type mice and in the $M_4^{-/-}$ mice, the effect was strongly attenuated in the $M_1^{-/-}$ mice. This is perhaps surprising in the light of the effects of scopolamine in the knockout mice, but may suggest that stimulation of postsynaptic M_1 receptors plays a more important role relative to M_4 receptors in attenuating the cocaine S^D.

4.4 Conclusions

Taken together, the present findings suggest that cocaine-like S^D effects of muscarinic antagonists are primarily mediated through muscarinic M_1 receptors, with a minor contribution of M_4 receptors. The data are in agreement with the notion that stimulation of M_1 receptors and M_4 receptors can each attenuate the S^D effect of cocaine, not only through direct pharmacological stimulation as shown previously ([2, 5]; [6]), but also through increased cholinergic tone due to blockade of M_2 autoreceptors. Finally, the findings also suggest that the cocaine-modulating effects of muscarinic receptor manipulations are likely not mediated entirely in the nucleus accumbens, but involve dorsal striatum or other brain regions as well.

Supplementary Material

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Highlights

- Muscarinic antagonists produce cocaine-like effects mainly via M_1 receptors
- M_2-preferring antagonists attenuate cocaine effects
- M_2 antagonist effects are likely mediated by M_1/M_4 receptors via increased acetylcholine

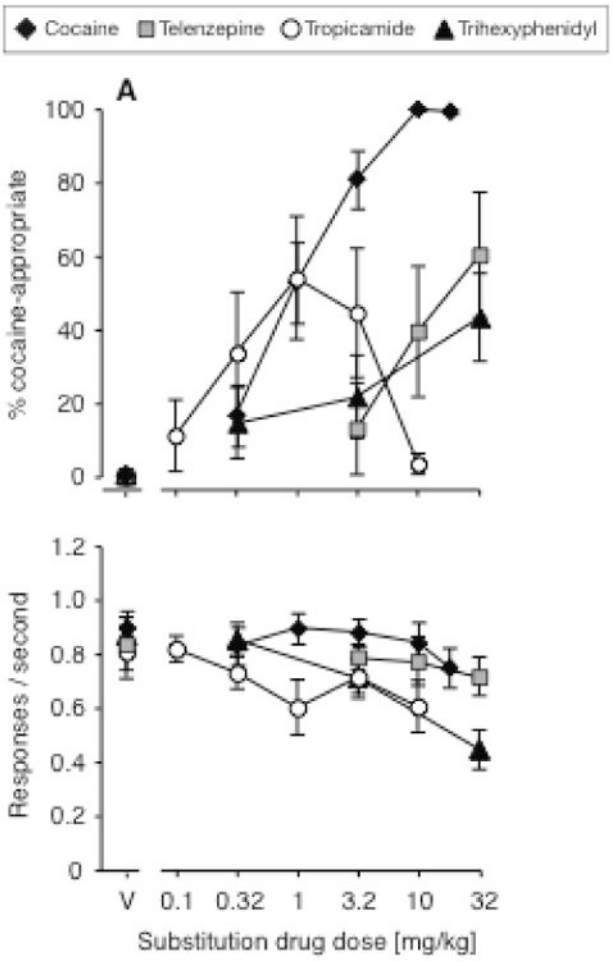

Figure 1.

Dose-effect functions for cocaine and muscarinic M_1/M_4 receptor -preferring antagonists in Swiss-Webster mice, tested as substitutions for the training dose of 10 mg/kg cocaine: telenzepine (N=8), tropicamide (N=9), trihexyphenidyl (N=14), and cocaine in the same cohort (N=19). Data are %DAR (top) and responses per second (bottom), as a function of substitution drug dose.

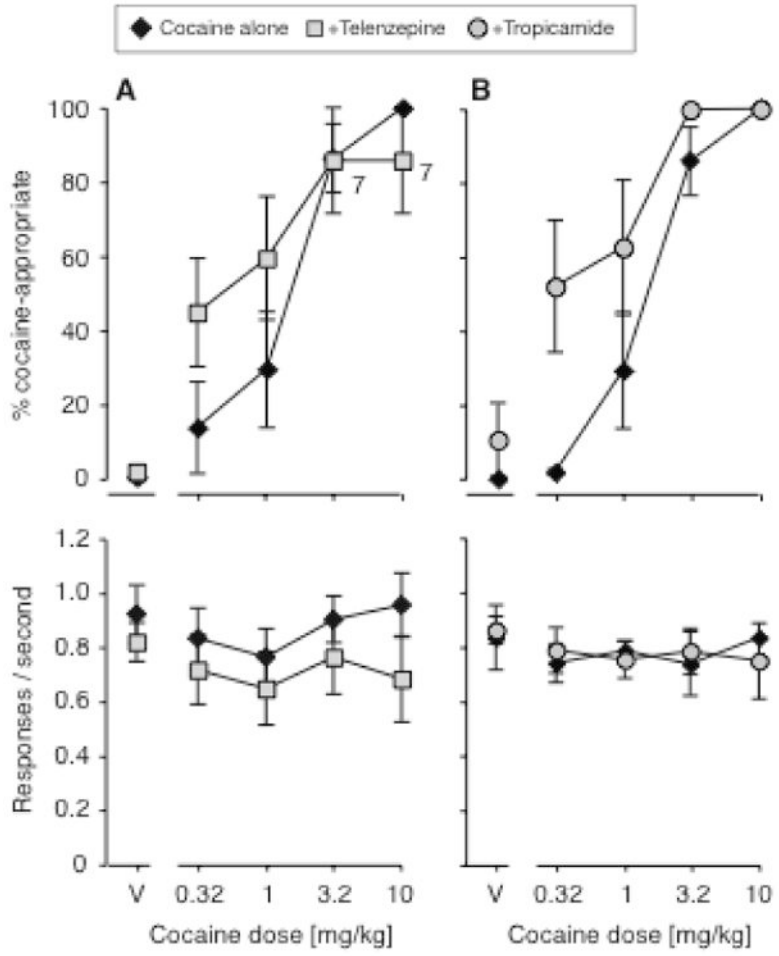

Figure 2.

Effects of adding 3.2 mg/kg telenzepine (A, N=9) or 0.1 mg/kg tropicamide (B, N=8) to cocaine in Swiss-Webster mice. Data are %DAR (top) and responses per second (bottom), as a function of cocaine dose. In the top panel, exceptions to the group sizes are indicated on the figure when some mice failed to respond at a given dose.

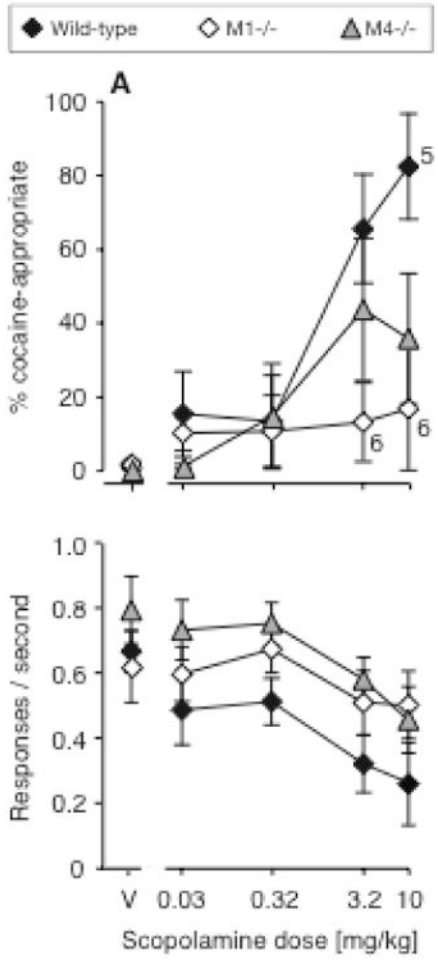

Figure 3.

Dose-effect functions for the non subtype-selective antagonist scopolamine in wild-type mice (WT, N=8), $M_1^{-/-}$ mice (N=7), and $M_4^{-/-}$ mice (N=7), tested as substitutions for the training dose of 10 mg/kg cocaine. No data in the $M_2^{-/-}$ mice due to toxicity. Data are %DAR (top) and responses per second (bottom), as a function of scopolamine dose. In the top panel, exceptions to the group sizes are indicated on the figure when some mice failed to respond at a given dose.

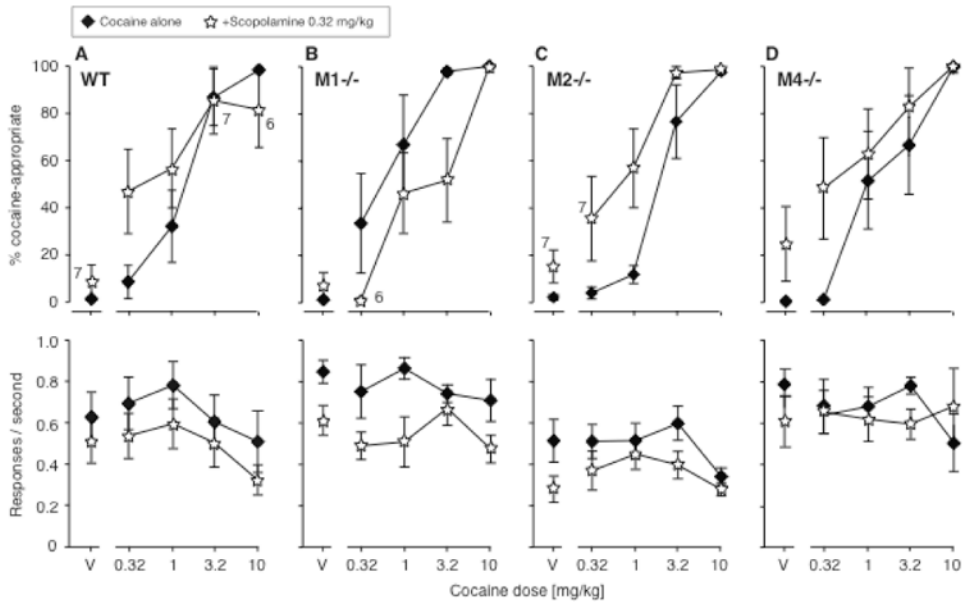

Figure 4.

Effects of adding 0.32 mg/kg scopolamine to cocaine in wild-type mice (A, N=8), $M_1^{-/-}$ mice (B, N=6), $M_2^{-/-}$ mice (C, N=6), and $M_4^{-/-}$ mice (D, N=8). Note the qualitatively different effect in the $M_1^{-/-}$ mice. Data are %DAR (top) and responses per second (bottom), as a function of cocaine dose. In the top panel, exceptions to the group sizes are indicated on the figure when some mice failed to respond at a given dose.

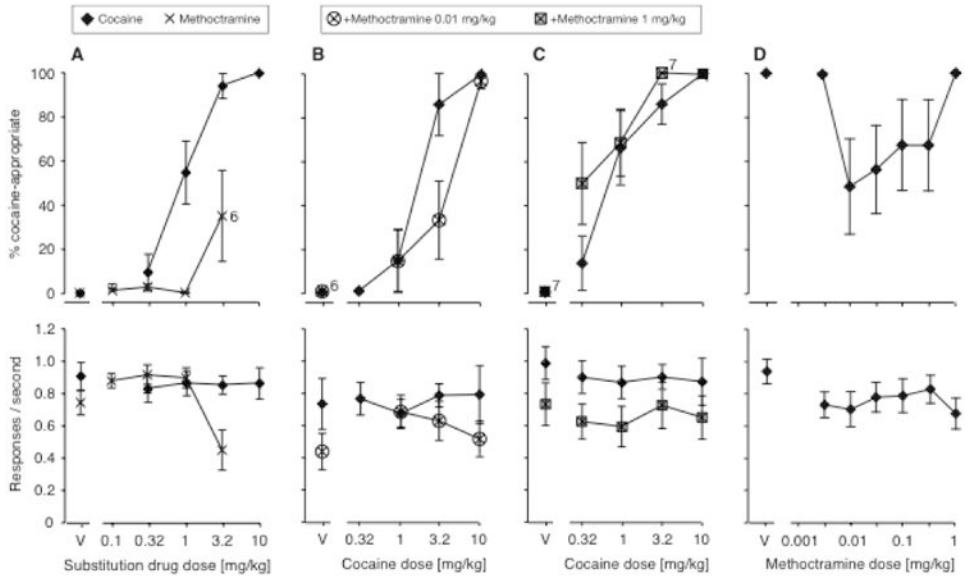

Figure 5.

A: Dose-effect functions for cocaine (N=12) and the muscarinic M_2 receptor-preferring antagonist methoctramine (N=8) in Swiss-Webster mice, tested as substitutions for the training dose of 10 mg/kg cocaine. B: Effect of adding a 0.01 mg/kg methoctramine to a range of cocaine doses (N=7). C: Effect of adding 1 mg/kg methoctramine to a range of cocaine doses (N=8). D: Dose-response relationship of adding methoctramine to 3.2 mg/kg cocaine in Swiss-Webster mice (N=6). Data are %DAR (top) and responses per second (bottom), as a function of cocaine dose or methoctramine dose. In the top panel, exceptions to the group sizes are indicated on the figure when some mice failed to respond at a given dose.

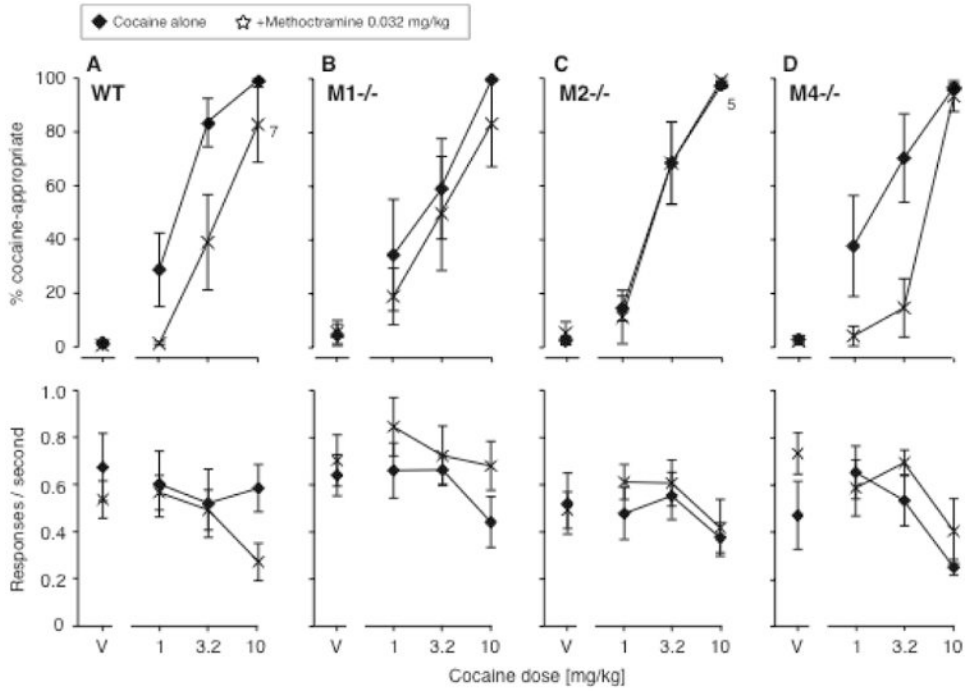

Figure 6.

Effects of adding 0.032 mg/kg methoctramine to cocaine in wild-type mice (A, N=8), $M_1^{-/-}$ mice (B, N=6), $M_2^{-/-}$ mice (C, N=6), and $M_4^{-/-}$ mice (D, N=6). Data are %DAR (top) and responses per second (bottom), as a function of cocaine dose. In the top panel, exceptions to the group sizes are indicated on the figure when some mice failed to respond at a given dose.