Behavioral/Systems/Cognitive

Reduced Cocaine Self-Administration in Muscarinic M_5 Acetylcholine Receptor-Deficient Mice

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The reinforcing effects of cocaine have been related to increased extracellular concentrations of dopamine in the ventral striatum. Several studies suggest that M_5 muscarinic receptors facilitate striatal dopamine release. We tested the hypothesis that the reinforcing effects of cocaine are decreased in M_5 receptor-deficient mice using chronic intravenous cocaine self-administration in extensively backcrossed mice. We also assessed whether operant performance generally, rather than cocaine self-administration specifically, was altered in the mutant mice. To this end, we evaluated both food-maintained operant behavior and cocaine self-administration under a fixed ratio 1 and a progressive ratio (PR) schedule of reinforcement. We also evaluated acquisition of self-administration in experimentally naive mice using several doses of cocaine. M_5 receptor deletion decreased self-administration of low to moderate doses of cocaine under a PR schedule of reinforcement and diminished acquisition of self-administration of a low dose in experimentally naive mice. We found no differences between genotypes in food-maintained behavior. The present study extends our previous findings using backcrossed mice and covering various experimental conditions. Our results indicate that M_5 receptor deletion diminished the reinforcing effects of low doses of cocaine and identified specific conditions under which this may be observed.

Key words: **muscarinic; M_5; acetylcholine; knock-out; self-administration; cocaine**

Introduction

The reinforcing effects of cocaine depend on dopamine systems that arise in the ventral tegmental area (VTA) and project to the nucleus accumbens (Roberts et al., 1977, 1980; Pettit and Justice, 1989; Caine and Koob, 1994). Dopaminergic VTA neurons receive cholinergic input from the laterodorsal tegmental (LDT) and pedunculopontine (PPT) nuclei (Bolam et al., 1991; Oakman et al., 1995; Blaha et al., 1996). The M_5 receptor is the only muscarinic acetylcholine receptor subtype detected in dopaminergic neurons in the VTA (Vilaro et al., 1990; Weiner et al., 1990). Ligands selective for the M_5 subtype are not available. However, the generation of M_5 receptor-deficient ($M_5^{-/-}$) mice has provided evidence that M_5 receptors facilitate striatal dopamine release. The prolonged accumbal dopamine release, observed after electrical stimulation of the LDT in intact animals (Forster and Blaha, 2000, 2003), was absent in $M_5^{-/-}$ mice (Forster et al., 2002).

$M_5^{-/-}$ mice were also found to be less responsive to abused drugs under some conditions. $M_5^{-/-}$ mice exhibited decreased dopamine release and conditioned place preference in response to morphine and blunted withdrawal symptoms compared with wild-type mice (Basile et al., 2002). We previously reported decreased cocaine-conditioned place preference and acute cocaine self-administration (in a single-session procedure) in $M_5^{-/-}$ mice (Fink-Jensen et al., 2003). However, it remains to be determined whether M_5-receptor deletion attenuates the reinforcing effects of cocaine in a chronic self-administration procedure and under a broad range of conditions, including manipulations of behavioral history and schedule of reinforcement. Moreover, the mice used in the previous studies were generated and maintained on a mixed genetic background (129 × CF_1). 129 substrains have been shown to differ from other strains in operant task performance, in the place conditioning effects of cocaine, and in the potency of cocaine as a reinforcer (Miner, 1997; Kuzmin and Johansson, 2000) (M. Thomsen and S. B. Caine, unpublished observations), all of which could have contributed to the phenotype of the $M_5^{-/-}$ mice (Gerlai, 1996; Kelly et al., 1998).

The present experiments were designed to further test whether M_5 receptor activity contributes to cocaine reinforcement and to extend previous findings in three areas. First, we used M_5 receptor mutant mice ($M_5^{-/-}$ and $M_5^{+/-}$) and wild-type littermates that were extensively backcrossed to minimize the influence of genetic background. Second, we evaluated food-maintained behavior to assess whether general operant performance, rather than the reinforcing efficacy of cocaine, was altered in the mutant mice. Third, we measured chronic cocaine self-administration under a broad range of conditions. In one exper-

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iment, cocaine self-administration was measured under a fixed ratio 1 (FR 1) schedule of reinforcement in mice previously trained with food reinforcement. In a second experiment, we measured acquisition of cocaine self-administration in experimentally naive mice using several cocaine doses. Finally, dose–effect functions were also determined using a progressive ratio (PR) schedule, in which self-administration under increasing ratio requirements served as a measure of the reinforcing strength of cocaine. We also included measures of general sensorimotor and behavioral characteristics to identify overt phenotype differences that might contribute to differences in operant behavior (Crawley, 1999).

Materials and Methods

Animals and housing. Homozygous ($M_5^{-/-}$) and heterozygous ($M_5^{+/-}$) M_5 muscarinic receptor knock-out mice and their wild-type littermates ($M_5^{+/+}$) were bred at Taconic Farms (Germantown, NY) and genotyped by PCR from tail DNA at the Molecular Signaling Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases/National Institutes of Health (Bethesda, MD). M_5 knock-out mice were generated as described previously using 129S6/SvEv embryonic stem cells (Yamada et al., 2001) and were backcrossed for 11 generations to C57BL/6NTac females to yield a high degree of congenicity. Animals were kept in a 12 h light/dark cycle (lights on at 7:00 A.M.) at 22°C and 55% humidity. Tap water was accessible *ad libitum*. Standard rodent chow was provided *ad libitum* until operant training started and was then delivered once daily after self-administration sessions, ~3.8 g/d per mouse. For enrichment purposes, variously flavored rodent "treats" were given twice weekly, and nesting material (cotton) and hiding/nesting devices (cardboard "shacks" or plastic "igloos") were provided. Exercise devices (running wheels) were available (but only before catheter implantation to avoid injuries caused by the protruding catheter base). Clean litter (pine wood shavings) was provided twice weekly. The air in each cage was actively circulated to improve respiratory conditions (40 changes per hour, HEPA-filtered air). Animals were group housed up to five per cage (genotypes mixed) and were left to acclimate to the housing facilities at least 7 d before experiments were initiated. During this time, the mice were also handled, and they were anesthetized once briefly while a microchip was inserted subcutaneously for unambiguous identification during experiments. The present data were collected from a total of 129 mice: 43 $M_5^{+/+}$ (21 male and 22 female), 47 $M_5^{+/-}$ (25 male and 22 female), and 39 $M_5^{-/-}$ (20 male and 19 female).

All animals were at least 8 weeks of age when testing began. All testing was conducted during the light phase of the circadian cycle, for practical reasons. All procedures were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and all protocols were approved by the Institutional Animal Care and Use Committee.

Basic sensorimotor characteristics and home-cage behaviors. General physical and behavioral characteristics of the animals were assessed in a standardized manner in 26–29 mice per genotype, after acclimatization and recovery from microchip implantation but before any other behavioral or surgical procedures. Body weight was measured, and the following were evaluated: appearance of fur (matting), signs of over-grooming (bald patches, missing or broken whisker), and home-cage sleep and nesting patterns, (observations at 8:00 A.M., 2:00 P.M., and 8:00 P.M.). The occurrence of spontaneous fighting was assessed when mice were returned to their home cage after sensorimotor testing (see below), and the presence of wounds from aggression was assessed. Reaction to handling (attempts to escape or to bite experimenter) was also rated. As a general assessment of sensorimotor function, the eye blink, the ear twitch, the whisker orientation, and the righting reflex were evaluated (as described by Crawley and Paylor, 1997; Crawley, 1999). Additional tests of sensorimotor skills were the "paw splaying" visual test, the "hanging wire" test, and evaluation of the hindpaw footprint pattern. In the visual test, the mouse was picked up by its tail and lowered toward the edge of a table with a smooth motion (modified from Crawley, 1999). The test was positive if the mouse splayed its paws toward the table when it came within close proximity, indicating visual detection of the table. For the hanging wire test (Crawley, 1999), an apparatus was made from a standard cage top (1.8 mm round stainless-steel rods, 7 mm apart). The time in seconds that the mouse hung from the apparatus without falling into a cushioned area below was recorded, with a cutoff value of 60 s. The footprint pattern assessment was modified from Barlow et al. (1996). Briefly, the hindpaws of the mouse were dipped in a nontoxic "ink" made of powdered red beet and water. The mouse was then allowed to walk over a sheet of white paper, confined in a "runway" ~10 cm wide. A nesting device containing palatable food placed at the end of the runway served to encourage the animals to traverse the runway. A minimum of three straight, uninterrupted traversals with at least five step cycles each were obtained from each animal. Patterns were evaluated by stride length, variation of stride length [(90th percentile minus 10th percentile) for each animal], and width of the base of support.

Operant test apparatus. Operant chambers as well as training and evaluation of food-maintained behavior under an FR schedule have been described in detail previously (Caine et al., 1999; Thomsen and Caine, 2005). Briefly, each operant chamber contained two nose-poke holes 10 mm above the grid floor, both equipped with photocells and a discriminative cue light (yellow), positioned on either side of a small dish-shaped plate into which liquid food could be delivered. In all tests, except the hole reversal procedure, responding in the right hole resulted in delivery of a reinforcer and illumination of the cue light for 20 s, during which additional responses were without scheduled consequences (i.e., postreinforcer timeout). Responses in the left hole were counted but had no scheduled consequences. The house light was turned on after a single noncontingent delivery of the reinforcer and stayed on until the session ended.

Overview of experiments. Mice of each sex and genotype were randomly assigned to one of two experimental designs as follows. In experiment 1, the mice were trained with liquid food as the reinforcer before being introduced to cocaine under the FR 1 schedule of reinforcement. This was done, first, to evaluate operant behavior maintained by a nondrug reinforcer and, second, to obviate possible differences in general performance that might influence cocaine self-administration. In experiment 2, acquisition of cocaine self-administration was evaluated in experimentally naive mice with various doses. Cocaine self-administration dose–effect functions under the PR schedule of reinforcement were subsequently determined in mice from both experiments. Because there was no significant difference or consistent trend between determinations of cocaine dose–effect functions under the PR schedule obtained from mice trained with food reinforcement and naive mice, these determinations were pooled.

Experiment 1: training and evaluation of behavior under the FR schedule. Seventy-two mice were trained and evaluated under the FR schedule (23 $M_5^{+/+}$, 14 male and 9 female; 29 $M_5^{+/-}$, 14 male and 15 female; 20 $M_5^{-/-}$, 10 male and 10 female). Animals were mildly food deprived before the first presentation of the liquid food (5 ml of Ensure protein drink) in the operant chamber (i.e., *ad libitum* dry food in the home cage was removed 18–21 h before the session; water remained available). When a minimum of 1.5 ml of the 5 ml available was consumed per 2 h session (typically within one or two sessions), the mice were placed in the operant chamber with one active (right) and one inactive (left) nose-poke hole, for one 2 h session daily, 6 d/week. During the acquisition phase, activation of the right nose-poke hole led to the presentation of a liquid food reinforcer according to an FR 1 timeout 20 s schedule. Acquisition lasted for at least five consecutive sessions and until criteria were met (minimum of 20 reinforcers earned, with no more than 20% variation over two sessions and at least 70% responses in the active hole). After acquisition criteria were met, water was substituted for at least three sessions and until responding was extinguished to <80% of food-maintained responding, followed by six sessions in which either food or water reinforced nose pokes in alternating sessions. Subsequently, a range of liquid food dilutions (water, 3, 10, 32, and 100%) was presented according to a Latin square design, determined twice in each mouse. Finally, a subset of these mice was studied under a progressive ratio schedule of food reinforcement before catheter implantation (see below, Progressive ratio schedule).

Catheter implantation surgery and maintenance. An indwelling catheter was implanted into the right or left external jugular vein under oxygen/isoflurane or oxygen/sevoflorane vapor anesthesia. The surgical procedure has been described in detail previously (Thomsen and Caine, 2005). Briefly, a catheter (SILASTIC tubing, 0.18 mm inner diameter, 0.41 mm outer diameter) was inserted 1.2 cm into the jugular vein and delicately anchored to the vein with suture. The catheter ran subcutaneously to the base located above the midscapular region. The mice were allowed 7 d of recovery, during which 0.02 ml of 0.9% saline containing heparin (30 United States Pharmacopeia U/ml) and antibiotic (cefazolin, 50 mg/ml) was infused daily through the catheter to forestall clotting and infection. Outside the self-administration sessions, the free end of the cannula guide was kept closed at all times. Catheter patency was confirmed after completion of an experimental phase by the infusion of 0.02–0.03 ml of 15 mg/ml ketamine plus 0.75 mg/ml midazolam in saline. Loss of muscle tone and clear signs of anesthesia within 3 s of infusion indicated catheter patency.

Cocaine self-administration behavior under an FR schedule. After jugular catheter implantation and recovery, intravenous cocaine was available as the reinforcer (1.0 mg/kg per infusion of cocaine HCl in 0.9% saline) in daily 3 h sessions until baseline criteria were met (minimum of 20 reinforcers earned, with no more than 20% variation over two consecutive sessions and at least 70% responses in the active hole). Saline was then substituted for cocaine in consecutive sessions until extinction criteria were met (<80% of the baseline responding for cocaine self-administration). Subsequently, dose–effect functions (saline, 0.03–3.2 mg/kg per infusion of cocaine, half-log intervals) were determined in each mouse according to a Latin square design. To prevent overdose, the total drug intake was limited to 30 mg/kg per session. In the reversal procedure, the positions of the active and inactive nose-poke holes were reversed for 5 d and then returned to their original configuration (always with 1.0 mg/kg per infusion as the reinforcer). Mice were regarded as having successfully switched to the newly active hole when at least 20 reinforcers were earned within a session, with a minimum of 70% active responses. This procedure was only used with cocaine and never with food to evaluate the reinforcing effects of cocaine based on acquisition of a new response (left nose poke) that had never previously been reinforced. Cocaine was provided by the National Institute on Drug Abuse (National Institutes of Health, Bethesda, MD).

Experiment 2: acquisition of cocaine self-administration behavior in experimentally naive mice. After jugular catheter implantation and recovery, experimentally naive mice were introduced to the operant chamber with one of three doses of intravenous cocaine as the reinforcer: 0.03, 0.32, or 1.0 mg/kg per infusion. Sessions were identical to the FR 1 schedule described under experiment 1, with the following two exceptions. First, the sessions were started immediately before introducing the animal into the chamber to ensure that the first nose pokes were reinforced. Second, there was no noncontingent infusion at the start of the sessions. Mice were allowed to self-administer cocaine for 10 sessions or until acquisition criteria were met, whichever occurred first. These mice were never food deprived. Criteria for acquisition of self-administration were as follows: (1) a minimum of 15 reinforcers earned per session for at least two of three consecutive sessions ("acquisition level"); (2) at least 70% responses in the active hole on the second day; (3) extinction of responding when saline was substituted for cocaine (i.e., <80% of the acquisition level); and (4) an increase in the active responses to or above the acquisition level when cocaine was again made available after extinction. To prevent overdose, the total drug intake was limited to 30 mg/kg per session. Catheter patency was verified at the end of each experimental phase; animals in which catheter patency could not be demonstrated through all phases were removed from the dataset (six to eight mice per genotype, 20 in all). Complete acquisition data were collected from 57 mice: 18 $M_5^{+/+}$ mice (9 male and 9 female), 24 $M_5^{+/-}$ mice (12 male and 12 female), and 15 $M_5^{-/-}$ mice (8 male and 7 female). After acquisition studies, mice were allowed to self-administer 1.0 mg/kg per infusion of cocaine to stable self-administration levels before introducing the PR schedule.

Progressive ratio schedule. Cocaine self-administration under a PR schedule of reinforcement was assessed in mice from both experiments 1 and 2. Also, mice from experiment 1 were tested with the liquid food reinforcer under the PR schedule before catheter implantation surgery. After stable responding was observed under the FR 1 schedule maintained by undiluted food or 1.0 mg/kg per infusion of cocaine, an FR 3 schedule was used as a transition from the FR 1 schedule before introduction of the PR schedule. For the PR schedule, the ratio for successive reinforcers was incremented in steps according to the following equation: ratio $= 19 \times [1 + \log(\text{step}/(7 - 0.3 \times \text{step}))]$, rounded to the nearest integer up to step 23, after which the ratio was increased linearly by 12 (ratios: 3, 9, 13, 16, 18, 20, 22, 24, 25, 27, 28, 29, 31, 32, 34, 35, 37, 39, 41, 44, 47, 52, 64. . .). The breaking point was defined as the step value associated with the last completed ratio (i.e., number of reinforcers earned) after a 60 min limited hold (i.e., period with no reinforcer earned). If a breaking point had not been reached within 6 h, the session was terminated to prevent health hazard, and the last reached ratio was used (<6% of the "breaking points" were recorded because of session expiration without a full 1 h limited hold). After a stable baseline had been achieved (two consecutive sessions with breaking points more than step 10 and with <20% variation), saline or water was substituted until responding extinguished to breaking points <10 and ≤50% of the baseline. Cocaine dose–effect curves (0.03, 0.32, 1.0, and 3.2 mg/kg per infusion of cocaine) and liquid food concentration–effect curves (0, 3, 10, 32, and 100% food in water) were determined according to a Latin square design, with each dose tested for two or three consecutive sessions (i.e., if the breaking points reached in the two first determinations varied by >20%, a third determination was made). Each mouse was tested with all cocaine doses or food concentrations to provide within-subjects curves.

Data analysis. Acquisition of operant behaviors was compared for food-maintained responding in experiment 1 and for each cocaine dose in experiment 2 using the log-rank test (survival statistics) with genotypes as groups (sexes combined) and with sexes as groups (genotypes combined). In experiment 2, mice that failed to meet acquisition criteria within 10 sessions were assigned a censored 10 session value (there was no time restriction in experiment 1). The number of sessions before criteria were met for different experimental phases was compared using a two-way ANOVA, with genotype and sex as between-subjects variables. For food concentration–effect functions and cocaine dose–effect functions, comparisons were made using a mixed-model ANOVA, with genotype and sex as between-subjects variables and food concentration or cocaine dose as within-subjects (repeated-measures) variables. Significant effects were followed when appropriate by the Student–Newman–Keuls test, *t* test, or *post hoc* ANOVA for simple effects. Significance level was set at $p < 0.05$. Unless stated otherwise, the Student–Newman–Keuls test was used for *post hoc* analysis.

Results

Basic sensorimotor characteristics and home-cage behaviors

Table 1 summarizes the general sensorimotor characteristics and home-cage behavior assessments of male and female $M_5^{-/-}$ and $M_5^{+/-}$ mutant mice and their wild-type littermates. Few measures indicated differences between the $M_5^{-/-}$ mice and their $M_5^{+/-}$ and $M_5^{+/+}$ littermates. The male $M_5^{-/-}$ mice were observed to fight more often and/or more violently than female mice or $M_5^{+/-}$ and wild-type male mice, and scores of wounds from fighting (i.e., bite wounds on tails and rumps) were higher for the male $M_5^{-/-}$ mice than for any other group. In both cases, *post hoc* analysis after significant effects of genotype, sex, and significant genotype by sex interaction revealed that the male $M_5^{-/-}$ mice had significantly higher scores for fighting and wounds ($p < 0.01$ vs all other groups), whereas all other groups of mice showed absence or near-absence of fighting and wounds. In scores of general health and appearance, only "fur matting" differed between groups. *Post hoc* analysis after a significant effect of genotype, sex, and significant genotype by sex interaction again indicated that male $M_5^{-/-}$ mice had significantly higher score for fur matting compared with all other genotype/sex groups ($p < 0.01$).

Females had lower body weight and shorter stride length com-

Table 1. General health, sensorimotor, and behavioral characteristics of female and male $M_5^{+/+}$, $M_5^{+/-}$, and $M_5^{-/-}$ mice

Parameter	+/+ Females ($n = 15$)	+/− Females ($n = 13$)	−/− Females ($n = 13$)	+/+ Males ($n = 14$)	+/− Males ($n = 13$)	−/− Males ($n = 13$)
General health						
Body weight (g)	21.0 ± 0.3	20.5 ± 0.2	20.7 ± 0.3	24.9 ± 0.5	25.3 ± 0.6	24.8 ± 0.5
Fur matting[a]	0	0	0	0.07 ± 0.07	0	0.31 ± 0.13
Balding[a]	0	0	0	0	0	0
Sores[a]	0	0	0	0	0	0.15 ± 0.15
Whiskers[a]	0	0	0	0	0	0
Home-cage behavior[a]						
Sleep						
8:00 A.M.	2.00 ± 1.15	1.33 ± 1.33	3.33 ± 0.67	1.33 ± 1.33	2.67 ± 1.33	4.00 ± 0.00
2:00 P.M.	2.67 ± 10.33	1.33 ± 1.33	4.00 ± 0.00	3.33 ± 0.67	4.00 ± 0.00	2.67 ± 0.67
8:00 P.M.	0	0	0	0	0	0
Huddled						
8:00 A.M.	2.33 ± 1.20	2.33 ± 1.20	4.00 ± 0.00	2.67 ± 0.67	4.00 ± 0.00	4.00 ± 0.00
2:00 P.M.	2.67 ± 1.33	2.67 ± 1.33	4.00 ± 0.00	3.33 ± 0.67	4.00 ± 0.00	3.00 ± 0.58
8:00 P.M.	0	0	0	0	0	0
In "igloo"						
8:00 A.M.	3.00 ± 1.00	2.33 ± 1.20	4.00 ± 0.00	2.67 ± 0.67	4.00 ± 0.00	3.33 ± 0.67
2:00 P.M.	0.33 ± 0.33	2.67 ± 1.33	4.00 ± 0.00	3.33 ± 0.67	4.00 ± 0.00	3.00 ± 0.58
8:00 P.M.	0	0.33 ± 0.33	0.33 ± 0.33	0.33 ± 0.33	0	1.00 ± 0.00
Fighting	0	0	0	0.33 ± 0.33	0.33 ± 0.33	2.00 ± 058
Wounds from fighting	0.13 ± 0.09	0	0	0	0	0.69 ± 0.24
Reaction to handling[a]						
Escape	0	0.08 ± 0.08	0.23 ± 017	0	0	0
Biting	0	0	0	0	0	0
Sensorimotor[b]						
Eye blink test	15/15	13/13	13/13	14/14	12/13	12/13
Ear twitch	15/15	13/13	13/13	14/14	13/13	13/13
Whisker orientation	14/15	13/13	11/13	14/14	13/13	13/13
Righting reflex	15/15	13/13	13/13	14/14	13/13	13/13
Vision test	15/15	13/13	13/13	14/14	13/13	13/13
Motor skills						
Hanging wire test (s)	58.13 ± 1.87	59.46 ± 0.54	55.85 ± 2.81	55.50 ± 2.30	57.62 ± 2.07	54.54 ± 3.20
Footprint pattern						
SL (mm)	52.95 ± 1.15	52.36 ± 1.34	51.17 ± 1.25	55.82 ± 1.30	57.19 ± 1.10	56.12 ± 1.07
Difference in SL (mm)	18. 85 ± 1.03	18.88 ± 1.49	16.38 ± 1.30	20.26 ± 1.35	19.91 ± 1.34	17.42 ± 1.35
Base of support (mm)	24.09 ± 0.38	24.49 ± 0.57	24.28 ± 0.60	24.72 ± 0.50	25.47 ± 0.41	24.51 ± 0.30

The male $M_5^{-/-}$ mice showed significantly higher scores than all other genotype/sex groups in incidence of fighting in the home cage, presence of wounds from fighting (bite wounds on tails and rumps), and fur matting (all $p < 0.01$). Regardless of genotype, female mice had lower body weight and shorter stride length than male mice (both $p < 0.001$). All other measures were similar between genotypes and sexes. SL, Stride length.

[a]Scores 0–4 (0, none; 4, as much as ever observed).

[b]Number of mice testing "normal"/total mice.

Table 2. Mean number of sessions to criteria in food-trained $M_5^{+/+}$, $M_5^{+/-}$, and $M_5^{-/-}$ mice (males and females combined)

	Genotype		
Sessions to criteria	+/+	+/−	−/−
Food FR 1 acquisition	3.17 ± 0.56 (23)	2.66 ± 0.11 (29)	2.80 ± 0.28 (20)
Food FR 1 extinction	1.35 ± 0.13 (23)	1.31 ± 0.12 (29)	1.75 ± 0.28 (20)
Food PR baseline	5.38 ± 0.69 (13)	5.25 ± 0.86 (16)	5.10 ± 0.71 (10)
Food PR extinction	2.92 ± 0.37 (13)	2.88 ± 0.92 (16)	1.20 ± 0.13 (10)
Cocaine FR 1 baseline	3.39 ± 0.88 (18)	2.71 ± 0.28 (24)	2.80 ± 0.39 (15)
Cocaine FR 1 extinction	5.22 ± 1.15 (18)	5.21 ± 1.28 (24)	3.27 ± 0.56 (15)

Data are group means ± SEM, with *n* in parentheses. All mice were trained with liquid food under an FR 1 schedule of reinforcement, and a randomly chosen subset was subsequently tested under a PR schedule. After recovery from the catheter implantation surgery, 1.0 mg/kg per infusion of cocaine was made available as the reinforcer. Some attrition occurred during or shortly after surgery. There was no significant effect of genotype or of sex in any of the measures.

pared with males ($p < 0.001$), with no significant effect of genotype or genotype by sex interaction. All other measures were similar between genotypes and between sexes.

Operant behavior as a function of liquid food concentration under FR 1 and PR schedules of reinforcement

In experiment 1, mice were first introduced to the operant apparatus with undiluted liquid food as the reinforcer to both evaluate performance maintained by a nondrug reinforcer and provide operant training before cocaine self-administration. The majority of the mice met acquisition criteria within five sessions ($M_5^{+/+}$, 95.7%; $M_5^{+/-}$, 100%; $M_5^{-/-}$, 95%). Comparison of acquisition using survival statistics revealed no effects with genotype as groups or with sex as groups. The first part of Table 2 summarizes the number of sessions necessary before criteria were met for various experimental phases of food-maintained behavior. There was no significant effect of genotype or of sex after ANOVA in any of the measures. In the

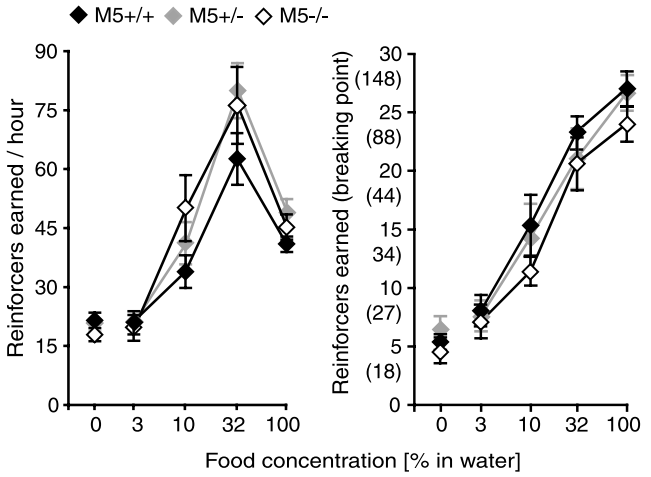

Figure 1. Food-maintained operant behavior in $M_5^{-/-}$ (white) and $M_5^{+/-}$ (gray) mutant mice and their wild-type littermates (black), males and females combined. Abscissas, Concentration of liquid food in water. Ordinates, Food reinforcers earned under the FR 1 (reinforcers per hour; left) and the PR (breaking point; right) schedules of reinforcement. The final ratio corresponding to each breaking point is indicated in parentheses for the PR schedule. Data are group means ± SEM of reinforcers earned. Group means were calculated from the average for each mouse of one (FR) or two to three (PR) determinations per concentration evaluated according to a Latin square design. Group sizes are as follows: FR schedule, $n = 20–29$; PR schedule, $n = 10–16$. The mutant mice did not differ from their wild-type littermates in food-maintained operant behaviors.

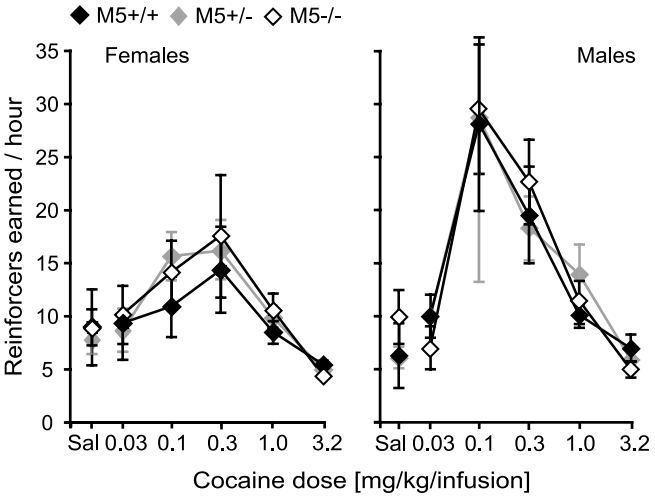

Figure 2. Intravenous cocaine self-administration under the FR 1 schedule of reinforcement in male and female $M_5^{-/-}$ (white) and $M_5^{+/-}$ (gray) mutant mice and their wild-type littermates (black) with previous food-maintained, operant experience. Data from female mice are shown in the left panel, and data from male mice are shown in the right panel. Abscissas, Unit dose of cocaine in saline (Sal) (milligrams per kilogram per infusion; evaluated according to a Latin square design). Ordinates, Cocaine reinforcers earned per hour. Data are group means ± SEM. Group sizes are as follows: females, $n = 5–8$; males, $n = 6–7$. Response rates did not differ significantly between genotypes. There was a significant sex by dose interaction ($p < 0.01$), but doses of 0.10 and 0.32 mg/kg per infusion maintained higher response rates than saline in both sexes ($p < 0.05$ or $p < 0.01$ vs saline; Student–Newman–Keuls test).

acquisition of food-maintained behavior under the FR 1 schedule, there was a significant genotype by sex interaction ($p = 0.007$), but none of the genotype/sex groups differed significantly from each other, and we observed no consistent trend between sexes or genotypes.

After acquisition, extinction, and a series of food–water alternations, operant behavior was evaluated for a range of food concentrations under the FR 1 and then under the PR schedule of reinforcement. Figure 1 shows the concentration–effect functions for $M_5^{-/-}$ and $M_5^{+/-}$ mice and their wild-type littermates under both schedules. Under both schedules, there was a significant effect of food concentration ($p < 0.0001$) but no significant effect of genotype, sex, or any significant interactions. *Post hoc* analysis of the food concentration effects revealed that concentrations of 10–100% maintained higher response rates than water ($p < 0.01$) under the FR 1 schedule (Fig. 1, left), and all concentrations maintained significantly higher breaking points than water under the PR schedule ($p < 0.05$ or $p < 0.01$).

Intravenous cocaine self-administration under an FR 1 schedule of reinforcement

As can be seen from Table 2, mice of all three genotypes trained with food reinforcement met criteria for stable cocaine self-administration under the FR schedule and for extinction of the behavior on saline substitution after comparable numbers of sessions. There was no significant effect of genotype or sex in either measure and no significant genotype by sex interaction. Figure 2 shows cocaine dose–effect functions in all three genotypes under the FR 1 schedule of reinforcement in female and male mice (left and right panels, respectively). ANOVA showed a significant effect of cocaine dose ($p < 0.0001$) and a significant sex by dose interaction ($p = 0.001$) but no significant effect of genotype, sex, or other significant interactions. *Post hoc* analysis of the sex by dose interaction revealed that female and male mice differed significantly only at the 0.10 mg/kg per infusion dose ($p < 0.001$). Because there was a significant sex by dose interaction, an ANOVA was then performed for each sex separately, with genotype as a between-subjects factor and dose as a within-subjects factor. The effect of dose was significant in both the female and male mice ($p < 0.0001$), whereas the effect of genotype did not approach significance in either sex. Despite the significant sex by dose interaction, *post hoc* analysis of the dose effect in the female and male mice, respectively, revealed that doses of 0.10 and 0.32 mg/kg per infusion of cocaine maintained significantly higher response rates relative to saline in both sexes ($p < 0.05$ or $p < 0.01$).

Five $M_5^{-/-}$ mice (three male, two female) were also tested in a reversal procedure. Once stable responding maintained by 1.0 mg/kg per infusion of cocaine was reestablished after dose–effect determinations, the active and inactive nose-poke holes were reversed for five sessions and then returned to their initial configuration for three consecutive sessions. The mean numbers of responses in the right and left nose-poke holes before, during, and after reversal are shown in Figure 3. All mice rapidly reallocated their behavior to the previously inactive left nose-poke hole, thus maintaining the number of self-administered cocaine injections to levels equivalent to their pre-reversal cocaine intake. Simultaneously, responding in the previously active right hole, although initially high, decreased over sessions. In the subsequent reversal to the original configuration, allocation of behavior was again adapted to self-administer cocaine. All five mice met criteria for successful reallocation of behavior (mean time to criteria, 2.20 ± 0.20 sessions). Because these results closely matched our previous findings with intact mice of the C57BL/6J strain [2.33 ± 0.21 session to criteria (Thomsen and Caine, unpublished observations)] and because all of the $M_5^{-/-}$ mice met criteria, additional testing in wild-type and heterozygous mice was not pursued.

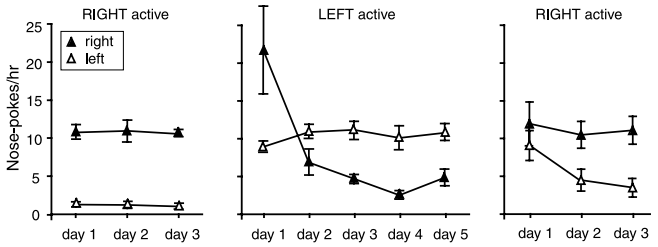

Figure 3. Active and inactive responses during the hole reversal procedure in $M_5^{-/-}$ mice, males and females combined. Abscissas, Session number in each phase. Ordinates, Response rate per hour. Active responses were reinforced by 1.0 mg/kg per infusion of cocaine under an FR 1 schedule, and inactive responses had no scheduled consequences. Filled symbols represent responses in the right nose-poke hole (reinforced or inactive), and open symbols represent responses in the left nose-poke hole (reinforced or inactive). The left panel shows responding before reversal (right hole active), the center panel shows responding with the nose-poke holes reversed (left hole active), and the right panel shows responding after the second reversal (right hole active). All points are group means ± SEM. Group size is $n = 5$. All mice successfully reallocated their behavior to maintain cocaine intake at rates comparable with pre-reversal levels, while decreasing nonreinforced responses over sessions. Results were similar to our previous assessment of the background strain C57BL/6J (see Results).

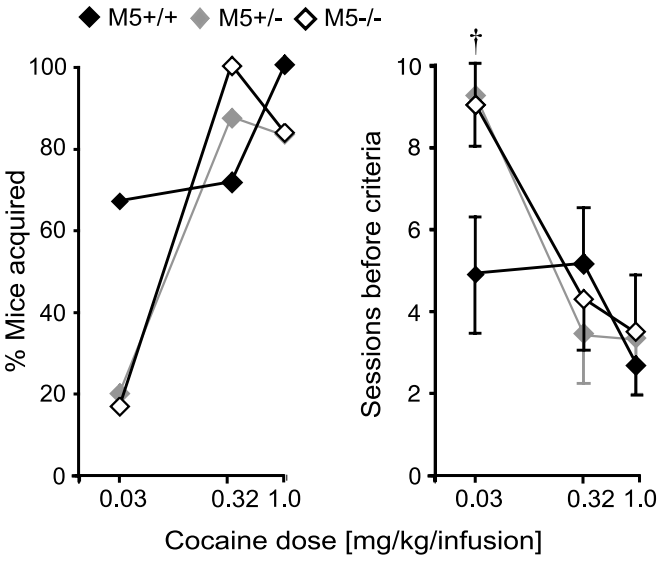

Figure 4. Acquisition of cocaine self-administration behavior in experimentally naive $M_5^{-/-}$ (white) and $M_5^{+/-}$ (gray) mutant mice and their wild-type littermates (black), males and females combined. Abscissas, Unit dose of cocaine in saline (milligrams per kilogram per infusion). Ordinates, Percentage of mice that acquired self-administration behavior (left) and sessions before criteria were met (right). Group sizes are as follows: $n = 6$–7 except $M_5^{+/-}$ mice at the 0.03 mg/kg per infusion dose, $n = 5$. Left, Percentage of mice that met criteria for cocaine self-administration. Right, Number of sessions before acquisition criteria were met. Data are group means ± SEM. The reduced acquisition by the mutant mice was reflected by a significant effect of genotype in the number of sessions before acquisition criteria were met at the lowest dose ($^{\dagger}p < 0.05$, log-rank test). Sex was not a significant factor at any dose.

Acquisition of cocaine self-administration behavior in drug- and training-naive mice

Experimentally naive mice were introduced to the operant chamber with one of three doses of cocaine available, 0.03, 0.32 or 1.0 mg/kg per infusion, until acquisition criteria were met or for 10 sessions, whichever occurred first. Figure 4 shows the percentage of mice that met criteria for cocaine self-administration (left panel) and the mean number of sessions before criteria were met (right panel). There was a significant effect of genotype at the 0.03 mg/kg per infusion dose, which supported acquisition of self-administration less efficiently in the $M_5^{-/-}$ and $M_5^{+/-}$ mice relative to the wild-type mice ($p < 0.05$, log-rank test). At doses of 0.32 and 1.0 mg/kg per infusion, the three genotypes did not differ significantly.

There was no significant effect of sex or genotype by sex interaction at any dose in any of the measures above, and we observed no consistent trend between sexes for different doses (data not shown).

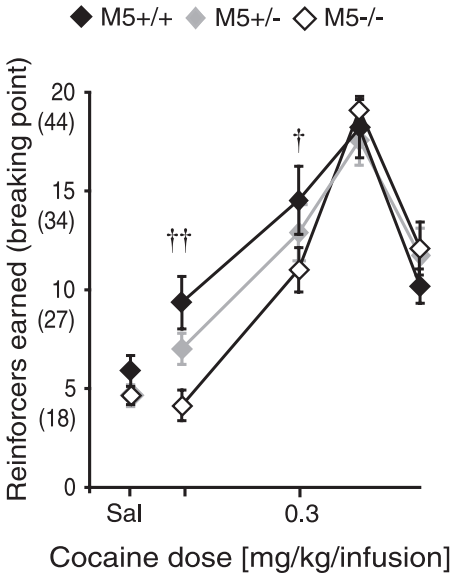

Figure 5. Intravenous cocaine self-administration under the PR schedule of reinforcement in $M_5^{-/-}$ (white) and $M_5^{+/-}$ (gray) mutant mice and their wild-type littermates (black), males and females combined. Abscissas, Unit dose of cocaine in saline (milligrams per kilogram per infusion). Ordinates, Breaking point (reinforcers earned), with the corresponding final ratio in parentheses. Data are group means ± SEM, calculated from the average for each mouse of two or three determinations per dose evaluated according to a Latin square design. Group sizes were $n = 8$–12. Sex was not a significant factor. $M_5^{-/-}$ mice reached lower breaking points than wild-type mice at doses of 0.03 and 0.32 mg/kg per infusion. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ versus wild type; *post hoc t* test.

Intravenous cocaine self-administration under a PR schedule of reinforcement

Cocaine dose–effect functions for all three genotypes under the PR schedule of reinforcement are shown in Figure 5 (sexes combined). We found a significant effect of cocaine dose ($p < 0.0001$) and a significant genotype by dose interaction ($p = 0.004$). There was no significant main effect of genotype, sex, or other significant interactions. *Post hoc* analysis revealed that the $M_5^{-/-}$ mice reached significantly lower breaking points relative to the wild-type mice at both the 0.03 and the 0.32 mg/kg per infusion dose ($p < 0.05$, *t* test), whereas the $M_5^{+/-}$ to wild-type comparison did not reach significance. In contrast to results with low cocaine doses, comparable breaking points were reached across genotypes when saline or high cocaine doses (1.0–3.2 mg/kg per infusion) were available. Because there was a significant genotype by dose interaction, an ANOVA was performed for each genotype separately, with sex as a between-subjects factor and dose as a within-subjects factor. Cocaine dose was a significant factor in all three genotypes ($p < 0.0001$). In the wild-type mice, all doses maintained significantly higher breaking points relative to saline ($p < 0.05$ or <0.01), whereas in the $M_5^{-/-}$ and $M_5^{+/-}$ mice, only doses of 0.32–3.2 mg/kg per infusion of cocaine maintained higher breaking points relative to saline (all $p < 0.01$). Thus, the ascending limb of the dose–effect curve was shifted to the right in both the $M_5^{-/-}$ and $M_5^{+/-}$ mice relative to

the wild-type mice. The effect of sex and the sex by dose interaction were not significant in any of the genotypes.

Discussion

The present study provides evidence that cocaine was less potent as a reinforcer in an extensively backcrossed line of M_5 receptor-deficient mice relative to their wild-type littermates. First, $M_5^{-/-}$ and $M_5^{+/-}$ mice failed to reliably acquire self-administration behavior maintained by a low cocaine dose that supported acquisition in wild-type mice. Second, low to moderate doses of cocaine were less reinforcing in $M_5^{-/-}$ mice than in wild-type mice under a progressive ratio schedule of reinforcement. No differences were observed between genotypes under a fixed ratio schedule or with high cocaine doses. These results corroborate our previous findings using a single-session self-administration acquisition procedure and identify additional experimental conditions under which attenuated cocaine reinforcement by M_5 receptor deletion was observed.

Dose–effect functions for cocaine self-administration under an FR 1 schedule of reinforcement in animals previously trained with food did not differ between genotypes. In contrast, experimentally naive $M_5^{-/-}$ and $M_5^{+/-}$ mice showed decreased acquisition of cocaine self-administration behavior compared with wild-type mice when a low dose of cocaine was available. There was no difference between genotypes in acquisition at higher doses, indicating a rightward shift in the function relating cocaine dose to acquisition of self-administration. Similarly, $M_5^{-/-}$ and $M_5^{+/-}$ mice required a higher cocaine dose than wild-type mice to maintain breaking points higher than those maintained by saline under a PR schedule. Thus, the ascending limb of the dose–effect function under the PR schedule was shifted to the right in the $M_5^{-/-}$ and $M_5^{+/-}$ mice, whereas the genotypes did not differ at high doses. Together, these data indicate that cocaine had decreased potency but comparable efficacy as a reinforcer in the M_5 receptor-deficient mice compared with wild-type mice during acquisition of cocaine self-administration in experimentally naive mice and also under a PR schedule of reinforcement.

A decreased potency but comparable reinforcing efficacy of cocaine in the $M_5^{-/-}$ mice is consistent with the existing data on striatal dopamine release in $M_5^{-/-}$ mice. The muscarinic agonist oxotremorine was less potent in increasing potassium-stimulated [^{3}H]dopamine release in striatal slices from $M_5^{-/-}$ mice compared with wild-type mice, whereas a high dose produced comparable effects in tissues from both genotypes (Yamada et al., 2001). Also, electrical stimulation of the LDT *in vivo* induced a triphasic change in accumbal dopamine release in intact mice, and only the prolonged dopamine release was absent in $M_5^{-/-}$ mice, whereas the initial dopamine "spike" was intact (Forster et al., 2002). Together with the present data, those findings suggest that M_5 receptors facilitate accumbal dopamine release in response to cocaine but that other mechanisms that contribute to stimulated dopamine release are sufficient to elicit a full cellular and behavioral effect at high cocaine doses. Although it is beyond the scope of the present paper to discuss additional muscarinic mechanisms in detail, it is worth mentioning that oxotremorine-enhanced release of dopamine from striatal slices was absent in tissues from $M_4^{-/-}$ mice (Zhang et al., 2002). Thus, both M_5 and M_4 receptors may modulate striatal dopamine release and the abuse-related effects of cocaine.

The contribution of M_5 receptors to the reinforcing effects of drugs of abuse is not limited to cocaine or psychostimulants. Abuse-related effects of morphine, in both cellular and behavioral measures, were attenuated in $M_5^{-/-}$ mice relative to wild-type mice, whereas morphine-induced analgesia remained unchanged (Basile et al., 2002). As in the present study with cocaine, conditioned place preference was not abolished at the highest morphine dose used. In addition, infusion of M_5 receptor antisense oligonucleotides into the VTA temporarily increased brain-stimulation reward thresholds in rats (Yeomans et al., 2000). Thus, M_5 receptors may contribute to the reinforcing effects of opioids and electrical brain stimulation in addition to psychostimulants, and this contribution seems surmountable at high stimulus magnitudes.

Whereas the reinforcing effects of low cocaine doses differed across M_5 genotypes using a measure of acquisition and under a PR schedule, this was not observed under an FR schedule in animals trained with food reinforcement. Similar observations were reported in another investigation involving cholinergic/psychomotor stimulant interactions. Alderson et al. (2004) lesioned the PPT in rats and observed decreased D-amphetamine self-administration in an acquisition procedure and under a PR schedule but not under an FR schedule in animals pretrained with food. One possible explanation is that the contribution of cholinergic systems to the reinforcing effects of psychomotor stimulants is most clearly observed at threshold levels of reinforcement. Relative to the FR schedule, the PR schedule yields a more graded ascending limb in dose–effect functions for cocaine self-administration, with greater resolution between ineffective and effective doses. Likewise, acquisition of self-administration of a low cocaine dose may be more susceptible to disruption than low-dose self-administration in pretrained animals. Regardless of the explanation, the present and previous findings suggest that contribution of cholinergic mechanisms to the reinforcing effects of cocaine in rodents is detectable using acquisition or PR procedures but not FR procedures after training with food.

We observed lower peak response rates in female than in male mice under the FR schedule but no difference between sexes in the acquisition or PR procedures. In previous studies, we found no sex differences in cocaine self-administration in dopamine D_2 receptor-mutant or wild-type mice under the FR 1 schedule nor in rats under low fixed ratio schedules (Caine et al., 2002, 2004). Sex differences in cocaine self-administration in rodents have been observed under some conditions, although usually higher rates in females than males were reported (for review, see Lynch et al., 2002). Accordingly, the slightly lower rates in females than males in the present study are not conclusive. More germane to the present investigation, we observed no sex by M_5 genotype interactions in cocaine self-administration.

We observed few differences between genotypes in general sensorimotor or behavioral characteristics. The incidence of fighting in the home cage and related measures were higher in male $M_5^{-/-}$ mice relative to $M_5^{+/-}$ and wild-type mice. Although these are preliminary measures, our observations suggest that M_5 receptors may be involved in aggressive behaviors. However, those differences likely did not affect operant behavior appreciably. The three genotypes did not differ in acquisition of food-maintained operant behavior or in food concentration–effect functions under FR or PR schedules. In addition, $M_5^{-/-}$ mice performed a reversal task in the present study with comparable efficiency to C57BL/6J mice in a previous study (Thomsen and Caine, unpublished observations). Collectively, those data suggest that learning of operant behavior and general operant

performance, including under a high response-requirement PR schedule, were similar in the mutant and wild-type mice. This is also consistent with our previous finding that $M_5^{-/-}$ and wild-type mice did not differ in a passive avoidance learning task (Fink-Jensen et al., 2003).

Even substantial backcrossing cannot produce absolutely congenic mice when the mutation was originally expressed on a mixed genetic background, making it difficult to exclude a contribution of 129 genes linked to the knock-out locus (Lathe, 1996). 129/SvEvTac (129S6) and C57BL/6 inbred strains used in the present study for the embryonic stem cells and backcrossing, respectively, have been shown to differ in some measures of the behavioral effects of cocaine (see Introduction). Nevertheless, the profile of food-maintained behavior and cocaine self-administration in the $M_5^{-/-}$ mice used in the present study differed from the strain-dependent profiles observed under a similar range of conditions in a recent study (Thomsen and Caine, unpublished observations). Specifically, food-maintained responding was markedly altered in 129S6 compared with C57BL/6J mice, and acquisition of food-maintained behavior and learning of new operant behaviors was slower in 129S6 mice. In contrast, in the present study, the mutant and wild-type mice did not differ in food-maintained behavior and operant performance and showed a phenotype similar to the C57BL/6J mice in the previous study. It is therefore unlikely that the observed differences in cocaine self-administration between M_5 genotypes are attributable to 129 genes linked to the knock-out locus.

In conclusion, we found that the $M_5^{-/-}$ and $M_5^{+/-}$ mice exhibited decreased reinforcing effects of cocaine at low to moderate doses in measures of acquisition of self-administration and under a PR schedule of reinforcement, whereas these differences were not observed at higher cocaine doses and under an FR 1 schedule in animals previously trained with food reinforcement. This decrease in the reinforcing potency of cocaine in the mutant mice was not likely attributable to impaired operant performance or to genetic contribution from the background strains. The use of constitutive knock-out mice is not free of caveats, because compensatory changes in gene expression could have influenced the observed phenotype. Future studies using inducible knock-out mutations or M_5-selective antagonists are needed to confirm the implications of the present findings. Our results nevertheless suggest that the muscarinic M_5 receptor may be a useful target for candidate treatments for cocaine abuse. Because the effect of M_5 receptor deletion could be overcome by higher cocaine doses, future M_5-selective ligands might be most effective in combination with other pharmacotherapeutic targets. Our data also highlight the influence of experimental conditions and suggest that acquisition procedures and PR schedules with low cocaine doses may be especially useful for detecting subtle differences in the reinforcing effects of cocaine between genotypes.

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