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Effects of muscarinic M₁ and M₄ acetylcholine receptor stimulation on extinction and reinstatement of cocaine seeking in male mice, independent of extinction learning

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Abstract

Rationale—Stimulating muscarinic M₁/M₄ receptors can blunt reinforcing and other effects of cocaine. A hallmark of addiction is continued drug seeking/craving after abstinence, and relapse.

Objectives—We tested whether stimulating M₁ and/or M₄ receptors could facilitate extinction of cocaine seeking, and whether this was mediated via memory consolidation.

Methods—Experimentally naïve C57BL/6J mice were allowed to acquire self-administration of intravenous cocaine (1 mg/kg/infusion) under a fixed-ratio 1 schedule of reinforcement. Then, saline was substituted for cocaine until responding extinguished to £30% of cocaine-reinforced responding. Immediately after each extinction session, mice received saline, the M₁/M₄ receptor-preferring agonist xanomeline, the M₁ receptor-selective allosteric agonist VU0357017, the M₄ receptor-selective positive allosteric modulator VU0152100, or VU0357017+VU0152100. In additional experiments, xanomeline was administered delayed after the session, or in the home cage before extinction training began. In the latter group, reinstatement of responding by a 10 mg/kg cocaine injection was also tested.

Results—Stimulating M₁ + M₄ receptors significantly expedited extinction from 17.2 sessions to 8.3 using xanomeline or 7.8 using VU0357017+VU0152100. VU0357017 alone and VU0152100 alone did not significantly modify rates of extinction (12.6 and 14.6 sessions). The effect of xanomeline was fully preserved when administered delayed after or unpaired from extinction sessions (7.5 and 6.4 sessions). Xanomeline-treated mice showed no cocaine-induced reinstatement.

Conclusions—These findings show that M₁/M₄ receptor stimulation can decrease cocaine seeking in mice. The effect lasted beyond treatment duration, and was not dependent upon

extinction learning. This suggests that M₁/M₄ receptor stimulation modulated or reversed some neurochemical effects of cocaine exposure.

Keywords

Addiction; abuse; preclinical; psychostimulant; extinction; relapse; cholinergic; C57BL/6; mouse

INTRODUCTION

Overuse of and dependence upon psychoactive substances, legal or illegal, are becoming recognized as a public health problem of epidemic proportions both in the USA and worldwide. Cocaine remains one of the most common substances to be used illegally, and there are no pharmacotherapies approved for treating addiction to cocaine or other psychostimulant drugs (Skolnick et al. 2015; Czoty et al. 2016). Improvement of cognitive functions through pharmacological agents, so called “cognitive enhancers”, have been proposed as adjunct treatments for psychostimulant addiction in conjunction with psychotherapeutic approaches, in the hopes of improving inhibitory control, decision-making, and other cognitive functions that may affect treatment outcome (Sofuoglu et al. 2013, 2016). In addition, the use of cognitive enhancers to facilitate extinction of conditioned responses to drug-associated cues has been suggested as a potential treatment strategy for drug addiction (Kaplan et al. 2011; Nic Dhonnchadha and Kantak 2011). The latter suggestion was largely modeled on the use of cognitive enhancers to facilitate extinction of responses to fearful cues in phobias, anxiety disorders, and post-traumatic stress disorder (Singewald et al. 2015). Cognitive enhancers most studied in the context of drug addiction include the NMDA receptor agonist D-cycloserine and cholinergic receptor stimulation by use of acetylcholinesterase inhibitors (e.g., tacrine, donepezil).

In laboratory animals, resistance to extinction of a behavior previously reinforced with a drug of abuse has been proposed to model aspects of craving in humans (Markou et al. 1993). D-cycloserine administered during extinction training can facilitate extinction of cocaine-conditioned place preference and cocaine self-administration in rodents (for review see Myers and Carlezon 2012). Post-session administration of the non subtype-selective muscarinic receptor agonist oxotremorine similarly facilitated extinction of amphetamine-conditioned place preferences in rats (Schroeder and Packard 2004). This facilitation of extinction is generally interpreted as being effected through enhancement of memory consolidation, because it appears to be dependent upon a temporal concordance between the extinction training and the drug treatment. For instance, in place conditioning experiments, facilitation of extinction was not observed when administration of D-cycloserine was delayed by 4 hours after the session or when administration of oxotremorine was delayed by 2 hours (Schroeder and Packard 2004; Botreau et al. 2006; see also Torregrossa et al. 2010).

Acetylcholinesterase inhibitors and non-selective muscarinic receptor agonists are limited both in their clinical use and as pharmacological tools by potentially opposing actions at different receptor populations, and by gastrointestinal side-effects that limit the dose that can be used and/or mask desired effects. The development of highly muscarinic receptor subtype-selective ligands (by targeting allosteric binding sites rather than the highly

conserved orthosteric site) has made it possible to investigate specific functions of muscarinic receptors (Nickols and Conn, 2014; Thal et al. 2016). Muscarinic receptors have long been known to play important roles in memory and cognitive functions, with muscarinic receptor antagonists like scopolamine being powerful amnesic drugs (Davis et al. 1978; Sitaram et al. 1978; Power et al. 2003; Bubser et al. 2012), while M_1/M_4 receptor agonists can produce measurable memory enhancing effects in humans and in laboratory animals (Bodick et al. 1997; Shekhar et al. 2008; Lebois et al. 2010; Bubser et al. 2014; Galloway et al. 2014; Gould et al. 2017). In addition to those cognitive effects, we have shown that stimulation of M_1 or M_4 receptors can decrease abuse-related effects of cocaine in rodents, including the direct reinforcing effects of cocaine in self-administration assays, and to some extent also “subjective” or conditioned effects, in drug discrimination and place conditioning assays (Thomsen et al. 2010a, 2012, 2014; Dencker et al. 2012; Dall et al. 2017). We therefore wanted to investigate whether M_1/M_4 receptor stimulation can also facilitate extinction of cocaine seeking, providing multiple mechanisms by which they may be efficacious in the treatment of cocaine addiction.

We hypothesized that muscarinic agonist treatment would facilitate extinction of nose poking previously reinforced with intravenous cocaine through a process involving memory consolidation. To test the first part of the hypothesis, that muscarinic agonist treatments would facilitate extinction (through any mechanism), we examined the effects of the muscarinic M_1/M_4 receptor-preferring agonist xanomeline, the M_1 receptor-selective allosteric agonist VU0357017, the M_4 receptor-selective positive allosteric modulator (PAM) VU0152100, and the combination of VU0357017 and VU0152100 on rates of extinction of nose-poking behavior previously reinforced with cocaine (Experiment 1). Male C57BL/6J mice were used, based on our observations that this strain showed more protracted extinction relative to other strains, providing a high baseline suited to detect changes in extinction behavior (Thomsen and Caine 2006, 2011). The test drugs were administered immediately after each extinction session, when memory consolidation is thought to take place (McGaugh 2000), and to avoid potential “false positive” results that could arise from any rate-altering effects of the treatment (i.e., behavior was measured under drug-free conditions). For the second part of the hypothesis, if the effect involved memory consolidation, M_1/M_4 stimulation would be expected to have no effect when unpaired from extinction training. We therefore examined the effects of M_1/M_4 receptor stimulation delayed 2.5 hours after the session (Experiment 2, Delayed administration), and administered during a period free of operant sessions (Experiment 2, Unpaired administration). We also examined the effect of xanomeline when the extinction was cued, i.e., the light cue that accompanied delivery of cocaine was not presented during the extinction sessions. Finally, cocaine prime-induced reinstatement of nose poking was evaluated in the Unpaired treatment groups, testing the hypothesis that a period of M_1/M_4 receptor stimulation would diminish cocaine seeking.

MATERIALS AND METHODS

Animals and housing.

Male C57BL/6J mice were acquired at 6 weeks of age from the Jackson Laboratories (Bar Harbor, ME), and were acclimated to the housing facilities at McLean Hospital at least 7 days before experiments were initiated. Only male mice were included in this study because we have not previously observed any sex differences in the extinction of cocaine seeking, in several cohorts of mice of a C57BL/6 genetic background (Thomsen et al. 2005; 2009a,b; Caine et al. 2007, 2012). Animals were kept on a 12-h light/dark cycle in a temperature- and humidity-controlled vivarium, group housed up to 4 per cage, with tap water and standard rodent food (rodent diet 5015; PMI Feeds, Inc., St. Louis, MO) freely available in the home cage. Nesting material/devices and variously flavored rodent “treats” were available for enrichment, as were exercise devices until catheter implantation (removed after surgery to avoid injuries caused by the protruding catheter base). All testing was conducted during the light phase of the circadian cycle, typically 5 days/week. All procedures were carried out in accordance with NIH Guide for the Care and Use of Laboratory Animals and were approved by the McLean Hospital Institutional Animal Care and Use Committee. One control experiment (see below) was conducted at the Laboratory of Neuropsychiatry, Copenhagen Denmark (AAALAC accredited facility) under comparable conditions, approved by the Animal Experiments Inspectorate under the Danish Ministry of Food, Agriculture in accordance with the EU directive 2010/63/EU.

Operant conditioning apparatus.

Mouse operant conditioning chambers (ENV-300, Med-Associates, Georgia, VT) were used, and have been described in detail (Caine et al. 1999; Thomsen and Caine 2005). Each chamber was equipped with two nose-poke holes each containing a yellow cue light (ENV-313M, Med-Associates). A low-torque liquid swivel (375/25; Instech Laboratories, Plymouth Meeting, PA) mounted on a balance arm was used for intravenous drug delivery in the freely moving animals. Each apparatus was enclosed in a sound-attenuating cubicle equipped with a syringe pump, a fan providing ventilation and background noise, and a 3-Watt white stimulus light (ENV-221, Med-Associates).

Catheter implantation and maintenance.

Surgical methods were as previously described (Thomsen and Caine 2005). A catheter (CamCaths model MIVSA, Cambridge, UK; Silastic tubing 0.2 mm inner diameter, 0.4 mm outer diameter) was inserted 1.2 cm into the jugular vein and anchored to the vein with silk suture. The catheter ran subcutaneously to the base located above the midscapular region. The mice were allowed 7 days recovery, during which catheters were flushed once daily with 0.02 ml of 0.9% saline containing heparin (30 USP units/ml) and antibiotic (cefazoline, 67 mg/ml) to forestall clotting and infection. After the post-operative recovery period, catheters were flushed with saline containing heparin immediately before and after self-administration sessions, and the free end of the catheter base was kept closed. Catheter patency was confirmed before initiation of cocaine self-administration and after acquisition criteria were met (or weekly in rare mice that did not meet acquisition criteria within a week) by the infusion of 0.02–0.03 ml of a ketamine + midazolam mixture (15 + 0.75 mg/ml

in saline). Loss of muscle tone and clear signs of anesthesia within 3 seconds of infusion indicated catheter patency. Mice that failed this test were removed from the study.

Acquisition of cocaine self-administration.

In all experiments with the exception of the food control described below, mice were allowed to acquire the nose-poke response reinforced by 1.0 mg/kg/infusion cocaine with no prior training or drug exposure, under an FR 1 schedule of reinforcement with a 20 second post-reinforcer timeout period including infusion time. Daily sessions lasted 3 hours or until 30 cocaine infusions were earned, whichever occurred first. The cubicle light was turned on at session start and remained illuminated until the session terminated. Responses in the active nose-poke hole resulted in a cocaine infusion, and illumination of the cue light through the infusion and timeout period. Responses in the inactive hole were recorded but had no scheduled consequences. Acquisition criteria were at least 15 cocaine infusions earned per session and at least 75% of responses in the active hole (not counting timeout responses in the active hole), with cocaine infusions earned varying by no more than 20% over at least two consecutive sessions. Cocaine intake at acquisition criteria (number of cocaine infusions earned averaged over sessions) was used as the baseline to determine extinction criteria in each mouse.

Extinction tests.

Once acquisition criteria were met, saline was substituted for cocaine and mice were tested for latency to extinguish nose-poking behavior over successive once-daily 3-hour sessions. Each mouse was tested with only one experimental condition and drug. In all experiments, extinction criteria were met when levels of responding decreased to 30% of the acquisition level, chosen as a low level very unlikely to be achieved spuriously out of variability of day to day responding (see for instance Thomsen and Caine 2011).

In Experiment 1 and 2, extinction sessions were identical to cocaine self-administration sessions with the exception that saline was substituted for cocaine. In Experiment 1, mice received injections of saline, 1.8 mg/kg xanomeline subcutaneously (SC), 5.6 mg/kg VU0357017 (SC), 1.0 mg/kg VU0152100 intraperitoneally (IP), or both VU0357017 and VU0152100, immediately after each extinction session. In Experiment 2, mice received 1.8 mg/kg xanomeline (SC) 2.5 hours after the session (Delayed administration), or received saline or 1.8 mg/kg xanomeline in the home cage once daily for 7 days while operant sessions were not conducted, after acquisition criteria were met and before extinction sessions began (Unpaired administration; no treatment during extinction).

In Experiment 3, we examined the effect of cued extinction, i.e., the availability of cocaine was indicated by the presence of light cues, so that extinction sessions (cue lights never on) differed from cocaine self-administration sessions (cue light on at cocaine delivery). Mice received injections of saline or 1.8 mg/kg xanomeline immediately after each extinction session. Two hypotheses were tested: a) primary: that M_1/M_4 receptor stimulation would also facilitate extinction under the cued condition b) secondary: that changing the cue conditions between cocaine and saline sessions would itself facilitate extinction by signaling the availability of cocaine.

Figure 1 shows a diagram of experimental procedures. Doses and routes of administration of the test compounds were determined based on previous results from drug discrimination and cocaine self-administration assays in mice (Thomsen et al. 2010a, 2012; Dall et al. 2017; unpublished observations). The 2.5-hour delay in Experiment 2 was chosen based on the 2h delay used by Schroeder and Packard (2004).

Reinstatement test.

The study was not originally designed to evaluate reinstatement of the previously cocaine-reinforced behavior because the variable number of drug administrations between animals made interpretation problematic. However, we did measure cocaine prime-induced reinstatement of nose poking in Experiment 2 in the Unpaired treatment, in which all mice received 7 injections of either saline or xanomeline. Once extinction criteria were met, mice were left one day with no testing or treatment. The next day, mice were tested in a single session after administration of 10 mg/kg cocaine (IP) 10 min. before the session. The session parameters were identical to the extinction sessions. Note that mice were tested in the absence of xanomeline on the reinstatement day.

Food-reinforced control experiment.

To control for a possible effect of M_1/M_4 receptor stimulation on general operant performance, separate cohorts of mice were allowed to acquire nose-poking reinforced with liquid food (Nutridrink® vanilla flavor, Nutricia, Glostrup, Denmark) under an FR 1 20 second timeout schedule of reinforcement, differing from the intravenous self-administration sessions only in that session duration was shortened to 2 hours, and that a maximum of 100 reinforcers were available per session. These experiments were performed at Psychiatric Center Copenhagen using operant chambers identical to those used for all other experiments at McLean Hospital. Once acquisition criteria were met (at least 20 reinforcers earned per session, and as described for cocaine self-administration), mice received 1.8 mg/kg xanomeline or saline in the home cage once daily for 7 days while operant sessions were not conducted, followed by one day with no drug administration or testing. Then, operant activity was measured in a session with either food available or no reinforcer (extinction condition using an empty syringe in the pump).

Drugs.

Cocaine hydrochloride was supplied by the National Institute on Drug Abuse (National Institutes of Health, Bethesda, MD). The centrally active M_1 receptor allosteric agonist VU0357017 (ethyl 4-(2-(2-methylbenzamido)ethylamino)-piperidine-1-carboxylate) and the centrally active M_4 receptor PAM VU0152100 (3-amino-N-(4-methoxybenzyl)-4,6-dimethylthieno[2,3-b]pyridine carboxamide) were synthesized at Vanderbilt University as previously described (Brady et al. 2008; Lebois et al. 2010). Xanomeline was synthesized at McLean Hospital according to published methods (Kane et al. 2008). Cocaine and xanomeline were dissolved in sterile 0.9% saline, VU0357017 in deionized sterile water. VU0152100 was dissolved in Tween80 by gentle heating then diluted to 5% Tween80 in sterile deionized water. Cocaine solution was prepared every few weeks and kept refrigerated, all other drug solutions were prepared fresh daily. Drug doses refer to the weights of the respective salts.

Data analysis.

The primary measure was latencies to meet extinction criteria, which is most appropriately analyzed by survival statistics. Number of sessions to criteria was compared between groups by Log-rank test, uncensored (i.e., all subjects were tested until extinction criteria were met). To verify that groups of mice were comparable at baseline, numbers of infusions earned at acquisition criteria and on the first day of extinction were each compared by ANOVA with treatment group as a between-subjects factor. Reinstatement was evaluated by ANOVA with treatment group as a between-subjects variable and phase (acquisition, extinction, reinstatement) as a repeated variable, followed by two-tailed paired-sample or unpaired-sample *t*-tests. For the food experiment, number of reinforcers earned on the last day of acquisition and following xanomeline treatment were compared by two-tailed paired-sample *t*-test. Significance level was set at $P < 0.05$. All significant findings were confirmed after controlling for false discovery rate using the Benjamini-Hochberg procedure with the false discovery rate limit set at 5%.

RESULTS

The different drug treatment groups did not differ significantly in cocaine intake or rates of responding at acquisition of cocaine self-administration, or in levels of responding during the first or last extinction session (see Figure 2), but see below for a pre-planned selected comparison of the unpaired-xanomeline group. To control for possible cohort variations, saline controls were included alongside each drug test, first xanomeline (saline cohort 1, Experiment 1), then VU0357017, VU0152100, VU0357017+VU0152100 combination, and delayed xanomeline in parallel (saline cohort 2, Experiment 1). Then, a cohort of mice received unpaired saline for 7 days in parallel with the unpaired xanomeline treatment group (saline cohort 3, Experiment 2). A separate cohort of mice received saline alongside the xanomeline-treated mice tested with cued extinction (saline cohort 4, Experiment 3). These saline cohorts ($N = 5, 14, 7$ and 8 , respectively) also did not differ significantly in cocaine intake or rates of responding at acquisition. Most importantly, cohorts tested under uncued extinction conditions (cohorts 1–3) did not differ in latencies to extinction or in responding on the first day of extinction (see Table 1). Therefore, all the saline-treated mice under uncued conditions were pooled into one control group for subsequent analyses ($N = 26$).

Experiments 1 and 2 are shown together to allow for easy comparison of effect sizes. Figure 3 shows the number of extinction sessions required to reach extinction criteria in each treatment group. Leftmost columns show groups in which saline, VU0357017, VU0152100, xanomeline, or the VU0357017+VU0152100 combination was administered immediately after each extinction session (Experiment 1). Rightmost columns show groups that received xanomeline delayed after the session or unpaired from the sessions (Experiment 2). Latency to extinction was related to treatment ($\chi^2 = 19.7$, $P = 0.003$). Comparison of each drug to saline control showed a significant effect of post-session xanomeline ($\chi^2 = 5.48$, $P = 0.02$) and the post-session VU0357017+VU0152100 combination ($\chi^2 = 5.04$, $P = 0.02$), which both reduced extinction time to about half relative to saline controls. Treatment with VU0357017 alone or VU0152100 alone did not affect extinction significantly. Fig. 4a shows the same data as survival curves, illustrating times to extinction criteria of individual mice in the

different groups. Because group sizes were not identical, data were transformed to percentage of mice meeting criteria.

In Experiment 2, we tested the hypothesis that the treatment required the temporal association with the extinction experience, i.e., that the effect was dependent on memory consolidation. We chose to test xanomeline, i.e., a single injection, rather than the two VU0357017+VU0152100 injections for simplicity and to minimize impact on the animals. As can be seen from the two rightmost columns in Figure 3, the effect of xanomeline treatment on extinction was fully preserved when delayed 2.5 hours after the extinction session ($\chi^2=6.97$, $P=0.008$ vs. control), and also when administered unpaired from extinction training ($\chi^2=13.1$, $P=0.0003$). Corresponding survival curves can be seen in Fig. 4b. A pre-planned comparison on the first day of extinction in the unpaired xanomeline group relative to saline control revealed significantly lower responding in the xanomeline-treated mice (see Fig. 2).

Fig. 5 shows baseline behaviors and sessions to extinction when saline or xanomeline was administered immediately following cued extinction sessions, i.e., cocaine-associated light cues remained off during extinction sessions (Experiment 3). Mice did not differ in cocaine intake or rates of responding at acquisition of cocaine self-administration, or in levels of responding during the first or last extinction session (Fig. 5a). An overall test on the extinction behavior showed the latency to extinction to be unequal among the groups ($\chi^2=15.6$, $P=0.001$), with significant effects of both xanomeline ($\chi^2=6.91$, $P=0.009$) and cue condition ($\chi^2=10.0$, $P=0.002$; Fig. 5b,c). Preplanned comparison showed that cueing the extinction sessions accelerated (facilitated) extinction in itself ($\chi^2=10.4$, $P=0.001$, cued saline vs. uncued saline). Unlike the uncued extinction in Experiment 1, xanomeline treatment in the cued condition did not affect latency to extinction ($P>0.8$, cued saline vs. cued xanomeline).

Reinstatement was tested in the Unpaired treatment group, in which all mice received the same number of muscarinic drug injections (Fig. 6). Cocaine priming caused reinstatement in the saline-treated mice, but not in the xanomeline-treated mice. ANOVA confirmed a significant effect of phase (acquisition, extinction, reinstatement; $[F(1,12)=13.0$, $P=0.004]$), and a significant phase by treatment interaction $[F(1,12)=9.49$, $P=0.01]$, while the main effect of treatment only approached significance ($P=0.06$). In the saline-treated mice, responding in the previously cocaine-reinforced nose-poke hole was higher after cocaine priming than at extinction criteria ($P=0.01$), and was also higher relative to the xanomeline-treated mice after priming ($P=0.02$). At the individual level, 6 out of 7 mice in the saline group were considered to have reinstated responding as defined by at least doubling active responses relative to extinction level, while only 1 out of 7 mice in the xanomeline group did so. Responses in the inactive nose-poke hole averaged 1.3 ± 0.5 to 3.1 ± 1.1 per session and did not differ significantly between treatments or phases.

To evaluate whether xanomeline treatment produced a general decrement in operant performance, additional cohorts of mice were allowed to acquire nose poking reinforced with a liquid food reinforcer. The mice were then treated once daily with 1.8 mg/kg xanomeline or saline for seven days, replicating conditions of the Unpaired treatment group.

Responding during the post-treatment extinction session did not differ between the xanomeline-treated mice and the saline-treated mice (Fig. 7). There was also no significant effect of xanomeline treatment on levels of responding comparing food reinforcers earned before and after 7 days of xanomeline treatment (6.2 ± 7.2 percent increase post-treatment, $N=7$).

DISCUSSION

Extinction of drug seeking is an active, learned process by which a previously reinforced behavior is not erased or forgotten, but inhibited (for review, see Millan et al. 2011). A large number of studies point to glutamatergic projections from the prelimbic and infralimbic prefrontal cortex to the nucleus accumbens as playing key roles in both extinction and reinstatement of drug seeking (Peters et al. 2008; Knacksted et al. 2010a; Stefanik et al. 2013; reviewed in Kalivas and Volkow 2005; Schmidt et al. 2005; Millan et al. 2011). Specifically, it has been proposed that cellular changes resulting from repeated drug use alter glutamatergic transmission in these corticostriatal circuits, resulting in impaired inhibitory control over drug taking and drug seeking behaviors (Baker et al. 2003; Kalivas 2009; Spencer et al. 2016). Indeed, in rats trained to self-administer cocaine, restoring glutamatergic transmission in the nucleus accumbens reduced cocaine seeking, with prime-induced reinstatement of lever pressing being reliably suppressed, while effects on cue-induced reinstatement were more variable (Knacksted et al. 2010b; Reissner et al. 2014; LaCrosse et al. 2016). Those findings are remarkably similar to the current results, in that daily treatments for 6–7 days during either extinction training or, to some extent, during abstinence without extinction, prevented or attenuated previously drug-reinforced responding. In those previous studies, rates of extinction were either not assessed or not significantly affected. However, extinction occurred much faster than in our investigation, most likely due to experimental parameters including, for some studies, cued extinction, and different subjects being used: rats in previous studies vs. the C57BL/6 mice that were selected specifically for their extinction-resistant phenotype in the present studies. In the present investigation, signaling extinction by different cues during cocaine vs. saline infusions significantly accelerated extinction, and xanomeline was ineffective in further reducing the number of sessions needed to meet extinction criteria. It is plausible that a “floor effect” accounts for the lack of facilitation of extinction in both previous and present experiments when extinction was rapid at baseline.

M₁ and M₄ receptors are widely expressed in the brain, including cortical and striatal regions (Levey et al. 1991; Bernard et al. 1992; Hersch et al. 1994). In striatal tissues, including the nucleus accumbens, M₄ receptors are expressed pre- and postsynaptically, on glutamatergic corticostriatal and thalamostriatal projections, cholinergic interneurons, and medium spiny neurons (MSNs, the major striatal output neurons), the latter predominantly in the dopamine D₁ receptor-expressing striatonigral population; M₁ receptors are expressed mainly postsynaptically, on both striatonigral and striatopallidal MSN (Weiner et al. 1990; Hersch and Levey 1995; Ince et al. 1997; Yan et al. 2001; Jeon et al. 2010). Both M₁ and M₄ receptors modulate corticostriatal glutamatergic signaling, its integration by MSNs, and neuroplasticity (long-term potentiation and long-term depression) in the system. Broadly, corticostriatal glutamatergic transmission is depressed by stimulation of presynaptic M₄

receptors (Shen et al. 2005, 2015; Narushima et al. 2007; Pakhotin and Bracci 2007; Bonsi et al. 2008; Higley et al. 2009; Pancani et al. 2014, 2015). M₁ receptor stimulation enhances excitability of striatopallidal MSN preferentially, making striatal activity more “responsive” to corticostriatal input and facilitating inhibition of behavior (Shen et al. 2007; Ding et al. 2010; for review see Oldenburg and Ding 2011; Goldberg et al. 2012). M₁ and M₄ receptor stimulation also modulates striatal dopamine signaling and can produce effects opposite to dopamine receptor stimulation, both at the cellular level in striatal tissues, and effects consistent with functional dopamine antagonism in behavioral assays (Threlfell et al. 2010; Threlfell and Cragg 2011; Foster et al. 2016; for review see Pisani et al. 2007; Oldenburg and Ding 2011). However, local infusion of dopamine receptor antagonists into the nucleus accumbens is not sufficient to block reinstatement induced by systemic cocaine administration (Cornish and Kalivas 2000). Therefore, this dopamine antagonist-like effect of muscarinic receptor agonists appears a less plausible explanation for the effects observed in the present investigation. Extinction can also be thought of as a form of reversal learning, beginning with the identification of a reward prediction error. While striatal dopamine plays a key role in initial reward learning, responses to changes in reinforcement contingency are most strongly related to activity in cholinergic interneurons (Yamanaka et al. 2017). Taken together, we propose that the present data may reflect muscarinic modulation of corticostriatal glutamatergic transmission, a hypothesis that we are exploring in ongoing studies.

One mechanism by which pharmacological interventions are thought to facilitate extinction of a behavior is by increasing the effect of extinction training through enhanced memory consolidation. We examined whether our effects could be explained by such cognitive enhancing effects in two experiments. We found that delaying xanomeline administration by 2.5 hours post-session did not prevent or diminish facilitation of extinction. In previous studies, D-cycloserine facilitated extinction of fear-potentiated startle when administered immediately after or 30 min after the extinction session, the effect was diminished when administered after a 2-hour delay, and absent if administered 4 hours after the session (Ledgerwood et al. 2003). Delaying oxotremorine administration by 2 hours after the extinction session was enough to lose the facilitating effect in an amphetamine-conditioned place preference assay (Schroeder and Packard 2004). Thus, the 2.5-hour delay used in the present investigation would be expected to at least attenuate the effect of xanomeline, although it could be argued that 2.5 hours was too brief a delay to fully avoid effects on memory. When xanomeline was administered during a 7-day period of abstinence with no extinction training (no exposure to the operant conditioning chambers) mice still met extinction criteria as fast as when xanomeline or VU0357017+VU0152100 was administered immediately post-session. In fact, responding in the cocaine-associated nose-poke hole was already decreased on the first extinction session, relative to saline controls. Those data strongly argue against the effect being mediated by enhancing the effect of extinction training. From the perspective of potential clinical use, we view this finding as an advantage, given the concerns about the use of cognitive enhancers to extinguish responses to drug cues (such as the potential to consolidate drug cue memories rather than promoting extinction), and the generally disappointing results using D-cycloserine in cocaine users so

far (Nic Dhonnchadha and Kantak 2011; Myers and Carlezon 2012; Price et al. 2013; Santa Ana 2015).

In the present investigation, stimulation of both M_1 and M_4 receptors facilitated extinction, whether in the form of an M_1/M_4 receptor-preferring agonist or in the form of combined administration of selective M_1 and M_4 ligands, while stimulation of either receptor alone had little or no effect. The doses of VU0357017 and VU0152100 used each attenuated the discriminative stimulus of cocaine in C57BL/6 mice in our previous investigations (Thomsen et al. 2012; Dall et al. 2017), and reduced cocaine self-administration to extinction levels in wild-type mice of a C57BL/6 genetic background ($N=5-7$, unpublished observations). Nevertheless, future studies should evaluate quantitatively whether contributions of M_1 and M_4 receptor stimulation to facilitation of extinction are synergistic or simply additive. This requires isobolographic analysis, with the determination of full dose-effect functions from ineffective doses to peak/plateau effect of each drug alone and the mixture (Grabovsky and Tallarida 2004). While the use of single dosing must be considered a weakness in any pharmacological study, isobolographic analysis, when applied to an assay such as intravenous self-administration, would be extremely resource-intensive, and may be better suited to higher-throughput (including non-behavioral) experimental approaches (see Thomsen et al. 2010a; Thomsen 2014). Studies using genetically engineered mice that lack M_1 , M_4 , or both subtypes, supported the involvement of both receptors in the acute attenuation of the discriminative stimulus effect of cocaine by xanomeline (Thomsen et al. 2010a, 2012). Mice lacking M_4 receptors extinguished responding previously reinforced with cocaine more slowly than wild-type mice, consistent with a role of M_4 receptors in promoting extinction (Schmidt et al. 2011; but see Okada et al. 2014). $M_1^{-/-}$ mice have not been tested in cocaine self-administration assays, and their generally hyperactive phenotype would complicate the interpretation of any increase in operant behavior (Gerber et al. 2001; Miyakawa et al. 2001). We also found that genetic deletion of both the M_1/M_4 subtypes, but not of either alone, caused deficits in sensory gating as measured by prepulse inhibition of the startle reflex (Thomsen et al. 2010b). Taken together, the findings indicate that combined manipulations of M_1 and M_4 receptor activity is not redundant, but rather can produce additive or synergistic effects.

The finding that the “unpaired” xanomeline treatment facilitated extinction and prevented cocaine-primed reinstatement of nose-poking behavior also implies that the effects are not attributable to acute pharmacological actions on the test days. Indeed, the last once-daily xanomeline administration was on average 7 days before the reinstatement test, ranging from 3 to 11 drug-free days. The duration of action of xanomeline in mice, measured by the time-course of suppression of cocaine self-administration in our laboratory and consistent with reported plasma half-life, is approximately two hours, making drug accumulation extremely unlikely (Mirza et al. 2003; Thomsen et al. 2010a). We have found that acute stimulation of M_1 , M_4 , or both receptor subtypes attenuated the discriminative stimulus effects of cocaine in mice (Thomsen et al. 2012, 2010a; Dall et al. 2017). However, mice were able to discriminate cocaine correctly on training sessions or vehicle pretreatment sessions after testing with muscarinic receptor ligands in those studies, making it unlikely that a long-lasting effect on discrimination accounts for the present findings in the reinstatement test. In addition, 7-day xanomeline treatment did not cause suppression of operant behavior under

extinction or food-reinforced conditions in mice trained to respond for a food reinforcer, indicating that the decreased responding in the cocaine experiments was not attributable to non-specific suppression of operant performance (e.g., sedation, motor function impairment, nausea). Our findings are in agreement with effects of a 4-day administration of the acetylcholinesterase inhibitor tacrine in rats: while tacrine did not affect (cued) extinction, it produced lasting decreases in cocaine self-administration, and prevented cocaine-induced reinstatement of lever pressing in a minority of rats (Grasing et al. 2015).

In summary, the present set of experiments tested the hypothesis that muscarinic M₁ and/or M₄ receptor agonist treatments would facilitate extinction of a response previously reinforced with cocaine. We found that stimulation of both M₁ and M₄ subtypes significantly shortened the number of extinction sessions needed to reach predetermined criteria. The effect could not be attributed to enhanced consolidation of extinction training memories, indicating a different, non-cognitive mechanism. M₁/M₄ receptor agonist treatment also prevented reinstatement of cocaine seeking by a cocaine “prime” injection several days after the treatment. In a food-reinforced control experiment, we verified that 7-day xanomeline treatment did not cause non-specific suppression of operant behavior, suggesting a specific effect on cocaine seeking.

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C.W.L. is an inventor on multiple pending and issued patents that protect composition of matter for M₁ and M₄ ligands. All other authors declare no conflict of interest.

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Fig. 1. Diagram of the experimental designs

The duration of the extinction phase was determined by the subject's behavior, and typical times are depicted (variable duration is indicated by dotted line at the end of the phase). Similarly, number of acquisition sessions was determined by the subject's behavior. Illumination of the cue light (symbolized by white star) accompanied cocaine infusions during acquisition in all experiments, and accompanied saline infusions in all but Experiment 3.

Fig. 2. Baseline behaviors in Experiment 1 and 2

Cocaine infusion earned at acquisition criteria, and saline infusions earned on the first day of extinction and at extinction criteria in Experiment 1 and 2. Ordinate: Infusions earned per 3h session. Data are group means, bars represent one s.e.m. Group sizes: pooled saline, N=26; xanomeline delayed, N=6; all others, N=8.

Fig. 3. Effects of muscarinic receptor M1/M4 stimulation on extinction of previously cocaine-reinforced behavior

Number of sessions required to meet extinction criteria in Experiments 1 and 2. Data are group means, bars represent one s.e.m. Group sizes as in Fig. 2. * $P < 0.05$, ** $P < 0.01$ vs. saline, uncensored Log-rank test.

Fig. 4. Survival curves of proportion of mice meeting extinction criteria

a. Experiment 1, groups receiving saline or drug immediately after the extinction sessions. b. Experiment 2, groups receiving xanomeline delayed after or unpaired from the extinction sessions. Each symbol represents individual mice, or, when more than one mouse met criteria after the same number of sessions, 2–3 mice. Abscissa: number of sessions to meet extinction criteria; axis was truncated at 30 sessions to improve visibility (longest latency: 64 sessions, saline group). Ordinates: cumulative percentage of mice meeting criteria in each group. Group sizes as in Fig. 2.

Fig. 5. Effect of post-session xanomeline in “cued” extinction

a. Cocaine intake at acquisition, and saline infusions earned on the first and last day of extinction, in Experiment 3. The “uncued” saline and xanomeline groups (Experiment 1) are included for comparison. Ordinate: infusions earned per 3h session. b. Latency to extinction in the same groups. Ordinate: number of sessions needed to meet extinction criteria. c. Survival curves showing individual events. Abscissa: number of sessions to meet extinction criteria; axis was truncated at 30 sessions for visibility (longest latency: 64 sessions, uncued saline group). Ordinates: cumulative percentage of mice meeting criteria in each group. Group sizes: pooled saline, N=26; all others, N=8.

Fig. 6. Effect of xanomeline on cocaine prime-induced reinstatement of previously cocaine-reinforced responding

Infusions earned at acquisition criteria (cocaine infusions), at extinction criteria (saline infusions), and after administration of 10 mg/kg cocaine IP (saline infusions). Ordinate: Infusions earned per 3h session. Data are group means, bars represent one s.e.m. Group sizes: N=7. * $P=0.01$ vs. extinction paired-sample t-test, † $P=0.02$ vs. saline, unpaired-sample t-test.

Fig. 7. Effect of xanomeline on extinction after food-reinforced responding

Food reinforcers earned and pump activations earned before and after 7 days treatment with 1.8 mg/kg/day xanomeline or saline. Ordinate: Pump activations earned per 2h session. Data are group means, bars represent one s.e.m. Group sizes: N=7.

Table 1

Control cohorts treated with saline under uncued conditions (Experiments 1 and 2) did not differ during acquisition or extinction of the self-administration behavior. Cohort 1 was tested alongside the initial experiment with xanomeline; cohort 2 was tested alongside groups receiving VU0357017 and/or VU0152100 or delayed xanomeline; cohort 3 received saline unpaired from the operant sessions alongside the “unpaired xanomeline” group.

	Saline cohort 1 (N=5)	Saline cohort 2 (N=14)	Saline cohort 3 (N=7)
Sessions to extinction	17.8 ± 4.1	17.5 ± 3.2	15.9 ± 9.6
Cocaine intake [mg/kg]	21.8 ± 2.2	26.5 ± 1.2	26.2 ± 1.8
Rate at acquisition	0.14 ± 0.03	0.19 ± 0.01	0.18 ± 0.02
Infusions on extinction day 1	51.6 ± 7.8	44.8 ± 7.2	42.3 ± 10.3
Rate on extinction day 1	0.32 ± 0.05	0.33 ± 0.04	0.26 ± 0.07
Infusions at extinction	5.80 ± 0.97	7.00 ± 0.66	5.57 ± 1.36
Rate at extinction	0.03 ± 0.01	0.04 ± 0.00	0.04 ± 0.01