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A novel mGluR5 antagonist, MFZ 10-7, inhibits cocaine-taking and cocaine-seeking behavior in rats

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ABSTRACT

Pre-clinical studies suggest that negative allosteric modulators (NAMs) of the metabotropic glutamate receptor subtype 5 (mGluR5), including 2-methyl-6-(phenylethynyl)pyridine (MPEP), 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP) and fenobam are highly effective in attenuating drug-taking and drug-seeking behaviors. However, both MPEP and MTEP have no translational potential for use in humans because of their off-target effects and short half-lives. Here, we report that 3-fluoro-5-[(6-methylpyridin-2-yl)ethynyl]benzonitrile (MFZ 10-7), a novel mGluR5 NAM, is more potent and selective than MPEP, MTEP and fenobam in both *in vitro* binding and functional assays. Similar to MTEP, intraperitoneal administration of MFZ 10-7 inhibited intravenous cocaine self-administration, cocaine-induced reinstatement of drug-seeking behavior and cocaine-associated cue-induced cocaine-seeking behavior in rats. Although MFZ 10-7 and MTEP lowered the rate of oral sucrose self-administration, they did not alter total sucrose intake. Further, MFZ 10-7 appeared to be more potent than MTEP in inducing downward shifts in the cocaine dose–response curve, but less effective than MTEP in attenuating sucrose-induced reinstatement of sucrose-seeking behavior. MFZ 10-7 and MTEP had no effect on basal locomotor behavior. These findings not only provide additional evidence supporting an important role for mGluR5 in cocaine reward and addiction, but also introduce a new tool for both *in vitro* and *in vivo* investigations with which to further characterize this role.

Keywords Cocaine, cue-induced cocaine seeking, MFZ 10-7, mGluR5, MTEP, reinstatement, self-administration, sucrose.

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INTRODUCTION

Glutamate neurotransmission is critically involved in drug reward and addiction (Olive *et al.* 2012). Glutamate signaling occurs via functional activation of ionotropic glutamate receptors and metabotropic glutamate receptors (mGluRs). Eight mGluRs are classified into three groups based on sequence homology and G protein interactions: group I (mGluR1, mGluR5), group II (mGluR2, mGluR3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8; Ferraguti & Shigemoto 2006). Activation of mGluR1 and mGluR5, which are G_q protein-coupled, induces mobilization of intracellular Ca^{2+} stores and activation of phospholipase C (Schoepp & Conn 1993). mGluR5 is expressed in multiple brain regions, including the mesolimbic structures such as the ventral tegmental area and the nucleus accumbens, that are critically involved in drug reward and addiction (Shigemoto *et al.* 1993; Romano, Pol & O'Malley 1996). mGluR5 is mostly located postsynaptically (Mitrano & Smith 2007) and co-localized with dopamine D2, adenosine A2A and N-methyl-D-aspartate (NMDA) receptors (Tebano *et al.* 2005).

mGluR5 became a major target of interest in medication development for treatment of addiction when it was reported that mice lacking mGluR5 do not self-administer cocaine and that blockade of mGluR5 by 2-methyl-6-(phenylethynyl)pyridine (MPEP), a negative allosteric modulator (NAM), inhibits cocaine self-administration (Chiamulera *et al.* 2001). Since then, a series of investigations have reported that MPEP and its analog 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine

(MTEP), as well as fenobam significantly inhibit behaviors associated with addiction in experimental animals, including cocaine self-administration (Tessari *et al*. 2004; Kenny *et al*. 2005; Lee *et al*. 2005; Paterson & Markou 2005; Martin-Fardon *et al*. 2009; Keck *et al*. 2013), cocaine-induced conditioned place preference (McGeehan & Olive 2003; Herzig & Schmidt 2004), cocaine-induced hyperactivity (McGeehan, Janak & Olive 2004), and cocaine-, cue- or stress-induced reinstatement of drug-seeking behavior (Lee *et al*. 2005; Backström & Hyytiä 2006; Kumaresan *et al*. 2009; Martin-Fardon & Weiss 2012; Keck *et al*. 2013; Wang *et al*. 2013). These data strongly suggest that mGluR5 plays an important role in cocaine abuse and addiction and that mGluR5 NAMs may have potential for the treatment of cocaine addiction in humans (Heidbreder *et al*. 2003; Olive *et al*. 2005).

However, MPEP and MTEP have not been tested in human trials for multiple reasons (Lindsley & Emmitte 2009). Off-target effects of MPEP include positive allosteric modulation of mGluR4 (Mathiesen *et al*. 2003), antagonism of NMDA receptors (O'Leary *et al*. 2000; Movsesyan *et al*. 2001), binding to the norepinephrine transporter (Heidbreder *et al*. 2003) and inhibition of monoamine oxidase A (Lea & Faden 2006). Although MTEP is more selective than MPEP for mGluR5 (Cosford *et al*. 2003; Lea & Faden 2006), it has been reported to inhibit cytochrome P450 1A2 (Green, Jiang & King 2004), produce social isolation in rats (Koros *et al*. 2007) and has reduced binding affinity for mGluR5 compared with MPEP (Keck *et al*. 2012). These off-target effects predict potential undesirable side effects or metabolic instability that were deemed too risky to advance these drugs into the clinic. In light of these limitations, extensive efforts have been undertaken to develop novel potent, selective and metabolically more stable mGluR5 NAMs for potential translation to clinical investigation (Emmitte 2011; Nicoletti *et al*. 2011; Rocher *et al*. 2011).

3-Fluoro-5-[(6-methylpyridin-2-yl)ethynyl]benzonitrile (MFZ 10-7) is a highly potent mGluR5 NAM (Keck *et al*. 2012). Structurally, MFZ 10-7 is an analog of MPEP, but has much higher mGluR5 binding affinity and mGluR5 antagonist potency *in vitro* than MPEP, MTEP or fenobam (Keck *et al*. 2012, 2013). In the present study, we evaluated MFZ 10-7 in a broad receptor screen to determine potential off-target effects, comparing its pharmacological profile with MTEP as well as the clinically investigated mGluR5 NAM fenobam. We then investigated and compared the effects of MFZ 10-7 and MTEP on cocaine self-administration maintained by a single dose or multiple doses, cocaine priming-induced reinstatement of drug-seeking behavior, and cocaine-associated cue-induced cocaine-seeking behavior in rats—three commonly used animal models to predict drug reward and relapse (O'Brien & Gardner 2005). In addition, we compared the effects of MFZ 10-7 and MTEP on oral sucrose self-administration and reinstatement of sucrose seeking behavior to determine whether both these mGluR5 NAMs selectively inhibit cocaine-taking and cocaine-seeking behavior versus sucrose-taking and sucrose-seeking behavior. Finally, we observed the effects of both compounds on open-field locomotor activity to determine whether either compound produced non-specific locomotor impairment that might underlie the inhibitory effects on cocaine- or sucrose-taking or -seeking behavior.

MATERIALS AND METHODS

Experiment 1: *In vitro* functional assay of mGluR5 NAMs

To compare the *in vitro* potency (IC_{50}) of MFZ 10-7, MPEP, MTEP and fenobam under the same experimental conditions, we used HEK293 cells stably expressing rat mGluR5 in a competitive immunoassay to evaluate G_q protein-mediated production of the intracellular messenger inositol 1,4,5-trisphosphate (IP3; detailed immunoassay methods in Keck *et al*. 2012; stable construct described in Romano *et al*. 1995). Briefly, the IP-One enzyme-linked immunosorbent assay (Cisbio US, Bedford, MA, USA) measures the accumulation of D-*myo*-inositol 1 phosphate (IP1), a degradation product of IP3, via an anti-IP1 monoclonal antibody (Anti-IP1 Mab) and IP1-horse-radish peroxidase (IP1-HRP) conjugate in the presence of LiCl. For each assay, test compounds were dissolved in 30% dimethyl sulfoxide (DMSO) and water to a concentration of 100 μM and serially diluted in 1X stimulation buffer; final test concentrations ranged from 10 μM to 10 pM. After 1-hour incubation with test compounds at 37°C, cells were lysed and the lysate was treated with Anti-IP1 Mab and IP1-HRP. The optical density (OD) of each treatment was determined at wavelengths of 450 nm and 620 nm (Spectramax M5 reader and Softmax Pro 5.3 software, Molecular Devices, Sunnyvale, CA, USA). IP1 levels for each treatment were determined by subtracting the OD at 620 nm from the OD at 450 nm and normalizing to vehicle-only control values. IC_{50} values for inverse agonism (no agonist present) were calculated from at least three independent experiments.

Experiment 2: *In vitro* binding assays of mGluR5 NAMs

Receptor binding data for MPEP, MTEP and fenobam have been reported previously (Gasparini *et al*. 1999; Porter *et al*. 2005), but the present experiment was designed to compare the mGluR5 binding affinity (K_i) of these compounds with MFZ 10-7 under identical experimental

conditions, using experimental procedures reported previously (detailed methods in Keck *et al.* 2012). Briefly, binding was performed in membranes prepared from the brains of male Sprague–Dawley rats. Whole brains minus the cerebellum were homogenized in cold assay buffer (50 mM Tris, 120 mM NaCl, 5 mM KCl, pH 7.4 at 25°C) and centrifuged at 50 000 g for 10 minutes at 4°C. The resulting pellet was re-suspended in cold assay buffer, re-centrifuged and re-suspended in buffer to a concentration of 75 mg/ml. Ligand binding experiments were conducted at room temperature for 60 minutes in glass assay tubes containing 0.5 ml total volume. Each reaction contained 4 nM [^{3}H]MPEP (American Radiolabeled Chemicals, St. Louis, MO, USA), 7.5 mg of brain tissue (original wet weight), and varying concentrations of test compounds. Non-specific binding was determined using 100 μM MPEP. Incubations were terminated by rapid filtration and washing with cold assay buffer. Filters were transferred to scintillation vials, scintillation fluid was added and the vials were counted in a liquid scintillation counter. Each compound was tested over full dose–response curves, with test compound concentrations at half-log units ranging from 10 pM to 100 μM final concentration, performed in triplicate. K_i values were determined from at least three independent experiments.

In a previous *in vitro* binding screen of 64 functional receptor/enzyme proteins, MFZ 10-7 bound to two off-target binding sites at a 10 μM concentration: the prostaglandin thromboxane A2 receptor (TXA2) and the peripheral monoamine oxidase-B enzyme (MAO-B; Keck *et al.* 2012). To assess the likelihood of these sites affecting the results of behavioral experiments, the binding affinities of MFZ 10-7, MTEP and fenobam at these sites were also determined [National Institute on Drug Abuse (NIDA) Contract N01DA-8-8877—Caliper LifeSciences].

Experiment 3: Cocaine self-administration

Animals

Male Long-Evans rats (250–300 g; Charles River Laboratories, Raleigh, NC, USA) were used for all experiments. They were individually housed in a climate-controlled room on a reverse light–dark cycle (lights on at 1900 hours, lights off at 0700 hours) with *ad libitum* access to food and water. All experimental procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (US National Academy of Sciences), and were approved by the Animal Care and Use Committee of the National Institute on Drug Abuse. The animal facility was fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Surgery

Intravenous (i.v.) catheterization of the right external jugular vein was performed under sodium pentobarbital (60 mg/kg, i.p.) anesthesia, utilizing standard aseptic surgical techniques as we reported previously (Xi *et al.* 2010, 2013). To help prevent clogging, the catheters were flushed daily with a gentamicin–heparin–saline solution (0.1 mg/ml gentamicin, 30 IU/ml heparin; ICN Biochemicals, Cleveland, OH, USA).

Apparatus

Self-administration chambers from MED Associates Inc. (Saint Albans, VT, USA) were the same as used previously (Xi *et al.* 2010, 2013).

Multiple-dose cocaine self-administration

To determine whether MFZ 10-7 or MTEP attenuates cocaine's rewarding efficacy, we observed the effects of both compounds on the cocaine dose–response self-administration curve. This is based upon the well-accepted view that a leftward or upward shift of a dose–response curve reflects an increase in pharmacological action, and vice versa (Hiranita *et al.* 2009). After recovery from surgery, each rat was placed into a test chamber and allowed to lever-press for i.v. cocaine (1 mg/kg/infusion) on a fixed ratio 1 (FR1) reinforcement schedule. Each cocaine infusion delivered a volume of 0.08 ml/infusion over 4.65 seconds and was paired with the simultaneous presentation of a stimulus light and tone (each lasting 4.65 seconds). Each session lasted 3 hours. FR1 reinforcement was used for 5 days to establish stable self-administration. Subjects were then allowed to continue cocaine self-administration (0.5 mg/kg/infusion) under FR2 reinforcement. After stable self-administration was established on the FR2 schedule (less than 10% variation in total infusions over 3 consecutive days), animals were switched to multiple-dose cocaine self-administration maintained by a full range of cocaine doses (0.03, 0.06, 0.125, 0.25, 0.5, 1.0 mg/kg/infusion) in a single dose–response session (Hiranita *et al.* 2009; Keck *et al.* 2013). Each dose–response session consisted of six sequential trials, beginning with a 30-minute extinction period (0 mg/kg cocaine) followed by six 20-minute components in which different cocaine doses were delivered. A 20-minute intertrial time-out period was included between each session to allow for changing the cocaine dose. Infusion volume, infusion duration and the presentation of a stimulus light and tone (each lasting 4.65 seconds) during the infusion remained constant across all trials. Self-administration continued until stable cocaine-maintained responding was achieved (i.e. a minimum of 10 mg/kg cocaine intake within a session and less than 10% variation in total number of cocaine

infusions for 3 consecutive days). Then, each rat ($n = 7$) randomly received either vehicle or one of two doses of MFZ 10-7 (3, 10 mg/kg, i.p.) or MTEP (3, 10 mg/kg, i.p.) 15 minutes prior to the test session. Animals then received an additional 2–3 days of self-administration of cocaine alone until the baseline response rate was reestablished prior to testing the next dose of MTEP or MFZ 10-7. The order of testing for the various doses of drug or vehicle was counterbalanced.

Single-dose cocaine self-administration

Given that the multiple-dose cocaine self-administration experiment lasted at least 4 hours per test session, while the pharmacological action of a test drug may last shorter than 4 hours, it is likely that a single injection of a test drug may inhibit self-administration maintained by initial lower doses of cocaine, but not by subsequent high doses of cocaine. Therefore, we used two additional groups of rats ($n = 7$–11 per group) to study whether a single injection of MFZ 10-7 or MTEP inhibits cocaine self-administration maintained by a single high dose (0.5 mg/kg/injection) of cocaine. The initial cocaine self-administration procedures were the same as described above. Each session lasted 3 hours. To avoid cocaine overdose during the self-administration period, each animal was limited to a maximum of 50 cocaine injections per 3-hour session. After stable self-administration was established on the FR2 schedule (less than 10% variation in total infusions over 3 consecutive days), the effects of MFZ 10-7 and MTEP on cocaine self-administration were assessed. Drugs were administered (i.p.) 15 minutes prior to testing. After each drug test, animals continued daily cocaine self-administration until stable self-administration was reestablished. The order of testing for various doses of each compound was counterbalanced.

Experiment 4: Sucrose self-administration

The procedures for sucrose self-administration were identical to the procedures for cocaine self-administration except for the following: (1) no surgery was performed on the animals; (2) active lever presses led to delivery of 0.1 ml of 5% sucrose solution into a liquid food tray on the operant chamber wall along with presentation of a stimulus light and tone; (3) 3-hour FR1 acquisition sessions were capped at 100 deliveries; and (4) test sessions were 90 minutes in length and were capped at 100 deliveries. After stable sucrose self-administration was established on the FR1 schedule (less than 10% variation in total deliveries over 3 consecutive days), the effects of MFZ 10-7 and MTEP on sucrose self-administration were determined in two separate groups of rats ($n = 7$ per group). Drug doses and administration methods were identical to the cocaine self-administration experiments.

Experiment 5: Sucrose-triggered reinstatement of sucrose-seeking behavior

Two additional group of rats ($n = 15$ total) were used to evaluate the effects of MFZ 10-7 and MTEP on sucrose-induced reinstatement of sucrose-seeking behavior. After stable sucrose self-administration was achieved, animals underwent extinction sessions until sucrose-seeking behavior was extinguished. To determine whether MFZ 10-7 or MTEP pretreatment inhibits relapse to sucrose-seeking behavior, extinguished animals were treated with vehicle, MFZ 10-7 or MTEP prior to reinstatement triggered by five non-contingent sucrose deliveries (which were subtracted from the total number of sucrose reinforcements for data analysis) within the initial 5 minutes of testing. The animals were divided into two dose groups. Group 1 ($n = 10$) randomly received either vehicle or one of two doses of MFZ 10-7 (3, 10 mg/kg, i.p.). Group 2 ($n = 5$) randomly received either vehicle or one of two doses of MTEP (3, 10 mg/kg, i.p.). The order of testing for the various doses of drug or vehicle was counterbalanced and there were two intervening extinction trials between each test. Lever presses during the reinstatement tests were recorded, but did not lead to either sucrose delivery or presentation of the conditioned cue-light and tone.

Experiment 6: Cocaine-primed reinstatement of cocaine-seeking behavior

After the completion of the single-dose cocaine self-administration study from Experiment 3, 11 animals tested with MTEP and 9 animals tested with MFZ 10-7 during cocaine self-administration were used in this experiment to observe the effects of MTEP or MFZ 10-7 on cocaine-induced reinstatement of drug-seeking behavior, respectively. This is based on our observation that the effects of MTEP or MFZ 10-7 on cocaine self-administration lasted only a few hours (3–4 hours) and was completely reversible 24 hours after the drug administration. There is no tolerance or sensitization to the biological effects observed after MTEP or MFZ 10-7 administration. To further exclude the potential effects of previous MFZ 10-7 treatment history on the effects of MFZ 10-7 on cocaine-induced reinstatement of drug-seeking behavior, we used eight additional animals without MFZ 10-7 treatment history during the self-administration phase in the MFZ 10-7 group of rats to compare the effects of MFZ 10-7 on cocaine-induced reinstatement of drug-seeking behavior between two groups of rats with or without MFZ 10-7 treatment history. Additional cocaine self-administration continued for at least 3–5 days until stable self-administration was reestablished. Then, the animals underwent extinction sessions, during which cocaine was replaced by saline

and the light and sound cues that previously accompanied cocaine infusions were turned off. After the drug-seeking behavior was extinguished, defined as ≤ 15 active lever presses during each 3-hour session for at least 3 consecutive days, the effects of MFZ 10-7 and MTEP on cocaine-primed reinstatement were determined. On the reinstatement test day, each group of animals received vehicle, MFZ 10-7 or MTEP 30 minutes prior to cocaine priming (10 mg/kg, i.p.). Then, the animals were placed into the operant chambers that were previously paired with cocaine self-administration. Reinstatement conditions were identical to those in the extinction sessions, i.e. active lever presses were recorded without cocaine infusions or accompanying cues for 3 hours. Effects of MFZ 10-7 and MTEP on cocaine-induced reinstatement were assessed by comparing the mean number of active lever presses per test session.

Experiment 7: Contextual cue-induced incubation of cocaine seeking

Two groups of rats ($n = 12$ each group) were used to examine the effects of MFZ 10-7 and MTEP, respectively, on cocaine-associated contextual cue-induced cocaine-seeking behavior. We used a within-subjects design to evaluate the effects of the drugs on cocaine-associated contextual cue-induced cocaine-seeking behavior following 3 weeks of withdrawal. This is based upon our recent finding that cue-induced drug-seeking behavior is relatively stable in this period of withdrawal from cocaine self-administration (Xi *et al*. 2013). Initial cocaine self-administration methods were the same as described above. Following establishment of stable cocaine self-administration, animals underwent 3 weeks of drug abstinence in which rats were left undisturbed in the housing facility. Then, on each subsequent test day, rats were placed into the same self-administration chambers in which they had been previously tested, and cocaine-associated contextual cue-induced cocaine-seeking behavior (i.e. active lever presses) was assessed under extinction conditions during which cocaine and cocaine-associated discrete cues (light and tone) were unavailable, and lever pressing resulted in no consequences. Each session lasted 3 hours. Each animal was tested three times with different drug doses, in a counterbalanced fashion, with MFZ 10-7 (0, 3, 10 mg/kg, i.p., 15 minutes prior to testing) or MTEP (0, 1, 10 mg/kg, i.p., 15 minutes prior to testing). The interval between drug tests was 2–3 days.

Experiment 8: Effects of MFZ 10-7 and MTEP on locomotor behavior

To determine whether the reduction in cocaine-taking and seeking behavior was due to non-specific locomotor impairment or sedative effects, we observed the effects of MFZ 10-7 and MTEP on basal locomotor behavior in two separate groups of rats ($n = 8$ each). Before testing, drug-naïve rats were habituated in a locomotor detection chamber (AccuScan, Columbus, OH, USA) for 1 hour each day on 2 consecutive days. On each test day, 1 hour basal levels of locomotor activity were recorded prior to drug or vehicle administration; drug-induced changes in locomotor behavior were recorded for an additional 3 hours. Each animal was tested three times with different drug doses. The time intervals between testing were 2–3 days. Drug doses and administration methods were identical to the cocaine self-administration experiments.

Drugs

MFZ 10-7 was synthesized at NIDA-IRP according to a recently published procedure (Keck *et al*. 2012). MPEP was purchased from Tocris Bioscience (Ellisville, MO, USA). MTEP and fenobam free base were provided by the Drug Supply Program of NIDA (NIDA DPMCDA; Bethesda, MD, USA). Cocaine HCl was provided by NIDA and dissolved in physiological saline. For *in vitro* tests, all compounds were dissolved in 30% DMSO and water. For behavioral tests, MFZ 10-7 was suspended in 1% Tween 80 and water for intraperitoneal (i.p.) administration; MTEP was dissolved in saline for i.p. administration.

Data analysis

All data are presented as means ± standard error of the mean and were analyzed by using GraphPad Prism software (San Diego, CA, USA). One-way analysis of variance (ANOVA) was used to determine the significance of the changes in reward-taking or reward-seeking behavior after each mGluR5 NAM administration. Whenever a significant main effect was found, individual group comparisons were carried out using pre-planned Bonferroni *t*-tests.

RESULTS

MFZ 10-7 is a highly potent and selective mGluR5 NAM

Figure 1 shows the chemical structures and the *in vitro* functional potencies of the mGluR5 NAMs: MPEP, MTEP, MFZ 10-7 and fenobam. MFZ 10-7 was approximately 13-, 46- and 188-fold more potent (lower IC_{50}) than MPEP, MTEP and fenobam, respectively. Table 1 shows the *in vitro* binding affinities of MTEP, MFZ 10-7 and fenobam. At mGluR5, MFZ 10-7 had approximately 63- and 330-fold higher binding affinity (lower K_i) than MTEP and fenobam, respectively. A recent screen of MFZ 10-7 for binding to 64 functional receptor/enzyme proteins (Keck *et al*. 2012; NIDA Contract N01DA-8-8877-Caliper

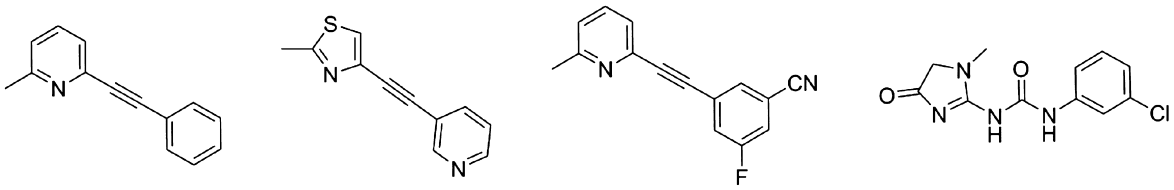

Figure 1 Chemical structures and *in vitro* functional potencies (IC_{50}) for MPEP, MTEP, MFZ 10-7 and fenobam. *Reported previously in Keck *et al.* (2012)

Table 1 Binding affinities of MTEP, MFZ 10-7 and fenobam at mGluR5, MAO-B, and TXA2.

Binding target	*MTEP (K_i μM)*	*MFZ 10-7 (K_i μM)*	*Fenobam (K_i μM)*
Rat mGluR5	0.042 ± 0.001*	0.00067 ± 0.00001*	0.221 ± 0.010
Rat peripheral MAO-B	12.9 ± 1.3	0.77 ± 0.16	> 20
Human TXA2	> 20	2.02 ± 0.85	5.63 ± 1.39

*Reported previously in Keck *et al.* (2012).
MAO-B, monoamine oxidase-B enzyme; TXA2, thromboxane A2 receptor.

LifeSciences) identified binding to only two off-target sites at which MFZ 10-7 bound with greater than 10 μM affinity: MAO-B and TXA2. Further analysis determined the binding affinities of MTEP, MFZ 10-7 and fenobam at MAO-B and TXA2 (Table 1). MFZ 10-7 had approximately 1150- and 3000-fold lower affinity for MAO-B and TXA2, respectively, compared with mGluR5 (Table 1). MTEP had no detectable affinity for TXA2 and was approximately 300-fold selective for mGluR5 over MAO-B (Table 1). Fenobam had no detectable affinity for MAO-B and was approximately 25-fold selective for mGluR5 over TXA2 (Table 1).

MFZ 10-7 and MTEP inhibit cocaine self-administration

Figure 2 illustrates the effects of MFZ 10-7 and MTEP on dose-dependent cocaine self-administration, demonstrating that a single injection of MFZ 10-7 (Fig. 2a) or MTEP (Fig. 2c) dose-dependently shifted the cocaine dose–response curve downward, suggesting a reduction in cocaine's rewarding effects after MFZ 10-7 or MTEP administration. Two-way repeated-measures ANOVA revealed a statistically significant treatment main effect of MFZ 10-7 (Fig. 2a; $F_{2,90} = 6.33$, $P < 0.01$), a statistically significant effect of cocaine dose ($F_{5,90} = 3.48$, $P < 0.01$) and a significant MFZ 10-7 × cocaine dose interaction ($F_{10,90} = 4.05$, $P < 0.001$). Individual group comparisons at each cocaine dose revealed a significant reduction in the number of cocaine infusions after 3 mg/kg MFZ 10-7 (0.06 mg/kg: $t = 2.91$, $P < 0.05$; 0.125 mg/kg: $t = 3.26$, $P < 0.05$) or 10 mg/kg MFZ 10-7 (0.06 mg/kg: t = 4.54, $P < 0.001$; 0.125 mg/kg: t = 5.33, $P < 0.001$; 0.25 mg/kg: t = 3.00, $P < 0.05$). Two-way repeated-measures ANOVA revealed no statistically significant treatment main effect of MTEP (Fig. 2c; $F_{2,90} = 3.06$, $P = 0.07$), a statistically significant effect of cocaine dose ($F_{5,90} = 5.35$, $P < 0.001$) and no significant MTEP × cocaine dose interaction ($F_{10,90} = 1.68$, $P = 0.10$). Individual group comparisons at each cocaine dose revealed a significant reduction in the number of cocaine infusions for two cocaine doses after 10 mg/kg MTEP (0.06 mg/kg: $t = 3.22$, $P < 0.05$; 0.125 mg/kg: $t = 3.05$, $P < 0.05$).

Based upon the data shown in Fig. 2a/c, it appears that MFZ 10-7 and MTEP more effectively attenuate cocaine self-administration maintained by initial lower doses (0.03, 0.06, 0.125, 0.25 mg/kg/infusion) of cocaine than by subsequent higher doses (0.5, 1.0 mg/kg/infusion) of cocaine. However, because the experimental drugs were given ~3 hours prior to 0.5 mg/kg cocaine self-administration testing, we hypothesized that the peak pharmacological effect of the drugs may have been over before high dose (0.5–1.0 mg/kg) of cocaine self-administration began. To test this hypothesis, we carried out additional experiments to reassess the effects of MFZ 10-7 and MTEP on single-dose cocaine self-administration maintained by 0.5 mg/kg/injection. Figure 2b/d illustrates that systemic (i.p.) administration of MFZ 10-7 or MTEP, when given 15 minutes prior to self-administration testing, produced a significant and dose-dependent reduction in cocaine self-administration. One-way repeated measures ANOVA revealed a statistically significant treatment main effect of MFZ 10-7 (Fig. 2b: $F_{2,6} = 10.19$, $P < 0.01$) or MTEP (Fig. 2d: $F_{2,18} = 7.23$, $P < 0.01$) on total cocaine infusions.

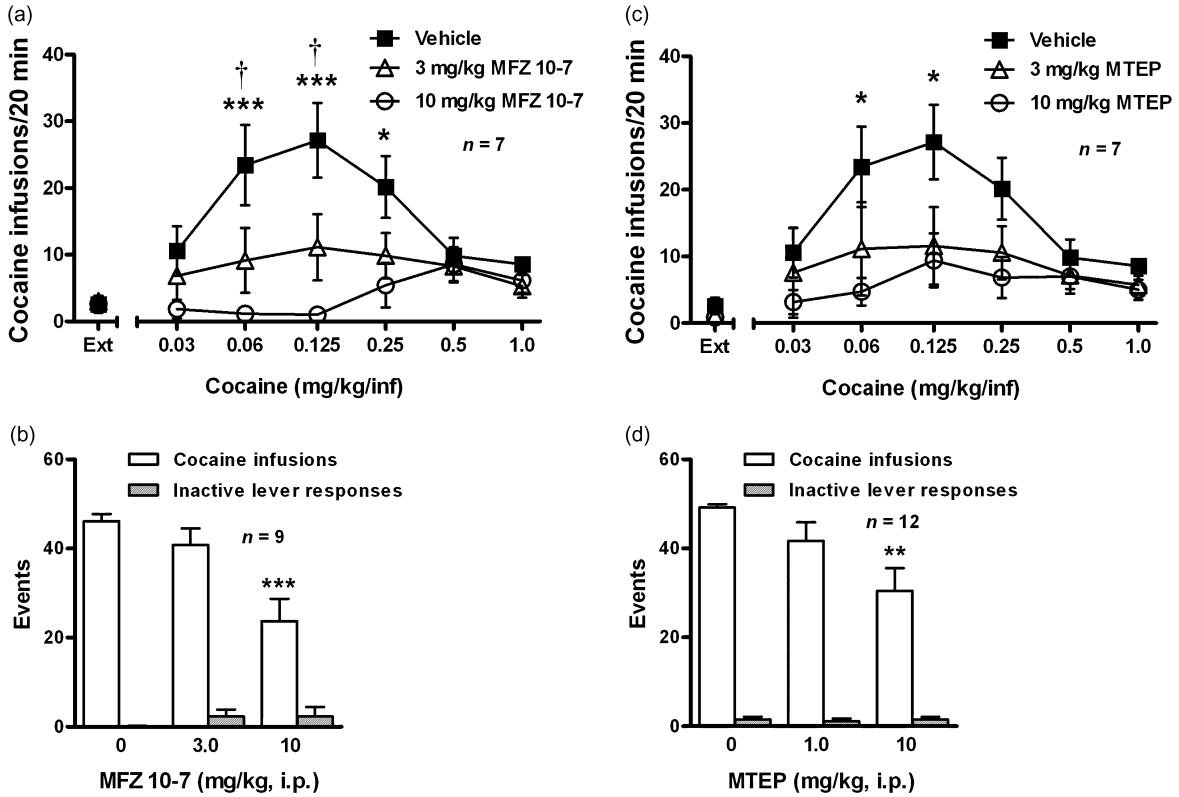

Figure 2 Effects of MFZ 10-7 and MTEP on cocaine self-administration behavior in rats. a, b: MFZ 10-7 (0, 3 or 10 mg/kg by i.p.) dose-dependently shifted the cocaine dose–response curve downward and inhibited cocaine self-administration maintained by high dose (0.5 mg/kg/infusion) of cocaine; c, d: MTEP (0, 1, 10 mg/kg, i.p.) shifted the cocaine dose–response curve downward and inhibited cocaine self-administration maintained by 0.5 mg/kg/infusion cocaine. 3 mg/kg MFZ 10-7 or MTEP: †$P < 0.05$ compared with vehicle. 10 mg/kg MFZ 10-7 or MTEP: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with vehicle

Individual group comparisons demonstrated that each drug, at the highest tested doses, significantly decreased cocaine infusions. This effect lasted for less than 24 hours as cocaine self-administration behavior returned to basal levels 24 hours after NAM administration. There were no significant differences in inactive lever responding across all treatments.

MFZ 10-7 and MTEP inhibit oral sucrose self-administration rate, but have no effect on total sucrose intake

Figure 3 illustrates the effects of MFZ 10-7 and MTEP on oral sucrose self-administration, illustrating that systemic administration of either one failed to alter the total number of sucrose deliveries (Fig. 3a: $F_{2,6} = 2.04$, $P > 0.05$; Fig. 3b: $F_{2,6} = 3.72$, $P > 0.05$). Because a limitation of 100 deliveries was set and the majority of animals completed the maximal number of deliveries during the 90-minute testing period, we renormalized the data to the rate of sucrose deliveries per hour (Fig. 3c,d). The normalized data show that systemic administration of MFZ 10-7 (Fig. 3c: $F_{2,6} = 25.25$, $P < 0.001$) or MTEP (Fig. 3d: $F_{2,6} = 5.96$, $P < 0.05$) significantly and dose-dependently inhibited the rate of oral sucrose self-administration. Individual group comparisons revealed a significant reduction in the rate of sucrose delivery after 10 mg/kg MFZ 10-7 ($t = 5.77$, $P < 0.001$) and 10 mg/kg MTEP ($t = 3.35$, $P < 0.05$). There were no statistically significant variations in inactive lever responding across treatments.

MFZ 10-7 and MTEP inhibit cocaine-primed reinstatement of cocaine-seeking behavior

Figure 4 illustrates the total number of active and inactive lever presses observed during the last session of cocaine self-administration, the last session of extinction, and the reinstatement test session in the three different dose groups for MFZ 10-7 (Fig. 4a) or MTEP (Fig. 4b). A single, non-contingent cocaine priming dose (10 mg/kg, i.p.) produced robust reinstatement of extinguished operant responding (i.e. active lever presses) in rats previously reinforced by i.v. cocaine infusions. Pretreatment with MFZ 10-7 or MTEP produced a significant reduction in cocaine-induced reinstatement of drug-seeking behavior. One-way ANOVA revealed a statistically significant treatment main effect of MFZ 10-7

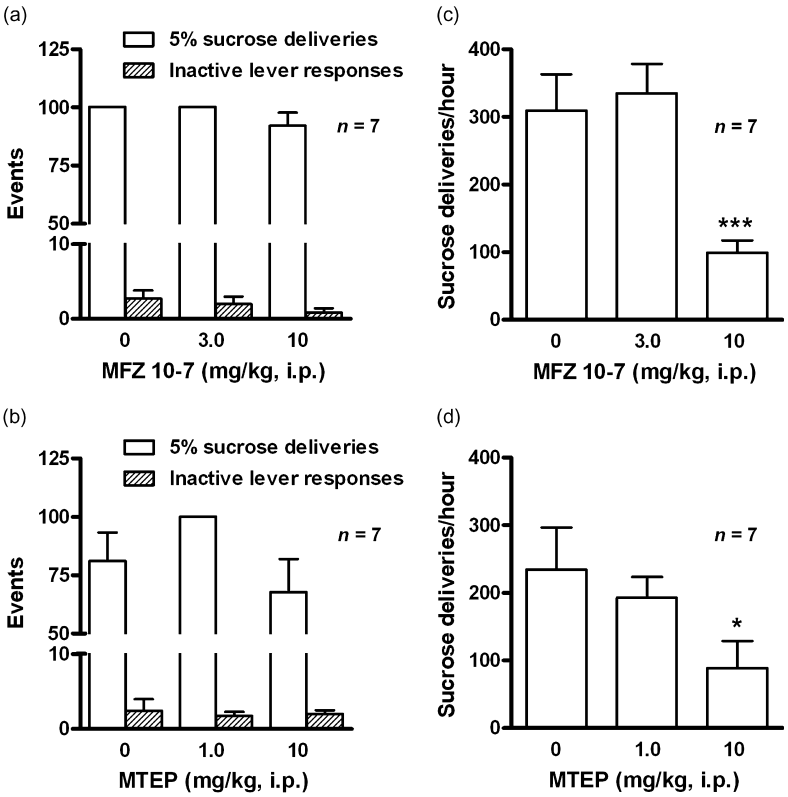

Figure 3 Effects of MFZ 10-7 and MTEP on oral sucrose self-administration. a, b: Total numbers of oral sucrose deliveries and inactive lever responses; c, d: Rates of sucrose self-administration (sucrose deliveries per hour), demonstrating that MFZ 10-7 (a) and MTEP (b) did not alter total sucrose rewards received or inactive lever responding but dose-dependently reduced the rate of sucrose delivery (c, d). $^{*}P < 0.05$, $^{***}P < 0.001$ compared with vehicle

(Fig. 4a: $F_{2,16} = 52.08$, $P < 0.001$) or MTEP (Fig. 4b: $F_{2,10} = 6.69$, $P < 0.01$) on active lever responding. Individual group comparisons revealed a significant reduction in drug-seeking after 3 mg/kg ($t = 9.42$, $P < 0.001$) or 10 mg/kg ($t = 8.11$, $P < 0.001$) MFZ 10-7 and after 1 mg/kg ($t = 2.87$, $P < 0.05$) or 10 mg/kg ($t = 3.89$, $P < 0.001$) MTEP, when compared with vehicle control groups. There were no statistically significant differences in inactive lever responding across treatments.

MTEP, but not MFZ 10-7, inhibits sucrose-primed reinstatement of sucrose-seeking behavior

Figure 5 illustrates the total number of active and inactive lever presses observed during the last three sessions of sucrose self-administration, the last three sessions of extinction training, and the reinstatement test session in the three different dose groups for MFZ 10-7 (Fig. 5a) or MTEP (Fig. 5b). Five non-contingent sucrose deliveries produced robust reinstatement of extinguished operant responding (i.e. active lever presses) in rats previously reinforced by oral sucrose self-administration. Pretreatment with MTEP, but not MFZ 10-7, produced a significant and dose-dependent reduction in sucrose-induced reinstatement of sucrose-seeking behavior. One-way ANOVA revealed no statistically significant treatment main effect of MFZ 10-7 (Fig. 5a: $F_{2,16} = 2.94$, $P > 0.05$), but a statistically significant treatment main effect of MTEP (Fig. 5b: $F_{2,10} = 42.91$, $P < 0.001$) on active lever responding. Individual group comparisons revealed no significant reductions in sucrose-seeking after 3 mg/kg ($t = 0.06$, $P > 0.05$) or 10 mg/kg ($t = 2.13$, $P > 0.05$) MFZ 10-7, but significant reductions in sucrose-seeking after 3 mg/kg ($t = 7.75$, $P < 0.001$) or 10 mg/kg ($t = 8.27$, $P < 0.001$) MTEP, when compared with vehicle control group. There were no statistically significant differences in inactive lever responding across treatments.

MFZ 10-7 and MTEP inhibit cocaine-associated cue-induced cocaine-seeking behavior

Following cocaine self-administration, animals were divided into two groups ($n = 12$ each) that were used to evaluate the effects of MFZ 10-7 and MTEP, respectively, on cocaine-seeking behavior (active lever responses) following 21 days of withdrawal (Fig. 6a,b). One-way ANOVA revealed statistically significant treatment main effects of MFZ 10-7 (Fig. 6a: $F_{2,11} = 17.72$, $P < 0.001$) and MTEP (Fig. 6b: $F_{2,11} = 13.19$, $P < 0.001$) on active lever presses. Individual group comparisons revealed significantly reduced active lever responding after 3 mg/kg ($t = 5.70$, $P < 0.001$) or 10 mg/kg ($t = 4.33$, $P < 0.001$) MFZ 10-7 and 10 mg/kg MTEP ($t = 5.13$, $P < 0.001$), when compared with vehicle control groups. Pretreatment with MFZ 10-7 ($F_{2,11} = 7.48$, $P < 0.01$; 10 mg/kg, $t = 3.24$, $P < 0.05$; 3 mg/kg, $t = 3.45$, $P < 0.01$) or MTEP ($F_{2,11} = 5.38$, $P < 0.05$; 10 mg/kg, $t = 2.61$, $P < 0.05$;

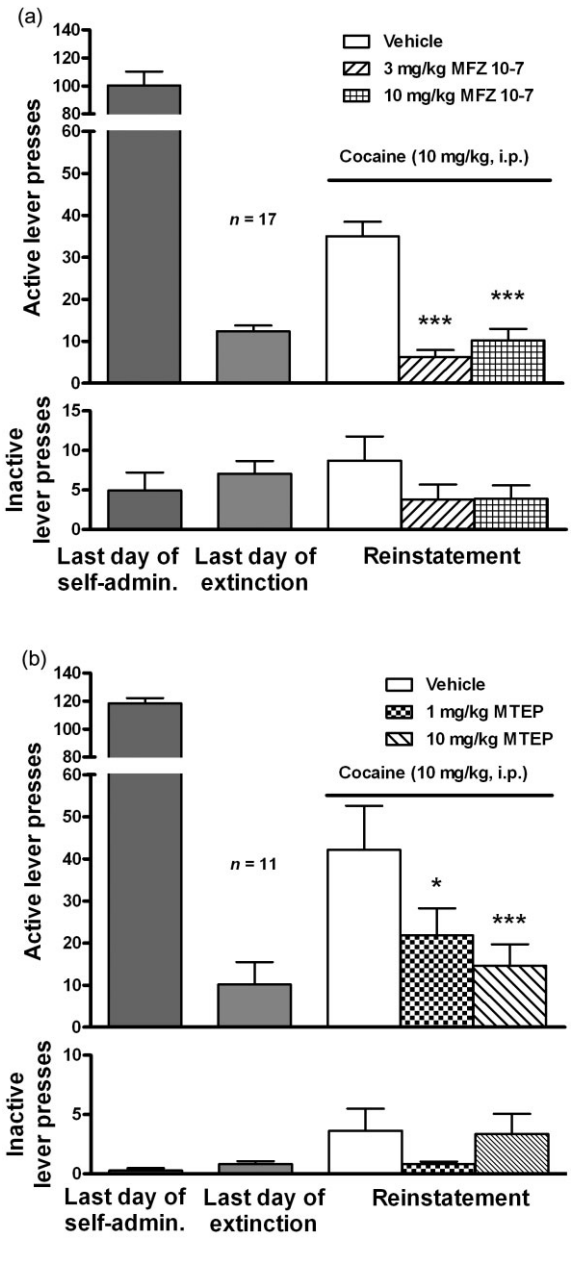

Figure 4 Effects of MFZ 10-7 and MTEP on cocaine-induced reinstatement of drug-seeking behavior. Pretreatment with MFZ 10-7 (0, 3 or 10 mg/kg, i.p.) (a) or MTEP (0, 1 or 10 mg/kg, i.p.) (b) dose-dependently inhibited cocaine-induced reinstatement of drug-seeking behavior in rats after cocaine-taking and cocaine-seeking behavior was extinguished. $^{*}P < 0.05$, $^{***}P < 0.001$, compared with vehicle

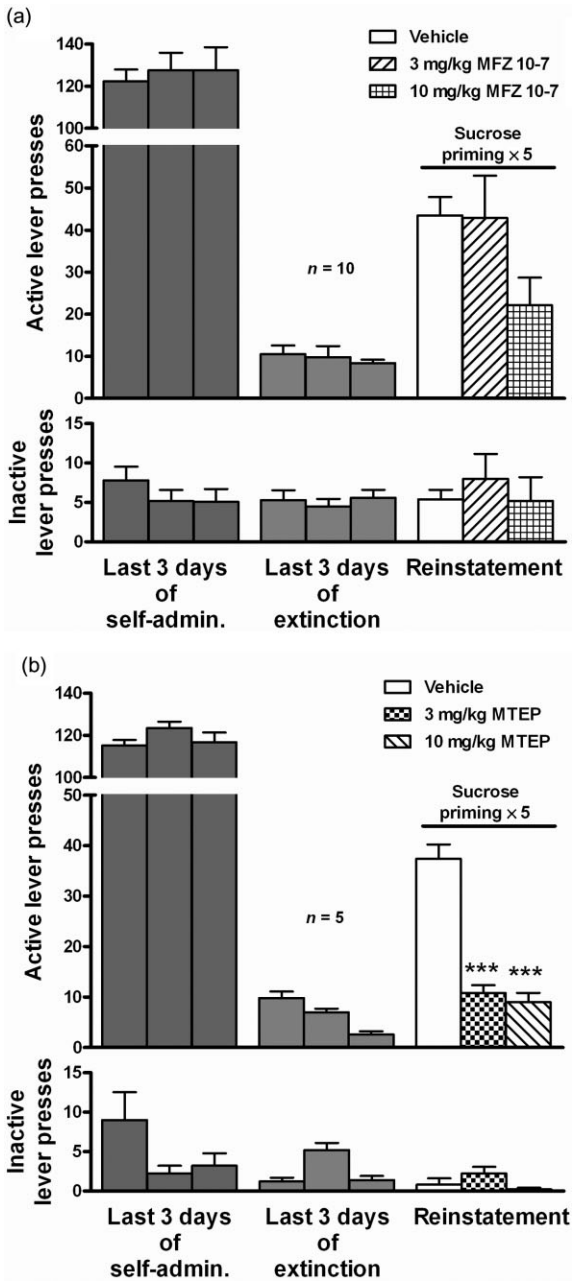

Figure 5 Effects of MFZ 10-7 and MTEP on sucrose-induced reinstatement of sucrose-seeking behavior. Pretreatment with MFZ 10-7 (3 or 10 mg/kg, i.p.) did not produce a statistically significant reduction (a), while pretreatment with MTEP (1 or 10 mg/kg, i.p.) dose-dependently inhibited sucrose-induced reinstatement of sucrose-seeking behavior (b). $^{***}P < 0.001$, compared with vehicle

3 mg/kg, $t = 0.42$, $P > 0.05$) also significantly inhibited inactive lever responding in forced drug-abstinent rats.

MFZ 10-7 and MTEP do not inhibit locomotor activity

Figure 7 shows locomotor behavior data from 20 minutes before to 180 minutes after MFZ 10-7 (Fig. 7a/b) or MTEP (Fig. 7c/d) administration, illustrating that neither MTEP nor MFZ 10-7 significantly altered locomotor behavior as assessed by either binned locomotor counts per unit time (10-minute intervals; Fig. 7a/c) or cumulative distance travelled (Fig. 7b/d). Two-way ANOVA for repeated measures over time for 3h following MFZ 10-7 administration revealed a significant main effect of time (Fig. 7a, $F_{17,357} = 14.44$, $P < 0.001$; Fig. 7b, $F_{17,357} = 66.02$, $P < 0.001$), a significant drug treatment effect of MFZ 10-7 only in the binned analysis (Fig. 7a, $F_{2,357} = 3.76$,

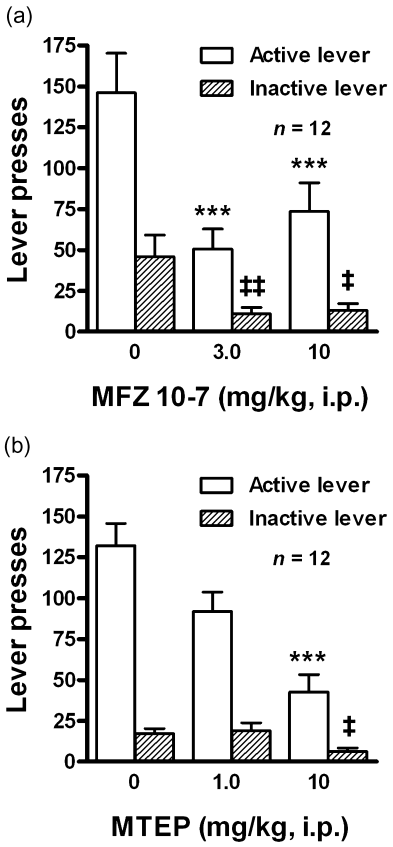

Figure 6 Effects of MFZ 10-7 and MTEP on contextual cue-induced cocaine-seeking behavior. Pretreatment with MFZ 10-7 (3 or 10 mg/kg, i.p.) (a) or MTEP (1 or 10 mg/kg, i.p.) (b) dose-dependently inhibited contextual cue-induced cocaine-seeking behavior (i.e. extinction responding). ***$P < 0.001$ compared with active lever after vehicle; ‡$P < 0.05$, ‡‡$P < 0.01$ compared with inactive lever after vehicle

$P < 0.05$; Fig. 7b, $F_{2,357} = 2.74$, $P > 0.05$), and a significant time × MFZ 10-7 interaction (Fig. 7a, $F_{34,357} = 1.99$, $P < 0.01$; Fig. 7b, $F_{34,357} = 2.57$, $P < 0.001$). However, individual group comparisons revealed no significant difference at any tested time-point between vehicle and either dose of MFZ 10-7. Two-way ANOVA for repeated measures over time for 3 hours following MTEP administration revealed a significant time main effect (Fig. 7c, $F_{17,357} = 14.67$, $P < 0.001$; Fig. 7d, $F_{17,357} = 30.21$, $P < 0.001$), but no significant MTEP treatment effect and no significant time × MTEP interaction. Individual group comparisons revealed a significant difference between vehicle and 1 mg/kg MTEP only in the first 10 minutes following drug administration in the binned analysis (Fig. 7c, $t = 4.49$, $P < 0.001$) and no significant differences in the cumulative analysis.

DISCUSSION

The present study compared the *in vitro* pharmacological profile of the novel mGluR5 NAM MFZ 10-7 with the prototypic NAMs MPEP, MTEP and fenobam. We found that MFZ 10-7 has a substantially higher *in vitro* potency and mGluR5 binding affinity compared with MPEP, MTEP and fenobam. MFZ 10-7 is also more than 1000-fold selective for mGluR5 over its only known off-targets, MAO-B and TXA2, making it the most mGluR5-selective NAM reported in *in vivo* models of drug abuse, to our knowledge.

We then compared the *in vivo* potency of MFZ 10-7 with MTEP in behavioral models of reward-taking and reward-seeking behaviors, using cocaine and sucrose as reinforcers. We found: (1) Systemic administration of either MFZ 10-7 or MTEP attenuated single-dose cocaine self-administration (0.5 mg/kg/infusion under FR2 reinforcement schedule), cocaine-induced reinstatement of drug-seeking behavior, and cocaine-associated contextual cue-induced cocaine-seeking behavior. (2) MFZ 10-7 appeared to be more potent than MTEP in shifting the cocaine dose–response curve downward. (3) Although MFZ 10-7 and MTEP both significantly lowered the rate of oral sucrose self-administration, they had no effect on the total number of sucrose deliveries per daily session. Additionally, we found that MFZ 10-7 was less effective than MTEP in attenuating sucrose-induced reinstatement of sucrose-seeking behavior, suggesting relatively higher selectivity of MFZ 10-7 in attenuation of cocaine-seeking over sucrose-seeking behavior. (4) The effects of MFZ 10-7 and MTEP on cocaine- and sucrose-seeking behavior are unlikely the result for sedation as there was no effect on locomotor behavior. Taken together, the present study not only provides additional evidence supporting an important role for mGluR5 in cocaine reward and addiction but also introduces a new tool for further *in vivo* and mechanistic investigations into the pharmacotherapeutic potential of mGluR5 NAMs.

Drug addiction is characterized by compulsive drug-taking and drug-seeking behavior following abstinence (Gawin & Kleber 1986; Satel, Southwick & Gawin 1991). Intravenous drug self-administration and reinstatement of drug-seeking behavior are commonly used animal models to study a drug's reinforcing effects and relapse to drug-seeking behavior (O'Brien & Gardner 2005). In addition, the animal model of incubation of craving is used to study contextual cue-induced drug-seeking behavior in which animals are forcibly withdrawn from cocaine self-administration without behavioral extinction of the previously reinforced operant responding for drug reward (Lu *et al.* 2004).

In the present study, we used multiple animal models of drug-taking and drug-seeking behavior to evaluate the pharmacological action of MFZ 10-7 in rats. We found that MFZ 10-7 and MTEP significantly inhibited cocaine-taking and cocaine-seeking behavior in rats, with MFZ 10-7 more potent than MTEP in attenuating

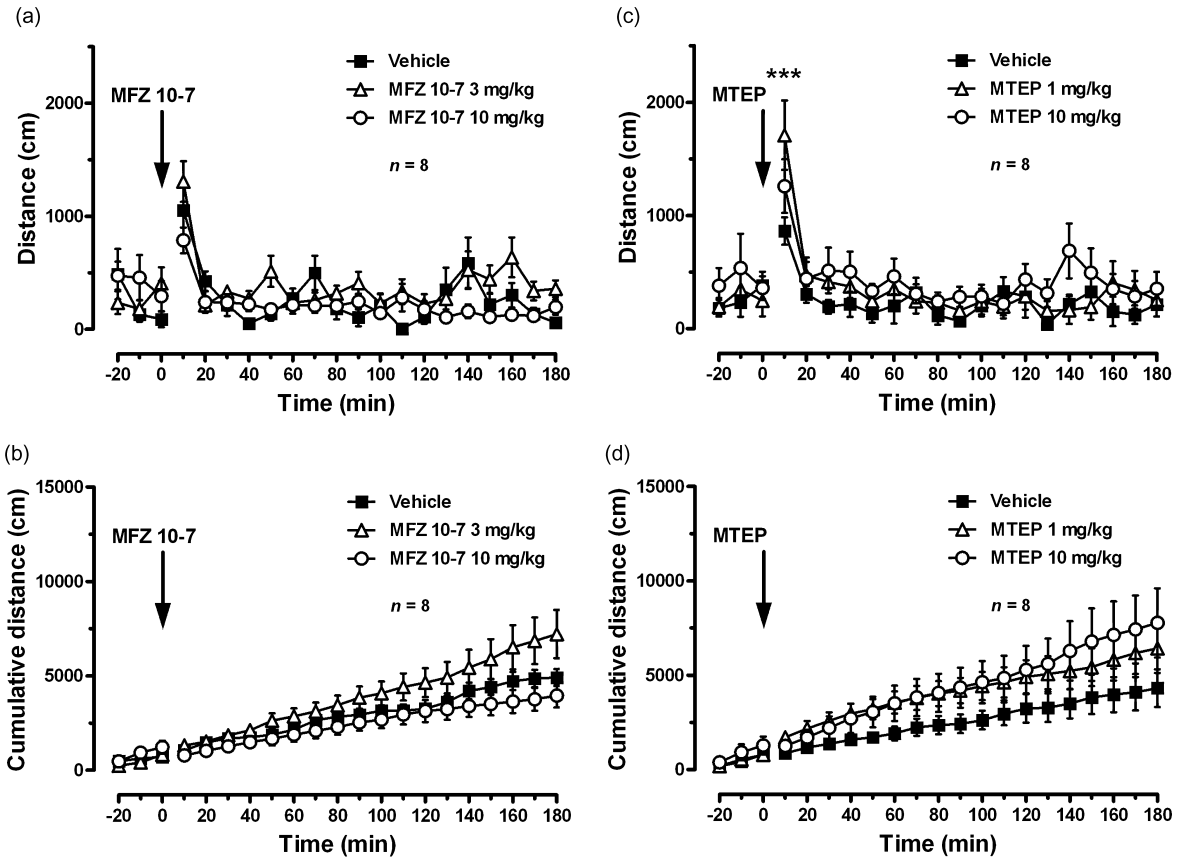

Figure 7 Effects of MFZ 10-7 and MTEP on locomotor activity. Following 1 hour of habituation, one dose of MFZ 10-7 (0, 3, or 10 mg/kg, i.p.) (a/b) or MTEP (0, 1, or 10 mg/kg, i.p.) (c/d) was given. 1 mg/kg MTEP produced a significant increase in locomotion only during the first 10 minutes immediately following drug administration (c). Neither treatment at any dose significantly altered overall locomotion compared with vehicle (b, d) locomotion. ***$P < 0.01$ compared with vehicle

multiple-dose cocaine self-administration. Attenuation of cocaine self-administration and a downward shift in the cocaine dose–response curve are generally interpreted as a reduction in cocaine's rewarding effects. In addition, we also found that both MFZ 10-7 and MTEP inhibited cocaine priming-induced reinstatement of drug-seeking behavior and contextual cue-induced incubation of cocaine seeking, suggesting that mGluR5 NAMs may be useful in preventing relapse to drug use after abstinence.

Because the same doses of the drugs failed to significantly alter basal levels of locomotion, the reduction in cocaine-taking and cocaine-seeking behaviors observed in this study are unlikely due to locomotor impairment following MTEP or MFZ 10-7 administration. Overall, our findings are congruent with prior reports that the mGluR5 NAMs MPEP or MTEP significantly inhibit cocaine-taking and cocaine-seeking behavior in both rodents and non-human primates. They are also congruent with our recent report that oral administration of fenobam sulfate significantly inhibits cocaine self-administration, cocaine-induced reinstatement of cocaine-seeking behavior and cocaine-associated cue-induced cocaine-seeking behavior (Keck *et al.* 2013). We note that MFZ 10-7 displayed much higher (30-fold) potency for mGluR5 than MTEP in the *in vitro* functional assays, but we did not see such a difference in the present *in vivo* behavioral assays. This may be related to relatively poor sensitivity of the presently used behavioral tests to detect a minor-to-moderate difference in the pharmacological actions of these drugs, and/or different pharmacokinetics, metabolism and/or blood-brain barrier penetration after systemic administration. Interestingly, MFZ 10-7 was up to 30-fold more potent than MPEP or MTEP in mouse models of anxiety (Keck *et al.* 2012). It is possible that the anxiolytic, antidepressive, and anti-aggression effects produced by mGluR5 NAMs (Varty *et al.* 2005; Navarro *et al.* 2006; Koros *et al.* 2007; Krystal *et al.* 2010) could contribute to their therapeutic benefit.

The neural mechanisms underlying the antagonism of cocaine reward by mGluR5 NAMs are not fully understood. Multiple studies suggest that mGluR5 blockade decreases brain reward functioning as measured by the intracranial self-stimulation reward paradigm (Kenny

et al. 2005; Cleva *et al.* 2012), suggesting that attenuated cocaine self-administration results from a diminished rewarding response to cocaine. For cocaine- or cue-induced reinstatement of cocaine-seeking behavior, previous studies suggest that re-exposure to cocaine-associated cues or cocaine-priming injections evoke glutamate release within the nucleus accumbens (NAc) and ventral tegmental area (VTA) (McFarland, Lapish & Kalivas 2003; Wang *et al.* 2005, 2007; Xi *et al.* 2006; You *et al.* 2007; Miguéns *et al.* 2008). Glutamate neurotransmission in these regions has been established to play an important role in relapse to drug-seeking behaviors (Knackstedt & Kalivas 2009; Kalivas & Volkow 2011). mGluR5 receptors are located primarily on somatodendritic domains of neurons within corticolimbic regions including cerebral cortex, olfactory tubercle, striatum, NAc and lateral septum (Ferraguti & Shigemoto 2006; Mitrano & Smith 2007; Mitrano, Arnold & Smith 2008). Thus, it is plausible that mGluR5 NAMs reduce postsynaptic mGluR5 signaling in regions such as the VTA and NAc, thereby attenuating cocaine- or cue-induced increases in glutamate transmission, thus reducing reinstatement of drug-seeking behavior.

In contrast to inhibition of cocaine self-administration, both MFZ 10-7 and MTEP failed to alter total sucrose intake, but reduced the rate of sucrose self-administration. The significance of these changes in sucrose self-administration is unclear. We note that rats displayed much higher rates of active lever responses for sucrose (> 100 deliveries per hour) than for cocaine (10–20 infusions per hour for 0.5 mg/kg cocaine), and that the majority of rats completed the maximally allowed 100 sucrose deliveries within 30–40 minutes. Thus, it is quite expected that a reduction in rate of sucrose self-administration failed to cause a reduction in total sucrose deliveries during the 90-minute test duration. The simplest interpretation of the present findings is that acutely administered MFZ 10-7 or MTEP attenuate sucrose's rewarding effects, lowering motivation for sucrose-taking and sucrose-seeking behavior. This is consistent with previous reports that MPEP, MTEP and fenobam decrease food or sucrose self-administration and reinstatement behavior in rats and non-human primates (Paterson & Markou 2005; Platt, Rowlett & Spealman 2008; Keck *et al.* 2013; Watterson *et al.* 2013; but see Martin-Fardon *et al.* 2009) and reports that mGluR5 signaling regulates general appetitive behaviors (Bradbury *et al.* 2005). We note, however, that while MTEP also inhibited sucrose-triggered reinstatement of sucrose-seeking behavior, MFZ 10-7 did not significantly alter this behavior. At the doses tested, there appears to be a therapeutic window within which MFZ 10-7 significantly alters cocaine-seeking behavior but not sucrose-seeking behavior.

As stated earlier, neither MPEP nor MTEP have translational potential for use in humans because of significant off-target actions and short half-lives. We have herein included *in vitro* comparisons with fenobam, a selective mGluR5 NAM (Porter *et al.* 2005; Montana *et al.* 2009) that has been previously tested in phase II clinical trials as a non-benzodiazepine anxiolytic (Friedmann *et al.* 1980; Pecknold *et al.* 1982) and as a treatment for fragile X syndrome (Berry-Kravis *et al.* 2009). Fenobam has been reported to attenuate cocaine- (Keck *et al.* 2013) and methamphetamine-seeking behavior in rats (Watterson *et al.* 2013).

Although MTEP and fenobam have previously been tested in multiple radioligand and enzyme assays to evaluate their selectivities for mGluR5 (Cosford *et al.* 2003; Porter *et al.* 2005), there is no report of these compounds binding to the two off-target sites—TXA2 and MAO-B. The very low relative binding affinity that MFZ 10-7 had for these two sites in comparison with mGluR5 casts serious doubt that these off-targets mediate the present findings. Furthermore, neither fenobam nor MTEP showed high-affinity binding to TXA2 or MAO-B. Hence, the only known high-affinity target common to the three structurally diverse ligands MFZ 10-7, MTEP and fenobam is mGluR5, and it is highly likely that negative allosteric modulation of this site drives the behavioral effects observed in the present study and in other preclinical studies utilizing mGluR5 NAMs to attenuate behaviors associated with drug abuse.

Finally, we note that the MTEP solutions used in the present study had a pH ranging between 3 and 4, which might cause abdominal pain and distress after i.p. injection, thereby potentially contributing to the reduction in cocaine-taking and cocaine-seeking behavior observed in the present study. However, we did not observe any signs of pain and/or distress in locomotion or body movement up to 3–4 hours after injection. This could be related to the small injection volume (1 ml/kg) or the analgesic effects of MTEP after systemic administration (Zhu *et al.* 2004; Varty *et al.* 2005).

In summary, MFZ 10-7 is a novel selective and highly potent mGluR5 NAM, efficacious in attenuating cocaine-taking and cocaine-seeking behaviors as assessed in several animal models of cocaine addiction. These preclinical data suggest that MFZ 10-7 is a new tool that can be used to determine whether mGluR5 is a viable target for medication development and with which mechanisms underlying mGluR5's role in addiction may be further elucidated.

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Disclosure/Conflict of Interest

All authors hereby declare no competing financial interests.

Authors' Contributions

TMK, ELG, Z-XX and AHN were responsible for the study concept and design. TMK, G-HB, X-FW, H-JY, H-YZ and RS contributed to the acquisition of animal data. M-FZ synthesized MFZ 10-7. TMK and Z-XX analyzed and interpreted the data, and drafted the manuscript. ELG and AHN provided critical revisions of the manuscript for important intellectual content. All authors critically reviewed the manuscript content and approved the final version for publication.

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