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Title: Methocinnamox (MCAM) produces long-lasting antagonism of the behavioral effects of μ opioid receptor agonists but not prolonged precipitated withdrawal in rats

Authors: Lisa R. Gerak, Vanessa Minervini, Elizabeth Latham, Saba Ghodrati, Katherine V. Lillis, Jessica Wooden, Alex Disney, Stephen M. Husbands, Charles P. France

Affiliations: Department of Pharmacology, University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA (LRG, VM, EL, SG, KVL, JW, CPF); Department of Psychiatry, University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA (CPF); Addiction Research, Treatment & Training Center of Excellence, University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA (LRG, VM, EL, SG, KVL, JW, CPF); Department of Pharmacy and Pharmacology, University of Bath, Bath, United Kingdom (SMH, AD)

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Running title: Antagonism by MCAM in rats

Corresponding Author: Charles P France, Department of Pharmacology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, Mail Code 7764, San Antonio, TX, 78229, USA. E-mail: france@uthscsa.edu; Tel: 210-567-6969; Fax: 210-567-0104

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Abstract

A novel μ opioid receptor antagonist, methocinnamox (MCAM), attenuates some abuse-related and toxic effects of opioids. This study further characterized the pharmacology of MCAM in separate groups of rats using procedures to examine antinociception, gastrointestinal motility, and withdrawal in morphine-dependent rats. Antinociceptive effects of opioid receptor agonists were measured before and after MCAM (1-10 mg/kg) using warm water tail withdrawal and sensitivity to mechanical stimulation in inflamed (CFA) paws. Before MCAM, morphine, fentanyl, and the κ opioid receptor agonist spiradoline dose-dependently increased tail-withdrawal latency from 50ºC water; MCAM attenuated the antinociceptive effects of morphine and fentanyl, but not spiradoline. Morphine increased sensitivity to mechanical stimulation and decreased gastrointestinal motility, and MCAM blocked both effects. These antagonist effects of 10 mg/kg MCAM were persistent, lasting for 2 weeks or longer. Withdrawal emerged after discontinuation of morphine treatment or administration of 10 mg/kg MCAM or 17.8 mg/kg naloxone; other than the day of antagonist administration when withdrawal signs were greater in rats that received antagonist, as compared with rats that received vehicle, there was no difference among groups in directly observable withdrawal signs or decreased body weight. These results confirm that MCAM is a selective μ opioid receptor antagonist with an exceptionally long duration of action, likely due to pseudoirreversible binding. Despite its sustained antagonist effects, the duration of withdrawal precipitated by MCAM is not different from that precipitated by naloxone, suggesting that the long duration of antagonism provided by MCAM could be particularly effective for treating opioid abuse and overdose.

Significance Statement

The opioid receptor antagonist MCAM attenuates some abuse-related and toxic effects of opioids. This study demonstrates that MCAM selectively antagonizes multiple effects mediated by μ opioid receptor agonists for 2 weeks or longer, and like naloxone, MCAM precipitates withdrawal in morphine-dependent rats. Despite this persistent antagonism, withdrawal signs precipitated by MCAM are not significantly different from signs precipitated by naloxone or occurring after discontinuation of morphine, suggesting that using MCAM for opioid abuse or overdose would not produce sustained withdrawal.

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Introduction

Currently, three pharmacological options are available for treating opioid use disorder (OUD). Two medications, methadone and buprenorphine, are μ opioid receptor agonists that mimic some effects of abused opioids. The third medication is the opioid receptor antagonist naltrexone, which blocks the effects of abused opioids. While effective in some patients, they each have limitations. For example, methadone and buprenorphine are diverted and abused, and both have adverse effects, including respiratory depression, which can be exacerbated by alcohol and benzodiazepines (Jones et al. 2012, 2014; Jones and McAninch 2015; Kintz 2001; Kriikku et al. 2018; Pelissier-Alicot et al. 2010; Pirnay et al. 2004). Although naltrexone is not abused and is safer than methadone and buprenorphine, induction of treatment must be done carefully to minimize the emergence of withdrawal. Poor compliance and a relatively short duration of action limit the usefulness of naltrexone for treating OUD, although recently developed extended release formulations might improve outcomes. However, because of its competitive, reversible binding to μ opioid receptors, the antagonist effects of naltrexone can be surmounted by large dose of agonists, such that reinforcing and respiratory-depressant effects of opioids can be achieved by taking more drug, thereby limiting protection by naltrexone. Thus, despite the effectiveness of these medications, the risk of relapse and overdose remains high, indicating that current options are not adequately addressing the opioid crisis (Volkow et al. 2019).

Naloxone is the only medication available for opioid overdose. Like naltrexone, it is a competitive, reversible antagonist at opioid receptors, it precipitates withdrawal in opioid-dependent individuals, and its antagonism can be surmounted by taking more drug.

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Moreover, it duration of action (≤1 hr) is shorter than many abused opioids, such that opioid-induced toxic effects can reemerge after rescue with naloxone. A medication that reverses and provides long-lasting protection from opioid overdose could be a significant improvement over naloxone.

Methocinnamox (MCAM) is an opioid receptor antagonist that might retain the positive features of naltrexone and naloxone (e.g., safety and no abuse liability) while reducing vulnerabilities associated with surmountability. While each antagonist has affinity for all three types of opioid receptors (μ, κ and δ) and most of the interactions between antagonist and receptors are reversible, MCAM differs from naltrexone and naloxone in that its interactions with μ opioid receptors are functionally irreversible (i.e. pseudoirreversible; Broadbear et al. 2000), suggesting that MCAM would selectively and insurmountably block μ opioid receptors. In contrast, the surmountable antagonism produced by naltrexone and naloxone is not selective. MCAM does not appear to have efficacy at any opioid receptor and, when given alone, does not produce antinociceptive effects or alter respiration, although it attenuates the reinforcing, antinociceptive, and respiratory-depressant effects of μ opioid receptor agonists in mice, rats, and nonhuman primates (Broadbear et al. 2000; Peckham et al. 2005; Gerak et al. 2019; Maguire et al. 2019). In the presence of MCAM, large doses of morphine can produce antinociceptive effects, although these effects might be mediated by other receptors (e.g. κ opioid; Peckham et al. 2005; Takemori and Portoghese 1987) rather than surmountability at μ opioid receptors. Moreover, these antagonist effects are persistent, lasting for a week or more in monkeys (Gerak et al. 2019; Maguire et al. 2019). In nonhuman primates, doses of MCAM larger than those that

attenuate the abuse-related and toxic effects of opioids do not attenuate self-administration of nonopioids (e.g., cocaine), decrease responding for food, or alter heart rate, blood pressure, body temperature or activity (Maguire et al. 2019), suggesting that MCAM is safe and not likely to produce unexpected adverse effects.

While MCAM could safely provide long-lasting protection against the abuse-related and toxic effects of opioids, little is known about the ability of MCAM to block other effects mediated by μ opioid receptors. Moreover, the impact of sustained, insurmountable blockade of μ opioid receptors is also unknown. One possible concern regarding the therapeutic use of MCAM for OUD would be how to provide pain relief because MCAM would block the analgesic effects of μ opioid receptor agonists. In addition, if used to rescue an opioid-dependent individual from overdose, MCAM would precipitate withdrawal, and precipitation of withdrawal by a pseudoirreversible antagonist like MCAM has not been studied extensively. This study addressed these potential concerns and examined the generality of sustained antagonism by MCAM to another class of μ opioid receptor agonists that are currently predominating the opioid crisis (fentanyl) using two procedures to measure antinociception and other procedures to examine gastrointestinal motility and changes in body temperature as well as precipitation of withdrawal in morphine-dependent rats.

Materials and methods

Subjects. Male Sprague-Dawley rats (Envigo Inc., Chicago, IL, USA) were maintained under a 14 hr light/10 hr dark cycle; experiments were conducted during the light cycle. Rats were housed individually in colony rooms that were maintained at a constant temperature and humidity. While in their home cage, they had continuous access to water and, with the

exception of one study (see below), unlimited access to food (Envigo Teklad, Madison, WI). The eight rats in which gastrointestinal transit was measured did not have unlimited access to food; instead, they were maintained at 375 ± 5 g body weight with daily food rations of 15 g, except on days preceding tests when the ration was decreased to 5 g. All animals used in these studies were maintained in accordance with the Institutional Animal Care and Use Committee at The University of Texas Health Science Center at San Antonio, and the guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council [Department of Health, Education and Welfare, publication No. (NIH) 85-23, revised 2011].

Apparatus. Antinociceptive effects were determined using two assays. For the warm water tail withdrawal procedure, three water baths (EW-14576-00, Cole-Parmer, Vernon Hills, IL) were maintained at constant temperatures (40, 50, or 55 °C) throughout the experiment, and latency for rats to remove their tails from water maintained at each temperature was measured using a stopwatch. For the paw inflammation procedure, rats were placed on a mesh stand (Model 410, IITC Inc. Life Science) in plastic enclosures (Model 435, IITC Inc. Life Science) for testing, and an electronic Von Frey probe (Model 2396, IITC Inc. Life Science) with a rigid tip (Model 2391, IITC Inc. Life Science) was used to measure paw withdrawal threshold in grams of force. Paw thickness was measured with a digital caliper (Fowler, Model 54-101-175), and body temperature was measured with a rectal thermometer (PhysiTemp Instruments, Clifton, NJ) before and after sessions.

Procedures.

Warm water tail withdrawal. Antinociceptive effects were assessed by measuring the

latency for rats to remove their tails from water maintained at 40, 50, or 55 °C. Experiments began with determination of baseline latencies for each of the three temperatures by gently restraining the rats and placing the lower portions of their tails into water baths. The remainder of the session was divided into 30-min cycles, each of which began with an injection and ended with redetermination of tail-withdrawal latencies from each of the three temperatures. Latencies were measured starting 28 min after each injection with the order of presentation of the different temperatures varying nonsystematically across cycles and across rats. The first cycle began immediately after obtaining baseline latencies with an injection of vehicle. During some sessions, only vehicle or sham injections were administered for up to 6 cycles. For other sessions, dose-effect curves were determined. An ineffective dose of drug was given at the beginning of the second cycle with the cumulative dose increasing in ½ log unit increments every cycle.

Three separate groups of eight rats participated in these studies. In the first group, the magnitude and duration of antagonism by MCAM was examined by determining morphine dose-effect curves in the absence of MCAM and at various times after administration of MCAM (1-10 mg/kg). Doses of MCAM were studied in ascending order and separated by 21 days. For each of these morphine dose-effect curves, dosing continued until tail-withdrawal latency from water maintained at 50°C was at least 13 sec, which insured that the latency reached 80% of the maximum possible effect. On days when MCAM was administered, rats received no other injections; tail-withdrawal latencies were measured 28 and 58 min after MCAM. Morphine dose-effect curves were redetermined one day later (i.e. 24 hr after MCAM) and every 4 days thereafter until the potency of morphine in the presence of MCAM

was not different from its potency in the absence of MCAM (see data analyses for details). When rats received 10 mg/kg MCAM, the morphine dose-effect curve remained shifted 21 days later; after determination of that dose-effect curve, the interval between tests with morphine increased to 8 days through day 53 and then to 16 days for one final test 69 days after MCAM administration. After day 21, control tail-withdrawal latencies were measured 4 days before each morphine test with rats receiving only vehicle injections during those intervening sessions.

In a second group of eight rats, 10 mg/kg MCAM was administered before assessing the antinociceptive effects of fentanyl. First, a dose range of fentanyl was identified in the absence of MCAM such that the smallest dose was ineffective and the largest dose increased tail-withdrawal latency from water maintained at 50°C to at least 13 sec. Thereafter, the dose range for each fentanyl test remained the same regardless of latencies obtained; sessions comprised four cycles, with rats receiving saline at the beginning of the first cycle and cumulative doses of 0.01, 0.032 and 0.1 mg/kg fentanyl at the beginning of the second, third and fourth cycles, respectively, and ended after the fourth cycle. This fixed dose range was used to measure the duration of antagonism while limiting the amount of agonist administered in order to avoid other effects that might develop after repeated administration of large doses (e.g., tolerance). On days when MCAM was administered, rats received no other injections and tail-withdrawal latencies were not measured. Fentanyl dose-effect curves were obtained one and five days later and every 8 days thereafter until the tail-withdrawal latency obtained with a cumulative dose of 0.1 mg/kg fentanyl in the presence of MCAM was not different from the same dose of fentanyl obtained in the absence of MCAM.

After day 5, control tail-withdrawal latencies were obtained 4 days before each fentanyl test with rats receiving only vehicle injections during those intervening sessions. For reasons unrelated to the study, one rat died between fentanyl dose-effect curves obtained 29 and 37 days after MCAM.

In a third group of eight rats, the antinociceptive effects of spiradoline and later of morphine were determined at various times after 10 mg/kg MCAM. First, dose-effect curves for spiradoline and morphine were obtained in the absence of MCAM up to the dose that increased tail-withdrawal latency from water maintained at 50°C to at least 13 sec. On the day that MCAM was administered, rats received no other injections and tail-withdrawal latencies were measured 28 and 58 min after MCAM. One day later (i.e. 24 hr after MCAM), spiradoline dose-effect curves were obtained during a four-cycle session beginning with saline and then using the dose-range of spiradoline that was identified before MCAM administration (1, 3.2, and 10 mg/kg). Five days after MCAM administration and four days after redetermination of the spiradoline dose-effect curve, the antinociceptive effects of morphine were assessed in a similar four-cycle session; morphine was studied in this manner every 8 days until the tail-withdrawal latency obtained with a cumulative dose of 17.8 mg/kg morphine in the presence of MCAM was not different from latency obtained with the same dose of morphine obtained in the absence of MCAM. After day 5, control tail-withdrawal latencies were obtained 4 days before each morphine test with rats receiving only vehicle injections during those intervening sessions.

Paw inflammation. Sixteen rats that weighed 250-270 g at the start of the experiment were randomly assigned to two groups with eight rats in each group. Baseline values for

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body temperature, paw thickness, and paw withdrawal threshold were determined in all rats before receiving hindpaw injections. Rats were briefly anesthetized with 2-4% isoflurane, and the plantar surface of the footpad was cleaned with betadine and 70% ethanol. Using a 27-G, ½-in needle, 0.1 ml of the Complete Freund's Adjuvant (CFA) emulsion or saline was injected s.c. to the footpad. The paw side (left or right) associated with CFA injection was counterbalanced across rats, and saline was injected into the other paw. One day after hindpaw injections, paw thickness was redetermined and then one group of rats received 10 mg/kg MCAM and the other group received vehicle. Paw withdrawal threshold was tested on days 1, 3, 15, 25, and 30 after MCAM or vehicle. In each of six cycles, an injection was given, and 15 min later, the force that resulted in paw withdrawal was determined three times for each paw.

One day after MCAM or vehicle, four rats from each group were tested with cumulative doses of morphine (1.78 – 17.8 mg/kg) and the other four rats from each group were tested with cumulative doses of meloxicam (0.56 – 5.6 mg/kg) to test the selectivity of MCAM. Three days after MCAM or vehicle, the rats that had initially received morphine were tested with meloxicam, and the rats that had initially received meloxicam were tested with morphine. Fifteen and 30 days after MCAM or vehicle all rats were tested with morphine, and 25 days after MCAM or vehicle, all rats were tested with saline given in all cycles.

Gastrointestinal transit. On test days, standard rodent chow (Enivgo Teklad; 75 g) was soaked in warm tap water (120 ml) until homogenized (approximately 90 min). Tests were conducted in eight rats in the home cage with bedding removed immediately prior to the session and began with 2 hr access to the wet chow. After 2 hr, any remaining chow was

removed from the cage and weighed. The weight of the remaining chow was subtracted from the initial weight to estimate consumption. Rats then received either saline or 10 mg/kg morphine, and fecal boli were collected, counted, and weighed hourly for the next 6 hr. The effects of saline and morphine on fecal output were determined twice with tests separated by at least 1 week. Thereafter, MCAM (10 mg/kg) was administered 5 min before morphine (10 mg/kg). Beginning five days after MCAM administration, morphine was tested every four days (i.e., days 5, 9, 13, 17, 21, 25, and 29) until results of three consecutive tests were not significantly different from results obtained before MCAM administration.

Dependence/withdrawal. Three groups of five rats received morphine twice daily at 0700 and 1700 hr. The 19-day treatment period began with a dose of 3.2 mg/kg/injection, and the dose increased every three days in ¼ log unit increments up to 56 mg/kg/injection twice daily. On the third day of treatment with the largest dose of morphine, rats received an injection of vehicle 100 min after the morning injection of morphine, withdrawal signs were monitored, and rats receiving 56 mg/kg morphine at 1700 hr. On the next day, rats received 56 mg/kg morphine at 0700 hr; a second injection was administered 100 min later with rats randomly assigned to receive vehicle, 10 mg/kg MCAM, or 17.8 mg/kg naloxone. Beginning at 1700 hr on that day and continuing for the next 5 days, rats received saline instead of morphine at 0700 and 1700 hr. Withdrawal signs were scored 1, 2, 3 and 5 days after the last dose of morphine.

Two individuals blind to the injection (i.e. vehicle, naloxone, or MCAM) given on the last day of morphine treatment monitored directly observable withdrawal signs. During observation periods, rats were in their home cage, which was moved into a different room

before observations began; food, but not water, was available during sessions. Three discrete observation periods were conducted for each rat beginning 30, 60 and 90 min after the injection of vehicle or antagonist including measurement of body weight and scoring of vocalization during handling. Thereafter, the remaining 13 signs (ptosis, teeth chattering, tongue protrusion, salivation, lacrimation, chromodacryorrhea, jumping, abdominal writhing, wet dog shake, rearing, paw biting, paw tremor, and diarrhea) were scored as present or absent during 4 15-sec intervals that were separated by 15 sec. As long as a sign was observed during at least one interval, that sign was recorded as present for the observation period. Thus, the maximum score for each observation period was 14.

Drugs. Morphine sulfate and fentanyl hydrochloride (Drug Supply Program, National Institute on Drug Abuse, Rockville, MD, USA) as well as spiradoline mesylate (Upjohn, Kalamazoo, MI, USA) were dissolved in normal saline and administered i.p. in a volume of 1 ml/kg body weight except when the largest dose of MCAM (10 mg/kg) was given before determination of morphine dose-effect curves up to doses that produced a maximum possible effect. Because very large doses were needed, morphine was dissolved in 10% β-cyclodextrin vehicle so that a larger concentration could be obtained, thereby limiting injection volumes at the largest doses to a maximum of 1.2 ml. Meloxicam sodium salt hydrate was purchased from Sigma-Aldrich Corp. (St. Louis, MO), dissolved in a vehicle containing equal volumes of polyethylene glycol 400 and saline, and administered i.p. MCAM hydrochloride, which was synthesized by two of the authors (AD, SMH) according to a previously established procedure (Broadbear et al. 2000), and naloxone hydrochloride (Drug Supply Program, National Institute on Drug Abuse, Rockville, MD, USA) were

dissolved in a vehicle of 10% w/v β-cyclodextrin in saline and administered s.c. Doses are expressed in the form listed above in mg/kg body weight. CFA containing 1 mg of heat-killed and dried Mycobacterium tuberculosis (strain H37Ra, ATCC 25177) per ml was purchased from Sigma Aldrich (product number F5881), and then diluted with saline (1:1 ratio) to a concentration of 0.5 mg/ml mycobacterium emulsion.

Data analyses. Graphs were constructed and analyses were conducted with GraphPad Prism version 7.03 for Windows (GraphPad Software, La Jolla, CA, USA). Significance was set at $P<0.05$.

Warm water tail withdrawal. Tail-withdrawal latencies were converted to a percentage of the maximum possible effect (15 sec) according to the following expression: [(test latency - control latency)/(15 sec - control latency)] x 100% and then averaged across seven or eight rats; mean latencies, expressed as a percentage of the maximum possible effect (± 1 SEM), were plotted as a function of dose. Whenever possible, ED_{50} values were determined by first fitting straight lines to dose-effect curves for individual rats using the largest dose for which tail-withdrawal latency remained below 25%, the smallest dose for which tail-withdrawal latency exceeded 75%, and all doses in between. ED_{50} values were then estimated from those straight lines using linear regression when three or more data points were available or by interpolation when only two points were available and plotted as a function of time since MCAM administration. Potency ratios were calculated for each rat by dividing the ED_{50} values for morphine or spiradoline obtained after MCAM administration by the ED_{50} values obtained in the absence of MCAM. A significant change in the potency of morphine or spiradoline was detected when the 95% confidence intervals of the potency ratios averaged

among rats did not include 1. For tests in which ED_{50} values could not be obtained because a limited dose range was studied and tail-withdrawal latencies did not exceed 50% of the maximum possible latency (i.e. 15 sec) from 50°C water, statistical significance was determined by comparing the percentage of the maximum possible latency obtained following administration of the largest cumulative dose of morphine or fentanyl using one-factor (time since MCAM administration), Geisser-Greenhouse corrected repeated-measures ANOVA followed by Dunnett's multiple comparisons test.

Paw inflammation. Paw thickness was similar before and after sessions and across the two groups (i.e. rats that received MCAM or vehicle 1 day after hindpaw injections). Consequently, measurements taken before sessions were averaged across groups (95% confidence intervals). The paw that received an injection of CFA was considered significantly inflamed if the thickness of that paw was outside of the 95% confidence interval of the thickness of the vehicle-injected paw. Similarly, paw withdrawal threshold was obtained at the beginning of each session 15 min after a saline injection and was considered significantly lower in the CFA paw when the mean withdrawal threshold was outside of the 95% confidence interval of the value obtained in the vehicle-injected paw. The effects of morphine on antinociception were not significantly different on day 3 compared with day 1 in either MCAM-treated rats or vehicle-treated rats (not shown); therefore the data are collapsed across those two tests, which allowed for a two-factor repeated measures ANOVA to be conducted with treatment (MCAM or vehicle) and time (days since MCAM or vehicle) as factors. Change in body temperature was determined by subtracting the body temperature before the session from the body temperature at the end of the session. The

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effects of morphine on body temperature were not significantly different on day 3 compared with day 1 in either MCAM-treated rats or vehicle-treated rats; therefore the data are collapsed across those two tests, which allowed for a two-factor repeated measures ANOVA to be conducted with treatment (MCAM or vehicle) and time (days since MCAM or vehicle) as factors.

Gastrointestinal transit. Fecal output (fecal boli/6 hr) and the estimated amount of wet chow consumed (g/2 hr) were plotted as a function of time since MCAM administration. Statistical significance was determined using a one-factor (time since MCAM administration) repeated measures ANOVA followed by Bonferroni's post-hoc test.

Dependence/withdrawal. Directly observable signs were combined to give a composite score for total withdrawal signs for each rat on each day. This score was determined by counting the number of signs recorded as present by at least one observer during each observation period and then adding across observation periods to obtain a withdrawal score for that day; the maximum possible score for one day was 42 ([3 observation periods x 14 signs]. Scores were determined for individual rats and then averaged (± 1 SEM) among rats within a treatment condition. Body weight was measured at the beginning of each observation period; the value obtained before the third observation period (i.e. 90 min after the injection of vehicle or antagonist on the last day of morphine treatment) is plotted as a function of time. Statistical significance was determined by comparing the number of withdrawal signs observed across all intervals in all three observations periods using two-factor repeated-measures ANOVA; one factor was the injection given before the first observation period on the last day of morphine treatment (MCAM, naloxone, or vehicle) and

the other factor was days since that injection. Dunnett's multiple comparisons test was used for *post hoc* analyses.

Results

Warm water tail withdrawal. Tail-withdrawal latencies (mean ± 1 SEM) obtained in the absence of drug were 15, 2.63 ± 0.18, and 1 sec from water maintained at 40, 50, and 55°C, respectively. When given alone, morphine, fentanyl and spiradoline dose-dependently increased latencies to >90% of control latencies at cumulative doses of 17.8, 0.1, and 10 mg/kg, respectively (filled circles, figures 1, 3 and 4). When given alone, MCAM (1-10 mg/kg) did not increase tail-withdrawal latencies 28 or 58 min after administration (data not shown).

In one group of rats, morphine dose-effect curves were determined at various times after administration of MCAM (1-10 mg/kg). When given one day earlier, MCAM dose-dependently attenuated the effects of morphine on tail withdrawal latency from water maintained at 50°C, with each larger dose of MCAM shifting the morphine dose-effect curve further rightward (compare filled squares with filled circles, all panels, figure 1). For example, the dose of morphine needed to increase tail-withdrawal latency to >90% was 56 mg/kg after 1 mg/kg MCAM (filled squared, top panel, figure 1) and 560 mg/kg after 10 mg/kg MCAM (filled squares, bottom panel, figure 1). The magnitude of antagonism diminished over time since MCAM administration. The morphine dose-effect curve recovered and was not different from the control curve within 5 and 17 days of administration of 1 (triangles, top panel, figure 1) and 3.2 mg/kg MCAM (open squares, middle panel, figure 1), respectively; in

contrast, the morphine dose-effect curve remained shifted to the right of the control curve 69 days after administration of 10 mg/kg MCAM (filled stars, bottom panel, figure 1). ED_{50} values for morphine also demonstrate that the magnitude and duration of antagonism increased with MCAM dose and decreased over time since MCAM administration (figure 2). Under these conditions, the potency of morphine returned to control after 1 and 3.2 mg/kg MCAM and remained significantly changed even 69 days after 10 mg/kg MCAM (table 1). For each of these 21 morphine dose-effect curves, dosing stopped when tail withdrawal latency from water maintained at 50°C was at least 13 sec, and at the largest dose of morphine tested under each of these conditions, average tail withdrawal latency from water maintained at 55° ranged from 5.4 ± 1.8 to 30.5 ± 5.3% of the maximum possible effect.

In a second group of rats the ability of 10 mg/kg MCAM to attenuate the antinociceptive effects of another μ opioid receptor agonist, fentanyl, was assessed. Because sensitivity to morphine did not recover fully in MCAM-treated rats that were tested with large doses of morphine (see above), a limited dose range was used to assess MCAM antagonism of fentanyl (i.e. to avoid the development of tolerance or other effects of large doses of fentanyl that could influence the recovery of sensitivity to fentanyl antinociception). MCAM antagonized the effects of a fixed dose range of fentanyl, shifting the dose-effect curve downward (figure 3). Tail-withdrawal latencies at 0.1 mg/kg fentanyl were still significantly decreased 37 days after MCAM and were not different from control 45 days after MCAM ($F[1.93, 11.6]=40.7$, $P<0.0001$; table 2).

Finally, in a third group of rats, MCAM did not shift the spiradoline dose-effect curve (top panel, figure 4); the ED_{50} values (± 1 SEM) for spiradoline obtained before and 1 day after

MCAM administration were 2.51 ± 0.53 and 2.87 ± 0.13 mg/kg, respectively, yielding a potency ratio (95% confidence interval) of 1.56 (0.78, 2.24). In contrast, MCAM antagonized the antinociceptive effects of morphine, as evidenced by the downward shift in the morphine dose-effect curve in these rats 5 days after MCAM (i.e., four days after the spiradoline test; bottom panel, figure 4). Tail-withdrawal latencies obtained after administration of 17.8 mg/kg morphine were significantly decreased for 29 days after MCAM and were no longer different from control 37 days after MCAM ($F[2.23, 15.6]=41.8$, $P<0.0001$; table 2).

Paw inflammation. Before hindpaw injections, there was no difference in thickness between the paw that later received an injection of saline and the paw that later received an injection of CFA. CFA but not saline significantly increased paw thickness for up to 31 days (Table 3). Paw withdrawal threshold in cycle 1 (i.e., 15 min after i.p. saline) was used to determine changes in paw sensitivity to mechanical stimulation across days. Before hindpaw injections, there was no difference in withdrawal threshold between the paw that later received an injection of saline and the paw that later received an injection of CFA (Table 3); CFA but not saline increased sensitivity to mechanical stimulation for up to 31 days after hindpaw injections, as indicated by a significantly lower withdrawal threshold for the CFA-injected paw compared with the saline-injected paw (Table 3).

Morphine, but not saline or meloxicam, dose-dependently increased paw withdrawal threshold in vehicle-treated rats (not shown). There was a significant main effect of treatment ($F[1,7]=6.7$, $P=0.036$), a significant main effect of time ($F[2,14]=16$, $P<0.001$), and a significant treatment x time interaction ($F[2,14]=6.6$, $P=0.009$). MCAM attenuated the effects of morphine on paw withdrawal threshold for at least 15 days (figure 5), as evidenced by a

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significant difference between groups on days 1 and 3 (t[14]=4.6, P=0.001) as well as on day 15 (t[14]=2.9, P=0.035), but not on day 30 (t[14]=0.49, P>0.999).

Morphine, but not saline or meloxicam, increased body temperature in MCAM-treated and vehicle-treated rats (not shown). There was a significant main effect of treatment (F[1,7]=7.7, P=0.027) but not time (F[2,14]=1.7, P<0.317), and a significant treatment x time interaction (F[2,14]=4.8, P=0.026). MCAM blocked the hyperthermic effects of morphine for at least 15 days (figure 6), as there was a significant difference between groups on days 1 and 3 (t[14]=4.3, P=0.002) and on day 15 (t[14]=3.1, P=0.021), but not day 30 (t[14]=0.08, P>0.999).

Gastrointestinal transit. Morphine significantly decreased fecal output compared with saline (t[63]=7.5, P<0.001; compare points above M and S, top panel, figure 7). On the day of MCAM administration, morphine did not significantly decrease fecal output (t[63]=0.9, P>0.99; point above 0, top panel, figure 7). The effects of morphine on days 0, 5, 9, 13, and 17 after MCAM administration were significantly different from the effects of morphine before MCAM administration (t[63]=8.4, P<0.001 for day 0; t[63]=6.0, P<0.001 for day 5; t[63]=4.1, P=0.001 for day 9; t[63]=4.8, P<0.001 for day 13; and t[63]=5.1, P<0.001 for day 17) whereas the effects of morphine on day 21 after MCAM administration was not significantly different from the effects of morphine before MCAM administration (t[63]=2.4, P=0.19; top panel, figure 7).

Estimated consumption of wet chow remained relatively stable across tests (bottom panel, figure 7). On days when morphine alone was tested, consumption was not different compared with saline (t[63]=2.0, P=0.50); however, consumption was slightly but

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significantly elevated on all tests after MCAM administration compared with the effects of morphine alone ($t[63]=4.3$, $P<0.001$ for day 0; $t[63]=5.7$, $P<0.001$ for day 5; $t[63]=6.1$, $P<0.001$ for day 9; $t[63]=3.0$, $P=0.031$ for day 13; $t[63]=6.5$, $P<0.001$ for day 17; $t[63]=5.2$, $P<0.001$ for day 21).

Dependence/withdrawal. During twice-daily treatment with 56 mg/kg morphine, withdrawal signs did not occur (points above C, top panel, figure 8) and body weight was not significantly different among groups (points above C, bottom panel, figure 8). MCAM (10 mg/kg) and naloxone (17.8 mg/kg) increased the number of withdrawal signs observed on the day they were administered, compared with the number of withdrawal signs observed before antagonist administration or the number of signs observed in the group that received vehicle instead of an antagonist before the first observation period (points above 0, top panel, figure 8). There was a significant main effect of the injection given before the observation periods (i.e. antagonist or vehicle; $F[2,8]=14.63$, $P=0.0021$), a significant main effect of days since antagonist or vehicle administration ($F[5,20]=29.77$, $P<0.0001$), and a significant interaction ($F[10,40]=13.43$, $P<0.0001$). *Post hoc* tests revealed that the group receiving MCAM and the group receiving naloxone showed significantly more withdrawal signs on the day antagonists were administered (Day 0, figure 8) compared with the group that received vehicle. No morphine was administered to any rats after Day 0; for all three groups, the number of withdrawal signs was significantly greater 1 day after the last dose of morphine compared with the number observed during morphine treatment (for each group, compare points above 1 to points above C, top panel, figure 8); however, there was no difference in withdrawal signs among the three groups on any day after discontinuation of

morphine treatment (top panel, figure 8). Body weight was also significantly decreased by administration of antagonist or discontinuation of morphine treatment (bottom panel, figure 8). There was a significant main effect of days since antagonist or vehicle administration ($F[5,20]=99.69$, $P<0.0001$) and a significant interaction ($F[10,40]=4.60$, $P=0.0002$), but no main effect of the injection given on the last day of morphine treatment. The largest decrease in body weight occurred 2 days after the last dose of morphine, and this decrease was not different among groups (figure 8).

Discussion

MCAM is an opioid receptor antagonist that might be useful for treating OUD and overdose because it retains the positive aspects of naltrexone and naloxone (e.g., safety and no abuse liability; Maguire et al. 2019) and its long duration of antagonist action would block the effects of abused opioids for an extended period, particularly compared with naloxone for which extended release formulations are not available. Although MCAM would be expected to provide long-term protection against the abuse-related and toxic effects of opioids, sustained blockade of μ opioid receptors could introduce other challenges. For example, MCAM will block the analgesic effects of μ opioid receptor agonists, rendering them ineffective, and using MCAM to reverse opioid overdose would precipitate withdrawal in opioid-dependent patients. This study examined antagonism of opioid agonists by MCAM using several different procedures in rats.

MCAM attenuated the acute effects of μ opioid receptor agonists in a dose- and time-related manner. Sustained antagonism by MCAM has been reported (Broadbear et al. 2000;

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Gerak et al. 2019; Maguire et al. 2019; Peckham et al., 2005); however, the current study extends previous findings in several ways. First, although MCAM was shown to attenuate the antinociceptive effects of morphine in rodents (Broadbear et al. 2000; Peckham et al. 2005), antagonism was monitored for only 2 days. In the current study, MCAM blocked the effects of morphine and fentanyl for several weeks. Two different procedures were used to assess the ability of MCAM to attenuate the antinociceptive effects of μ opioid receptor agonists (warm water tail withdrawal and CFA-induced hypersensitivity), with 10 mg/kg MCAM antagonizing these effects of morphine for more than 2 weeks. Morphine and other μ opioid receptor agonists decrease gastrointestinal motility and alter body temperature, producing hypothermia or hyperthermia depending on the conditions. In this study, morphine significantly decreased fecal output and increased body temperature, on average by more than 2.5°C. MCAM produced long-lasting antagonism of these effects of morphine on gastrointestinal motility and body temperature. Thus, MCAM produces sustained antagonism of multiple effects of μ opioid receptor agonists.

Using the warm water tail withdrawal procedure, the long duration of action of MCAM was observed. In one study, doses of morphine producing a predetermined level of effect were given frequently to rats that received MCAM; under these conditions, there was progressive and complete recovery of sensitivity to morphine in rats treated with 1 or 3.2 mg/kg MCAM. Although sensitivity to morphine recovered partially in rats treated with 10 mg/kg MCAM, the morphine dose-effect curve did not return fully to control 69 days after MCAM administration. Because MCAM is thought to bind pseudoirreversibly to μ opioid receptors, its duration of action might be determined by the availability of newly synthesized

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μ receptors (Zernig et al. 1994; Zernig et al. 1996). One possible explanation for sensitivity to morphine not recovering fully to control after treatment with 10 mg/kg MCAM is that the very large doses of morphine tested relatively frequently (i.e., up to 560 mg/kg every four days) desensitized newly synthesized μ receptors as they became expressed. Although the reason sensitivity to morphine did not recover fully is not evident from this experiment, given this outcome, the duration of action of a single dose of 10 mg/kg MCAM was likely overestimated in this study. Nevertheless, in patients receiving MCAM for opioid abuse or overdose, use or abuse of opioid receptor agonists would not be expected to decrease, and might substantially increase, the time needed for agonist effects to return to control after MCAM administration, which would effectively lengthen the antagonist activity of a large dose of MCAM.

Because sensitivity to morphine did not recover fully in rats treated with 10 mg/kg MCAM and tested with large doses of morphine, the agonist dose range was limited for subsequent experiments, with the largest doses studied being 0.1 mg/kg for fentanyl (figure 3) and 17.8 mg/kg for morphine (figure 4). Moreover, to reduce further possible changes in sensitivity resulting from repeated testing, agonists were tested less frequently (every 8 days). Under these conditions, MCAM antagonized fentanyl and morphine for at least 30 days, suggesting that a single large dose of MCAM could provide long-term protection against the effects of μ opioid receptor agonists, regardless of the use of opioids in the presence of MCAM. Recently, there has been an increase in overdose deaths caused by fentanyl and its analogs (Colon-Berezin et al. 2019; Spencer et al. 2019; Zoorob 2019), resulting in concerns, particularly in the lay press, that currently available opioid receptor

antagonists (naltrexone and naloxone) are less effective in attenuating the effects of fentanyl and its analogs, compared with their ability to attenuate the effects of other opioids (e.g., heroin). Antagonism across different classes of μ opioid receptor agonists (morphine-like and fentanyl-like) indicates that MCAM would be expected to be equally effective in attenuating the effects of all μ opioid receptor agonists. Reported differences between fentanyl and other μ opioid receptor agonists are likely due to the amount taken (e.g., inadvertently) as well as the potency of the opioids agonists, and not to qualitative differences in effectiveness between morphine-like and fentanyl-like opioids or their susceptibility to antagonism by MCAM, naltrexone, or naloxone.

MCAM binds pseudoirreversibly to μ opioid receptors (Broadbear et al. 2000), and several predictions can be made based on this type of interaction between drugs and receptors. First, because MCAM does not readily dissociate from μ opioid receptors, its antagonist effects would be expected to be persistent, and in the current study, the effects of the largest dose of MCAM lasted at least several weeks. In addition, antagonism by MCAM would be expected to be insurmountable; however, large doses of morphine produced maximal effects the day after administration of a large dose of MCAM. One possible explanation is that the antinociceptive effects of very large doses of morphine are mediated by receptors other than μ opioid receptors (e.g. κ opioid receptors; Stoller et al. 2007; Takemori and Portoghese 1987). This possibility is consistent with the effects of spiradoline (i.e., unchanged) in the presence of MCAM. Although MCAM also binds to κ and δ opioid receptors, its interactions with those receptors are reversible (Broadbear et al. 2000) and MCAM would not still be binding to those receptors the day after administration. That the

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antinociceptive effects of spiradoline are not changed the day after MCAM suggests that κ opioid receptors are unchanged and could be mediating the effects of morphine in MCAM-treated rats (Toll et al., 1998).

In morphine-dependent subjects, administration of a μ opioid receptor antagonist precipitates characteristic signs of withdrawal. In the current study, both MCAM and naloxone precipitated withdrawal in morphine-treated rats; however, the number of withdrawal signs and decreased body weight on subsequent days were not different in rats that received MCAM, naloxone, or vehicle, suggesting that withdrawal precipitated by MCAM is not qualitatively different from either withdrawal precipitated by naloxone or withdrawal after discontinuation of morphine treatment, consistent with results obtained with the irreversible μ opioid receptor antagonist β-funaltrexamine in morphine-dependent nonhuman primates (Gmerek and Woods, 1985).

Male rats were used in this study. Female rats are less sensitive than male rats to the antinociceptive effects of morphine, and this potency difference is not due to variations in the number of μ opioid receptors, binding affinity of morphine for those receptors, or ability of morphine to stimulate G proteins (Peckham et al. 2005). Moreover, when given 24 hr before testing, 0.32 mg/kg MCAM decreased the number of μ opioid receptors by 50%, producing a 3-fold shift to the right in the morphine dose-effect curve in female and male rats; while larger doses of MCAM produced greater rightward shifts in the morphine dose-effect curve in both sexes, they decreased the maximum effect produced by morphine in female, but not in male, rats (Peckham et al. 2005). Similar effects of MCAM were obtained in male rats in the current study. Given that a 30-fold smaller dose of MCAM reduced the number of μ opioid

receptors by 50%, few μ opioid receptors would be available to interact with morphine 1 day after administration of 10 mg/kg MCAM, and males are more sensitive than females to antinociceptive effects of drugs acting at κ opioid receptors (Craft and Bernal 2001).

Because MCAM attenuates the effects of morphine and fentanyl, but not those of spiradoline, persistent antagonism by MCAM is selective for μ opioid receptor agonists. While these behavioral effects are predicted for a drug with the pharmacological properties of MCAM *in vitro*, these results also indicate that acute pain could be treated through mechanisms other than μ opioid receptors in patients taking MCAM for opioid abuse or overdose. These results extend those of previous studies on MCAM (Broadbear et al., 2000; Gerak et al., 2019; Maguire et al., 2019; Peckham et al., 2005) to other measures of μ opioid receptor agonism and to subjects physically dependent on morphine. Taken together, studies in mice, rats, and nonhuman primates provide compelling support for the potential use of MCAM for treating OUD as well as opioid overdose.

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Authorship contributions

Participated in research design: Gerak, Minervini, Wooden, France

Conducted experiments: Gerak, Minervini, Latham, Ghodrati, Lillis, Wooden

Contributed new reagents or analytic tools: Husbands, Disney

Performed data analysis: Gerak, Minervini

Wrote or contributed to the writing of the manuscript: Gerak, Minervini, Wooden, Husbands, Disney, France

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Footnotes

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Figure legends

Figure 1. Antinociceptive effects of morphine in the absence of MCAM (filled circles) and at various times after MCAM administration. Each panel shows the antagonist effects of a different dose of MCAM. Ordinates: latency to remove tails from 50°C water, expressed as a percentage of control (mean ± 1 SEM) and averaged across eight rats. Abscissae: vehicle (S) or morphine dose in mg/kg body weight.

Figure 2. ED_{50} values obtained from morphine dose-effect curves at various times after MCAM administration (1-10 mg/kg; same data as figure 1 and table 1). The gray bar represents the 95% confidence interval of the mean ED_{50} from the control morphine dose-effect curves (filled circles, fig 1). Ordinates: estimates of ED_{50} values obtained by linear regression of morphine dose-effect curves in mg/kg body weight. Abscissa: days since MCAM administration.

Figure 3. Antinociceptive effects of fentanyl in the absence of MCAM (filled circles) and at various times after 10 mg/kg MCAM administration. The dose range for fentanyl tests remained the same, regardless of latencies obtained. Ordinates: latency to remove tails from 50°C water, expressed as a percentage of control (mean ± 1 SEM) and averaged across seven rats for the dose-effect curve determined 45 days after MCAM and across eight rats for all other dose-effect curves. Abscissae: vehicle (S) or fentanyl dose in mg/kg body weight.

Figure 4. Antinociceptive effects of spiradoline (top panel) and morphine (bottom panel) in

the absence of MCAM (filled circles) and at various times after 10 mg/kg MCAM administration. The dose range for each agonist test remained the same, regardless of latencies obtained. Ordinates: latency to remove tails from 50°C water, expressed as a percentage of control (mean ± 1 SEM) and averaged across eight rats. Abscissae: vehicle (S) or agonist dose in mg/kg body weight.

Figure 5. Effects of morphine on paw withdrawal threshold in rats with an inflamed paw. The top panel shows a morphine dose-effect curve obtained 1-3 days after administration of vehicle in rats with an inflamed paw. In the bottom panel, the effects of a cumulative dose of 17.8 mg/kg of morphine on paw withdrawal threshold are shown in rats with an inflamed paw that received either vehicle or MCAM. Ordinate: paw withdrawal threshold (g) averaged across eight rats; error bars represent ± 1 SEM. The gray bar represents the 95% CI for the saline paw in the first cycle of sessions in which a morphine dose-effect curve was determined. * indicates that the effects of 17.8 mg/kg morphine were significantly different ($p<0.05$) in rats that received 10 mg/kg MCAM as compared with rats that received vehicle. Abscissae: days since administration of MCAM or vehicle.

Figure 6. Changes in body temperature produced by 17.8 mg/kg morphine in rats with an inflamed paw that received either vehicle or MCAM. Ordinate: change in body temperature (ºC) averaged across eight rats; error bars represent ± 1 SEM. *Indicates that the effects of 17.8 mg/kg morphine were significantly different ($p<0.05$) in rats that received 10 mg/kg MCAM compared with rats that received vehicle. Abscissae: days since administration of MCAM or vehicle.

Figure 7. Fecal output and amount of wet chow consumed in 8 rats that received 10 mg/kg MCAM and 10 mg/kg morphine. Ordinates: fecal output (fecal boli/6 hr) and wet chow consumed (g/12 hr); error bars represent ± 1 SEM. *p<0.05 compared with the effects of 10 mg/kg morphine obtained in the absence of MCAM. Abscissae: days since administration of MCAM or vehicle.

Figure 8. Number of withdrawal signs and body weight in 5 rats that received vehicle, naloxone or MCAM on the last day of morphine treatment. Ordinates: number of signs or body weight (g); error bars represent ± 1 SEM. *p<0.05 compared with effects obtained on the last day of morphine treatment. Abscissae: days since administration of MCAM or vehicle.

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Table 1. ED_{50} values (±1 SEM) for morphine alone and after MCAM in a warm water tail withdrawal procedure measuring the latency to remove tails from water maintained at 50°C. Dose ratios (95% confidence intervals) are ED_{50} values for morphine after MCAM divided by ED_{50} values for morphine before MCAM.

Dosing condition	ED_{50} (mg/kg)	Dose ratio
Morphine alone	7.52 ± 0.80	
Morphine after 1 mg/kg MCAM		
1 day	17.14 ± 2.20	2.38 (1.65, 3.08)*
5 days	7.95 ± 0.53	1.14 (0.85, 1.41)
Morphine alone	7.52 ± 0.80	
Morphine after 3.2 mg/kg MCAM		
1 day	68.61 ± 8.95	10.21 (5.77, 14.29)*
5 days	29.27 ± 0.55	4.47 (2.90, 5.79)*
9 days	27.38 ± 1.58	4.15 (2.67, 5.39)*
13 days	17.38 ± 2.73	2.66 (1.28, 3.85)*
17 days	8.80 ± 0.14	1.33 (0.90, 1.69)

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Morphine alone	7.30 ± 0.56	
Morphine after 10 mg/kg MCAM		
1 day	187.68 ± 7.56	26.76 (21.44, 31.81)*
5 days	61.68 ± 6.01	8.97 (6.01, 11.65)*
9 days	41.75 ± 3.90	6.11 (3.94, 8.10)*
13 days	91.67 ± 10.56	12.65 (9.54, 15.59)*
17 days	98.95 ± 2.71	14.13 (11.36, 16.78)*
21 days	100.23 ± 3.90	14.23 (11.60, 16.77)*
29 days	64.75 ± 8.59	9.29 (5.71, 12.39)*
37 days	55.06 ± 9.72	7.58 (4.63, 10.15)*
45 days	35.40 ± 3.06	4.95 (4.05, 5.81)*
53 days	29.77 ± 0.59	4.25 (3.44, 5.02)*
69 days	25.73 ± 3.20	3.83 (2.02, 5.53)*

*potency of morphine was significantly changed as evidenced by 95% confidence intervals of the potency ratios that did not include 1.

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Table 2. Tail-withdrawal latency from water maintained at 50°C, expressed as a percentage of the maximum possible effect (± 1 SEM), obtained after administration of the largest cumulative dose of fentanyl (0.1 mg/kg; n=7) or morphine (17.8 mg/kg; n=8) alone and at various times after administration of 10 mg/kg MCAM.

Dosing condition	% maximum possible effect after 0.1 mg/kg fentanyl	% maximum possible effect after 17.8 mg/kg morphine
Agonist alone	95.2 ± 4.8	99.0 ± 1.0
Agonist after 10 mg/kg MCAM		
1 day	5.2 ± 2.1*	n.s.
5 days	0*	3.8 ± 1.5*
13 days	10.3 ± 4.0*	32.7 ± 6.8*
21 days	13.9 ± 2.9*	52.3 ± 8.4*
29 days	33.5 ± 5.0*	37.6 ± 11.7*
37 days	65.4 ± 7.6*	97.9 ± 1.4
45 days	68.1 ± 8.9	n.s.

n.s. morphine dose-effect curves were not determined under these conditions

*p<0.05 as compared with the effects of the agonist in the absence of MCAM

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Table 3: Paw thickness determined before sessions and paw withdrawal threshold determined during the first cycle of the session with saline administered at the beginning of that cycle. Each value represents the mean (95% confidence intervals) in 8 rats.

	Paw thickness (mm)		Withdrawal threshold (g)	
Days since hindpaw injections	Saline paw	CFA paw	Saline paw	CFA paw
Before	3.26 (3.02, 3.49)	3.17 (2.95, 3.39)	50.73 (40.95, 60.50)	50.34 (40.48, 60.19)
1	3.34 (3.15, 3.53)	7.55 (7.26, 7.84)*	n/a	n/a
2	3.24 (3.11, 3.36)	7.61 (7.28, 7.94)*	52.47 (45.34, 59.60)	17.59 (13.19, 21.98)*
31	3.22 (3.13, 3.30)	5.91 (5.62, 6.20)*	37.37 (31.66, 43.09)	18.54 (13.83, 23.25)*

*paw thickness or withdrawal threshold were significantly changed when the value of the inflamed (CFA) paw was outside of the 95% confidence interval of the noninflamed (saline) paw.

Figure 1

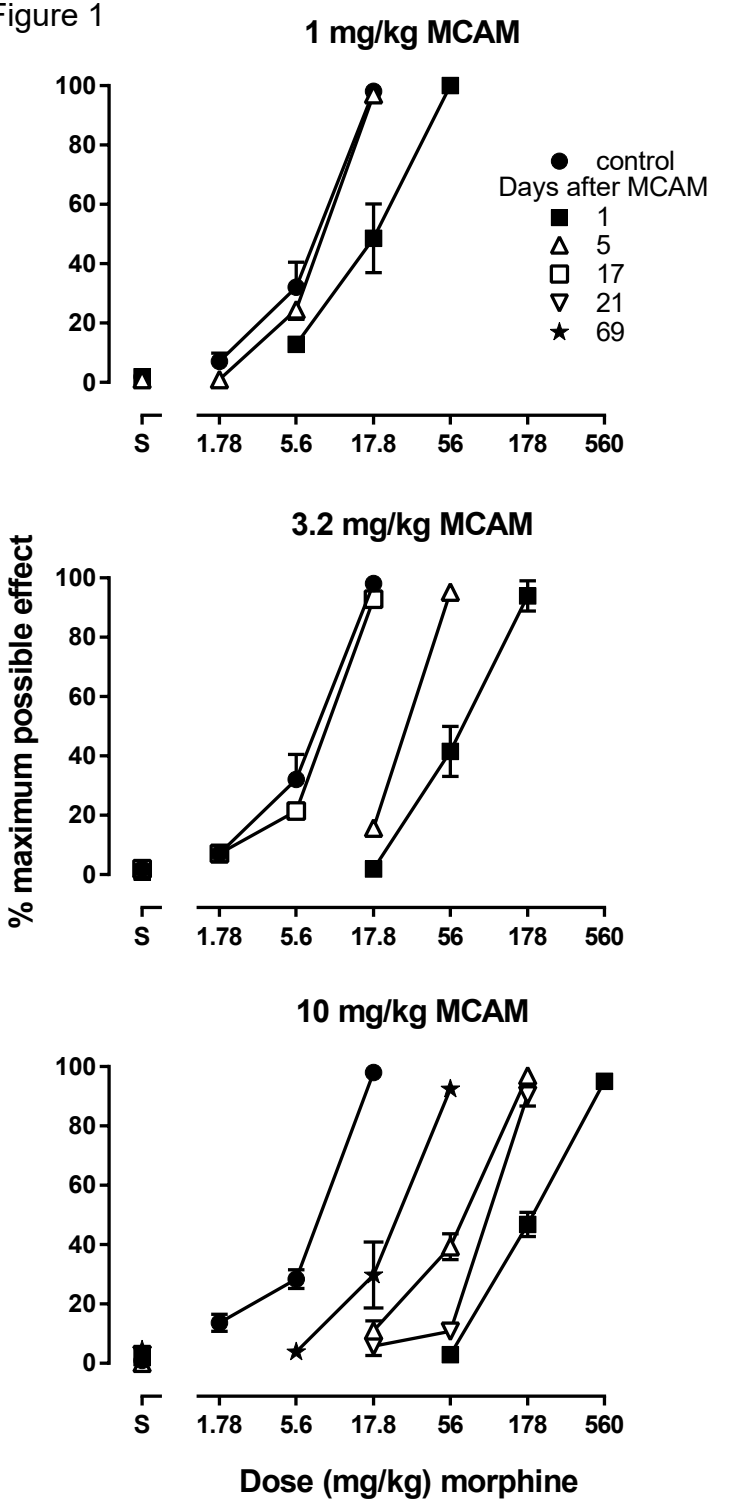

1 mg/kg MCAM
3.2 mg/kg MCAM
10 mg/kg MCAM
% maximum possible effect
100
80
60
40
20
0
control
Days after MCAM
1
5
17
21
69
S
1.78
5.6
17.8
56
178
560
Dose (mg/kg) morphine

Figure 2

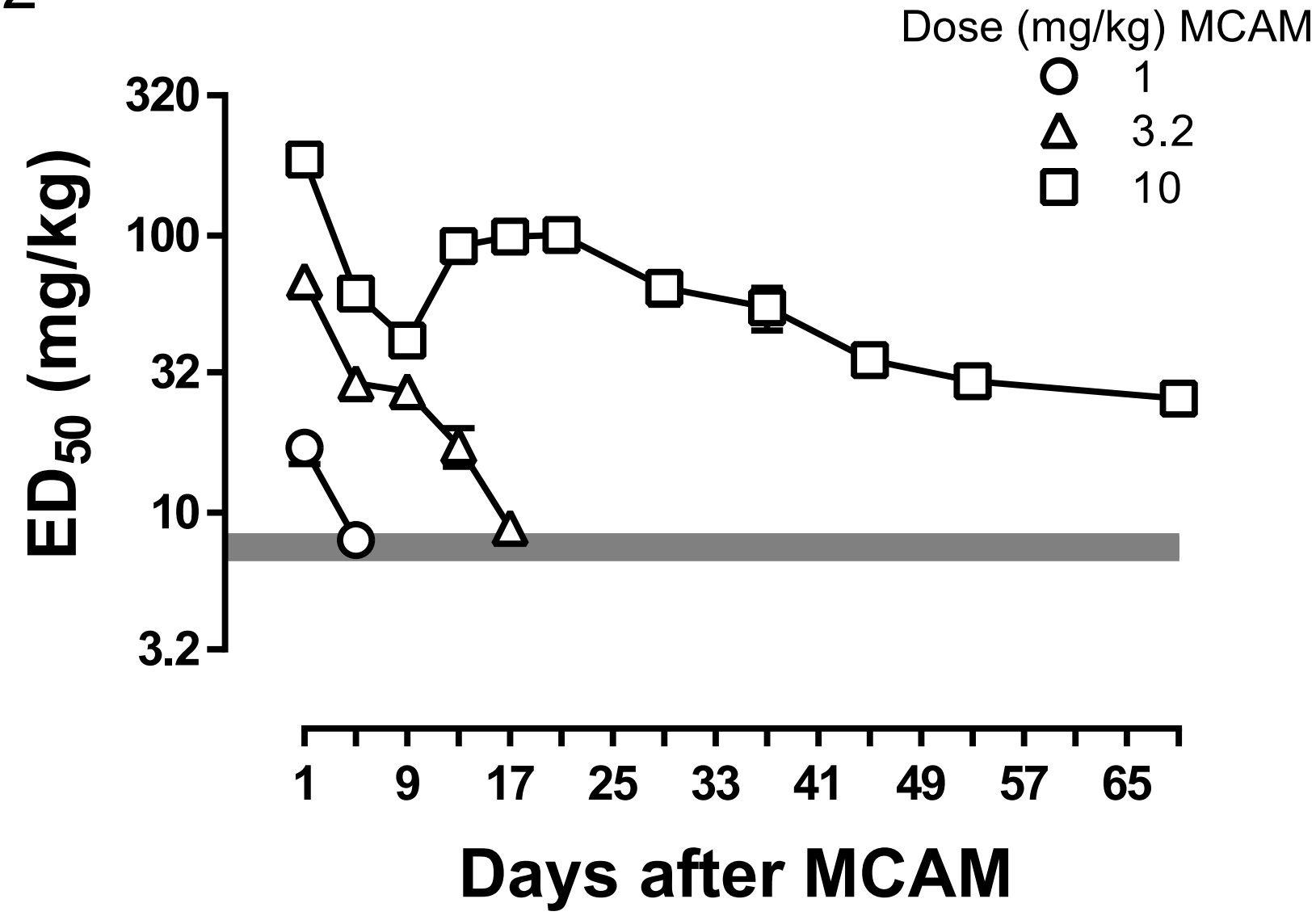

Dose (mg/kg) MCAM
1
3.2
10
320
100
32
10
3.2
ED50 (mg/kg)
1
9
17
25
33
41
49
57
65
Days after MCAM

Figure 3

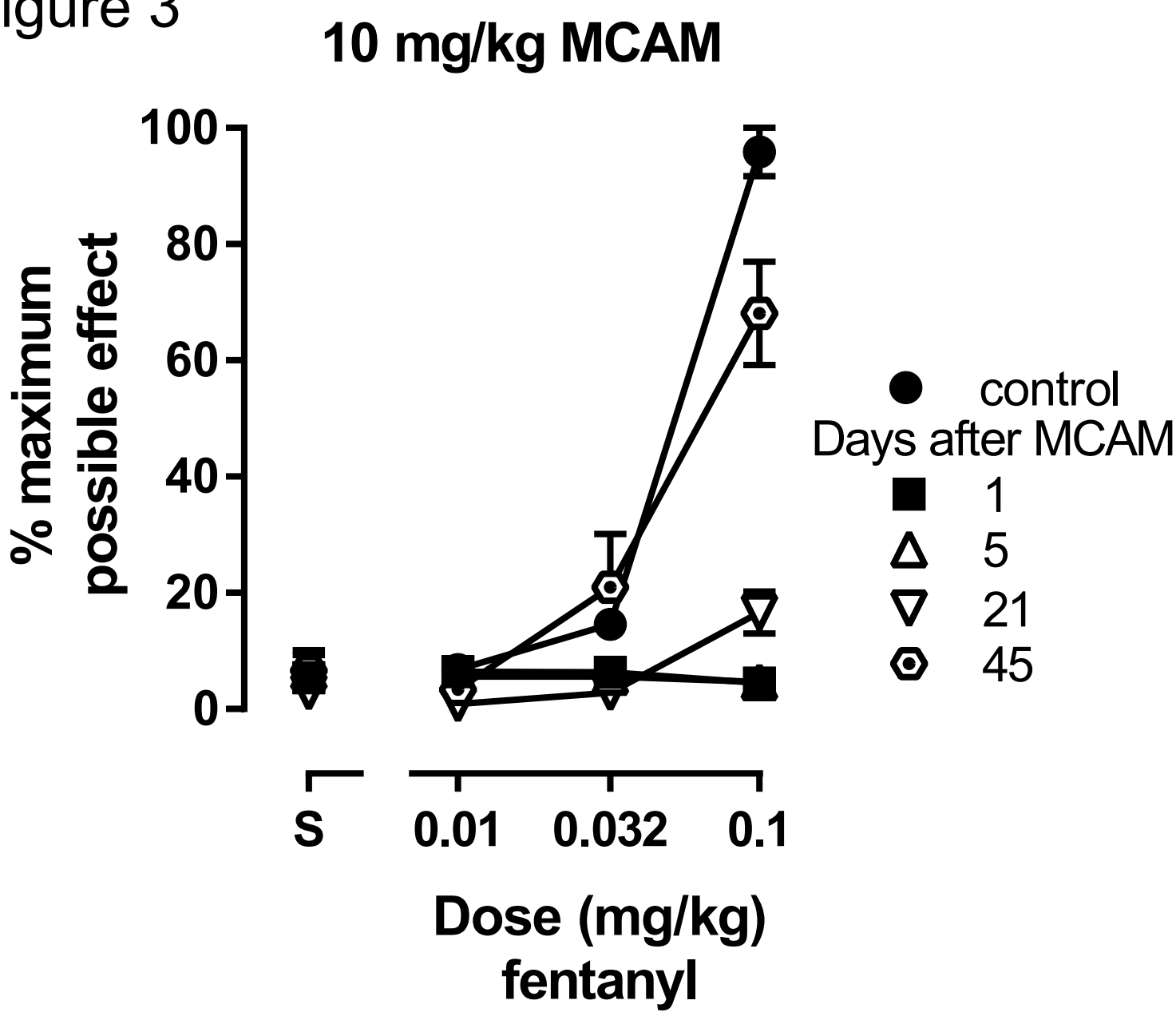

10 mg/kg MCAM
% maximum possible effect
100
80
60
40
20
0
S
0.01
0.032
0.1
Dose (mg/kg) fentanyl
control
Days after MCAM
1
5
21
45

Figure 4

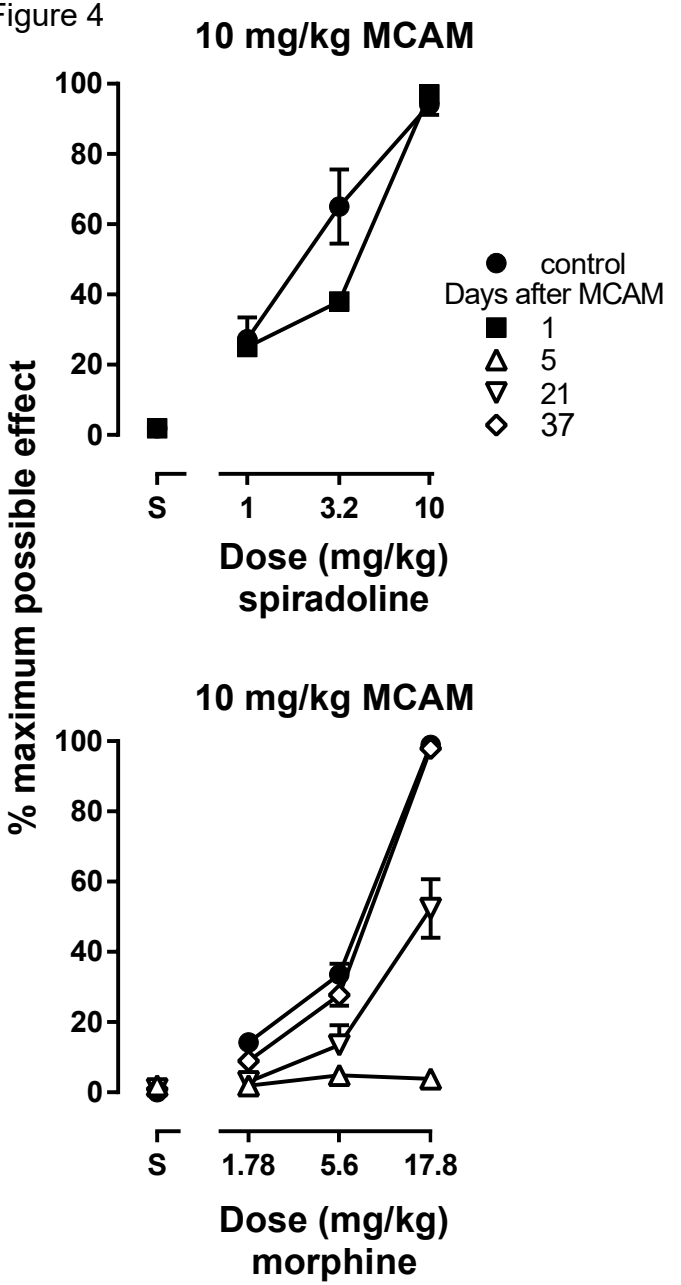
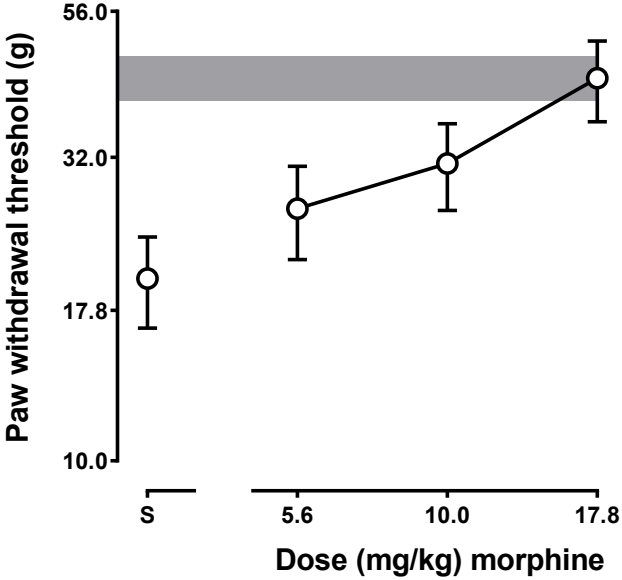

10 mg/kg MCAM
100
80
60
40
20
0
% maximum possible effect
control
Days after MCAM
1
5
21
37
S
1
3.2
10
Dose (mg/kg)
spiradoline
10 mg/kg MCAM
100
80
60
40
20
0
S
1.78
5.6
17.8
Dose (mg/kg)
morphine

Figure 5


56.0
32.0
17.8
10.0
Paw withdrawal threshold (g)
S
5.6
10.0
17.8
Dose (mg/kg) morphine

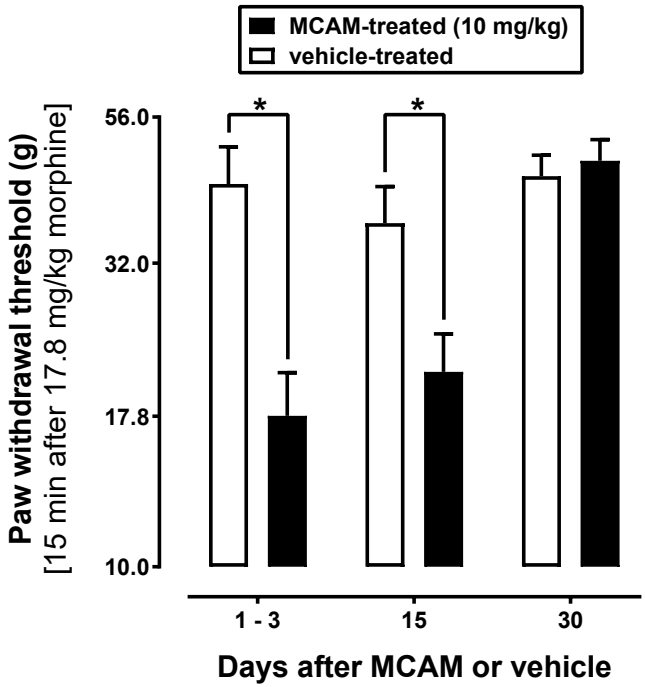

MCAM-treated (10 mg/kg)
vehicle-treated
*
*
56.0
32.0
17.8
10.0
Paw withdrawal threshold (g)
[15 min after 17.8 mg/kg morphine]
1 - 3
15
30
Days after MCAM or vehicle

Figure 6

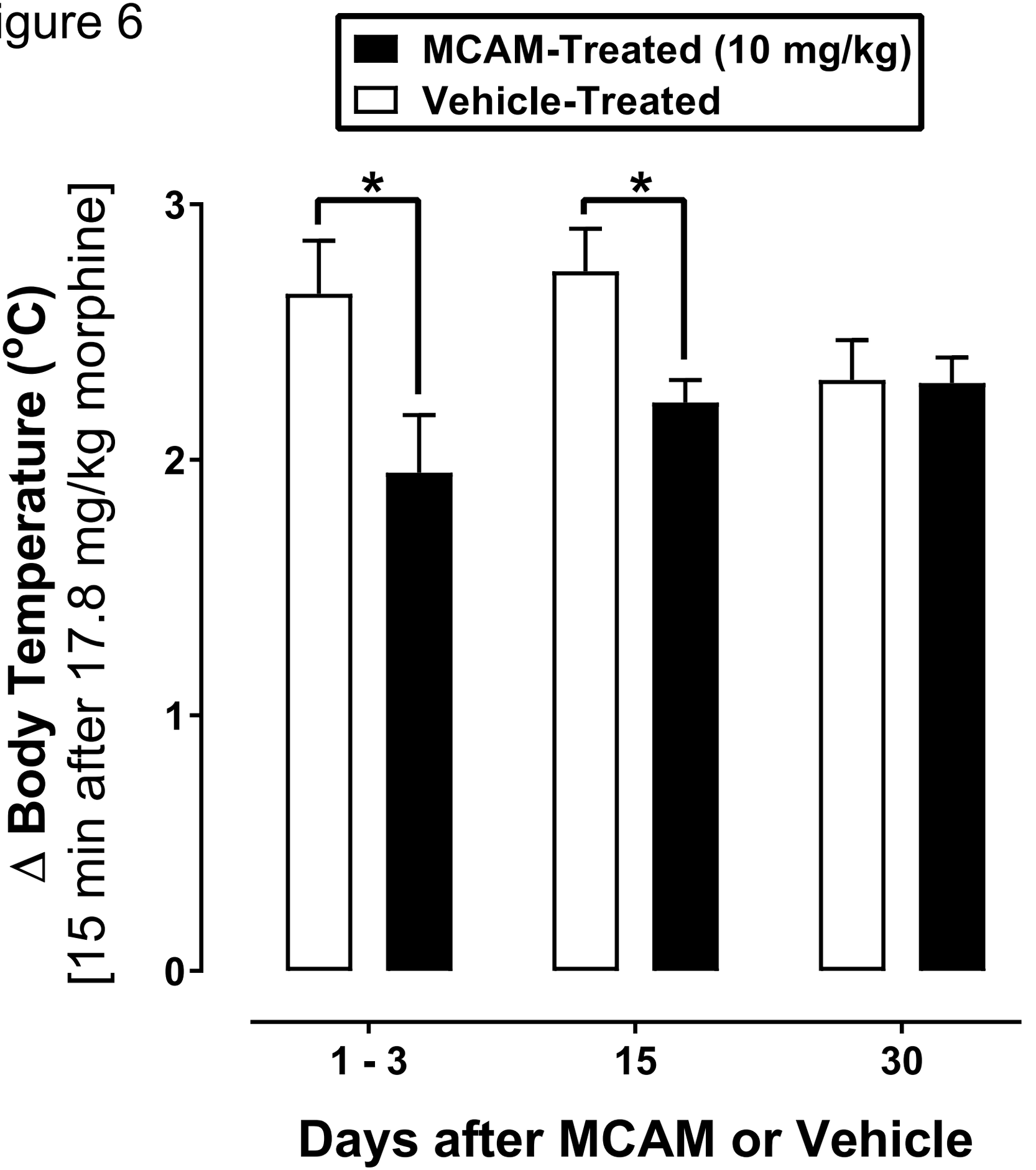

MCAM-Treated (10 mg/kg)
Vehicle-Treated
*
*
3
2
1
0
Δ Body Temperature (°C)
[15 min after 17.8 mg/kg morphine]
1 - 3
15
30
Days after MCAM or Vehicle

Figure 7

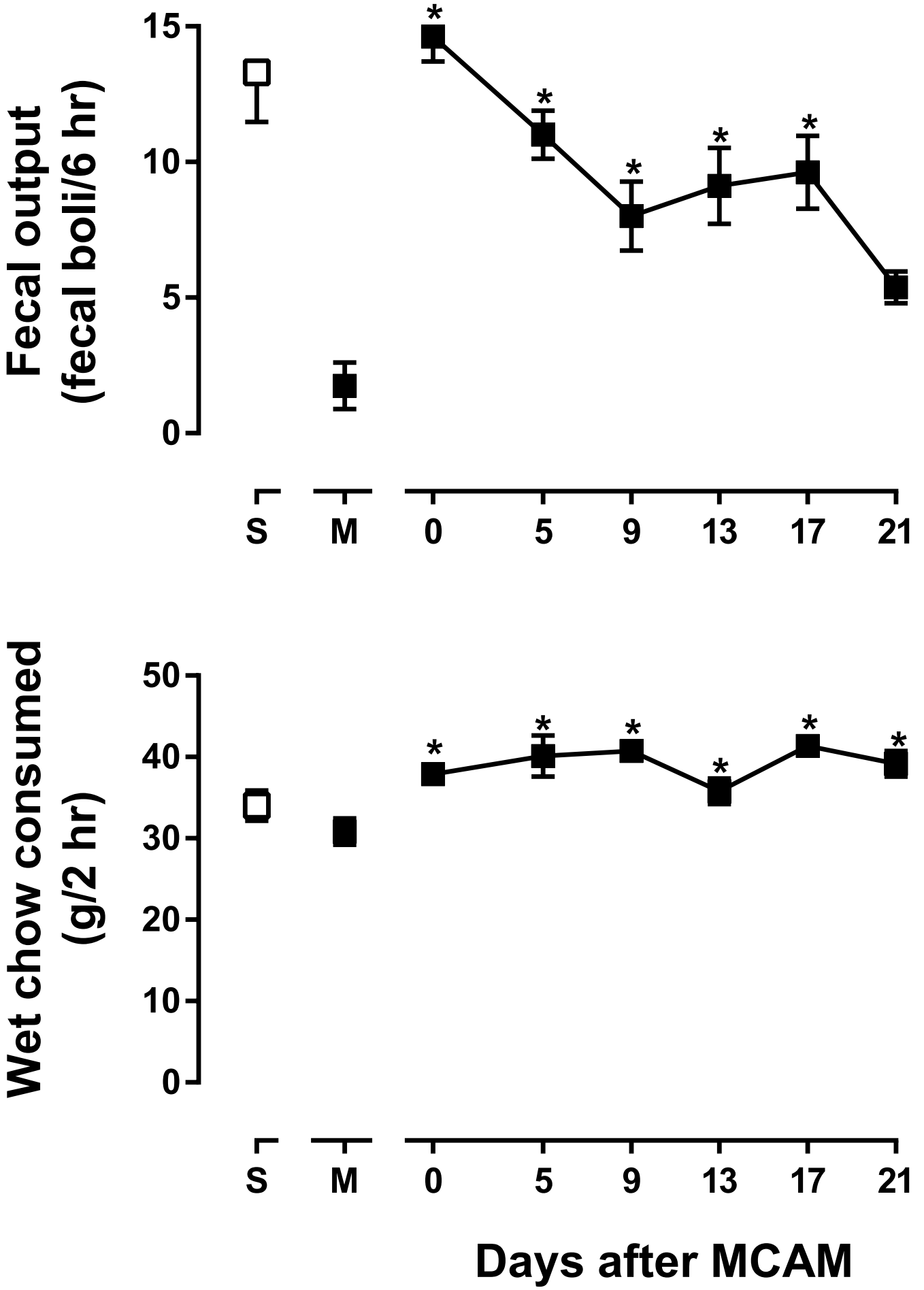
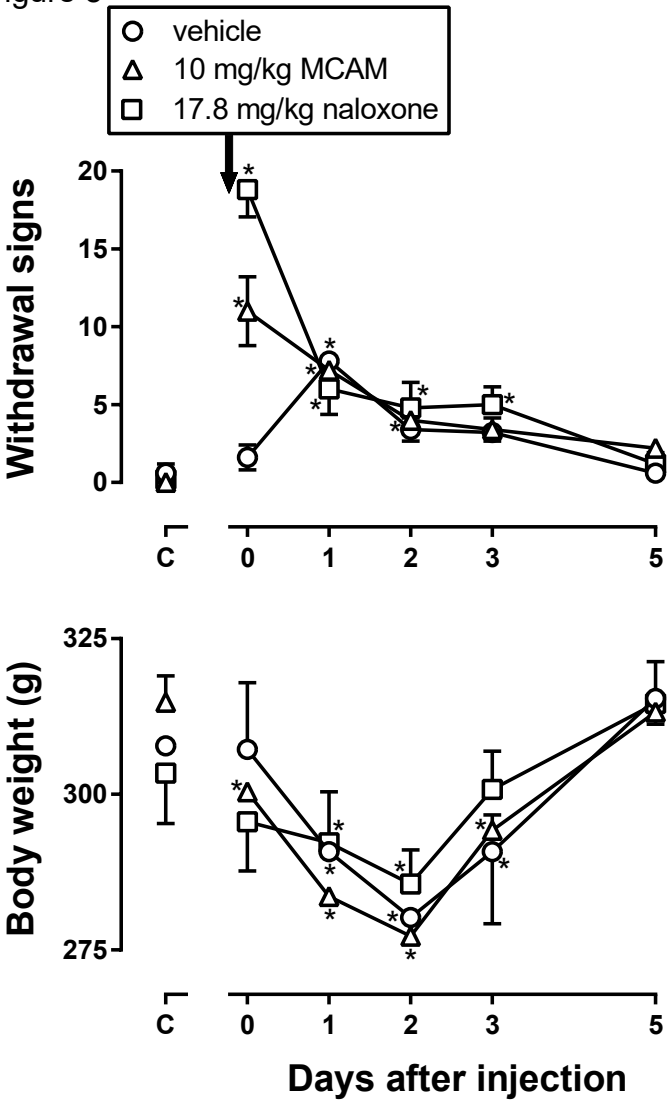

Fecal output
(fecal boli/6 hr)
15
10
5
0
S
M
0
5
9
13
17
21
Wet chow consumed
(g/2 hr)
50
40
30
20
10
0
S
M
0
5
9
13
17
21
Days after MCAM

Figure 8


vehicle
10 mg/kg MCAM
17.8 mg/kg naloxone
Withdrawal signs
20
15
10
5
0
C
0
1
2
3
5
Body weight (g)
325
300
275
C
0
1
2
3
5
Days after injection