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The role of 6-acetylmorphine in heroin-induced reward and locomotor sensitization in mice

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Abstract

We have previously demonstrated that heroin's first metabolite, 6-acetylmorphine (6-AM), is an important mediator of heroin's acute effects. However, the significance of 6-AM to the rewarding properties of heroin still remains unknown. The present study therefore aimed to examine the contribution of 6-AM to heroin-induced reward and locomotor sensitization. Mice were tested for conditioned place preference (CPP) induced by equimolar doses of heroin or 6-AM (1.25-5 μmol/kg). Psychomotor activity was recorded during the CPP conditioning sessions for assessment of drug-induced locomotor sensitization. The contribution of 6-AM to heroin reward and locomotor sensitization was further examined by pretreating mice with a 6-AM specific antibody (anti–6-AM mAb) 24 hours prior to the CPP procedure. Both heroin and 6-AM induced CPP in mice, but heroin generated twice as high CPP scores compared with 6-AM. Locomotor sensitization was expressed after repeated exposure to 2.5 and 5 μmol/kg heroin or 6-AM, but not after 1.25 μmol/kg, and we found no correlation between the expression of CPP and the magnitude of locomotor sensitization for either opioid. Pretreatment with anti–6-AM mAb suppressed both heroin-induced and 6-AM–induced CPP and locomotor sensitization. These findings provide evidence that 6-AM is essential for the rewarding and sensitizing properties of heroin; however, heroin caused stronger reward compared with 6-AM. This may be explained by the higher lipophilicity of heroin, providing more efficient drug transfer to the brain, ensuring rapid increase in the brain 6-AM concentration.

KEYWORDS

6-acetylmorphine, antibody, CPP, heroin, opioid, reward, sensitization

1 | INTRODUCTION

Heroin is considered one of the most addictive illicit drugs and is involved in numerous fatal drug intoxications worldwide each year.[1] Heroin induces strong euphoric and rewarding effects, and its rapid onset of action has been associated with the high abuse potential.[2] The intake of heroin can promote maladaptive changes in brain circuitries related to reward and reinforcement, which may render an individual more sensitive upon subsequent drug exposure.[3,4] This drug-induced neuroplasticity is presumably an important aspect of the transition from drug use to drug addiction.[5,6]

Drug-induced conditioned place preference (CPP) is a commonly used behavioral model to study the rewarding properties of drugs of abuse. The method is based on repeated drug exposure in which the animal learns to associate a specific environment with the drug effects.[7-9] A preference or avoidance of the drug-paired environment in a drug-free test is considered to be a reliable indicator of a rewarding or aversive drug effect, respectively.[8,10] Upon repeated drug

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administrations to rodents, a progressive increase in drug-induced locomotor activity can be observed, termed locomotor sensitization. This phenomenon has been suggested to reflect neuroplastic changes induced by the drug, however, whether these changes are involved in the development of addiction is debated.[4,11-15] Both heroin-induced CPP and locomotor sensitization have been reported in rodents even after low doses of heroin.[16,17]

The high addiction potential of heroin may be due to its pharmacokinetic properties, in particular its lipophilicity, which provides rapid passage across the blood-brain-barrier (BBB).[18] However, heroin itself is suggested to be a pro-drug acting mainly through its metabolites 6-acetylmorphine (6-AM) and morphine.[19] In rodents, 6-AM is the predominant metabolite measured in blood and brain the first 30 minutes after administration, whereas heroin becomes undetectable within a few minutes.[20-22] It has also been reported that a single 6-AM injection leads to profound increases in locomotor activity and striatal dopamine release in rodents.[20,23,24] Furthermore, we recently showed that a monoclonal antibody against 6-AM (anti–6-AM mAb) reduced heroin-induced locomotor activity and brain 6-AM levels in mice.[24,25] Altogether, these reports imply that 6-AM is an important mediator of the acute actions of heroin. Previous work by Raleigh et al.[26] suggested that 6-AM is also essential for heroin reinforcement, however, the significance of 6-AM to the rewarding properties of heroin still remains elusive.

The present study aimed to investigate the implication of 6-AM in heroin-induced reward and locomotor sensitization. Therefore, the acquisition of CPP and simultaneous measurements of locomotor sensitization were examined in mice conditioned with either heroin or 6-AM. Furthermore, pretreatment with anti–6-AM mAb was used to characterize the contribution of 6-AM to the observed heroin-induced behavioral effects.

2 | MATERIALS AND METHODS

2.1 | Materials

Drugs: Heroin-HCl (mol.wt. 421.91) and 6-AM–HCl (mol.wt. 417.88) were purchased from Lipomed AG (Arlesheim, Switzerland) and dissolved in 0.9% NaCl. Opioid doses were chosen based on previous pharmacokinetic and behavioral studies in mice.[16,20,24] Antibody: Anti–6-AM mAb (human immunoglobulin G1; IgG1) was provided by Affitech Research AS (Oslo, Norway). The properties of the mAb have been described in more detail previously.[25,27] The mAb was dialyzed against phosphate buffer and endotoxins removed. The mAb was diluted in 0.9% NaCl and stored at −80°C. A NOVEX ELISA kit provided by Thermo Fisher Scientific Inc (Waltham, Massachusetts) was used for antibody quantification in blood. mAb doses for the present study were chosen based on opioid:mAb ratio experiments reported in Kvello et al.[24]

2.2 | Animals

Male C57BL/6J mice (7-8 wk old, 20-25 g; Taconic, Ejby, Denmark) were housed four to eight per cage in the animal facility at the Norwegian Institute of Public Health (Oslo, Norway). C57BL/6J mice are widely used for drug abuse research and have been studied in our laboratory for behavioral and pharmacokinetic studies after opioid exposure for more than 20 years.[20,24,28,29] The animals were housed in plexiglass cages containing wooden bedding and small plastic houses for environmental enrichment, and acclimatized for at least 5 days prior to the experiments. Temperature, humidity, and lights were regulated (22°C ± 1°C, 50% ± 10% humidity, lights on from 7 AM to 7 PM), and commercial mouse pellets and water were available ad libitum. The experiments were carried out during the light period of the day under dimmed lighting. The animal experimental protocols comply with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health. All experimental protocols were approved by the Norwegian Animal Research Authority and conducted in accordance with the ARRIVE guidelines.[30]

2.3 | Experiment I. Heroin-induced and 6-AM–induced CPP and locomotor sensitization

2.3.1 | The CPP apparatus

Cages measuring 40 × 40 cm, divided into two distinct compartments measuring 20 × 40 cm, were used for the CPP procedure. The two compartments were connected by an opening in the center of the box, which was closed during the conditioning sessions. One compartment had white walls with vertical black stripes and a wrinkled plastic floor; the other compartment had black walls with horizontal white stripes and a perforated metal floor. Both compartments had a transparent plastic ceiling. The animal's position in the cage was registered by infrared beams (spaced 2.5 cm apart) situated on the lateral walls at floor level. The sensors were connected to a Versamax animal activity monitoring system (AccuScan Instruments Inc, Colombus). As no significant preference for either of the two compartments was found in drug-naïve mice, the CPP apparatus was considered unbiased.

2.3.2 | The CPP procedure

Conditioning (days 1-3): Mice were randomly assigned to different groups and conditioned with either heroin (n = 50) or 6-AM (n = 54) for three consecutive days. The mice received opioid injection in the morning and saline injection in the afternoon (6 h later). For each individual mouse, one compartment of the CPP chamber was always paired with heroin or 6-AM injection (1.25-5 μmol/kg, corresponding to 0.5-2.1 mg/kg, 10 mL/kg, subcutaneous (s.c.); morning session), and the other compartment was paired with saline injection (0.9%, 10 mL/kg, s.c.; afternoon session). A counterbalanced CPP paradigm was used in which 50% of the mice were drug conditioned to the white compartment and 50% were drug conditioned to the black compartment. A control group was also included, receiving saline injections both at morning and afternoon sessions (n = 10). The maximum psychomotor effect, as well as the brain C_{max} of 6-AM, appears simultaneously upon heroin and 6-AM injection in rodents.[20,23] Therefore, no delay was implemented between the time of drug injection and the conditioning sessions in the CPP chambers. Each conditioning session lasted for 20

minutes, and the animals were returned to their home cage immediately after each session.

CPP test (day 4): On the morning of the fourth day, the animals were tested for place preference in a drug-free state. The mice were injected with saline (0.9%, 10 mL/kg, s.c.) and immediately placed in the opening between the two compartments of the test cage, having free access to both compartments. The residence time in each compartment was measured for 20 minutes.

2.3.3 | Measuring locomotor activity to assess locomotor sensitization during conditioning (days 1-3)

To assess locomotor sensitization to heroin and 6-AM, the locomotor activity of the mice was recorded during conditioning, as previously shown by others.[13,16,31-33] For all six conditioning sessions, the animals' locomotor activity was measured during the 20-minute session, using the Versamax animal activity monitoring system. Dose-response relationships with locomotor activity measured per 5 minutes bin (cm/5 min) and total distance travelled during each session (cm/20 min) were assessed after opioid injections (sessions 1, 3, and 5, morning) and after saline injections (sessions 2, 4, and 6, afternoon).

2.4 | Experiment II. The effect of anti–6-AM mAb on heroin-induced and 6-AM–induced CPP and locomotor sensitization

2.4.1 | Prestudy: The effect of a single pretreatment with anti–6-AM mAb upon repeated heroin injections

Mice were pretreated with a single saline (0.9%, 7 mL/kg, i.p., n = 20) or anti–6-AM mAb (10 mg/kg, 7 mL/kg, i.p., n = 20) injection 4 hours prior to the first heroin injection. The animals then received one daily heroin injection (2.5 μmol/kg, 10 mL/kg, s.c.) for one, two, or three consecutive days. Twenty-five minutes after the final heroin injection, the mice were anesthetized with isoflurane before blood and brain sampling. Brain samples were temporarily stored at −80°C and prepared for analyses as described by Kvello et al.[24] The 6-AM, morphine, morphine-3-glucuronide (M3G), and morphine-6-glucuronide (M6G) concentrations in brain samples were quantified within 24 hours after sampling by a LC-MS/MS method.[34] Blood sampling by heart puncture and quantification of human IgG1 levels by ELISA were performed as described in more detail by Kvello et al.[24]

2.4.2 | Heroin-induced and 6-AM–induced CPP and locomotor sensitization after pretreatment with anti–6-AM mAb

Mice received either no pretreatment or saline (0.9%, 7 mL/kg, i.p., controls), or a single injection of anti–6-AM mAb (10-200 mg/kg, 7 mL/kg, i.p.), 24 hours prior to the first conditioning with heroin or 6-AM (2.5 μmol/kg, 10 mL/kg, s.c.). The mice were tested for 6-AM–induced and heroin-induced CPP and locomotor sensitization exactly as described for Experiment I. Statistical analyses revealed no differences in CPP scores or locomotor activity in mice pretreated with saline (n = 13) compared with mice receiving no pretreatment (n = 15) prior to heroin or 6-AM (2.5 μmol/kg) conditioning, and these were therefore combined as control groups, for heroin or 6-AM, respectively, and designated as "0 mg/kg mAb."

2.5 | Data and statistical analysis

An established CPP was defined as significantly more time spent in the drug-paired compartment compared with time spent in the saline-paired compartment for each individual animal during the CPP test. CPP score was defined as time (s) spent in the drug-paired compartment minus time spent in the saline-paired compartment. The saline group was used to control for possible bias of the CPP apparatus.[35] To assess locomotor sensitization, the total distance travelled during the first opioid conditioning session was compared with the distance travelled during the third opioid conditioning session. Locomotor sensitization was defined as a significantly increased locomotor activity after the third opioid injection compared with the first opioid injection. Data are presented as mean + S.E.M. unless stated otherwise.

Statistical tests were performed using SPSS, version 23 (SPSS Inc, Chicago, Illinois). Each dataset was checked for normal distribution using histograms and stem-and-leaf plots. The data were to a large extent not normally distributed, and nonparametric statistical tests were performed. Wilcoxon signed rank test for related samples was used for comparison within groups to assess establishment of CPP and locomotor sensitization. Mann-Whitney *U* test for independent samples was used to check for difference in CPP score and difference in total locomotor activity between groups. *P* values less than 0.05 were considered as statistically significant. Correlation analysis (Spearman rho rank correlation coefficient [ρ]) and figures were generated using GraphPad Prism version 7 (GraphPad Software, Inc, San Diego, California).

3 | RESULTS

3.1 | Experiment I. Heroin-induced and 6-AM–induced CPP and locomotor sensitization

3.1.1 | Heroin-induced and 6-AM–induced CPP

All mice conditioned with heroin (1.25-5 μmol/kg) expressed significant CPP ($P < 0.05$); however, no dose-response relationship was observed (Figure 1). The heroin CPP scores ranged from 272 ± 106 seconds for 1.25 μmol/kg to 381 ± 62 seconds for 2.5 μmol/kg. 6-AM doses of 2.5 and 5 μmol/kg induced significant CPP, with scores of 168 ± 70 seconds and 159 ± 49 seconds, respectively ($P < 0.05$, Figure 1). Although 1.25 μmol/kg 6-AM produced a CPP score of 192 ± 88 seconds, the time spent in the saline compartment versus the 6-AM compartment was not statistically significant for this group; i.e., no CPP was established. No dose-response relationship was observed for 6-AM–induced CPP. Heroin induced a two times higher CPP score after 2.5 μmol/kg ($P < 0.05$) and 5 μmol/kg ($P = 0.058$) compared with equimolar doses of 6-

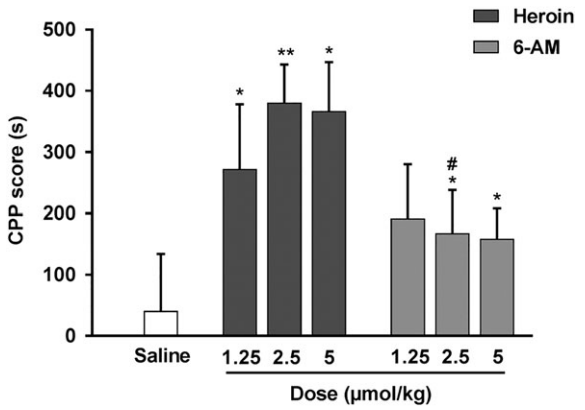

FIGURE 1 Opioid-induced conditioned place preference (CPP). Daily injections of saline, heroin, or 6-AM (1.25-5 μmol/kg, corresponding to 0.5-2.1 mg/kg, s.c.) were paired with either of two chambers for three consecutive conditioning days. The CPP test (20 min, 1200 s) was conducted after injection of saline on the fourth day. Residence time (s) in the drug-paired chamber minus time in the saline-paired chamber was calculated (CPP score). Values are expressed as mean + SEM, n = 9-15. *P < 0.05, **P < 0.01 time spent in the drug-paired chamber versus saline-paired chamber within a group (Wilcoxon signed rank test for related samples), #P < 0.05 CPP score compared with equimolar heroin dose (Mann-Whitney U test)

AM (Figure 1). The animals in the saline control group displayed no significant preference for either of the two compartments (Figure 1).

3.1.2 | Heroin-induced and 6-AM–induced locomotor sensitization

Both heroin and 6-AM produced a dose-response in locomotor activity recorded during the conditioning sessions (Figure 2). Heroin and 6-AM administration of 2.5 and 5 μmol/kg, but not 1.25 μmol/kg, induced locomotor sensitization, as defined by a significant increase in total locomotor activity from the first to the third opioid injection (P < 0.01, Figure 3A). For 2.5 and 5 μmol/kg of heroin and 6-AM, there was a 30% to 34% increase in total locomotor activity from the first injection to the third injection; however, the magnitude of sensitization did not differ between the two opioids (Figure 3A). In the opioid-exposed mice, saline injections received on afternoon conditioning sessions (2, 4, and 6) did not increase locomotor activity, rather the activity significantly decreased across repeated saline sessions (P < 0.05, Figure 3B). Mice in the control group receiving only saline injections had significantly lower locomotor activity compared with all opioid-treated groups across all morning sessions (P < 0.001, statistical symbols omitted in Figure 3A), and their total locomotor activity was significantly reduced after the third compared with after the first injection (P < 0.01, Figure 3A).

3.1.3 | The relationship between CPP and locomotor sensitization induced by heroin and 6-AM

No significant correlation was found between the CPP score and the magnitude of locomotor sensitization induced by heroin or 6-AM for each individual mouse (Figure 4).

3.2 | Experiment II. Heroin-induced and 6-AM–induced CPP and locomotor sensitization after pretreatment with anti–6-AM mAb

3.2.1 | The duration of the effect of a single anti-6-AM mAb injection on repeated heroin exposure

A single mAb pretreatment given 4 hours prior to the first heroin injection significantly reduced 6-AM brain concentrations measured after the final of either one, two, or three heroin injections (P < 0.01, Figure 5A). The anti–6-AM mAb concentration in mouse blood, measured as human IgG1 concentration, was close to the theoretical concentration of 143 μg/mL after one and two heroin injections and was reduced by 38% after three heroin injections (Figure 5B).

The brain concentrations of 6-AM (Figure 5A), morphine, and M3G in saline-pretreated mice were 0.34 to 0.39, 0.11 to 0.13, and 0.01 to 0.02 nmol/g, respectively, 25 minutes after a single or repeated heroin administration (2.5 μmol/kg/d), while no M6G was detected (LOQ 0.004 nmol/g, results not shown).

3.2.2 | Heroin-induced and 6-AM–induced CPP after pretreatment with anti–6-AM mAb

Mice received pretreatment with a single mAb dose (10-200 mg/kg) 24 hours prior to the first heroin or 6-AM injection and were submitted to the CPP procedure. Pretreatment with the highest mAb dose (200 mg/kg) inhibited the establishment of heroin-induced CPP and resulted in a 78% reduction in CPP score compared with control mice (mAb 0 mg/kg, P < 0.05, Figure 6). A nonsignificant tendency for lower heroin-induced CPP scores was observed for mice pretreated with 10 and 50 mg/kg mAb compared with controls; however, these groups still expressed significant CPP (P < 0.05, Figure 6). Pretreatment with 50 mg/kg mAb inhibited the establishment of 6-AM–induced CPP, although the CPP score was not significantly reduced compared with the control group (0 mg/kg mAb, Figure 6). As 50 mg/kg mAb was sufficient to prevent the induction of CPP and locomotor sensitization in mice injected with 2.5 μmol/kg 6-AM, higher doses of mAb were not used.

3.2.3 | Heroin-induced and 6-AM–induced locomotor sensitization after pretreatment with anti–6-AM mAb

A single mAb-pretreatment dose-dependently reduced heroin-induced and 6-AM–induced locomotor activity measured during the conditioning sessions (Figure 7). Heroin-induced (2.5 μmol/kg) locomotor activity was significantly reduced in all mAb-pretreated groups (10-200 mg/kg) compared with controls (mAb 0 mg/kg), on all three conditioning sessions (P < 0.05, Figure 8A). While 2.5 μmol/kg heroin induced a sensitized locomotor activity, with a 30% increase in activity from the first to the third heroin injection, a single pretreatment with mAb (10-200 mg/kg) completely abolished heroin-induced locomotor sensitzation (Figure 8A). 6-AM–induced (2.5 μmol/kg) locomotor activity was significantly reduced in all mAb-pretreated mice (10-50

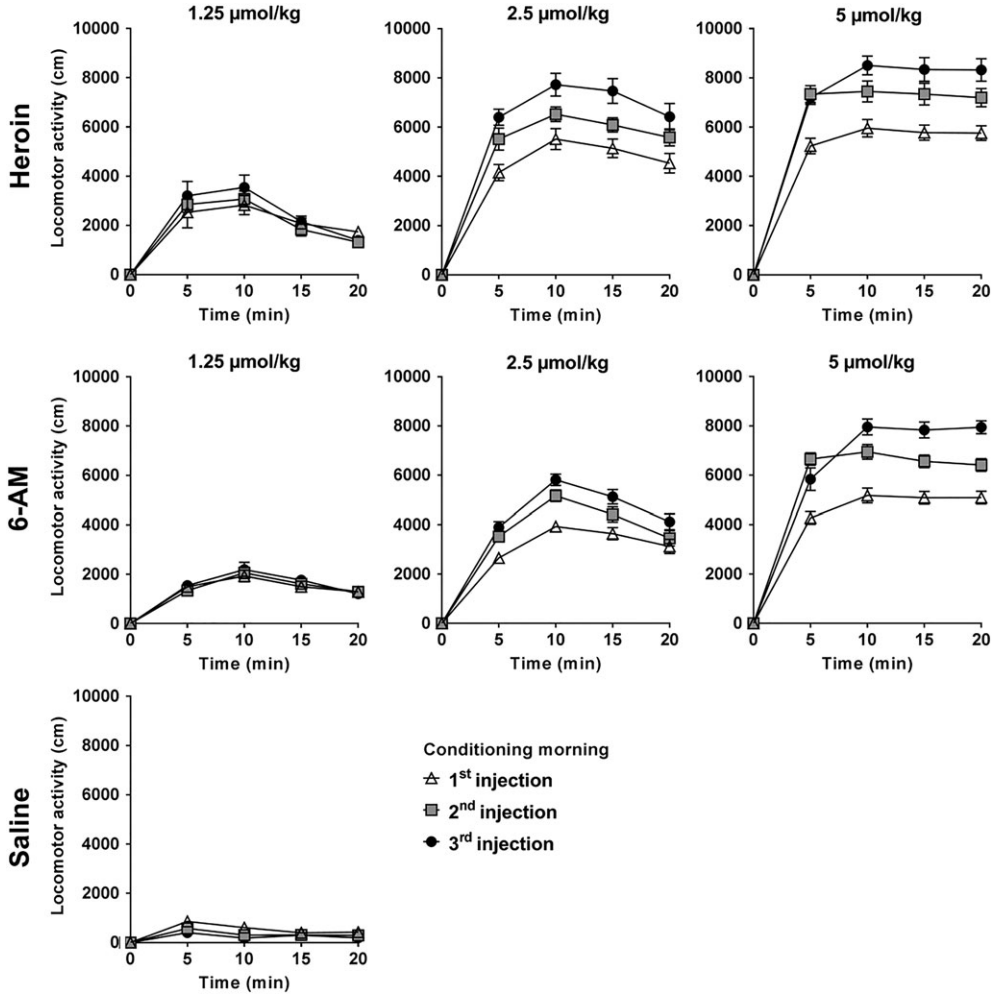

FIGURE 2 Locomotor activity (cm per 5 min bin) measured after daily injection of heroin or 6-AM (1.25-5 µmol/kg, corresponding to 0.5-2.1 mg/kg, s.c.) or saline for three consecutive days. The locomotor activity was measured during the drug conditioning sessions (morning). Values are expressed as mean ± SEM, n = 9-15. Statistical symbols are omitted for clarity

mg/kg) compared with controls (mAb 0 mg/kg), on all three conditioning sessions ($P < 0.05$, Figure 8A). While 2.5 µmol/kg 6-AM induced a 30% increase in locomotor activity from the first to the third 6-AM injection, pretreatment with 50 mg/kg mAb abolished 6-AM–induced locomotor sensitization (Figure 8A). The saline injections received on afternoon conditioning sessions (2, 4, and 6) did not increase locomotor activity, rather the activity significantly decreased across repeated saline sessions ($P < 0.05$, Figure 8B).

4 | DISCUSSION

The present study examined the contribution of heroin's first metabolite, 6-AM, to heroin-induced reward and locomotor sensitization. Our main findings show that both heroin and 6-AM induced CPP and locomotor sensitization in mice. To our knowledge, CPP caused by 6-AM exposure has not been investigated previously. Heroin generated nearly twice as high CPP scores and a more pronounced acute locomotor activity compared with 6-AM. However, pretreatment with anti–6-AM mAb inhibited both heroin-induced and 6-AM–induced CPP and locomotor sensitization, providing evidence that 6-AM is important for the rewarding and sensitizing properties of heroin.

Drug-induced CPP can be considered an indirect measure of the rewarding properties of a drug.[9] We found that both heroin and 6-AM induced CPP in mice, suggesting that both opioids have rewarding properties. While others have reported CPP after injection of 1.25 to 10 mg/kg heroin in C57BL/6J mice,[16,17,36] we found CPP after heroin doses of 1.25 to 5 µmol/kg, corresponding to 0.5 to 2.1 mg/kg.

Previous reports have indicated that heroin is a prodrug with effects mediated by the metabolites 6-AM and morphine.[19,20,23] Still, heroin induced approximately twice as high CPP scores as 6-AM, suggesting that heroin elicits a stronger reward compared with 6-AM. One explanation for the increased reward of heroin compared with 6-AM might be the higher lipophilicity of heroin, providing an efficient transfer across the BBB, and thereby a more rapid increase in the brain 6-AM concentration. We previously showed that the psychomotor stimulating effect, measured as increased locomotor activity, is stronger and emerges faster after heroin injection than after 6-AM injection[24] and that heroin provides higher brain 6-AM concentration compared with an equimolar dose of injected 6-AM.[20,24] Thus, mice might develop a stronger CPP after heroin than after 6-AM injection

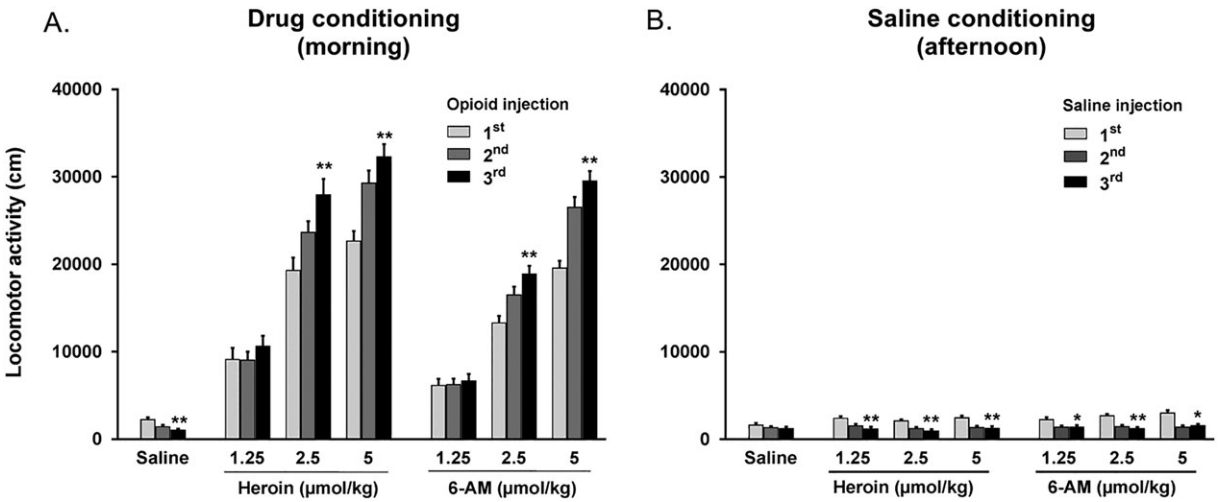

FIGURE 3 Opioid-induced locomotor sensitization. Total locomotor activity (cm per 20 min) was measured during the conditioning sessions on three consecutive days after (A) heroin or 6-AM injection (1.25-5 μmol/kg, corresponding to 0.5-2.1 mg/kg, s.c.) in the morning and (B) saline injection in the afternoon. The saline group received saline injections only for all conditioning sessions. Values are expressed as mean + SEM, n = 9-15. *P < 0.05, **P < 0.01 difference between first and third injection within groups (Wilcoxon signed rank test for related samples)

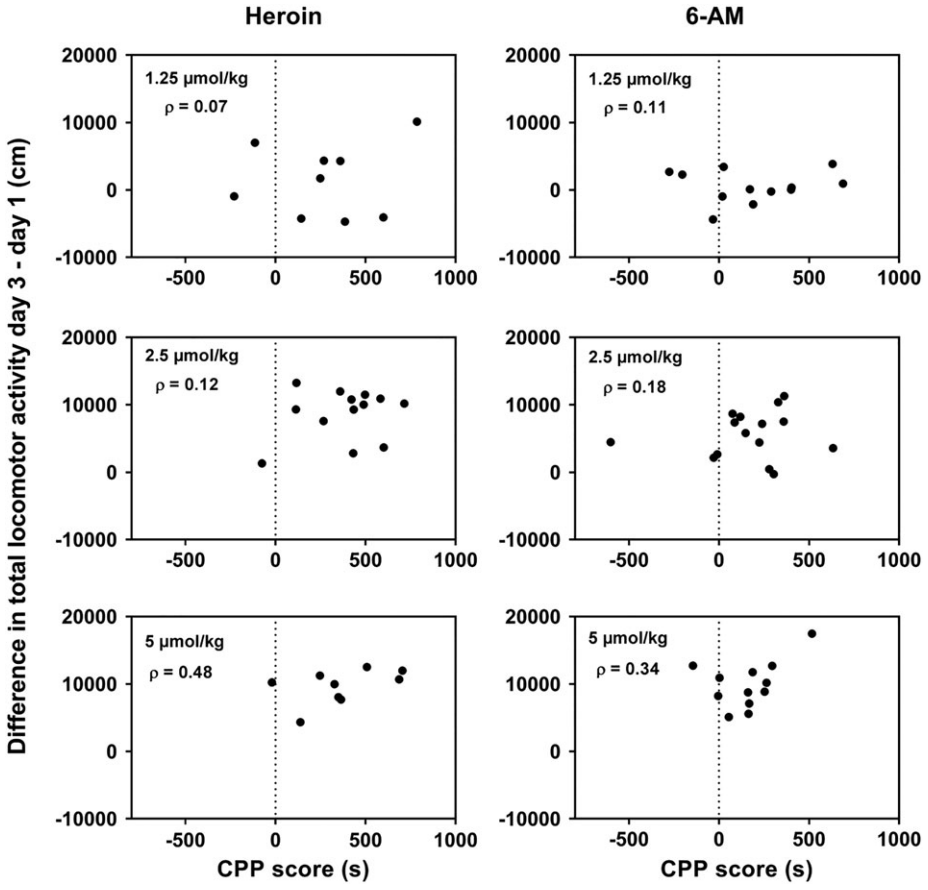

FIGURE 4 The relationship between CPP score (s) and difference in total locomotor activity (day 3 minus day 1, cm) in individual mice conditioned with daily injections of heroin or 6-AM (1.25-5 μmol/kg, corresponding to 0.5-2.1 mg/kg, s.c.), with Spearman rho (ρ), n = 9-15

due to a more efficient increase in brain 6-AM levels. Interestingly, in this respect, it has been suggested that rapid delivery of a drug to the brain may predict a high rewarding and addictive potential.[2,37] Another explanation that cannot be excluded is that reward induced by heroin may be mediated through a different mechanism than 6-AM. We know from previous studies that minor structural differences, such as removal or addition of a single acetyl group, may have profound effects on the signaling pathways initiated upon μ-opioid receptor binding.[38,39] However, the very low levels of heroin found in the brain after s.c. or intravenous (i.v.) administration of heroin[20,22,23] would imply an extremely high potency of heroin through an hitherto undescribed mechanism.

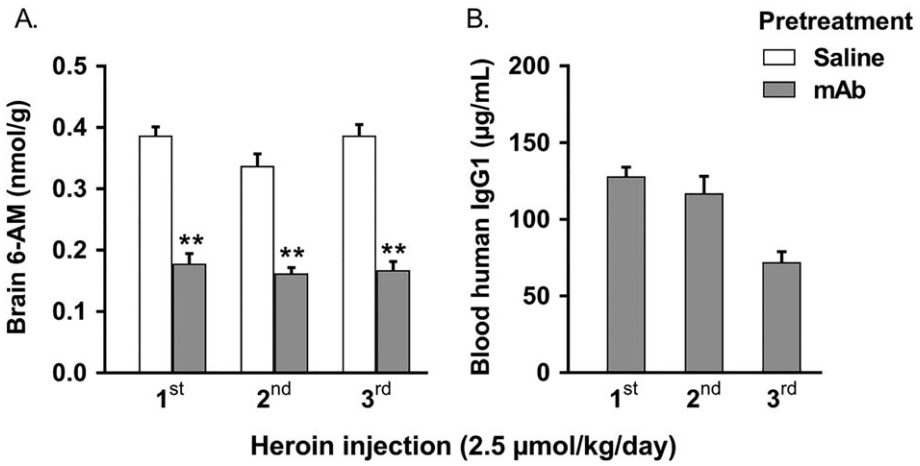

FIGURE 5 The effect of anti–6-AM mAb pretreatment upon repeated heroin injections. (A) Brain 6-AM concentration and (B) blood human IgG1 concentration after a daily injection of heroin (2.5 µmol/kg, corresponding to 1.05 mg/kg, s.c.) for up to three consecutive days, in mice pretreated with a single injection of saline or mAb (10 mg/kg, i.p.). Brain 6-AM concentration and blood IgG1 concentration were measured in samples collected 25 min after the final heroin injection. (A) n = 6-8; (B) n = 4-7. Values are expressed as mean + SEM. $^{**}P < 0.01$ against saline (Mann-Whitney *U* test)

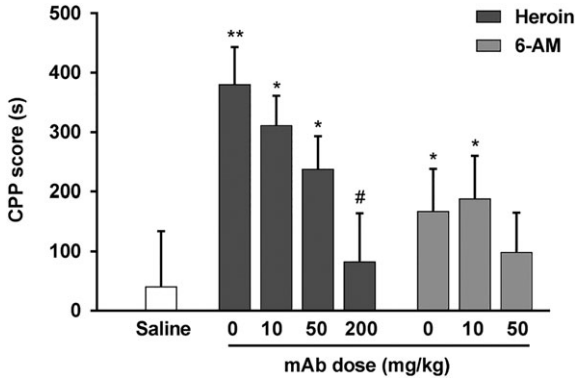

FIGURE 6 Opioid-induced conditioned place preference (CPP) in anti–6-AM mAb pretreated mice. Mice received either no pretreatment (controls, mAb 0 mg/kg) or a single injection of mAb (10-200 mg/kg, i.p.) 24 h prior to the first opioid injection. Daily injections of saline, heroin or 6-AM (2.5 µmol/kg, corresponding to 1.05 mg/kg, s.c.) were paired with either of two chambers for three consecutive conditioning days. The CPP test (20 min, 1200 s) was conducted after saline injection on the fourth day. Residence time (s) in the drug-paired chamber minus time in the saline-paired chamber was calculated (CPP score). Values are expressed as mean + SEM, n = 5-15. $^{*}P < 0.05$, $^{**}P < 0.01$ time spent in the drug-paired chamber versus saline-paired chamber within a group (Wilcoxon signed rank test for related samples), $\#P < 0.05$ CPP score against control (mAb 0 mg/kg) (Mann-Whitney *U* test)

Since the CPP procedure implies repeated drug administrations, drug-induced locomotor sensitization was examined during the CPP conditioning sessions, as shown in previous studies.[13,16,31-33] Both heroin and 6-AM generated a sensitization of the locomotor response. Heroin caused higher total locomotor activity in each test session compared with the same dose of 6-AM. However, there was no difference in the magnitude of sensitization between the two opioids, i.e., the relative increase in activity upon repeated injections. The lack of sensitization after 1.25 µmol/kg heroin demonstrated in our study coincides with other reports,[16,17] implying that low doses of heroin may produce a modest psychomotor activating effect that does not result in locomotor sensitization. Thus, low opioid doses may induce a slight increase in dopamine release, which is probably insufficient to promote long-lasting neuroplastic changes associated with a sensitized drug effect.[3] In our study, we demonstrate that the repeated saline injections did not induce locomotor sensitization, emphasizing that the sensitized effect is caused by heroin and 6-AM exposure per se.

It has been disputed whether locomotor sensitization reflects drug-induced neuroplasticity involving mechanisms related to the development of addiction.[4,11-15,40,41] To explore a potential relationship between heroin-induced and 6-AM–induced CPP and locomotor sensitization, we examined the correlation between these behaviors. The CPP scores and magnitude of sensitization did not correlate for any of the heroin or 6-AM doses, suggesting either different underlying mechanisms or brain-area specific effects. Previous studies have proposed that opioid-induced locomotor sensitization and CPP are separate responses regulated by distinct mechanisms.[13,14,33] Shabat-Simon et al.[14] reported that opioid-induced reward is dependent on glutamatergic transmission in the anterior ventral tegmental area (VTA), whereas locomotor sensitization is mediated by glutamatergic transmission in the posterior VTA. Urs et al.[42] suggested that a Dopamine 1 (D1) receptor–dependent beta arrestin2/pERK signaling complex plays an important role in morphine-induced locomotor sensitization, but not in morphine-induced reward. Thus, the findings of our study support that opioid-induced CPP and locomotor sensitization are dissociated behaviors, representing different underlying neural substrates.

For further investigation of 6-AM's contribution to heroin-induced reward and sensitization, we used a 6-AM–specific mAb that acts by sequestering 6-AM in the blood, thereby preventing its passage to the brain.[24,25] mAb pretreatment suppressed both heroin-induced and 6-AM–induced CPP and locomotor sensitization in mice, emphasizing the importance of 6-AM for these heroin-induced behavioral effects. This is in accordance with another study indicating that 6-AM is a key mediator of heroin reinforcement.[26] A recently published study of self-administration in rats found the reinforcing effects of 6-AM to be similar to those of heroin, including the ability to trigger

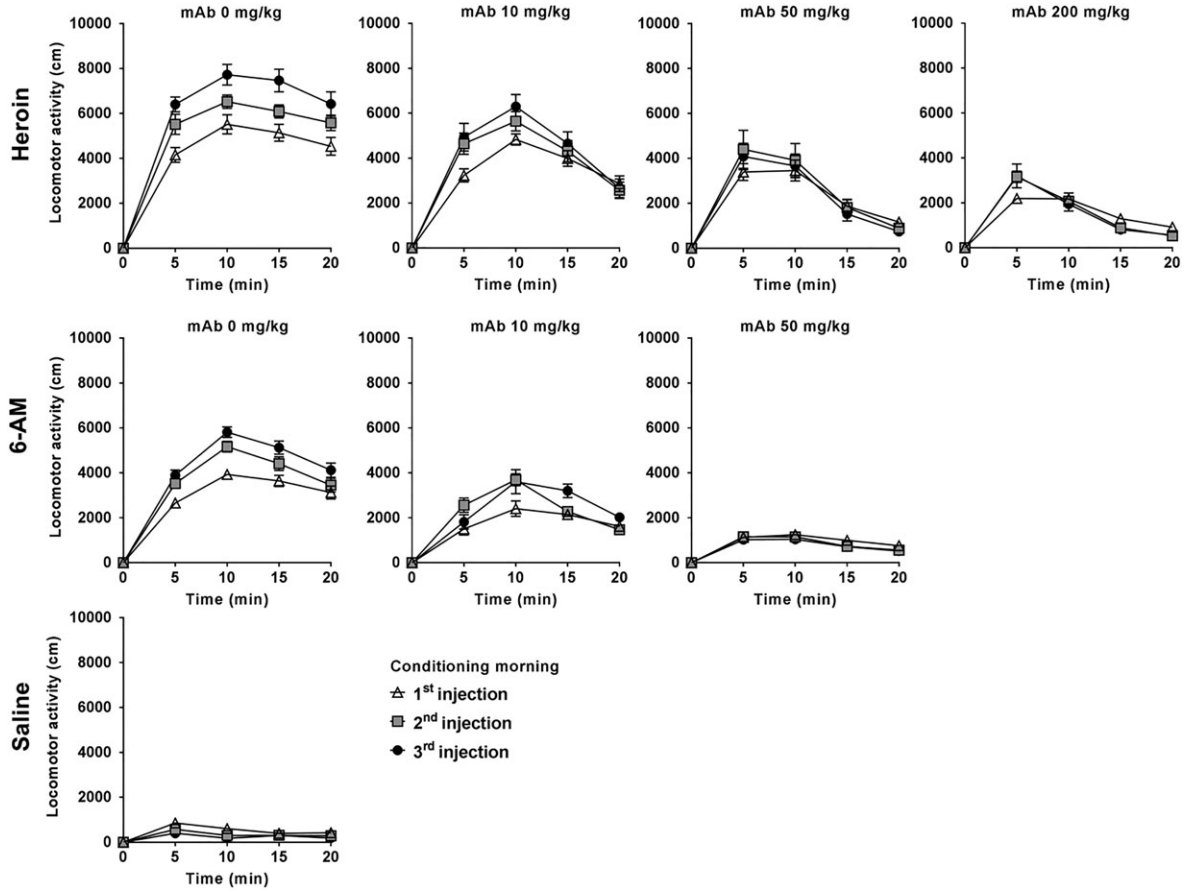

FIGURE 7 Locomotor activity (cm per 5 min bin) measured after daily injections of heroin or 6-AM (2.5 μmol/kg, corresponding to 1.05 mg/kg, s.c.) or saline for three consecutive days in control mice (0 mg/kg mAb) and mice pretreated with anti–6-AM mAb (10-200 mg/kg, i.p.). The locomotor activity was measured during the drug conditioning sessions (morning). Values are expressed as mean ± SEM, n = 5-15. Statistical symbols are omitted for clarity

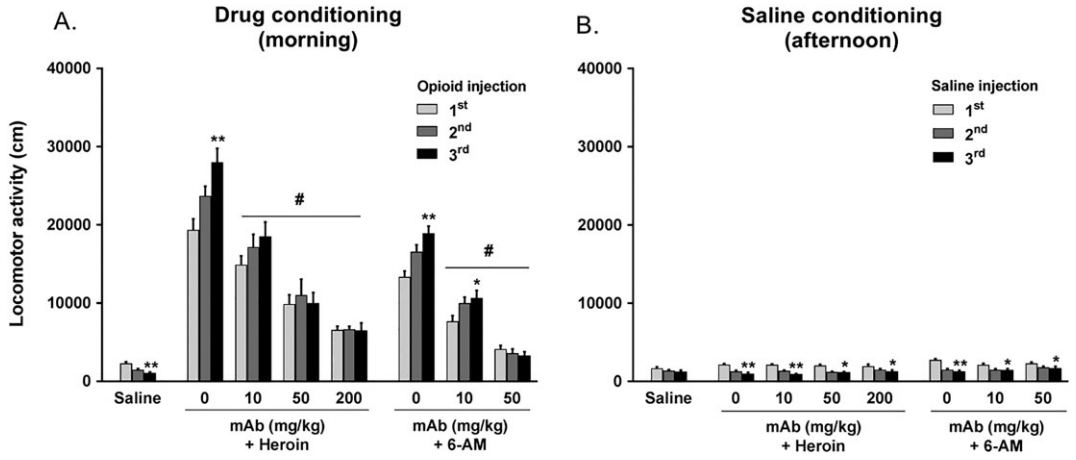

FIGURE 8 The effect of anti–6-AM mAb pretreatment on opioid-induced locomotor sensitization. Total locomotor activity (cm per 20 min) was measured in control mice (0 mg/kg) and mice pretreated with anti–6-AM mAb (10-200 mg/kg, i.p.), during the conditioning sessions on three consecutive days after (A) heroin or 6-AM injection (2.5 μmol/kg, corresponding to 1.05 mg/kg, s.c.) in the morning and (B) saline injection in the afternoon. The saline group received saline injections only for all conditioning sessions. Values are expressed as mean + SEM, n = 5-15. *P < 0.05, ** P < 0.01 difference between first and third injection within groups (Wilcoxon signed rank test for related samples), #P < 0.05 against control (mAb 0 mg/kg) for the same session (Mann-Whitney U test)

relapse into drug seeking after a period of abstinence. However, while treatment with the anti–6-AM mAb blocked relapse to a low 6-AM dose, it was ineffective against heroin-induced relapse, possibly because the mAb dose used was too low.[43] Indeed, we observed that a higher mAb dose was required to attenuate heroin-induced CPP as compared with 6-AM–induced CPP and that increasing amounts of mAb reduced heroin-induced CPP in a dose-dependent manner. Furthermore, a higher dose of anti–6-AM mAb was needed to block 6-

AM–induced locomotor sensitization and CPP, as compared to obtain a significant reduction in 6-AM–induced locomotor activity.[24,25]

We have previously shown that the locomotor activity and brain levels of 6-AM are significantly higher (145%-180%) after heroin injection than after 6-AM injection and that the difference is most profound during the first 10 to 15 minutes after injection.[23,24] The differences in 6-AM brain concentrations have been observed both after s.c. and i.v. injections, implying that differences in absorption from the injection site into the blood is not a major reason for the poorer efficacy of 6-AM. Our previous experiments have indicated that most of the injected heroin is metabolized to 6-AM prior to brain entry,[44] while a minor, but important, fraction passes directly to the brain where it is further metabolized to 6-AM.[24] With increasing doses of mAb, more of the 6-AM formed peripherally will be sequestered in blood and thus prevented from brain entry. In addition, the mAb appears to reduce the rewarding effects of heroin by efficient sequestration of 6-AM, which has been transferred from the brain to the blood, possibly due to a shift in the drug concentration gradient across the BBB, promoting drug diffusion back into the blood, as previously suggested by Janda and Treweek.[45]

In vitro characterization revealed that anti–6-AM mAb is also able to bind heroin to a minor degree.[25] However, in vivo studies have reported that heroin enters the rodent brain in equal amounts in the presence and absence of mAb.[24,46] This suggests that heroin disappears from the blood circulation too rapidly for extensive antibody binding to occur. The mAb is unable to bind morphine,[25] but the doses of heroin and 6-AM (2.5 μmol/kg; 1 mg/kg) used in the current study were probably too low to provide brain morphine concentrations required for morphine-induced behavioral effects in mice.[20]

In conclusion, we provide evidence that heroin's first metabolite, 6-AM, is a major mediator of heroin-induced CPP and therefore important for heroin reward. However, heroin appears to hold a higher reward potential compared with 6-AM, which could be explained by the higher lipophilicity of heroin providing a more efficient transfer across the BBB and a rapid increase in the brain 6-AM concentration. We also show that 6-AM mediates heroin-induced locomotor sensitization. No significant correlation was found between opioid-induced CPP and locomotor sensitization, indicating that these behaviors are dissociated with different underlying mechanisms.

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CONFLICT OF INTEREST

None.

AUTHORS CONTRIBUTION

Andersen, Bogen, Boix, Kvello, and Mørland participated in the research design. Andersen, Bogen, Boix, and Kvello conducted the experiments. Bogen, Boix, and Kvello performed the data analysis. Andersen, Bogen, Boix, Kvello, and Mørland wrote or contributed to the writing of the manuscript.

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