Title: The role of 6-acetylmorphine in heroin-induced reward and behavioral sensitization in mice

Running title: Role of 6-acetylmorphine in heroin reward

Authors: Anne Marte Sjursen Kvello[1,2], Jannike Mørch Andersen[1,2], Fernando Boix[1], Jørg Mørland[3] and Inger Lise Bogen[1,4]

[1]*Section for Drug Abuse Research, Department of Forensic Sciences, Oslo University Hospital, Norway*

[2]*School of Pharmacy, Faculty of Mathematics and Natural Sciences, University of Oslo, Norway*

[3]*Norwegian Institute of Public Health, Oslo, Norway*

[4]*Institute of Basic Medical Sciences, Faculty of Medicine, University of Oslo, Norway*

Corresponding author:

Anne Marte Sjursen Kvello

Address: PO Box 4950 Nydalen, N-0424 Oslo, Norway

Phone: 0047 41412989

E-mail: rmanmk@ous-hf.no

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 behavioral 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 behavioral sensitization. The contribution of 6-AM to heroin reward and behavioral sensitization was further examined by pretreating mice with a 6-AM specific antibody (anti-6-AM mAb) 24 h prior to the CPP procedure. Both heroin and 6-AM induced CPP in mice, but heroin generated twice as high CPP scores compared to 6-AM. Psychomotor sensitization was expressed after repeated exposure to 2.5 and 5 μmol/kg heroin or 6-AM, but not after 1.25 μmol/kg. Pretreatment with anti-6-AM mAb suppressed both heroin- and 6-AM-induced CPP and locomotor sensitization. No correlation was found between the expression of CPP and the magnitude of locomotor sensitization. We provide evidence that 6-AM is essential for the rewarding and sensitizing properties of heroin, however, heroin caused stronger reward compared to 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: Antibody, CPP, heroin, opioid, reward, sensitization.

Introduction

Heroin is considered one of the most addictive illicit drugs and is involved in numerous fatal drug intoxications worldwide each year (UNODC, 2017). Heroin induces strong euphoric and rewarding effects, and its rapid onset of action has been associated with the high abuse potential (Samaha and Robinson, 2005). As with other drugs of abuse, heroin mediates reward by extensive dopamine release in the midbrain (Wise, 1989; Fields and Margolis, 2015; Koob and Volkow, 2016). The initial drug intake can promote maladaptive changes in dopaminergic neurons and related circuitries, which may render an individual hypersensitive upon subsequent drug exposure (Robinson and Berridge, 1993; Kauer and Malenka, 2007). This drug-induced neuroplasticity is presumably an important aspect of the transition from drug use to drug addiction (Robinson and Berridge, 2008; Mazei-Robison and Nestler, 2012).

Drug-induced conditioned place preference (CPP) and behavioral sensitization are two commonly used behavioral models to study the motivational and rewarding properties of drugs in animals (Robinson and Berridge, 1993; Tzschentke, 1998; Solecki et al., 2009; Bailey et al., 2010; Guegan et al., 2016), and may be linked to the neuroplastic adaptations following drug exposure (Robinson and Berridge, 1993; Kauer and Malenka, 2007). CPP is based on classic pavlovian conditioning using repeated drug exposure in which the animal learns to associate a specific environment with the motivational properties of the drug (Bardo et al., 1995; Tzschentke, 1998). Behavioral sensitization can manifest as a progressive increase in locomotor activity after repeated drug administration (Robinson and Berridge, 1993; Vezina, 2004). Both heroin-induced CPP and locomotor sensitization have been reported in rodents even after low doses of heroin (Schlussman et al., 2008; Bailey et al., 2010).

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) (Oldendorf et al., 1972). However, heroin itself is suggested to be a pro-drug acting mainly through its metabolites 6-acetylmorphine (6-AM) and morphine (Inturrisi et al., 1983). In rodents, 6-AM is the predominant metabolite measured in blood and brain the first ~30 min after administration, whereas heroin becomes undetectable within a few minutes (Andersen et al., 2009; Gottas et al., 2012; Gottas

et al., 2013). It has also been reported that a single 6-AM injection leads to profound increases in locomotor activity and striatal dopamine release in rodents (Andersen et al., 2009; Gottas et al., 2014; Kvello et al., 2016). 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 (Bogen et al., 2014; Kvello et al., 2016). Altogether, these reports imply that 6-AM is an important mediator of the acute actions of heroin. Previous work by Raleigh and coworkers (2014) 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 behavioral 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.

Materials and Methods

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 (Andersen et al, 2009; Bailey et al, 2010; Kvello et al, 2016). 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 (Moghaddam et al., 2003; Bogen et al., 2014). 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, MA, USA) 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 (2016).

Animals

Male C57BL/6J mice (7-8 weeks old, 20-25 g; Taconic, Ejby, Denmark) were housed 4-8 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 (Grung et al., 1998; Handal et al., 2002; Andersen et al., 2009; Kvello et al., 2016). The animals were housed in plexi glass cages containing wooden bedding and small plastic houses for environmental enrichment, and acclimatized for at least five days prior to the experiments. Temperature, humidity and lights were regulated (22±1°C, 50±10% humidity, lights on from 7 AM-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 U.S. National Institutes of Health. All experimental protocols were approved by the Norwegian Animal Research Authority and conducted in accordance with the ARRIVE guidelines (McGrath and Lilley, 2015).

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

The CPP apparatus.

Cages measuring 40×40 cm, divided into two distinct compartments, 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, USA). As no significant preference for either of the two compartments was found in drug-naïve mice, the CPP apparatus was considered unbiased.

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 (6h 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, 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 for all 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 (Andersen et al, 2009; Gottas et al. 2014,). 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 min, 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 min.

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

To assess behavioral sensitization to heroin and 6-AM, the locomotor activity of the mice was recorded during conditioning, as previously shown by others (Orsini et al., 2005; Seymour and Wagner, 2008; Vindenes et al., 2009; Bailey et al., 2010; Niikura et al., 2013). For all six conditioning sessions, the animals' locomotor activity was measured during the 20 min session, using the Versamax animal activity monitoring system (AccuScan Instruments Inc., Colombus, USA). Dose-response relationships with locomotor activity measured per 5 min 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).

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

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 four 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. 25 min after the final heroin injection, the mice were anaesthetized 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. (2016). The 6-AM concentration in brain samples was quantified within 24 h after sampling by a LC-MS/MS method (Karinen et al., 2009). Blood sampling by heart puncture and quantification of human IgG1 levels by ELISA were performed as described in more detail by Kvello et al. (2016).

Heroin- 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 h 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- 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 to 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”.

Data and statistical analysis

An established CPP was defined as significantly more time spent in the drug-paired compartment compared to time spent in the saline-paired compartment within the same 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 (Cunningham et al., 2006). To assess locomotor sensitization, the total distance travelled during the first opioid conditioning session was compared to 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 to 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, IL, USA). Each dataset was checked for normal distribution using histograms and stem-and-leaf plots. The data was to a large extent not normally distributed, and non-parametric statistical tests were performed. Wilcoxon Signed Ranks 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's rho rank correlation coefficient (ρ)) and figures were generated using GraphPad Prism version 7 (GraphPad Software, Inc., San Diego, CA, USA).

Results

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

a) Heroin- and 6-AM-induced CPP

All mice conditioned with heroin (1.25-5 µmol/kg) expressed significant CPP ($P < 0.05$), however, no significant dose-response relationship was observed (Fig. 1). The heroin CPP scores ranged from 272±106 s for 1.25 µmol/kg to 381±62 s for 2.5 µmol/kg. 6-AM doses of 2.5 and 5 µmol/kg induced significant CPP, with scores of 168±70 s and 159±49 s, respectively ($P < 0.05$, Fig. 1). Although 1.25 µmol/kg 6-AM produced a CPP score of 192±88 s, 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 to equimolar doses of 6-AM (Fig. 1). The animals in the saline control group displayed no significant preference for either of the two compartments (Fig. 1).

b) Heroin- and 6-AM-induced locomotor sensitization

Both heroin and 6-AM produced a dose-response in locomotor activity recorded during the conditioning sessions (Fig. 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 ($P < 0.01$, Fig. 3A). For 2.5 and 5 µmol/kg heroin and 6-AM, there was a 30-34% 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 (Fig. 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 ($P < 0.05$, Fig. 3B). Mice in the control group receiving only saline injections had significantly lower locomotor activity compared to all opioid treated groups across all morning sessions ($P < 0.001$, statistical symbols omitted in Fig. 3A), and their total locomotor activity was significantly reduced after the third compared to after the first injection ($P < 0.01$, Fig. 3A).

c) 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 (Fig. 4).

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

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

A single mAb pretreatment given four 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 ($P < 0.01$, Fig. 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 (Fig. 5B).

b) Heroin- and 6-AM-induced CPP after pretreatment with anti-6-AM mAb

Mice recieved pretreatment with a single mAb dose (10-200 mg/kg) 24 h prior to the first heroin/6-AM injection, and were submitted for 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 to control mice (mAb 0 mg/kg, $P < 0.05$, Fig. 6). A non-significant tendency for lower heroin-induced CPP scores was observed for mice pretreated with 10 and 50 mg/kg mAb

compared to controls, however, these groups still expressed significant CPP ($P < 0.05$, Fig. 6). Pretreatment with 50 mg/kg mAb inhibited the establishment of 6-AM-induced CPP, although the CPP score was not significantly reduced compared to the control group (0 mg/kg mAb, Fig. 6).

c) Heroin- and 6-AM-induced locomotor sensitization after pretreatment with anti-6-AM mAb

A single mAb-pretreatment dose-dependently reduced heroin- and 6-AM-induced locomotor activity measured during the conditioning sessions (Fig. 7). Heroin-induced (2.5 µmol/kg) locomotor activity was significantly reduced in all mAb-pretreated groups (10-200 mg/kg) compared to controls (mAb 0 mg/kg), on all three conditioning sessions ($P < 0.05$, Fig. 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 (Fig. 8A). 6-AM-induced (2.5 µmol/kg) locomotor activity was significantly reduced in all mAb-pretreated mice (10-50 mg/kg) compared to controls (mAb 0 mg/kg), on all three conditioning sessions ($P < 0.05$, Fig. 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 (Fig. 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 ($P < 0.05$, Fig. 8B).

Discussion

The present study examined the contribution of heroin's first metabolite, 6-AM, to heroin-induced reward and behavioral sensitization. Our main findings show that both heroin and 6-AM induced CPP and locomotor sensitization in mice. To our knowledge, this study is the first to examine the effect of 6-AM on CPP and locomotor sensitization. Heroin generated nearly twice as high CPP scores and a more pronounced acute locomotor activity compared to 6-AM. However, pretreatment with anti-6-AM mAb inhibited both heroin- 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 (Tzschentke, 1998). 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 1.25-10 mg/kg heroin in C57BL/6J mice (Schlussman et al., 2008; Solecki et al., 2009; Bailey et al., 2010), we found CPP after heroin doses of 1.25-5 µmo/kg, corresponding to 0.5-2.1 mg/kg.

Previous reports have indicated that heroin is a prodrug with effects mediated by the metabolites 6-AM and morphine (Inturrisi et al., 1983; Andersen et al., 2009; Gottas et al., 2014). Still, heroin induced approximately twice as high CPP scores as 6-AM, suggesting that heroin elicits a stronger reward compared to 6-AM. One explanation for the increased reward of heroin compared to 6-AM might be the higher lipophilicity of heroin, providing an efficient transfer across the BBB, and thereby a 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 (Kvello et al., 2016), and that heroin provides higher brain 6-AM concentration compared to an equimolar dose of injected 6-AM (Andersen et al., 2009; Kvello et al., 2016). Thus, mice might develop a stronger CPP after heroin than after 6-AM injection 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 (Gossop et al., 1992; Samaha and Robinson, 2005). Another explanation which 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 (Frolich et al., 2011; Pasternak and Pan, 2013). However, the very low levels of heroin found in the brain after s.c. or i.v. administration of heroin (Andersen et al., 2009; Gottas et al., 2013; Gottas et al., 2014) would imply an extremely high potency of heroin through an hitherto undescribed mechanism, and lacks support in the literature.

In the present study, drug-induced locomotor sensitization was examined during the CPP conditioning sessions to gain as much information as possible from the experimental set up, as previously shown (Orsini et al., 2005; Seymour and Wagner, 2008; Vindenes et al., 2009; Bailey et al., 2010; Niikura et al., 2013). Both heroin and 6-AM generated a sensitization of the locomotor response, which may reflect drug-induced neuroplasticity, involving mechanisms related to the development of addiction (Wise and Bozarth, 1987; Robinson and Berridge, 1993; Vezina, 2004). Heroin caused higher total locomotor activity in each test session compared to 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 for 1.25 μmol/kg heroin demonstrated in our study coincides with other reports (Schlussman et al., 2008; Bailey et al., 2010) 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 (Kauer and Malenka, 2007). 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.

To explore a potential relationship between heroin- 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. A number of previous studies have proposed different underlying mechanisms for morphine-induced locomotion and reward (Tzschentke, 1998; Borgkvist et al., 2007; Vindenes et al., 2009; Bailey et al., 2010). Urs and colleagues (2011) found a dopamine receptor dependent beta arrestin2/pERK signaling complex with an important role in

morphine-induced locomotion, but not in morphine reward. Another study demonstrated that serotonergic lesions in nucleus accumbens attenuated morphine-induced CPP but increased analgesia and locomotor activity in rats (Spyraki et al., 1988). Our current finding therefore coincides with several studies reporting that opioid-induced CPP and locomotor sensitization are not correlated behaviors.

For further investigation of 6-AM's contribution to heroin-induced reward and sensitization, we used a 6-AM specific mAb which acts by sequestering 6-AM in the blood, thereby preventing its passage to the brain (Bogen et al., 2014; Kvello et al., 2016). mAb pretreatment suppressed both heroin- 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 (Raleigh et al., 2014). We observed that a higher mAb dose was required to attenuate heroin-induced CPP as compared to 6-AM-induced CPP, and that increasing amounts of mAb reduced heroin-induced CPP in a dose-dependent manner. We have previously shown that brain levels of 6-AM are significantly higher (145 to 180%) after heroin injection than after 6-AM injection, and that the difference is most profound during the first 10 to 15 min after injection (Gottas et al., 2014; Kvello et al., 2016). This finding has been observed both after s.c. and i.v. injection, and eliminates a more rapid transport of heroin from the s.c. injection site to the blood as a possible explanation. Our previous mAb experiments indicated that most of the injected heroin is metabolized to 6-AM prior to brain entry, while a minor, but important, fraction passes directly to the brain (Kvello et al., 2016). 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 (2012).

In vitro characterization revealed that anti-6-AM mAb is also able to bind heroin to a minor degree (Bogen et al., 2014). However, in vivo studies have reported that heroin enters the rodent brain in equal amounts in the presence and absence of mAb (Raleigh et al., 2013; Kvello et al., 2016). This

suggests that heroin disappears from the blood circulation too rapidly for extensive antibody binding to occur. The mAb is unable to bind morphine (Bogen et al., 2014), but the doses of heroin and 6-AM (2.5 µmol/kg; 1 mg/kg) used in the current study were too low to provide brain morphine concentrations required for morphine-induced behavioral effects in mice (Andersen et al., 2009).

In conclusion, we provide evidence that heroin's first metabolite, 6-AM, is an important mediator of heroin-induced CPP and locomotor sensitization, and is therefore essential to heroin reward. No significant correlation was found between opioid-induced CPP and locomotor sensitization. We show that heroin holds a higher rewarding potential compared to 6-AM, which may be explained by the higher lipophilicity of heroin, providing an efficient transfer across the BBB, and a rapid increase in the brain 6-AM concentration.

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Authorship Contributions

Participated in research design: Andersen, Bogen, Boix, Kvello and Mørland

Conducted experiments: Andersen, Bogen and Kvello

Performed data analysis: Bogen, Boix and Kvello

Wrote or contributed to the writing of the manuscript: Andersen, Bogen, Boix, Kvello and Mørland

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Figure legends

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 more time spent in the drug-paired chamber versus saline-paired chamber within a group (Wilcoxon Ranks test for related samples), #*P* < 0.05 reduced CPP score compared to equimolar heroin dose (Mann-Whitney U test).

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.

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 1st and 3rd injection within groups (Wilcoxon Ranks 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's rho (ρ), n = 9-15.

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. $^{**}P < 0.05$ 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. $^{*}P < 0.05$, $^{**}P < 0.01$ more time spent in the drug-paired chamber versus saline-paired chamber within a group (Wilcoxon Ranks test for related samples), $\#P < 0.05$ reduced CPP score against control (mAb 0 mg/kg) (Mann-Whitney U test).

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. $^{*}P < 0.05$ difference between 1^{st} and 3^{rd} injection within groups (Wilcoxon Ranks test for related samples), $\#P < 0.05$ against control (mAb 0 mg/kg) for the same session (Mann-Whitney U test).