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Interactions between morphine and the morphine-glucuronides measured by conditioned place preference and locomotor activity

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ABSTRACT

After intake of heroin or morphine, active metabolites are formed in the body. The two most important morphine metabolites are morphine-6-glucuronide (M6G) and morphine-3-glucuronide (M3G). M6G and M3G are present for longer time periods and in higher concentrations than the parent drug, but their potential contribution to reward and to development of dependence and addiction is not clear.

We tested the effects of morphine and M6G separately (doses of 10, 20, 30 and 50 μmol/kg), administered together, and also in combination with with 200 μml/kg M3G in male C57BL/6J-Bom mice. M3G in doses of 50, 100, 200, 300 and 400 μmol/kg were also tested alone. We evaluated the rewarding effects in a conditioning place preference (CPP) model and the psychomotor stimulating effects by recording locomotor activity.

Mice were subjected to three consecutive conditioning days with drugs or saline before testing. Changes in locomotor activity from conditioning day one to day three were also compared to the expression of CPP on the test day.

This study revealed that coadministration of morphine and M6G induced CPP of similar magnitude to the sum of equimolar doses of these compounds alone, and different ratios of the two drugs did not affect the results. M3G did not cause CPP and reduced the CPP induced by both morphine and M6G when coadministered with these drugs. Morphine induced locomotor activity was reduced by coadministration of M3G, but this was not seen when M3G was co-injected with M6G. The changes in locomotor activity during the conditioning periods did not correlated with the expression of CPP.

This study revealed that the morphine-glucuronides in different and complex ways can influence the pharmacological effects of psychomotor activation and reward observed after intake of morphine.

1. Introduction

Heroin and morphine can exhibit their actions through several metabolites. In man, heroin is metabolised after a few minutes to the μ-opiate receptor agonist 6-mono-acetylmorphine, and further to morphine, which is considered to mediate a substantial proportion of the effects seen after heroin administration (Rook et al., 2006). Morphine is conjugated to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), and the metabolites are present in higher concentrations and for longer than the parent drug (Lawrence et al., 1992; Christrup, 1997). The contribution these two morphine metabolites may make to the rewarding and psychomotor stimulating effects seen after intake of heroin or morphine, and how they might interact with each other, is, however, not clear.

M6G is a potent antinociceptive agent (Kilpatrick and Smith, 2005) and has rewarding properties similar to morphine (Abbott and Franklin, 1991; Vindenes et al., 2006, 2008). The role of M3G is more complex. Studies have shown that M3G has very low affinity for the μ-opioid receptor (Pasternak et al., 1987; Christrup, 1997) and does not have analgesic or ventilatory depressive effects (Milne et al., 1996). Some animal studies have shown that M3G can antagonize morphine analgesia (Smith et al., 1990; Gong et al., 1992; Ekblom et al., 1993), but other studies have not confirmed this (Suzuki et al., 1993; Ouellet and Pollack, 1997). M3G has been reported to have excitatory behavioural effects, which are mediated by non-opioid receptors (Smith, 2000). M3G alone does not increase locomotor activity in mice (Morland et al., 1994; Handal et al., 2002; Vindenes et al., 2006), but influences locomotor activity after treatment with morphine or M6G (Handal et al., 2007). We have previously looked at the effect of M3G in a biased conditioned place preference (CPP) model, and found that M3G did not induce CPP (Vindenes et al., 2006). For M6G, we have shown that induction of CPP is affected by different time schedules during the

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conditioning phase. The greatest CPP was induced for morphine when 20 minute conditioning sessions were performed directly after drug injection, but for M6G the best result was seen when the 20 minute conditioning sessions were started 15 minutes after treatment (Vindenes et al., 2008).

Until now, the rewarding effects of morphine and M6G have only been studied separately in the CPP model. However, after intake of heroin or morphine in humans, morphine, M3G and M6G are all found in the blood at the same time (Kilpatrick and Smith, 2005). In C57BL mice the half-lives of morphine, M6G and M3G are reported to be 28, 25 and 27 minutes respectively, and the subcutaneous bioavailability is approximately one (Handal et al., 2002).

The concentration ratios of the different drugs in blood vary between subjects and depend on the site of administration and the time since drug intake, with the glucuronides generally present in higher concentrations (Lotsch, 2005). It has generally been shown that the ratio between two drugs can affect their potency, with some combinations leading to additive properties and other combinations leading to superadditive properties. This has also been shown for opioid agonists (Adams et al., 1993). However, the additive analgesic effect of M6G after administration of morphine in humans is variable, and has only been demonstrated in a few clinical studies (Skarke et al., 2003; Andersen et al., 2003).

No studies, as far as we know, have investigated CPP in rodents mimicking the human situation after administration of morphine, with the parent drug and the morphine-glucuronides present at the same time. Mice metabolise morphine to M3G, but they are not able to produce M6G (Christrup, 1997; Handal et al., 2002). This makes it possible to compare the effects of morphine and M6G.

The aim of this study was to investigate how the rewarding properties of morphine and M6G were influenced when both drugs were administered together and also in combination with M3G. Different combinations of these substances were tested in the CPP model and locomotor activity during conditioning was also measured to evaluate possible effects on psychomotor stimulation.

2. Material and methods

2.1. Animals

A total of 253 C57BL/6J-Bom male, adult (6–8 weeks) mice (from Bomholt, Denmark), weighing 17–25.3 g at testing, were included in the experiments. At least 5 days prior to the experiments, six mice were housed together in standard plastic cages, containing small red shelters to enrich the environment. Except during the behavioural tests, food and water were available ad libitum. The colony room was illuminated with a 12-hour light–dark schedule, with the light period from 07:00 to 19:00. The temperature was maintained at 23 ± 1 °C. The experiments were carried out during the light cycle of the day under dimmed light. The mice were not handled prior to the experiments and each mouse was tested only once. The experimental protocol of this study was approved by the Norwegian National Animal Research Authority.

2.2. Recording of CPP and locomotor activity

Place preference and locomotor activity were measured using a Versamax animal activity monitoring system (AccuScan Instruments Inc., Colombus, USA). The cage size was 40×40 cm with infrared beams at 2.5 cm spacings. Each cage was divided into two distinct compartments connected by an opening in the centre of the box, which remained closed during the conditioning sessions. One compartment had white walls with a meshed metal plate as its floor. The other compartment had vertical black and white stripes (2 cm wide) on the walls and a metal plate with holes (4 mm Ø) as the floor. Both compartments had a transparent ceiling. The apparatus was considered to be unbiased, since no significant preference was recorded in drug-naïve mice for either of the two compartments (Vindenes et al., 2008).

2.3. Drugs

Morphine hydrochloride was purchased from Norsk Medisinaldepot (Oslo, Norway). Morphine-6-β-D-glucuronide hydrate and morphine-3-β-D-glucuronide hydrate were purchased from Lipomed (Arlesheim, Switzerland). The drugs were dissolved in 0.9% saline. When combinations of different drugs in different doses were prepared, the substances were dissolved together. The drugs were easy to dissolve and did not crystallize. The injection volumes were 0.01 ml/g mouse.

2.4. Experimental design

The mice were randomly assigned to the different treatment groups. The CPP experiments included six conditioning sessions followed by a test session. Conditioning was carried out twice daily for 3 consecutive days, one after drug and the other after saline injection, with a 6-hour interval in between. Half of the mice in each group were conditioned with drug (paired compartment) to the striped compartment and the other half to the white compartment. The saline injections before the mice were placed in the unpaired compartment followed the same procedure as the drug injections for the paired compartment. All the injections were subcutaneous, on the back, about 1 cm cranial from the tail. The locomotor activity during all the daily conditioning sessions was recorded.

On day 4, the mice were injected with saline and tested for 20 minutes in a drug-free state. During testing, the mice had free access to both the white and the striped compartments. All the animals, whether conditioned immediately or with a delay after the injections, followed the same procedures during the testing session as during conditioning.

A control group ($n = 12$ mice) was injected with only saline before conditioning in both the paired and unpaired compartments, and conditioned for 40 minutes immediately after the injections.

Three different experiments were conducted:

2.4.1. CPP and locomotor activity after injection of morphine or M6G alone or in combination

Different doses of morphine, M6G or the combination of both were administered as described in Table 1 (in total 13 different groups). Forty minute conditioning sessions immediately after treatment were used for all the groups in this experiment. This time was chosen because of the results from a previous study (Vindenes et al., 2008) where 20 minute conditioning, 15 minutes after treatment, induced the highest CPP for M6G, while immediate conditioning for 20 minutes yielded the best result after morphine treatment. The 40 minute conditioning included the period where acquisition of CPP took place for both drugs. So we could compare the results across the different groups, they all followed the same time schedule for the conditioning sessions. Locomotor activity was recorded during all conditioning sessions. Each dose group consisted of 6 mice.

Table 1
The different doses (μmol/kg) of morphine, M6G, and the different combinations of both drugs, which have been injected subcutaneously.

Separate administration of:		Coadministration of: morphine and M6G
Morphine	M6G	
10	10	
20	20	Morphine 10 + M6G 10
30	30	Morphine 10 + M6G 20 Morphine 20 + M6G 10
50	50	Morphine 20 + M6G 30 Morphine 30 + M6G 20

2.4.2. CPP and locomotor activity for M3G in different time schedules

To enable us to choose a suitable dose and time schedule for the interaction studies with M3G and morphine or M6G, M3G was investigated alone in doses of 50, 100, 200, 300 or 400 μmol/kg.

In a previous CPP study testing M3G we used 120 minute conditioning sessions (Vindenes et al., 2006). The time schedules (20 and 40 minutes) for the present experiments were chosen based on the results for morphine and M6G from our preceding study (Vindenes et al., 2008). Twenty minute conditioning sessions both immediately and with a 15 minute delay after drug injection were thus investigated for M3G 50, 100, 200, 300 or 400 μmol/kg. To be able to compare the M3G results with the results after coadministration of M3G with morphine or M6G, the 40 minute time schedule was also tested with M3G 200 and 400 μmol/kg. Locomotor activity was recorded during all conditioning sessions. Each dose group consisted of 6–9 mice.

2.4.3. CPP and locomotor activity after injection of M3G in combination with morphine or M6G

Co-injection of 10 or 30 μmol/kg morphine or M6G with 200 μmol/kg M3G was studied in the CPP model, using the 40 minute conditioning schedule.

In a previous study the most marked CPP was found when 20 minute conditioning sessions were performed either direct after morphine or 15 minutes delayed after M6G injections (Vindenes et al., 2008). Therefore, we also wanted to explore if these time-schedule induced CPP if M3G was co-injected with 30 μmol/kg morphine or M6G.

Locomotor activity was recorded during all the conditioning sessions. Each dose group consisted of 6–8 mice.

2.5. Data presentation and statistical analysis

Time spent in the drug-paired compartment minus time spent in the unpaired compartment was used as a measure of preference for the drug-paired compartment. A positive result was interpreted as CPP. Statistical significant differences between the different treatment groups were evaluated using ANOVA with dose as the between subjects factor followed by Dunnetts post hoc test to compare each dose with the saline treated animals. Student's independent *t*-test was performed if only two groups were compared. The CPP results from the whole 20 minute test period were used for statistical analysis.

The locomotor activity data are shown from conditioning day one, either as distance travelled (cm/5 minutes) or as total distance travelled (cm/40 minutes). The groups conditioned for 20 minutes directly after treatment or after a time delay were not compared because of the different recording periods after drug administration. The groups that were conditioned for 40 minutes after treatment with 30 μmol/kg morphine or M6G were analysed. To compare the change in total distance travelled from conditioning day one to three, paired sample *t*-test was used for each dose. The interaction between M3G and morphine or M6G on the locomotor activity was investigated during conditioning day one. The change in locomotor activity after treatment with 30 μmol/kg morphine or M6G alone or in coadministration with M3G were compared using Student's *t*-tests.

The change in total distance travelled from conditioning day one to day three was compared to the CPP during testing for each mouse, to see if the two measurements were related to each other using correlation analysis. A comparison of locomotor activity and CPP was done for mice that were conditioned for 40 minutes after treatment with morphine, M6G, the combination of both or when M3G was co-injected with morphine or M6G.

p values less than 0.05 were taken to be statistically significant. Data is presented as mean ± S.E.M. The statistical analyses were conducted using the statistical package SPSS 14.0.

3. Results

3.1. CPP and locomotor activity after injection of morphine or M6G alone or in combination

3.1.1. CPP

The 13 different treatment groups are shown in Table 1. Coadministration of morphine and M6G induced the same degree of CPP as administration of a dose equimolar to the drug combination of either of the drugs alone, and did not reveal any significant difference whether morphine or M6G was the higher drug dose in the combination (Fig. 1). The combinations of morphine and M6G that equalled 30 μmol/kg (Fig. 1B) induced a statistically significant CPP [$F(4, 34) = 4.4$, $p<0.01$], and post hoc tests revealed that all the doses were statistically significant compared to saline; morphine ($p = 0.05$), M6G ($p = 0.01$) and both combinations of morphine and M6G ($p<0.01$). The combinations of morphine and M6G that equalled 20 μmol/kg (Fig. 1A) did not induce statistically significant CPP [$F(3, 28) = 2.6$, $p = 0.08$], and neither did the group treated with the 50 μmol/kg (Fig. 1C) doses [$F(4, 34) = 1.0$, $p = 0.4$].

3.1.2. Locomotor activity

An increase in locomotor activity during conditioning day one (Fig. 2) was seen after treatment with different doses of morphine [$F(4,35) = 58.25$, $p<0.001$] (Fig. 2A). Post hoc testing revealed that all the doses increased locomotor activity significantly from saline ($p = 0.04$ for morphine 10 μmol/kg and $p<0.001$ for morphine 20, 30 and 50 μmol/kg).

The different doses of M6G increased the locomotor activity during conditioning day one [$F(4,41) = 16.43$, $p<0.001$] and post hoc testing revealed that M6G 30 ($p = 0.002$) and 50 ($p<0.001$) μmol/kg were significant from saline (Fig. 2B).

Coadministration of morphine and M6G increased the locomotor activity [$F(5, 53) = 14.51$, $p<0.001$] during conditioning day one (Fig. 2C). Post hoc tests revealed that the combinations of morphine and M6G that equalled 30 or 50 μmol/kg increased the activity significantly from saline ($p<0.001$), while the combination of morphine 10 and M6G 10 μmol/kg was not different from saline ($p = 0.41$).

The change in total distance from conditioning day one to conditioning day three showed that 10 μmol/kg of both morphine ($t_5 = 3.66$, $p<0.015$) and M6G ($t_5 = 3.45$, $p<0.018$) led to a reduction in locomotor activity (tolerance). In contrast morphine 30 ($t_5 = -5.24$, $p = 0.003$) and 50 μmol/kg ($t_5 = -5.25$, $p = 0.003$) and 30 μmol/kg M6G ($t_5 = -9.06$, $p<0.001$) increased the activity (sensitisation). No significant change in total distance travelled were seen for 20 μmol/kg morphine ($t_5 = 0.28$, $p = 0.8$) or M6G in doses of 20 ($t_5 = 1.71$, $p = 0.1$) or 50 μmol/kg ($t_5 = -0.79$, $p = 0.5$).

When morphine and M6G were injected in combination, an increase in locomotor activity on day three compared to day one was observed for the combination of morphine 20 and M6G 30 μmol/kg ($t_5 = -3.56$, $p = 0.016$), for morphine 30 and M6G 20 μmol/kg ($t_5 = -4.24$, $p<0.008$ for) as well as for the combination of morphine 20 and M6G 10 μmol/kg ($t_5 = -4.53$, $p = 0.001$). No change in activity was seen for the combination of morphine 10 and M6G 10 μmol/kg ($t_5 = -0.53$, $p = 0.6$) or the combination of morphine 10 and M6G 20 μmol/kg ($t_5 = 0.95$, $p = 0.4$).

3.2. CPP for M3G in different time schedules

M3G did not induce CPP (Fig. 3A) either after 20 minute conditioning, immediately after the injections [$F(5, 54) = 0.73$, $p = 0.6$], or 15 minutes after the drug injections [$F(5, 55) = 1.1$, $p = 0.4$]. Data from the mice injected with M3G 200 μmol/kg are shown in Figs. 4 and 5. Conditioning for 40 minutes with 200 or 400 μmol/kg M3G immediately after drug injections (Fig. 3B) did not induce statistically significant CPP [$F(2, 29) = 0.7$, $p = 0.5$].

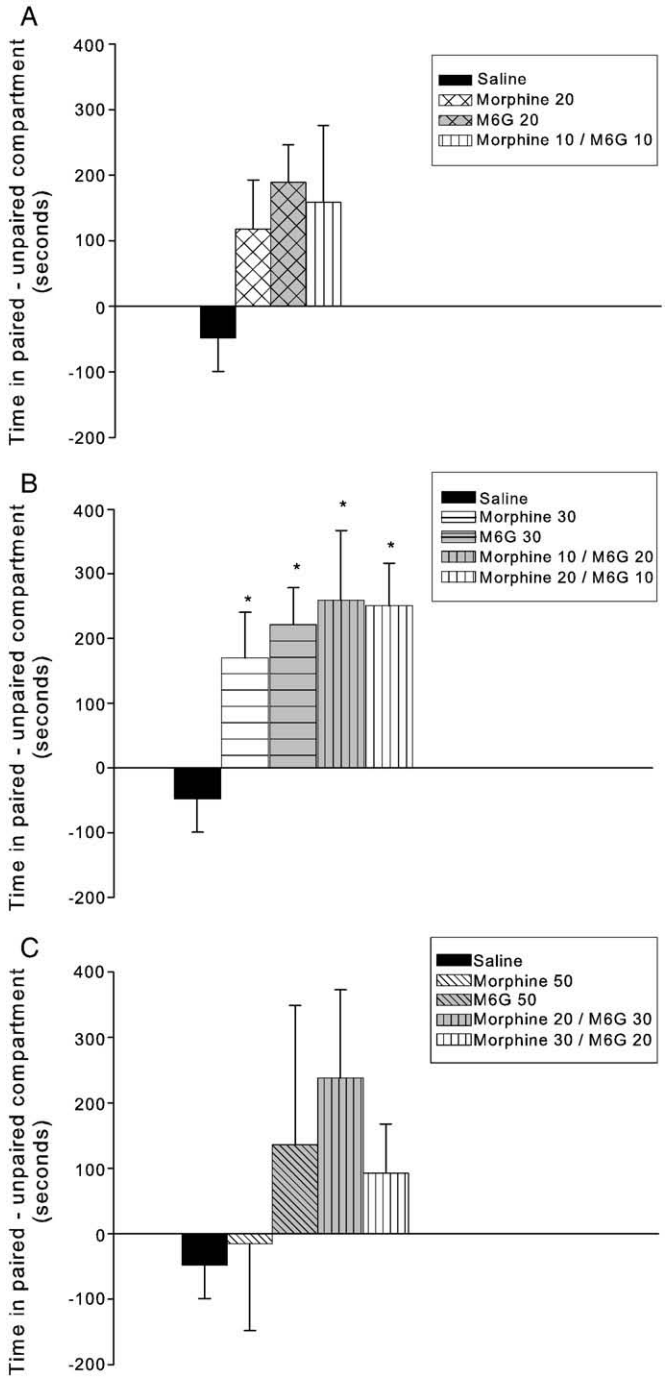

Fig. 1. CPP after 40 minute' conditioning directly after treatment with morphine or M6G alone or in different combinations. Doses are expressed in μmol/kg. Bars illustrate mean ± SEM. $n = 6$–8 mice for each drug treatment compared to the saline group ($n = 12$ mice). *$p < 0.05$.

As we did not find a dose–response relationship for the M3G doses we tested, and because of the possibility that an interaction with morphine or M6G could shift the M3G effects either to the left or right, we selected the middle dose of 200 μmol/kg M3G for the interaction studies. The 20 minute conditioning sessions, either immediately or 15 minutes after drug injection, did not result in higher CPP than the 40 minute conditioning (Fig. 3). To be able to compare the results with the morphine–M6G interaction studies and to investigate the interaction on locomotor activity during conditioning, we chose the 40 minute time schedule for the following experiments.

3.3. CPP and locomotor activity after injection of M3G in combination with morphine or M6G

3.3.1. CPP

Statistically significant CPP was seen after 40 minute conditioning with morphine 10 ($t_{16} = -3.55$, $p = 0.007$; Fig. 4A) or 30 μmol/kg ($t_{16} = -2{,}47$, $p = 0.03$; Fig. 4B). Co-injection of M3G prevented the significant CPP induced by 10 μmol/kg morphine ($t_{16} = -0.38$, $p = 0.7$; Fig. 4A), but not the CPP induced by 30 μmol/kg morphine

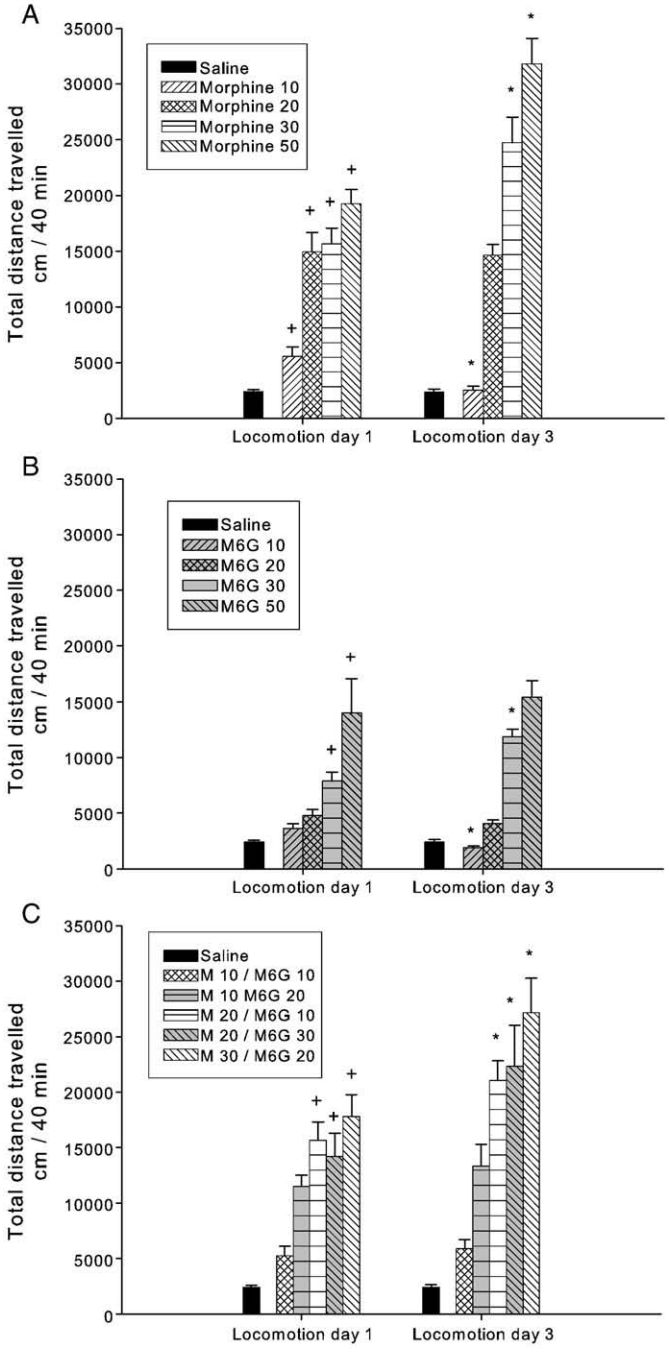

Fig. 2. Total distance travelled in cm/40 minutes (mean ± SEM) after treatment with different doses (μmol/kg) of morphine (A), M6G (B) or the combination of both morphine and M6G (C) on conditioning day one and day three. $^{+}p < 0.05$ when locomotor activity during conditioning day one is different from saline. *$p < 0.05$ when the locomotor activity during conditioning day one is different from conditioning day three. $n = 6$ mice for each drug treatment and $n = 12$ mice for the saline group.

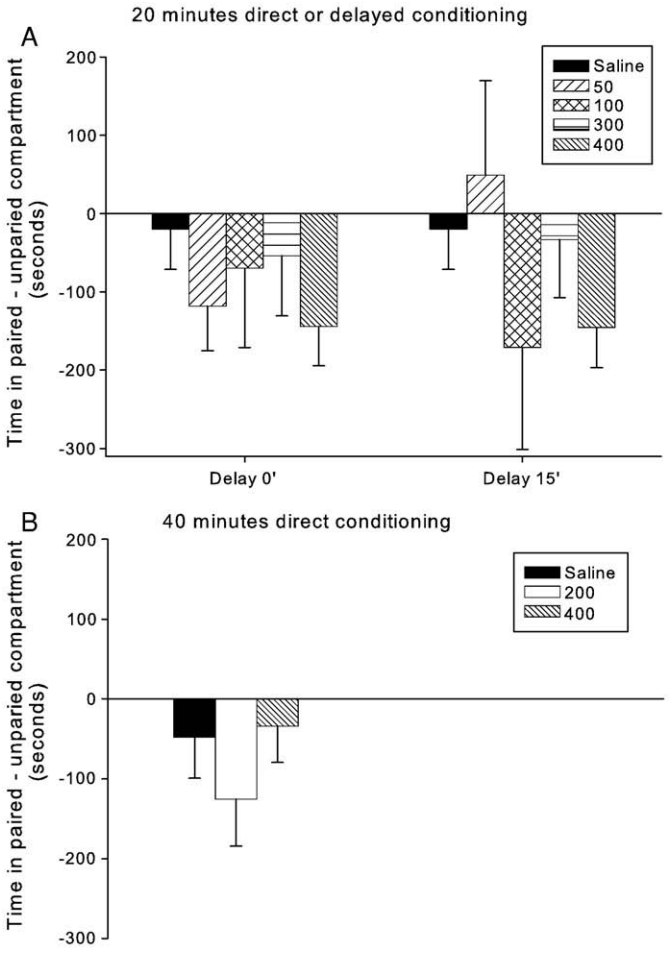

Fig. 3. CPP after subcutaneous injection with different doses (μmol/kg) of M3G for 20 minutes immediately (direct) or 15 minutes (delay) after injection (A), and for 40 minutes immediately after injection (B), compared to the saline group ($n=12$ mice). Bars illustrate mean ± SEM. $n=6$–9 mice for each drug treatment.

($t_{16}=-2.68$, $p=0.03$; Fig. 4B). Conditioning for 20 minutes directly after injection with 30 μmol/kg morphine induced statistically significant CPP ($t_{18}=-4.23$, $p=0.001$), which was attenuated by the co-injection of 200 μmol/kg M3G ($t_{16}=-1.14$, $p=0.3$; Fig. 4C).

Animals that were conditioned for 40 minutes did not show statistically significant CPP after treatment with 10 μmol/kg M6G ($t_{16}=-1.60$, $p=0.2$; Fig. 5A), but did show statistically significant CPP after treatment with 30 μmol/kg M6G ($t_{16}=-3.51$, $p=0.004$; Fig. 5B). CPP was not seen when M3G was co-injected with M6G 10 ($t_{16}=-1.82$, $p=0.1$) or 30 ($t_{16}=-1.69$, $p=0.1$) μmol/kg (Fig. 5A and B).

When 20 minute conditioning was performed 15 minutes after injection with M6G 30 μmol/kg statistically significant CPP was induced ($t_{16}=-2.47$, $p=0.03$), but not when M6G was co-injected with M3G ($t_{16}=-1.35$, $p=0.2$; Fig. 5C).

3.3.2. Locomotor activity

Different doses of M3G induced the same magnitude of total distance as the saline treated animals, but only the time-course for mice treated with 200 μmol/kg M3G for 40 minutes ($t_{16}=1.06$, $p=0.3$ compared to saline) is shown (Fig. 6).

An overall ANOVA comparing total distance travelled after treatment with morphine 30, M6G 30, M3G 200 or the combination of M3G with morphine 30 or M6G 30 showed that there was a significant difference between the groups [$F(5,36)=66.88$, $p<0.001$]. Post hoc tests revealed that 30 μmol/kg of morphine ($p<0.001$) or M6G ($p<0.001$) and the combination of 200 μmol/kg M3G and morphine 30 μmol/kg ($p<0.001$) increased the total distance travelled compared to saline. A tendency to increased locomotor activity was seen for the combination of M3G 200 μmol/kg with 30 μmol/kg M6G ($p=0.057$).

A statistically significant reduction in the distance travelled was seen when M3G was co-injected with morphine ($t_{10}=4.07$, $p=0.002$; Fig. 6A), but not after coadministration with M6G ($t_{10}=1.06$, $p=0.3$; Fig. 6B), compared to the animals treated with only morphine or M6G respectively, using an independent Students *t*-test.

An overall ANOVA comparing total distance travelled during conditioning day one after treatment with M3G 200 alone or the

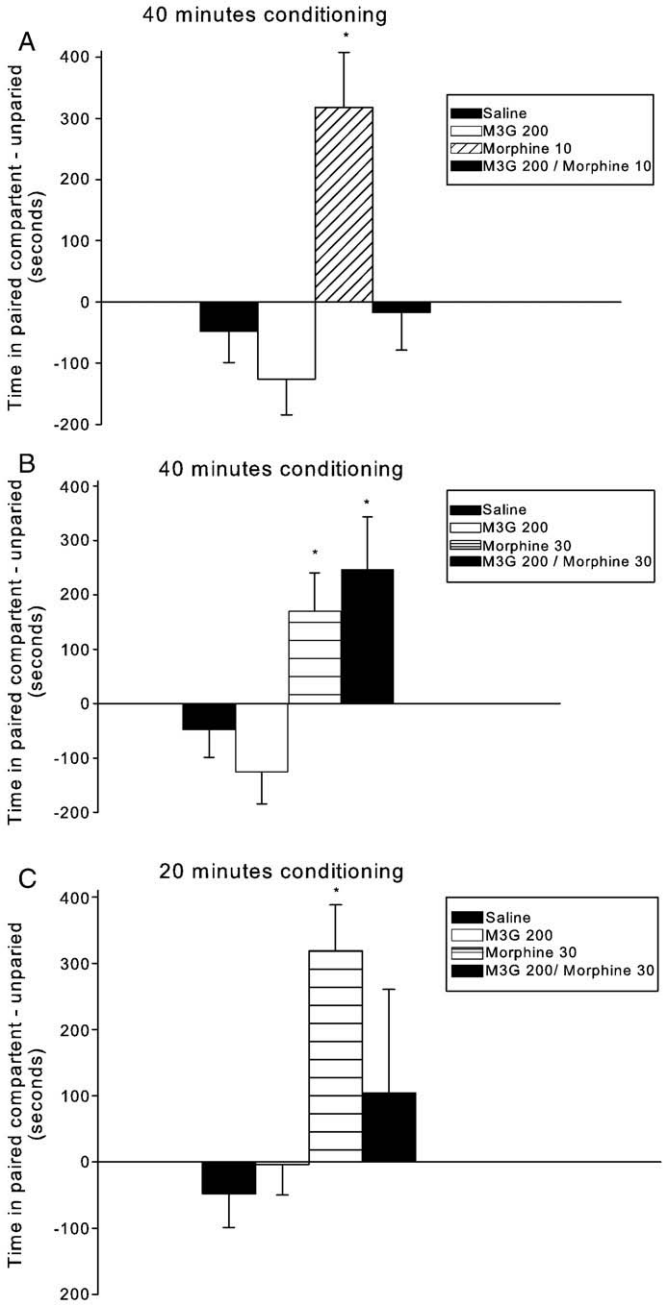

Fig. 4. CPP after subcutaneous treatment with M3G, morphine or the combination of both when conditioning was performed for 40 minutes (A and B) or 20 minutes (C) directly after injections compared to the saline group ($n=12$ mice). Doses are expressed in μmol/kg. Bars illustrate mean ± SEM. *$p<0.05$. $n=6$–8 mice for each drug treatment.

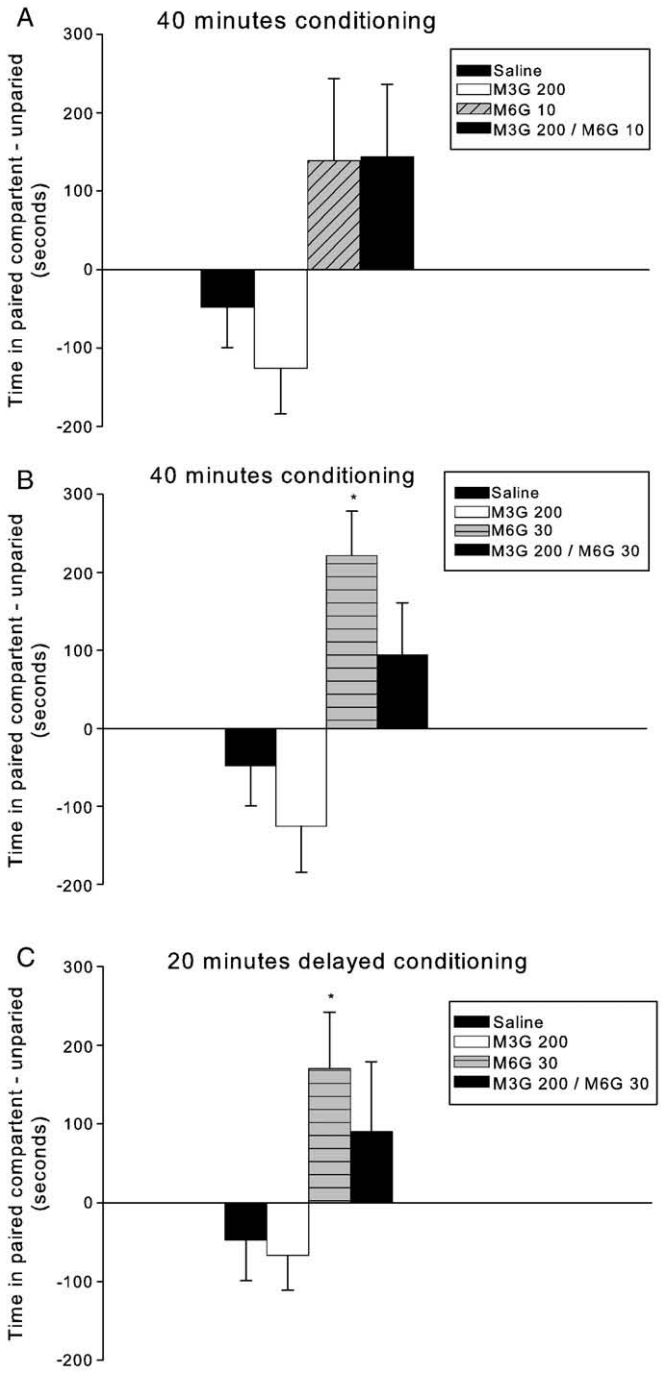

Fig. 5. CPP after subcutaneous treatment with M3G, M6G or the combination of both when conditioning was performed for 40 minutes immediately after drug injections (A and B) or for 20 minutes 15 minutes after injections (C), compared to the saline group ($n=12$ mice). Doses are expressed in μmol/kg. Bars illustrate mean ± SEM. *$p<0.05$. $n=6$–8 mice for each drug treatment.

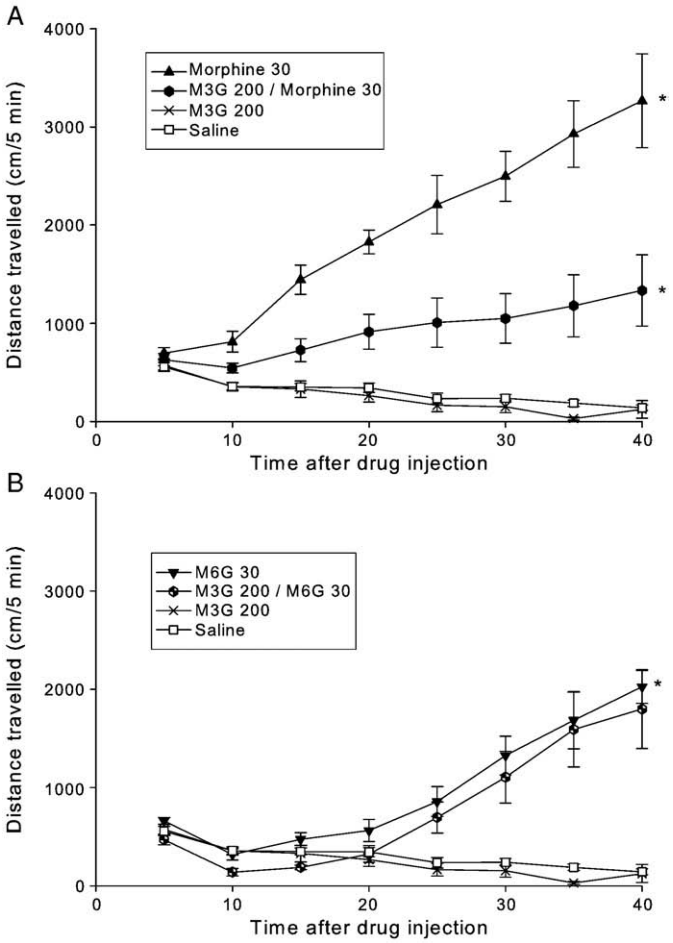

Fig. 6. Distance travelled (cm/5 minutes) during conditioning day one after treatment with morphine, M3G or the combination of both (A) and after treatment with M6G, M3G or the combination of both (B), compared to the saline group ($n=12$). Doses are expressed in μmol/kg. The results are mean ± SEM. $n=6$–8 mice for each drug treatment. *$p<0.05$ when total distance travelled for drug treated animals are different from the saline group.

combination of M3G with 10 or 30 μmol/kg of morphine or M6G showed that there was a significant difference between the groups [$F(5,41)=5.97$, $p<0.001$]. Post hoc testing showed that an increase in locomotor activity compared to saline was seen when M3G was co-injected with 30 μmol/kg morphine ($p<0.001$) or M6G ($p=0.005$), but not for the other doses.

The total distance travelled during conditioning day one was not different from day three neither after treatment with 200 μmol/kg M3G ($t_5=-1.22$, $p=0.12$) or 200 μmol/kg M3G in combination with morphine 10 ($t_5=0.74$, $p=0.5$), 30 ($t_5=-1.22$, $p=0.28$), M6G 10 ($t_5=1.58$, $p=0.18$) or 30 ($t_5=0.56$, $p=0.6$) μmol/kg (Fig. 7).

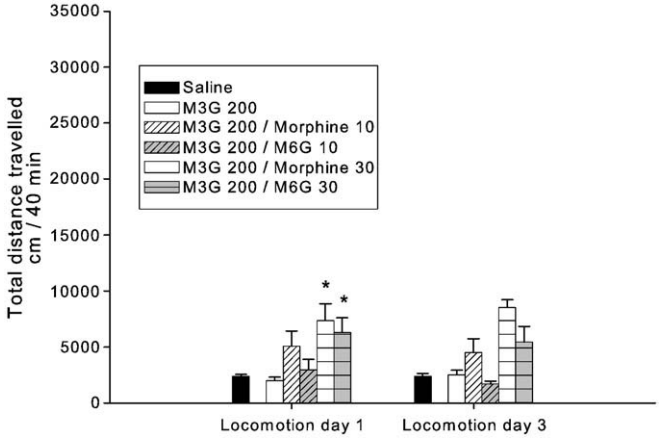

Fig. 7. Total distance travelled in cm/40 minutes (mean ± SEM) after treatment with a combination of M3G and morphine or M6G during conditioning day one and day three. *$p<0.05$ when the locomotor activity during conditioning day one is different for the drug treated animals compared to the saline group. There was no statistically difference between the activity during conditioning day one and day three. $n=6$ mice for each drug treatment and $n=12$ mice for the saline group.

3.4. Relationship between CPP and change in locomotor activity

The seventeen different treatment groups of mice were sorted by their change in locomotor activity from day one to day three (tolerance, no change or sensitisation). A positive preference for the drug-paired compartment was seen for 75% (27 of 36 mice) of the animals in the groups with sensitisation of locomotor activity, 76% (41 of 54 mice) in the groups with no change in activity and 83% (10 of 12 mice) of the mice in the groups with tolerance. Correlation analysis did not reveal any statistically significant correlation between the change in locomotor activity and CPP in the different groups (data not shown). Moreover, the correlations coefficients varied from positive to negative between groups treated with different dose of the same drug, without showing any consistent pattern. A regression analysis with CPP as dependent variable and treatment and change in locomotor activity as independent variable showed that change in locomotor activity can explain only about 4% of the CPP variance. The activity during the test sessions was not different for mice that showed tolerance, sensitisation or no change in activity from conditioning day one to day three.

4. Discussion

This study shows that treatment with 30 μmol/kg doses of morphine and M6G, as well as a combination of morphine and M6G that equalled 30 μmol/kg, induced CPP. No difference was seen for different ratios of morphine and M6G in combination. Other doses did not result in statistically significant CPP. No rewarding effect was seen for M3G, but for several of the M3G doses the mice spent most time in the unconditioned compartment. When M3G was co-injected with morphine or M6G, reduced CPP was seen for both drugs. M3G reduced morphine induced locomotor activity, but this reduction was not seen for M6G. The lowest dose of morphine and M6G induced tolerance while higher doses caused sensitisation in locomotor activity on conditioning day three compared to day one, but these changes were not seen for animals co-injected with M3G. The tolerance or sensitisation of the total distance travelled was not correlated to the expression of CPP during testing.

4.1. Interaction between morphine and M6G

The UDP glucuronosyl transferase 2B7 catalyzes the glucuronidation of morphine at both the 3- and 6-positions in humans, resulting in production of M3G and M6G, respectively (Lotsch, 2005). The M6G to morphine ratio is normally reported to range between 1 and 9 in humans and it depends on the administration route, time after drug intake and varies between different people (Andersen et al., 2003; Kilpatrick and Smith, 2005; Lotsch, 2005). Pasternak et al. have reported that morphine and M6G bind to different subtypes of μ-opioid receptors (2004), and altered rate in the biotransformation may have consequences for the pharmacological effects. Antonilli et al. have shown that after repeated heroin intake, the M6G/M3G ratios in street addicts are higher compared to opiate naïve patients after acute or repeated treatment with morphine (2003). The ratio has also been studied in rats and rat hepatocytes, and like heroin, prolonged treatment with morphine leads to reduction in M3G synthesis and increased M6G formation, although in a more modest way than for heroin (Graziani et al., 2008). As far as we know, there are no studies on opiate reward or psychostimulant activation that have investigated the administration of both M6G and morphine in combination, but the influence of M6G on the analgesic action after treatment with morphine has been addressed in several studies (Andersen et al., 2003). So far only a few studies have been able to show that M6G contributes to the analgesia after morphine administration in humans, and this contribution is reported to vary from zero to 66% (Faura et al., 1996; Klepstad et al., 2000; Lotsch, 2005). Our result indicated that morphine and M6G had additive rewarding effects only for the doses that equalled 30 μmol/kg, but in previous studies we have, however, shown that a dose–response relationship is not necessarily seen for different doses in the CPP model (Vindenes et al., 2006, 2008), and additive rewarding effects can therefore be difficult to reveal in the CPP model.

The locomotor activity during conditioning day one revealed that the total distance travelled after administration of M6G was lower compared to equimolar doses of morphine. When morphine and M6G were injected in combination, the activity was thus lower for the combination of both drugs, compared to an equimolar dose of morphine alone. This shows that for some doses, M6G does not add any activity, but for other doses, an additive effect on locomotor activity might be seen.

Studies on the μ-opioid receptors, where both morphine and M6G exert their effect, have shown that the combination of morphine and methadone produces a synergy on analgesia that is far beyond what would be expected from simple additive interactions (Bolan et al., 2002), and superadditive interactions on analgesia between δ agonists and morphine have been reported both in mice and rats (Adams et al., 1993).

4.2. Interactions with M3G

A limited number of studies from our research group have investigated the effect of M3G on reward, as revealed by CPP, and on locomotor activity and its sensitisation (Handal et al., 2002, 2007, 2008; Vindenes et al., 2006). Handal et al. have shown that M3G pretreatment, administered 30 minutes before treatment, decreased morphine induced locomotor activity and, in contrast to our results, increased M6G induced activity (2007). The mechanisms underlying those interactions were assumed to be pharmacodynamic for morphine and pharmacokinetic for M6G (Handal et al., 2007). Pretreatment with M3G led to increased serum and brain concentrations of M6G, a mechanism that might be due to the two compounds' competing for the same transporters, which again could explain the increased locomotor activity. Those results are therefore not incompatible with our results when there is no period between M3G and M6G administration to allow M6G levels to increase. The interval where locomotor activity is measured is also different between these studies, and this might also lead to different results.

From our results it seems like M3G might be able to reduce the rewarding potential of morphine, an effect that would have been very interesting to take advantage of in a clinical setting. Handal et al. have however shown that mice pretreated with M3G showed a sensitised response to morphine 6 days after M3G pretreatment (two injections at 6 days intervals), a result that was not seen for the mice challenged with M6G (2008). The behaviour sensitisation phenomenon, which is not only related to the change in locomotor activity, is considered by some authors to be an important, basic neurobiological mechanism for the development of addiction and dependence (Robinson and Berridge, 2008). A situation where M3G is present in the body alone, without M6G or morphine, is however not realistic in real life, and the significance of this result for humans is not known.

It is not clear which receptor systems the M3G interactions with morphine and M6G take place in. Both morphine and M6G are known to be μ-opioid receptor agonists, but M3G has a very low affinity to these receptors and the neuroexcitatory M3G effects like myoclonus, hyperaesthesia and allodynia are assumed to be mediated through non-opioid receptors (Christrup, 1997). Some studies in rats have shown that M3G can antagonize the analgesic effect of morphine (Smith et al., 1990) and M6G (Gong et al., 1992). Other studies have not confirmed this but have shown a prolonged and increased analgesia after coadministration of M3G and morphine (Christrup, 1997). Both the rewarding and psychomotor stimulating effects of the opioids are mediated by the mesocortical dopamine system (Tzschentke, 1998). The opioids suppress GABA inhibitory interneurons in the ventral tegmental

area leading to increased dopamine release in nucleus accumbens, accompanied by an inhibition of glutamate release (Vanderschuren and Kalivas, 2000; Shabat-Simon et al., 2008). In addition, activation of toll-like receptors (TLR) located on microglia and astrocytes not only influence analgesia and the reward properties of morphine, but also contribute to the development of morphine tolerance and dependence (Hutchinson et al., 2007). Morphine is a TLR-receptor agonist, and it has been reported that M3G also has agonist effects on this receptor (personal communication from Susannah S. Lewis). Which of these different receptor systems that is mainly responsible for the M3G interaction with morphine and M6G needs further exploration. A complicated interaction between the drugs is to be expected and, with the knowledge we have today, the contribution of M3G to short and long term effects observed after heroin or morphine intake in humans is difficult to predict.

4.3. CPP and locomotor sensitisation

The different dose–response curves and interactions seen for CPP and locomotor activity emphasize that these models do not display the same drug effects, and this is reported in several studies (Tzschentke, 1998; Cadoni and Di Chiara, 2000). Expression of CPP is assumed to reflect the rewarding and incentive properties of a drug while locomotor activity represents the psychostimulant effects, and despite the involvement of similar neurotransmitter systems in these responses, the models are not measuring the same effect (Tzschentke, 1998). The CPP paradigm is not an optimal model to investigate changes in locomotor activity after repeated drug treatments, but the results achieved might provide important information about the simultaneous development of these different drug effects (Cunningham et al., 2002; Orsini et al., 2005; Shabat-Simon et al., 2008).

It is well known that both tolerance and sensitisation for different drug effects take place with repeated drug intake and these are believed to occur at the same time (Robinson and Berridge, 2008). However, we did not observe this effect on locomotor activity in previous experiments using different apparatus (Vindenes et al., 2006) or time schedules (Vindenes et al., 2008).

Drugs of abuse induce a sensitised psychostimulant effect, frequently measured as sensitisation of locomotor activity, but in these studies the response was measured several days after pretreatment (Vanderschuren and Kalivas, 2000; Handal et al., 2008). Behavioural sensitisation has also been reported to take place during conditioning with morphine in the CPP model (Orsini et al., 2005), but an interesting finding from our study is the induction of tolerance for the low dose and sensitisation for the higher doses. Grung et al. have shown that both 20 and 30 μmol/kg morphine induce tolerance for the locomotor activity after 7 days treatment, but this was not seen after administration of either 10 or 40 μmol/kg morphine (2000). Previously sensitised mice have been reported to display increased CPP (Shippenberg and Heidbreder, 1995), but, in such a protocol, the sensitised drug response had developed before the conditioning was started, and cannot be compared to experiments where change in locomotor activity is recorded during conditioning. Our results, however, agree with earlier findings that susceptibility to induce sensitisation of locomotor activity does not predict drug-induced CPP after treatment with ethanol (Cunningham et al., 2002), morphine or amphetamine (Orsini et al., 2005), or when cocaine-treated mice are challenged 6 days after testing (Seymour and Wagner, 2008). Our results also showed that development of behavioural tolerance during conditioning is not correlated to the rewarding properties. A relationship between sensitisation of psychomotor activation and the susceptibility to induce CPP has been reported for amphetamine (Orsini et al., 2004) as well as methamphetamine and cocaine (Shimosato and Ohkuma, 2000). Environmental factors from the apparatus can interfere with the results (Tzschentke, 1998), and it is not unlikely that the inconsistent results are due to different apparatus (Shimosato and Ohkuma, 2000).

5. Conclusion

This study reveals that morphine and M6G injected alone or in combination induces CPP, but it is unclear if morphine and M6G have additive effects on reward. M3G seems to reduce the rewarding properties of both drugs and was also able to inhibit the development of both tolerance and sensitisation for locomotor activity after treatment with different doses of morphine or M6G. This strengthens the assumption that M3G can influence different effects of morphine and M6G. Such interactions between the parent drug and the metabolites are important to study in animal models, so we can obtain more information about the basic pharmacological mechanisms underlying reward, addiction and dependence after intake of heroin or morphine. However, under normal circumstances, mice do not metabolise morphine to M6G, and the interactions after administration of two drugs in combination might not be the same as when the metabolites are formed in the body. The length of time the drugs are present in the body might affect how they interact with each other. More studies that investigate the interaction of the combination of these drugs are needed. It may be of great importance to reveal if the change in ratio between M3G and M6G changes the rewarding and addictive properties of morphine and heroin.

We have shown that the morphine metabolites M3G and M6G can interact with the short term pharmacological effects observed after intake of heroin or morphine, and could, therefore, be of importance in the development of addiction and dependence.

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