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Behavioural sensitization in mice induced by morphine-glucuronide metabolites

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ABSTRACT

Sensitization is thought to be involved in central aspects of drug addiction. Both morphine-3-glucuronid (M3G) and morphine-6-glucuronid (M6G) are rapidly formed in high concentrations shortly after heroin and morphine consumption. Their role in the development of sensitization has not previously been studied. In our study, mice received three injections of M6G or morphine at six day intervals. M6G induced locomotor sensitization comparable to morphine as early as the first injection. In a second experiment two injections of M6G or morphine were given, separated by 6, 12, 18, 24 or 30 days. A sensitized response was observed for both morphine and M6G up to 18 days after the first injection. In a third experiment with two injections, the first with M6G and the second with morphine, or the opposite sequence, M6G did not induce cross-sensitization to morphine although morphine induced cross-sensitization to M6G. Finally, pretreatment with M3G induced sensitization of morphine locomotor activity but not M6G. In conclusion M6G induced long-lasting sensitization similar but not identical to morphine. M3G was shown to sensitize morphine induced locomotor activity in a similar way to morphine pretreatment. This suggests that morphine-glucuronide metabolites may play a role in the development of addiction to morphine.

1. Introduction

Heroin and morphine are well known drugs of abuse with rewarding and reinforcing properties. In humans, heroin is rapidly metabolized to 6-monoacetylmorphine and then to morphine, which is again metabolized into two main metabolites: morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). Shortly after heroin or morphine intake, M3G and M6G will be the most abundant circulating opiates (Sawe et al., 1985; Osborne et al., 1990). Both these glucuronides may cross the blood-brain barrier, although to a lesser extent than morphine (Bickel et al., 1996; Wu et al., 1997). If they do have relevant pharmacological effects, they may play a role in addiction arising from morphine and heroin intake.

Morphine-6-glucuronide is known to be a μ-opioid agonist which can cause analgesia and ventilatory depression (Kilpatrick and Smith, 2005). The drug is currently being tested in a phase 3 study as an analgesic drug (Romberg et al., 2007). M3G, however, has very low affinity for the opioid receptors and is not considered to have typical opioid effects (Skarke et al., 2005).

Given that M6G is an opioid receptor agonist, a few studies have considered whether the metabolite has rewarding or psychomotor stimulating effects similar to morphine. We have shown previously that M6G induces condition place preference (CPP) in mice and this suggests that the metabolite might induce reward (Vindenes et al., 2006). M6G also induced locomotor activity comparable to morphine suggesting that it affects the mesolimbic reward system (Morland et al., 1994; Grung et al., 1998; Handal et al., 2002).

In earlier experiments, we found that M3G did not induce CPP (Vindenes et al., 2006) or locomotor activity in mice (Handal et al., 2007). It has also been shown that M3G does not precipitate an opiate abstinence syndrome in morphine-dependent rats and this suggests that M3G is not involved in the physical component of opioid addiction (Salem and Hope, 1997). However, pretreatment with M3G reduced the psychomotor stimulating effect of morphine, but increased the effect of M6G because of pharmacodynamic and pharmacokinetic interactions respectively (Handal et al., 2007). This suggests that M3G might influence the effect of morphine and M6G in the mesolimbic reward system.

To explore further the role of morphine glucuronides in the development of heroin and morphine addiction, we wanted to study behavioural sensitization. Behavioural sensitization is considered to be linked to central aspects of the development of drug addiction, such as drug craving and the persistence of compulsive drug-seeking

Abbreviations: M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide.

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behaviour (Robinson and Berridge, 1993). As far as we know, no previous studies have investigated whether the two morphine glucuronides could induce (or interfere with) behavioural sensitization.

2. Materials and methods

2.1. Animals

C57BL/6J-Bom adult (7–8 weeks old), drug-naive, male mice (16.4–28.2 g body weight at testing) from Bomholt, Denmark were used for the experiments. The animals were housed eight to ten per cage at the National Institute of Public Health, Oslo, Norway, at a room temperature of 21–26 °C. The animals were kept on a 12/12 h light/dark schedule with the light period from 07:00 to 19:00 h. The mice were housed for at least 5 days prior to experiments, with free access to food and water throughout this acclimatization period. They were fasted overnight before the experiments. The animals were housed in their home cage between experiments. The Norwegian Review Committee for the Use of Animal Subjects approved the experimental protocol of this study.

2.2. Materials

Morphine hydrochloride (mol.wt. 375.9) was purchased from Norsk Medisinaldepot (Oslo, Norway), morphine-6-β-D-glucuronide hydrate (mol.wt. 461.47) and morphine-3-β-D-glucuronide (mol.wt. 461.47) from Lipomed (Arlesheim, Switzerland). The drugs were dissolved in 0.9% saline. Acetonitril from Labscan ltd. (Dublin, Ireland) was HPLC-grade. All other reagents were analytical grade.

2.3. Experimental design

In this study, we carried out five different experiments. The designs of the experiments are described below and a schematic chart of the experiments is shown in Fig. 1.

2.3.1. Sensitization induced by morphine and M6G

2.3.1.1. Repeated injections at 6 day intervals (experiment A). Drug induced locomotor activity: Each animal received three sc bolus injections at 6 day intervals (day 0, day 6 and day 12) with morphine (n=9–12) or M6G (n=11–15) in four different doses; 10, 15, 20 or 30 µmol/kg (3.8, 5.6, 7.5 or 11.3 mg/kg for morphine and 4.6, 6.9, 9.2 or 13.8 mg/kg for M6G) (Fig. 1). The injections were given in total volumes of 10 ml/kg. The control group received saline injections (n=5). Locomotor activity was registered for 180 min after each injection (drug induced locomotor activity).

We also wanted to study the drug free locomotor activity of the mice in this experiment to see whether the mice which had only received saline so far had different spontaneous activity during habituation from the mice which had already had injections of drug on various timescales. So we studied the locomotor activity of the different groups of mice during their habituation periods before they received their injection on days 0, 6 and 12.

2.3.1.2. Pharmacokinetic study (experiment B). To exclude the possibility that increased locomotor activity was due to changes in pharmacokinetics, we did a small pharmacokinetic experiment. Each animal received two sc bolus injections, on days 0 and 6, with either 30 µmol/

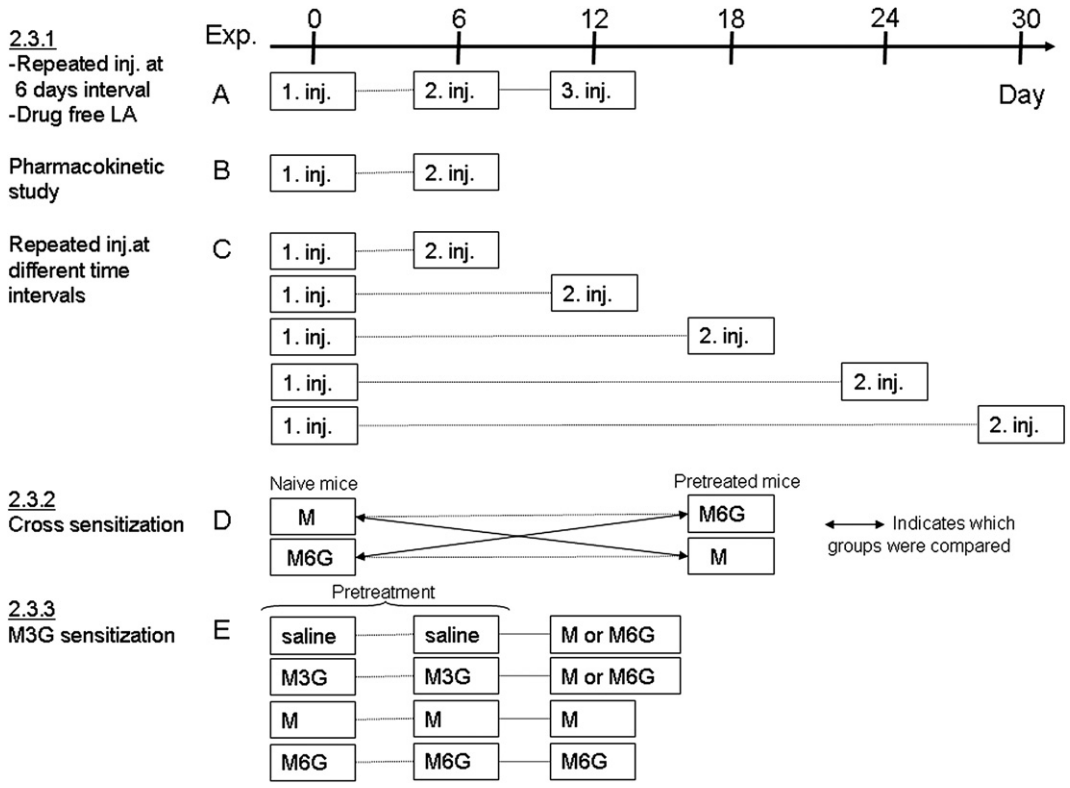

Fig. 1. Flow chart of the experimental design of the five different experiments (A–E) of the study. The number of injections and the interval between injections in the different experiments are shown. The underlined number to the left in the figure refers the section in the Experimental design section that describes the design of the experiment. In the "Sensitization induced by morphine and M6G" section (Section 2.3.1) at the head of the list, the chart only shows what days the injections were given but not what drug that was given. In experiment A the mice received either three injection of morphine, M6G or saline. Morphine and M6G were given in different doses. In this experiment the locomotor activity (LA) was also registered during the habituation period as indicated by "Drug free LA". In the pharmacokinetic study of morphine (experiment B) the first injection was either saline or morphine, while the second was morphine and corresponding for M6G. In experiment C the mice received two injections of morphine, M6G or saline. A set of control groups received one injection of morphine or M6G (day 0) and one injection of saline as the second injection at different time intervals after the first injection. In the "Cross-sensitization" (Section 2.3.2) and "M3G sensitization" sections (Section 2.3.3) both the drugs administered and the time interval between injections are shown.

kg morphine (11.3 mg/kg) or M6G (13.8 mg/kg) (Fig. 1). The control groups received saline as the first injection. The injections were given in total volumes of 10 ml/kg. Five to six animals were killed 30 min after the second injection. We have shown previously that the concentrations in serum and brain are relatively high after 30 min and behavioural differences between groups have previously been observed at this time (Handal et al., 2007). From the results in experiment A, we also knew that, at 30 min, the locomotor activity of mice which had just received their second drug injection was markedly increased compared to the mice which had just received their first drug injection (Fig. 4). Blood samples were taken and the brains were removed for further analysis (description below).

2.3.1.3. Repeated injections with different time intervals between injections (experiment C). To study the duration of locomotor activity sensitization, we administered two sc bolus injections with morphine (n=12) or M6G (n=13–14) with an increasing time interval between the injections. One set of control groups received two injection with saline (n=6–7) at the same time intervals. Another set of control groups received morphine or M6G as the first injection and saline as the second injection at the same time intervals (n=2–6). The intervals between the two injections were 6, 12, 18, 24 or 30 days (Fig. 1). Morphine and M6G were given in a dose of 30 µmol/kg (11.3 and 13.8 mg/kg respectively). The injections were given in total volumes of 10 ml/kg. Before each injection, locomotor activity was registered for 90 min (habituation). Because results from earlier experiments showed that locomotor activity lasted longer after M6G than morphine injection, we chose to register locomotor activity for 240 min following M6G injections as opposed to 180 min following morphine injections (Handal et al., 2007).

2.3.2. Cross-sensitization (experiment D)

At day 0, two groups of mice received one bolus injection of morphine (n=13) or M6G (n=19) and locomotor activity was registered. Eighteen days later (day 18) the two groups received the opposite drug (morphine-treated mice received M6G and M6G mice received morphine) (Fig. 1). We registered their locomotor activity again after the second treatment. Morphine induced locomotor activity was compared between the group that received morphine at day 0 (naïve mice) and the group that received morphine at day 18 (mice pretreated on day 0 with M6G). Similarly, M6G induced locomotor activity was compared between the group that received M6G at day 0 (naïve mice) and the group that received M6G at day 18 (mice pretreated on day 0 with morphine) (Fig. 1). If the pretreatment increased the locomotor activity compared to the locomotor activity in naïve mice, this was interpreted as cross-sensitization. Morphine and M6G were given as sc injections in doses of 30 µmol/kg (11.3 and 13.8 mg/kg respectively). The injections were given in total volumes of 10 ml/kg. Locomotor activity was registered for 90 min before each injection and for 240 min following each injection.

2.3.3. Morphine and M6G sensitization after M3G pretreatment (experiment E)

Morphine and M6G sensitization were studied in mice after different pretreatments. The four different pretreatments consisted of two sc injections at 6 day intervals (day 0 and day 6) with either saline, M3G, morphine or M6G. Repeated injections of the pretreatment were used to strengthen the effect of the pretreatment, especially M3G. Morphine and M6G were administered as sc bolus injections 6 days after the last pretreatment (i.e., on day 12) (Fig. 1). There were 6–10 animals in each group. The morphine and M6G doses were 30 µmol/kg (11.3 and 13.8 mg/kg respectively), while the M3G dose was 500 µmol/kg (231 mg/kg). The injections of saline, morphine and M6G were given in total volumes of 10 ml/kg, while M3G was given in an injection volume of 20 ml/kg. Locomotor activity was registered for 90 min before each injection and for 240 min after each injection.

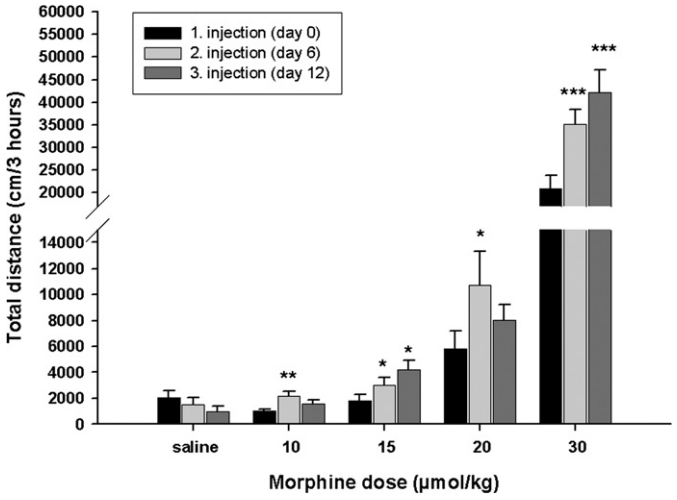

Fig. 2. Locomotor activity induced by repeated injection of saline (n=5) or different doses of morphine (n=9–12) given as sc bolus injections on day 0, 6 and 12 in experiment A (Fig. 1). The locomotor activity is given as the total distance travelled in the 3 h following drug injection. Each bar represents mean activity±SEM. *p<0.05, **p<0.01, ***p<0.001 versus the locomotor activity on day 0, by paired Student's t-test.

2.4. Blood and brain sampling, sample purification and HPLC analysis

Animals were killed by heart blood sampling under CO_2-anaesthesia. The brains were removed immediately after blood sampling. They were washed in ice-cold physiological saline, blotted on a filter paper and instantly frozen in liquid nitrogen. They were stored at −18 °C until analysis. Before HPLC analysis, the brains were homogenized with an Ultra Turrax T8 homogenizer (IKA, Jake & Kunkle, Germany) in ice-cold water to a final concentration of 0.33 g tissue/litre homogenate as described in a previous article (Handal et al., 2007). After 60 min at room temperature, the blood samples were centrifuged for 10 min at 1670 ×g and serum was stored at −18 °C until analyses were performed. Brain homogenate and serum was subjected to solid phase extraction and HPLC analysis modified after Svensson (1986) as described earlier by Handal et al. (2007). The limits of detection in serum for M6G and morphine were 0.2 µM and 0.3 µM respectively, and 0.2 µM for M3G. The limits of detection in brain homogenates for M6G and morphine were 0.06 nmol/g and 0.6 nmol/g for M3G.

2.5. Locomotor activity

Each animal was tested individually in the activity chamber of a Digiscan optical animal activity monitoring system (Omnitec Electonics Inc., Columbus, USA). The chamber size was 20×20 cm with infrared beam spacing of 2.5 cm. Each animal was individually habituated in the activity chamber for 90 min before each injection. After habituation, the mouse was gently removed from the activity chamber and given its treatment in another room. Immediately following the injection, it was gently returned to the same activity chamber as used for habituation. Locomotor activity induced by the treatment was then measured as described for each experiment. Each animal's score was expressed as activity counts per 5-min period or as a total sum of activity counts per hour(s). A battery of different activities were measured as described earlier (Grung et al., 1998). We focused on one activity, namely the horizontal distance travelled.

2.6. Data analysis

Statistical comparisons between locomotor activities in groups of mice studied on several occasions were performed by paired Student's t-test. Comparison between different groups of mice (i.e., mice subject to a different treatment regime) were performed by one-way analysis

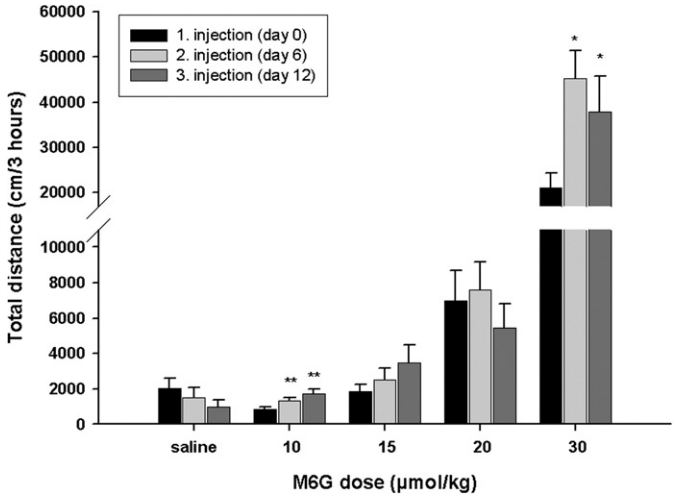

Fig. 3. Locomator activity induced by repeated injection of saline (n=5) or different doses of M6G (n=11–15) given as sc bolus injections on day 0, 6 and 12 in experiment A (Fig. 1). The locomotor activity is given as the distance travelled in the 3 h following drug injection. Each bar represents mean activity±SEM. *p<0.05, **p<0.01 versus the locomotor activity on day 0, by paired Student's t-test.

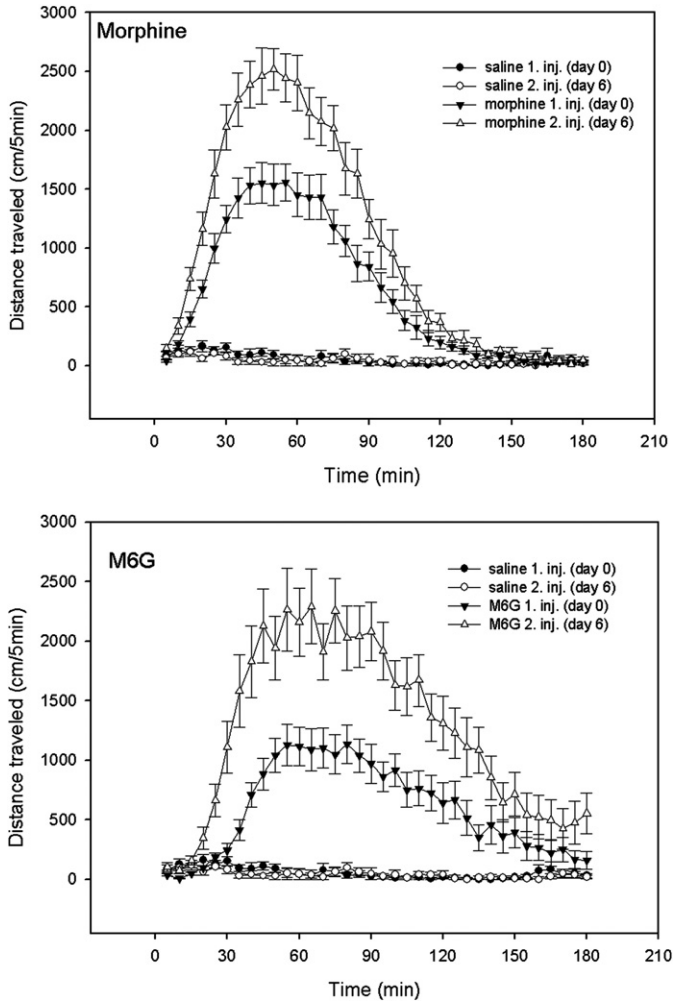

Fig. 4. The activity time curves of saline and one of the doses of morphine and M6G (30 µmol/kg (11.3 and 13.8 mg/kg respectively)) shown in Figs. 1 and 2 respectively. Locomotor activity time curves after the first and second injections given at 6 day interval (day 0 and day 6) of morphine (upper panel, n=11) and M6G (lower panel, n=11) or saline (both panels, n=5). The curves show the mean locomotor activity± SEM. The time scales are given in relation to the moment of drug injections.

of variance (ANOVA) followed by Bonferroni test when there were more than two groups. Serum and brain concentrations were compared with the control group by independent Student's t-test.

Data are presented as mean±SEM unless otherwise stated.

P values of less than 0.05 were regarded as statistically significant.

Statistical analyses were performed using SPSS version 12.0 statistical software.

3. Results

3.1. Sensitization induced by morphine and M6G

3.1.1. Repeated injections at 6 day intervals (experiment A)

Administration of the second dose of morphine, 6 days after the first morphine dose, markedly increased the locomotor activity at all doses tested (Fig. 2). The groups given a third injection of morphine (at day 12), of either 15 or 30 µmol/kg (5.6 and 11.3 mg/kg respectively) showed a further increase in locomotor activity. However the groups given a third morphine dose of 10 or 20 µmol/kg (3.8 and 7.5 mg/kg respectively), showed no further increase in activity.

When we administered different doses of M6G at 6 day intervals, a similar picture was observed as for morphine (Fig. 3). The locomotor activity was higher following the second injection although this only reached statistical significance following 10 and 30 µmol/kg (4.6 and 13.8 mg/kg respectively) of M6G. After the third injection (at day 12) it was still only the two doses mentioned above that caused a statistically significant increase in activity.

Three injections of saline at 6 day intervals (days 0, 6 and 12) did not induce any increase in locomotor activity (Figs. 2 and 3).

An activity time curve for the mice which received saline and the highest dose of morphine or M6G (30 µmol/kg (11.3 and 13.8 mg/kg respectively)) on day 0 and 6, is shown in Fig. 4. Morphine induced an immediate increase in locomotor activity after administration, while the increase following M6G administration was not obvious until 35 min after the first injection (day 0) and 20 min after the second injection (day 6).

The activity during habituation prior to drug injections was markedly lower the second and third time (on days 6 and 12) compared to the first time (day 0) the mice were in the activity chamber (Fig. 5). The activity during habituation was similar in all groups on day 6 and day 12, i.e. the activity was not related to the administration of either saline or a drug 6 days earlier.

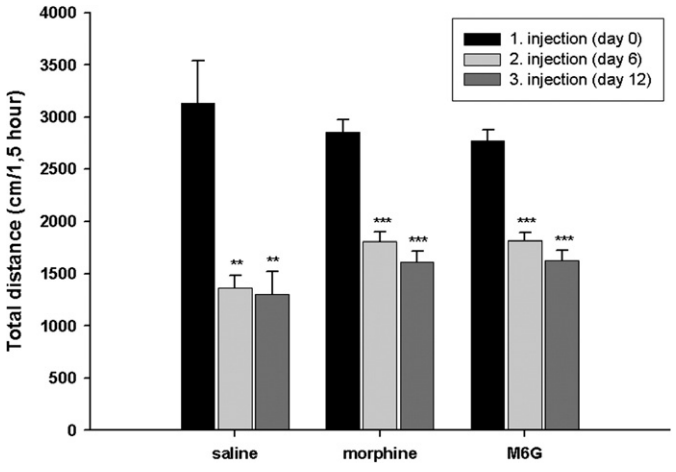

Fig. 5. The total locomotor activity during the 1.5 h habituation periods before the three drug injections at day 0, 6 and 12 respectively in experiment A (Fig. 1). Each bar represents mean activity±SEM (n=5 in the saline group, 44 in the morphine group and 54 in the M6G group). The animals were placed in groups depending on the drug they would receive later in the experiment. **p<0.01, ***p<0.001 when comparing locomotor activity on day 6 and 12 respectively versus day 0 in each group separately, by paired Student's t-test.

Table 1
Serum and brain concentrations of morphine and M6G in mice that received two sc injections at 6 days interval

Drug injected	Serum concentration at day 6 (μM)	Brain concentration at day 6 (nmol/g)
Pretreatment/treatment	Morphine	
Saline/morphine (n=5)	4.1±0.2	0.83±0.07
Morphine/morphine (n=6)	3.8±0.3	0.86±0.05
	M6G	
Saline/M6G (n=6)	23.6±1.6	0.26±0.02
M6G/M6G (n=5)	23.2±1.4	0.23±0.02

The concentrations were measured 30 min following one injection of 30 μmol/kg morphine or M6G given at day 6 in experiment B (Fig. 1). The pretreatment was given 6 days earlier (at day 0). Concentrations are given as mean±SEM.
There were no statistical differences detected in either serum or brain when comparing the two groups that received morphine or M6G as the second injection respectively by independent sample t-test.

3.1.2. Pharmacokinetic study (experiment B)

No differences were observed in serum or brain concentrations of the drug 30 min after morphine or M6G administration (30 μmol/kg (11.3 or 13.8 mg/kg respectively)) on day 6 when we compared animals that had received morphine or M6G on day 0 to those which had received saline (Table 1).

3.1.3. Repeated injections with increasing time intervals between injections (experiment C)

Increased locomotor activity was observed up to 18 days after the first morphine injection (Fig. 6).

Following M6G injections, an increased locomotor activity was observed at all intervals even though this increase was only statistically significant at 6 and 18 days (Fig. 7). In contrast to the mice injected with morphine, the mice on M6G injections demonstrated a tendency to show sensitization 24 and 30 days after the first injection.

There was no significant change in locomotor activity for the mice receiving a second saline injection compared to when they received the first, irrespective of the time interval between injections (data not shown).

When saline was given as the second injection, there were no differences in locomotor activity irrespective of whether the mice had received saline or drug as pretreatment (data not shown).

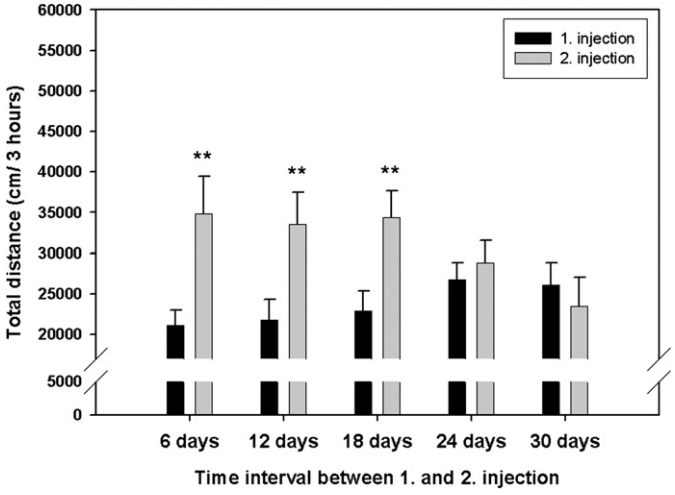

Fig. 6. Sensitization of locomotor activity induced by 30 μmol/kg (11.3 mg/kg) morphine at increasing time intervals between injections (sc) in experiment C (Fig. 1). The bars represent the locomotor activity after the first and second injection of morphine respectively. Each bar represents mean activity±SEM (n=12 in all groups). **p<0.01 versus the locomotor activity after the first injection in each group, by paired Student's t-test.

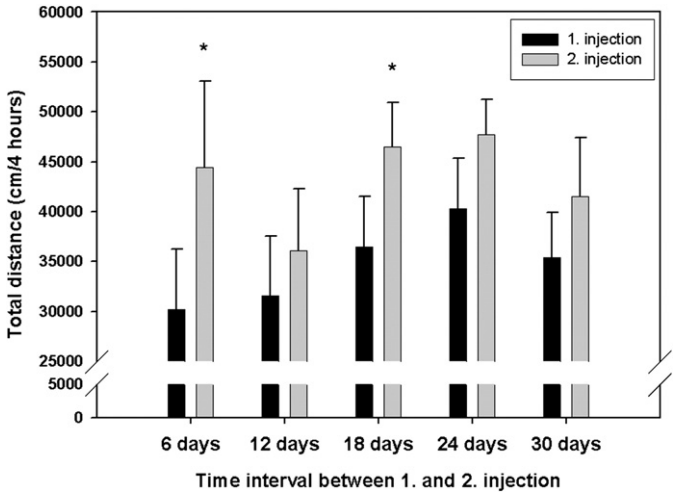

Fig. 7. Sensitization of locomotor activity induced by 30 μmol/kg (13.8 mg/kg) M6G at increasing intervals between injections (sc) in experiment C (Fig. 1). The bars represent the locomotor activity after the first and second injection of M6G respectively. Each bar represents mean activity±SEM (n=13–14). *p<0.05 versus the locomotor activity after the first injection in each group, by paired Student's t-test.

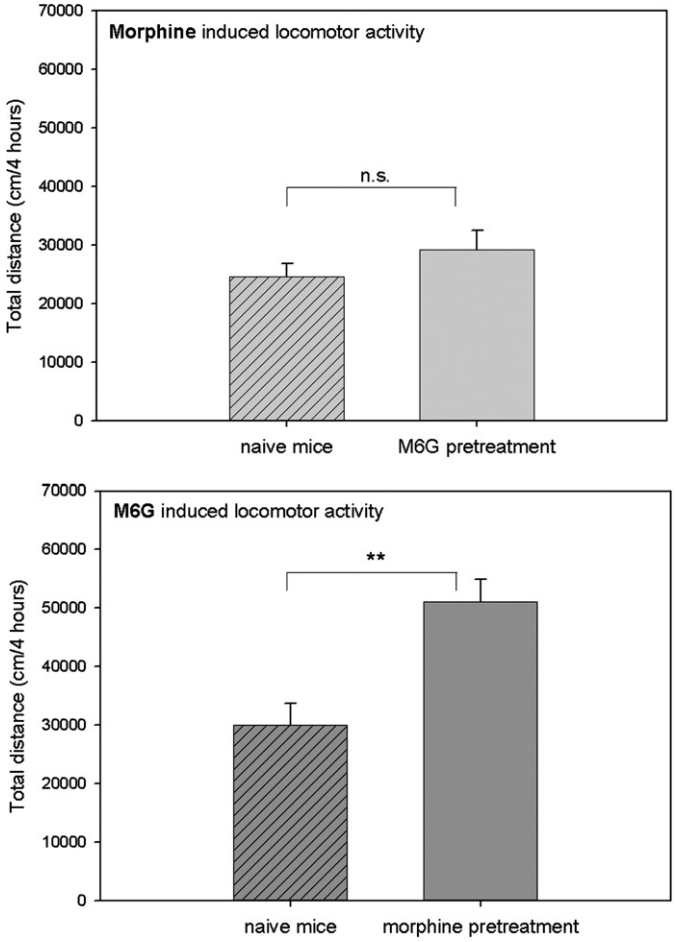

Fig. 8. Locomotor activity induced by morphine (upper panel) or M6G (lower panel) in experiment D (Fig. 1). In the upper panel the patterned bar represents morphine induced locomotor activity in naïve mice (at day 0) (n=13). The other bar represents morphine induced locomotor activity at day 18 after pretreatment with M6G at day 0 (n=19). In the lower panel the patterned bar represents M6G induced locomotor activity in naïve mice (at day 0) (n=19). The other bar represents M6G induced locomotor activity at day 18 after pretreatment with morphine at day 0 (n=13). The drug dose in all cases was 30 μmol/kg (11.3 and 13.8 mg/kg of morphine and M6G respectively). Each bar represents mean activity±SEM. The drug induced locomotor activities were compared by independent samples Student's t-test. **p<0.01.

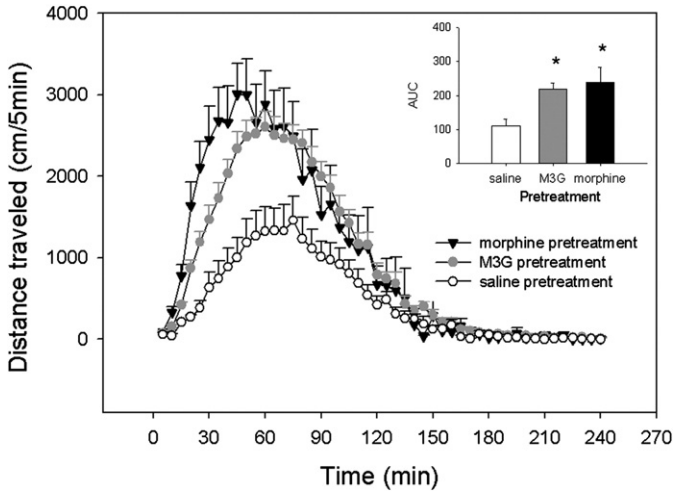

Fig. 9. Morphine induced locomotor activity after different pretreatments in experiment E (Fig. 1). Morphine (30 µmol/kg (11.3 mg/kg)) was administered at day 12, 6 days after the last pretreatment and the mean morphine induced locomotor activity+SEM in the different pretreatment groups are shown in the figure. The three different pretreatments consisted of two injections at 6 day intervals (day 0 and day 6) with either saline (n=6), M3G (n=10) or morphine (n=6) respectively. The time scales are given in relation to the moment of drug injections. Locomotor activity results during the 90 min habituation period were omitted for clarity. The inset figure shows the area under the locomotor activity curves (AUC) from 0 to 240 min following morphine injection in the three groups that had received different pretreatments. Each bar represents mean AUC (×1000 cm×min)±SEM. One-way ANOVA; $F(2,19)=5.4$ ($p=0.014$) followed by post hoc analysis (Bonferroni). *$p<0.05$.

3.2. Cross-sensitization (experiment D)

M6G pretreatment did not result in increased locomotor activity of morphine compared to morphine induced locomotor activity in naïve mice (Fig. 8, upper panel). However morphine pretreatment did increase locomotor activity of M6G compared to M6G induced activity in naïve mice (Fig. 8 lower panel).

3.3. Morphine and M6G sensitization after M3G pretreatment (experiment E)

Morphine-3-glucuronide pretreatment (two M3G injections at 6 day intervals on days 0 and 6) significantly increased the locomotor activity induced by morphine on day 12, nearly as much as pretreatment with morphine itself (Fig. 9). The M6G induced locomotor activity also tended to be somewhat higher after M3G pretreatment but not to the same degree as for morphine, and the increase was not significant (Fig. 10).

4. Discussion

The present findings show that one single injection of morphine or M6G induced behavioural sensitization of the same magnitude. The induced sensitization after a single dose of morphine was observed 6, 12 and 18 days later, and the sensitization induced by a single injection of M6G lasted for at least as long. Morphine pretreatment resulted in increased locomotor activity induced by M6G 18 days later, but M6G pretreatment did not result in a corresponding increase in locomotor activity induced by morphine. This difference indicates that mechanisms behind the development of sensitization might be different for the two opiates. M3G did not increase locomotor activity. Pretreatment with M3G resulted in increased locomotor activity induced by morphine, but M3G pretreatment did not significantly affect M6G induced locomotor activity.

As far as we know, there have been no studies exploring the possibility that the morphine glucuronides could induce behavioural sensitization. Our findings demonstrate, for the first time, that M6G induces pronounced sensitization and that M3G induces sensitization of morphine. Behavioural sensitization is thought to be linked to central aspects of drug addiction such as drug craving and the persistence of compulsive drug-seeking behaviour (Robinson and Berridge, 1993). The induction of behavioural sensitization has also been reported to be predictive of other behaviours related to the development of addiction, such as drug self-administration (Vezina, 2004) and reinstatement of drug-seeking behaviour (De Vries et al., 1998). We have shown earlier that M6G demonstrates reward effects by inducing CPP (Vindenes et al., 2006). Morphine-6-glucuronides' ability to induce sensitization adds further evidence to the idea that the metabolite contributes to the development of heroin and morphine addiction.

One injection of M6G was sufficient to induce sensitization. Drugs of abuse alter neuronal circuits by changing gene expression, and after repeated treatment the changes become more long-lasting because more permanent changes in transcription factors and chromatin structure occur, resulting, among other things, in sensitization (McClung and Nestler, 2008). This neuronal plasticity in the brain is important for the learning and memory which is necessary in the development of addiction. The changes in the brain have been reported to be very persistent; repeated morphine pretreatment has been reported to induce sensitization which was observed for up to 8 months (Babbini et al., 1975).

One single injection of M6G induced sensitization that lasted for at least as long as one injection of morphine. The long-lasting sensitization of morphine's locomotor activating effect supports results from Vanderschuren et al. that morphine induced sensitization of locomotor activity in rats which lasted 3 weeks after one single exposure (Vanderschuren et al., 2001). One possible explanation of the tendency of more prolonged sensitization after M6G administration is that M6G and morphine may induce different signal transduction cascades. This could result in different activation of transcription factors, which again might result in changes in gene expression of different duration (McClung and Nestler, 2008). Since mice do not

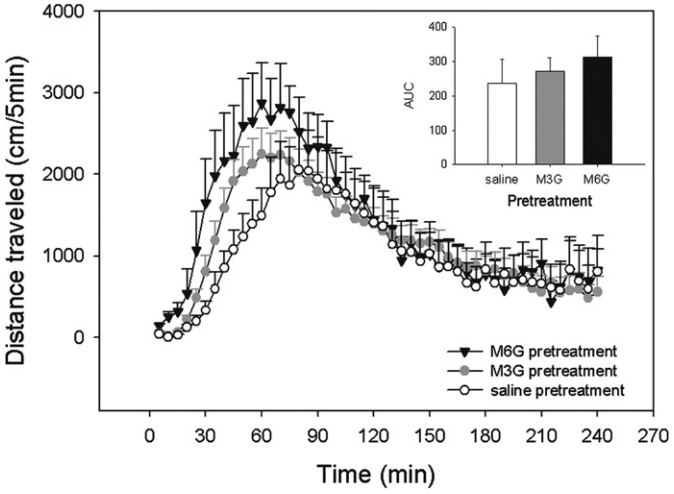

Fig. 10. Morphine-6-glucuronide induced locomotor activity after different pretreatments in experiment E (Fig. 1). Morphine-6-glucuronide (30 µmol/kg (13.8 mg/kg)) was administered at day 12, 6 days after the last pretreatment injection and the mean M6G induced locomotor activity+SEM in the different pretreatment groups are shown in the figure. The three different pretreatments consisted of two injections at 6 day intervals (day 0 and day 6) with either saline (n=7), M3G (n=9) or M6G (n=6) respectively. The time scales are given in relation to the moment of drug injections. Locomotor activity results during the 90 min habituation period were omitted for clarity. The inset figure shows the area under the locomotor activity curves (AUC) from 0 to 240 min following M6G injection in the three groups that had received different pretreatments. Each bar represents mean AUC (×1000 cm×min)±S.E.M. One-way ANOVA; F (2, 19)=0.4 (p=0.66) followed by post hoc analysis (Bonferroni).

metabolize morphine to M6G, the morphine sensitization observed has to be a pure morphine effect. Consequently, since we found that both morphine and M6G induced sensitization of comparable size after only one previous dose, there is reason to believe that their simultaneous presence, in individuals that metabolize morphine to M6G, could result in stronger or more permanent influence on gene expression than one of the drugs alone.

Cross-sensitization refers to the phenomenon that pretreatment with a given drug results in sensitization to the behavioural effects of another drug (Stewart and Badiani, 1993). This phenomenon has been reported in rats where pre-exposure to morphine has been shown to induce long-lasting sensitization to amphetamine (as well as to morphine) (Vanderschuren et al., 1997). Although both morphine and M6G induce sensitization of their own locomotor activity, M6G pretreatment did not induce cross-sensitization to morphine. Similarly it has been reported that pretreatment with amphetamine does not induce cross-sensitization with morphine (Vanderschuren et al., 1999). Those observations were thought to reflect partially distinct neuronadaptive phenomena behind the sensitization of opioids and psychostimulants. Our observations may similarly indicate partially distinct neuroadaptive mechanisms behind morphine and M6G sensitization. If different intracellular signal transduction cascades are activated by morphine and M6G, this could result in activation of different transcription factors or in different activation of the same factors. This could explain the observed lack of cross-tolerance.

The primary site of action of both M6G and morphine is thought to be the μ-opioid receptor. Morphine-6-glucuronide has a slightly lower affinity for the μ-opioid receptor, but may have a slightly higher efficacy than morphine resulting in approximately equal potencies in *in vitro* functional studies (Kilpatrick and Smith, 2005). However, the effects of morphine and M6G on feeding and analgesia, using oligodeoxynucleotides, are reported to be mediated via coupling to different G-proteins (Rossi et al., 1995; Silva et al., 2000). This might indicate that the intracellular signal cascades could be different. It has also been reported that morphine and M6G have different binding affinities at different μ-receptor subtypes (μ_1 and μ_2) even though the results are not unambiguous. With respect to locomotor activity, we have shown earlier that the μ_1-opioid receptor specific antagonist, naloxonazine, reduced the locomotor activity of morphine more powerfully than it reduced the M6G activity (Grung et al., 1998). Another indication of receptor differences is the reported lack of cross-tolerance between morphine and M6G with respect to locomotor activity (Grung et al., 2000). An M6G selective, high affinity splice variant of the μ-receptor site has been reported in studies using either antisense probes, a selective antagonist and knock-out mice (Rossi et al., 1996; Brown et al., 1997; Schuller et al., 1999) although these results have not been confirmed by other groups (Kitanaka et al., 1998; Connor et al., 1999). Even though it has been proposed that morphine to a lesser extent exerts its analgesic effect through this M6G selective receptor, studies of self-administration have implied that this receptor might still play a role in morphine's reinforcing properties (Walker et al., 1999). The possibility that different receptor subtypes might be important when considering other effects of opiates than analgesia limit our knowledge about the differences between morphine and M6G receptors and intracellular signalling, although several “behavioural” measures indicate that such differences could well exist.

The most common procedure to induce sensitization is by repeated treatment with morphine over several days. The magnitude of the reported sensitization in rodents in such studies did not differ substantially from that found in our study (Bartoletti et al., 1987; Airio and Ahtee, 1997). After M6G treatment, the two medium doses (15 and 20 μmol/kg (6.9 and 9.2 mg/kg)) did not result in significant sensitization although a clear tendency towards increased activity was observed. The reason for this might be that the inter-individual variation in locomotor activity induced by M6G was quite pronounced and greater than that observed by morphine. This difference in variation is clearly demonstrated in larger SEM's of locomotor activity induced by M6G compared to morphine, depicted in Fig. 4. Differences in M6G induced locomotor activity between different groups of mice receiving the same treatment can also be observed by looking at the results of the first injection of M6G (black bars) in Fig. 7.

We excluded the possibility that the sensitization of locomotor activity observed after morphine or M6G treatment could be caused by increased serum or brain concentrations of the two drugs as no changes in pharmacokinetics could be observed after repeated morphine or M6G administrations.

There is little previous evidence to suggest that M3G would be involved in development of sensitization. This metabolite did not demonstrate reward effects in the CPP model (Vindenes et al., 2006) and it did not induce locomotor activity itself (Handal et al., 2002). However, we have shown earlier that M3G pretreatment 30 min before one single injection of morphine, reduced morphine induced locomotor activity and this suggested that there was a negative interaction between the two drugs with respect to the acute psychomotor stimulating effects. Our results indicate that M3G might also interfere with the more long-lasting processes in the development of addiction to morphine.

The mechanism behind M3G's ability to induce morphine sensitization is intriguing. We could exclude deglucuronidation to morphine because of results from a previous study (Handal et al., 2002). Morphine-3-glucuronide has very low affinity for opioid receptors (Pasternak et al., 1987). However Halliday et al. showed that a selective μ-opioid receptor antagonist reduced the excitatory effects of M3G (Halliday et al., 1999). This suggests that at least some of M3G's effects are mediated through the μ-opioid receptor. Behavioural sensitization is generally considered to be the result of a functional reorganization in a circuit consisting of dopaminergic, glutamatergic and GABAergic projections between ventral tegmental area, nucleus accumbens, prefrontal cortex, hippocampus and amygdala (Vanderschuren and Kalivas, 2000). It has been suggested that the effects of M3G are mediated through non-opioid mechanisms. Both increased excitatory transmitter release of glutamate or decreased inhibitory transmitter release of glycine and GABA have been suggested (Skarke et al., 2005). Both these mechanisms could explain how M3G could influence the circuits important in the development of sensitization.

Activation of glia cells has recently been suggested to play a part in rewarding and reinforcing actions of morphine and this activation has been shown to occur though toll-like receptors (TLR) (Hutchinson et al., 2007). Morphine has been shown to be a toll-like receptor 4 (TLR4) agonist (Hutchinson et al., 2007) and it has been suggested that the rewarding and reinforcing effects of morphine are partly mediated by down regulation of glial glutamate transporters (Nakagawa and Satoh, 2004). These transporters regulate extra cellular glutamate levels, and it has been shown that an inhibition of the transporter facilitates CPP. Recently M3G was reported to be an even more effective TLR4 agonist than morphine (Susannah S Lewis, personal communication). These findings offer a possible mechanism for how M3G might influence glutamate levels in the reward circuit and in this way influence morphine sensitization. It has also been stated that a pure TLR4 signal is not rewarding alone, but requires traditional neuronal opioid signals as well (Larson, 2006). This fits very well with our observations that M3G does not induce CPP even though it is a TLR4 agonist, probably because it does not activate the traditional opioid receptors (or does so to a very limited extent) because of very low affinity for the μ-opioid receptors.

We performed a set of control experiments to explore the possibility that the observed sensitization was caused, not by the drugs (morphine/M6G), but by the experimental procedures themselves. We used repeated injections with saline at both 6 day intervals and increasing intervals (12, 18, 24, 30 days) without any observed increase in activity. The animals that had received drugs previously did not show any increased activity during the habituation period compared to the saline controls. Finally mice that had received drugs as the first injection and then got saline the second

time did not show any increased activity compared to their saline controls. This suggests that the observed sensitizations were not caused by the experimental procedures, and that the cause of sensitization in the study was actually the drugs.

We have shown that in our mice model both of the morphine-glucuronides possess properties that might be of importance in the development of heroin/morphine addiction. In the case of M6G this was an important, but maybe not surprising, finding. However for M3G, which has often been considered an inactive metabolite, this was not easily foreseen. After intake of heroin or morphine both the metabolites and the parent drug(s) will be present in the brain at the same time and the net effect is not easily predicted. In addition, Antonilli et al. have reported that heroin addicts show higher M6G/M3G concentration ratios relative to morphine-treated control subjects (Antonilli et al., 2003). In rats, the same group reported that repeated administration of heroin reduced the formation of M3G, but induced the formation of M6G that is usually undetectable in the rat (Antonilli et al., 2005). This group concluded that the increased production of M6G may lead to a more prolonged effect of each single heroin exposure. This is probably true, but in the light of our results the reduction in M3G levels reported might complicate the picture and at least decrease the sensitization to morphine found in our study.

In conclusion, this study shows that the morphine-glucuronides, metabolites which are present in high concentrations following heroin or morphine administration in humans, could be of importance in the development of addiction. This study suggests that M6G may have an abuse potential that is comparable to or even more pronounced than morphine and that this is probably related to a somewhat different neurobiological mechanism. This should be considered if M6G is introduced as an anti-analgesic drug. Morphine-3-glucuronide had an effect on morphine sensitization that was almost comparable to the sensitizing effect of morphine itself. The repeated use of heroin or morphine might therefore lead to sensitization by both morphine and the two morphine-glucuronides possibly by different mechanisms which could further interact in a complicated way.

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