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Morphine-3-glucuronide inhibits morphine induced, but enhances morphine-6-glucuronide induced locomotor activity in mice☆

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Abstract

The main metabolite of morphine, morphine-3-glucuronide (M3G) has no opioid effects. Some studies have rather indicated that it antagonizes the antinociceptive and respiratory depressive effects of both morphine and the active metabolite morphine-6-glucuronide (M6G). We studied the possible influence of M3G on the psychostimulant properties of morphine and M6G measured by locomotor activity. Mice were given two injections, one with either 80, 240 or 500 μmol/kg M3G or saline followed by an injection of 20 or 30 μmol/kg morphine or M6G. M3G influenced the locomotor activity induced by both morphine and M6G, but in opposite directions. M3G reduced the morphine induced locomotor activity during the first hour following morphine injection in a concentration dependent manner. M3G pretreatment did not significantly influence brain concentrations of morphine indicating that the interaction was of a pharmacodynamic type. In contrast M3G pretreatment increased the M6G induced locomotor activity. M3G pretreatment increased serum and brain M6G concentrations to an extent indicating that this interaction was mainly of a pharmacokinetic type. In conclusion our results disclose complicated interactions between morphine and its two metabolites with respect to induction of locomotor activity and possibly also with respect to mechanisms related to drug reward.

Keywords: Morphine; Morphine-3-glucuronide; Morphine-6-glucuronide; Locomotor activity; Pharmacokinetics; Antagonism; Potentiation; Mice

1. Introduction

Morphine causes a spectrum of effects ranging from analgesia and respiratory depression to euphoria. These effects are predominantly mediated by μ-opioid receptors. Morphine also has an effect on the mesencephalic dopamine neurons resulting in locomotor stimulation (Joyce and Iversen, 1979). In humans, the main metabolic pathway of morphine is glucuronidation with the formation of morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). The plasma concentrations of these glucuronides by far exceed the concentration of morphine shortly after single dose administrations as well as during chronic treatment (Sawe et al., 1985; Osborne et al., 1990). Glucuronides are generally considered as highly polar metabolites unable to cross the lipid layers of the blood–brain barrier. However, it has been reported that M6G, and to a lesser extent M3G, are more lipophilic than predicted and not much less lipophilic than morphine itself (Carrupt et al., 1991). There is also direct evidence that the morphine glucuronides pass the blood–brain barrier (Aasmundstad et al., 1995; Xie et al., 2000).

M6G is an active metabolite of morphine causing analgesia, ventilatory depression and inhibition of gastrointestinal transit (Milne et al., 1996). M3G does not have analgesic or ventilatory depressive effects in either animals (Milne et al., 1996) or man (Penson et al., 2000), but is suggested to have effects that counteract those of morphine. These effects ranges from

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decreasing morphine analgesia (Smith et al., 1990; Gong et al., 1992; Ekblom et al., 1993) to behavioral excitation (Yaksh et al., 1986).

Mice metabolize morphine to M3G, but produce no M6G (Handal et al., 2002). This makes mice an experimental model where the effects of morphine can be studied undisturbed of M6G formation, which could otherwise add its pharmacodynamic effects to the effects of morphine. We have previously demonstrated that systemic administration of M6G can cause locomotor activation similar to morphine. M3G did not elicit locomotor activation in mice (Morland et al., 1994; Grung et al., 1998; Grung et al., 2000; Handal et al., 2002).

The present experiments were designed to study the possible influence of M3G, which does not induce locomotor activity itself, on the psychomotor stimulating properties of morphine and M6G measured by locomotor activity. Furthermore we wanted to study whether possible interactions could be pharmacokinetic in nature.

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 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 h 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. Each animal was tested once. 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). Because of change in purchase routines both morphine-6-β-D-glucuronide dihydrate (mol. wt. 497.5 from Ultrafine Chemicals (Manchester, England)) and morphine-6-β-D-glucuronide hydrate (mol. wt. 461.47 from Lipomed (Arlesheim, Switzerland)) were used in the experiments. Morphine-3-β-D-glucuronide (mol. wt. 461.47) was purchased 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

Four different types of experiments were conducted.

2.3.1. Dose response studies

Each animal was given one subcutaneous (sc) bolus injection of saline, morphine or M6G. Morphine and M6G were given in four different doses: 10, 15, 20 and 30 μmol/kg (equivalent to 3.8, 5.6, 7.5 and 11.3 mg/kg for morphine and 4.6, 6.9, 9.2 and 13.8 mg/kg for M6G). Each treatment group consisted of a minimum of four animals. The injections were given in total volumes of 10 ml/kg. Locomotor activity was registered for 180 min (description below).

2.3.2. M3G locomotor interaction studies

Each animal received two bolus injections with 30-min interval, one intraperitoneal (ip) with M3G or saline followed by one sc with morphine, M6G or saline. The injections were given by two different administration routes to avoid any interactions at the administration site. M3G was given in three different doses; 80, 240 or 500 μmol/kg (equivalent to 37, 111, 231 mg/kg), morphine and M6G were given in two doses; 20 or 30 μmol/kg (7.5 or 11.3 mg/kg for morphine and 9.2 or 13.8 mg/kg for M6G). The agonist doses were chosen based on the dose response study and our previous work demonstration that these doses are in the lower segment of the steepest part of the sigmoid shaped dose response curve (0–120 μmol/kg) (Grung et al., 1998). The ip injections were given in total volumes of 20 ml/kg, and the sc injections were given in total volumes of 10 ml/kg. There were 9–10 animals in each treatment group. Locomotor activity was thereafter registered for 180 min (20 μmol/kg) or 300 min (30 μmol/kg).

2.3.3. M3G pharmacokinetic interaction studies

Each animal received two bolus injections at 30-min interval, one ip with M3G (500 μmol/kg (231 mg/kg)) or saline and one sc with morphine (20 μmol/kg (7.5 mg/kg)) or M6G (20 μmol/kg (9.2 mg/kg)) as described above. Three to five animals were killed at 15, 30, 45, 60 and 120 min following the second injection. At each time point, as well as after 180 min (from animals included in Experiment 2.3.2), blood samples were taken and the brains were removed for further analysis (description below).

2.3.4. Comparative studies of concentrations (serum and brain) and locomotor activity following 20 and 30 μmol/kg (9.2 and 13.8 mg/kg) M6G respectively

To make it possible to interpret the relationship between serum and brain concentrations of M6G and the corresponding locomotor activity following pretreatment with M3G, we had to explore how M6G pharmacokinetics influenced M6G locomotor activity. Therefore two groups of animals received one sc injection of either 20 or 30 μmol/kg M6G. The injections were given in total volumes of 10 ml/kg. Three to five animals were killed at 15, 30, 45, 60 and 120 min following the injection. Only one animal receiving each dose was killed at 180 min following the injection. At each time point blood samples were taken and the brains were removed for M6G analysis (description below). Locomotor activity was registered during the time period between the injection and the time of blood and brain sampling. The locomotor activity curve and the total distance travelled were calculated based on the mean activity at each 5-min interval.

2.4. Blood and brain sampling

Animals were killed by heart blood sampling under CO_2-anesthesia. 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. After 60 min at room temperature, the blood samples were centrifuged for 10 min at 1670 ×*g* and serum was removed and stored at − 18 °C until analyses were preformed.

2.5. Serum and brain sample purification

2.5.1. Serum

Solid-phase extraction and sample injection modified from Svensson et al. (Svensson et al., 1982; Svensson, 1986) were performed by an ASPEC (Automated Sample Preparation with Extraction Columns) robot (Gilson, Medical Electronics, Villiers de Ble, France). The extraction cartridges, Oasis HLB 1 cc/30 mg (Waters, U.S.A.), were washed with 1 ml methanol and 1 ml water. Fifty microliters of sample was mixed with 1.6 ml 0.5 M ammonium sulfate (pH 9.3) and 50 µl internal standard (normorphine). Even though normorphine has been detected in small amounts in urine from mice (Yeh et al., 1977), we have not, in previous experiments, been able to detect it in serum or brain even following administration of high morphine doses (80 µmol/kg) to C57BL/6J-Bom mice. Normorphine could therefore be used as an internal standard without influencing the other measured concentrations. The sample was then passed through the cartridge and subsequently washed with 3 ml 5 mM ammonium sulfate (pH 9.3) and 75 µl 10% acetonitril (ACN) in 10 mM NaH_2PO_4 (pH 2.1). Morphine and its metabolites were eluted with 500 µl 10% ACN in 10 mM NaH_2PO_4 (pH 2.1). This 500 µl sample was added and mixed with 5 mM ammonium sulfate (pH 9.3) and passed through a second cartridge and subsequently washed with 2 ml 5 mM ammonium sulfate (pH 9.3). Morphine and its metabolites were eluted with 500 µl 10% ACN in 10 mM NaH_2PO_4 (pH 2.1). A 50 µl extract was injected into the HPLC.

2.5.2. Brain

Preceding analysis the brains were homogenized with an Ultra Turrax T8 homogenizer (IKA, Jake and Kunkle, Germany) in ice-cold water to a final concentration of 0.33 g tissue/l homogenate. Five hundred microliter water was added to a sample of 600 µl brain homogenate and 50 µl internal standard (normorphine). This sample was frozen and thawed two times and then centrifuged for 10 min at 1670 ×*g*. The supernatant was mixed with 1.2 ml 5 mM ammonium sulfate (pH 9.3) and then subjected to the same procedure of solid-phase extraction and sample injection as described above.

2.6. HPLC analysis

The HPLC analysis was an automated modification of the method described by Svensson et al. (Svensson et al., 1982; Svensson, 1986). The system consisted of an ASPEC robot mentioned above, HP-1050 isocratic pump (Hewlett Packard, Waldbronn, Germany), Spectra System UV2000 detector (Spectras Physics Analytical, San Jose, CA, U.S.A.) in series with an ESA Coulochem model 5100A with ESA model 5010 analytical cell (ESA, Bedford, MA, U.S.A). Drug concentrations were determined from curve area by integration with EZChrom (Scientific Software, Inc., San Ramon, CA, USA) or Totalchrom (PerkinElmer Instruments LLC, Shelton, CT, U.S.A.). The column used was Chrompac ODS-2 (10 cm × 0.46 cm i.d., 3 µm particle size) (Waters, Milford, Massachusetts, U.S.A.). The mobile phase consisted of 24% ACN (v/v) with the ion-paring agent sodium dodecyl sulphate (4 mM) in a mixture of 10 mM NaH_2PO_4 and 10 mM H_3PO_4 (pH 2.1). The flow rate was 0.8 ml/min. The UV wavelength used was 210 nm. The electrochemical detector operated at electrode potentials of 0.3 and 0.42 V and only the second electrode was used for quantification.

2.6.1. Method performance

2.6.1.1. Serum. The analytical recoveries in serum were 70% for M3G, 79% for M6G and 85% for morphine. The inter-assay variability of the automated extraction was less than 10% for all compounds. The limits of detection (LOD) for the electrochemical detector (M6G and morphine) were 0.2 µM and 0.3 µM respectively, and for the UV detector (M3G) 0.2 µM. When the initial morphine concentration was below 5 µM, M6G and morphine were quantified by the electrochemical detector. Higher concentrations of morphine and M6G and M3G were quantified by UV detection.

2.6.1.2. Brain. The analytical recoveries in brains were 55% for M3G and 75% for M6G and morphine. The inter-assay variability of the automated extraction was less than 12% for all compounds. The limits of detection (LOD) for M6G and morphine were 0.06 nmol/g (electrochemical detector) and 0.6 nmol/g for M3G (UV detector).

2.7. Locomotor activity

Each animal was tested individually in an 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 injections. After habituation the mouse was gently removed from the activity chamber and given its treatment in another room. If the treatment consisted of more than one injection the animal was kept in its home cage between the two injections. Immediately following the complete treatment it was gently returned to the same activity chamber as used for habituation. Locomotor activity was measured for different time periods after treatment 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 was measured as

described earlier (Grung et al., 1998). We focus on one activity, the horizontal distance travelled, to present our results.

2.8. Data analysis

Statistical comparison between groups were preformed by one-way analysis of variance (ANOVA) followed by Bonferroni test or by Student's unpaired *T*-test, as appropriate.

Data from the concentration time curves were analyzed using regression analysis where the concentrations represented the dependent variable and pretreatment was coded as the independent predictor variable (a dummy variable). In addition the time variable was coded as a second dummy variable. The result of the analysis is presented as an unstandardized coefficient.

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

Statistical analyses were performed with SPSS version 12.0 statistical software.

Data are presented as mean ± SEM unless otherwise stated.

Calculations of the area under the curve (AUC) were performed by use of the trapezoidal rule.

3. Results

3.1. Dose response

The dose response experiments demonstrated that doses higher than 15 μmol/kg morphine were necessary to significantly increase locomotor activity. Escalating doses of M6G induced increasing locomotor activity reaching statistical significance at 30 μmol/kg M6G. Equal doses of morphine and M6G induced locomotor activity of similar magnitude (Fig. 1).

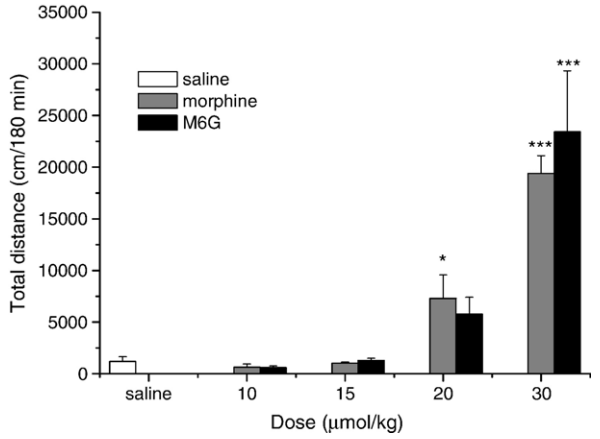

Fig. 1. Dose response relationship as the sum of the total distance travelled during a 180-min registration period induced by a sc bolus dose of saline or increasing doses of morphine or M6G. Results are mean activity ± S.E.M. (n = 4–5). One-way ANOVA (saline and morphine); $F(4, 16) = 41.11$ ($p < 0.001$). One-way ANOVA (saline and M6G); $F(4, 16) = 13.48$ ($p < 0.001$). Post hoc analysis (Bonferroni) compared morphine and M6G to saline. Equal doses of morphine and M6G were compared by Student's T test without any statistical significant results. *$p < 0.05$, ***$p < 0.001$.

3.2. M3G induced behavior

In the experiments where we studied the M3G influence on locomotor activity induced by morphine and M6G, we first studied the locomotor activity of M3G per se. None of the M3G doses did, when administered before saline, induce any change in locomotor activity when compared with the group that received two saline injections. Nor did any M3G-dose induce other behaviors like stereotypic behavior or vertical activity (data not shown).

3.3. M3G influence on morphine induced locomotor activity

Pretreatment with M3G showed the same tendency to, in a dose dependent manner, reduce the locomotor activity of both 20 and 30 μmol/kg morphine (Fig. 2A and C). From the figures it is apparent that the effect of M3G was most pronounced during the first hour following morphine treatment. The M3G effect reached statistical significance in the group of mice treated with 500 μmol/kg M3G and 30 μmol/kg morphine when observing the first 60 min of registration (Fig. 2D). The maximal effect of M3G pretreatment reduced the locomotor activity induced by both 20 and 30 μmol/kg morphine during the first 60 min of registration to about 50% of the saline pretreated controls (Fig. 2B and D).

3.4. Serum and brain concentrations in morphine (20 μmol/kg) treated mice

Although statistically significant there was only a very small increase in serum morphine concentrations in mice pretreated with M3G compared to saline pretreated controls. There were no differences in morphine brain concentrations between the two pretreatment groups (Fig. 3A and C). This was also reflected in the AUC of the morphine concentration time curves being slightly increased in serum but not in the brain when comparing the M3G pretreatment group with the saline controls (Table 1).

The concentrations of M3G were also measured in serum and brain (Fig. 3B and D). In the saline pretreated mice M3G was detected as a metabolite of morphine in serum but not in brain. The serum AUC of M3G as a metabolite was only 5% of the AUC of M3G when M3G was given as pretreatment (Fig. 3B and Table 1).

In the M3G pretreated mice the brain M3G concentration fell below the brain morphine concentration after 60 min (compare Fig. 3C and D).

No M6G was detected in serum or brain in the morphine treated mice.

3.5. M3G influence on M6G induced locomotor activity

Contrary to the effect on morphine, M3G increased the locomotor activity induced by 20 and 30 μmol/kg M6G in a dose dependent manner (Fig. 4A and C). The increase in activity was evident during the whole registration periods. The maximal effect of M3G pretreatment increased the locomotor activity of 20 and 30 μmol/kg M6G to about 400% and 125% of saline pretreated mice respectively (Fig. 4B and D). The maximal increase of 30 μmol/kg M6G induced locomotor activity was

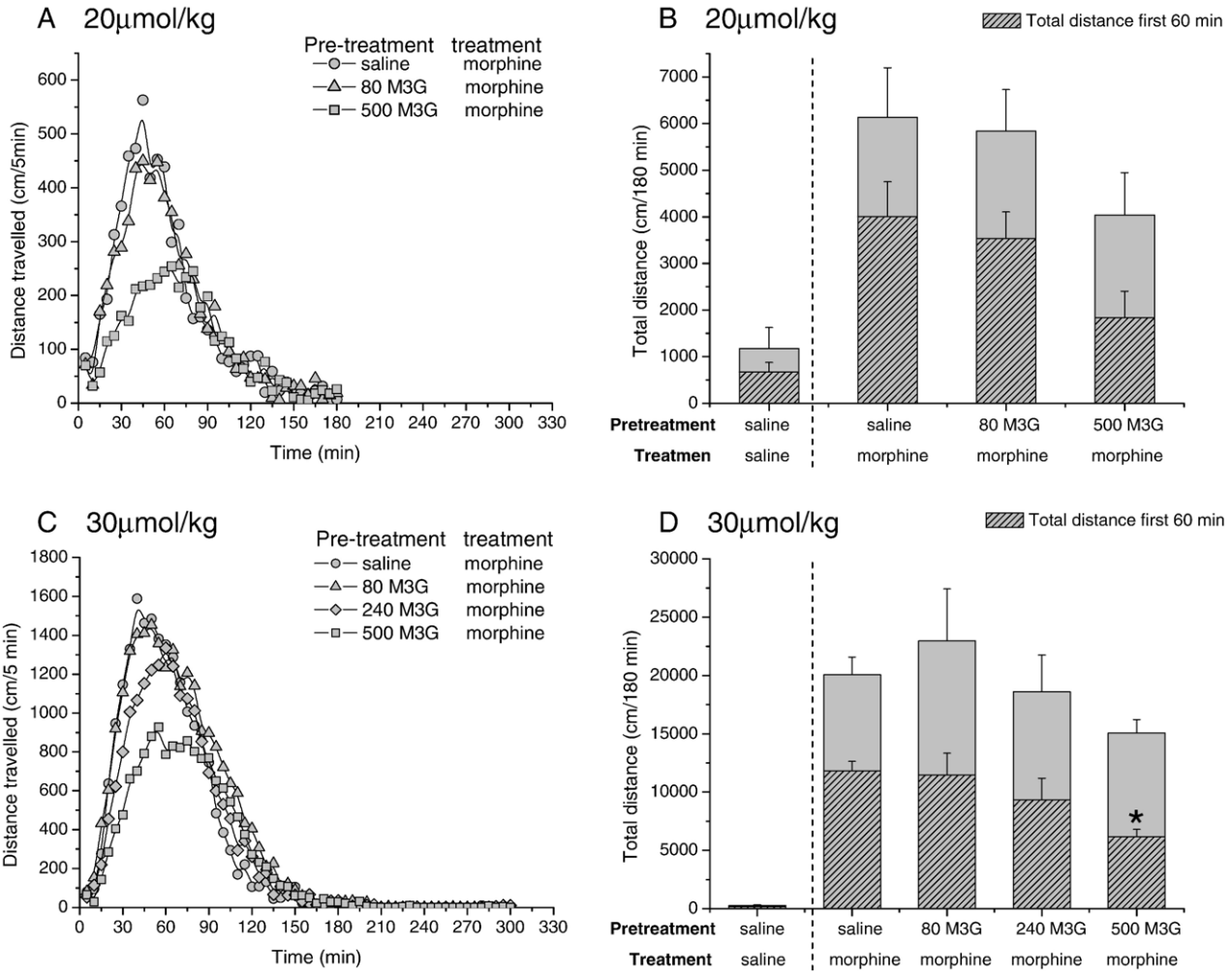

Fig. 2. Locomotor activity induced by a sc bolus dose of morphine (20 or 30 µmol/kg) in mice pretreated ip with saline or increasing doses of M3G (80 and 500 µmol/kg in panels A and B or 80, 240 and 500 µmol/kg in panels C and D). Control groups receiving two doses of saline are also shown in panels B and D. M3G was given 30 min before morphine injections. The left panels show the time course change of the different pretreatment groups in mean distance travelled per 5 min at each 5-min interval following injections with 20 µmol/kg (A) or 30 µmol/kg (C) morphine respectively. The time scales are given in relation to the moment of morphine injections. Locomotor activity results during the 90-min habituation period and S.E.M. bars were omitted for clarity. In the right panels the total sum of locomotor activity during 180 min following injection with 20 µmol/kg (B) or 30 µmol/kg (D) morphine respectively are shown. The activities of the first 60 min following morphine injection are indicated with a different fill pattern of each bar. Each bar represents mean activity ± S.E.M. (n = 9–10). Panel B one-way ANOVA (180 min); $F(2, 25) = 1.36$ ($p = 0.28$) and one-way ANOVA (60 min); $F(2, 25) = 3.15$ ($p = 0.062$). Panel D one-way ANOVA (180 min); $F(3, 35) = 1.189$ ($p = 0.33$) and one-way ANOVA (60 min); $F(3, 35) = 3.13$ ($p = 0.038$) followed by post hoc analysis (Bonferroni) comparing saline pretreatment with M3G pretreatment. *$p < 0.05$.

observed following the M3G pretreatment dose of 240 µmol/kg and increasing this dose to 500 µmol/kg did not result in a further increase in locomotor activity. The shape of the effect time curve (Fig. 4C) following this treatment was also somewhat different from the other curves, with a lower effect maximum and a somewhat longer duration of the locomotor activity.

3.6. Serum and brain concentrations in M6G (20 µmol/kg) treated mice

Following pretreatment with 500 µmol/kg M3G there was an increase in M6G serum and brain concentrations compared to the saline pretreated controls (Fig. 5A and C). This was also reflected in M3G pretreatment increasing the AUCs of both the M6G serum and brain concentration time curves compared to saline pretreatment (Table 2). However, the M3G pretreatment did not seem to change the ratio between AUC of the M6G concentration time curves in brain divided by the corresponding AUC in serum when compared to the saline pretreatment, the AUC ratio (brain/serum) being of the same order of magnitude (Table 2).

Contrary to what was observed in the morphine treated mice the M3G concentrations in the brain remained higher than M6G brain concentrations during the whole experiment (Fig. 3C and D compared to Fig. 5C and D).

No M3G (Table 2) or morphine was detected in serum or brain in the M6G treated mice pretreated with saline.

3.7. Relation between M6G concentration and induced locomotor activity

The magnitude of the increase in M6G serum and brain concentrations when the M6G dose was increased from 20 to

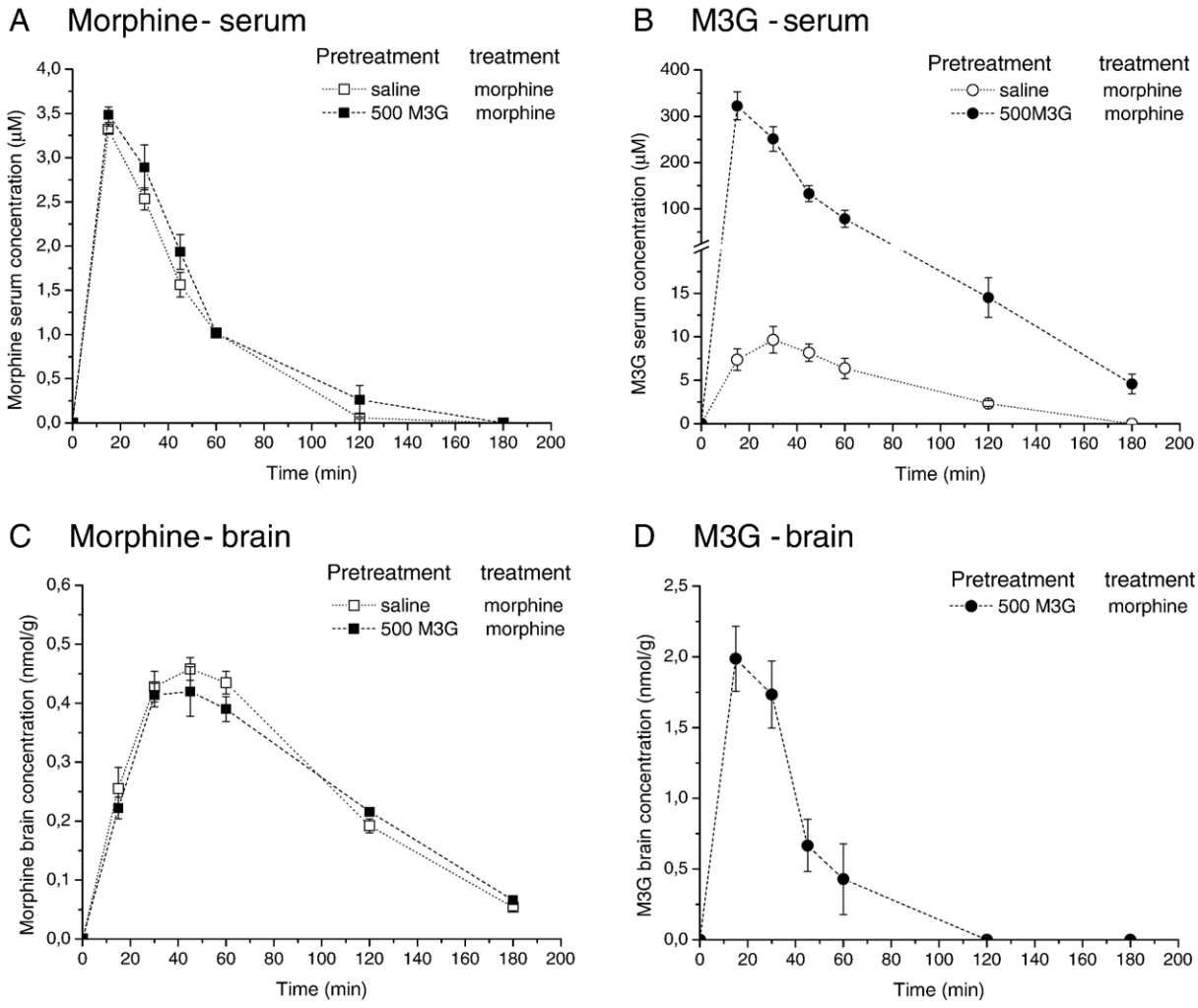

Fig. 3. Left panels show morphine serum (A) and brain (C) concentrations versus time curves in mice pretreated with saline or M3G (500 μmol/kg) ip 30 min before a sc bolus dose of morphine (20 μmol/kg). Right panels show M3G serum (B) and brain (D) concentrations in the same treatment groups. Results are mean concentrations ± S.E.M. (n = 4–5). Regression analysis: panel A, effect of pretreatment; unstandardized coefficient = 0.18 (CI = 0.04–0.33), p = 0.016. Panel B, effect of pretreatment; unstandardized coefficient = 124 (CI = 87–162), $p < 0.001$. Panel C, effect of pretreatment; unstandardized coefficient = − 0.01 (CI = − 0.04–0.01), n.s.

30 μmol/kg M6G is shown in Fig. 6A and C. Following 30 μmol/kg M6G the AUC in serum was nearly the double of the AUC following 20 μmol/kg M6G (Table 3). In brain the corresponding increase in AUC was 1.6 (Table 3). The M6G induced locomotor activity following the higher dose (30 μmol/kg) was increased to approximately 800% of the

Table 1
Area under the curve (AUC) of the concentration time curves (0–180 min) in serum and brain of morphine and M3G respectively in morphine (20 μmol/kg) treated mice pretreated with either M3G (500 μmol/kg) or saline 30 min before morphine injection

Concentrations time curves of:	Morphine (M)			M3G		
	AUC_{mean} (AUC_{min}–AUC_{max})		AUC ratio [a] (M3G M/saline M)	AUC_{mean} (AUC_{min}–AUC_{max})		AUC ratio [a] (M3G M/saline M)
	M3G M	Saline M		M3G M	Saline M	
Serum (μmol·min)/l	178 (159–198)	153 (147–159)	1.2	14,550 (12,571–16,529)	755 (635–875)	19
Brain (nmol·min)/g	45 (43–48)	47 (44–49)	1.0	82 (63–101)	n.d. [b]	–
AUC ratio [a] (brain/serum)	0.25	0.31				

AUC_{mean} was calculated based on mean concentrations (C_{mean}) at each time point.
AUC_{min} was calculated based on C_{mean} − SEM at each time point.
AUC_{max} was based on C_{mean} + SEM at each time point.
[a] Ratios were based on AUC_{mean} values.
[b] Not detected.

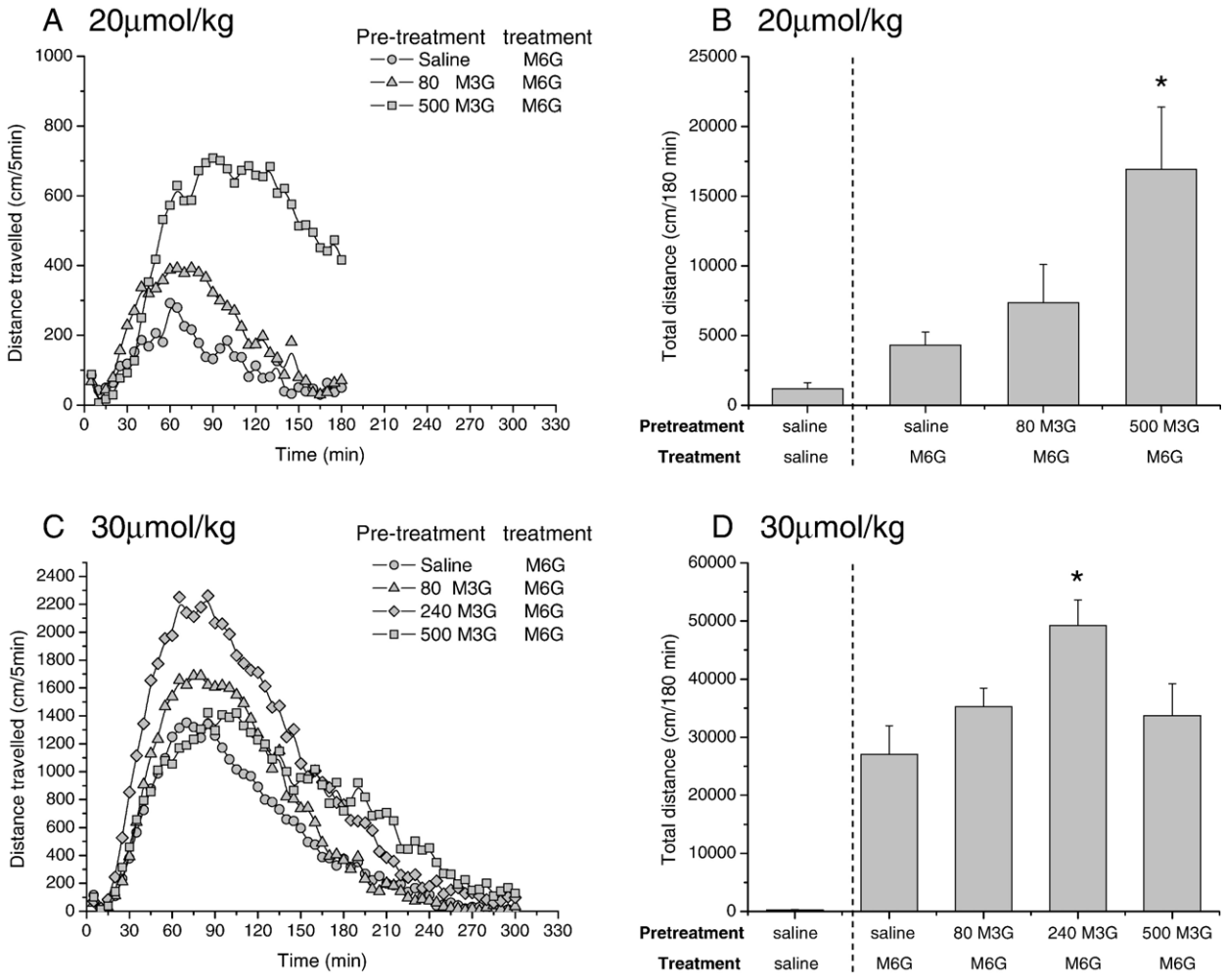

Fig. 4. Locomotor activity induced by a sc bolus dose of M6G (20 or 30 µmol/kg) in mice pretreated ip with saline or increasing doses of M3G (80 and 500 µmol/kg in panels A and B or 80, 240 and 500 µmol/kg in panels C and D). Control groups receiving two doses of saline are also shown in panels B and D. Pretreatment was given 30 min before M6G injections. The left panels show the time course change of the different pretreatment groups in mean distance travelled per 5 min at each 5-min interval following injections with 20 µmol/kg (A) or 30 µmol/kg (C) M6G respectively. The time scales are given in relation to the moment of M6G injections. Locomotor activity results during the 90-min habituation period and S.E.M. bars were omitted for clarity. In the right panels the total sum of locomotor activity during the first 180 min following injection with 20 µmol/kg (B) or 30 µmol/kg (D) M6G respectively are shown. Each bar represents mean activity ± S.E.M. (n = 9–10). Panel B one-way ANOVA; $F(2, 24) = 4.58$ ($p = 0.02$) followed by post hoc analysis (Bonferroni) comparing saline pretreatment with M3G pretreatment. Panel D one-way ANOVA; $F(3, 34) = 3.95$ ($p = 0.02$) followed by post hoc analysis (Bonferroni) comparing saline pretreatment with M3G pretreatment. *$p < 0.05$.

locomotor activity induced by the lower dose (20 µmol/kg) (Fig. 6B and D).

4. Discussion

We found that M3G, although inactive as a locomotor activity agonist when administered alone, influenced the locomotor activity induced by both morphine and M6G, but in opposite directions. M3G antagonized the locomotor activity of morphine in a dose dependent manner, but gave no reduction in morphine brain concentrations. This indicated that the mechanism behind the antagonism was of a pharmacodynamic type. M3G potentiated the locomotor activity of M6G. Simultaneously both the serum and brain concentrations of M6G were increased, and the observed increase in locomotor activity could well be explained by the increased brain concentrations. This indicated that the M3G potentiation of M6G induced locomotor activity was mainly due to a pharmacokinetic interaction.

The reduction in morphine induced locomotor activity was most pronounced during the first hour of the registration period. This corresponded with the time period when the M3G concentrations were higher than the morphine concentrations in brain. This supports that there was some kind of pharmacodynamic antagonistic relationship between M3G and morphine respectively.

To our knowledge, there are no other studies on M3G-morphine interaction regarding psychomotor stimulating effects. However, M3G has been shown to antagonize morphine analgesia in animals (Smith et al., 1990; Gong et al., 1992; Ekblom et al., 1993), although this antagonism has not been shown by others (Suzuki et al., 1993; Ouellet and Pollack, 1997). It has even been reported that M3G increased and prolonged the analgesic effect of morphine in rats (Lipkowski

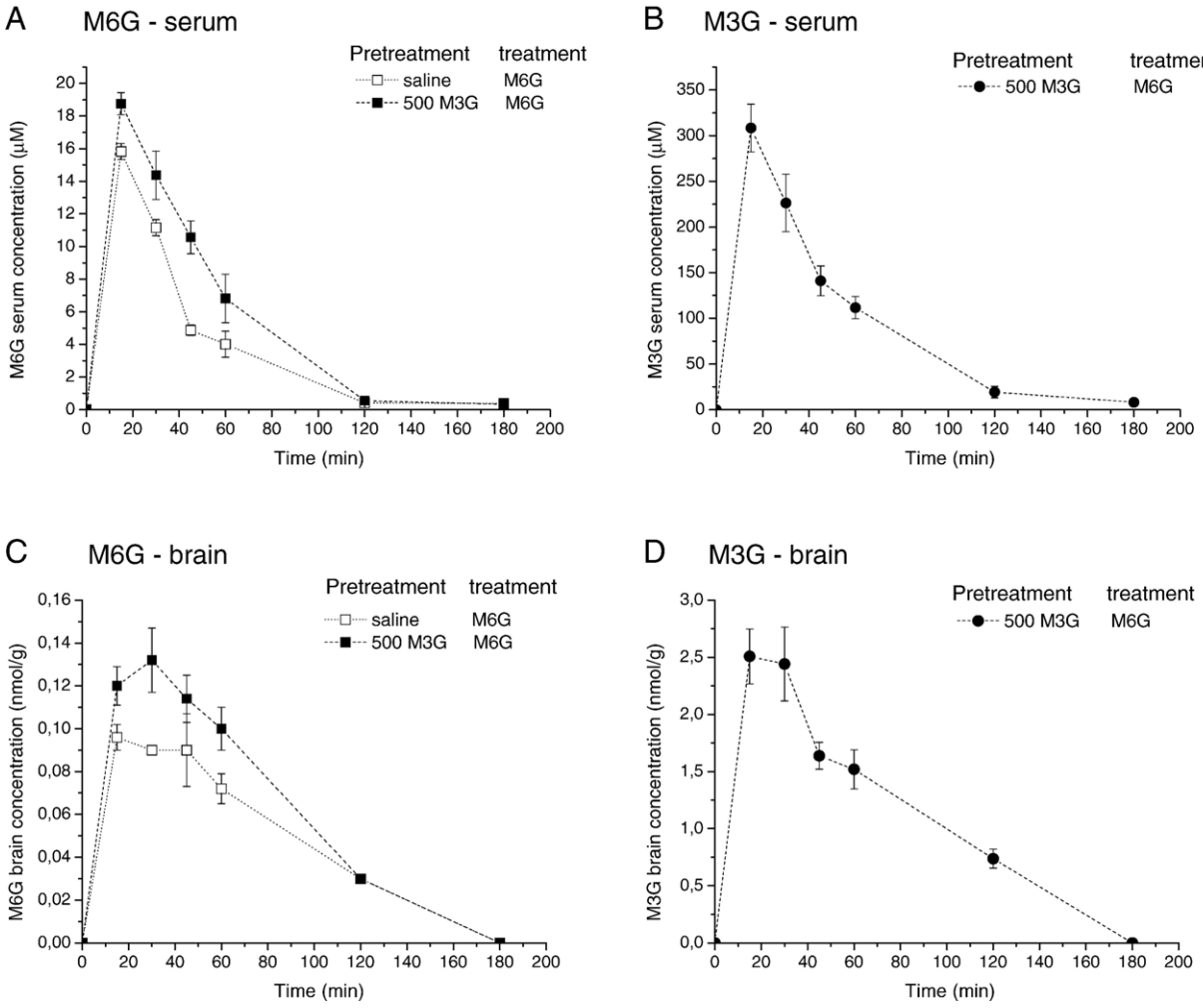

Fig. 5. Left panels show M6G serum (A) and brain (C) concentrations versus time curves in mice pretreated with saline or M3G (500 µmol/kg) ip 30 min before a sc bolus dose of M6G (20 µmol/kg). Right panels show M3G serum (B) and brain (D) concentrations in the same treatment groups. Results are mean concentrations± S.E.M. (n = 3–5). Regression analysis: panel A, effect of pretreatment; unstandardized coefficient = 2.5 (CI = 1.5–3.5), $p < 0.001$. Panel C, effect of pretreatment; unstandardized coefficient = 0.02 (CI = 0.01–0.03), $p < 0.001$.

et al., 1994). Two small clinical trials in humans did not show antagonism of M3G on morphine analgesic or respiratory depressive effects (Penson et al., 2000; Penson et al., 2001). All of these groups, however, have studied interactions in pain and/or respiration models.

M3G has a very low affinity for opioid receptors (Pasternak et al., 1987). However, Halliday et al. showed that the selective µ-opioid receptor antagonist, beta-funaltrexamine, reduced the excitatory effects of M3G (Halliday et al., 1999). This indicates that at least some of M3G's effects are mediated through the µ-opioid receptor. An interaction at this receptor might therefore be a possible explanation of the observed interaction in our study.

M3G has been shown to cause dose dependent behavioral excitation when administered to rats or mice by the intrathecal (Yaksh and Harty, 1988) or intracerebroventricular routes (Smith et al., 1990). The increasing M3G doses in the present study resulted in very high concentrations in the brain. Despite this we observed no change in any conceivable behavior with respect to neuroexcitatory effects when compared with saline.

Contrary to what we found regarding the M3G-morphine interaction, pretreatment with M3G increased the M6G induced locomotor activity during the whole registration period. At the same time pretreatment with M3G resulted in an increase in serum and brain concentrations of M6G. To explore whether this increase in brain concentrations could explain the observed increase in locomotor activity, we compared the effects of two doses of M6G from the steep part of the sigmoid shaped dose response curve. Increasing the M6G dose from 20 to 30 µmol/kg resulted in a 1.6 times increase in the AUC of the brain concentration time curve and a corresponding 7 times increase locomotor activity. Based on this one would expect that the observed AUC of the brain concentration time curves ratio (M3G pretreatment/saline pretreatment) of 1.3 would result in a corresponding locomotor activity increase of at least 3 times as observed. This indicated that the M3G–M6G interaction was mainly of a pharmacokinetic type.

A conceivable reason for the increase in serum M6G concentrations might be that M3G interfered with the systemic excretion of M6G in the kidneys or into the bile. The most

Table 2
Area under the curve (AUC) of the concentration time curves (0–180 min) in serum and brain of M6G and M3G respectively in M6G (20 μmol/kg) treated mice following pretreatment with either M3G (500 μmol/kg) or saline 30 min before M6G injection

Concentrations time curves of:	M6G			M3G	
	AUC_{mean} (AUC_{min}–AUC_{max})		AUC ratio [a] (M3G M6G/saline M6G)	AUC_{mean} (AUC_{min}–AUC_{max})	
	M3G M6G	Saline M6G		M3G M6G	Saline M6G
Serum (μmol·min)/l	955 (827–1083)	665 (613–717)	1.4	15,727 (13,773–17,680)	n.d. [b]
Brain (nmol·min)/g	11 (10–12)	8.6 (8.0–9.2)	1.3	200 (178–222)	n.d. [b]
AUC ratio [a] (brain/serum)	0.012	0.014			

AUC_{mean} was calculated based on mean concentrations (C_{mean}) at each time point.
AUC_{min} was calculated based on C_{mean} − SEM at each time point.
AUC_{max} was based on C_{mean} + SEM at each time point.
[a] Ratios were based on AUC_{mean} values.
[b] Not detected.

probable explanation would be a competition for a common secretion mechanism. Both *p*-glycoprotein (MDR1) (Letrent et al., 1998; Lotsch et al., 2002; Bourasset et al., 2003; Bourasset and Scherrmann, 2005), multidrug resistance protein 3 (Mrp3) (Zelcer et al., 2005) and the organic anion transporters (Oats) (Xie et al., 2000; Tunblad et al., 2005) have been studied.

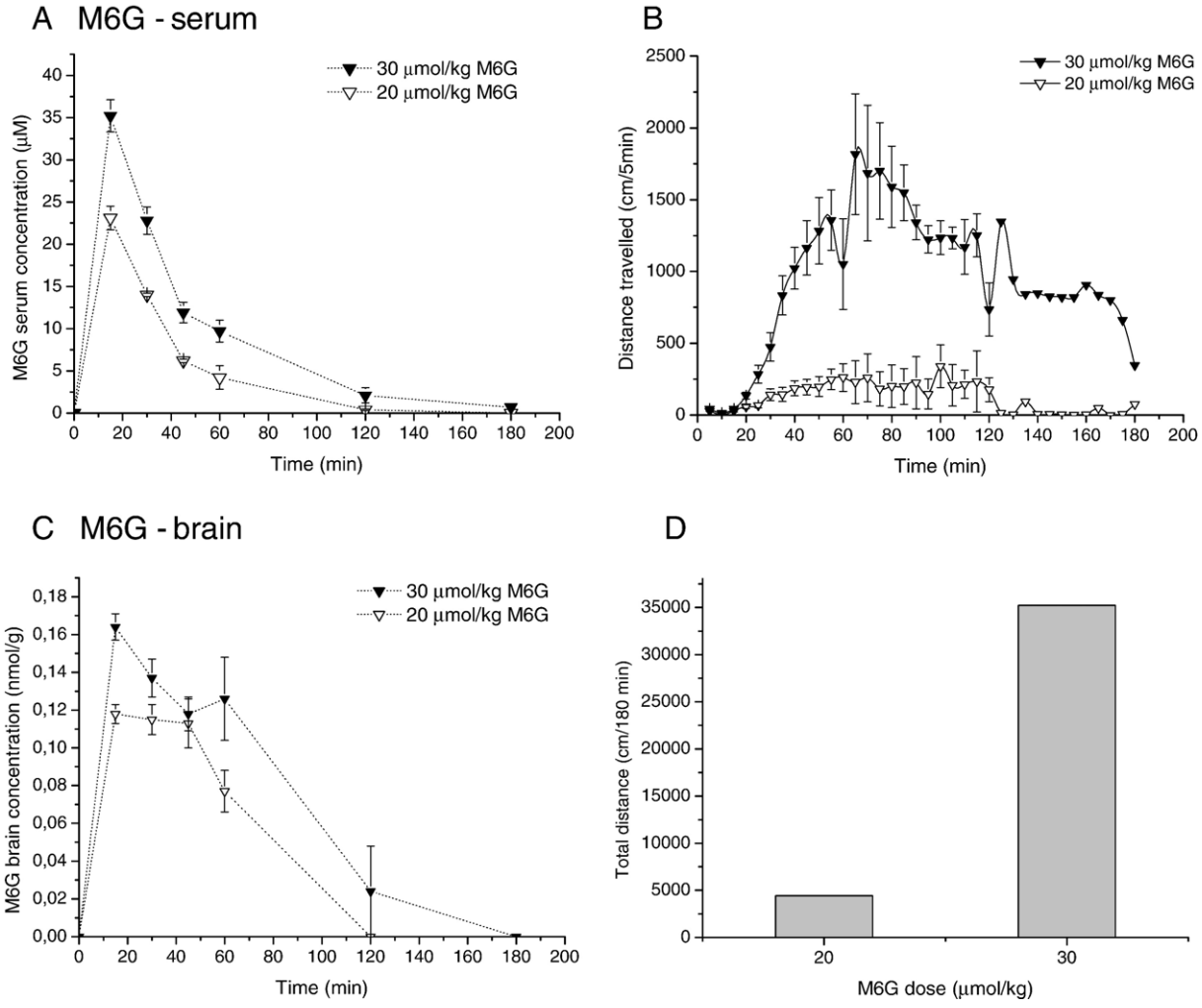

Fig. 6. The left panels show M6G serum (A) and brain (C) concentrations versus time curves in mice treated sc with two different bolus doses of M6G (20 and 30 μmol/kg). Results are mean concentrations ± S.E.M and $n = 3$–5 unless at 180 min where $n = 1$. Regression analysis: panel A, effect of dose; unstandardized coefficient = 6.3 (CI = 4.3–8.3), $p < 0.001$. Panel C, effect of dose; unstandardized coefficient = 0.03 (CI = 0.01–0.05), $p = 0.002$. The right panels show the locomotor activity of the same mice as in panels A and C. Panel B shows the time course change of the two different M6G doses as the mean total distance travelled per 5 min of the animals still alive at that time at each 5-min interval following the M6G injections. The time scales are given in relation to the moment of M6G injections. Locomotor activity results during the 90-min habituation period were omitted for clarity. Panel D shows the total sum of locomotor activity during 180 min following M6G injections with 20 or 30 μmol/kg respectively. Because of the nature of the data no statistical analyses were preformed on data from panel D.

Table 3
Area under the curve (AUC) of the concentration time curves (0–180 min) in serum and brain of M6G following injection of either 20 or 30 μmol/kg M6G

Concentrations time curves of:	AUC_{mean} (AUC_{min}–AUC_{max})		AUC ratio [a] (M6G 30/M6G 20)
	M6G (20)	M6G (30)	
Serum (μmol·min)/l	835 (750–920)	1557 (1388–1727)	1.9
Brain (nmol·min)/g	8.1 (7.3–8.9)	12 (9.8–15)	1.6
AUC Ratio [a] (brain/serum)	0.010	0.008	

AUC_{mean} was calculated based on mean concentrations (C_{mean}) at each time point.
AUC_{min} was calculated based on C_{mean} − SEM at each time point.
AUC_{max} was based on C_{mean} + SEM at each time point.
[a] Ratios were based on AUC_{mean} values.

However, it is not unambiguously determined which transporters that move morphine glucuronides across biological membranes.

In conflict with the above suggestions of an active transport of morphine glucuronides in the kidneys, it has been reported that this was not the case in isolated rat kidney (Van Crugten et al., 1991). This does not exclude a competition in other parts of the systemic elimination of the glucuronides.

The observed increase in M6G brain concentrations could be considered secondary to the increased serum concentrations, or it could be considered a result of a competition on a transport molecule in the BBB. Various groups have reported that both M6G and M3G brain concentrations largely depended upon the respective serum or plasma levels (Tunblad et al., 2005; Zelcer et al., 2005). We observed similar AUC ratios (brain/serum) of M6G in the M3G and saline pretreated mice respectively, indicating that M3G did not affect the BBB transport of M6G. This is in agreement with the difficulty in finding unambiguous evidence for a common M3G and M6G transporter in the BBB.

Our results do not exclude the possibility that in addition to a pharmacokinetic interaction there is a component of a pharmacodynamic antagonism of M3G on the M6G induced locomotor activity. Few other groups have studied the interaction between M3G and M6G. Gong et al. reported that M3G might functionally antagonize M6G induced antinociception and ventilatory depression in rats (Gong et al., 1992), but lack of antagonism has also been reported (Suzuki et al., 1993).

We used pretreatment with rather high M3G doses. As shown in both serum and brain concentrations the pretreatment with the highest M3G dose, reached concentrations far above what was observed when M3G was formed as a metabolite. However, our results demonstrate that the lower doses gave similar although less pronounced effects. We also showed that the effects of M3G would depend on the concentration of the interacting opiate. Thus a low dose of M3G could possibly interact markedly with the effects of a low concentration of either morphine or M6G.

The high M3G pretreatment dose resulted in a ratio in serum of M3G to morphine of about 80 which is very high. In contrast the same ratio in the saline pretreated mice was five. Route of administration, renal function and age are some factors that may influence the ratio. We have earlier reported that morphine administered by different routes of administration to C57BL mice resulted in ratios from 4.6 to 10.5 (Handal et al., 2002). Faura et al. did a systematic review of factors affecting the ratio of morphine and its major metabolites and reported that across all studies the M3G to morphine ratio in humans varied from 0.001–504 (Faura et al., 1998).

The amount of serum needed for the HPLC analysis in the pharmacokinetic parts of this study did not allow us to take serial blood samples of each mouse. This makes it impossible to follow the fate of each substance in one animal. Consequently we had to use mean serum concentrations from several animals per time point to draw the concentration time curve. This makes statistical analysis of the AUC data very difficult. The AUC values, however, give a reasonable estimate of the magnitude of change in concentrations.

In conclusion our results point at complicated interactions between morphine, its main metabolite M3G, which itself does not induce locomotor activity, and its active metabolite M6G with respect to induction of locomotor activity and possibly mechanisms related to drug reward and reinforcement. M3G antagonize the morphine induced locomotor activity, but potentiate the M6G induced locomotor activity. The M3G–morphine interaction was of a pharmacodynamic type, while the M3G–M6G interaction mainly was of a pharmacokinetic type. This complexity makes it more difficult to predict the consequences of an increase in M3G concentrations, than would have been the case if M3G had influenced the effects of the parent drug (morphine) and the active metabolite (M6G) in the same direction.

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References

Aasmundstad TA, Morland J, Paulsen RE. Distribution of morphine 6-glucuronide and morphine across the blood-brain barrier in awake, freely moving rats investigated by in vivo microdialysis sampling. J Pharmacol Exp Ther 1995;275:435–41.

Bourasset F, Cisternino S, Temsamani J, Scherrmann JM. Evidence for an active transport of morphine-6-beta-d-glucuronide but not P-glycoprotein-mediated at the blood–brain barrier. J Neurochem. 2003;86:1564–7.

Bourasset F, Scherrmann JM. Carrier-mediated processes at several rat brain interfaces determine the neuropharmacokinetics of morphine and morphine-6-beta-D-glucuronide. Life Sci 2005.

Carrupt PA, Testa B, Bechalany A, el Tayar N, Descas P, Perrissoud D. Morphine 6-glucuronide and morphine 3-glucuronide as molecular chameleons with unexpected lipophilicity. J Med Chem 1991;34:1272–5.

Ekblom M, Gardmark M, Hammarlundudenaes M. Pharmacokinetics and pharmacodynamics of morphine-3-glucuronide in rats and its influence on the antinociceptive effect of morphine. Biopharm Drug Dispos 1993; 14:1–11.

Faura CC, Collins SL, Moore RA, McQuay HJ. Systematic review of factors affecting the ratios of morphine and its major metabolites. Pain 1998; 74:43–53.

Gong QL, Hedner J, Bjorkman R, Hedner T. Morphine-3-glucuronide may functionally antagonize morphine-6-glucuronide induced antinociception and ventilatory depression in the rat. Pain 1992;48:249–55.

Grung M, Skurtveit S, Aasmundstad TA, Handal M, Alkana RL, Morland J. Morphine-6-glucuronide-induced locomotor stimulation in mice: role of opioid receptors. Pharmacol Toxicol 1998;82:3–10.

Grung M, Skurtveit S, Ripel A, Morland J. Lack of crosstolerance between morphine and morphine-6-glucuronide as revealed by locomotor activity. Pharmacol Biochem Behav 2000;66:205–10.

Halliday AJ, Bartlett SE, Colditz P, Smith MT. Brain region-specific studies of the excitatory behavioral effects of morphine-3-glucuronide. Life Sci 1999;65:225–36.

Handal M, Grung M, Skurtveit S, Ripel A, Morland J. Pharmacokinetic differences of morphine and morphine-glucuronides are reflected in locomotor activity. Pharmacol Biochem Behav 2002;73:883–92.

Joyce EM, Iversen SD. The effect of morphine applied locally to mesencephalic dopamine cell bodies on spontaneous motor activity in the rat. Neurosci Lett 1979;14:207–12.

Letrent SP, Pollack GM, Brouwer KR, Brouwer KL. Effect of GF120918, a potent P-glycoprotein inhibitor, on morphine pharmacokinetics and pharmacodynamics in the rat. Pharm Res 1998;15:599–605.

Lipkowski AW, Carr DB, Langlade A, Osgood PF, Szyfelbein SK. Morphine-3-glucuronide: silent regulator of morphine actions. Life Sci 1994;55:149–54.

Lotsch J, Schmidt R, Vetter G, Schmidt H, Niederberger E, Geisslinger G, et al. Increased CNS uptake and enhanced antinociception of morphine-6-glucuronide in rats after inhibition of P-glycoprotein. J Neurochem 2002; 83:241–8.

Milne RW, Nation RL, Somogyi AA. The disposition of morphine and its 3- and 6-glucuronide metabolites in humans and animals, and the importance of the metabolites to the pharmacological effects of morphine. Drug Metab Rev 1996;28:345–472.

Morland J, Jones BL, Palomares ML, Alkana RL. Morphine-6-glucuronide: a potent stimulator of locomotor activity in mice. Life Sci 1994;55:L163–8.

Osborne R, Joel S, Trew D, Slevin M. Morphine and metabolite behavior after different routes of morphine administration: demonstration of the importance of the active metabolite morphine-6-glucuronide. Clin Pharmacol Ther 1990;47:12–9.

Ouellet DM, Pollack GM. Effect of prior morphine-3-glucuronide exposure on morphine disposition and antinociception. Biochem Pharmacol 1997;53: 1451–1457.

Pasternak GW, Bodnar RJ, Clark JA, Inturrisi CE. Morphine-6-glucuronide, a potent mu agonist. Life Sci 1987;41:2845–9.

Penson RT, Joel SP, Bakhshi K, Clark SJ, Langford RM, Slevin ML. Randomized placebo-controlled trial of the activity of the morphine glucuronides. Clin Pharmacol Ther 2000;68:667–76.

Penson RT, Joel SP, Clark S, Gloyne A, Slevin ML. Limited phase I study of morphine-3-glucuronide. J Pharm Sci 2001;90:1810–6.

Sawe J, Kager L, Svensson Eng JO, Rane A. Oral morphine in cancer patients: in vivo kinetics and in vitro hepatic glucuronidation. Br J Clin Pharmacol 1985;19:495–501.

Smith MT, Watt JA, Cramond T. Morphine-3-glucuronide—a potent antagonist of morphine analgesia. Life Sci 1990;47:579–85.

Suzuki N, Kalso E, Rosenberg PH. Intrathecal morphine-3-glucuronide does not antagonize spinal antinociception by morphine or morphine-6-glucuronide in rats. Eur J Pharmacol 1993;249:247–50.

Svensson JO. Determination of morphine, morphine-6-glucuornide and normorphine in plasma and urine with high-performance liquid chromatography and electrochemical detection. J Chromatogr 1986;375:174–8.

Svensson JO, Rane A, Sawe J, Sjoqvist F. Determination of morphine, morphine-3-glucuronide and (tentatively) morphine-6-glucuronide in plasma and urine using ion-pair high-performance liquid chromatography. J Chromatogr 1982;230:427–32.

Tunblad K, Hammarlund-Udenaes M, Jonsson EN. Influence of probenecid on the delivery of morphine-6-glucuronide to the brain. Eur J Pharm Sci 2005;24:49–57.

Van Crugten JT, Sallustio BC, Nation RL, Somogyi AA. Renal tubular transport of morphine, morphine-6-glucuronide, and morphine-3-glucuronide in the isolated perfused rat kidney. Drug Metab Dispos 1991;19:1087–92.

Xie R, Bouw MR, Hammarlund-Udenaes M. Modelling of the blood–brain barrier transport of morphine-3-glucuronide studied using microdialysis in the rat: involvement of probenecid-sensitive transport. Br J Pharmacol 2000;131:1784–92.

Yaksh TL, Harty GJ. Pharmacology of the allodynia in rats evoked by high dose intrathecal morphine. J Pharmacol. Exp Ther 1988;244:501–7.

Yaksh TL, Harty GJ, Onofrio BM. High dose of spinal morphine produce a nonopiate receptor-mediated hyperesthesia: clinical and theoretic implications. Anesthesiology. 1986;64:590–7.

Yeh SY, McQuinn RL, Gorodetzky CW. Biotransformation of morphine to dihydromorphinone and normorphine in the mouse, rat, rabbit, guinea pig, cat, dog, and monkey. Drug Metab Dispos 1977;5:335–42.

Zelcer N, van de WK, Hillebrand M, Sarton E, Kuil A, Wielinga PR, et al. Mice lacking multidrug resistance protein 3 show altered morphine pharmacokinetics and morphine-6-glucuronide antinociception. Proc Natl Acad Sci U S A 2005;102:7274–9.