The Excitatory Behavioral and Antianalgesic Pharmacology of Normorphine-3-glucuronide after Intracerebroventricular Administration to Rats

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ABSTRACT
In the adult male Sprague-Dawley rat, a species commonly used to study tolerance to the antinociceptive effects of morphine, 10% of the morphine dose is metabolized to normorphine-3-glucuronide (NM3G). In contrast, NM3G is a relatively minor metabolite of morphine in human urine reportedly accounting for 1% of the morphine dose. To date, the pharmacology of NM3G has been poorly characterized. Therefore, our studies were designed to determine whether the intrinsic pharmacology of NM3G is similar to that of morphine-3-glucuronide (M3G), the major metabolite of morphine, which has been shown to be a potent central nervous system (CNS) excitant and to attenuate the intrinsic antinociceptive effects of morphine in rats. The CNS excitatory potency of NM3G was found to be approximately half that of M3G, inducing convulsions in rats at intracerebroventricular (i.c.v.) doses of 16.8 nmol. When administered before morphine (70 nmol i.c.v.), NM3G (8.9 nmol i.c.v.) attenuated antinociception for up to 2 hr, but when administered after morphine, no significant attenuation of morphine antinociception was observed. Thus, after i.c.v. administration, NM3G like M3G, is a potent CNS excitant and antianalgesic in the rat. NM3G may therefore play a role in the development of tolerance to the antinociceptive effects of morphine in the rat as has been proposed previously for M3G.

SD rats readily become tolerant to the pain-relieving effects of chronically administered morphine and hence are commonly used for studies designed to investigate the mechanisms underlying this phenomenon (Smith and Smith, 1995). In SD rats, morphine is metabolized primarily to morphine-3-glucuronide (M3G) (Milne et al., 1996). However, recent studies using in situ isolated perfused adult male SD rat livers have reported that in addition to M3G, 10% of an administered dose of morphine is secreted into the bile as NM3G (Evans and Shanahan, 1995). After systemic morphine dosing in humans, <1% of the total dose of morphine is excreted into urine as NM3G, suggesting that NM3G is a minor morphine metabolite in humans (Yeh et al., 1977).

M3G has been shown to induce potent dose-dependent behavioral excitation when administered to adult male SD rats by the i.c.v. route (Bartlett et al., 1994a). Specifically, the administration of M3G (15 nmol i.c.v.) induced severe myoclonus and status epilepticus within 15 min of administration. In addition, supraspinal administration of M3G (5.4–6.5 nmol i.c.v.) has been shown to markedly attenuate the antinociceptive effects of both i.c.v. morphine (70 nmol; Smith et al., 1990) and i.c.v. morphine-6-glucuronide (0.02–0.5 nmol; Smith et al., 1990; Gong et al., 1992; Faura et al., 1996).

Currently, the CNS pharmacology of NM3G has not been well characterized, with only one brief report in the published literature (Oguri et al., 1989), in which, after i.c.v. administration, NM3G induced significant antinociception in three of eight mice in a dose of 2 nmol and induced convulsions at higher doses (doses not defined). Because the purity of the synthesized NM3G was not described, it is conceivable that it may have contained significant normorphine contamination, which may have been responsible for the observed antinociception.

Due to the structural similarity between M3G and NM3G, one could speculate that after supraspinal administration, NM3G may induce similar excitatory behavioral and antianalgesic effects to M3G. Therefore, the aims of this study were to (1) characterize the intrinsic CNS excitatory behavioral pharmacology of NM3G; (2) compare the excitatory behavioral pharmacology of NM3G with that previously reported for M3G (Bartlett et al., 1994a); and (3) determine whether i.c.v. administration of NM3G attenuates the antinociceptive effects of i.c.v. morphine in the rat in a manner analogous to that previously reported for M3G (Smith et al., 1990).

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ABBREVIATIONS: NM3G, normorphine-3-glucuronide; M3G, morphine-3-glucuronide; i.c.v., intracerebroventricular; SD, Sprague-Dawley; AUC, area under the response vs. time curve; CNS, central nervous system; %MPE, percentage of the maximum possible antinociceptive effect.
Experimental Procedures

Materials

Water for injection was purchased from Astra (Brisbane, Australia). Ketamine (Ketav) and xylazine (Xylazil) were purchased from Abbott (Sydney, Australia). Benzathine penicillin (Bicillin) was purchased from Wyeth Pharmaceuticals (Sydney, Australia). Dental acrylic and cold-cure powder were purchased from Regional (Brisbane, Australia). NM3G was synthesized and purified using a method developed in our laboratory (Smith et al., 1997).

Animals

Adult male SD rats (225–275 g) were purchased from the Faculty of Medicine Animal House, The University of Queensland (Brisbane, Australia). Rats were housed in cages with unlimited access to food and water for at least 3 days before surgery in a temperature-controlled room (21 ± 2°C) maintained on a 12-hr/12-hr light-dark cycle. Ethical approval for the studies described herein was obtained from the Animal Experimentation Ethics Committee of the University of Queensland.

Intracerebroventricular Cannula Placement

Guide cannulae were cut from 21-gauge stainless steel needles to a length of 8 mm and filed to a 40° bevel. Stainless steel plugs that extended 0.5 mm beyond the tip of the guide cannulae were cut from 25-gauge needles. Deep and stable anesthesia was induced and maintained by the intraperitoneal injection of ketamine (120 mg/kg) using 25-gauge needles. Deep and stable anesthesia was induced and maintained by the intraperitoneal injection of ketamine (120 mg/kg) and xylazine (12 mg/kg). An incision was made slightly to the left of the cranial midline, the skin was retracted, and underlying tissue was removed. The skull was exposed, cleaned with sterile water for injection and allowed to dry. A stainless steel guide cannula then was implanted to a depth of 1 mm above the left lateral ventricle, according to the stereotaxic coordinates (L 1.5 mm, P 0.8 mm, V 3.2 mm) of Paxinos and Watson (1986), and secured in position with dental cement. After cannula implantation, 60 mg of penicillin was administered intramuscularly to provide prophylactic antibiotic cover.

Drugs were dissolved in water for injection, and i.c.v. drug administration was performed using a 10-μl SGE syringe. At the conclusion of each experiment, 5 μl of malachite green dye (100 mg/ml) was injected using the same syringe. After the animals were killed with morphine (7 nmol i.c.v., n = 6); (3) morphine hydrochloride (70 nmol i.c.v., n = 6); and (4) control rats received either 10 μl of water for injection (n = 3), or 5 μl of water for injection followed 15 min later by 5 μl of water for injection (n = 3).

The degree of antinociception achieved was assessed using the tail flick latency test (D’Amour and Smith, 1941) as detailed below.

Intracerebroventricular administration of NM3G. Rats received i.c.v. NM3G (8.9 nmol) in 10 μl of water for injection (n = 6). The degree of antinociception achieved was assessed using the tail flick latency test as detailed below.

M3G administration. Rats received i.c.v. M3G (8.9 nmol) in 10 μl of water for injection (n = 6). Rats were assessed for signs of behavioral excitation using the behavioral paradigm described by Bartlett et al. (1994a).

Assessment and Quantification of the Intrinsic Excitatory Behavioral Pharmacology of NM3G

Rats were placed individually into a clear Perspex observation chamber (45 × 25 × 25 cm) containing sawdust and food pellets. After a 30-min acclimatization period, each rat received i.c.v. drug or vehicle administration and was observed for the next 80 min. The behavioral paradigm used to assess the excitatory response of each animal was based on that of Bartlett et al. (1994a). Rats were observed before dosing and then at 5, 15, 25, 35, 50, 65 and 80 min after i.c.v. injection, and 14 excitatory behaviors were scored. Four behaviors (myoclonus, chewing, wet-dog shakes and rearing) were scored on an absolute number of incidents over a 30-sec period (0 = no incidents, 1 = 1–5 incidents, 2 = 6–10 incidents, 3 = 11–15 incidents and 4 = 16 incidents). Tonic-clonic convulsions, explosive motor behavior, grooming, exploring, general activity and eating were each scored on a 5-point scale where 0 = none, 1 = mild, 2 = moderate, 3 = severe and 4 = extreme incidence. Ataxia was scored on a 3-point scale (0 = none, 1 = mild and 2 = severe ataxia), whereas righting reflex was scored on the basis of its presence or absence (1 = present, 2 = absent). The two remaining behaviors were scored as follows: body posture (0 = normal, 1 = abnormal posture, 2 = severely abnormal posture) and touch-evoked agitation (0 = no avoidance of a non-noxious stimulus, 1 = mild avoidance behavior, 2 = biting at probe, 3 = vocalization, 4 = profound avoidance behavior).

Our preliminary studies of the intrinsic behavioral excitation of NM3G have been published previously (Smith et al., 1997).

Data Analysis for the Excitatory Behavioral Paradigm

Scores for each behavior were plotted against time, and the AUC was calculated using trapezoidal integration. The individual AUC values for each behavior were summed to give a total excitation score for each animal ($\text{AUC}_{i-14}$). Total excitation scores for each animal at each dose were then converted to a percentage of the maximum possible excitation score ($\text{AUC}_{\text{max}} = 5577.5$). ED$_{50}$ doses for M3G and NM3G were calculated using the GraphPAD Prism computer program (GraphPAD Software, San Diego, CA).

Tail Flick Latency Test

The degree of antinociception achieved in the rats was assessed using the tail flick latency test as described by D’Amour and Smith (1941). At least 15 min before tail flick latency determination, rats were placed in restraining cages for acclimatization. Radiant heat was focused on the lower third of the dorsum of the tail at an intensity that gave predrug reaction times of ~2.0 to 3.0 sec. The preinjection tail-flick latency was the average of three measurements determined at 5-min intervals. Antinociceptive testing was performed at 15, 30, 45, 60, 75, 90, 120, 150 and 180 min after dosing. A maximum tail flick latency of 9.0 sec was used to minimize tissue damage to the tail.
The degree of antinociception developed was expressed as a percentage of maximum possible effect (%MPE) according to the formula (Brady and Holtzmann, 1982):

\[
\% \text{ MPE} = \frac{\text{Postdrug latency} - \text{Predrug latency}}{\text{Maximum latency (9.0 sec)} - \text{Predrug latency}} \times 100\%
\]

Differences between groups were determined by comparing the AUCs for each group, using the Kruskal-Wallis one-way analysis of variance by rank with a statistical significance criterion of \( P < .05 \).

### Results

**Dose-response relationship for NM3G.** The i.c.v. administration of NM3G to rats produced a dose-dependent increase in the total behavioral excitation score (fig. 1, table 1). Thus, rats dosed with 2.2 nmol of NM3G exhibited only a mild increase in general activity and exploration such that the mean ± S.E.M. total excitation score (ΣAUC\textsubscript{1–14}) was 214 ± 18. At a dose of 5.6 nmol, general activity and exploration increased, as did the onset of myoclonus, principally of the right hind limb, giving a mean ± S.E.M. ΣAUC\textsubscript{1–14} value of 335 ± 28. The administration of 11.2 nmol of NM3G intensified the excitatory behaviors observed at the lower doses, and additional behaviors, including chewing and excessive grooming, were seen. More generalized myoclonus was observed, as was the onset of mild ataxia and alterations in body-posture such that the mean ± S.E.M. ΣAUC\textsubscript{1–14} was 672 ± 76.

Tonic-clonic convulsions were observed subsequent to the administration of 16.8 nmol of NM3G, as was the onset of a moderate degree of explosive motor behavior and an increase in general activity and exploration. Loss of righting reflex associated with tonic-clonic convulsions was also observed such that the mean ± S.E.M. ΣAUC\textsubscript{1–14} was 732 ± 108. After doses of 22.4 nmol, the maximum mean ± S.E.M. excitatory response was observed (ΣAUC\textsubscript{1–14} = 1247 ± 150), which was not significantly different from the mean ± S.E.M. excitatory response observed after i.c.v. administration of 33.6 nmol of NM3G (ΣAUC\textsubscript{1–14} = 967 ± 131). These doses of NM3G induced intense but relatively short periods of tonic-clonic convulsions that occurred intermittently with explosive motor behavior, increased exploration, general activity, chewing and wet-dog shakes. A profound effect on body posture leading to the animal lying prostrate on the floor of the observation chamber also was noted. A pronounced response to non-noxious stimuli also was observed, particularly at the 22.4 nmol dose.

The ED\textsubscript{50} value for cumulated behavioral excitation induced by NM3G was determined to be 16.8 nmol compared with a calculated ED\textsubscript{50} value for cumulated behavioral excitation for M3G of 9.3 nmol (from the data of Bartlett et al., 1994a).

**Excitatory behavioral effects observed after i.c.v. M3G administration.** The administration of M3G (8.7 nmol i.c.v.) to adult male SD rats (positive control experiments) induced excitatory behaviors similar to those described previously by Bartlett et al. (1994a). Importantly, the mean ± S.E.M. total excitation score (ΣAUC\textsubscript{1–14} = 750 ± 250) reported by Bartlett et al. (1994a) was not significantly different (\( P > .05 \)) from that determined in this study (862 ± 32), thereby ensuring that valid comparisons can be made between the relative excitatory potencies of NM3G and M3G.

**Attenuation of morphine antinociception by the prior administration of NM3G.** Rats dosed with 70 nmol of i.c.v. morphine achieved high levels of antinociception (%MPE > 70%) within 15 min of morphine dosing, which were maintained for the 180-min observation period (Fig. 2). However, after i.c.v. administration of NM3G (8.9 nmol) 15 min before the i.c.v. administration of morphine (70 nmol), marked attenuation of morphine-induced antinociception was observed for the first 120 min of the 180-min observation period (fig. 2). Based on a comparison of the mean ± S.E.M. area under the degree of antinociception vs. time curve (AUC) achieved in this group of rats for up to 120 min after NM3G administration, no significant difference (\( P > .05 \)) was seen from the base-line levels of antinociception observed in control rats. At 150 and 180 min after i.c.v. NM3G administration, the mean levels of antinociception achieved in rats dosed with NM3G before morphine were not significantly different from the mean %MPE values achieved in rats dosed with morphine (70 nmol) alone, indicating that the attenuation of morphine-induced antinociception was of a relatively short duration (2 hr).

**Lack of attenuation of morphine antinociception by the administration of NM3G after morphine.** In contrast to our findings when i.c.v. NM3G was administered before i.c.v. morphine, the mean AUC achieved in rats that received 8.9 nmol of i.c.v. NM3G 15 min after i.c.v. morphine (70 nmol) was not significantly different from that achieved in rats dosed with morphine (70 nmol i.c.v.) alone (fig. 3). When the i.c.v. dose of NM3G administered after morphine was increased to 11 nmol, myoclonic behavior (e.g., tail movement) that may have confounded unambiguous interpretation of the tail-flick latency test was induced, thereby precluding administration of this dose of NM3G after morphine.

**Rats dosed with i.c.v. vehicle.** The mean ± S.E.M. levels of antinociception achieved in control animals dosed with i.c.v. vehicle did not differ significantly (\( P > .05 \)) from pre-dosing base-line values of antinociception for the entire duration of the 3-hr observation period (figs. 2 and 3), indicating that the experimental procedure itself did not affect the levels of antinociception observed in these experiments.
larger than 16.8 nmol, although these effects were of only lesser severity and shorter duration than those induced by the same receptor mechanism in the CNS. Quantitatively, the excitatory behaviors produced by i.c.v. NM3G were of a relatively short duration (15–30 min), with rats recovering completely by the end of the 80-min study period. In contrast, previous studies in our laboratory have shown that i.c.v. administration of M3G in a dose of 7 μg (15.1 nmol) induced irreversible status epilepticus (Bartlett et al., 1994a). The shorter duration of excitatory action of NM3G relative to that of M3G may be due to increased hydrophilicity, resulting from its more basic secondary amine, and thus facilitating more rapid removal by the cerebrospinal fluid from its site of action within the brain.

Qualitatively, the excitatory behaviors produced by i.c.v. NM3G in this study (table 1) did not differ from those evoked by i.c.v. M3G (Bartlett et al., 1994a), strongly indicating that NM3G and M3G elicit their excitatory behavioral effects through the same receptor mechanism in the CNS. Quantitatively, the excitatory behaviors evoked by NM3G were of a lesser severity and shorter duration than those induced by equimolar doses of i.c.v. M3G. This finding was not due to differences in the scoring methods of the different observers.

**Table 1**

Dose-dependent excitatory behaviors observed after i.c.v. administration of NM3G to adult male Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Behavior</th>
<th>NM3G dose (i.c.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.2 nmol</td>
</tr>
<tr>
<td>Myoclonus</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Chewing</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Wet-dog shakes</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Rearing</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Convulsions</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Explosive motor behavior</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Grooming</td>
<td>2.1 ± 1.4</td>
</tr>
<tr>
<td>Exploring</td>
<td>2.1 ± 1.8</td>
</tr>
<tr>
<td>General activity</td>
<td>32.9 ± 10.3</td>
</tr>
<tr>
<td>Eating</td>
<td>1.4 ± 1.4</td>
</tr>
<tr>
<td>Ataxia</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Righting reflex</td>
<td>80.0 ± 0.0</td>
</tr>
<tr>
<td>Posture</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Touch-evoked agitation</td>
<td>3.2 ± 1.5</td>
</tr>
</tbody>
</table>

Control rats received i.c.v. saline. Values represent the mean ± S.E.M. area under the excitation score vs. time curve (AUC) for each behavior.
NM3G: Excitation and Antianalgesia

The adult male SD rat is commonly used in studies of the development of antinociceptive tolerance to morphine. In this rat model, morphine is avidly metabolized to two excitatory and antianalgesic metabolites, NM3G and M3G, accounting for ~10% and 55% of the morphine dose, respectively (Evans and Shanahan, 1995; Milne et al., 1996). Considerable indirect evidence strongly suggests that M3G plays a significant role in the development of antinociceptive tolerance to morphine by progressively attenuating morphine’s antinociceptive effects (Barjavel et al., 1995; Smith and Smith, 1995). By analogy, the results of this study suggest that NM3G will also contribute significantly to the development of antinociceptive tolerance to morphine in the SD rat.

In conclusion, the findings of this study suggest that NM3G is a potent CNS excitant in a manner analogous to M3G, the major metabolite of morphine in both rats and humans. NM3G evokes behavioral excitation in a dose-dependent manner with convulsions of short duration occurring at an i.c.v. dose of 16.8 nmol. The intrinsic excitatory potency of NM3G in the rat appears to be ~50% that of M3G. In addition, supraspinal administration of NM3G before, but not after, morphine results in significant attenuation of morphine antinociception, suggesting that in the rat, NM3G, like M3G (Smith et al., 1990; Gong et al., 1992; Smith and Smith, 1995; Barjavel et al., 1995), may have a significant role in the development of antinociceptive tolerance to morphine.

References


Bartlett SE, Cramond T and Smith MT (1994a) The excitatory effects of morphine-3-glucuronide are attenuated by LY274614, a competitive NMDA receptor antagonist, and by midazolam, an agonist at the benzodiazepine site on the GABAA receptor complex. Life Sci 54:687–694.


involved in these two studies because the total behavioral excitation scores obtained for rats that received i.c.v. M3G (4 μg; 8.7 nmol) in this study (ΣAUC1–14 = 862 ± 32, mean ± S.E.M.) were not significantly different from the respective scores (ΣAUC1–14 ~ 750 ± 250, mean ± S.E.M.) for rats administered the same dose of i.c.v. M3G in the study reported by Bartlett et al. (1994a).

The administration of NM3G (8.9 nmol i.c.v.) 15 min before but not after i.c.v. morphine (70 nmol) potently attenuated morphine’s antinociceptive effects for the first 2 hr of the 3-hr observation period. In contrast, previous studies in our laboratory (Smith et al., 1990) have shown that i.c.v. M3G markedly attenuates the antinociceptive effects of the same dose of i.c.v. morphine regardless of whether M3G was given before or after morphine. The reason for this difference between M3G and NM3G is unclear, particularly because the CNS mechanism through which NM3G and its close structural analog, M3G, elicit their excitatory and/or antianalgesic effects is currently unknown.

It is unlikely that M3G and NM3G attenuate morphine antinociception by competitive inhibition of opioid receptors because M3G (and presumably NM3G) have a very low affinity for classic inhibitory opioid receptors of any class (mu, delta and kappa) (Bartlett et al., 1994b; Löser et al., 1996). However, it is currently unknown whether M3G and NM3G induce CNS excitation by interacting with the recently cloned excitatory opioid receptor ORL1 (Anton et al., 1996; Nothacker et al., 1996). Similarly, in vitro binding studies indicate that M3G (and NM3G) are unlikely to produce CNS excitation and/or antianalgesia through disinhibition of inhibitory glycinergic or GABAergic neurotransmission or through activation of N-methyl-D-aspartate receptors in the CNS (Bartlett et al., 1994b). In vitro studies using rat brain synaptosomes also indicate that M3G (and presumably NM3G) are unlikely to evoke CNS excitation by augmentation of excitatory amino acid (glutamate) neurotransmitter release or inhibition of inhibitory amino acid neurotransmitter (γ-aminobutyric acid) release (Bartlett and Smith, 1996).

It is possible, however, that M3G and NM3G produce CNS excitation by augmenting the putative endogenous antiopioid system, proposed to exist in a tonic balance with the inhibitory opioidergic system in the CNS (Rothman, 1992). The administration of i.c.v. NM3G before i.c.v. morphine could potentiate the antiopioid system, thereby attenuating morphine’s antinociceptive effects. In contrast, i.c.v. administration of morphine before NM3G could potentiate the inhibitory opioidergic system to such an extent that NM3G may be insufficiently potent to attenuate morphine’s antinociceptive effects. Clearly, further studies are required to investigate the mechanistic basis for M3G and NM3G induced excitation and antianalgesia.

The results of this study also clearly show that NM3G in a dose of 8.9 nmol is devoid of antinociceptive activity in a manner analogous to that reported previously for M3G (Yoshimura et al., 1973; Labella et al., 1979; Gong et al., 1991). On this basis, the “apparent” antinociception reportedly observed in three of eight mice dosed with NM3G in a preliminary report (Oguri et al., 1989) is likely to have been due to contamination of the NM3G with the potent antinociceptive agent normorphine. In contrast, the purity of the NM3G used in our studies was quantified and found to be >99% with <0.3% normorphine contamination (Smith et al., 1997).
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