Sex-Related Differences in Antinociception and Tolerance Development following Chronic Intravenous Infusion of Morphine in the Rat: Modulatory Role of Testosterone via Morphine Clearance

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ABSTRACT

This study investigated possible sex-related differences in levels of antinociception and the rate of development of tolerance to the antinociceptive effects following prolonged (48 h) intravenous (i.v.) morphine administration in the rat. Groups of adult intact male, castrated male, female, and testosterone-pretreated female Sprague-Dawley rats received prolonged (48 h) infusions of i.v. morphine (5 or 10 mg/day) plus intra-arterial (i.a.) saline or i.v. morphine (5 mg/day) plus i.a. chloramphenicol (300 mg/day). Antinociception was quantified using the hot-plate test. Serum concentrations of morphine and morphine-3-glucuronide (M3G) were assayed using high performance liquid chromatography with electrochemical detection, whereas the serum testosterone concentrations were quantified using an enzyme-linked immunosorbent assay method. Consistent with our previous findings in intact male rats, prolonged coinfusion of chloramphenicol with morphine produced a marked increase in the extent and duration of morphine antinociception in all experimental groups. Additionally, female and castrated male rats developed tolerance more slowly than either intact male or testosterone-pretreated female rats, when coinfused with parenteral morphine plus chloramphenicol. However, mean levels of antinociception were not significantly correlated with either the mean serum morphine or M3G concentrations, but were significantly inversely correlated with the mean values of the M3G/morphine serum molar concentration ratio. Testosterone pretreatment of female rats for 1 week before chronic morphine infusion abolished antinociception and markedly reduced both the serum morphine and M3G concentrations. These latter findings imply that testosterone modulates antinociception evoked by prolonged morphine infusion in rats via a mechanism that appears to involve modulation of morphine metabolism.

In humans, morphine is metabolized predominantly to two glucuronide metabolites, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), both of which are pharmacologically active (see review by Milne et al., 1996). M6G is a more potent analgesic than morphine following central administration (Milne et al., 1996), but it is not formed in detectable quantities in Wistar and Sprague-Dawley rats (Coughtrie et al., 1989; Tan et al., 1989). By contrast, M3G, the principal metabolite of morphine in both rats and human, has no pain-relieving effects. However, following intracerebroventricular (i.c.v.) or intrathecal administration to rats, M3G evokes a range of neuroexcitatory behaviors in a dose-dependent manner (LaBella et al., 1979; Yaksh et al., 1986; Bartlett et al., 1994). Additionally, supraspinal (but not spinal; Hewett et al., 1993; Suzuki et al., 1993) M3G has been shown to potently attenuate the antinociceptive effects of i.c.v. morphine (Smith et al., 1990) and M6G (Smith et al., 1990; Gong et al., 1992; Faura et al., 1996), suggesting that M3G is an anti-analgesic metabolite of morphine.

Biological sex differences in the sensitivity of rats to the antinociceptive effects of chronically administered morphine and the rate of development of antinociceptive tolerance are not well defined in the literature. Previous studies that have examined this issue have shown that male rats develop antinociceptive tolerance to repeated subcutaneous morphine administration more rapidly than do female rats (Kasson and

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ABBREVIATIONS: AUC, area under the curve; CNS, central nervous system; M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; MOR AUC, area under the serum morphine versus time curve; %MPE, percentage maximum possible antinociceptive effect; SD, Sprague-Dawley; UGT, UDP-glucuronosyltransferase.
George, 1984; Craft et al., 1999). However, the possible influence of M3G, the putatively anti-analgesic metabolite of morphine, on the levels of antinociception evoked, was not assessed because serum morphine and M3G concentrations were not quantified in the same rats as used for antinociceptive testing. Clearly, if the circulating serum concentrations of M3G significantly influence either the levels of antinociception produced, and/or the rate of antinociceptive tolerance development, in rats dosed chronically with morphine, then it is important to undertake systematic studies involving quantification of antinociception together with the serum concentrations of morphine and M3G in the same rats.

Recent studies in our laboratory (Smith et al., 2000) have shown that coadministration of parenteral chloramphenicol (an inhibitor of morphine glucuronidation; Miners et al., 1988) with intravenous (i.v.), but not i.c.v. morphine, increased the extent and duration of morphine antinociception by 5.5-fold relative to these effects in Sprague-Dawley (SD) rats dosed with i.v. morphine alone. These findings indicate that the mechanism through which chloramphenicol enhances i.v. morphine antinociception in the rat does not directly involve supraspinal opioid receptors. Importantly, following chronic coadministration of parenteral chloramphenicol and morphine for 48 h, there was no significant change in the area under the serum morphine versus time curve (MOR AUC), despite the 5.5-fold concomitant increase in morphine antinociception. Thus, our previous findings (Smith et al., 2000) clearly indicate that factors other than serum morphine concentrations contribute significantly to the levels of antinociception produced. A possible explanation for the lack of effect of chloramphenicol on the MOR AUC is that there was a compensatory increase in the N-demethylation of morphine to normorphine, a metabolic pathway that normally accounts for 20% of the morphine dose in adult male rat livers (Evans and Shanahan, 1995). Additionally, antinociceptive tolerance to morphine developed more slowly in rats coadministered chloramphenicol, even though the serum morphine concentrations were not significantly altered. Thus, our previous studies are consistent with the proposal that in vivo inhibition of the formation of the putatively anti-analgesic, M3G, would increase antinociception and delay development of tolerance (Smith et al., 2000).

In vitro studies have shown that morphine, chloramphenicol, and testosterone are all substrates for the UGT2B1 isoform of UDP-glucuronosyltransferase (UGT) (Pritchard et al., 1994; Coffman et al., 1996). Generally, the expression of UGT appears to be higher in male rats compared with female and prepubescent male rats. Importantly, the expression of UGT was reduced in gonadectomized male rats, but recovered to match that of adult male rats when gonadectomized male rats were given a testosterone supplement. Although these data suggest that circulating testosterone concentrations appear to regulate expression of UGT in the SD rat, especially UGT2B1 expression at a pretranslational level (Strasser et al., 1997), this role is not absolutely conclusive because testosterone was not administered to female rats to determine whether similar changes in UGT isoform expression and activity could be produced.

Thus, the aims of this study were to investigate possible sex-related differences in the development of antinociceptive tolerance to chronically administered i.v. morphine relative to that observed in rats coadministered morphine plus chloramphenicol in intact male, castrated male, female, and testosterone-pretreated female SD rats, and to determine whether the serum concentrations of morphine and/or M3G were significantly correlated with levels of antinociception in the same rats.

Materials and Methods

Experimental Animals. Approval from the Animal Experimentation Ethics Committee of The University of Queensland was obtained for all of the studies described herein. Adult male and female SD rats were purchased from The University of Queensland Medical School Animal Breeding Facility (Brisbane, Australia). Adult male rats castrated before puberty were purchased from Monash University Animal Breeding House (Melbourne, Australia).

Reagents and Materials. Disposable, 1-μl inoculating loops were purchased from Difco Laboratories (Detroit, MI). Isoflurane (Fortane) was purchased from Abbott Australasia Pty. Ltd. (Sydney, Australia). Polyethylene tubing (o.d. 1 mm, i.d. 0.5 mm) was purchased from Dural Plastics and Engineering Pty. Ltd. (Sydney, Australia) and silastic tubing from Auburn Plastics and Engineering (Sydney, Australia). Silk sutures were Dysisilk Black Braided Siliconised Silk purchased from Dynek Pty. Ltd. (Hendon, Australia). Minivials (Eppendorf) were purchased from Disposable Products (Brisbane, Australia). Graseby Medical infusion pumps (Model MS16A, Graseby Medical, Watford, UK) were used for both intravenous and intra-arterial drug administration. Morphine sulfate ampoules (30 mg/ml) were purchased from David Bull Laboratories (Melbourne, Australia). Chloramphenicol succinate injection vials (1.2 g) were purchased from Parke-Davis Pty. Ltd. (Sydney, Australia) and were diluted to the required concentrations with heparinized saline (50 IU/ml) (Astra Pharmaceuticals Pty. Ltd., Sydney, Australia). Testosterone enanthate (Ropel; 75 mg/ml) was a generous gift from Dr. J. Keast (Brisbane, Australia). Testosterone was diluted to a concentration of 1.5 mg/ml using organic sesame oil from Melrose Laboratories (Melbourne, Australia).

Experimental Methods

Female Estrous Cycle. Cervical smears were taken from female rats and examined microscopically to ensure that experimentation was initiated when female rats were in the estrus/diestrus stage of the estrus cycle.

Surgery

Jugular Vein and Femoral Artery Cannulation. Rats underwent jugular vein and femoral artery cannulation while under general anesthesia with 3% isoflurane:97% oxygen. The cannulae were externalized through a subcutaneous tunnel to the back of the neck and were protected by a stainless-steel spring. Following surgery, the animals were allowed to recover overnight before experiments were initiated and were housed in a temperature-controlled room (21°C ± 2°C) with a 12/12-h light/dark cycle; food and water were available ad libitum.

Drug Dosage

Testosterone Supplementation of Female Rats. Testosterone-pretreated female rats received a subcutaneous injection of testosterone enanthate (1.5 mg/kg) at the same time each day for 7 days before experimentation.

Morphine and Chloramphenicol. Preliminary experiments established that infusion of morphine in a dose of 5 mg/day was the maximum tolerable by female and castrated male rats when coadministered chloramphenicol. Groups of adult male and female SD rats received an initial bolus dose of morphine (2.1 mg/kg), followed by an infusion for 48 h of either (i) morphine (5 mg/day) plus saline or (ii) morphine (5 mg/day) plus chloramphenicol (bolus 100 mg then
300 mg/day); corresponding groups of control rats received chronic parenteral infusions of (iii) saline plus saline or (iv) saline plus chloramphenicol (bolus 100 mg then 300 mg/day). Groups of testosterone-pretreated female SD rats received similar dosing regimens to those described above for male and female rats, except that the infusions were terminated at 6 h. Morphine or saline was infused via the jugular vein cannula (i.v.), whereas chloramphenicol or saline was infused via the femoral artery cannula (intra-arterial). For rats that received combinations (ii) and (iv), chloramphenicol succinate (100 mg) was administered via the jugular vein cannula, 30 min before i.v. administration of morphine or saline, in a manner analogous to that used in our previous study (Smith et al., 2000). Similar groups of castrated male rats received an initial bolus dose of morphine (4.2 mg/kg), followed by prolonged (48 h) coadministration of parenteral morphine (10 mg/day) plus saline. All infusion solutions were administered at a constant flow rate of 4.5 ml/24 h, and all morphine doses are expressed as the base.

Quantitation of Antinociception. Briefly, antinociception was quantified using the hotplate latency test (55 ± 0.5°C) (Eddy and Leimbach, 1953). A maximum hotplate latency of 30.0 s was used to prevent tissue damage to the rat’s paws. Predosing latencies were determined on at least three occasions (5 min apart with the three measurements being within ± 1 s) before the administration of drugs or heparinized saline. To correct for individual differences in baseline latencies, the antinociceptive data (hotplate latencies) were normalized to the percentage maximum possible effect (%MPE) using the following equation (Brady and Holtzman, 1982):

\[
\%\text{MPE} = \frac{(\text{Post-drug latency}) - (\text{pre-drug latency})}{(\text{Maximum latency}) - (\text{pre-drug latency})} \times 100
\]

Antinociceptive Testing and Blood Sample Collection. For groups of male, female, and castrated male rats, antinociceptive testing and blood sample (0.4 ml) collection were performed immediately predosing and at 0.25, 0.5, 1, 2, 3, 6, 12, 24, 30, 36, and 48 h after commencement of the morphine or saline infusion. For testosterone-pretreated female rats, antinociceptive testing and blood samples collected were discontinued at the same time (6 h) as the infusion was ceased. Blood samples were collected into Eppendorf tubes via the femoral artery cannula to prevent possible contamination with the morphine infusion solution. After centrifugation, the serum was separated and stored at ~20°C, before assay.

Quantification of Morphine and M3G in Rat Serum. Serum concentrations of morphine and M3G were quantified using solid-phase extraction and high-performance liquid chromatography with electrochemical detection (Wright and Smith, 1998). Briefly, morphine and M3G were separated from endogenous components of serum using “classic” solid-phase extraction cartridges (Sep-paks, Millipore-Waters) installed in a Vac-Elut vacuum filtration system (Analytichem International, Harbor City, CA). Aliquots of serum (100 µl) were added to 10 ml polypropylene tubes, followed by 100 µl of internal standard (M6G, 2 ng/µl) and 1.0 ml of 0.05 M phosphate buffer (pH 7.5). After vortex mixing for 10 s, samples were loaded onto extraction columns that had been preconditioned with methanol (5 ml), followed by 0.05 M phosphate buffer (pH 7.5) (3–5 ml), under gentle vacuum (~1 inch Hg). The extraction columns were then washed with 10 ml of 0.05 M phosphate buffer (pH 7.5) and dried under increased vacuum (5–10 inches Hg) for 2–4 min. The cartridges were washed with 3 ml of 5% methanol:H2O, facilitating the removal of endogenous components of rat serum, and then dried again under vacuum. Morphine, M3G, and the internal standard were eluted with 1.6 ml of methanol and collected into Eppendorf tubes. After methanol was removed under high-purity nitrogen at 65°C, the residue was dissolved in 100 µl of mobile phase by vortexing for 30 s, and 80 µl was then injected onto the high-performance liquid chromatography. The retention times of M3G, morphine, and internal standard were approximately 8.4, 11.4, and 15.6 min, respectively. The extraction efficiencies of morphine and M3G were 94 and 93%, respectively (Wright and Smith, 1998) and the lower limits of quantification for morphine and M3G were 2.3 and 5 ng injected on-column, respectively. Chromatograms of extracted serum samples from control rats (dosed chronically with saline/saline or chloramphenicol/saline) did not contain any peaks that cochromatographed with either morphine or M3G, including the chloramphenicol and putative chloramphenicol glucuronide peaks (Fig. 1).

Standard curves comprising six to seven concentrations of morphine and M3G were chromatographed in random order with each batch of rat serum samples. Peak height ratios of either morphine or M3G relative to that of the internal standard were plotted against concentration. Regression analysis was used to produce standard curves, which were accepted if the correlation coefficients were >0.99. Additionally, control serum samples in two different concentrations (17.0 or 170 ng of morphine and 46.9 or 469 ng of M3G per sample) were included in each chromatographic run at a frequency of approximately one “seed” per five samples. Within-run coefficients of variation for low and high control samples containing morphine and M3G were <5.6 and <3.9%, respectively (Wright and Smith, 1998). Interday within-run coefficients of variation for the low and high control samples incorporated into each chromatographic run were <9 and <13%, respectively.

Quantification of Testosterone and Creatinine Concentrations in Rat Serum. Serum testosterone concentrations were quantified in intact male, castrated male, female, and testosterone-pretreated female rats (n = 3 per group), on a fee-for-service basis. The testosterone assays were performed by The University of Queensland Veterinary Pathology service using a validated enzyme-linked immunosorbent assay method. Serum creatinine concentrations were assayed by the Royal Brisbane Hospital Pathology Department.

![Fig. 1.](image-url)
Pharmacodynamic and Pharmacokinetic Analyses. The extent and duration of antinociception were quantified by calculating the area under the degree of antinociception versus time curve (%MPE AUC values) using trapezoidal integration; the corresponding units are %MPE · h. To facilitate comparison of %MPE AUC0–6 h values between groups of rats, these values were normalized to correct for small differences in body weight between some groups of male and female rats. This was done by dividing the %MPE AUC0–6 h by the morphine dose (expressed as μmol/kg) given in the 6-h infusion period; the corresponding units are %MPE · h/μmol/kg. Using a similar approach, the areas under the serum morphine and M3G plasma concentration versus time curves for the first 6 h of the infusion (MOR AUC0–6 h and M3G AUC0–6 h values, respectively) were also dose-normalized; the corresponding units are h · mg/liter.

The mean total body clearance of morphine, CL, was determined from the relationship:

\[ \text{CL} = \frac{\text{infusion rate of morphine}}{C_{\text{steady-state}}} \]

where \( C_{\text{steady-state}} \) = mean serum morphine concentration in interval, 24 to 48 h, for intact male, female, and castrated male rats; or the serum morphine concentration at 6 h for testosterone-pretreated female rats.

Statistical Analyses. Comparisons of the %MPE AUC data and the corresponding morphine and M3G AUC values between experimental groups were performed using the Wilcoxon rank-sum test as implemented in the Minitab statistical analysis program. Regression analysis was used to determine the relationship between mean levels of antinociception and the mean serum morphine or M3G concentrations, or the mean values of the serum molar concentration ratio, M3G/MOR, for each experimental group. The degree of correlation between mean levels of antinociception and the corresponding mean morphine concentrations or the mean values of the serum molar concentration ratio, M3G/MOR, for each experimental group was determined using the sigmoidal curve-fitting program in GraphPad Prism (version 2.0). The statistical significance criterion was \( p < 0.05 \).

Results

Antinociception

Morphine Plus Saline Infusions (48 h). Intravenous infusion of morphine (5 mg/day for 48 h) produced significant antinociception in female (%MPE values ≥40%, \( n = 8 \), Fig. 2A), but not male rats (%MPE values ≤20%, \( n = 6 \), Fig. 2C). However, the %MPE values in untreated female rats (Fig. 2A) returned to baseline by 2 to 3 h, which then persisted for the remainder of the 48-h experimental period. Furthermore, testosterone pretreatment of female rats (Fig. 2B) essentially abolished the antinociception (%MPE values ≤10% MPE).

In a previous study in our laboratory (Smith et al., 2000), i.v. infusion of a higher dose of morphine (10 mg/day for 48 h) in intact male rats produced relatively high levels of antinociception (%MPE ≈ 60%) for approximately 30 min. Additionally, these same rats became completely tolerant to the antinociceptive effects of morphine by 3 h. However, when the same dose of i.v. morphine (10 mg/day for 48 h) was administered to male rats castrated before puberty (\( n = 7 \)) in the present study, maximal antinociception (%MPE ≈ 100) was found, together with a marked attenuation in the rate of tolerance development, such that baseline levels of antinociception were not attained until 12–24 h after initiation of the morphine infusion (Fig. 2D). Calculation of the area under the %MPE versus time curve, revealed that the mean extent and duration of morphine antinociception (%MPE AUC0–48 h) for castrated male rats was approximately twice that (\( p < 0.05 \)) reported previously by our laboratory (Smith et al., 2000) for intact male rats (Table 1).

Morphine Plus Chloramphenicol Infusions (48 h). Consistent with our previous findings (Smith et al., 2000), parenteral coadministration of chloramphenicol (300 mg/day for 48 h) with morphine (5 mg/day for 48 h) significantly (\( p < 0.05 \)) increased both the extent and duration of antinociception (%MPE AUC values) for all experimental groups, relative to comparable rats that received morphine (5 mg/day for 48 h) alone (Table 1). Moreover, the magnitude of this effect was large, particularly for intact male and female rats where coadministration of chloramphenicol increased the morphine %MPE AUC values by approximately 30-fold (Table 1). Visual inspection of Fig. 2 reveals that, for intact male rats (\( n = 7 \)), mean levels of antinociception were >70% MPE for 3 h (Fig. 2C), whereas for female (\( n = 8 \)) and castrated male rats (\( n = 7 \)), high levels of antinociception (>70% MPE) were maintained for approximately 12 h (Fig. 2, A and D, respectively). Thereafter, levels of antinociception decreased such that intact male rats were completely tolerant (baseline %MPE values) by 24 to 30 h after initiation of the morphine infusion.

Although a longer period was required (~48 h), castrated male rats also became completely tolerant to the antinociceptive effects of morphine (Fig. 2D). By contrast, the mean %MPE values remained ≈60% for the majority of female rats at 48 h (Fig. 2A), indicating that chloramphenicol had markedly slowed the development of antinociceptive tolerance in females. However, the levels of antinociception produced by the same doses of chloramphenicol plus morphine in testosterone-pretreated female rats were much lower (%MPE < 30%) than in comparable untreated female rats (Fig. 2B), indicating that testosterone pretreatment of female rats had significantly (\( p < 0.05 \)) attenuated the extent and duration of antinociception by >100-fold (Table 1). Taken together, our findings show that female and castrated male rats experienced high levels of antinociception for a significantly (\( p < 0.05 \)) longer duration than did either intact male or testosterone-pretreated female rats following chronic coadministration of the same parenteral doses of morphine plus chloramphenicol (Table 1). Additionally, although chloramphenicol was less effective in intact males or testosterone-pretreated female rats, it nevertheless did augment the magnitude of antinociception and attenuated tolerance in the latter groups.

Control Rats. For rats in each control group (Fig. 2, E and F), viz. intact male, female, castrated male, and testosterone-pretreated rats (saline/saline, \( n = 3 \); saline/chloramphenicol, \( n = 3 \)), baseline levels of antinociception (%MPE < 5%) were observed throughout the 48-h study period. Male and female rats were equally sensitive to the hotplate latency test, because there were no significant differences in the mean (± S.E.M.) baseline latency values between intact male (4.1 ± 0.2 s) and female rats (3.7 ± 0.2 s) or between intact male and castrated male rats (4.7 ± 0.4 s). Although the mean (± S.E.M.) baseline latency value for testosterone-pretreated female rats (2.7 ± 0.1 s) was significantly lower than that for female rats (3.7 ± 0.2 s), the magnitude of this difference was too small to have accounted for the major differences in morphine antinociception observed between female and testosterone-pretreated female rats.
Serum Morphine and M3G Concentrations

Morphine Plus Saline Infusions (48 h). For rats infused chronically with i.v. morphine plus saline, the mean measured maximum serum morphine concentration ($C_{max}$) was approximately three times larger in female rats relative to the respective $C_{max}$ concentrations in testosterone-pretreated female and intact male rats (Table 2; Fig. 3), and this was consistent with the high initial levels of antinociception observed in female rats. The morphine concentrations decreased slowly from peak to a mean steady-state concentration of $\sim1.0 \, \mu M$ for female rats (Fig. 3A) and $\sim0.6 \, \mu M$ for intact male and testosterone-pretreated female rats (Fig. 3, C and B). Additionally, the dose-normalized MOR AUC$_{0-6 \, h}$ values in female rats were approximately twice ($p < 0.05$) the corresponding values in intact male and testosterone-pretreated female rats (Table 2). Consistent with these findings, the mean ($\pm$S.E.M.) total body clearance of morphine in female rats ($2.47 \pm 0.37 \, l/kg/h$) was approximately one-half ($p < 0.05$) that for intact male rats ($5.35 \pm 0.62 \, l/kg/h$) and testosterone-pretreated female rats ($3.95 \pm 0.45 \, l/kg/h$) dosed with morphine alone (Table 2).

The concomitant mean ($\pm$S.E.M.) $C_{max}$ concentration for M3G in female rats ($5.7 \pm 1.1 \, \mu M$) was also significantly ($p < 0.05$) larger (3- to 8-fold) than the corresponding values in testosterone-pretreated females ($1.7 \pm 0.2 \, \mu M$) and male rats ($0.7 \pm 0.1 \, \mu M$; Table 3). Consistent with these findings, the mean steady-state serum M3G concentrations and the mean M3G AUC values were significantly...
TABLE 1
Mean (±S.E.M.) maximum levels of antinociception (%MPE) and area under the %MPE versus time curve (%MPE AUC values) for male, female, and testosterone-pretreated female rats dosed chronically with i.v. morphine in the presence and absence of parenteral chloramphenicol.

<table>
<thead>
<tr>
<th>Dosing Regimen</th>
<th>Experimental Group</th>
<th>Maximum Antinociception (%MPE)</th>
<th>Time to Reach Baseline</th>
<th>%MPE AUC_{0-6} h</th>
<th>%MPE AUC_{0-48} h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male 1.25 mg/kg bolus + infusion (5 mg/24 h)</td>
<td>Male 19.2 ± 4.1</td>
<td>1</td>
<td>12.1 ± 3.6</td>
<td>0.75 ± 0.23</td>
<td>26.3 ± 12.7</td>
</tr>
<tr>
<td></td>
<td>Female 39.9 ± 13.4</td>
<td>3</td>
<td>66.9 ± 16.2</td>
<td>3.19 ± 0.96</td>
<td>124.0 ± 32.2</td>
</tr>
<tr>
<td></td>
<td>Testosterone-pretreated female</td>
<td>8.7 ± 1.8</td>
<td>0</td>
<td>6.3 ± 0.28</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>Male 1.25 mg/kg bolus + infusion (10 mg/24 h)</td>
<td>Male 67.3 ± 33</td>
<td>6</td>
<td>82.6 ± 14.9</td>
<td>2.40 ± 0.48</td>
<td>248.8 ± 46.3</td>
</tr>
<tr>
<td></td>
<td>Castrated male</td>
<td>100 ± 0</td>
<td>24</td>
<td>290.6 ± 73</td>
<td>7.05 ± 1.76</td>
</tr>
<tr>
<td>Male 2.5 mg/kg bolus + infusion (5 mg/24 h)</td>
<td>Male 91.0 ± 9.0</td>
<td>30</td>
<td>448.0 ± 56</td>
<td>24.42 ± 3.0</td>
<td>852.3 ± 196</td>
</tr>
<tr>
<td></td>
<td>Female 100 ± 0</td>
<td>&gt;48</td>
<td>508.2 ± 13.4</td>
<td>24.08 ± 0.56</td>
<td>3589.5 ± 493</td>
</tr>
<tr>
<td></td>
<td>Testosterone-pretreated female</td>
<td>25 ± 3.8</td>
<td>1</td>
<td>30.5 ± 5.4</td>
<td>1.28 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>Castrated male</td>
<td>100 ± 0</td>
<td>&gt;48</td>
<td>523.7 ± 40.7</td>
<td>24.72 ± 1.96</td>
</tr>
</tbody>
</table>

Significant differences (p < 0.05) shown as: * male > testosterone female; b female > testosterone female; c female > male; d data from Smith et al. (2000); e castrated male > male; f Morphine/chloramphenicol > morphine/saline; g female > castrated male; h castrated male > testosterone female.

TABLE 2
Mean (±S.E.M.) maximum serum concentration of morphine (C_{max}, μM) and area under the serum morphine concentration versus time curve (MOR AUC) for male, female, testosterone-pretreated female, and castrated male rats dosed with either morphine plus saline or morphine plus chloramphenicol.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>T_{max} h</th>
<th>C_{max} μM</th>
<th>AUC_{0-6} h</th>
<th>AUC_{0-48} h (Dose-Normalized)</th>
<th>AUC_{0-48} h</th>
<th>CL l/kg/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male 1.25 mg/kg bolus + infusion (5 mg/24 h)</td>
<td>0.5</td>
<td>1.5 ± 0.6</td>
<td>6.1 ± 2.4</td>
<td>0.24 ± 0.12</td>
<td>38.5 ± 14.8</td>
<td>5.35 ± 0.62</td>
</tr>
<tr>
<td>Female 1.25 mg/kg bolus + infusion (5 mg/24 h)</td>
<td>0.5</td>
<td>5.3 ± 1.1</td>
<td>10.0 ± 1.2</td>
<td>0.49 ± 0.05</td>
<td>61.9 ± 11.8</td>
<td>2.47 ± 0.37</td>
</tr>
<tr>
<td>Testosterone-pretreated female</td>
<td>0.25</td>
<td>1.5 ± 0.1</td>
<td>4.8 ± 0.5</td>
<td>0.22 ± 0.02</td>
<td>Not available</td>
<td>3.95 ± 0.45</td>
</tr>
<tr>
<td>Male 2.5 mg/kg bolus + infusion (10 mg/24 h)</td>
<td>0.25</td>
<td>2.9 ± 0.3</td>
<td>6.9 ± 1.9</td>
<td>0.23 ± 0.07</td>
<td>37.2 ± 8.7</td>
<td>6.03 ± 1.16</td>
</tr>
<tr>
<td>Castrated male</td>
<td>0.25</td>
<td>4.1 ± 1.2</td>
<td>10.5 ± 3.5</td>
<td>0.26 ± 0.10</td>
<td>61.2 ± 7.2</td>
<td>4.20 ± 0.69</td>
</tr>
<tr>
<td>Male 2.5 mg/kg bolus + infusion (5 mg/24 h)</td>
<td>0.25</td>
<td>3.0 ± 0.9</td>
<td>14.6 ± 4.0</td>
<td>0.80 ± 0.22</td>
<td>44.4 ± 12.2</td>
<td>6.73 ± 2.72</td>
</tr>
<tr>
<td>Female 2.5 mg/kg bolus + infusion (5 mg/24 h)</td>
<td>0.25</td>
<td>5.1 ± 0.7</td>
<td>22.6 ± 1.6</td>
<td>0.95 ± 0.18</td>
<td>157.7 ± 16.5</td>
<td>1.08 ± 0.24</td>
</tr>
<tr>
<td>Testosterone-pretreated female</td>
<td>0.25</td>
<td>2.4 ± 0.2</td>
<td>6.3 ± 0.5</td>
<td>0.26 ± 0.02</td>
<td>Not available</td>
<td>4.49 ± 0.47</td>
</tr>
<tr>
<td>Castrated male</td>
<td>2</td>
<td>1.7 ± 0.8</td>
<td>8.0 ± 2.4</td>
<td>0.37 ± 0.11</td>
<td>56.2 ± 13.7</td>
<td>5.33 ± 2.52</td>
</tr>
</tbody>
</table>

T_{max} time of maximum plasma concentration; C_{max} maximum plasma concentration; CL clearance of morphine from serum.

Significant differences (p < 0.05) shown as: * female > male; b female > testosterone female; c morphine/saline > morphine/chloramphenicol; d female < male; e data from Smith et al. (2000); f castrated male > male; g castrated male > female; h morphine/chloramphenicol > morphine/saline; i male > testosterone female; j female > castrated male.

larger (p < 0.05) for female relative to intact male rats (compare Fig. 4, A and C; Table 3). Comparison of the dose-normalized M3G AUC_{0-6} h values between female and testosterone-pretreated female rats revealed that testosterone pretreatment reduced the serum M3G AUC_{0-6} h by >50%. For castrated male rats dosed with morphine (10
mg/day for 48 h) alone, the mean values of \(C_{\text{max}}\), \(\text{AUC}_{0–6\ h}\), and \(\text{AUC}_{0–48\ h}\) for M3G were approximately 4- to 5-fold larger than the respective values reported previously (Smith et al., 2000) by our laboratory for intact male rats (compare Fig. 4, D and C; Table 3).

**Morphine Plus Chloramphenicol Infusions (48 h).** Parenteral coadministration of chloramphenicol (300 mg/day) and morphine (5 mg/day) resulted in a doubling of the dose-normalized mean (±S.E.M.) serum MOR \(\text{AUC}_{0–6\ h}\) in female rats (0.95 ± 0.18 h·kg/l). Similarly, there was an approximately 2.5-fold increase in the mean (±S.E.M.) serum MOR \(\text{AUC}_{0–48\ h}\) in female rats (157.7 ± 16.5 \(\mu\)M·h; Table 2) to a value approximately 3.5-fold larger \((p < 0.05)\) than that for intact male rats (44.4 ± 12.2 \(\mu\)M·h). Additionally, in female rats, the mean (±S.E.M.) total body clearance of morphine (1.08 ± 0.24 l/kg/h) was less than one-half \((p <

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**TABLE 3**

Mean (±S.E.M.) maximum serum concentration of M3G (\(C_{\text{max}}, \mu M\)) and area under the serum M3G concentration versus time curve (M3G AUC) for male, female, testosterone pretreated female, and castrated male rats dosed with either morphine plus saline or morphine plus chloramphenicol.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>(T_{\text{max}}, h)</th>
<th>(C_{\text{max}}, \mu M)</th>
<th>(\text{AUC}_{0–6\ h}, \mu M\cdot h)</th>
<th>(\text{AUC}_{0–6\ h}) (Dose-normalized)</th>
<th>(\text{AUC}_{0–48\ h}, \mu M\cdot h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphine/Saline</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.1 mg/kg bolus + infusion (5 mg/24 h)</td>
<td>0.5</td>
<td>0.7 ± 0.1</td>
<td>1.4 ± 0.3</td>
<td>0.08 ± 0.02</td>
<td>12.5 ± 4.4</td>
</tr>
<tr>
<td>Male</td>
<td>0.5</td>
<td>5.7 ± 1.1 &lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>16.0 ± 2.7 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.72 ± 0.14 &lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>82.4 ± 10.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Female</td>
<td>0.25</td>
<td>1.7 ± 0.2</td>
<td>7.1 ± 1.2</td>
<td>0.32 ± 0.05</td>
<td>Not available</td>
</tr>
<tr>
<td>Testosterone-treated female</td>
<td>0.25</td>
<td>1.8 ± 0.2</td>
<td>4.6 ± 0.8</td>
<td>0.14 ± 0.03</td>
<td>35.6 ± 8.1</td>
</tr>
<tr>
<td>4.25 mg/kg bolus + infusion (10 mg/24 h)</td>
<td>0.5</td>
<td>10.1 ± 2.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>28.4 ± 5.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.70 ± 0.15&lt;sup&gt;d&lt;/sup&gt;</td>
<td>158.3 ± 9.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Castrated male</td>
<td>1</td>
<td>1.5 ± 0.08</td>
<td>4.3 ± 1.6</td>
<td>0.23 ± 0.09&lt;sup&gt;e&lt;/sup&gt;</td>
<td>34.3 ± 16.6&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Morphine/chloramphenicol</strong></td>
<td>12</td>
<td>1.7 ± 0.2</td>
<td>7.6 ± 0.8</td>
<td>0.31 ± 0.03</td>
<td>Not available</td>
</tr>
<tr>
<td>2.1 mg/kg bolus + infusion (5 mg/24 h)</td>
<td>12</td>
<td>4.7 ± 0.6&lt;sup&gt;a,b,f&lt;/sup&gt;</td>
<td>13.8 ± 3.0&lt;sup&gt;b,f&lt;/sup&gt;</td>
<td>0.58 ± 0.12&lt;sup&gt;b,f&lt;/sup&gt;</td>
<td>255.6 ± 31.1&lt;sup&gt;a,b,f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Male</td>
<td>1</td>
<td>1.7 ± 0.2</td>
<td>7.6 ± 0.8</td>
<td>0.31 ± 0.03</td>
<td>Not available</td>
</tr>
<tr>
<td>Female</td>
<td>30</td>
<td>2.4 ± 0.5</td>
<td>6.1 ± 2.1</td>
<td>0.29 ± 0.11</td>
<td>47.5 ± 15.8</td>
</tr>
<tr>
<td>Testosterone-treated female</td>
<td>30</td>
<td>2.4 ± 0.5</td>
<td>6.1 ± 2.1</td>
<td>0.29 ± 0.11</td>
<td>47.5 ± 15.8</td>
</tr>
</tbody>
</table>

\(T_{\text{max}}\) time of maximum plasma concentration; \(C_{\text{max}}\) maximum plasma concentration.

Significant differences \((p < 0.05)\) shown as: \(<sup>a</sup>\) female > male; \(<sup>b</sup>\) female > testosterone female; \(<sup>c</sup>\) data from Smith et al. (2000); \(<sup>d</sup>\) castrated male > male; \(<sup>e</sup>\) morphine/chloramphenicol > morphine/saline; \(<sup>f</sup>\) female > castrated male.
the respective rate observed in female rats dosed with morphine alone (2.47 ± 0.37 l/kg/h), and only 20% of the comparable rate for intact male rats coadministered morphine/chloramphenicol (5.35 ± 0.62 l/kg/h). Although there was a trend for an increase in the mean value of the serum MOR AUC0–48 h and in the morphine clearance for intact male rats coadministered with morphine plus chloramphenicol, these trends were not statistically significant (p > 0.05). For testosterone-pretreated female rats, the mean (±S.E.M.) maximum serum morphine concentration (Cmax, 2.4 ± 0.2 μM) was significantly greater (p < 0.05) than that for comparable rats that received morphine alone (1.5 ± 0.1 μM; Fig. 3B, Table 2). However, there was no significant (p > 0.05) difference in the mean value of the dose-normalized MOR AUC0–6 h in testosterone-pretreated female rats coadministered chloramphenicol plus morphine compared with testosterone-pretreated female rats dosed with morphine alone (0.22 ± 0.02 h · kg/l). Importantly, testosterone pretreatment of female rats coadministered parenteral chloramphenicol and morphine resulted in a significant (p < 0.05) decrease in the mean serum concentration of morphine compared with testosterone-pretreated female rats dosed with morphine alone (0.22 ± 0.02 h · kg/l).

The corresponding mean (±S.E.M.) M3G AUC0–48 h values for rats coadministered morphine and chloramphenicol increased approximately 3-fold (p < 0.05) in both intact male (34.3 ± 16.6 μM · h) and female rats (255.6 ± 31.1 μM · h), compared with comparable groups of rats dosed with morphine alone (12.5 ± 4.4 and 82.4 ± 10.8 μM · h, respectively; Table 3). Irrespective of whether rats were coadministered chloramphenicol, the mean M3G AUC0–48 h values were approximately 7-fold larger (p < 0.05) in female relative to intact male rats. For testosterone-pretreated female rats dosed with morphine plus chloramphenicol (Fig. 4B), the mean (±S.E.M.) dose-normalized M3G AUC0–6 h value (0.32 ± 0.05 h · kg/l) did not differ significantly from the corresponding value in rats dosed with morphine alone (0.31 ± 0.03 h · kg/l; Table 3). Although there was a trend for the mean (±S.E.M.) M3G AUC0–6 h value for castrated male rats (47.5 ± 15.8 μM · h) to be larger than that for intact male rats (34.3 ± 16.6 μM · h; Table 3), it was not statistically significant (p > 0.05).

Lack of Correlation between Mean Levels of Antinociception and the Mean Serum Concentrations of Morphine and M3G. Mean levels of antinociception were poorly correlated with either the mean serum concentrations of morphine or M3G for groups of intact male, castrated male, female, and testosterone-pretreated female rats that were coadministered morphine plus saline or morphine plus chloramphenicol (Fig. 5, A–D).

Significant Inverse Correlation between Mean Levels of Antinociception and the Mean Values of the Serum M3G/MOR Molar Concentration Ratio. For rats that achieved significant antinociception, the mean %MPE values were inversely correlated (p < 0.05), with the mean values of the serum [M3G]/[MOR] molar concentration ratio (Fig. 5, E and F). Moreover, close inspection of these data reveals that, although similar inverse correlations were found for female and castrated male rats (Fig. 5E), and between intact male and testosterone-pretreated female rats (Fig. 5F), the two inverse correlations were quite different. Specifically, for female and castrated male rats, the inverse correlation between levels of antinociception and the mean value of the M3G/MOR molar concentration ratio appears to be shifted to the right by 3- to 4-fold relative to the respective
Fig. 5. A, mean (±S.E.M.) levels of antinociception were not significantly correlated with the mean serum morphine concentrations for female (▲) and castrated male (■) rats that were coadministered morphine (5 mg/day) plus chloramphenicol by chronic parenteral infusion for 48 h or for female (○) rats that received morphine (5 mg/day for 48 h) alone. B, mean (±S.E.M.) levels of antinociception were not significantly correlated with the mean serum morphine concentrations for intact male (●) and testosterone-pretreated female (●) rats infused chronically with parenteral morphine (5 mg/day for 48 h) plus either saline or chloramphenicol. Similarly, mean (±S.E.M.) levels of antinociception were not significantly correlated with the mean serum morphine concentrations for female rats (○) infused for 48 h with parenteral morphine/saline. C, lack of significant inverse correlation between mean (±S.E.M.) levels of antinociception and the mean serum M3G concentrations for female (▲) and castrated male (■) rats that were coadministered morphine (5 mg/day) plus chloramphenicol by chronic parenteral infusion for 48 h or for female (○) rats that received morphine (5 mg/day for 48 h) alone. D, lack of significant inverse correlation between mean (±S.E.M.) levels of antinociception and the mean serum M3G concentrations for intact male (●) and testosterone-pretreated female (●) rats dosed chronically with parenteral morphine (5 mg/day for 48 h) plus either saline or chloramphenicol. Similarly, mean (±S.E.M.) levels of antinociception were not significantly inversely correlated with the mean serum M3G concentrations for female rats (○) infused for 48 h with parenteral morphine/saline. E, significant inverse correlation between mean levels of antinociception and the mean serum M3G/MOR concentrations for intact male (●) and testosterone-pretreated female (●) rats that were coadministered morphine (5 mg/day for 48 h) plus chloramphenicol. (%MPE = 95.886 - 5.759 x [M3G/MOR]; r = 0.67, n = 21, p < 0.05). F, significant inverse exponential correlation (r² = 0.87) between the mean levels of antinociception and the mean serum testosterone concentrations quantified in intact male (●), female (○), and testosterone-pretreated female (●) rats infused for 48 h with parenteral morphine/saline. E, significant inverse correlation between mean levels of antinociception and the mean serum M3G/MOR concentrations for intact male (●) and testosterone-pretreated female (●) rats that were coadministered morphine (5 mg/day for 48 h) plus chloramphenicol. (%MPE = 95.886 - 5.759 x [M3G/MOR]; r = 0.67, n = 21, p < 0.05). F, significant inverse exponential correlation (r² = 0.87) between the mean levels of antinociception and the mean serum testosterone concentrations quantified in intact male (●), female (○), and testosterone-pretreated female (●) rats infused for 48 h with parenteral morphine/saline. E, significant inverse correlation between mean levels of antinociception and the mean serum M3G/MOR concentrations for intact male (●) and testosterone-pretreated female (●) rats that were coadministered morphine (5 mg/day for 48 h) plus chloramphenicol. (%MPE = 95.886 - 5.759 x [M3G/MOR]; r = 0.67, n = 21, p < 0.05). F, significant inverse exponential correlation (r² = 0.87) between the mean levels of antinociception and the mean serum testosterone concentrations quantified in intact male (●), female (○), and testosterone-pretreated female (●) rats infused for 48 h with parenteral morphine/saline. E, significant inverse correlation between mean levels of antinociception and the mean serum M3G/MOR concentrations for intact male (●) and testosterone-pretreated female (●) rats that were coadministered morphine (5 mg/day for 48 h) plus chloramphenicol. (%MPE = 95.886 - 5.759 x [M3G/MOR]; r = 0.67, n = 21, p < 0.05).
inverse correlation for intact male and testosterone-pretreated female rats (compare Fig. 5, E and F).

**Serum Creatinine and Testosterone Concentrations.** The creatinine concentrations, quantified in the final serum sample collected from each rat, were within the normal range (data not shown), and were indicative of normal kidney function. The mean serum testosterone concentration in testosterone-pretreated female rats (14.6 nM), was approximately twice the respective mean concentration (6.3 nM) in male rats. In both female and castrated male rats (Strasser et al., 1997), the serum testosterone concentrations were below the limit of quantification (<0.7 nM). Coadministration of chloramphenicol and morphine in testosterone-pretreated female rats resulted in a significant decrease \( p < 0.05 \) in the mean circulating serum testosterone concentration from 14.6 to 5.3 nM. Importantly, in a separate study (our unpublished results), we have found that concentrations of testosterone as high as 1 \( \mu \)M do not significantly inhibit the glucuronidation of morphine to M3G in either male or female rat liver microsomes.

**Significant Inverse Correlation between Mean Levels of Antinociception and the Mean Serum Testosterone Concentration.** Intriguingly, there was a significant inverse quasi-exponential correlation between the group mean %MPE AUC values and the group mean serum testosterone concentrations quantified in intact male, female, and testosterone-pretreated female rats \( r^2 = 1.0; \) Fig. 5G) dosed with morphine alone.

**Behavioral Effects.** Testosterone pretreatment of female rats for 1 week before morphine dosing had no discernible effect on behavior or on the apparent functioning of the estrus cycle. Control rats that received chronic infusions of saline/saline or chloramphenicol/saline were behaviorally indistinguishable from rats that received no treatment. Additionally, intact male and testosterone-pretreated female rats dosed chronically with morphine (5 mg/day for 48 h) alone were behaviorally similar to comparable groups of control rats. By contrast, significant antinociception was produced in female and castrated male rats dosed with morphine (5 mg/day for 48 h) alone. They were also sedated for the first 1 to 2 h; but, by 6 h, they all exhibited normal feeding, grooming, and exploring behaviors. Intact male, castrated male, and female rats coadministered morphine (5 mg/day for 48 h) plus chloramphenicol were sedated for approximately 3 to 6 h, 6 to 12 h, and 12 to 24 h, respectively. Thereafter, all intact male, castrated male, and some female rats recovered slowly to their predosing levels of alertness and activity. Approximately 12 h after initiation of the combined morphine/chloramphenicol infusion, castrated male and female rats appeared to experience respiratory depression resulting in death in some cases \( n = 3/8 \) and 5/11, respectively). Also, some rats in these latter groups exhibited abnormal excitatory behavior, such as intermittent myoclonic jerks of the head and biting at the bottom of the cage. Any rat that appeared distressed was immediately euthanized. In contrast, testosterone-pretreated female rats that received morphine plus chloramphenicol were behaviorally indistinguishable from control rats.

**Discussion**

Prolonged parenteral infusion of morphine or morphine plus chloramphenicol for 48 h produced consistently higher levels of antinociception in female and castrated male rats relative to intact males (Fig. 2; Table 1). However, our findings differ from those of most previous studies, whereby higher levels of antinociception have generally been reported in male relative to female rats after acute single-dose administration of morphine (Romero and Bodnar, 1986; Romero et al., 1988a,b; Kepler et al., 1989, 1991; Islam et al., 1993; Cicero et al., 1996, 1997; Craft et al., 1999). The only exceptions are found in a report by Kasson and George (1984) and our own recent data (unpublished data) that showed either a lack of sex-related differences in the antinociceptive effects of acutely administered morphine or that sex differences in acute morphine antinociception are strictly dose- and antinociceptive test-selective. Importantly, following acute administration of the bolus dose of morphine administered before the initiation of the chronic morphine infusions in the studies described herein, there were no sex-related differences in morphine antinociception over a 3-h study period (our unpublished data). Clearly, the marked disparity in sex-related differences in morphine antinociception between acute and chronic dosing illustrates the dangers inherent in the extrapolation of results from the acute to the chronic dosing setting and vice versa.

In the present studies, tolerance to the antinociceptive effects of morphine developed more slowly in female and castrated male rats, consistent with previous reports that male rats develop antinociceptive tolerance at a faster rate than do female rats (Kasson and George, 1984; Craft et al., 1999). Additionally, consistent with our previous findings (Smith et al., 2000), coadministration of chloramphenicol with morphine significantly \( p < 0.05 \) delayed the development of antinociceptive tolerance in all experimental groups compared with the corresponding groups that received morphine alone. As we have previously shown (Smith et al., 2000) that chloramphenicol does not significantly alter the levels of antinociception evoked by i.e.v. morphine, the current findings strongly indicate that parenteral chloramphenicol augments the antinociceptive effects of chronically administered i.e.v. morphine via a mechanism that does not directly involve supraspinal opioid receptors in the rat CNS.

Significant antinociception was not produced by testosterone, chloramphenicol, or the experimental procedures themselves, because baseline levels of antinociception were maintained for the 48-h experimental period in all control rats. Additionally, female rats were standardized for the diestrus/estrus stage of the estrus cycle to ensure that any change in morphine antinociception by chloramphenicol in female rats was not due to coincident changes in morphine antinociception that have been shown to occur in the proestrus stage of the estrus cycle (Islam et al., 1993).

We found that testosterone pretreatment of female rats abolished morphine antinociception, irrespective of whether rats were dosed with morphine/saline or morphine/chloramphenicol. By contrast, prepubertal castration of male rats resulted in a marked enhancement of morphine antinociception reminiscent of the high levels of antinociception observed in female rats. Intriguingly, the mean changes in %MPE AUC values (Table 1) were significantly inversely correlated with the mean changes in serum testosterone concentrations for rats dosed with morphine (5 mg/day) alone (Fig. 5G), but the mechanism is unlikely to involve a direct effect of testosterone on opioid receptor function in the CNS,
because our recent studies (our unpublished results) have shown that testosterone pretreatment of female rats does not significantly alter the antinociceptive effects of morphine given by the i.c.v. route.

Quantification of the serum concentrations of morphine and its (neuroexcitatory) metabolite, M3G, in the same rats used to assess antinociception, revealed that the serum MOR and M3G AUC values were significantly larger in females and castrated males relative to the comparable group of intact male rats dosed with morphine alone (Tables 2 and 3). These findings are even more prominent in female rats co-administered morphine/chloramphenicol, consistent not only with the higher levels of antinociception, longer duration of sedation, and apparent respiratory depression observed in females, but also the neuroexcitatory behavior consistent with the markedly greater serum M3G AUC values found.

Pretreatment of female rats with testosterone not only abolished antinociception, but also significantly (p < 0.05) increased the total body clearance of morphine, irrespective of whether rats received morphine alone or were coadministered morphine plus chloramphenicol. Our findings taken together imply that the clearance of morphine is modulated by testosterone such that high testosterone concentrations (intact males and testosterone-pretreated females) induce a greater morphine clearance, whereas low testosterone concentrations (castrated males and females) are associated with a lesser morphine clearance. In support of this proposal, the clearance of morphine was found to be greater in isolated perfused livers from male than from female rats (Evans and Shanahan, 1995). However, the same study also showed that there were no sex differences in the partial clearance of morphine to M3G. This is consistent with our finding that even a very high concentration of testosterone (1 μM) does not significantly alter the glucuronidation of morphine to M3G in rat liver microsomes (our unpublished results). Rather, Evans and Shanahan (1995) showed that ~20% of the morphine dose was N-demethylated to normorphine in livers from male rats, but that normorphine was undetectable in livers from female rats (Evans and Shanahan, 1995).

Importantly, the hepatic N-demethylation of morphine is testosterone-dependent in SD rats, such that female and castrated male rats have a markedly reduced capacity (15- to 22-fold) to N-demethylate morphine to the weak opioid agonist, normorphine, compared with intact male rats (Blanck et al., 1990). Consequently, the markedly elevated serum testosterone concentrations observed in testosterone-pretreated female rats are likely to have induced the expression of the CYP450 N-demethylation pathway, in a manner analogous to that which occurs in the liver (Blanck et al., 1990) and brain of intact male rats at puberty (Anandatheerthavarada and Ravindranath, 1991). Once formed in the liver, normorphine is rapidly glucuronidated to normorphine-3-glucuronide (NM3G), with this pathway accounting for up to 20% of the administered morphine dose in the livers of intact male rats (Evans and Shanahan, 1995).

For the male, female, and castrated male rats that achieved significant antinociception for extended periods of time following chronic coadministration of parenteral morphine plus chloramphenicol in the present study, mean levels of antinociception were not correlated with either the mean serum concentrations of morphine (Fig. 5, A and B) or of M3G (Fig. 5, C and D). Rather, levels of antinociception were highly inversely correlated (p < 0.05), with the mean values of the serum M3G/MOR, molar concentration ratio, but the values for intact male rats (Fig. 5F) are leftward shifted relative to the corresponding values for female and castrated male rats (Fig. 5E). As the relationship between the mean %MPE values and the mean values of the M3G/MOR serum molar concentration ratio for intact male and testosterone-pretreated female rats dosed with morphine/chloramphenicol is similar to that reported previously by our laboratory for intact male rats (Smith and Smith, 1995; Smith et al., 2000) and to the relationship between %MPE values and the M3G/MOR ratio values in brain extracellular fluid reported by Barjavel et al. (1995), our current findings imply that one or more factors additional to the serum morphine and M3G concentrations may have contributed to the antinociception observed in the adult male and testosterone-pretreated female rats.

From the foregoing, these additional factors are likely to be the products of morphine N-demethylation, a metabolic pathway that is highly testosterone-dependent in rats. Thus, the very low levels of antinociception observed in testosterone-pretreated female rats are almost certainly due to enhanced N-demethylation of morphine with subsequent metabolism to NM3G, another putatively anti-analgesic metabolite of morphine (Smith and Smith, 1998). Thus, the significant inverse correlation observed between %MPE AUC values and the serum testosterone concentrations in the present studies is almost certainly due to a modulatory effect of testosterone on phase I and not phase II morphine metabolism.

To gain additional insight into the factors underlying the greater clearance of morphine in both male and testosterone-pretreated female rats relative to female and castrated male rats, both normorphine and NM3G would need to be quantified in future studies. Moreover, studies involving in vivo microdialysis sampling of brain extracellular fluid in rats dosed with morphine and morphine/chloramphenicol, together with quantification of antinociception, would be beneficial in understanding the complex interplay of mechanisms responsible for these intriguing observations.

In summary, clear sex-related differences in the antinociceptive effects of chronically administered i.v. morphine were found, such that the %MPE AUC values in female and castrated male rats were significantly larger than the respective values in male rats. Importantly, mean levels of antinociception were not significantly correlated with the mean serum morphine or M3G concentrations, but were highly inversely correlated with the mean value of the M3G/MOR molar concentration ratio in those rats that achieved significant antinociception. Pretreatment of female rats with testosterone for 1 week before initiation of the morphine infusion abolished antinociception with a concomitant large reduction in the serum morphine concentrations to levels approaching those observed in intact male rats. Sex-related differences were also found in the rate of development of tolerance to the antinociceptive effects of coadministered morphine plus chloramphenicol, with female and castrated male rats developing tolerance more slowly than either intact male or testosterone-pretreated female rats. Taken together, our findings clearly suggest that the circulating testosterone concentration is an important determinant of the levels of antinociception evoked by morphine infused intravenously for 48 h in rats,
via a mechanism that appears to involve modulation of phase I morphine metabolism rather than a direct effect at opioid receptors in the CNS.

References


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