Comparison of the Antinociceptive Response to Morphine and Morphine-Like Compounds in Male and Female Sprague-Dawley Rats

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ABSTRACT
Male rats are more sensitive to the antinociceptive effects of morphine than female rats. This difference is seen across several rat strains using a variety of nociceptive stimuli. However, the literature in regard to sex differences in antinociceptive responses to μ-opioids other than morphine is less consistent. The present study was designed to examine whether there is a structure-activity rationale that determines which μ-opioids will show a differential antinociceptive response between male and female rats. A series of morphinans closely related in structure to morphine, namely, codeine, heroin, hydrocodone, hydromorphone, oxymorphone, and oxycodone, were examined for their antinociceptive activity in male and female Sprague-Dawley rats and compared with the structurally unrelated μ-opioid agonists methadone and fentanyl. Antinociception was measured by the warm-water tail-withdrawal assay. The results show that morphine is more potent in males compared with females > hydromorphone = hydrocodone = oxymorphone, but there was no observable sex difference in the antinociceptive potency of codeine, heroin, oxycodone, methadone, or fentanyl. The potency to stimulate guanosine 5′-O-(3-[(35)S]thio)triphosphate ([35S]GTPγS) binding and binding affinity of the various morphinans was compared in rat glioma C6 cells expressing the rat μ-opioid receptor; relative efficacy was also compared by stimulation of [35S]GTPγS binding in slices of rat brain thalamus. The presence of a sex difference in antinociceptive responsiveness was not related to drug potency, efficacy, or affinity. Consequently, it is likely that differential metabolism of the opioid, possibly by glucuronidation, determines the presence or absence of a sex difference.

Male rats are more sensitive to the antinociceptive effects of morphine than female rats. These results seem to be consistent over several types of antinociceptive assays using a variety of nociceptive stimuli and behavioral endpoints. Differences have been reported in the warm-water tail-withdrawal assay, which measures primarily a spinal response (Cicero et al., 1996; Cook et al., 2000; Peckham et al., 2005); the hot-plate assay, which measures primarily a supraspinal response (Cicero et al., 1996; Bartok and Craft, 1997); and the abdominal constriction test, which measures visceral pain induced by a chemical stimulus and is thought to be mediated by higher brain mechanisms (Cicero et al., 1996). All of these studies show a 2- to 3-fold higher potency for morphine in males compared with females. Although these findings may be complicated by the estrous cycle of the female rats, the literature on this is not consistent (Banerjee et al., 1983; Kepler et al., 1989; Stoffel et al., 2003). Moreover, our studies using the same strain of rats as used in the current work have found no significant differences in morphine antinociception in rats in different stages of the estrous cycle (Peckham et al., 2005). Overall, there seems to be a male versus female sex difference in antinociception to morphine over and above any changes because of estrous cycling.

Although the sex difference in response to the antinociceptive properties of morphine is consistent and well documented, this difference may not be general to all μ-opioids. For example, in response to the peptidic μ-opioid agonist [D-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin (DAMGO) given directly into brain, Sprague-Dawley males are reported to be more sensitive in the tail-flick test (i.c.v. administration) (Kepler et al., 1991), less sensitive in the tail-flick test (intraventricular periaqueductal gray administration) (Tershner et al., 2000), or equal in sensitivity in the shock jump test (i.c.v. administration) compared with females (Kepler et al., 1991). This inconsistency in response is also observed for

**ABBREVIATIONS:** DAMGO, [D-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin; [35S]GTPγS, guanosine 5′-O-(3-[(35)S]thio)triphosphate; CHO, Chinese hamster ovary; MPE, maximal possible effect; M6G, morphine-6-glucuronide; M3G, morphine-3-glucuronide.
buprenorphine. In Sprague-Dawley rats using the hot-plate test, F-344 rats using the tail-withdrawal and paw pressure tests, and Lewis rats using the tail-withdrawal test, males are reported to be more sensitive than females (Cook et al., 2000; Craft et al., 2001), whereas Sprague-Dawley males have an equal response to females to buprenorphine in the tail withdrawal and hot-plate when tested by another group (Bartok and Craft, 1997; for review, see Craft, 2003).

The goal of the present study was to test the hypothesis that the structure of the μ-agonist determines whether a sex difference is observed. Since μ-agonists exert their antinociceptive effect through activation of the same receptor, this would be particularly true if the difference in potencies between male and female rats was because of pharmacokinetic and metabolism differences. A variety of μ-agonists, with similar (codeine, heroin, oxymorphone, hydrocodone, and hydrocodone) or different structures (methadone and fentanyl) from morphine (Fig. 1) were tested for antinociception using the warm-water tail-withdrawal assay in male and female Sprague-Dawley rats. In addition, the compounds were compared for binding affinity and selectivity for the μ-opioid receptor; for their ability to activate G proteins using the [35S]GTP·S assay (Traynor and Nahorski, 1995); and for lipophilicity, to determine whether there was a relationship between any of these parameters and any observable sex difference in antinociception. The results confirm a sex difference in the potency of morphine to elicit antinociception in the warm-water tail-withdrawal assay and demonstrate a smaller but significant difference in potency in response to hydrocodone and hydromorphone, with a trend to a difference with oxymorphone. In vitro biochemical measures of affinity, potency, or efficacy or the lipophilicity of the compounds was not predictive of a sex difference in vivo.

Materials and Methods

Subjects
Female and male Sprague-Dawley rats (9–10 weeks of age; approximately 200 and 300 g, respectively) were purchased from Harlan (Indianapolis, IN). Our previous studies have shown that estrous cycle does not alter antinociceptive response in these animals (Peckham et al., 2005), so we used females that were not controlled for estrous cycle. For each warm-water tail-withdrawal assay, six male and six female rats were used. Rats were housed three to a cage with free access to standard laboratory diet chow and water. Animal quarters were maintained at 71–74°F on a 12:12-h light/dark cycle, with lights on at 6:30 AM. Studies were performed in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. Experimental animal protocols were approved by the University of Michigan University Committee on the Use and Care of Animals.

Chemicals and Drugs

[35S]GTP·S (1250 Ci/mmol), [3H]DAMGO (42 Ci/mmol), and [3H]diprenorphine (50 Ci/mmol) were from PerkinElmer Life and Analytical Sciences (Boston, MA). 14C-Standard was from Amersham Biosciences Inc. (Piscataway, NJ). Tissue culture medium, Geneticin, fetal bovine serum, and trypsin were from Invitrogen (Carlsbad, CA). Morphine, methadone, codeine, oxymorphone, oxycodone, hydromorphone, and hydrocodone were obtained through the Narcotic Drug and Opioid Peptide Basic Research Center at the University of Michigan (Ann Arbor, MI). Adenosine deaminase was from Calbiochem (San Diego, CA). Kodak BioMax MR film was purchased from Fisher Scientific (Pittsburgh, PA). DAMGO, GDP, GTP·S, 2-methylbutane, and all other biochemicals were from Sigma-Aldrich (St. Louis, MO) and were of analytical grade. Ecolume scintillation fluid was from MP Biomedicals (SOLON, OH). All drugs were dissolved in sterile water.

Behavioral Assays

Warm-Water Tail-Withdrawal Assay. Rats received s.c. injections of vehicle (sterile water) or the opioid being assayed. A cumulative dosing schedule was used. Animals first received sterile water and then up to five cumulative doses of morphine. After injection (30 min), the tail was placed in a warm water bath at 50°C, and the latency (seconds) to withdraw the tail was measured, with a cut-off time of 20 s to prevent damage to the tail. Separate groups of six animals per group were used for each experiment. All testing began at 10:00 AM.

Biochemical Assays

Cell Culture. C6 glioma cells stably expressing the rat μ-opioid receptor (C6μ cells) or the rat δ-receptor (C6δ cells) (Lee et al., 1999) and CHO cells stably expressing the human κ-opioid receptor (CHOκ cells) (Zhu et al., 1997) were used. C6 cells were grown in Dulbecco’s...
modified Eagle’s medium containing 10% fetal bovine serum under 5% CO₂ in the presence of 0.25 mg/ml Genetin to maintain expression of the opioid receptor in a Genetin-resistant plasmid. CHOx cells were grown in Dulbecco’s modified Eagle’s medium/F-12 containing 10% fetal bovine serum under 5% CO₂ in the presence of 0.25 mg/ml Genetin.

**Membrane Preparation.** C6α, C6β, and CHOx cells were grown to confluence. Media were removed, and cells were rinsed twice with ice-cold phosphate-buffered saline (0.9% NaCl, 0.61 mM Na₂HPO₄, and 0.38 mM KH₂PO₄, pH 7.4), detached from the plate by incubation with harvesting buffer (20 mM HEPEs, pH 7.4, 150 mM NaCl, and 0.68 mM EDTA) at room temperature, and pelleted by centrifugation at 200g for 3 min. The cell pellet was resuspended in ice-cold Tris-HCl (50 mM Tris, pH 7.4) and homogenized with a Tissue-Tearor (BioSpec Products, Inc., Bartlesville, OK) for 20 s at setting 4, and 20 more milliliters of Tris was added. The homogenate was centrifuged at 20,000g at 4°C for 15 min, and the pellet was resuspended in ice-cold 50 mM Tris-HCl buffer, pH 7.4, with a Tissue-Tearor for 5 to 10 s at setting 2 and centrifuged at 20,000g at 4°C for 15 min. The final pellet was resuspended in 50 mM Tris-HCl buffer, pH 7.4, aliquoted, and frozen at −80°C. To determine protein concentration, membrane samples were dissolved with 1 N NaOH for 30 min at room temperature, neutralized with 1 M acetic acid, and assayed by the method of Bradford (1976) using bovine serum albumin as the standard.

**Ligand Binding Assays.** Membranes (25 μg) were incubated with 0.2 nM [³H]diprenorphine and unlabeled ligand (0.3 nM–1 μM) for competition assays in 50 mM Tris buffer, pH 7.4, for 90 min in a shaking water bath at 25°C. Nonspecific binding was defined with 10 μM naloxone. Samples were filtered through glass fiber filters (45 μm; Schleicher & Schuell, Keene, NH) mounted in a cell harvester (Brandel Inc., Gaithersburg, MD) and rinsed three times with ice-cold 50 mM Tris, pH 7.4. Radioactivity retained on the filters was counted by liquid scintillation counting in 4 ml of EcoLume. The homogenate was centrifuged at 20,000g at 4°C for 15 min, and the pellet was resuspended in ice-cold 50 mM Tris-HCl buffer, pH 7.4, with a Tissue-Tearor for 5 to 10 s at setting 2 and centrifuged at 20,000g at 4°C for 15 min. The final pellet was resuspended in 50 mM Tris-HCl buffer, pH 7.4, aliquoted, and frozen at −80°C. To determine protein concentration, membrane samples were dissolved with 1 N NaOH for 30 min at room temperature, neutralized with 1 M acetic acid, and assayed by the method of Bradford (1976) using bovine serum albumin as the standard.

**[³²S]GTPγS Assay.** Membranes (20 μg) were incubated with 50 mM Tris, pH 7.4, 1 mM EDTA, 5 mM MgCl₂, 100 mM NaCl, 1 mM dithiothreitol (freshly prepared), 30 μM GDP, 0.1 nM [³²S]GTPγS, and 0.001 to 10 μM ligand in a total volume of 0.2 ml for 60 min in a shaking water bath at 25°C. Samples were collected by filtration as described above (washed with ice-cold buffer made up of 50 mM Tris, 5 mM MgCl₂, and 100 mM NaCl, pH 7.4) and counted as described above.

**Agonist-Stimulated [³²S]GTPγS Autoradiography.** Agonist-stimulated [³²S]GTPγS autoradiography was performed as described previously (Sim et al., 1995; Jutkiewicz et al., 2005). In brief, naive rats were decapitated, and brains were rapidly removed and placed in 2-methylbutylate (Sigma-Aldrich) on dry ice. Coronal sections (20 μm) of thalamus were sliced on a cryostat maintained at −18°C, mounted on gelatin subbed slides, and stored at −80°C for less than 4 weeks until use. Thalamic sections were rinsed in TME buffer (50 mM Tris-HCl, 3 mM MgCl₂, 0.2 mM EDTA, 100 mM NaCl, and 0.1% bovine serum albumin, pH 7.4) at room temperature for 10 min. After this first incubation, slices were incubated in TME buffer containing 2 mM GDP and 9.5 mM/μl adenosine deaminase at room temperature for 15 min. Then, slices were incubated in TME buffer containing GDP, adenosine deaminase, 40 pM [³²S]GTPγS, and agonist or no agonist or 10 μM GTP (to determine nonspecific binding) for 2 h at room temperature. After the 2-h incubation, slices were rinsed twice for 2 min in ice-cold 50 mM Tris, pH 7.4, and once in distilled water for 1 min and then air-dried for 2 h and exposed to film for 48 h in film cassettes together with ¹⁴C-standards. Images were digitized, and densitometric analysis was conducted using NIH ImageJ software. All compounds were tested in duplicate thalamic slices from six rats.

**Lipophilicity**

Structures were drawn in their (un)protonated form and minimized in MOE (Molecular Operating Environment version 2004.03; Chemical Computing Group, Montreal, ON, Canada) using the MMFF94x force field. LogP (o/w) and slogP were calculated using the QuaSAR-Descriptor calculator implemented in Molecular Operating Environment, and the average of the two values was taken.

**Data Analysis**

All date were analyzed using Prism (GraphPad Software Inc., San Diego, CA). Tail-withdrawal latencies (seconds) were expressed as %MPE, defined as follows: %MPE = latency to respond − baseline (cut-off time − baseline) × 100. Nonlinear regression analysis was used to determine ED₅₀ values. Two-way analysis of variance was used to analyze differences in dose-effect curves between males and females, and Student’s t test was used to analyze differences in ED₅₀ values.

For the competition and saturation binding studies, data were fitted to a one-site curve to determine Kᵦ, Bₘax, and Kᵦ values. Differences were analyzed using Student’s t test. For the [³²S]GTPγS stimulation assays, data were fitted to a sigmoidal dose-response curve to determine maximal binding and EC₅₀ values. Concentration-effect curves were compared by two-way analysis of variance.

**Results**

**Warm-Water Tail-Withdrawal Assay.** All of the μ-opioids examined dose dependently increased latency to response in the warm-water tail-withdrawal assay and were able to produce maximal antinociceptive effects when administered s.c. in both male and female rats. However, the potency of the compounds varied considerably (Table 1). Fentanyl was the most potent compound in both male and female rats, with an ED₅₀ value of 0.06 mg/kg in both sexes, followed by oxymorphone > hydromorphone > heroin > oxycodone = methadone = morphine = hydrocodone = codeine, which was the least potent compound with an ED₅₀ value of 46 mg/kg in both males and females.

There was no difference in the rank order of potency across male and female rats, although morphine was equipotent with hydrocodone in males but less potent than hydrocodone in females. This was because, as reported previously (Peckham et al., 2005), morphine was more potent in males than in females.
females \( F(4,50) = 6.29; P = 0.0004 \) (Table 1). In addition to morphine, dose-response curves were different between males and females only for hydrocodone \( F(4,86) = 6.65; P = 0.0001 \), hydromorphone \( F(6,96) = 3.26; P = 0.0059 \), and oxymorphone \( F(4,86) = 2.78; P = 0.0316 \) (Fig. 2, a–c). When \( \text{ED}_{50} \) values were compared, significant potency differences were observed for hydromorphone \( P < 0.05 \) and hydrocodone \( P < 0.01 \) with a trend to significance for oxymorphone, but these were much less robust than the difference seen with morphine \( P < 0.001 \) (Table 1).

**Ligand Binding Assays.** All the compounds were tested in a competitive binding assay to determine the affinity (\( K_{i} \)) as determined by displacement of the nonselective opioid ligand \(^{3} \text{H} \text{diprenorphine} \) in membranes from C6 cells transfected with \( \mu \) or \( \delta \) opioid receptors and CHO cells transfected with \( \kappa \) opioid receptors (Table 2). The order of decreasing affinity was similar at \( \mu \) and \( \delta \) receptors, hydromorphone > fentanyl = oxymorphone > morphine > methadone > hydrocodone > oxycodone > heroin > codeine, but it was different at the \( \kappa \) opioid receptor with morphine having the second highest affinity after hydromorphone; there was no other change in rank order. All of the compounds were selective for the \( \mu \) opioid receptor, but selectivity varied from 18 to 124 times over the \( \delta \) opioid receptor and from 26 to 493 over the \( \kappa \) opioid receptor. Methadone was the most selective for \( \mu \), and hydromorphone was the least selective. At the \( \mu \) opioid receptor, the 3-methoxy (codeine derivatives) had less affinity than their 3-hydroxy counterparts, although this varied considerably, from morphine, which had 425-fold higher affinity than codeine, to oxymorphone, which had only 45 times higher affinity than oxycodone.

**\(^{35} \text{S} \text{GTP} \gamma \text{S Assay.}** The relative efficacy as determined by comparison with the maximum effect for DAMGO, of the various \( \mu \) agonists to stimulate of \(^{35} \text{S} \text{GTP} \gamma \text{S} \) binding was examined in C6 cells (Table 3). Methadone caused the highest maximal degree of stimulation compared with DAMGO followed by oxymorphone = morphine = heroin = fentanyl > codeine = oxycodone = hydromorphone = hydrocodone. The potency of the compounds did not correlate with their relative efficacy, but instead varied in the order hydromorphone > oxymorphone > morphine > methadone = fentanyl > oxycodone > hydrocodone > heroin > codeine.

The \( \mu \) opioid receptor expressed in rat glioma C6 cells is the rat \( \mu \) opioid receptor. However, this still is a heterologous expression system. Therefore, \( G \) protein binding was also measured in thalamic slices from male and female rat brain using \(^{35} \text{S} \text{GTP} \gamma \text{S} \) autoradiography (Table 4). The efficacy of nonpeptidic compounds relative to DAMGO was considerably less than at the transfected \( \mu \) opioid receptor, but the same rank order as observed in the cell expression system was maintained, namely, methadone > fentanyl > morphine > heroin = oxymorphone = oxycodone = codeine > hydromorphone > hydrocodone. There was no sex difference in the stimulation of \(^{35} \text{S} \text{GTP} \gamma \text{S} \) binding in thalamic slices from male and female rats.

**Lipophilicity.** Calculated lipophilicity data are presented in Table 5. Morphine analogs were relatively similar, the difference between the most lipophilic (heroin) and the least lipophilic (oxymorphone) was 15-fold. In contrast, methadone > fentanyl had more than 3 orders of magnitude greater lipophilicity than morphine.

**Discussion**

The present study confirmed, using Sprague-Dawley rats, that male rats are more sensitive to the antinociceptive action of morphine administered s.c. than female rats. A statistically significant difference in dose-response curves between males and females was also observed for hydromorphone, hydrocodone, and oxymorphone, but this was to a lesser degree than the difference seen with morphine and agrees with the 1.5-fold leftward shift in the potency of hydromorphone in males compared with females recently reported by Stoffel et al. (2005). No significant sex difference was observed in the potency of the morphine congeners oxycodone, heroin, and codeine or the structurally dissimilar fentanyl and methadone. Thus, the presence or absence of a
TABLE 4
Affinity values of morphine and analogs in C6γ, CHOγ, and C6α membranes

<table>
<thead>
<tr>
<th>Drug</th>
<th>C6γ</th>
<th>CHOγ</th>
<th>C6α</th>
<th>Selectivity</th>
<th>Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kᵢ nM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydromorphone</td>
<td>0.50 ± 0.03</td>
<td>12.9 ± 1.40</td>
<td>9.08 ± 1.95</td>
<td>26</td>
<td>18</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>0.71 ± 0.27</td>
<td>8.60 ± 2.40</td>
<td>51.16 ± 12.98</td>
<td>121</td>
<td>72</td>
</tr>
<tr>
<td>Oxydornorphine</td>
<td>0.98 ± 0.06</td>
<td>8.00 ± 2.50</td>
<td>84.20 ± 26.19</td>
<td>82</td>
<td>86</td>
</tr>
<tr>
<td>Morphine</td>
<td>1.70 ± 0.50</td>
<td>6.55 ± 22.6</td>
<td>104.57 ± 27.18</td>
<td>38</td>
<td>61</td>
</tr>
<tr>
<td>Methadone</td>
<td>2.90 ± 0.90</td>
<td>1427 ± 249</td>
<td>355 ± 119</td>
<td>493</td>
<td>124</td>
</tr>
<tr>
<td>Hydromorphone</td>
<td>3.62 ± 2.19</td>
<td>2212 ± 502</td>
<td>1238 ± 332</td>
<td>68</td>
<td>38</td>
</tr>
<tr>
<td>Oxycodone</td>
<td>4.39 ± 7.04</td>
<td>5943 ± 671</td>
<td>2180 ± 397</td>
<td>135</td>
<td>49</td>
</tr>
<tr>
<td>Heroin</td>
<td>158 ± 22.0</td>
<td>5634 ± 790</td>
<td>3895 ± 871</td>
<td>37</td>
<td>25</td>
</tr>
<tr>
<td>Codeine</td>
<td>727 ± 128</td>
<td>25,411 ± 10,015</td>
<td>52,207 ± 25,421</td>
<td>35</td>
<td>72</td>
</tr>
</tbody>
</table>

TABLE 3
Stimulation of [35S]GTPγS binding in C6γ cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>% DAMGO Maximum EC₅₀ nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methadone</td>
<td>100 ± 7.0 123.0 ± 9.1</td>
</tr>
<tr>
<td>Oxydornorphine</td>
<td>86.6 ± 2.4</td>
</tr>
<tr>
<td>Morphine</td>
<td>83.8 ± 1.0 78.4 ± 19.0</td>
</tr>
<tr>
<td>Heroin</td>
<td>83.4 ± 6.4 2735 ± 57.9</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>77.1 ± 6.0 128 ± 25.3</td>
</tr>
<tr>
<td>Codeine</td>
<td>67.1 ± 5.9 21,015 ± 6090</td>
</tr>
<tr>
<td>Oxycodone</td>
<td>66.1 ± 3.6 373 ± 125</td>
</tr>
<tr>
<td>Hydromorphone</td>
<td>65.1 ± 2.2</td>
</tr>
<tr>
<td>Hydrodorphone</td>
<td>59.5 ± 4.1</td>
</tr>
</tbody>
</table>

TABLE 4
[35S]GTPγS stimulation by maximum concentrations (10 μM) of μ-opioid agonists in the thalamus of coronal brain slices of naïve rats

<table>
<thead>
<tr>
<th>Drug</th>
<th>% DAMGO Maximum Male</th>
<th>% DAMGO Maximum Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methadone</td>
<td>58.5 ± 5.6</td>
<td>45.2 ± 9.6</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>50.9 ± 7.3</td>
<td>59.4 ± 10.7</td>
</tr>
<tr>
<td>Morphine</td>
<td>42.8 ± 5.3</td>
<td>41.7 ± 8.1</td>
</tr>
<tr>
<td>Heroin</td>
<td>39.2 ± 5.9</td>
<td>42.8 ± 6.4</td>
</tr>
<tr>
<td>Oxydornorphine</td>
<td>37.1 ± 6.4</td>
<td>35.4 ± 6.1</td>
</tr>
<tr>
<td>Oxycodone</td>
<td>36.5 ± 4.9</td>
<td>37.4 ± 4.5</td>
</tr>
<tr>
<td>Codeine</td>
<td>31.4 ± 5.2</td>
<td>25.5 ± 6.0</td>
</tr>
<tr>
<td>Hydromorphone</td>
<td>25.6 ± 4.9</td>
<td>34.3 ± 5.2</td>
</tr>
<tr>
<td>Hydrodorphone</td>
<td>17.8 ± 5.8</td>
<td>20.3 ± 3.7</td>
</tr>
</tbody>
</table>

least potent compound, codeine, was 21-fold weaker than morphine in males, but only 8-fold weaker in females, and the most potent compound, fentanyl, was 39 times more potent than morphine in males but 100 times more potent in females. This was solely because of the enhanced potency of morphine in the males since both fentanyl and codeine had similar potency in males and females such that the difference in potency between the fentanyl and codeine was the same in both sexes (827-fold in males and 776-fold in females).

We compared the ability of the various opioids to bind to and activate the μ-opioid receptor as an explanation for the differential observation of a sex difference across the compounds. As expected, the 3-hydroxy-substituted compounds hydromorphone, oxymorphone, and morphine had higher affinity for the μ-opioid receptor than their 3-methoxy-substituted counterparts hydrocodone, oxycodone, and codeine. Likewise, heroin had 90-fold less affinity than morphine. On the other hand, morphine was intermediate among the compounds in binding affinity at the μ-opioid receptor and had similar affinity to fentanyl at the δ- and κ-receptors, which did not show a sex difference in antinociception, suggesting a differential μ-opioid receptor affinity or selectivity across the compounds is not responsible. Likewise, there was no evidence that the sex difference observed in antinociceptive potency in the present study was dependent on efficacy or in vitro potency, since the compounds that exhibited a sex difference, namely, morphine, hydromorphone, hydrodorphone, and oxymorphone ranged in relative efficacy and potency as measured by the [35S]GTPγS assay in both C6γ cells and rat thalamic slices. This contrasts with reports (Cook et al., 2000; Terner et al., 2003) that a sex difference is more evident with lower efficacy μ-opioids. However, although Terner and colleagues also used the rat tail-withdrawal assay, they compared very low efficacy compounds, namely, buprenor-
phine, butorphanol, and nalbuphine with morphine, and this may explain the discrepancy with the present findings. The lipophilicity of the opioid did not seem to be a determining factor in whether a sex difference was observed. Lipophilicity is likely to impact the pharmacokinetics of the compound, and so this agrees with findings that similar levels of morphine are observed following morphine administration to male and female rats. No sex difference in the brain or plasma levels of morphine was found 60 min after 2.5 to 15 mg/kg morphine administered s.c. (Cicero et al., 1997). In a time-course study following administration of 20 mg/kg s.c. morphine, males reported consistently higher plasma levels of morphine than females, but significant differences were not observed until 3 h after morphine administration (Baker and Ratka, 2002), long after peak antinociception and at a time when no difference in male and female antinociceptive responses are observed (E. M. Peckham and J. R. Traynor, manuscript submitted for publication).

It was not unexpected that the in vivo and in vitro data did not correlate for compounds that need to undergo metabolism to more active species. Nevertheless, it was surprising that codeine, oxycodone, and heroin did not show a differential response in male and female rats since it is generally accepted that these are prodrugs for morphine, oxymorphene, and 6-monoacetylmorphine, respectively. Heroin is deacetylated, and codeine is metabolized in the rat by the cytochrome P450 enzyme CYP2D1 (Mikus et al., 1991; Cleary et al., 1994). In contrast, a sex difference was seen with both hydrocodone and its metabolic product hydromorphone. This may relate to the fact that inhibition of CYP2D1 in Wistar rats did not affect the antinociceptive effects of hydrocodone, suggesting the metabolism of hydrocodone is not required for its activity (Tomkins et al., 1997). These findings suggest that the requirement for metabolic activation by phase 1 metabolism overcomes the different responsiveness of the sexes.

The two major metabolites of morphine are morphine-6-glucuronide (M6G) and morphine-3-glucuronide (M3G) formed by diphosphate glucuronosyl transfersases 1A3 and 2B7. In humans, UGT2B7 is considered to produce M6G and UGT1A3 produces M3G, although there is not absolute specificity (Coffman et al., 1997; Green et al., 1998). M6G is a more potent antinociceptive agent than morphine in rodents (Frances et al., 1992; Gong et al., 1992), although it is detected in only small quantities after morphine injection in the rodent (Toy’oka et al., 2001). In contrast, M3G is produced in larger amounts but has little to no affinity for the μ-opioid receptor and has even been proposed to antagonize the antinociceptive effects of morphine and M6G (Smith et al., 1990). In humans, UGT2B7 activity is higher in males than in females (for review, see Anderson, 2002). If this is also true in the rat, the increased enzyme activity could result in an increased production of M6G and thus an increased potency following morphine administration. In support of this, Baker and Ratka (2002) report a higher ratio of M3G to M6G in female rats than male rats and suggest this ratio is responsible for the sex difference observed. Certainly in the present study, the most robust sex difference is seen with morphine, the only compound studied that has 3- and 6-hydroxy functions for conjugation without the need for phase 1 metabolism.

In summary, the present study shows that a robust sex difference was seen with morphine with a minor difference with hydrocodone, hydromorphone, and oxymorphone. No difference was observed with methadone or fentanyl. This differential response to the antinociceptive effect of morphine does not seem to correlate with the in vitro efficacy or μ-opioid receptor binding affinity of the agonist nor does it seem that there is a robust structure-activity relationship. The need for both 3- and 6-hydroxy functions and therefore the ability to form both 3- and 6-glucuronides seems a plausible explanation for the more robust difference seen with morphine and may explain why a smaller difference is seen with the 6-keto compounds. It may be too simplistic to expect one parameter to explain the differences observed. Nonetheless, it is important to understand the basis behind this sex difference, which may have important implications for pain management in women (Cepeda and Carr, 2003; Miller and Ernst, 2004).

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