Kappa_2 opioid receptor subtype binding requires the presence of the DOR-1 gene

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1. ABSTRACT

Over the past several years substantial evidence has documented that opioid receptor homo- and heterodimers form in cell lines expressing one or more of the opioid receptors. We used opioid receptor knockout mice to determine whether in vivo pharmacological characteristics of kappa_1 and kappa_2 opioid receptors changed following knockout of specific opioid receptors. Using displacement of the general opioid ligand diprenorphine, we observed that occupancy or knockout of the DOR-1 gene increases the binding density of kappa_1 receptors and eliminates kappa_2 opioid receptors in crude membrane preparations while the total density of kappa opioid binding sites is unchanged. Further, the analgesic potency of U69,593 in cumulative dose response curves is enhanced in mice lacking the DOR-1 gene. These results demonstrate that the DOR-1 gene is required for the expression of the kappa_2 opioid receptor subtype and are consistent with the possibility that a KOR-1/DOR-1 heterodimer mediates kappa_2 pharmacology.

2. INTRODUCTION

Opiate drugs are the treatment of choice for moderate to severe pain. However, their use is limited by the adverse side effects including, among others, respiratory depression, constipation, analgesic tolerance, and physical dependence (1). Most opiate analgesics currently in clinical use are thought to exert their biological effects through the _µ_ opioid receptor-1 gene (MOR-1) (2-6). As an alternative, initial studies using _κ_ ligands to produce analgesia were promising (7) though subsequent work has been less conclusive. For example, highly specific _κ_ agonists such as U50,488 and U69,593 generally produce reduced analgesic action (7, 8), whereas _κ_ drugs that produce greater antinociception have less selectivity for _κ_ receptors and cause more side effects possibly due to action at alternate sites (7, 8). See Ikeda and Matsumoto, 2001 for review (9). The molecular basis for these differences is not well understood.

_κ_ opioid receptor ligands have been divided into
two classes. One class is represented by highly specific ligands such as U69,593 and U50,488 that recognize only κ opioid receptors while less selective κ opioid ligands such as ethylketocyclazocine (EKC) recognize both κ receptors as well as other opioid receptors. κ receptors have been further divided into two classes. The κ1 receptor subtype is defined as the U69,593-sensitive κ opioid receptor and the κ2 receptor subtype includes opioid receptors sensitive to general κ ligands such as EKC but insensitive to κ1, μ and δ opioid drugs (10, 11). Studies in κ opioid receptor-1 gene (KOR-1) knockout mice demonstrate that mutation of the KOR-1 gene results in loss of both κ1 opioid receptor analgesia and binding (12, 13). Thus, κ1 opioid receptors require the KOR-1 gene. However, study of κ2 opioid receptor pharmacology has been limited due to the lack of a drug exhibiting high specificity for only this receptor. One recent study demonstrated that all opioid receptor binding, including that characteristic of κ2 opioid receptors, is lost following deletion of all three classic opioid receptor genes (14). Thus, the κ2 opioid receptor must be either a product of one or more of the three known opioid receptor genes or is concurrently ablated following knockout of these genes. Over the past several years substantial evidence has documented that G protein coupled receptors can form homo- and heterodimers in cell lines expressing different G protein coupled receptors. Evidence shows dimerization of GABAA receptors, 5HT receptors, adrenergic receptors, opioid receptors as well as others in vitro (15-18).

Studies in cell lines suggest that the κ opioid receptor can form homodimers and also heterodimerize with δ opioid receptors (17, 19, 20). These studies show coexpression of KOR-1 and DOR-1 genes in a single cell produce activities with characteristics of the pharmacologically defined κ2 opioid receptor subtype. In contrast, expression of the KOR-1 gene alone produces only the pharmacologically defined κ1 opioid receptor subtype (17). Behavioral studies confirm that κ and δ opioid ligands can functionally modulate each other in vivo (21, 22). Furthermore, recent in vitro and in vivo studies of the novel κ ligand 6’GNTI, suggest that this compound may be a selective κ/δ opioid receptor heterodimer agonist (19). Thus, both in vitro and in vivo data suggest that κ/δ opioid receptor heterodimers may form and have functional relevance.

We here have attempted to determine if the pharmacological consequences of dimerization observed in cell lines could be used to indicate potential receptor dimerization in vivo using KO models. Since studies examining opioid receptor binding in single and combinatorial opioid receptor knockout mice suggest that both κ1 and κ2 opioid receptors are present in mice as well as rats and require the opioid system (14), we examined the relationship between potential dimers and the classically defined κ subtypes. Finally, we confirm and extend studies suggesting that κ/δ heterodimers may have in vivo relevance by examining in vivo δ ligand modulation of κ agonist function in the presence and absence of a functional DOR-1 gene.

3. MATERIALS AND METHODS

3.1. Mice

All experiments were performed on male wild type and opioid receptor KO mice maintained on a 129S6 inbred background. Inbred strains bearing one opioid receptor deletion were constructed by mating original heterozygous male chimera (5, 12, 23) with 129S6 inbred females to produce isogenic mice heterozygous for the deletions. Mating heterozygous mice produced homozygous mutant mice and mating single homozygous mutant mice ultimately produced combinatorial mutants.

3.2. Binding studies

Crude membranes were isolated by standard procedures (23). Briefly, whole brain or three pooled spinal cords from wild type, DOR-1 KO, MOR-1 KO or MOR-1/DOR-1 KO 1296 inbred mice was mechanically homogenized in 25 ml 50 mM Tris HCL, pH 7.4. Homogenates were pelleted via centrifugation at 30,000Xg for 15 minutes at 4°C and resuspended in 25 ml 50 mM Tris HCL, pH 7.4. Resuspended pellets were then recentrifuged and resuspended again in 25 ml 50 mM Tris HCL, pH 7.4. Finally resuspended pellets were incubated 30 min at 37°C to dissociate endogenous ligand then repelleted and stored at -70°C until use.

3.2.1. Saturation studies

For saturation binding, –150 μg aliquots of frozen homogenate were incubated with 6 concentrations in duplicate of 3H diprenorphine (Specific Activity 50-60 Ci/mmol; Perkin Elmer Life Sciences, Waltham, MA) in 50 mM Tris HCL, pH 7.4 in the presence or absence of 10 μM DPDPE for 1 hr at room temperature. 10 μM naloxone was used to define nonspecific binding. Samples were rapidly filtered onto Whatman GF/C filter paper presoaked in 0.3% BMI, washed three times with ice-cold 50 mM Tris HCL, pH 7.4 using a Brandel Cell Harvester (Brandel, Gaithersberg, MD) and counted on a Beckman LS 6500 scintillation counter (Beckman Coulter, Fullerton, CA). The resulting Kd from the saturation binding experiments was used to calculate Ki and Bmax for displacement experiments.

3.2.2. Displacement studies

For displacement assays saturating concentrations (~2 nM) of 3H diprenorphine were used. 3H diprenorphine was incubated in the presence and absence of 11 concentrations of cold U69,593, DPDPE or EKC in triplicate for 2 hr at room temperature. 10 μM naloxone was used to define nonspecific binding. Samples were prepared the same as for saturation binding experiments. Displacement curves were generated using GraphPad Prizm (Graphpad, La Jolla, CA). Curves were generated for both a one-site displacement curve as well as a two-site displacement curve. Data was assumed to be a two-site displacement only if the two-site displacement curve significantly (p<0.05) fit the data better than a one-site displacement curve. Using a Kd for diprenorphine which was empirically determined in concurrent 3H diprenorphine saturation binding experiments, Ki were determined using the Cheng-Prusoff equation (24). The Kd for diprenorphine
needed to be determined for each individual experiment due to slight changes in $K_d$ between experiments. In addition, trends were observed in $K_d$ (as would be expected due to differences in receptors present) dependent on the tissue and genotype. Binding density for $^3$H diprenorphine in displacement studies was determined using the binding in the absence of displacing ligand, in the presence of 10 µM naloxxone and the empirically determined $K_d$ for $^3$H diprenorphine.

3.3. Behavioral studies
All analgesia testing was performed using the radiant heat tailflick assay of nociception. Intensity of the beam was adjusted to yield baseline tailflick latencies between 2-3 seconds. A cut off of 10 seconds was employed to reduce tissue damage. Percent maximum possible effect (%MPE) was determined according to the following formula: (post-latency-pre-latency)/ (10-pre-latency)X100.

U69,593 was initially dissolved in 10% ethanol then further diluted in normal saline (so that the final concentration of ethanol was less than 0.1%) and all other drugs were dissolved in saline. Intrathecal (i.t.) injections were performed as described elsewhere (25). All doses were administered in a final volume of 5 ul. Mice were injected with drug and tested for analgesia 15 minutes afterwards. For dose response curves, immediately after testing mice were injected with the next highest dose. This procedure was repeated until all doses had been administered. ED50 values were determined for each animal using a non-linear regression fit to a variable slope sigmoidal dose response curve (Graphpad Prizm, La Jolla, CA). When two drugs such as U69,593 and DPDPE were administered to the same animal, both U69,593 and DPDPE were diluted to the appropriate concentration in the same tube prior to injection.

3.4. Statistical analysis
All binding experiments were performed a minimum of 3 separate times using different membrane preparations. For derived binding density values, such as EKC-sensitive binding unaccounted for by DPDPE-sensitive binding and U69,593-sensitive binding, values were obtained by subtracting the means of the DPDPE-sensitive and U69,593-sensitive $B_{max}$ values from the EKC-sensitive $B_{max}$ value. Variance in these derived values was determined by calculating the square root of the sum of the squared variances of the individual values used to calculate the derived value. The “n” value used for statistical analysis was the smallest “n” of the individual $B_{max}$ values used to determine the derived binding density. All affinities and $B_{max}$ values are presented as mean ± standard error of the mean. For experiments with three or more conditions, significance was determined using 2-way ANOVA with K dop and/or $\kappa$ receptors, all subsequent displacement binding experiments were conducted in mice containing a deletion of the MOR-1 gene, which decreased total opioid binding density by ~50%, but increasing the percentage of opioid binding which is $\kappa$ from ~20% to ~40%. Although using mice with a deletion of the MOR-1 gene excludes the possibility that $\mu$/$\kappa$ heterodimers could contribute to the $\kappa_2$ opioid receptor subtype in vivo, no biochemical evidence to date suggests that $\mu$/$\kappa$ heterodimers can form in vitro (17).

4. RESULTS
Preliminary experiments using wild type and DOR-1 knockout mice demonstrated that the presence of high densities of $\mu$ opioid receptor in whole brain and spinal cord tissue reduced the sensitivity of $^3$H diprenorphine binding displacement assays such that identification of $\kappa$ opioid receptor subtypes was not possible (data not shown). To eliminate the possibility that the concentration of $\mu$ receptor antagonist required to block all the $\mu$ opioid receptor binding could crossreact with $\delta$ and/or $\kappa$ receptors, all subsequent displacement binding experiments were conducted in mice containing a deletion of the MOR-1 gene, which decreased total opioid binding density by ~50%, but increasing the percentage of opioid binding which is $\kappa$ from ~20% to ~40%.

Next we wanted to determine if any $\kappa_2$ opioid receptors could be detected in whole brain membranes from MOR-1 KO mice. Since no specific $\kappa_2$ opioid receptor ligand exists, we instead determined the total number of opioid sites by EKC displacement of $^3$H-diprenorphine and the number of $\delta$ opioid receptor sites by DPDPE displacement of $^3$H-diprenorphine. The DPDPE displacement curve is also biphasic (Figure 1A) and Hill coefficients obtained were significantly less than one, indicating two non-interacting binding sites (Table 1). The high affinity site detected is, by definition, the $\kappa_2$ opioid receptor subtype due to its sensitivity to $\delta$ opioid receptor antagonist required to block all the $\mu$ opioid receptor binding could crossreact with $\delta$ and $\kappa$ receptors, all subsequent displacement binding experiments were conducted in mice containing a deletion of the MOR-1 gene, which decreased total opioid binding density by ~50%, but increasing the percentage of opioid binding which is $\kappa$ from ~20% to ~40%. Although using mice with a deletion of the MOR-1 gene excludes the possibility that $\mu$/$\kappa$ heterodimers could contribute to the $\kappa_2$ opioid receptor subtype in vivo, no biochemical evidence to date suggests that $\mu$/$\kappa$ heterodimers can form in vitro (17).

4.1. Characterization of $\kappa_2$-like opioid receptor binding
We first determined the characteristics of the $\kappa_2$-like opioid receptor binding by displacing $^3$H-diprenorphine binding in whole brain membranes from MOR-1 knockout mice with increasing concentrations of U69,593. Displacement curves were biphasic (Figure 1A) and Hill coefficients obtained were significantly less than one, indicating two non-interacting binding sites (Table 1). The high affinity site detected is, by definition, the $\kappa$ opioid receptor subtype due to its sensitivity to U69,593 (10, 11, 26, 27), while the low affinity site must then comprise all other opioid receptor binding sites potentially including $\kappa_2$ sites.

For behavioral experiments, significance was determined for U69,593 treatment alone or in combination with $\delta$ ligands using 2-way ANOVA with genotype and treatment as factors with post-hoc analysis via Fisher’s exact test. Significance was assigned only if $p<0.05$. U69,593 dose response curves were considered significant only if their 95% confidence intervals did not overlap.
**κ₂ receptor subtype requires DOR-1 in vivo**

Table 1. Both κ₁ and κ₂ opioid receptor exist in whole brain membranes from MOR-1 but not MOR-1/DOR-1 KO mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Displacing Agent</th>
<th>Blocking Agent</th>
<th>Kᵢ (High)</th>
<th>Kᵢ (Low)</th>
<th>% Affinity</th>
<th>High</th>
<th>Bₘₐₓ (fmoles/mg)</th>
<th>Hill Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOR-1 KO</td>
<td>U69,593</td>
<td>None</td>
<td>4.0 ± 1.6</td>
<td>0.8 ± 0.1</td>
<td>78.8 ± 1.2</td>
<td>32.2 ± 2.6</td>
<td>0.36 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>MOR-1 KO</td>
<td>DPDPE</td>
<td>None</td>
<td>3.7 ± 0.9</td>
<td>10</td>
<td>63.5 ± 1.3</td>
<td>61 ± 3.1</td>
<td>0.34 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>MOR-1 KO</td>
<td>EKC</td>
<td>None</td>
<td>9.0 ± 2.0</td>
<td>100</td>
<td>101 ± 2.9</td>
<td>0.83 ± 0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOR-1 KO</td>
<td>U69,593</td>
<td>10 μM DPDPE</td>
<td>13.9 ± 3.5</td>
<td>100</td>
<td>47.2 ± 3.1</td>
<td>0.73 ± 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOR-1 KO</td>
<td>U69,593</td>
<td>100 nM TIPPψ</td>
<td>14.7 ± 3.8</td>
<td>100</td>
<td>40.5 ± 3.6</td>
<td>0.85 ± 0.11</td>
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<td></td>
</tr>
<tr>
<td>MOR-1/DOR-1 KO</td>
<td>U69,593</td>
<td>None</td>
<td>6.5 ± 1.8</td>
<td>100</td>
<td>44.3 ± 2.7</td>
<td>0.69 ± 0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOR-1/DOR-1 KO</td>
<td>U69,593</td>
<td>10 μM DPDPE</td>
<td>5.6 ± 1.0</td>
<td>100</td>
<td>39.5 ± 2.0</td>
<td>0.73 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOR-1/DOR-1 KO</td>
<td>U69,593</td>
<td>100 nM TIPPψ</td>
<td>14.7 ± 5.0</td>
<td>100</td>
<td>47.0 ± 6.4</td>
<td>0.83 ± 0.14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

U69593 and DPDPE displace 28% (κ₁) and 64% (δ) of ³H diprenorphine respectively in crude whole brain membranes prepared from MOR-1 KO mice leaving ~10% which is insensitive to U69593 and DPDPE (κ₂). All diprenorphine binding in membranes from MOR-1/DOR-1 KO mice is displaced by U69593 at nanomolar affinities (κ₁) ¹<p<0.01 vs MOR-1 + 10μM DPDPE and MOR-1/DOR-1 using 2-way ANOVA with genotype and presence or absence of a blocking reagent as factors and Fisher’s exact tests

To further verify that κ₂ opioid receptors can be detected, we displaced ³H-diprenorphine binding with U69,593 in the presence of 10μM DPDPE. If κ₂ opioid receptors require occupancy of δ opioid receptors (as is the case for μ/δ heterodimers in vitro (28)), then 10μM DPDPE would be sufficient to eliminate all diprenorphine binding to δ opioid receptors and convert any κ₂ opioid receptor binding to a U69,593 sensitive form (17). As predicted, U69,593 displacement of ³H-diprenorphine in the presence of DPDPE is monophasic (Figure 1D) with a Hill coefficient approaching 1. Furthermore, we observed no significant differences in the density of binding following blockade with DPDPE (Table 1). Thus, these data indicate that δ opioid receptor occupancy.

**4.2. Knockout of DOR-1 gene eliminates κ₂ opioid receptor binding**

Next we examined the possible role of δ opioid receptors in the κ₁ and κ₂ opioid receptor subtypes. Using a similar rationale as in experiments performed in whole brain membranes from MOR-1 KO mice, we displaced ³H-diprenorphine with U69,593 in the both the presence and absence of DPDPE in membranes from MOR-1/DOR-1 KO mice. U69,593 displacement of ³H-diprenorphine was monophasic in whole brain membranes from MOR-1/DOR-1 KO mice independent of the presence of DPDPE (Figure 2A & B) with a Hill coefficient approaching 1. Furthermore, we observed no significant differences in the density of binding following blockade with DPDPE (Table 1). Therefore, these data indicate that δ opioid receptors are required for the expression of the κ₂ opioid receptor subtype. Furthermore, the number of κ₁ opioid receptors is elevated in whole brain membranes from MOR-1/DOR-1 KO mice compared to whole brain membranes from MOR-1 KO mice (Table 1).

Similar studies using saturation binding of ³H-diprenorphine to whole brain membranes from MOR-1 and MOR-1/DOR-1 KO mice in the presence and absence of 10 μM DPDPE showed a significant decrease in total opioid binding following knockout of the DOR-1 gene, but no change in the total density of κ₁ opioid receptors (Table 2). Interestingly, the density of U69,593-sensitive sites in whole brain membranes from MOR-1/DOR-1 KO mice (44.3 ± 2.7) is significantly greater than U69,593-sensitive sites measured in the absence of DPDPE from MOR-1 KO mice (32.2 ± 2.6), but almost identical to the number of sites measured in the presence of DPDPE (47.2 ± 3.1) or TIPPψ (40.5 ± 3.6).

**4.3. Knockout of the DOR-1 gene increases κ₁ opioid receptor binding in spinal cord membranes**

We next examined if a similar change in κ₁ and κ₂ opioid receptor number occurs in the spinal cord of...
Figure 1. Representative curves of U69,593 (1A), DPDPE (1B), EKC (1C) and U69,593 + 10µM DPDPE (1D) displacement of 3H-diprenorphine binding in whole brain membranes from MOR-1 KO male mice.

4.4. Knockout of the DOR-1 gene increases potency of intrathecally injected U69,593

We undertook an additional series of experiments to determine if the pharmacological changes in κ opioid receptor subtypes observed following knockout of the DOR-1 gene produced any behavioral consequences. Previous experiments examining the analgesic potency of κ ligands in DOR-1 KO mice were performed only at a single near-maximal dose (23). Thus, we first performed cumulative dose response curves for U69,593 analgesia following i.t. injection in wild type, MOR-1, DOR-1, KOR-1 and MOR-1/DOR-1 KO mice. Consistent with the increase in κ opioid receptors seen by radioligand binding studies, the i.t. potency of U69,593 significantly increases following knockout of the DOR-1 gene. The ED50 for both DOR-1 and MOR-1/DOR-1 KO mice is significantly lower than that for wild type mice and MOR-1 KO mice, but similar to each other (Table 4). In KOR-1 KO mice all antinociceptive activity of U69593 is lost indicating that the KOR-1 gene is required for the κ1 opioid receptor (Table 4). (12)

4.5. Co-injection of κ agonists with δ ligands enhances κ agonist intrathecal potency

Finally, we attempted to determine whether co-administration in wild type mice of a κ1 agonist with δ ligands could mimic the enhanced U69,593 spinal analgesia observed in DOR-1 KO mice. As seen in figure 4, co-administration of U69,593 with either a sub-activating dose of DPDPE or a non-activating dose of TIPPψ, but not naltriben, enhances U69,593 potency to approximately the same level as U69,593 injected i.t. in DOR-1 KO mice alone. In contrast, in DOR-1 KO mice, co-administration of U69,593 with any of the tested δ ligands did not change the antinociceptive potency of U69,693.

5. DISCUSSION

The loss of the κ2 opioid receptor subtype in binding studies performed in membranes from MOR-1 KO mice in the presence of 10µM DPDPE or 100nM TIPPψ, or following deletion of the DOR-1 gene in MOR-1/DOR-1 KO mice indicates that the κ2 opioid receptor subtype is dependent upon the δ opioid receptor. Additionally, the fact
κ₂ receptor subtype requires DOR-1 in vivo

Table 2. Knockout of the DOR-1 gene reduces the total number of ³H diprenorphine binding sites in crude whole brain membranes, but does not alter the total number of κ opioid binding sites

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Blocking Agent</th>
<th>Kᵢ (nM)</th>
<th>Bₘₐₓ (fmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOR-1 KO</td>
<td>None</td>
<td>0.8 ± 0.1</td>
<td>100.7 ± 1.4</td>
</tr>
<tr>
<td>MOR-1 KO</td>
<td>10 nM TIPP</td>
<td>0.9 ± 0.3</td>
<td>39.8 ± 3.2</td>
</tr>
<tr>
<td>MOR-1/DOR-1 KO</td>
<td>None</td>
<td>0.7 ± 0.2</td>
<td>39.5 ± 3.3</td>
</tr>
<tr>
<td>MOR-1/DOR-1 KO</td>
<td>10 µM DPDPE</td>
<td>0.6 ± 0.1</td>
<td>36.9 ± 2.0</td>
</tr>
<tr>
<td>MOR-1/DOR-1 KO</td>
<td>100 nM TIPP</td>
<td>0.9 ± 0.1</td>
<td>36.6 ± 6.4</td>
</tr>
</tbody>
</table>

*p<0.01 vs MOR-1 all other groups using 2-way ANOVA with genotype and presence or absence of a blocking reagent as factors and Fisher’s exact tests

Table 3. Knockout of the DOR-1 gene elevates the density of U69593 sensitive sites in spinal cord membranes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Displacing Agent</th>
<th>Kᵢ (High) (nM)</th>
<th>Kᵢ (Low) (µM)</th>
<th>% Affinity</th>
<th>High Bₘₐₓ (fmol/mg)</th>
<th>Hill Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOR-1 KO</td>
<td>U69.593</td>
<td>4.4 ± 1.6</td>
<td>0.7 ± 0.3</td>
<td>32.4 ± 4.3</td>
<td>23.0 ± 3.4⁴</td>
<td>0.47 ± 0.04</td>
</tr>
<tr>
<td>MOR-1/DOR-1 KO</td>
<td>U69.593</td>
<td>17.3 ± 7.0</td>
<td></td>
<td>100</td>
<td>35.2 ± 1.1</td>
<td>0.72 ± 0.06</td>
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</tbody>
</table>

U69593 displaces 28% and 100% of ³H diprenorphine in spinal cords from MOR-1 KO and MOR-1/DOR-1 KO male mice respectively. ¹=p<0.01 vs MOR-1/DOR-1 using unpaired t-test

Table 4. Knockout of the DOR-1 gene enhances the potency of intrathecally injected U69593

<table>
<thead>
<tr>
<th>Genotype</th>
<th>EC₅₀ (µg)</th>
<th>Maximal Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>3.7 (1.4-10.2)</td>
<td>100%</td>
</tr>
<tr>
<td>DOR-1 KO</td>
<td>4.1 (2.0-8.3)</td>
<td>100%</td>
</tr>
<tr>
<td>MOR-1 KO</td>
<td>1.1 (0.9-1.2)</td>
<td>100%</td>
</tr>
<tr>
<td>MOR-1/DOR-1 KO</td>
<td>Not active</td>
<td>0%</td>
</tr>
</tbody>
</table>

¹=non-overlapping 95% confidence intervals compared to male mice with knockout in the DOR-1 gene

Figure 2. Representative curves of U69,593 (2A) and U69,593 + 10µM DPDPE (2B) displacement of 3H-diprenorphine binding in whole brain membranes from MOR-1/DOR-1 KO male mice.
κ2 receptor subtype requires DOR-1 in vivo

Figure 3. Representative curves of U69,593 displacement of 3H-diprenorphine binding in spinal cord membranes from MOR-1 KO (3A) and MOR-1/DOR-1 KO (3B) male mice.

Figure 4. Co-administration of sub-activating doses of selective δ ligands can potentiate U69,593 spinal potency. Wild type (N=41-17) and DOR-1 KO (20-7) mice were injected intrathecally with 1 µg U69,593 alone (white bars), U69593 and 0.2 nmole DPDPE (black bars), U69,593 and 1 nmole naltriben (light grey bars) or U69,593 and 10 nmole TIPPψ (dark grey bars). *=p<0.05 vs wild type U69,593 alone.

of δ and κ agonists would have a synergistic effect on their antinociceptive potency. We obtain just such a result upon co-injection of an activating dose of U69,593 and subactivating doses of DPDPE in agreement with earlier observations made by Miaskowski, et al. in 1990 (21). Additionally, the inverse interaction also appears to be relevant. In a study by Portoghese and Lunzer, norbinaltorphimine (a κ antagonist) antagonized IT injected DPDPE, suggesting that κ/δ heterodimers not only have physiological interaction with κ agonists but also δ agonists (22). More recently, a putative κ/δ heterodimer specific ligand, 6'-GNTI, was shown to have potency spinaly but not supraspinally suggesting that κ/δ dimers may not be expressed in the brain (19). In contrast we detect the presence of the κ2 opioid receptor subtype in membranes prepared from whole brain tissue (Table 1) suggesting that κ/δ dimers exist supraspinally (see also (19)), though we see no change in U69,593 potency in wild type and DOR-1 KO mice when administered supraspinally (data not shown). These results suggest that although the κ2 opioid receptor subtype is expressed supraspinally, supraspinal κ2 opioid receptors have no detectable role in modulation of nociception.

Although the current experiments do not biochemically demonstrate that the κ1 opioid receptor subtype is a κ/κ homodimer and that the κ2 opioid receptor is a κ/δ heterodimer, these results are consistent with this possibility. One alternative hypothesis is that both κ1 and κ2 opioid receptors are independent of the DOR-1 gene but their expression is coincidentally regulated by knockout of the DOR-1 gene. Thereby, knockout of the DOR-1 gene would effect an upregulation of κ1 comparable to the level of κ2 opioid receptor expression in wild type mice while simultaneously eliminating all κ2 opioid receptor expression. Although such a reciprocal alteration in κ1 and κ2 opioid receptor expression seems unlikely, it cannot be ruled out. Likewise, the κ2 opioid receptor may represent κ1 opioid receptors uncoupled from G-protein or coupled to an alternative pathway, thereby having an altered affinity for the κ agonist, U69,593. Theoretically, then, the knockout of the DOR-1 gene or occupancy of the δ opioid receptor could free a sufficient number of G proteins to couple to the κ2 opioid receptor and convert it to a κ1 opioid receptor. In vivo, this possibility is less likely than in the initial studies done in heterologous cell lines. In vivo there exists a host of other G-protein coupled receptors in the membrane preparation that should be able to absorb the free G-proteins released due to the knockout or occupancy of the δ opioid receptor which would likely cause minimal change in the coupling status of the κ opioid receptor. Additionally, one can make the argument that observed increase in κ1 binding is in fact only a more accurate sampling of the actual κ1 binding density due to the elimination of δ binding sites. If this hypothesis were correct, one could assume that the missing binding density would be proportional to the actual binding density. The percentage increase in binding density would thus be constant between tissues even if the absolute binding density difference varied. In contrast, we observe a significant difference in binding density increases between tissue with an increase of 35% in U69,593 binding density in whole brain membranes from MOR-1/DOR-1 KO mice.
kappa receptor subtype requires DOR-1 in vivo

compared to MOR-1 KO mice but an increase of 48% in U69,593 binding density in spinal cord membranes. These results suggest that the missing binding density is not uniform between tissues and thus not likely due to a systematic error introduced by the methodology. Finally, since we did not commonly precipitate homodimers and heterodimers from the mice, we cannot conclusively demonstrate that kappa opioid receptors are heterodimers in vivo. Nonetheless, the pharmacological changes from earlier experiments in cell lines that accompany loss of the kappa/beta heterodimer do occur. In MOR-1/DOR-1 KO mice where kappa/beta heterodimers cannot exist, no kappa opioid receptors are detectable. Likewise in MOR-1 KO mice where kappa/beta dimers can exist kappa opioid receptors are detected. In addition, because our definition of the kappa opioid receptor subtype also corresponds to what is defined as the delta opioid receptor subtype (19,22), we also suggest that the kappa opioid receptor subtype and delta opioid receptor subtype may be the same receptor complex with delta ligands recognizing the delta portion of a kappa/beta heterodimer.

In most regards, our results match the results obtained by Jordan and Devi (17) in cell lines. The one difference we notice when comparing in vivo membranes rather than in vitro membranes from cell lines is the altered response of U69,593 binding to TIPPPY treatment. Unlike in vitro studies, TIPPPY blockade of delta opioid receptors results in conversion of kappa binding sites to kappa binding sites in a manner similar to treatment with DPDPE (Table 1). Our results are internally consistent in that both DPDPE and TIPPPY coinjection with U69,593 show enhancement of analgesic potency (Table 4). Rather, it would be more surprising if TIPPPY did not enhance U69,593 potency considering that in other studies of opioid receptor heterodimers, such as mu/beta heterodimers, TIPPPY does enhance both binding affinity and drug potency (30,31). The exact reason for this difference is unknown.

In conclusion, we have provided substantial evidence consistent with the possibility that kappa opioid receptors require the DOR-1 gene and potentially may be beta heterodimers. After verifying the presence of both kappa1 and kappa2 opioid receptor subtypes in MOR-1 KO mice, we have been able to observe the loss of all kappa opioid receptor binding in MOR-1/DOR-1 KO mouse, which would be incapable of expressing a kappa/beta heterodimer, or following occupation of all delta opioid receptors. Finally, we demonstrate that knockout of the DOR-1 gene or inclusion of selective delta ligands enhances the potency of a kappa opioid receptor agonist injected IT consistent with the hypothesis of in vivo kappa heterodimers. These results support a possible simple alteration to enhance the clinical relevance of kappa ligands renewing the promise of the kappa opioid system as an alternative receptor system to induce antinociception and posist that co-treatment with both kappa and delta ligands may be a viable method to enhance drug potency without enhancement of pharmacological side effects such as tolerance.

6. ACKNOWLEDGEMENT

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Abbreviations: MOR-1: μ opioid receptor-1 gene, KOR-1: κ opioid receptor-1 gene, DOR-1: δ opioid receptor-1 gene, KO: knockout, U50,488: (trans)-3,4-dichloro-N-methyl-N-(2-(1-pyrrolidinyl)-cyclohexyl) benzeneacetamide methane-sulfonate hydrate, U69,593: (+)-(5α,7α,8β)-N-Methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro (4,5)dec-8-yl)-benzeneacetamide, EKC: Ethylketocyclazocine, DPDPE: (D-Pen(2),D-Pen(5))-enkephalin, i.t: intrathecally

Key Words: Opioid, Dimer, U69,693, Intrathecal, Knockout

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