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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$_2$ 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 that 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 κ_1 and κ_2 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 chimeras (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 disassociate 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 ^{3}H 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 ^{3}H diprenorphine were used. ^{3}H 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 K_d for diprenorphine which was empirically determined in concurrent ^{3}H diprenorphine saturation binding experiments, Kis were determined using the Cheng-Prusoff equation (24). The K_d 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 naloxone 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 genotype and the absence or presence of a blocking ligand as factors with post-hoc analysis via Fisher's exact test. If only two conditions were compared, significance was determined using an unpaired t-test. Significance was assigned only if $p<0.05$.

For behavioral experiments, significance was determined for U69,593 treatment alone or in combination with δ 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.

4. RESULTS

Preliminary experiments using wild type and DOR-1 knockout mice demonstrated that the presence of high densities of μ opioid receptor in whole brain and spinal cord tissue reduced the sensitivity of ^{3}H diprenorphine binding displacement assays such that identification of κ opioid receptor subtypes was not possible (data not shown). To eliminate the possibility that the concentration of μ receptor antagonist required to block all the μ opioid receptor binding could crossreact with δ and/or κ 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 κ from ~20% to ~40%. Although using mice with a deletion of the MOR-1 gene excludes the possibility that μ/κ heterodimers could contribute to the κ_2 opioid receptor subtype *in vivo*, no biochemical evidence to date suggests that μ/κ heterodimers can form *in vitro* (17) .

4.1. Characterization of κ_2-like opioid receptor binding

We first determined the characteristics of the κ_1-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, indicting two non-interacting binding sites (Table 1). The high affinity site detected is, by definition, the κ_1 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 κ_2 sites.

Next we wanted to determine if any κ_2 opioid receptors could be detected in whole brain membranes from MOR-1 KO mice. Since no specific κ_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 δ opioid receptor sites by DPDPE displacement of ^{3}H-diprenorphine. The DPDPE displacement curve is also biphasic (Figure 1B) with Hill coefficients less than one, indicating two non-interacting binding sites (Table 1). The high affinity site (Kd=3.7 nM) represents displacement of ^{3}H-diprenorphine from δ opioid receptors. The low affinity site represents binding to all remaining opioid receptors. Finally, EKC displacement of ^{3}H-diprenorphine was monophasic with an affinity of 9.0 nM (Figure 1C) and with Hill coefficient approaching one, indicative of a single binding site (Table 1). This single site represents binding to all opioid receptors present in whole brain membranes from MOR-1 KO mice. Empirically determined binding density values (Table 1) for κ_1 opioid

Table 1. Both κ_1 and κ_2 opioid receptor exist in whole brain membranes from MOR-1 but not MOR-1/DOR-1 KO mice

Genotype	Displacing Agent	Blocking Agent	K_i (High) (nM)	K_i (Low) (μM)	% High Affinity	B_{max} (High) (fmoles/mg)	Hill Coefficient
MOR-1 KO	U69.593	None	4.0 ± 1.6	0.8 ± 0.1	27.8 ± 1.2	32.2 ± 2.6[1]	0.36 ± 0.03[1]
MOR-1 KO	DPDPE	None	3.7 ± 0.9	>10	63.5 ± 1.3	61.1 ± 3.1	0.34 ± 0.02
MOR-1 KO	EKC	None	9.0 ± 2.0	----------	100	101.2 ± 2.9	0.83 ± 0.07
MOR-1 KO	U69,593	10 μM DPDPE	13.9 ± 3.5	----------	100	47.2 ± 3.1	0.73 ± 0.05
MOR-1 KO	U69,593	100 nM TIPPψ	14.7 ± 3.8	----------	100	40.5 ± 3.6	0.85 ± 0.11
MOR-1/DOR-1 KO	U69,593	None	6.5 ± 1.8	----------	100	44.3 ± 2.7	0.69 ± 0.06
MOR-1/DOR-1 KO	U69,593	10 μM DPDPE	5.6 ± 1.0	----------	100	39.5 ± 2.0	0.73 ± 0.08
MOR-1/DOR-1 KO	U69,593	100 nM TIPPψ	14.7 ± 5.0	----------	100	47.0 ± 6.4	0.83 ± 0.14

U69593 and DPDPE displace 28% (κ_1) and 64% (δ) of ^{3}H diprenorphine respectively in crude whole brain membranes prepared from MOR-1 KO mice leaving ~10% which is insensitive to U69593 and DPDPE (κ_2). All diprenorphine binding in membranes from MOR-1/DOR-1 KO mice is displaced by U69593 at nanomolar affinities (κ_1) [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

receptors (32.2 fmoles/mg) from U69,593 displacement curves and δ opioid receptors (61.1 fmoles/mg) from DPDPE displacement curves is less than the total density of opioid receptors determined by EKC displacement (101.2 fmoles/mg). These additional EKC binding sites (7.9 fmoles/mg) indicate κ_2 opioid receptor binding and indicate that the κ_2 opioid receptor subtype can be detected in whole brain membranes from MOR-1 KO mice.

To further verify that κ_2 opioid receptors can be detected, we displaced ^{3}H-diprenorphine binding with U69,593 in the presence of 10μM DPDPE. If κ_2 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 κ_2 opioid receptor binding to a U69,593 sensitive form (17). As predicted, U69,593 displacement of ^{3}H-diprenorphine in the presence of DPDPE is monophasic (Figure 1D) with a Hill coefficient approaching one. Furthermore, the density of binding sites displaced by U69,593 is significantly elevated in the presence of 10 μM DPDPE compared to the density of U69,593 sensitive sites detected in the absence of DPDPE (Table 1). These results confirm that, in the presence of blocking concentrations of DPDPE, κ_2 opioid receptors respond to U69,593 in a manner indistinguishable from κ_1 opioid receptors. Furthermore, the increase in U69,593 sensitive binding (15.0 ± 4.0 fmoles/mg) is similar to the difference between the total density of opioid receptors measured by EKC displacement of ^{3}H-diprenorphine and the sum of the density of κ_1 and δ opioid receptors measured by U69,593 and DPDPE displacement respectively (7.9 ± 5.0 fmoles/mg; p=0.33 EKC sensitive binding remaining following subtraction of DPDPE and U69,593 sensitive binding vs. increase in U69,593 sensitive binding in the presence of DPDPE via t-test). A similar increase was also obtained when the δ opioid receptor antagonist, TIPPψ (8.3 ± 4.5fmoles/mg; p=0.96_EKC sensitive binding remaining following subtraction of DPDPE and U69,593 sensitive binding vs. increase in U69,593 sensitive binding in the presence of TIPPψ via t-test) was used as a blocking reagent instead of DPDPE (Table 1). Thus, these data indicate that both κ_1 and κ_2 opioid receptor subtypes exist in whole brain membranes from MOR-1 KO mice and κ_2 opioid receptor subtype is influenced by δ opioid receptor occupancy.

4.2. Knockout of DOR-1 gene eliminates κ_2 opioid receptor binding

Next we examined the possible role of δ opioid receptors in the κ_1 and κ_2 opioid receptor subtypes. Using a similar rationale as in experiments performed in whole brain membranes from MOR-1 KO mice, we displaced ^{3}H-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 ^{3}H-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). Thus, these data indicate thatδ opioid receptors are required for the expression of the κ_2 opioid receptor subtype. Furthermore, the number of κ_1 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 ^{3}H-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 κ 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 κ_1 opioid receptor binding in spinal cord membranes

We next examined if a similar change in κ_1 and κ_2 opioid receptor number occurs in the spinal cord of

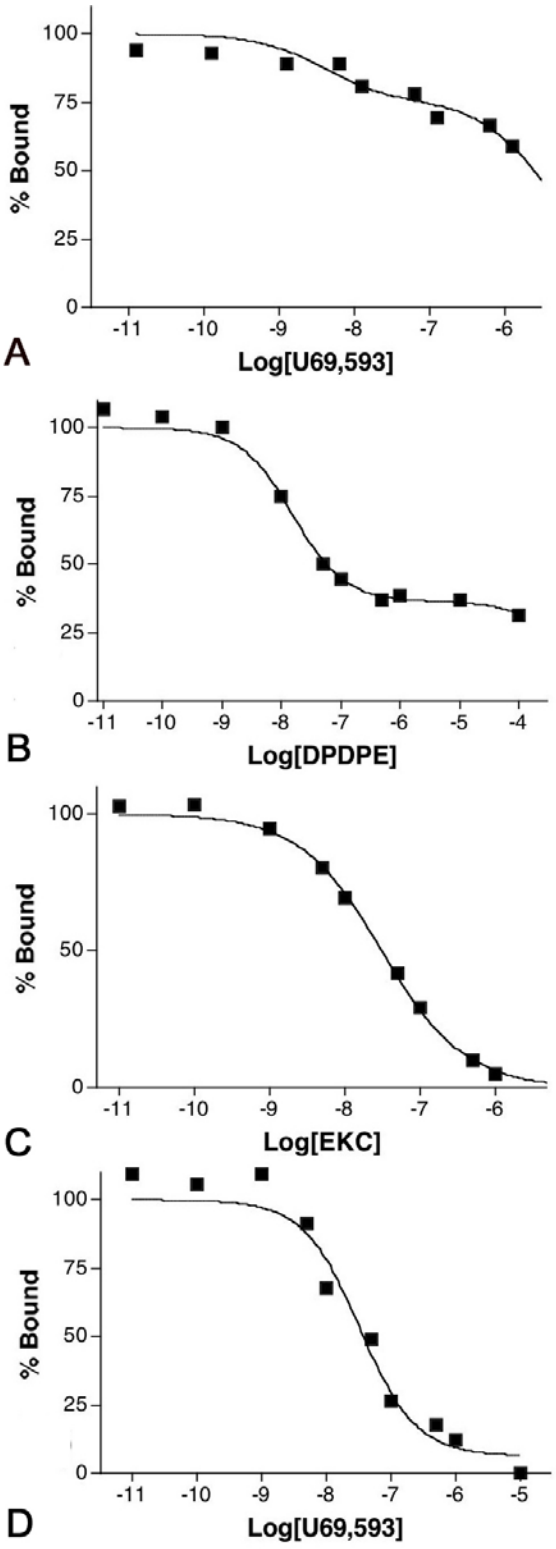

Figure 1. Representative curves of U69,593 (1A),DPDPE (1B), EKC (1C)and U69,593 + 10μM DPDPE (1D) displacement of 3H-diprenorphine binding in whole brain membranes from MOR-1 KO male mice.

MOR-1 and MOR-1/DOR-1 KO mice. As in brain membranes, U69,593 displacement in the spinal cord of MOR-1 KO mice is biphasic (Figure 3A) with a density of 23.0 fmoles/mg for U69,593-sensitive sites (Table 3). In MOR-1/DOR-1 KO mice U69,593 displacement of ^{3}H-diprenorphine is monophasic (Figure 3B) and the density of U69,593-sensitive sites is significantly elevated to 35.2 fmoles/mg (Table 3). The differences in κ_1 binding density are greater in magnitude compared to differences in κ_1 binding density in whole brain membrane preparations as expected by the greater apparent colocalization of δ and κ opioid receptors in the spinal cord (29), but both findings suggest the same mechanism; κ_2 opioid receptors are expressed in the spinal cord of MOR-1 KO mice but are converted to κ_1 opioid receptors following knockout of the DOR-1 gene.

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_1 opioid receptors seen by radioligand binding studies, the i.t. potency of U69,593 significantly increases following knockout of the DOR-1 gene. The ED_{50} 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 κ_1 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 κ_1 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 κ_2 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 κ_2 opioid receptor subtype is dependent upon the δ opioid receptor. Additionally, the fact

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

Genotype	Blocking Agent	K_i (nM)	B_{max} (fmoles/mg)
MOR-1 KO	None	0.6 ± 0.1	100.7 ± 1.4[1]
MOR-1 KO	10 μM DPDPE	0.9 ± 0.3	39.8 ± 3.2
MOR-1 KO	100 nM TIPPψ	1.0 ± 0.1	34.1 ± 7.0
MOR-1/DOR-1 KO	None	0.7 ± 0.2	39.5 ± 3.3
MOR-1/DOR-1 KO	10 μM DPDPE	0.6 ± 0.1	36.9 ± 2.0
MOR-1/DOR-1 KO	100 nM TIPPψ	0.9 ± 0.1	36.6 ± 6.4

[1]=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

Genotype	Displacing Agent	Blocking Agent	K_i (High) (nM)	K_i (Low) (μM)	% High Affinity	B_{max} (High) (fmoles/mg)	Hill Coefficient
MOR-1 KO	U69.593	None	4.4 ± 1.6	0.7 ± 0.3	32.4 ± 4.3	23.0 ± 3.4[1]	0.47 ± 0.04[1]
MOR-1/DOR-1 KO	U69.593	None	17.3 ± 7.0	----------	100	35.2 ± 1.1	0.72 ± 0.06

U69593 displaces 28% and 100% of ^{3}H 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

Genotype	EC_{50} (μg)	Maximal Effect
Wild Type	3.7 (1.4-10.2)[1]	100%
DOR-1 KO	0.8 (0.5-1.3)	100%
MOR-1 KO	4.1 (2.0-8.3)[1]	100%
MOR-1/DOR-1 KO	1.1 (0.9-1.2)	100%
KOR-1 KO	Not active	0%

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

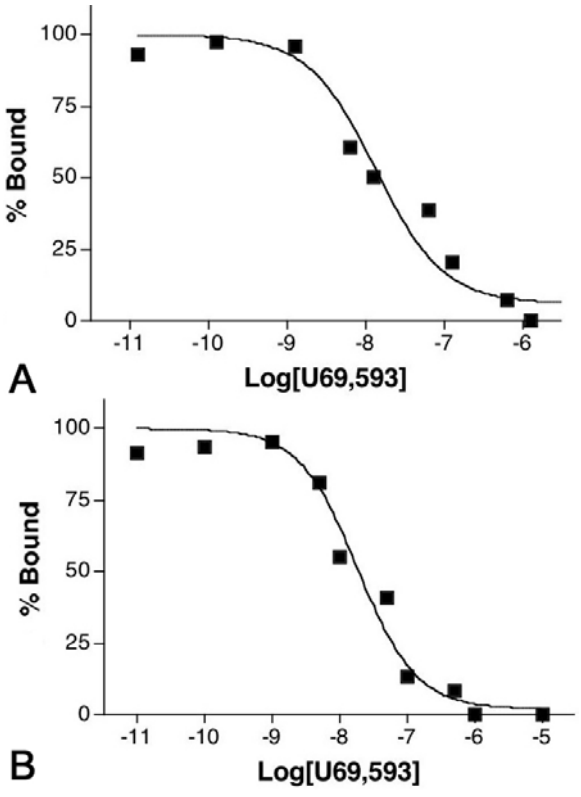

Figure 2. Representative curves of U69,593 (2A) and U69,593 + 10μM DPDPE (2B) displacement of 3H-diprenorphine binding in whole brain membranes from MOR-1/DOR-1 KO male mice.

that κ_1 opioid receptor binding increases by almost the exact extent as the amount of κ_2 opioid receptor binding lost suggests that κ_2 opioid receptors are converted to κ_1 opioid receptors following occupation or deletion of the δ opioid receptor. These results are consistent with previous findings in cell lines (17) where co-expression of the DOR-1 and KOR-1 genes produced cells expressing pharmacologically defined κ_2 opioid receptors, while expression of KOR-1 alone produced only the κ_1 opioid receptor subtype and expression of DOR-1 alone produced only the δ receptor pharmacology. We demonstrate here that a similar phenomenon occurs *in vivo*. In MOR-1 KO mice, which express both DOR-1 and KOR-1, both κ opioid receptor subtypes (κ_1 and κ_2) are present, while in MOR-1/DOR-1 KO mice, which express only KOR-1, only κ_1 opioid receptors are present. Thus, the κ_2 opioid receptor subtype requires the presence of the δ opioid receptor. Furthermore, the elevation of κ_1 opioid receptor binding observed in the presence of 10μM DPDPE would be predicted based on *in vitro* studies that show that in the presence of DPDPE only κ_1 opioid receptor binding is detectable.

The pharmacological evidence suggesting that the κ_2 opioid receptor subtype is converted to a κ_1 opioid receptor following mutation of the DOR-1 gene led us to test whether the potency of U69,593 is enhanced in DOR-1 KO mice. As seen in Table 4, the potency of U69,593 injected i.t is enhanced in MOR-1/DOR-1 and DOR-1 KO mice compared to wild type mice. These results are consistent with the pharmacological data indicating a 48% increase in the density of U69,593 sensitive sites in the spinal cord following knockout of the DOR-1 gene. Furthermore, these results would predict that co-injection

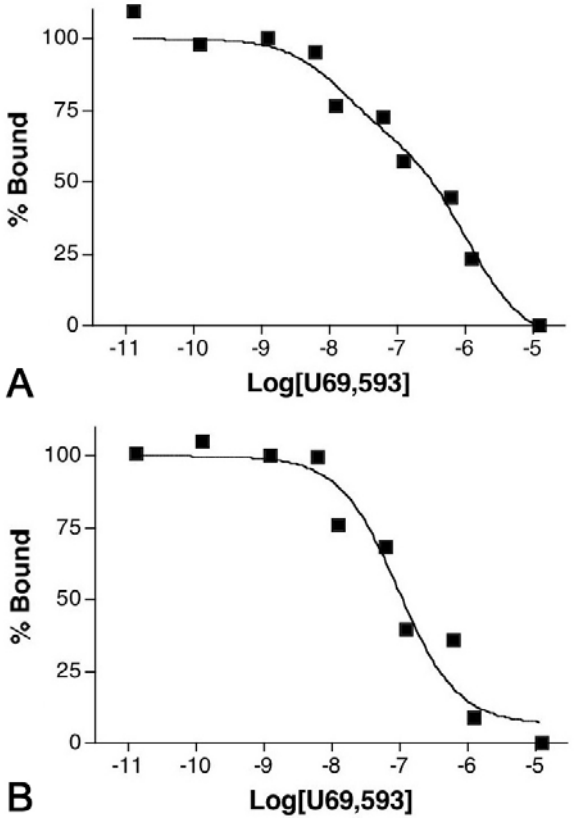

Figure 3. Representative curves of U69,593 displacement of 3H-diprenorphine binding in spinal cord membranes from MOR-1 KO (3A) and MOR-1/DOR-1 KO (3B) male mice.

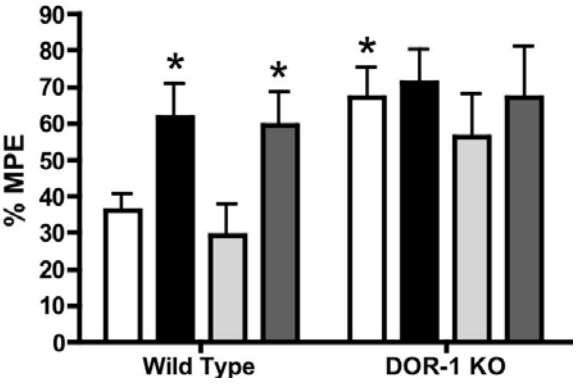

Figure 4. Co-administration of sub-activating doses of selective δ ligands can potentiate U69,593 spinal potency. Wild type (N=41-17) and DOR-1 KO (20-7) mice were injected intrathecally with 1 μg U69,593 alone (white bars), U69593 and 0.2 nmole DPDPE (black bars), U69,593 and 1 nmole naltriben (light 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 spinally but not supraspinally suggesting that κ/δ dimers may not be expressed in the brain (19). In contrast we detect the presence of the κ_2 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 κ_2 opioid receptor subtype is expressed supraspinally, supraspinal κ_2 opioid receptors have no detectable role in modulation of nociception.

Although the current experiments do not biochemically demonstrate that the κ_1 opioid receptor subtype is a κ/κ homodimer and that the κ_2 opioid receptor is a κ/δ heterodimer, these results are consistent with this possibility. One alternative hypothesis is that both κ_1 and κ_2 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 κ_1 comparable to the level of κ_2 opioid receptor expression in wild type mice while simultaneously eliminating all κ_2 opioid receptor expression. Although such a reciprocal alteration in κ_1 and κ_2 opioid receptor expression seems unlikely, it cannot be ruled out. Likewise, the κ_2 opioid receptor may represent κ_1 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 κ_2 opioid receptor and convert it to a κ_1 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 κ_1 binding is in fact only a more accurate sampling of the actual κ_1 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

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 coimmunoprecipitate homodimers and heterodimers from the mice, we cannot conclusively demonstrate that κ_2 opioid receptors are heterodimers *in vivo*. Nonetheless, the pharmacological changes from earlier experiments in cell lines that accompany loss of the κ/δ heterodimer do occur. In MOR-1/DOR-1 KO mice where κ/δ heterodimers cannot exist, no κ_2 opioid receptors are detectable. Likewise in MOR-1 KO mice where κ/δ dimers can exist κ_2 opioid receptors are detected. In addition, because our definition of the κ_2 opioid receptor subtype also corresponds to what is defined as the δ_1 opioid receptor subtype (19,22), we also suggest that the κ_2 opioid receptor subtype and δ_1 opioid receptor subtype may be the same receptor complex with δ_1 ligands recognizing the δ portion of a κ/δ heterodimer.

In most regards, our results match the results obtained by Jordan and Devi (17) in cell lines. The one difference we observe using *in vivo* membranes rather than *in vitro* membranes from cell lines is the altered response of U69,593 binding to TIPPΨ treatment. Unlike *in vitro* studies, TIPPΨ blockade of δ opioid receptors results in conversion of κ_2 binding sites to κ_1 binding sites in a manner similar to treatment with DPDPE (Table 1). Our results are internally consistent in that both DPDPE and TIPPΨ coinjection with U69,593 show enhancement of analgesic potency (Table 4). Rather, it would be more surprising if TIPPΨ did not enhance U69,593 potency considering that in other studies of opioid receptor heterodimers, such as μ/δ heterodimers, TIPPΨ 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 κ_2 opioid receptors require the DOR-1 gene and potentially may be κ/δ heterodimers. After verifying the presence of both κ_1 and κ_2 opioid receptor subtypes in MOR-1 KO mice, we have been able to observe the loss of all κ_2 opioid receptor binding in MOR-1/DOR-1 KO mouse, which would be incapable of expressing a κ/δ heterodimer, or following occupation of all δ opioid receptors. Finally, we demonstrate that knockout of the DOR-1 gene or inclusion of selective δ ligands enhances the potency of a κ_1 opioid receptor agonist injected IT consistent with the hypothesis of *in vivo* κ heterodimers. These results support a possible simple alteration to enhance the clinical relevance of κ ligands renewing the promise of the κ opioid system as an alternative receptor system to induce antinociception and posit that co-treatment with both κ and δ ligands may be a viable method to enhance drug potency without enhancement of pharmacological side effects such as tolerance.

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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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