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Contribution of mu and delta opioid receptors to the pharmacological profile of kappa opioid receptor subtypes

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Abstract

Molecular cloning has identified three opioid receptors: mu (MOR), delta (DOR) and kappa (KOR). Yet, cloning of these receptor types has offered little clarification to the diverse pharmacological profiles seen within the growing number of novel opioid ligands, which has led to the proposal of multiple subtypes. In the present study, utilizing *in vitro* and *in vivo* methods including the use of opioid receptor knockout mice, we find that certain antinociceptive effects of the KOR-1 and KOR-2 subtype-selective ligands (+)-(5 α ,7 α ,8 β)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzene-acetamide (U69, 593) and 4-[(3,4-Dichlorophenyl)acetyl]-3-(1-pyrrolidinylmethyl)-1-piperazine-carboxylic acid methyl ester fumarate (GR89, 696), respectively, are potentiated by antagonism of MOR and DOR receptors. We believe that our findings can be best explained by the existence of KOR-DOR and KOR-MOR heteromers. We only find evidence for the existence of these heteromers in neurons mediating mechanical nociception, but not thermal nociception. These findings have important clinical ramifications as they reveal new drug targets that may provide avenues for more effective pain therapies.

1. Introduction

Opioids are among the most effective analgesics used for the treatment of moderate to severe pain. Three opioid receptor genes have been identified: mu (MOR), delta (DOR) and kappa (KOR). The majority of clinically relevant opioid analgesics exert their effects primarily through the MOR (Matthes et al., 1996). However, these MOR-selective drugs are hampered by side effects including tolerance, dependence and addiction (Inturrisi, 2002; Raehal and Bohn, 2005). Consequently, there has been renewed interest in the DOR and KOR as targets for the treatment of pain (Vanderah, 2010). KOR agonists, like MOR agonists, are potent analgesics, participating in the control of tactile and thermal pain (Millan, 1990). Initially, KOR agonists appeared more beneficial than their MOR

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Conflicts of interest

The authors declare no conflicts of interest.

counterparts because they did not induce euphoria, reward and/or respiratory depression (Kivell and Prinszano, 2010). However, as research advanced, some KOR selective ligands, in addition to being analgesic, were shown to induce side effects such as dysphoria and sedation (Pfeiffer et al., 1986; Carlezon and Miczek, 2010), and thus their initial attraction for clinical use dwindled.

Pharmacological subtypes of each of the opioid receptors have been proposed based on the specific pharmacological profiles of individual ligands (Dietis et al., 2011). At least two subtypes (KOR-1 and KOR-2) have been distinguished on the basis of sensitivity to various KOR-selective compounds (Attali et al., 1982). KOR-1 is defined by its preferential binding for arylacetamide-like agonists such as U69, 593 (Lahti et al., 1985), U50,488H (Clark et al., 1989) and the KOR antagonist norbinaltorphimine (nor-BNI) (Portoghese et al., 1991). KOR-2 is characterized as being U69, 593 insensitive and exhibits a 100-fold lower affinity for nor-BNI than KOR-1 (Zukin et al., 1988). Bremazocine and GR89, 696 are the prototypical KOR-2 agonists (Tiberi and Magnan, 1990; Herrero and Headley, 1993; Ho et al., 1997; Butelman et al., 2001). A third KOR subtype has been defined displaying high affinity to the antagonist naloxone benzoylhydrazone (Clark et al., 1989), but its existence remains highly speculative (Connor and Kitchen, 2006).

Some investigators have proposed that receptor–receptor interactions could form the basis for several of the opioid receptor subtypes (Porreca et al., 1992; Xu et al., 1993; Jordan and Devi, 1999). Opioid receptors were initially thought to function as monomeric units. However, this notion has been revised over the last decade by a number of studies showing that opioid receptors (Cvejic and Devi, 1997; George et al., 2000) can form oligomeric structures. Furthermore, there is mounting *in vivo* evidence that both DOR-MOR (Gomes et al., 2004; Gupta et al., 2010; He et al., 2011) and DOR-KOR heteromers exist and form functionally relevant targets for pain (Portoghese and Lunzer, 2003; Waldhoer et al., 2005; Chakrabarti et al., 2010) and alcohol consumption (van Rijn and Whistler, 2009). A recent study suggesting that MOR and DOR are uniquely expressed in neurons mediating thermal and mechanical nociception, respectively (Scherrer et al., 2009), provided an interesting approach to test the hypothesis whether putative KOR subtype-selective agonists require the presence of MOR or DOR to produce either thermal or mechanical antinociception.

2. Methods

2.1 Calcium mobilization assay

HEK-293 cells stably expressing KOR, DOR or MOR were maintained at 37 °C humidified in 7% CO₂/93% air atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum. In order to test the potency of opioid agonists, we employ a high throughput Ca²⁺-mobilization assay. We made use of a chimeric G-protein 6-Gqi4-myr (Kostenis et al., 2005) that couples to G_i-coupled receptors, such as the opioid receptors, but transduces its signals as if activated by a G_q-coupled receptor. In other words, opioid agonists that now induces Ca²⁺-mobilization rather than inhibits cAMP production was measured in HEK-293 cells stably expressing the respective receptors that had been transiently transfected with chimeric G-protein 6-Gqi4-myr (Kostenis et al., 2005) (100 ng for every 60,000 cells). One day after transfection, cells were loaded with a Ca²⁺-

fluorophore dye (FLIPR calcium assay kit; Molecular Devices, Sunnyvale, CA, USA) for 60 min and stimulated with increasing amounts of ligand to obtain dose–response curves. For each compound, the 50% effective concentration (EC₅₀) was measured. All values are presented as the means ± standard error of the mean. Experiments were carried out a minimum of three times in triplicate.

2.2 Animals and housing

Wild-type (WT) (Taconic, Oxnard, CA, USA), MOR-, DOR- and KOR knockout (KO) C57BL/6 mice (male, 20–25 g) were housed (maximally five per cage) in ventilated plexiglass cages at ambient temperature (21 °C) in a room maintained on a 12L:12D cycle (lights on at 08:00 h, lights off at 20:00 h). Food and water were provided *ad libitum*. The mice were given 1 week to acclimatize before the start of the experiments. All animal procedures were pre-approved by the Gallo Center Institutional Animal Care and Use Committee, performed in our Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) certified facility and were in accordance with National Institutes for Health Guide for Care and Use of Laboratory Animals. Mice were not deprived of food and water at any time.

2.3 Intrathecal injection

Mice were anaesthetized with 2% isoflurane 5 min prior to injection. A 28 ½ gauge needle was used and the insertions were made at the lumbar level (L4–L6). A volume of 5 µL per animal was used (Hylden and Wilcox, 1980). Upon correct insertion of the needle, a clear tail flick response was observed. Mice would wake up from the anaesthesia 2–3 min after injection and would be fully mobile and alert upon time of measurement.

2.4 Antinociception assays

2.4.1 von Frey assay—Mechanical sensitivity was measured using a set of von Frey monofilaments (0.07–2 g; Stoelting, IL, USA), ranging from 0.07 to 2 g. The largest filament (2 g) was used as cut-off. One day prior to testing, mice were placed in Plexiglas cubicles with a metal mesh floor to habituate for 1 h. On test day, mice were placed in the chambers for an hour prior to testing. The monofilaments were applied perpendicular to the mid-plantar surface of each hind paw with adequate force to produce a slight buckling of the filament and remained in this position for 2 s. Monofilaments were applied in ascending order, and the smallest monofilament that elicited a foot withdrawal response in two out of three tests was considered the threshold stimulus.

2.4.2 Tail flick assay—Thermal antinociception was determined by using the radiant heat tail flick assay. The intensity of the light was adjusted so that baseline tail flick latencies ranged from 1.8 to 3.0 s. A cut-off time of three times the baseline latency was set to minimize damage to the tail. The longer the latency for the mouse to move its tail away from the source of radiant heat (in comparison to basal values) was indicative of antinociceptive response.

Animals were tested for antinociception 10 min after drug administration. Associated data are displayed as the ‘maximum possible effect’ (%MPE): $100\% \times [(\text{drug response time} -$

basal response time)/(cut-off time-basal response time)]. All values are presented as the means $\pm$ standard error of the mean. Statistical evaluations were determined using Student's *t*-test, and one-way and two-way analysis of variance with either a Tukey or Bonferroni *post hoc* analysis. Whenever possible, experiments were performed with the experimenters 'blind' to the drug administered.

2.5 Drugs

17,17'- (dicyclopropylmethyl)-6,6',7,7'-6,6'-imino-7,7'-binorphinan-3,4',14,14'-tetrol nor-BNI, (+)-(5 α ,7 α , 8 β)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5] dec-8-yl]-benzeneacetamide (U69, 593) and CTAP were purchased from Sigma Chemical Co. (St Louis, MO, USA). 4-[(3,4-Dichlorophenyl)acetyl]-3-(1-pyrrolidinylmethyl)-1-piperazinecarboxylic acid methyl ester fumarate (GR89696), (+)- and Naltriben mesylate (NTB) were purchased from Tocris Bio-science (Ellisville, MO, USA). All compounds were dissolved in saline, with the exception of NTB, which was dissolved in 5% dimethyl sulfoxide. All drugs were prepared immediately prior to i.t. injection. The drug concentrations were selected based on previous reports on the antinociceptive properties of the tested compounds (Millan et al., 1989; Bilsky et al., 1994; Ho et al., 1997; Eliav et al., 1999; van Rijn et al., 2011). Two concentrations were used to provide a better indication of the agonist potency to reduce thermal and mechanical nociception and to ensure that potential synergistic or inhibitory effects could be readily observed.

3. Results

3.1 The putative KOR-1 agonist U69, 593 and putative KOR-2 agonist GR89, 696 are both selective for KOR

We first characterized the specificity of U69, 593 and GR89, 696 *in vitro* in HEK-293 cells recombinantly expressing KOR, DOR or MOR. We found that the putative KOR-1 selective agonist U69, 593 and the putative KOR-2 agonist GR89, 696 can potently induce calcium mobilization in HEK cells singly expressing KORs and a chimeric G_{gi4} protein (see Methods). Both agonists are nearly 1000-fold selective for the KOR over MOR and DOR (Table 1). In addition, we found that GR89, 696 is almost 10-fold more potent than U69, 593, consistent with previous results using alternative signalling readouts (Butelman et al., 2001). However, the KOR-2 selective agonist GR89, 696, while displaying KOR selectivity, also shows sub-micromolar activity at both the DOR and the MOR (Table 1), suggesting that at high doses, this drug could lose specificity. It has been shown that binding of [³H]bremazocine, another KOR-2 selective agonist, is decreased in the presence of DOR and MOR-selective ligands (Caudle et al., 1997).

3.2 Disruption of KOR does not completely abolish the antinociceptive abilities of GR89, 696

To investigate potential molecular mechanisms responsible for KOR subtypes *in vivo*, we injected the KOR-1 agonist U69, 593 and KOR-2 agonist GR89, 696 in the spinal cord of WT and KOR KO C57BL/6 mice. We measured both thermal and mechanical antinociception, as the neuronal circuits mediating pain and mechanical sensitivity express either MOR but not DOR or DOR but not MOR, respectively (Scherrer et al., 2009). Both

KOR agonists elicited thermal as well as mechanical antinociception (Figure 1A and B). Thermal antinociception induced by U69, 593 and GR89, 696 was completely abolished in KOR KO mice (Figure 1A). Interestingly, disruption of KOR did not fully eliminate GR89, 696-induced mechanical antinociception (Figure 1B). However, disruption of either DOR or MOR had no effect ($F_{2,58} = 0.02$; $p = 0.98$) on the antinociceptive effects of GR89, 696 on mechanical sensitivity (Figure 2A).

3.3 Mechanical antinociception induced by GR89, 696 is potentiated by the DOR antagonist NTB only in WT mice

A potential downside of using receptor KO mice is that not only monomers of that receptor would be eliminated but any potential heteromers would be as well (Figure 2B). As mentioned earlier, several reports have suggested opioid receptors can form heteromers both *in vitro* (Cvejic and Devi, 1997; Jordan and Devi, 1999; George et al., 2000; Wang et al., 2005) and *in vivo* (Portoghese and Lunzer, 2003; Waldhoer et al., 2005; Chakrabarti et al., 2010; Gupta et al., 2010; He et al., 2011) and that these heteromers could possibly account for the different phenotypes observed for the pharmacologically defined KOR subtypes (Bhushan et al., 2004; Daniels et al., 2005; Ansonoff et al., 2010). To further examine the potential involvement of the DOR or MOR in the actions of GR89, 696, we determined whether we could block or modulate the antinociceptive effects of GR89, 696 with a DOR or MOR-selective antagonist. Interestingly, we found that the mechanical antinociception induced by GR89, 696 is significantly ($F_{2,64} = 13.96$; $p < 0.0001$) enhanced by the DOR antagonist, NTB, but not the MOR antagonist CTAP (Figure 3A). However, NTB administered alone did not induce either thermal or mechanical antinociception (Supporting Information Fig. S1). The observed potentiation required the presence of both KOR and DOR (Figure 3B). Specifically, in mice with disruption of KOR the residual antinociceptive effect of GR89, 696 is blocked by NTB (Figure 3C), suggesting that in the absence of KOR, GR89, 696 can bind to DORs (Figure 3D). However, in contrast to the effect of co-administration of GR89, 696 and NTB in WT, we do not observe a potentiated effect in the KOR KO. Furthermore, in mice with a disruption of DOR the effects of GR89, 696 are not affected by the DOR antagonist NTB (Figure 3C), suggesting that NTB does not bind to KOR (Figure 3D).

3.4 GR89, 696-induced thermal antinociception is not affected by disruption or antagonism of DOR or MOR

We next compared the effects of antagonism or complete KO of DOR or MOR on the thermal antinociceptive effects of GR89, 696. Similar to the results obtained in the mechanical sensitivity assay, we found that disruption of either DOR or MOR did not affect ($F_{2,52} = 1.62$; $p = 0.21$) GR89, 696-induced thermal antinociception (Figure 4A). In addition, while NTB potentiated the antinociceptive effects of GR89, 696 for mechanical sensitivity, NTB had no effects ($F_{2,54} = 1.01$; $p = 0.37$) on GR89, 696-induced thermal antinociception, nor did CTAP (Figure 4B).

3.5 Mechanical antinociception by U69, 593 is enhanced by co-administration of a MOR antagonist

We next tested the consequence of disruption or antagonism of DOR and MOR on U69, 593-induced antinociception. We found that neither disruption ($F_{2,27} = 0.20$; $p = 0.82$) nor antagonism ($F_{2,27} = 1.32$; $p = 0.29$) of DOR or MOR influenced U69, 593-induced thermal antinociception (Figure 5A and B). Similarly, disruption of DOR or MOR did not change ($F_{2,59} = 0.98$; $p = 0.38$) U69, 593-induced mechanical antinociception (Figure 4C). Unlike what was seen with GR89, 696 (Figure 3A), the effects of U69, 593 on mechanical sensitivity were not potentiated by NTB (Figure 5D). Instead, the effects of U69, 593 were significantly enhanced ($F_{2,63} = 9.59$; $p = 0.0002$) by co-administration of the MOR antagonist CTAP (Figure 5D). Importantly, CTAP administered alone did not induce either thermal or mechanical antinociception (Supporting Information Fig. S1). The observed potentiation required the presence of KOR as co-administration of U69, 593 with CTAP in KOR KO mice did not result in a synergistic response (Supporting Information Fig. S2).

4. Discussion

The current study provides a better understanding of the molecular mechanisms that underlie the antinociceptive actions of KOR subtype-selective agonists. We found that in circuits mediating mechanical sensitivity, there are pharmacological differences between KOR-1 and KOR-2 agonists. Specifically, we show that the KOR-2 agonist GR89, 696 targets a KOR that is potentiated by the presence of a DOR antagonist. Whereas we find that the KOR-1 agonist U69, 593 targets a KOR that is instead potentiated by a MOR antagonist.

We find that the mechanical antinociception induced by U69, 593 but not GR89, 696 is completely eliminated in KOR KO mice (Figure 1B). This suggests that in addition to targeting KOR, GR89, 696 may associate with other receptors in circuits mediating mechanical antinociception receptors, although this may only occur at high concentrations or in the absence of KOR (Figure 2B). Our *in vitro* findings show that GR89, 696 exhibits submicromolar potency at DOR and MOR, which implies that the residual antinociception observed in the KOR KO mice may come as a result of GR89, 696 associating with MOR and/or DOR. In order to test this hypothesis, we injected GR89, 696 in mice with a disruption in DOR or MOR, with the understanding that if these receptors did contribute to the mechanical antinociception induced by GR89, 696 by eliminating these potential targets, there would be a significant decrease in the antinociception observed in the WT mice. However, there was no change in mechanical antinociception, thus it is unlikely that the antinociception induced by GR89, 696 in WT mice is a result of a combination of actions on KOR and MOR and/or DOR.

Interactions among opioid receptors have been proposed as the basis for some of the putative opioid receptor subtypes, and has led to studies that focused on opioid receptor co-localization, association and oligomerization as possible mechanisms to explain the opioid receptor subtypes (Erez et al., 1982; Rothman and Westfall, 1982; Portoghesi et al., 1986; Porreca et al., 1992; Traynor and Elliott, 1993). With this in mind, the residual antinociception observed in KOR KO mice could arise from GR89, 696 targeting a novel functional unit comprised of KOR and MOR and/or DOR. However, by using KO mice we

disrupt not only the specific receptor but interactions between multiple receptors that GR89, 696 may target. In order to circumvent the elimination of such a functional unit, we co-administered GR89, 696 with either a MOR or DOR antagonist. We proposed that the antinociception induced by GR89, 696 depends on its interactions with KOR homomers and a target that requires associations with KOR and MOR or DOR. Therefore, the administration of a DOR or MOR antagonist should affect the binding potential of GR89, 696 to this target. Interestingly, we find that the mechanical antinociception observed in WT mice was potentiated upon co-administration with a DOR-, but not a MOR-selective antagonist. Having the ability to test the antagonists in the opioid receptor KO mice then allowed us to further investigate the binding mechanisms of GR89, 696.

GR89, 696 signalling via DOR may account for the residual mechanical antinociception observed in KOR KO mice, as evidenced by the decreased response upon the co-administration of GR89, 696 and NTB in KOR KO mice. However, apparently in the presence of both KOR and DOR a novel functional unit is formed with a unique pharmacological profile. Specifically, the DOR antagonist NTB potentiates the effects of GR89, 696 but *only* in WT mice (Figure 2B), not in mice with a disruption of either DOR or KOR (Figure 3B). It is possible that the potentiation of GR89, 696 comes as a result of NTB binding to KOR, suggesting that the potentiation is a result of two ligands associating with the same receptor. However, this is unlikely, since in DOR KO mice the antinociception induced by GR89, 696 does not increase with the administration of the DOR antagonist NTB. Thus, it is improbable that NTB is binding to KOR in an allosteric manner (Figure 3D). There is a possibility that a population of active 'pro-nociceptive' DORs is activated by GR89, 696 which when blocked by NTB could increase the antinociception. Yet, if this were the case, we would expect to see an increased effect of GR89, 696 in DOR KO mice, which we do not. We can not exclude that adaptive changes in the KO mice could account for some of our findings. It is therefore possible that rather than forming heteromers KOR-selective agonists, mediated antinociception could be enhanced by DOR or MOR antagonists through an overlapping circuitry mechanism, e.g., if pre-synaptic DORs are present on a dynorphin releasing terminal that projects onto neurons containing post-synaptic KORs. However, no changes in opioid expression level and G-protein coupling were observed in the KO mice (Matthes et al., 1998; Simonin et al., 1998; Filliol et al., 2000). Hence, we favour the hypothesis that GR89, 696 accesses both KOR homomers and KOR/DOR heteromers to mediate antinociception, and that binding of NTB to the KOR/DOR heteromer in the presence of GR89, 696 increases either agonist affinity and/or potency.

Here we propose a model whereby neurons mediating mechanical sensitivity express KOR-DOR heteromers that can be more potently activated by a KOR agonist in the presence of a DOR antagonist. A similar phenomenon has been reported to occur in the context of MOR-DOR heteromers whereby MOR-mediated antinociception is potentiated by a DOR antagonist (Gomes et al., 2000). Similarly, Han and colleagues have shown that maximal functional stimulation of a dopamine D₂ receptor dimer was achieved when a dopamine receptor agonist was co-administered with an antagonist (Han et al., 2009). We propose that the effects of GR89, 696 are equivalent on KOR homomers and on KOR-DOR heteromers

in which the DOR is unoccupied. Since there is a residual activity of GR89, 696 in KOR KO mice that is blocked (rather than potentiated) by NTB, we propose that the loss of the high affinity KOR binding site ‘frees up’ GR89, 696 that can now bind and activate some DORs, which would explain why we observe residual antinociception in KOR KO mice.

The pharmacological profile of GR89, 696 shown here is consistent with previous studies implicating the KOR-DOR heterodimer in KOR-2 pharmacology (Bhushan et al., 2004; Ansonoff et al., 2010). However, we show that GR89, 696 can act on KOR homomers as well and behave similar to the KOR-1 agonist U69, 593 depending on the associated circuit. We propose that the circuit regulating thermal nociception contains only KOR homomers, which can be activated by both U69, 593 and GR89, 696 (Figure 6A). The spinal circuit regulating mechanical sensitivity contains KOR homomers and heteromers (Figure 6B) that can be activated by the KOR agonists, but depending on the heteromer a combination of a KOR1 agonist and MOR antagonist or KOR2 agonist and DOR antagonist can result in enhanced antinociception.

Our observation that the mechanical antinociception induced by U69, 593 is enhanced in the presence of the MOR antagonist CTAP is at odds with recent findings by Scherrer and co-workers, who only found MOR expression in neurons mediating thermal but not mechanical nociception (Scherrer et al., 2009). However, we have recently shown that functional MORs are also expressed in mechanical nociception mediating neurons (van Rijn et al., 2011). Those findings, together with our current data, suggest that neurons mediating thermal nociception express MOR and KOR, whereas mechanical nociceptive neurons express MOR, KOR and DOR. Our findings would suggest that MOR and KOR may not be co-localized in neurons or interconnected in a circuit involved in thermal nociception.

By further examining the pharmacological profile of KOR subtypes, we may be able to find one subtype which when activated will still induce the same potent antinociception without the associated side effects. This has been shown with the MOR: Two subtypes of MOR have been proposed, MOR-1 and MOR-2 (McGilliard and Takemori, 1978; Wolozin and Pasternak, 1981), with only compounds that selectively target MOR-2 inducing respiratory depression (Ling et al., 1984). Our findings are potentially of significant therapeutic importance because they suggest a strategy for the development of KOR-selective ligands whose efficacy could be enhanced by either DOR or MOR antagonists with a possible decrease in known side effects. Similar findings with co-administration of DOR- and MOR-selective drugs have provided hopes of circumventing drug-associated side effects (Dietis et al., 2009). Indeed, one strategy that could be further pursued would be the use of ligands that have higher potency and/or efficacy on the heteromer than the respective homomer, or the development of bivalent ligands with mixed agonist-antagonist pharmacophores.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The KOR-1 agonist U69,696 and KOR-2 agonist GR89,696 produce antinociception primarily through the KOR. Wild-type (WT) and KOR KO C57BL/6mice ($n = 10$) were injected i.t. with an analgesic dose of the KOR-1 selective agonist U69,593 (25 nmol) or the KOR-2 selective agonist GR89,696 (1 nmol). Thermal antinociception was measured 10 min after injection using a radiant heat tail flick assay (A). Mechanical sensitivity was measured 10 min after injection using von Frey filaments (B). Data are displayed as the 'maximum possible effect' (%MPE): $100\% \times [(drug\ response\ time - basal\ response\ time) / (cut-off\ time - basal\ response\ time)]$. Student's t -tests were performed to determine significance. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 2.

GR89, 696-induced mechanical antinociception is not altered in DOR or MOR KO mice. Wild-type (WT), DOR KO and MOR KO C57BL/6mice ($n = 9-15$) were injected i.t. with 0.1 or 1 nmol GR89, 696 and mechanical sensitivity was measured (A). In WT mice, GR89, 696 activates KORs that are part of a KOR homomer or KOR-DOR heteromer with similar effect. Disruption of KOR results in a large enough concentration of unbound GR89, 696 to activate DORs producing moderate mechanical antinociception. Disruption of DOR disrupts the KOR-DOR heteromer into KOR homomers/monomers, thereby having no net effect on the mechanical antinociception produced by GR89, 696 (B). Data are displayed as the 'maximum possible effect' (%MPE): $100\% \times [(\text{drug response time} - \text{basal response time}) / (\text{cut-off time} - \text{basal response time})]$. A two-way analysis of variance was performed to determine significance.

Figure 3.

Naltriben potentiates GR89, 696-induced mechanical antinociception only when both DOR and KOR are present. Wild-type (WT) C57BL/6mice ($n = 10-15$) were injected with increasing doses of GR89, 696 i.t. in the absence or presence of a DOR (0.5 nmol NTB) or MOR (0.2 nmol CTAP) antagonist. Mechanical sensitivity was measured using von Frey filaments 10 min after injection (A). Simultaneous occupation of the KOR-DOR heteromer with a KOR agonist and DOR antagonist stabilizes a more efficacious conformation of the heteromer, leading to a potentiation in the response induced by GR 89, 696 in WT mice (B). WT, KOR KO and DOR KO C57BL/6 mice ($n = 10-15$) were injected with an analgesic dose of 1 nmol GR89, 696 i.t. in the absence or presence of the DOR antagonist NTB (0.5 nmol) Mechanical sensitivity was measured using von Frey filaments (C). Disruption of KOR results in a large enough concentration of unbound GR89, 696 to activate DORs producing moderate mechanical antinociception, which can be blocked. In DOR KO mice, NTB has no effect on the GR 89, 696-induced antinociception (D). Data are displayed as the 'maximum possible effect' (%MPE): $100\% \times [(\text{drug response time} - \text{basal response time}) / (\text{cut-off time} - \text{basal response time})]$. Student's t -tests and two-way analyses of variance were performed to determine significance. $*p < 0.05$; $**p < 0.01$.

Figure 4.

Thermal antinociception induced by the KOR-2 agonist GR 89, 696 is mediated solely via KOR. Wild-type (WT), DOR KO and MOR KO C57BL/6mice ($n = 9-10$) were injected i.t. with increasing doses of GR 89, 696. Thermal antinociception was measured using a radiant heat tail flick assay 10 min after injection (A). WT C57BL/6mice ($n = 10$) were injected i.t. with increasing doses of GR 89, 696 in the absence or presence of the DOR antagonist, NTB (0.5 nmol), or the MOR antagonist, CTAP (0.2 nmol), and thermal antinociception was measured (B). Data are displayed as the 'maximum possible effect' (%MPE): $100\% \times [(\text{drug response time} - \text{basal response time}) / (\text{cut-off time} - \text{basal response time})]$. Two-way analyses of variance were performed to determine significance.

Figure 5.

Thermal antinociception induced by KOR-1 agonist U69,593 is mediated solely via KOR, whereas mechanical sensitivity may involve a KOR-MOR heteromer. Wild-type (WT), DOR KO and MOR KO C57BL/6mice ($n = 10$) were injected i.t. with increasing doses of U69,593. Thermal antinociception was measured using a radiant heat tail flick assay 10 min after injection (A). WT C57BL/6mice ($n = 10$) were injected i.t. with increasing doses of U69,593 in the absence or presence of the DOR antagonist, NTB (0.5 nmol), or the MOR antagonist, CTAP (0.2 nmol), and thermal antinociception was measured (B). WT, DOR KO and MOR KO C57BL/6mice ($n = 10$) were injected i.t. with increasing doses of U69,593. Mechanical sensitivity was measured using von Frey filaments 10 min after injection (C). WT C57BL/6mice ($n = 10-15$) were injected i.t. with increasing doses of U69,593 in the absence or presence of the DOR antagonist, NTB (0.5 nmol), or the MOR antagonist, CTAP (0.2 nmol), and mechanical sensitivity was measured (D). Data are displayed as the 'maximum possible effect' (%MPE): $100\% \times [(\text{drug response time} - \text{basal response time}) / (\text{cut-off time} - \text{basal response time})]$. Two-way analyses of variance (ANOVAs) were performed to determine significance. $*p < 0.05$. Two-way ANOVAs were performed to determine significance.

Figure 6.

Subtype selectivity of KOR agonists depends on the presence of DOR and MOR in a circuit.

In neurons mediating thermal nociception in naïve mice, KOR agonists produce thermal antinociception solely by activating KORs (A). In neurons mediating mechanical sensitivity, KOR-1 agonists can bind to both KOR homomers and KOR-MOR heteromers, whereas KOR-2 agonists interact with KOR homomers and KOR-DOR heteromers and potentially DOR homomers (B).

Table 1

Potency ($\text{pEC}_{50} \pm$ standard error of the mean) of the putative KOR-1 agonist U69, 593 and putative KOR-2 agonist GR89, 696 to induce Ca^{2+} -mobilization on HEK293 cells expressing KOR, DOR or MOR and a chimeric $\text{G}_{\text{q}14}$ protein. Experiments were performed at least three times in triplicate.

Ligands	<u>pEC_{50} (M)</u>		
	KOR	DOR	MOR
U69, 593	9.3 ± 0.2	<5	<5
GR89, 696	10.1 ± 0.2	7.3 ± 0.3	7.2 ± 0.2