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Title:

The delta-opioid receptor is sufficient, but not necessary, for spinal opioid-adrenergic analgesic synergy.

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Running title: Role of DOR in opioid-adrenergic analgesic synergy

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Abstract:

Spinal administration of opioid and α_2-adrenergic receptor (α_2AR) agonists produce analgesia and interact synergistically when co-administered. The molecular mechanism underlying this synergy is largely unknown. Pharmacological studies have identified both the delta and the mu opioid receptors (DOR and MOR) as candidate receptors capable of interacting synergistically with α_2AR agonists. However, recent studies attribute the antinociceptive effect of DOR agonists to actions at the MOR, calling the role of DOR in opioid-adrenergic synergy into question. Other studies suggesting that DOR is implicated in morphine antinociception raise the possibility that DOR is nonetheless required for morphine synergy with α_2AR agonists. This study aimed to determine whether DOR activation is sufficient and necessary to mediate opioid-adrenergic synergistic interactions in the spinal cord. The antinociceptive effects of clonidine, [D-Ala^2]-deltorphin II (DeltII), morphine and [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin (DAMGO) were evaluated using the substance P (SP) behavioral assay in wild type (WT) and DOR-knockout (KO) mice. Opioid-adrenergic drug interactions were evaluated following spinal co-administration of clonidine with DeltII, morphine or DAMGO. Isobolographic analyses of dose-response curves determined whether interactions were synergistic or additive. The absence of DeltII antinociceptive effect in DOR-KO confirmed its selectivity in the SP assay. While DeltII+clonidine interacted synergistically in WT mice, no interaction with clonidine was observed in DOR-KO mice. Clonidine was synergistic with morphine in both mouse strains. DAMGO did not synergize with clonidine in either strain. These findings confirm that while other opioid receptors can interact synergistically with α_2AR agonists, DOR is sufficient for spinal opioid-adrenergic interactions.

Introduction

Patients with acute or chronic pain need better analgesic therapies to provide currently unmet pain relief. Opioid and α_2-adrenergic receptor (α_2AR) agonists are potent analgesic drugs, but their use is limited by their side effects or lack of efficacy in certain clinical conditions. Compared to the analgesic effects of drugs acting independently, analgesic synergy arising from drug combinations is advantageous since it produces adequate analgesia at lower doses, which can potentially reduce side effects and tolerance (Walker et al., 2002). Pain management using opioid-α_2AR agonist combinations reduces side effects associated with both α_2AR and opioid treatments (Eisenach et al., 1994) and such combinations may also be effective in treating chronic pain conditions with reduced opioid sensitivity (Eisenach et al., 1995). In rodents, the opioid agonist morphine and the α_2AR agonist clonidine synergize when co-administered at the spinal level, suggesting that beneficial opioid-adrenergic drug interactions occur at the spinal cord (Alguacil and Morales, 2004). The clinical benefits of opioid-adrenergic combinations remain largely underexploited because the development of new therapies taking advantage of the synergistic interaction is hindered, in part, by a lack of understanding of the underlying mechanism.

In order to gain mechanistic insight, it is necessary to know the receptor subtypes required for synergy. Opioid agonists can mediate their analgesic action at the spinal cord by activating the δ (DOR), μ (MOR) and κ (KOR) opioid receptor subtypes. [D-Ala^2]-deltorphin II (DeltII), a DOR-selective peptide agonist, can synergize with the α_2AR agonists clonidine (Overland et al., 2009), ST-91 (Stone et al., 2007), moxonidine (Fairbanks et al., 2000; Fairbanks et al., 2002), and UK 14,304 (Stone et al., 1997) when

co-administered in rodents at the spinal cord. Similarly, another DOR-selective agonist, [D-Pen^2,D-Pen^5]enkephalin (DPDPE), has been shown to synergize with clonidine (Ossipov et al., 1990b; Roerig et al., 1992), norepinephrine (Roerig et al., 1992), and UK 14,304 (Guo et al., 2003), and this latter synergistic interaction persists in MOR-KO mice (Guo et al., 2003). Thus, opioid-adrenergic interactions resulting in analgesic synergy are possible when activating DOR and can occur in the absence of MOR. However, reports that DeltII and DPDPE retain their antinociceptive action in DOR-KO mice and that MOR mediates this effect questions the selectivity of DOR agonists (Scherrer et al., 2004; van Rijn et al., 2012). This could mean that opioid-adrenergic synergistic interactions studied with DOR agonists are mediated by MOR and therefore justify further evaluation of the role of DOR in these interactions.

Morphine has been shown to interact synergistically with the α_2AR agonists clonidine (Ossipov et al., 1990a; Roerig et al., 1992; Fairbanks and Wilcox, 1999b), moxonidine (Fairbanks et al., 2000), norepinephrine (Roerig et al., 1992) and ST-91 (Monasky et al., 1990). The antinociceptive effect of morphine is considered to be MOR-mediated (Matthes et al., 1996), which is consistent with its relative selective affinity for MOR in expression systems (Raynor et al., 1994). Taken together, these studies would suggest that MOR mediates morphine's synergistic interactions with α_2AR agonists. However, interactions between MOR and DOR have been shown to modulate morphine response *in vivo* and *in vitro* (Costantino et al., 2012). For example, the cellular response following morphine treatment is more potent in cells co-expressing MOR and DOR rather than MOR alone (Yekkirala et al., 2010). Furthermore, DOR-selective ligands potentiate morphine analgesia *in vivo* (Gomes et al., 2004), and DOR is involved in the development of analgesic tolerance to morphine (Zhu et al., 1999). Morphine also

upregulates the expression of surface DOR through its action at the MOR (Cahill et al., 2001; Morinville et al., 2003; Gendron et al., 2006). It is currently unknown if DOR participates in morphine's synergistic interaction with α_2AR agonists.

The aim of this study was to disambiguate the role of DOR in the synergistic interaction between opioid and α_2AR agonists administered spinally. We therefore determined if DOR activation is sufficient to produce synergy and if DOR modulates MOR-mediated synergistic interactions with clonidine, the only α_2AR agonist approved for epidural analgesic use. The antinociceptive interaction between clonidine and DeltII, morphine or the MOR-specific agonist DAMGO was compared between wild type (WT) and DOR-KO mice, which have a genetic deletion in the *Oprd1* gene (Filliol et al., 2000). We observed that the synergistic interaction between DeltII and clonidine required DOR. In contrast, the interaction between morphine and clonidine remained synergistic in DOR-KO mice, and DAMGO failed to synergize with clonidine in either strain. Our results demonstrate that DOR is sufficient, but not necessary to mediate opioid-α_2 adrenergic analgesic synergy at the spinal cord.

Materials and Methods

Animals

Mice with a targeted gene deletion introducing a genetic deletion in exon 1 of the delta opioid receptor gene (*Oprd1*) were developed on a mixed C57Bl/6 x FVB/129 background (Filliol et al., 2000). Congenic mice backcrossed to a standard C57Bl/6 background were obtained from Jackson Laboratory (Bar Harbor, Main, B6.129S2-*Oprd1*^{tm1}*Kff*/J, stock #007557). Mice with a targeted gene deletion introducing a premature stop codon in the third transmembrane domain of the α_{2A}AR gene (*Adra2a*) were developed on a mixed C57Bl/6 x FVB/129 background (Altman et al., 1999). Congenic mice backcrossed to a standard C57Bl/6 background were obtained from Jackson Laboratory (Bar Harbor, Main, stock #004367). Commercially available C57BL/6 mice (Charles River, Quebec, Canada) were purchased and used as wild-type (WT). All strains were bred in house and genotyping controls were performed on parent breeders to monitor the stability of the colony.

Mice were maintained on a regular 12 hour light/dark cycle and given access to food and water *ad libitum.* Aged-matched 3-6 month old WT, DOR-KO and α_{2A}AR-KO males were used in this study and experimenters were blind to both genotype and treatment. All procedures were approved by the Animal Care Committee at McGill University, and conformed to ethical guidelines of the Canadian Council on Animal Care.

Drugs

Substance P (SP, Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) was purchased from AnaSpec (CA, USA) and concentrated stocks were dissolved in acidified saline (0.9% NaCl, 0.05 M acetic acid). Morphine sulfate (Medisca Pharmaceuticals, Montreal,

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QC) was dissolved in saline. [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin (DAMGO) and clonidine HCl (*N*-(2,6-dichlorophenyl)-4,5-dihydro-1*H*-imidazol-2-amine; R&D Systems, MN, USA) were dissolved in saline. [D-Ala^2]-deltorphin II (DeltII; R&D Systems) was dissolved in acidified saline. Drug stocks were diluted in saline to working concentrations.

Pharmacological Treatment

Intrathecal (i.t.) drug administration was done by direct lumbar puncture in a volume of 5 µl according to the method of Hylden and Wilcox (1980) in conscious mice. DeltII, morphine, DAMGO and clonidine doses are expressed as total nmol or pmol, and drug combination doses are graphed as total drug doses (i.e. the sum of both drugs) in 5 µl and expressed in nmol. Drugs were administered simultaneously with 15 ng SP in a single 5 µl volume. Following randomization, each mouse was reused with a minimum of 5 day between testing session to allow for drug wash out.

SP Behavioral Assay

The antinociceptive action of single drugs and their combination was tested in the substance P (SP) behavioral assay developed by Hylden and Wilcox (1981). Briefly, 15 ng of SP was administered i.t. alone (control) or co-administered with a single drug or a drug combination in a volume of 5 µl. The number of caudally directed biting, licking and scratching behaviors were counted for 1 min and results are expressed as the percent inhibition of SP-induced behaviors:

$$\% \text{ Inhibition} = \frac{\text{Control} - \text{Experimental}}{\text{Control}} \times 100$$

Dose-Response Analysis:

Dose-response graphs were generated with GraphPad Prism 6.0 (GraphPad Software, Inc.). A minimum of 5 animals was used per dose and each dose point is expressed as the mean % inhibition with S.E.M. For drug combinations, the dose-response curve was graphed according to the dose of clonidine present in the mixture. Drugs had to reach at least 50% inhibition to be considered effective. ED_{50} values and 95% confidence intervals (CI) were calculated using a minimum of three doses in the linear portion of each dose-response curve following the method of Tallarida and Murray (1987). Statistical comparisons of potencies based on the confidence limits of the ED_{50} values were calculated to obtain the relative potency ratio between two drugs.

Isobolographic Analysis:

Drug combination ratios were chosen according to the relative potency of each drug by determining an approximately equally effective potency ratio between the agonists based on their respective ED_{50} values. When two drugs were equally potent, they were mixed in a 1:1 (i.e. equieffective and equimolar) ratio. If a drug was 10 times more potent than the other, drugs were mixed in a 1:10 (i.e. equieffective) ratio. Since the relative drug potency of the drug pairs used in this study differed between WT and DOR-KO mice, different drug ratios were tested in each strains and the experimental ED_{50} value for the drug combination was determined. To test for interactions between agonists, the ED_{50} values and S.E.M. of all dose-response curves were arithmetically arranged around the ED_{50} value using equation $(\ln(10) \times ED_{50}) \times (\text{S.E. of log } ED_{50})$ (Tallarida, 1992). This manipulation was required to perform an isobolographic analysis, the appropriate method to evaluate if an interaction is synergistic, additive or sub-additive (Tallarida, 1992). When testing an interaction between two drugs, a theoretical additive ED_{50} value is calculated for the combination based on the dose-response curve of each drug

administered separately. This theoretical value is then compared by a Student's *t* test with the observed experimental ED_{50} value of the combination. An interaction is considered synergistic if the experimental ED_{50} is significantly less ($P < 0.05$) than the calculated theoretical additive ED_{50}.

Visualization of drug interactions can be facilitated by graphical representation of isobolographic analysis. This representation depicts the ED_{50} value of each drug as the *x*- or *y*-intercept. The line connecting these two points depicts the dose combination expected to yield 50% efficacy if the interaction is purely additive and is called the theoretical additive line. The theoretical additive ED_{50} is determined mathematically and plotted on this line with its CI spanning perpendicularly from the line. The experimental ED_{50} for the combination is plotted at the corresponding *x*,*y* co-ordinates along with its 95% confidence interval for comparison with the theoretical additive ED_{50} value.

All dose-response and isobolographic analyses were performed with the FlashCalc 4.5.3 pharmacological statistics software package generously supplied by Dr. Michael Ossipov.

Results

Comparable SP-evoked nocifensive behaviors are measured in WT and DOR-KO mice

We used the substance P (SP) behavioral assay to measure the antinociceptive effect of opioid agonists and clonidine at the spinal level. Intrathecal (i.t.) administration of SP induces a characteristic set of behaviors (biting, licking and scratching) directed at the abdomen and hind portion of the mouse receiving the exogenous SP (Hylden and Wilcox, 1981). Analgesic drugs acting at the spinal cord inhibit these nocifensive behaviors in a dose-dependent manner. There was no significant difference in SP-induced behaviors between WT (37 ± 4, n = 13), DOR-KO (34 ± 3, n = 22) and α_{2A}AR-KO (29 ± 3, n = 18) mice (unpaired one-way ANOVA, $P > 0.05$) upon intrathecal injection of 15 ng SP. Thus, there is no strain difference in sensitivity to SP nociception.

Antinociceptive action of spinally administered DeltII, DAMGO, morphine and clonidine in WT, DOR-KO and α_{2A}AR-KO mice

DeltII, morphine, DAMGO and clonidine were administered spinally (i.t.) in the SP behavioral assay in both WT and DOR-KO mice. All drugs inhibited SP-induced nocifensive behaviors in a dose-dependent manner (Figure 1, Table 1), except for DeltII, which was effective in WT but not DOR-KO mice (Figure 1B). Clonidine inhibition of SP-induced behaviors was 3-fold more potent in DOR-KO mice compared to WT mice (Figure 1A, Table 1). The inhibitory action of morphine was similar in both WT and DOR-KO mice at lower doses. As morphine dose increases, its efficacy was reduced in DOR-KO mice compared to WT mice (Figure 1C). Nevertheless, there is no significant potency difference between WT and DOR-KO mice (Table 1). No strain difference was

observed with the MOR agonist DAMGO (Figure 1D). In α_{2A}AR-KO mice, clonidine efficacy was reduced to less than 50% inhibition and potency was reduced by 20-fold compared to WT mice (Figure 1E). Together, this data indicate that DOR mediates DeltII antinociception in the SP assay, that morphine and clonidine antinociception are slightly altered in DOR-KO mice and that clonidine antinociception is largely mediated by the α_{2A}AR.

Deltorphin II-clonidine spinal antinociceptive synergy requires DOR

We tested the DOR-selective agonist, DeltII, and clonidine alone or in combination in the SP behavioral assay in WT and DOR-KO mice. In WT mice, DeltII and clonidine inhibited SP-induced nocifensive behaviors in a dose-dependent manner and with similar potency (Figure 2A). Thus, we tested DeltII in combination with clonidine at an equieffective ratio that also corresponds to an equimolar drug ratio (1:1). The inhibition of SP-induced behaviors by the drug combination shifted the dose-response curve leftward. Isobolographic analysis revealed that the experimental ED_{50} value of the drug combination was significantly lower than the theoretical additive ED_{50} value; the drug interaction is therefore synergistic (Figure 2B, Table 2).

In DOR-KO mice, DeltII was ineffective in the SP assay at all doses tested. We therefore used the same equimolar (1:1) drug ratio to compare the DeltII+clonidine interaction in DOR-KO mice that was used in the WT mice. Because DeltII was not efficacious, isobolographic analysis was not possible. However, the inhibition of SP-induced behaviors by the DeltII+clonidine combination was equivalent to clonidine alone, i.e. DeltII did not shift the clonidine dose-response curve in DOR-KO mice, suggesting that there is no interaction between DeltII and clonidine in DOR-KO mice (Figure 2C).

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Morphine-clonidine spinal antinociceptive synergy persists in the absence of DOR

Since DOR activation is sufficient to produce synergy using a DOR-selective agonist, we assessed its necessity for the synergistic interaction between morphine and clonidine.

In WT mice, spinally administered morphine and clonidine inhibited SP-induced behaviors in a dose-dependent manner. Calculated ED_{50} values obtained for each drug were within one order of magnitude, hence we combined morphine+clonidine at an equieffective and equimolar (1:1) ratio. The drug combination also inhibited SP behaviors in a dose-dependent manner and the dose-response curve was shifted to the left compared to the single doses (Figure 3A). The isobolographic analysis demonstrated that the morphine+clonidine interaction in WT mice was synergistic (Figure 3B, Table 2).

We then assessed the interaction between morphine and clonidine in DOR-KO mice. Because the difference in ED_{50} values between morphine and clonidine in DOR-KO mice was more than one order of magnitude (Table 1), we tested an equieffective drug ratio of 1 part clonidine + 10 parts morphine (1:10). The drug combination dose-dependently inhibited SP behaviors in DOR-KO mice and the dose-response curve was shifted to the left compared to each drug alone (Figure 3C). Isobolographic analysis showed that the experimental ED_{50} value is significantly lower than the theoretical additive ED_{50} value (Figure 3D), indicating that the interaction is synergistic (Table 2).

Taken together, these results show that equieffective doses of morphine and clonidine interact synergistically in both WT and DOR-KO mice.

DAMGO-clonidine interaction is additive in both WT and DOR-KO mice

The retention of morphine+clonidine synergy in DOR-KO mice suggests that opioid-adrenergic synergy can be mediated by MOR in the absence of DOR. To understand the

requirements for MOR-mediated synergy with clonidine, we tested the combination of clonidine with the MOR-selective peptide agonist, DAMGO, in WT and DOR-KO mice. In WT mice, DAMGO inhibited SP-induced behaviors with an ED_{50} value 120-fold more potent than clonidine (Table 1). We therefore tested a combination of clonidine and DAMGO at a 100:1 ratio, which also inhibited SP-induced behaviors in a dose-dependent manner (Figure 4A). The isobolographic analysis revealed that this interaction was additive (Figure 4B, Table 2).

The potency difference between DAMGO and clonidine in DOR-KO mice required the use of a 1:10 drug ratio corresponding to equieffective doses in this strain. The resulting drug interaction was also additive (Figure 4C, D, Table 2).

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Discussion

This study addressed the role of DOR in spinal opioid-adrenergic synergistic interactions. We first compared the antinociceptive response of clonidine, DeltII, morphine and DAMGO between WT and DOR-KO mice in the SP behavioral assay. The observed lack of DeltII efficacy in DOR-KO mice confirms its DOR selectivity in the SP behavioral assay. The addition of clonidine to DeltII resulted in a synergistic interaction in WT but not in DOR-KO mice. In contrast, a synergistic interaction between clonidine and morphine was observed in both strains and the interaction between clonidine and the MOR-selective agonist DAMGO was additive in both strains. These data demonstrate that DOR is sufficient, but not necessary, for opioid-adrenergic synergistic interaction at the spinal cord.

Mechanisms mediating opioid-adrenergic synergy

Opioid-adrenergic synergy could arise from pharmacokinetic interactions. In addition to their antinociceptive effects, α_2AR agonists like clonidine have a local vasoconstrictive effect (Asada and Lee, 1992; Iida et al., 1999) that can reduce drug clearance from the site of injection. Clonidine could therefore enhance the effect of another drug by maintaining it at a high local drug concentration. Following this logic, clonidine should interact synergistically with other drug classes. For example, in a post-operative pain model, clonidine interacts synergistically following intrathecal injection with gabapentin and with an allosteric adenosine receptor modulator (Cheng et al., 2000; Obata et al., 2004). However, reports of synergistic interactions with some, but not all, opioids makes altered drug clearance an unlikely mechanism. Furthermore, ST-91, an α_2AR agonist with hypertensive effects (Yasuoka and Yaksh, 1983; Nagasaka and Yaksh, 1990), interacts synergistically with morphine but does not affect morphine clearance from the

spinal cord (Monasky et al., 1990). Thus, the pharmacokinetic actions of α_2AR agonists are unlikely mediators of spinal opioid-adrenergic synergy.

The diffusion of intrathecally-administered drugs to supraspinal sites is negligible over a short period of time (Hylden and Wilcox, 1980). Rather, intrathecally-administered compounds act locally on the spinal cord and nociceptors, which have opioid and adrenergic receptors capable of inhibiting the transmission of afferent nociceptive signals. Pharmacodynamic interactions between opioids and α_2AR agonists resulting in analgesic synergy could involve spinal intercellular or intracellular mechanisms. Since spinal MOR, DOR and α_2AR are distributed on both primary afferent and spinal neurons (Stone et al., 1998; Wall et al., 2006), it is possible that the cumulated neuronal inhibition in the nociceptive circuit is supra-additive. Synergistic interactions could also result from the co-expression of opioid and adrenergic receptors in the same cells where their simultaneous activation results in synergistic output as proposed by Overland et al. (2009).

DOR is sufficient to produce opioid-adrenergic synergy

Previous studies have shown that spinal co-administration of the DOR-selective peptide agonists DPDPE and DeltII with clonidine results in a synergistic interaction in both the tail flick and SP behavioral assays (Ossipov et al., 1990b; Roerig et al., 1992; Roerig, 1995; Overland et al., 2009). These interactions persist in MOR-KO mice, suggesting that MOR is not required (Guo et al., 2003). However, the analgesic effects of DeltII have been attributed to MOR in some behavioral assays such as the tail flick assay (Scherrer et al., 2004; van Rijn et al., 2012), raising the possibility that these synergistic interactions with α_2AR agonists are MOR-mediated rather than DOR-mediated. In the current study,

DeltII antinociception was absent in DOR-KO mice, validating the use of the SP behavioral assay as a tool to examine the role of DOR in synergistic interactions. As a result, the absence of DeltII-clonidine synergy in DOR-KO mice confirms that DOR activation is sufficient to interact synergistically with α_2AR agonists. Assays should be carefully validated when studying DOR-mediated synergistic interactions as the current findings may not generalize to different assays.

In the spinal cord, clonidine has been shown to interact with both α_2AR and imadazoline binding sites, but the antinociceptive effect of clonidine is mediated by α_2AR rather than imadazoline receptors (Monroe et al., 1995a; Monroe et al., 1995b). Our data further suggests that the α_{2A}AR is a key mediator of clonidine antinociception. This observation is consistent with previous studies showing the loss of clonidine antinociceptive efficacy observed in mice expressing a dysfunctional α_{2A}AR (Fairbanks and Wilcox, 1999a). The α_{2A}AR is also necessary for analgesic synergy between DeltII and the α_2AR agonist UK 14,304 (Stone et al., 1997). DOR and α_{2A}AR can both exert their analgesic action by inhibiting transmitter release from primary afferent terminals (Glaum et al., 1994; Kawasaki et al., 2003). Data suggest that the synergistic interaction between DeltII and clonidine is maintained at the level of the primary afferent nerve terminal; for example, this drug combination inhibited KCl-induced CGRP release synergistically from spinal cord slices (Overland et al., 2009). Thus, peptidergic primary afferent neurons are a potential site of action of opioid-adrenergic synergy. We have demonstrated that DOR and α_{2A}AR receptors are co-expressed in primary afferent neurons and highly co-localize in SP-immunoreactive neurons and isolated nerve terminals (Riedl et al., 2009). While the localization of DOR in SP neurons is debated (Scherrer et al., 2009; Wang et al., 2010), the above-mentioned physiological and anatomical evidence support the presence

of DOR and α_{2A}AR in peptidergic neurons where they would be positioned to inhibit neurotransmitter release in a synergistic manner.

DOR is not necessary to mediate morphine-clonidine synergy

The synergistic interaction between morphine and clonidine is well documented in rodents using different assays (Fairbanks et al., 2009). Because the interaction is stronger when the drugs are administered intrathecally compared to intravenously, it has been proposed to be mediated largely at the level of the spinal cord (Ossipov et al., 1990a). Spinal MOR and DOR can interact together and form heteromeric complexes with altered signaling properties upon morphine treatment (Costantino et al., 2012), which led us to hypothesize that morphine+clonidine synergistic interaction may require DOR. Our data demonstrate that in DOR-KO mice, a synergistic interaction between morphine and clonidine is still present, allowing us to conclude that DOR is not the only opioid receptor able to mediate opioid-adrenergic analgesic synergistic interactions. Since morphine is not efficacious in the SP behavioral assay in MOR-KO compared to WT mice (Guo et al., 2003), the activation of MOR likely mediates the synergistic interaction between morphine and clonidine.

Synergistic interactions involving MOR are assay-dependent and ligand-biased

Co-activation of MOR with an α_2AR agonist produces different interactions depending on the experimental conditions and agonists used. While the interaction between DAMGO and clonidine is either additive (Figure 4) or subadditive in the SP behavioral assay (Roerig et al., 1992), this drug combination is synergistic in the tail flick assay (Roerig, 1995). Furthermore, the combination of DAMGO with different α_2AR agonists in the SP behavioral assay can produce either synergistic (Stone et al., 1997) or sub-

additive (Fairbanks et al., 2000) interactions. Thus, depending on the assay and ligands used, MOR activation may or may not contribute to spinal opioid-adrenergic synergistic interactions.

The contrasting results obtained using two agonists that activate MOR suggest the mechanism underlying these interactions may involve ligand-biased signaling. Morphine and DAMGO engage different downstream signaling cascades upon binding and activation of MOR. While DAMGO produces robust βarrestin-dependent MOR translocation and desensitization, morphine produces PKC-dependent desensitization (Johnson et al., 2006; Chu et al., 2008). In cultured sensory neurons, DAMGO cross-desensitizes clonidine's inhibition of Ca^{2+} currents and produces co-internalization of MOR with α_{2A}AR through the βarrestin 2 and p38 MAPK signaling pathway; morphine produces neither of these effects (Tan et al., 2009). This cross-desensitization between DAMGO and clonidine could explain why their interaction is typically not synergistic *in vivo*. On the other hand, morphine activates PKCε in HEK 293 cells expressing MOR, but not DAMGO (Chu et al., 2010). Interestingly, synergistic interactions arising from morphine-clonidine (Wei and Roerig, 1998) and DeltII-clonidine (Overland et al., 2009) combinations are PKC-dependent. This signaling event is unconventional for the opioid and adrenergic receptors that are usually coupled to the pertussis toxin (PTX)-sensitive Gi/o signaling pathway. Intrathecal PTX treatment decreases morphine and clonidine potency, but does not block their synergistic interaction (Roerig and Howse, 1996; Wei et al., 1996). Therefore, morphine-clonidine synergy probably arises from a signaling pathway independent of the pathways activated by the drugs alone and involves PKC activation. The direct interaction between MOR and the α_{2A}AR demonstrated in

expression systems support the hypothesis that these interactions could occur via heteromeric GPCR interactions (Vilardaga et al., 2008).

Conclusion

Our data supports that activation of DOR is sufficient, but not necessary, to produce analgesic synergy when α_2AR are also activated. Therefore, the synergistic interaction between different opioid-α_2 adrenergic agonists is mediated via different opioid receptor pathways; one of these pathways uses DOR and another pathway is likely using MOR.

Currently clinically used opioids act through MOR, which mediates both their analgesic and side effects. Despite the benefits of mixing morphine and clonidine, side effects are still an issue for some patients. We therefore encourage the development and use of DOR-selective ligands in combination with α_2AR agonists as an alternative to currently available opioid agonists.

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Authorship Contributions:

Participated in research design: Anne-Julie Chabot-Doré, Laura S Stone, Magali Millecamps

Conducted experiments: Anne-Julie Chabot-Doré, Laura S Stone, Magali Millecamps

Contributed new reagents or analytic tools:

Wrote or contributed to the writing of the manuscript: Anne-Julie Chabot-Doré, Laura S Stone

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Footnotes

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Figure legends

Figure 1: Clonidine, DeltII, DAMGO and morphine dose-response curves in the SP behavioral assay.

Dose-response curves showing the effects of clonidine, DeltII, DAMGO**,** and morphine in WT (■), DOR-KO (△) and α_{2A}AR-KO (○) mice where drugs were co-administered intrathecally (i.t.) with 15 ng of SP. **(A)** Clonidine was more potent in DOR-KO mice compared to WT mice. **(B)** DeltII inhibited SP behaviors in WT mice, but lacked efficacy in DOR-KO mice. **(C)** Morphine potency was not significantly different from WT mice. **(D)** DAMGO inhibition of SP behaviors was unchanged in DOR-KO mice compared to WT mice. (E) Clonidine efficacy and potency decreased in α_{2A}AR-KO mice compared to WT mice. Each data point represents the mean % inhibition ± SEM, n = 5 – 15 mice. The calculated ED_{50} value for each curve obtained in WT and DOR-KO mice, and their potency ratio are reported in Table 1.

Figure 2: The interaction between DeltII and clonidine is synergistic in WT mice, but not in DOR-KO mice in the SP behavioral assay.

(A) Dose-response curves of the spinal antinociceptive effect of deltorphin II (DeltII, ▲), clonidine (Clon, ●), and their combination at an equieffective 1:1 ratio graphed as the dose of clonidine present in the mixture (Clon (+DeltII; 1:1), ○). **(B)** Isobolographic analysis of the interaction between DeltII and clonidine in WT mice depicts the DeltII ED_{50} value with lower CI along the *y* axis, and the clonidine ED_{50} value with lower CI along the *x* axis. The measured experimental ED_{50} value for the drug combination (●) is lower than the calculated theoretical additive ED_{50} value (○), indicating that DeltII and

clonidine interact in a synergistic manner. **(C)** In DOR-KO mice, spinal administration of DeltII (▲) was inefficacious at inhibiting SP-elicited behaviors. The dose-response curves of clonidine (Clon, ●) and of clonidine in the presence of DeltII (Clon (+DeltII; 1:1), ○) overlapped, showing that adding DeltII to clonidine did not change its potency. Isobolographic analysis of this data set was not possible since the ED_{50} value for DeltII was incalculable. The calculated ED_{50} value for the experimental and theoretical DeltII+clonidine combinations are reported in Table 2.

Figure 3: Morphine and clonidine synergy persists in DOR-KO mice in the SP behavioral assay.

(A) Dose-response curves of spinal morphine (Mph, ▲) and clonidine (Clon, ●) in WT mice. The dose-response curve of their equieffective 1:1 ratio combination was graphed as the dose of clonidine present in the mixture (Clon (+Mph; 1:1), ○). **(B)** Isobolographic analysis of the interaction between morphine and clonidine in WT mice depicts the morphine ED_{50} value with lower CI along the *y* axis, and the clonidine ED_{50} value with lower CI along the *x* axis. The measured experimental ED_{50} value (●) for the drug combination was lower than the theoretical additive ED_{50} value (○), indicating that morphine and clonidine interact in a synergistic manner. **(C)** Dose-response curves of spinal clonidine (Clon, ●) and morphine (Mph, ▲) in DOR-KO mice. The dose-response curve of their 10:1 ratio combination was graphed as the dose of clonidine present in the mixture (Clon (+Mph; 1:10), ○) to show the relative leftward shift in potency caused by the addition of morphine. **(D)** Isobolographic analysis of the interaction between morphine and clonidine in DOR-KO mice depicts the morphine ED_{50} values with lower CI along the *y* axis, and the clonidine ED_{50} value with lower CI along

the *x* axis. The measured experimental ED_{50} value (●) for the combination of morphine and clonidine (10:1 ratio) and the theoretical additive ED_{50} value (○) are graphed with their upper and lower CI. The measured experimental ED_{50} value for the drug combination is below the calculated theoretical additive ED_{50} value, indicating that morphine and clonidine interact in a synergistic manner. The calculated ED_{50} value for the experimental and theoretical morphine+clonidine combinations are reported in Table 2.

Figure 4: DAMGO and clonidine are additive in WT and DOR-KO mice.

(A, C) Dose-response curves of spinal DAMGO (▲) and clonidine (Clon ●) in WT and DOR-KO mice. **(A)** In WT mice, DAMGO+clonidine were combined at an equieffective dose ratio of 1:100 and the dose-response curve was graphed as the dose of clonidine present in the mixture (Clon (+DAMGO; 100:1), ○). **(C)** In DOR-KO mice, DAMGO+clonidine were combined at an equieffective 1:10 ratio (Clon (+DAMGO; 10:1), ○). **(B, D)** Isobolographic analysis of the interaction between DAMGO and clonidine in WT and DOR-KO mice depicts the DAMGO ED_{50} value with lower CI along the *y* axis, and the clonidine ED_{50} value with lower CI along the *x* axis. The measured experimental ED_{50} value for the drug combination (●) and the theoretical additive ED_{50} value (○) are graphed with their upper and lower CI. In both strains, the measured experimental ED_{50} value overlaps the calculated theoretical ED_{50} value, indicating that the interactions are additive. The calculated ED_{50} value for the experimental and theoretical DAMGO+clonidine combinations are reported in Table 2.

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Table 1: Comparison of calculated ED_{50} (95% CI) values for single drugs administered intrathecally in the SP assay in WT and DOR-KO mice.

Single Drug	**WT**	**DOR-KO**
Clonidine (nmol)	0.4 (0.2-0.7)	0.12 (0.06-0.27)*
DeltII (nmol)	0.15 (0.05-0.44)	No efficacy
Morphine (nmol)	0.9 (0.5-1.7)	2.1 (1.0-4.1)
DAMGO (pmol)	3.4 (1.7-7)	6.0 (3.2-11)

* ED_{50} significantly different, WT vs. DOR-KO, Student's *t*-test ($P < 0.05$)

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Table 2: Calculated ED_{50} values (nmol (± 95% SEM)) for drug combinations administered intrathecally in SP assay in WT and DOR-KO mice.

Drug	WT				DOR-KO			
	Ratio	**Experimental**	**Theoretical**	**Interaction**	**Ratio**	**Experimental**	**Theoretical**	**Interaction**
DeltII + Clonidine	1:1	0.0010 (±0.0005)	0.22 (±0.19)	Synergistic*	1:1	0.20 (±0.23)	0.25 (±0.20)	No interaction [a]
Morphine + Clonidine	1:1	0.058 (±0.029)	0.56 (±0.27)	Synergistic*	10:1	0.085 (±0.050)	0.85 (±0.49)	Synergistic*
DAMGO + Clonidine	1:100	0.14 (±0.056)	0.19 (±0.09)	Additive	1:10	0.022 (±0.0084)	0.044 (±0.022)	Additive

*Indicates that the experimental ED_{50} value < theoretical ED_{50} value (Student's *t* test $P < 0.05$)

[a] Since DeltII was ineffective in DOR-KO mice, we compared the ED_{50} value for DeltII+clonidine combination to the ED_{50} value of clonidine alone (Student's *t* test, $P > 0.05$) instead of running an isobolographic analysis.

Figure 1

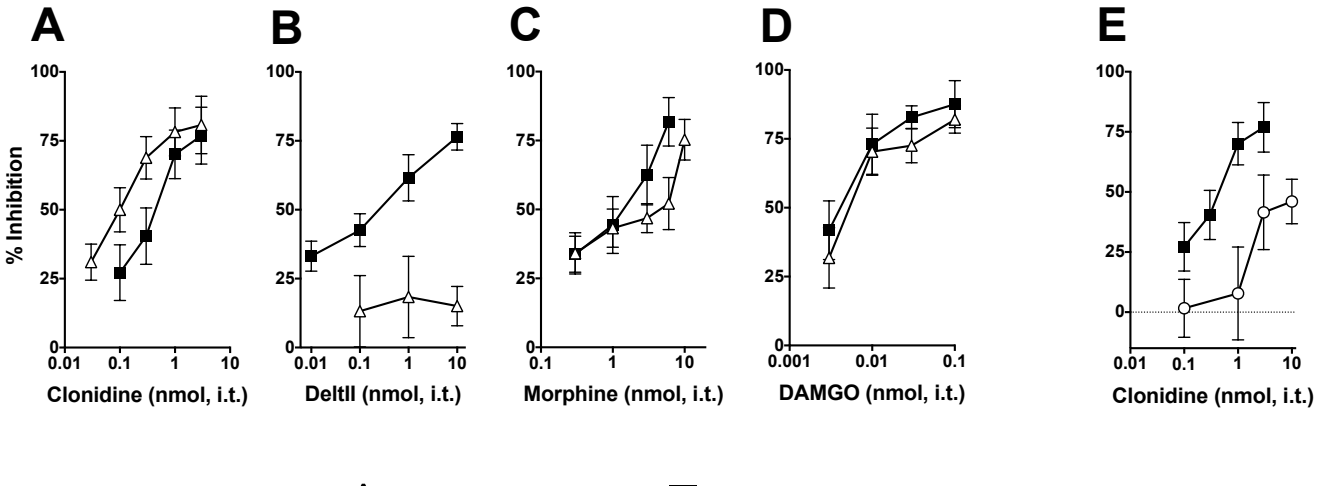
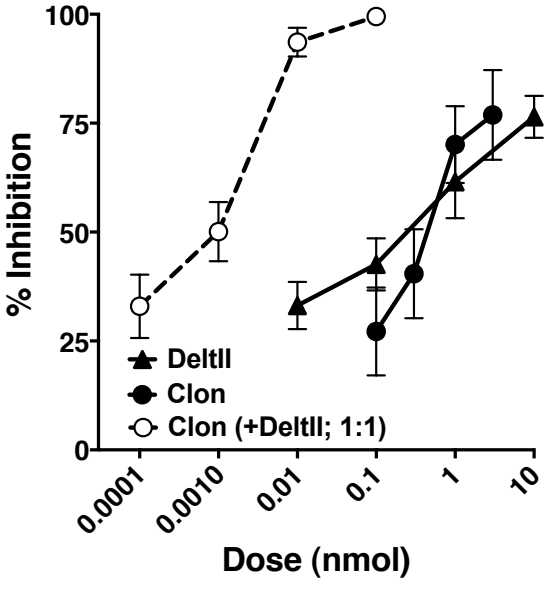
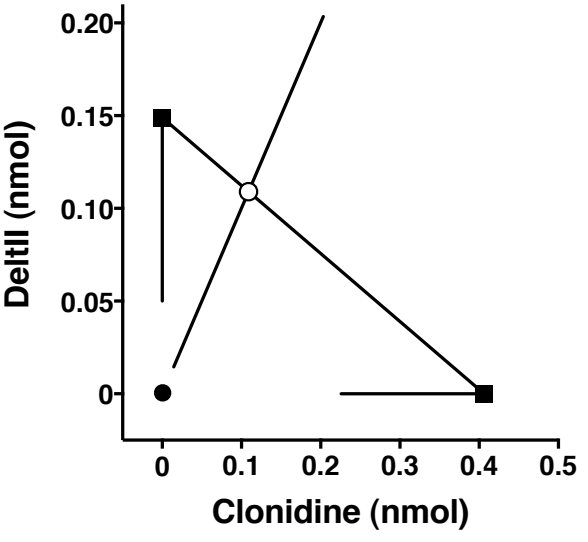

A
B
C
D
E
% Inhibition
100
75
50
25
0
0.01
0.1
1
10
0.001
Clonidine (nmol, i.t.)
DeltII (nmol, i.t.)
Morphine (nmol, i.t.)
DAMGO (nmol, i.t.)
Clonidine (nmol, i.t.)
DOR-KO
WT
α2AAR-KO

Figure 2

A: WT mice



B: WT mice



C: DOR-KO mice

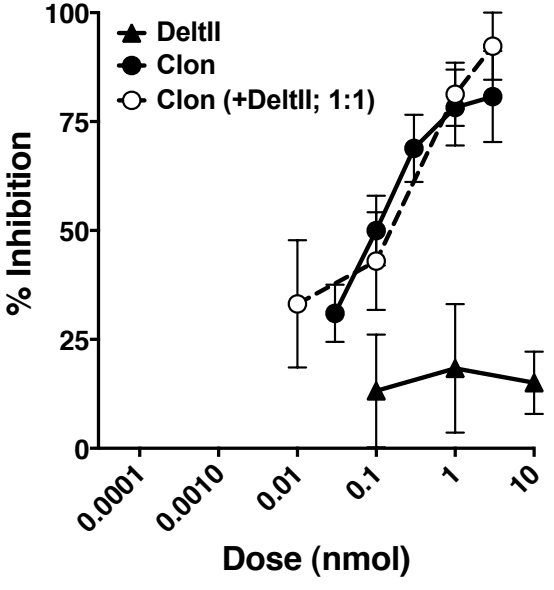
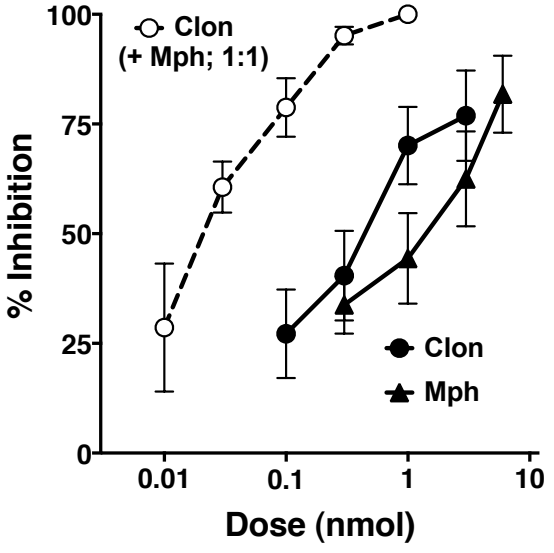
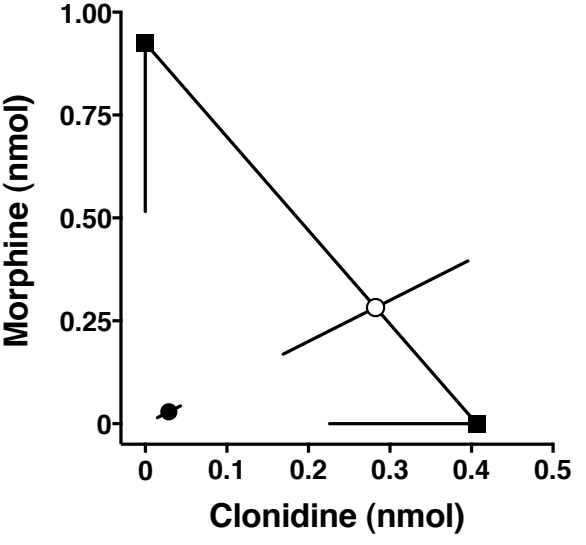


Figure 3

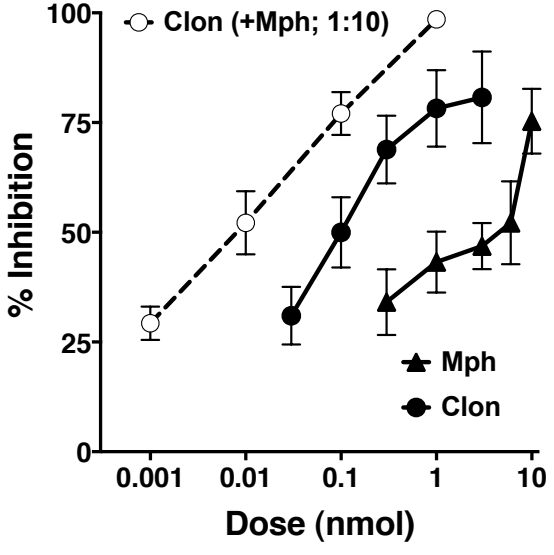
A: WT mice



B: WT mice



C: DOR-KO mice



D: DOR-KO mice

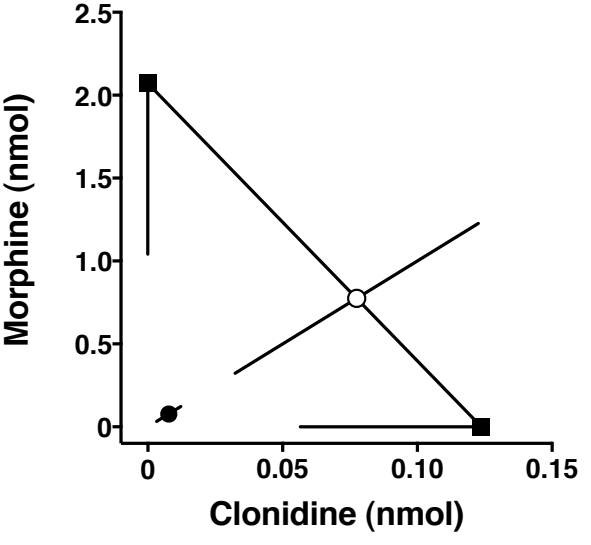
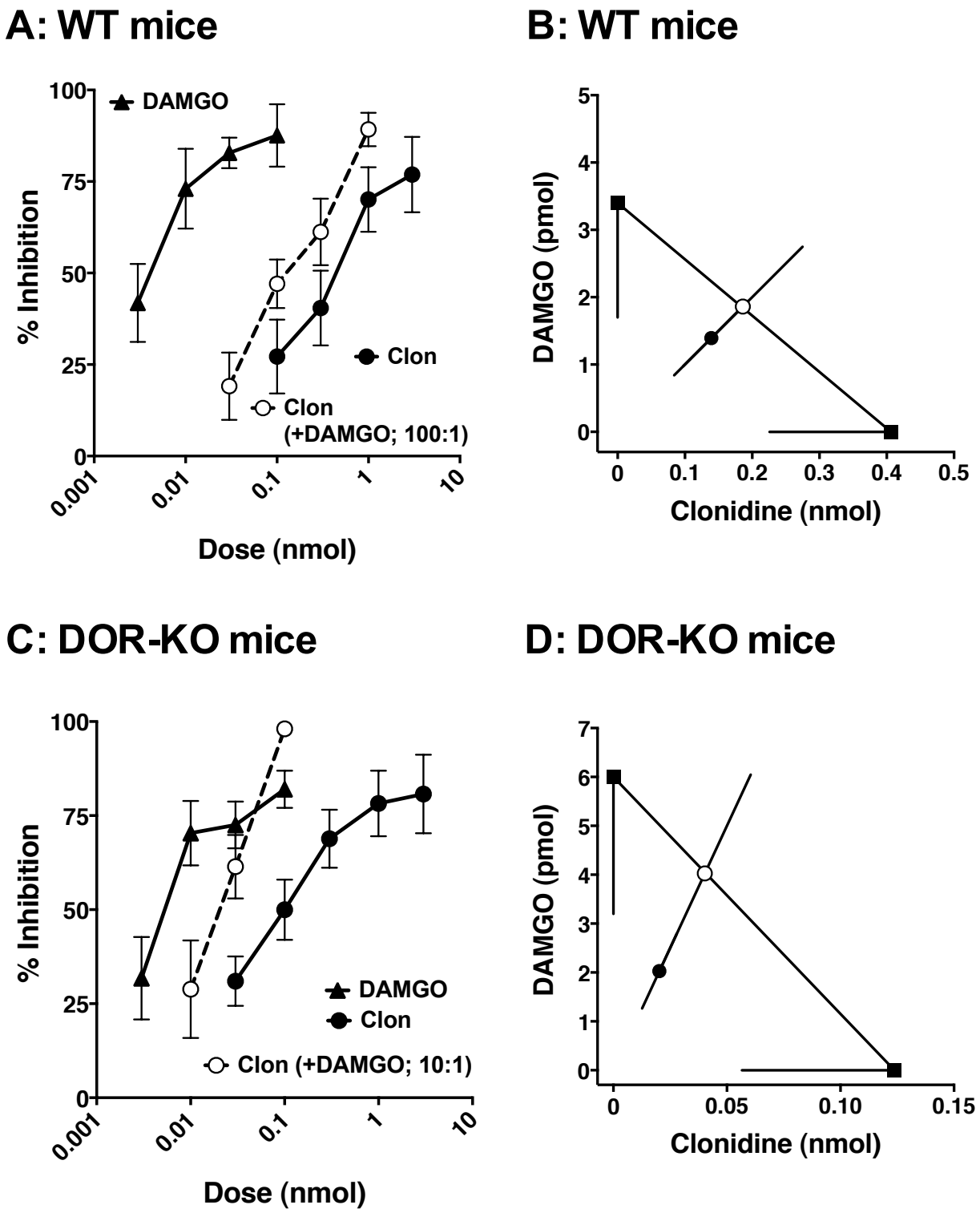


Figure 4


A: WT mice
DAMGO
Clon
Clon
(+DAMGO; 100:1)
% Inhibition
Dose (nmol)
B: WT mice
DAMGO (pmol)
Clonidine (nmol)
C: DOR-KO mice
DAMGO
Clon
Clon (+DAMGO; 10:1)
% Inhibition
Dose (nmol)
D: DOR-KO mice
DAMGO (pmol)
Clonidine (nmol)