The Delta-Opioid Receptor Is Sufficient, but Not Necessary, for Spinal Opioid-Adrenergic Analgesic Synergy

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ABSTRACT

Spinal administration of opioid and α2-adrenergic receptor (α2AR) agonists produces analgesia, and agonists interact synergistically when coadministered. 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 α2AR 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 involved in morphine antinociception raise the possibility that α2AR agonists interact synergistically with δ-opioid receptor (DOR) 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-Ala2]-deltorphin II (DeltII), morphine, and [D-Ala2, N-Me-Phe4, Gly-ol5]-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 after spinal coadministration of clonidine with DeltI, morphine, or DAMGO. Isobolographic analyses of dose-response curves determined whether interactions were synergistic or additive. The absence of DeltII antinociceptive efficacy in DOR-KO confirmed its selectivity in the SP assay. Although 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 although other opioid receptors can interact synergistically with α2AR 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 α2-adrenergic receptor (α2AR) agonists are potent analgesic drugs, but their use is limited by their side effects or lack of efficacy in certain clinical conditions. Compared with the analgesic effects of drugs acting independently, analgesic synergy arising from drug combinations is advantageous because it produces adequate analgesia at lower doses, which can potentially reduce side effects and tolerance (Walker et al., 2002). Pain management using opioid-α2AR agonist combinations reduces side effects associated with both α2AR 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 α2AR agonist clonidine synergize when coadministered 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 unexplored 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.

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 delta- (DOR), mu- (MOR), and kappa-opioid receptor subtypes. [D-Ala2]-Deltorphin II (DeltII), a DOR-selective peptide agonist, can synergize with the α2AR agonists clonidine (Overland et al., 2009), ST-91 [2-(2,6-diethylphenylamino)-2-imidazoline hydrochloride] (Stone et al., 2007),

ABBREVIATIONS: α2AR, α2-adrenergic receptor; CI, confidence interval; DAMGO, [D-Ala2, N-Me-Phe4, Gly-ol5]-enkephalin; DeltII, [D-Ala2]-deltorphin II; DOR, delta-opioid receptor; DPDPE, [D-Pen2, D-Pen5]enkephalin; KO, knockout; MOR, mu-opioid receptor; Oprd1, mouse opioid receptor delta gene; SP, substance P; WT, wild type.
moxonidine (Fairbanks et al., 2000, 2002), and UK 14,304 (5-
Bromo-6-(2-imidazolin-2-ylamino)quinoxaline) (Stone et al.,
1997) when coadministered in rodents at the spinal cord. Similarly, another DOR-selective agonist, [δ-Pen²,δ-Pen⁵]
enkaphalin (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
activating DOR and can occur in the absence of MOR.
interactions resulting in analgesic synergy are possible when
MOR-KO mice (Guo et al., 2003). Thus, opioid-adrenergic
interactions studied with clonidine (Ossipov et al., 1990a; Roerig et al.,
1992), 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
expression systems (Raynor et al., 1994). Taken together,
these studies would suggest that MOR mediates morphine’s
synergistic interactions with α₂AR 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 after morphine treatment is
more potent in cells coexpressing MOR and DOR 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 whether DOR participates in
morphine’s synergistic interaction with α₂AR agonists.

The aim of this study was to disambiguate the role of DOR in
the synergistic interaction between opioid and α₂AR agonists administered spinally. Therefore, we determined whether DOR
activation is sufficient to produce synergy and whether DOR
modulates MOR-mediated synergistic interactions with clonidine,
the only α₂AR 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-α₂ 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×FVB/129 background (Filliol et al.,
2000). Congenic mice backcrossed to a standard C57Bl/6 background were obtained from The Jackson Laboratory (B6.129S2-Oprd1tm1Klf1
J, stock #007557; Bar Harbor, ME). Mice with a targeted gene deletion
introducing a premature stop codon in the third transmembrane
domain of the α₂AR gene (Adra2a) were developed on a mixed C57Bl/
6×FVB/129 background (Altman et al., 1999). Congenic mice back-
crossed to a standard C57Bl/6 background were obtained from The
Jackson Laboratory (stock #004367). Commercially available C57Bl/6
mice (Charles River Laboratories, 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- to 6-month-
old WT, DOR-KO, and α₂AR-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 the ethical guidelines of the Canadian
Council on Animal Care.

Drugs. Substance P (SP; Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-
Met-NH₂) was purchased from AnaSpec (Fremont, CA), and concentrated
stocks were dissolved in acidiﬁed saline (0.9% NaCl, 0.05 M acetic acid).
Morphine sulfate (Medisca Pharmaceuticals, Montreal, QC, Canada) was
dissolved in saline. [D-Ala²,N-Me-Phe⁴,Gly-ol⁵]–Enkephalin (DAMGO)
and clonidine HCl [(N-[2,6-dichlorophenyl]-4,5-dihydro-1H-imidazol-
2-amine; R&D Systems, Minneapolis, MN) were dissolved in saline. [D-
Ala²]-deltorphin II (DeltII; R&D Systems) was dissolved in acidiﬁed
saline. Drug stocks were diluted in saline to working concentrations.

Pharmacological Treatment. Intrathecal 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
nanomoles or picomoles, and drug combination doses are graphed as
total drug doses (i.e., the sum of both drugs) in 5 μl and expressed in
nanomoles. Drugs were administered simultaneously with 15 ng of SP
in a single 5-μl volume. After randomization, each mouse was reused
with a minimum of 5 days 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). In brief, 15 ng of SP
was administered intrathecally alone (control) or coadministered
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 minute and results are expressed as the percent inhibition of
SP-induced behaviors.

% Inhibition = Control – Experimental × 100
Control

Dose-Response Analysis. Dose-response graphs were generated
with GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA). A
minimum of five animals was used per dose, and each dose point is
expressed as the mean percent 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. ED₅₀ 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 ED₅₀ 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 ED₅₀ 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

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mixed in a 1:10 (i.e., equieffective) ratio. Because 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 strain, 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 $\ln(10) \times ED_{50} \times (S.E. \ of \ log \ ED_{50})$ (Tallarida, 1992). This manipulation was required to perform an isobolographic analysis, the appropriate method to evaluate if an interaction is synergistic, additive, or subadditive (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 coordinates 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 SP behavioral assay to measure the antinociceptive effect of opioid agonists and clonidine at the spinal level. Intrathecal 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 (Hyden 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 ($37 \pm 4, n = 13$), DOR-KO ($34 \pm 3, n = 22$), and $\alpha_{2A}$AR-KO ($29 \pm 3, n = 18$) 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 DeltIIR, DAMGO, Morphine, and Clonidine in WT, DOR-KO, and $\alpha_{2A}$AR-KO Mice.** DeltIIR, morphine, DAMGO, and clonidine were administered intrathecally in both WT and DOR-KO mice. All drugs inhibited SP-induced nocifensive behaviors in a dose-dependent manner (Fig. 1; Table 1), except for DeltIIR, which was effective in WT but not DOR-KO mice (Fig. 1B). Clonidine inhibition of SP-induced behaviors was 3-fold more potent in DOR-KO mice compared with WT mice (Fig. 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 with WT mice (Fig. 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 (Fig. 1D). In $\alpha_{2A}$AR-KO mice, clonidine efficacy was reduced to less than 50% inhibition and potency was reduced by 20-fold compared with WT mice (Fig. 1E). Together, these data indicate that DOR mediates DeltIIR antinociception in the SP assay, morphine and clonidine antinociception are slightly altered in DOR-KO mice, and clonidine antinociception is largely mediated by the $\alpha_{2A}$AR.

**Deltorphin II-Clonidine Spinal Antinociceptive Synergy Requires DOR.** We tested the DOR-selective agonist DeltIIR and clonidine alone or in combination in the SP behavioral assay in WT and DOR-KO mice. In WT mice, DeltIIR and clonidine inhibited SP-induced nocifensive behaviors in a dose-dependent manner with similar potency (Fig. 2A). Thus, we tested DeltIIR 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 (Fig. 2B; Table 2).

![Fig. 1.](image-url) Clonidine, DeltIIR, DAMGO, and morphine dose-response curves in the SP behavioral assay. Dose-response curves showing the effects of clonidine, DeltIIR, DAMGO, and morphine in WT (■), DOR-KO (●), and $\alpha_{2A}$AR-KO (○) mice where drugs were coadministered intrathecally with 15 ng of SP. (A) Clonidine was more potent in DOR-KO mice compared with WT mice. (B) DeltIIR 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 with WT mice. (E) Clonidine efficacy and potency decreased in $\alpha_{2A}$AR-KO mice compared with WT mice. Each data point represents the mean % inhibition ± S.E.M., 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.
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 (Fig. 2C).

**Morphine-Clonidine Spinal Antinociceptive Synergy Persists in the Absence of DOR.** Because 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 with the single doses (Fig. 3A). The isobolographic analysis demonstrated that the morphine + clonidine interaction in WT mice was synergistic (Fig. 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 with each drug alone (Fig. 3C). Isobolographic analysis showed that the experimental ED$_{50}$ value is significantly lower than the theoretical additive ED$_{50}$ value (Fig. 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 behaviors with an ED$_{50}$ value 120-fold more potent than clonidine (Table 1). Therefore, we tested a combination of clonidine and DAMGO at a 100:1 ratio, which also inhibited SP-induced behaviors in a dose-dependent manner (Fig. 4A). The isobolographic analysis revealed that this interaction was additive (Fig. 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 (Fig. 4, C and D; Table 2).

**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 mice.

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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

<table>
<thead>
<tr>
<th>Single Drug</th>
<th>WT</th>
<th>DOR-KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clonidine (nmol)</td>
<td>0.4 (0.2–0.7)</td>
<td>0.12 (0.06–0.27)*</td>
</tr>
<tr>
<td>DeltII (nmol)</td>
<td>0.15 (0.05–0.44)</td>
<td>No efficacy</td>
</tr>
<tr>
<td>Morphine (nmol)</td>
<td>0.9 (0.5–1.7)</td>
<td>2.1 (1.0–4.1)</td>
</tr>
<tr>
<td>DAMGO (pmol)</td>
<td>3.4 (1.7–7)</td>
<td>6.0 (3.2–11)</td>
</tr>
</tbody>
</table>

*ED$_{50}$ significantly different, WT versus DOR-KO, Student’s $t$ test ($P < 0.05$).

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**Fig. 2.** The interaction between DeltII and clonidine is synergistic in WT mice but not in DOR-KO mice in the spinal 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-elicted behaviors. The dose-response curves of clonidine (●) and of clonidine in the presence of DeltII (○) overlapped, showing that adding DeltII to clonidine did not change its potency. Isobolographic analysis of this data set was not possible because 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.
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, α2AR agonists such as 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 synergistically with other drug classes. For example, in a postoperative pain model, clonidine interacts synergistically after 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 α2AR 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 α2AR 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 ofafferent nociceptive signals.

Pharmacodynamic interactions between opioids and α2AR agonists resulting in analgesic synergy could involve spinal intracellular or intracellular mechanisms. Because spinal MOR, DOR, and α2AR are distributed on both primary afferent and

**TABLE 2**

Calculated ED<sub>50</sub> values [nmol (± 95% S.E.M.)] for drug combinations administered intrathecally in SP assay in WT and DOR-KO mice.

<table>
<thead>
<tr>
<th>Drug Combination</th>
<th>WT</th>
<th>DOR-KO</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Ratio</td>
<td>Experimental</td>
</tr>
<tr>
<td>DeltII + Clonidine</td>
<td>1:1</td>
<td>0.0010 (±0.0005)</td>
</tr>
<tr>
<td>Morphine + Clonidine</td>
<td>1:1</td>
<td>0.058 (±0.029)</td>
</tr>
<tr>
<td>DAMGO + Clonidine</td>
<td>1:100</td>
<td>0.14 (±0.056)</td>
</tr>
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*Because DeltII was ineffective in DOR-KO mice, we compared the ED<sub>50</sub> value for DeltII + clonidine combination to the ED<sub>50</sub> value of clonidine alone (Student's t test, P > 0.05) instead of running an isobolographic analysis.

*Experimental ED<sub>50</sub> value < theoretical ED<sub>50</sub> value (Student's t test P < 0.05).

**Fig. 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 (t-Mph; 1:1); ●]. (B) Isobolographic analysis of the interaction between morphine and clonidine in WT mice depicts the morphine ED<sub>50</sub> value with lower CI along the y-axis and the clonidine ED<sub>50</sub> value with lower CI along the x-axis. The measured experimental ED<sub>50</sub> value (●) for the drug combination was lower than the theoretical additive ED<sub>50</sub> value (○), indicating that morphine and clonidine interact in a synergistic manner. (C) Dose-response curves of spinal clonidine (●) and morphine (▲) 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 (○) 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<sub>50</sub> values with lower CI along the y-axis and the clonidine ED<sub>50</sub> value with lower CI along the x-axis. The measured experimental ED<sub>50</sub> value (●) for the combination of morphine and clonidine (10:1 ratio) and the theoretical additive ED<sub>50</sub> value (○) are graphed with their upper and lower CI. The measured experimental ED<sub>50</sub> value for the drug combination is below the calculated theoretical additive ED<sub>50</sub> value, indicating that morphine and clonidine interact in a synergistic manner. The calculated ED<sub>50</sub> value for the experimental and theoretical morphine+clonidine combinations are reported in Table 2.

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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 coexpression 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 coadministration 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 \( \alpha_2 \)AR agonists are MOR mediated rather than DOR mediated. In the current study, DOR 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 DOR-clonidine synergy in DOR-KO mice confirms that DOR activation is sufficient to interact synergistically with \( \alpha_2 \)AR agonists. Assays should be carefully validated when studying DOR-mediated synergistic interactions because the current findings may not generalize to different assays.

In the spinal cord, clonidine has been shown to interact with both \( \alpha_2 \)AR and imidazoline binding sites, but the antinociceptive effect of clonidine is mediated by \( \alpha_2 \)AR rather than imidazoline receptors (Monroe et al., 1995a,b). Our data further suggest that the \( \alpha_2 \)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 \( \alpha_2 \)AR (Fairbanks and Wilcox, 1999a). The \( \alpha_2A \)AR is also necessary for analgesic synergy between DeltII and the \( \alpha_2 \)AR agonist UK 14,304 (Stone et al., 1997). DOR and \( \alpha_2 \)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 demonstrated that DOR and \( \alpha_2A \)AR receptors are coexpressed in primary afferent neurons and highly colocalize in SP-immunoreactive neurons and isolated nerve terminals (Riedl et al., 2009). Although the localization of DOR in SP neurons is debated (Scherrer et al., 2009; Wang et al., 2010), the above-mentioned physiologic and anatomic evidence support the presence of DOR and \( \alpha_2A \)AR in peptidergic neurons where they would be positioned to inhibit neurotransmitter release in a synergistic manner.

**Fig. 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 and 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.
**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 with 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 the 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. Because morphine is not efficacious in the SP behavioral assay in MOR-KO compared with 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.** Coactivation of MOR with an α2AR agonist produces different interactions depending on the experimental conditions and agonists used. Although the interaction between DAMGO and clonidine is either additive (Fig. 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 α2AR agonists in the SP behavioral assay can produce either synergistic (Stone et al., 1997) or subadditive (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. Although DAMGO produces robust β-arrestin-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 Ca2+ currents and produces cointernalization of MOR with α2aAR through the β-arrestin 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 PKCe in HEK 293 cells expressing MOR, but not DAMGO (Chu et al., 2010). It is noteworthy that 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-sensitive Gαo signaling pathway. Intrathecal pertussis toxin 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 α2AR demonstrated in expression systems support the hypothesis that these interactions could occur via heteromeric G protein-coupled receptor interactions (Vilardaga et al., 2008).

**Conclusion**

Our data support that activation of DOR is sufficient, but not necessary, to produce analgesic synergy when α2AR are also activated. Therefore, the synergistic interaction between different opioid-α2 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 α2AR agonists as an alternative to currently available opioid agonists.

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**Authorship Contributions**

**Participated in research design:** Chabot-Doré, Stone, Millecamps.  
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