

RESEARCH ARTICLE

# Methadone Reverses Analgesic Tolerance Induced by Morphine Pretreatment

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## Abstract

**Background:** Opiates such as morphine are the most powerful analgesics, but their protracted use is restrained by the development of tolerance to analgesic effects. Recent works suggest that tolerance to morphine might be due to its inability to promote mu opioid receptor endocytosis, and the co-injection of morphine with a mu opioid receptor internalizing agonist like [D-Ala<sup>2</sup>,N-Me-Phe<sup>4</sup>,Gly-ol<sup>5</sup>]enkephalin reduces tolerance to morphine. So far, no studies have been conducted to evaluate the ability of methadone to reduce morphine tolerance in morphine-pretreated animals, a treatment sequence that could be encountered in opiate rotation protocol. We investigated the ability of methadone (a mu opioid receptor internalizing agonist used in therapy) to reverse morphine tolerance and the associated cellular mechanisms in the periaqueductal gray matter, a region involved in pain control.

**Methods:** We measured analgesic response following a challenge dose of morphine in the hot plate test and investigated regulation of mu opioid receptor (coupling and endocytosis) and some cellular mechanisms involved in tolerance such as adenylate cyclase superactivation and changes in N-methyl-D-aspartate receptor subunits expression and phosphorylation state.

**Results:** A chronic treatment with morphine promoted tolerance to its analgesic effects and was associated with a lack of mu opioid receptor endocytosis, adenylate cyclase overshoot, NR2A and NR2B downregulation, and phosphorylation of NR1. We reported that a methadone treatment in morphine-treated mice reversed morphine tolerance to analgesia by promoting mu opioid receptor endocytosis and blocking cellular mechanisms of tolerance.

**Conclusions:** Our data might lead to rational strategies to tackle opiate tolerance in the frame of opiate rotation.

**Keywords:** morphine, methadone, analgesia, endocytosis, mu opioid receptor, adenylate cyclase, NMDA receptor

## Introduction

Opiates such as morphine are the most powerful drugs used in pain relief. However, the development of tolerance to their analgesic effects, in particular, limits their long-term use (Huxtable et al., 2011). Among the 3 types of opioid receptors, mu opioid receptor (MOPr) has been demonstrated to be the major

molecular target of opiate-mediated analgesia (Matthes et al., 1996). Opioid receptors belong to the G-protein coupled receptor family and are mainly coupled to G<sub>q/o</sub> resulting in adenylate cyclase (ACase) inhibition. They mainly follow the regulation pathways of the beta2-adrenergic receptor, the prototype of the

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class A of G-protein coupled receptor. Following agonist binding, receptor is phosphorylated and becomes a substrate for beta-arrestins. Binding of beta-arrestin promotes a functional uncoupling between the receptor and its cognate G proteins, resulting in desensitization and mediate receptor internalization. Once the receptors are internalized, they are targeted to lysosomes for degradation (resulting in desensitization potentiation by reducing number of active receptors) or recycled in an active state allowing resensitization (counteracting desensitization) (Allouche et al., 2014).

Tolerance is a highly complex phenomenon involving numerous mechanisms. During the past few years, Whistler and co-workers proposed a mechanism based on some particular properties of morphine to regulate MOPr (He et al., 2002; He and Whistler, 2005; Martini and Whistler, 2007). Indeed, contrary to some opiates such as methadone, morphine is unable to promote MOPr internalization (Arden et al., 1995; Keith et al., 1996). Following a prolonged treatment, these authors suggested that the persistent presence of the receptor at the plasma membrane will recruit signaling pathways responsible for tolerance such as ACase superactivation and alteration in N-methyl-D-aspartate (NMDA) receptor levels (Finn and Whistler, 2001; He and Whistler, 2005; He et al., 2009). Indeed, ACase superactivation, which occurs after chronic opiate exposure (Avidor-Reiss et al., 1995), has been suggested to be a cellular marker of tolerance and contributes to its development as a protein kinase A (PKA) inhibitor reduced morphine-induced tolerance to analgesia (Javed et al., 2004). NMDA receptors have been involved in opiate tolerance; for instance, NMDA receptor expression is altered in morphine-tolerant animals (Inoue et al., 2003; He et al., 2009; Rodriguez-Munoz et al., 2012; Zhao et al., 2012) and NMDA receptor antagonists block morphine tolerance (Trujillo and Akil, 1991; Ko et al., 2008). From these observations, a strategy consisting of coadministering morphine with a receptor internalizing agonist has been developed to reduce surface morphine-MOPr complex and thereby preventing tolerance-related signaling pathways and tolerance itself (He et al., 2002; He and Whistler, 2005).

However, to our knowledge, no studies have evaluated the effect of methadone (a MOPr internalizing agonist used in therapeutics) on morphine tolerance in morphine-pretreated animals. This question is of particular importance, as opiate rotation protocols are used rather than opiate agonist coadministration to reduce tolerance issues in pain management. Opiate rotation is based on incomplete cross-tolerance between opiates. It also allows clearance of toxic metabolites (Mercadante and Portenoy, 2001).

Moreover, despite its clinical efficacy, the mechanisms for reduced tolerance as a result of opiate rotation need to be clarified. In this study, we investigated the effects of a short methadone treatment in mice previously treated with morphine and tolerant to its analgesic effects. We measured analgesic response following a challenge dose of morphine in the hot plate test and investigated regulation of MOPr (coupling and endocytosis) and some cellular mechanisms involved in tolerance such as ACase superactivation and changes in NMDA receptor subunits expression and phosphorylation state.

## Materials and Methods

### Animals

Male Swiss mice (Janvier, France) weighing 23 to 25 g at the beginning of the experiments were housed 5/cage on a 12-hour-light/dark cycle in a temperature-controlled room (21±2°C)

with food and water available ad libitum. Animal experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) as well as French law, with the standard ethical guidelines, and under the control of the Ethical Committee of the faculty (N° CEEA34. NM.117.12). Every effort was made to reduce the number of animals used and their discomfort.

### Treatments

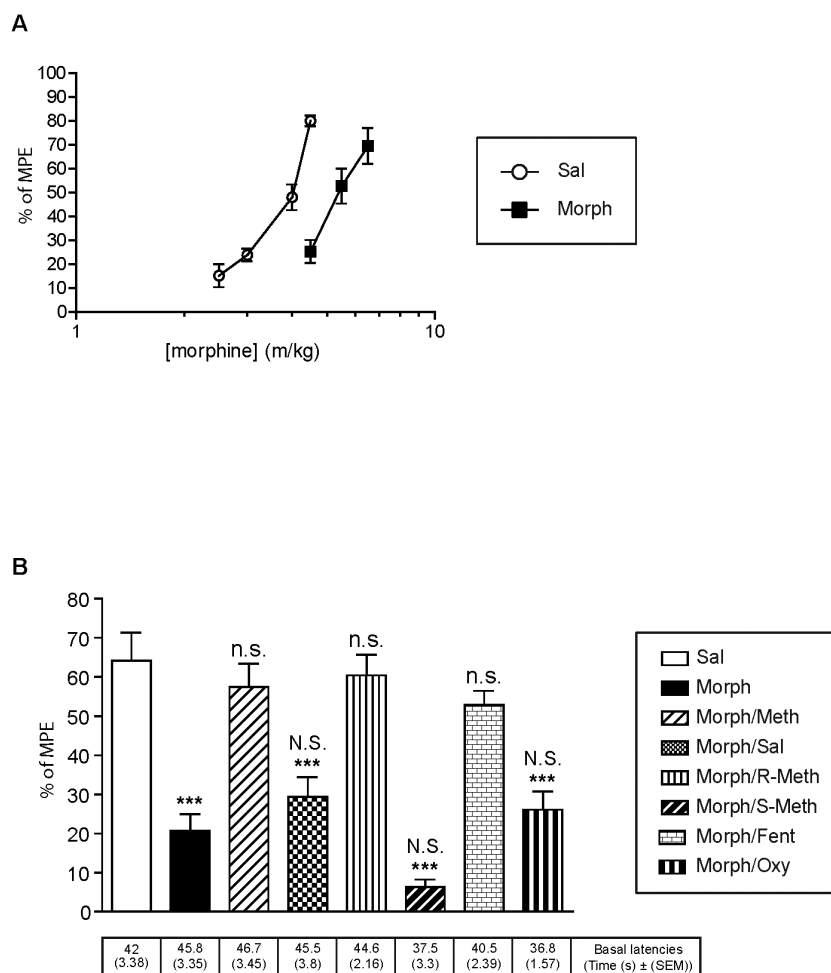
Morphine, methadone, oxycodone (Francopia, France), and fentanyl (Sigma Aldrich, France), all hydrochloride, were dissolved in saline solution (0.9% NaCl) and injected via i.p. route. All animals received 0.1 mL/10 g of bodyweight. Chronic treatment consisted of 2 daily injections (at 9:00 AM and 5:00 PM) with morphine (10 mg/kg), methadone (2.5 mg/kg), fentanyl (0.25 mg/kg), or oxycodone (1 mg/kg). These doses are equally effective in the locomotor activity assay (supplementary Figure 1). This test was chosen as it has a larger range of dose response than the hot-plate, where 10 mg/kg of morphine results in 100% of analgesia (Figure 1). Control groups received saline under the same conditions.

### Analgesia Measurement with the Hot Plate Test

Analgesia was measured with the hot plate test 20 minutes after opiate agonist injection. As previously described (Eddy and Leimbach, 1953), a glass cylinder (25 cm high, 20 cm diameter) was used to keep the mouse on the heated surface of the plate, which was kept at a temperature of 52°C (Panlab, Barcelona, Spain). The latency period until the mouse jumped was registered by means of a stop-watch (cut-off time 180 seconds). Percentage of analgesia was calculated using the following equation: % of MPE (maximal possible effect) = [(postdrug latency (s) – baseline latency (s))/(cutoff value (s) – baseline latency (s))] × 100. For each condition, 2 different groups received saline (baseline latency) and drug (postdrug latency). No habituation to the apparatus was done to avoid any learning effects (Bardo and Hughes, 1979; Hunskaar et al., 1986). These experiments were conducted by someone blind to the drug treatments.

### MOPr Coupling Assay

Agonist-stimulated [<sup>35</sup>S]-GTPγS (guanosine 5'-O-(3-thiotriphosphate)) binding assay was used to measure MOPr coupling to G proteins (Hilf et al., 1989; Contet et al., 2008). Mice were sacrificed by CO<sub>2</sub> inhalation, and brains were immediately extracted. Periaqueductal gray matter (PAG) was extracted using a tissue punch (2 PAG punches were pooled) and homogenized in 0.25 M sucrose and centrifuged (1000 g for 10 minutes at 4°C). Supernatant was suspended in 50 mM Tris-HCl (pH 7.4)/1 mM EDTA and centrifuged (28000 g for 60 minutes at 4°C). Pellet was suspended in 0.32 M sucrose and protein concentration was determined using the Bradford assay. Then 50 μL of homogenate (2.5 μg protein) was incubated in assay buffer (50 mM Tris-HCl [pH 7.4], 3 mM MgCl<sub>2</sub>, 100 mM NaCl, 0.2 mM EGTA, 50 μM GDP (guanosine 5'-diphosphate), 0.1 nM [<sup>35</sup>S]-GTPγS [specific activity 1250 Ci/mmol] Perkin Elmer) with increasing concentrations of [D-Ala<sup>2</sup>, N-MePhe<sup>4</sup>, Gly<sup>5</sup>-ol]-enkephalin (DAMGO; Bachem, Germany) in a total volume of 200 μL for 2 hours at 25°C. Reaction was terminated by rapid filtration through Whatman GF/B filters. The filters were washed twice with ice-cold 50 mM Tris-HCl (pH 7.4)/50 mM NaCl/5 mM MgCl<sub>2</sub> and scintillation liquid was added (Ultima Gold MV, Perkin Elmer). Radioactivity



**Figure 1.** Effects of methadone on morphine induced-analgesia. Animals were treated with i.p. injection, twice daily, for 4 days with morphine (10mg/kg) followed by 1 day of treatment with morphine (10mg/kg, Morph), methadone (2.5mg/kg, Morph/Meth), saline (Morph/Sal), (R)-Methadone (1.25mg/kg, Morph/R-Meth), (S)-Methadone (1.25mg/kg, Morph/S-Meth), fentanyl (0.25 mg/kg, Morph/Fent), oxycodone (1mg/kg, Morph/Oxy), or for 5 days with saline (Sal). On the sixth day, the analgesic effect of an acute injection of morphine (various doses [A] or a unique dose of 5.5mg/kg [B]) was determined with the hot plate test. Dots (A) or bars (B) are the means ± SEM of the percentage of maximum possible effect (MPE) produced by the acute injection of morphine. The basal latencies are given in the table. (A) EC<sub>50</sub> (Sal) = 3.91 mg/kg (3.78–4.039), EC<sub>50</sub> (Morph) = 5.47 mg/kg (5.21–5.72) (n = 7 to 13 animals/group). (B) One-way ANOVA for MPE,  $F_{7,61} = 17.67$ ,  $P < .0001$ . \*\*\* $P < .001$ , n.s. (not significant) vs Sal group; N.S. (not significant) vs Morph group. Bonferroni posthoc test, n = 7 to 10 animals/group. One-way ANOVA for basal latencies,  $F_{7,72} = 2.109$ ,  $P = .0533$ , n = 10 animals/group.

was counted with a liquid scintillation analyzer Tricarb 2810 TR (Perkin Elmer). Nonspecific binding was measured in the presence of 10  $\mu$ M unlabeled GTP $\gamma$ S. Results are expressed as percentage of basal [<sup>35</sup>S]-GTP $\gamma$ S binding. Basal values are given in [supplementary Table 1](#).

### Immunohistochemistry

Mice were deeply anesthetized by an i.p. injection of sodium pentobarbital and brains were fixed with intracardiac perfusion of freshly prepared, ice-cold 4% paraformaldehyde (Electron Microscopy Sciences) in 0.1M phosphate buffer for 15 minutes at 10mL/min using a peristaltic pump. Brains were dissected and postfixed in 4% paraformaldehyde/0.1M phosphate buffer for 1 hour at 4°C then transferred to phosphate buffered saline (PBS). After 2 washes in PBS, brain coronal sections containing the PAG were collected in PBS by sectioning the brain into 50- $\mu$ m slices using a vibratome VT 1000E (Leica, Germany).

Brain sections were incubated in immunohistochemistry (IHC) buffer (PBS, 5% bovine serum albumin [BSA], 0.02%

Triton X-100) for 4 hours at room temperature (RT) then with the following primary antibodies in IHC buffer for 24 hours at 4°C: rabbit anti-MOPr ([Georgescu et al., 2003](#); [Davis and Puhl, 2011](#); [Ena et al., 2013](#)) at 1:400 dilution (Immunostar, cat no. 24216) and mouse anti-NeuN (1:2000 dilution to label neurons, Millipore, cat no. MAB377) ([Mullen et al., 1992](#)) ([supplementary Figure 2](#)). After 3 washes (10 minutes each) with IHC buffer, sections were incubated with Alexa Fluor 488-labeled goat anti-rabbit and Alexa Fluor 594-labeled goat anti-mouse antibodies (Life Technologies) at 1:500 dilution for 24 hours at 4°C. After 3 washes (10 minutes each) with IHC buffer (without BSA), the slices were mounted in a glycerol-based mounting medium Mowiol containing 1,4-Diazabicyclo[2.2.2]octane (Sigma Aldrich) as an antifading reagent. MOPr distribution was examined in neurons (cells positive for the specific neuronal marker NeuN) with a confocal microscope (Leica SP2) with a  $\times 63$  oil-immersion objective. Slides were coded and vesicles (intracellular punctuations) counted by an experimenter blind to the experimental conditions. At least 10 cells from 3 animals were counted.

## Measurement of Adenylate Cyclase Activity

Mice were sacrificed by CO<sub>2</sub> inhalation and brains were immediately extracted. PAG was extracted using a tissue punch (3 PAG punches were pooled), homogenized using Polytron (Kinematica, Switzerland) in ice-cold homogenization buffer (50mM Tris-HCl [pH 7.4], 25mM KCl, 5mM MgCl<sub>2</sub>, 250mM sucrose), and centrifuged (20000 *g* for 15 minutes at 4°C). The pellet was resuspended in 2mM Tris-HCl (pH 7.4)/2mM EGTA, and protein concentration was determined using the Bradford assay (Sigma Aldrich). Tissue homogenate (10 µg in 10 µL) was added on ice to assay tubes (final volume of 60 µL) in 80mM Tris-HCl (pH 7.4)/1mM MgSO<sub>4</sub>/0.8mM EGTA/3mM NaCl/0.25mM ATP/10 µM GTP. Triplicate samples for each treatment were incubated at 30°C for 10 minutes. ACase activity was terminated by placing the tubes into boiling water for 2 minutes, and the amount of cyclic adenosine monophosphate (cAMP) formed was determined by a [<sup>3</sup>H]-cAMP protein binding assay (Brown et al., 1971; Noble and Cox, 1995). [<sup>3</sup>H]-cAMP (Perkin Elmer, final concentration 4nM) in citrate-phosphate buffer (pH 5.0) and then binding protein prepared from bovine adrenal glands were added to each sample. Additional samples were prepared, without tissue, containing known amounts of cAMP; they served as standards for quantification. The binding reaction was allowed to reach equilibrium for 90 minutes at 4°C. The assay was terminated by quick filtration through Whatman GF/B glass fiber filters. The filters were washed twice with 5 mL of ice-cold 50mM Tris-HCl (pH 7.4) and scintillation liquid was added (Ultima Gold MV, Perkin Elmer). Radioactivity was counted with a liquid scintillation analyzer Tricarb 2810 TR. Radioactivity was converted to picomoles of cAMP by comparison with the curve derived from the standards.

## Western Blotting and Immunoblotting

Mice were sacrificed by CO<sub>2</sub> inhalation and brains were immediately extracted. PAG was extracted using a tissue punch (3 PAG punches were pooled), homogenized using a glass dounce tissue grinder in ice-cold homogenization buffer (10mM Tris-HCl [pH 7.4]/10% sucrose, 1mM EDTA, 0.1mM Na<sub>3</sub>VO<sub>4</sub>, protease inhibitors cocktail; Roche), and centrifuged (1000 *g* for 10 minutes at 4°C). Supernatant was centrifuged (28000 *g* for 45 minutes at 4°C) and the resulting pellet was sonicated in solubilization buffer (10mM Tris-HCl [pH 7.4], 1mM EDTA, 0.1% SDS, 0.1% NP-40, 150mM NaCl, protease inhibitors cocktail, Roche). Proteins were quantified with Bradford assay (Sigma Aldrich) and samples were stored at -80°C until further used. Proteins were resolved on 10% SDS-PAGE after heat denaturation (10 minutes, 70°C) in sample buffer (62.5mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.001% bromophenol blue) (Laemmli, 1970), transferred to PVDF membrane, and cut into 2 pieces (the top was used for NMDA receptor probing and the bottom for actin detection).

The membranes were washed in Tris-buffered saline (TBS), incubated in blocking buffer for 1 hour at RT, and then with the primary antibody diluted in the blocking buffer (anti-NR1 (Zhang et al., 2014), Millipore cat no. MAB363, diluted at 1:3000 in TBS/0.05% Tween-20 (TBS/T)/3% nonfat dried milk; anti-pNR1(Ser890) (Jarabek et al., 2004), Cell Signaling, cat no. 3381, diluted at 1:1000 in TBS/T/5% BSA; anti-pNR1(Ser896) (Hida et al., 2015), Millipore cat no. ABN88, diluted at 1:3000 in TBS/T/3% nonfat dried milk; anti-pNR1(Ser897) (Hida et al., 2015), Millipore cat no. ABN99, diluted at 1:1000 in TBS/T/5% nonfat dried milk; anti-NR2A (Aoki et al., 2009), Millipore cat no. 07-632, diluted at

1:2000 in TBS/T/2% nonfat dried milk; anti-NR2B (Xie et al., 2012), Abcam, cat no. ab28373, diluted at 1:2000 in TBS/T/2% nonfat dried milk) overnight at 4°C. After washes with TBS/T, membranes were incubated with horseradish-peroxidase-conjugated anti-rabbit or anti-mouse antibody (1:10000 dilution in TBS/T, GE Healthcare) for 1 hour at RT. Secondary antibody was revealed using chemiluminescence reagent (Bio-Rad). PVDF membranes were probed with 2 different NMDA receptor antibodies. Indeed, after the first revelation, PVDF membrane was stripped for 30 minutes at 65°C in stripping buffer (62.5mM Tris-HCl, pH 6.8, 2% SDS, 100mM β-mercaptoethanol). The bottom of the membrane was probed with mouse horseradish-peroxidase-conjugated anti-β-actin antibody (Sigma Aldrich) at 1:20000 dilution in TBS/T for 1 hour and was revealed using chemiluminescence reagent (GE Healthcare). Proteins bands were visualized with Chemidoc XRS and quantified with Quantity One software (Bio-Rad). Full Western blots are shown in [supplementary Figure 3](#).

## Results

### Effects of Methadone on Tolerance to Morphine Analgesia

First of all, we determined the ability of a short morphine chronic treatment to promote tolerance in the hot plate test. [Figure 1A](#) showed a significant shift of the dose-response curve to the right after 5 days of morphine treatment. This shift was due to morphine treatment, as no difference in morphine-induced analgesia was noted before or after the chronic saline treatment ([supplementary Figure 4](#)). The dose of 5.5mg/kg of morphine (corresponding to the EC<sub>50</sub> in the Morph group) was chosen in the subsequent experiments to be able to detect any variation in morphine tolerance. **On the fifth day, morphine was replaced by methadone, and it restored the ability of morphine to promote analgesia as measured on the sixth day (Figure 1B).** This effect is specific to methadone, as tolerance was still observed when saline replaced morphine the last day of treatment. **The methadone used in clinic is a racemic mixture of (R)-methadone and (S)-methadone, with the (R) enantiomer being the most active and with the higher affinity on MOPr (Scott et al., 1948; Pert and Snyder, 1973). Figure 1B demonstrates that (R)-methadone (used at one-half the dose of the racemate) was able to reverse morphine-induced tolerance to analgesia as the racemate, suggesting that the methadone reversal of morphine tolerance to analgesia is MOPr dependent. Indeed, when on the fifth day S-methadone replaced morphine, tolerance was still observed (Figure 1B). Finally, on the fifth day, morphine was replaced by fentanyl, a MOPr internalizing agonist (Minnis et al., 2003), and no tolerance was measured. In contrast, tolerance to morphine analgesia was still observed on the fifth day, when morphine was replaced by oxycodone, a noninternalizing agonist (Koch et al., 2009).** It is noteworthy that the baseline latencies were not significantly different among groups ([Figure 1B](#)) and that mice were probably not in withdrawal state, as no weight loss was measured after morphine treatment ([supplementary Figure 5B](#)). Finally, a 1-day methadone treatment was not sufficient to promote morphine tolerance ([supplementary Figure 5A](#)).

### Mechanisms of Methadone Reversal Tolerance to Morphine Analgesia

In the second set of experiments, we investigated the mechanisms that would be involved in methadone reversal tolerance to morphine analgesia in the PAG, a key structure in pain control



(Reynolds, 1969). This structure is enriched in MOPr, and their activation through endogenous opioid release inhibits nociceptive signal (Barbaro, 1988). Moreover, NMDA receptors are expressed in PAG neurons (Commons et al., 1999; Narita et al., 2008).

#### MOPr Coupling

We first investigated MOPr coupling using [<sup>35</sup>S]-GTPγS binding assay, as it has been suggested that receptor uncoupling by promoting receptor desensitization might contribute to tolerance (Allouche et al., 2014). As depicted in Figure 2, DAMGO was able to increase [<sup>35</sup>S]-GTPγS binding in a dose-dependent manner in all 4 groups of animals. Analysis of efficacy and potency revealed no significant differences between treatments (Table 1), demonstrating that chronic treatment with opiates did not modify MOPr coupling.

#### MOPr Endocytosis

In most of the studies, morphine was unable to promote MOPr internalization (Arden et al., 1995; Keith et al., 1996) conversely to methadone (Borgland et al., 2003; Celver et al., 2004). We therefore investigated if methadone was able to promote MOPr endocytosis in morphine-treated animals. MOPr internalization in PAG neurons was visualized using immunohistochemistry. As depicted in Figure 3, MOPr are mainly localized at the plasma membrane in PAG neurons of the saline-treated animals. When mice were treated for 5 days with morphine, no internalization was observed. However, when mice received methadone the fifth day, we observed MOPr endocytosis, evidenced by the dramatic increase of MOPr-containing intracellular vesicles. This effect was specific to methadone; indeed, if animals received saline treatment the fifth day, the vast majority of MOPr were still localized at the plasma membrane (Figure 3).

#### Adenylate Cyclase Activity

Following chronic exposure to opiate, an increase in cAMP production (a phenomenon also known as ACase overshoot or superactivation) is frequently observed (Avidor-Reiss et al., 1995). This cellular adaptation has been suggested to contribute to tolerance (He and Whistler, 2005; Mohammed et al., 2013), so we determined if it occurs in our model by measuring ACase activity in PAG tissue. Figure 4 showed an increase of ACase activity following the 5-day treatment with morphine. This ACase superactivation was completely abolished when methadone was substituted for morphine on the fifth day of the chronic treatment.

When mice received saline instead of methadone, increase of ACase activity was still observed but to a lesser extent than in the morphine-treated animals.

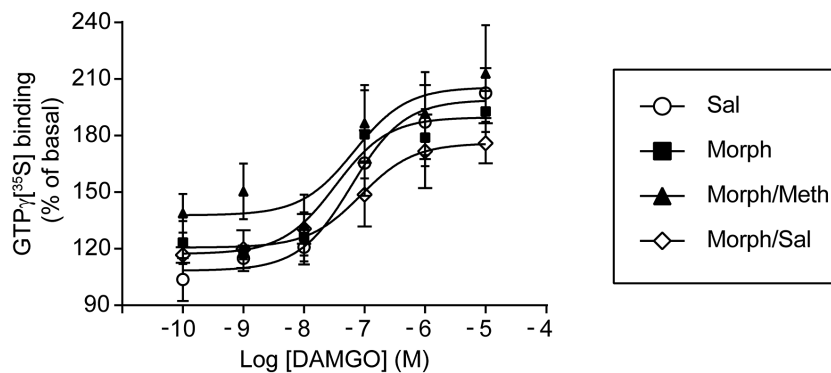
#### NMDA Receptor Regulation

Among the markers of morphine tolerance, regulation of NMDA receptor subunit has been demonstrated to be of particular interest (Trujillo, 2000). We first examined NMDA receptor level in PAG. As shown in Figure 5A, NR1 quantity was not modified among the different groups, whereas NR2A and NR2B were downregulated following morphine treatment (Figure 5B-C). This downregulation was completely abolished when methadone was substituted for morphine on the fifth day of treatment. This effect was specific to methadone, as in groups where saline replaced methadone on the fifth day of treatment, NR2A and NR2B levels remained at the morphine group level (Figure 5B-C). We also examined phosphorylation levels of the main residues in NR1 subunit: Ser-890 and Ser-896, both phosphorylated by protein kinase C (PKC) and Ser-897 phosphorylated by PKA (Tingley et al., 1997; Sanchez-Perez and Felipo, 2005). We found that the phosphorylation level of Ser-890 was not modified among the treated groups (Figure 6), conversely to Ser-896 and Ser-897. Indeed, whereas Ser-896 phosphorylation was not modified with morphine or morphine/methadone treatment, it was increased in the morphine/saline group (Figure 6B). Regarding the phosphorylation level of Ser-897, it increased after 5 days of morphine treatment, and this increase was blocked when methadone was substituted for morphine on the fifth day of the chronic treatment. This effect was specific to methadone treatment, as this increase was still observed when saline replaced methadone on the fifth day of treatment (Figure 6C).

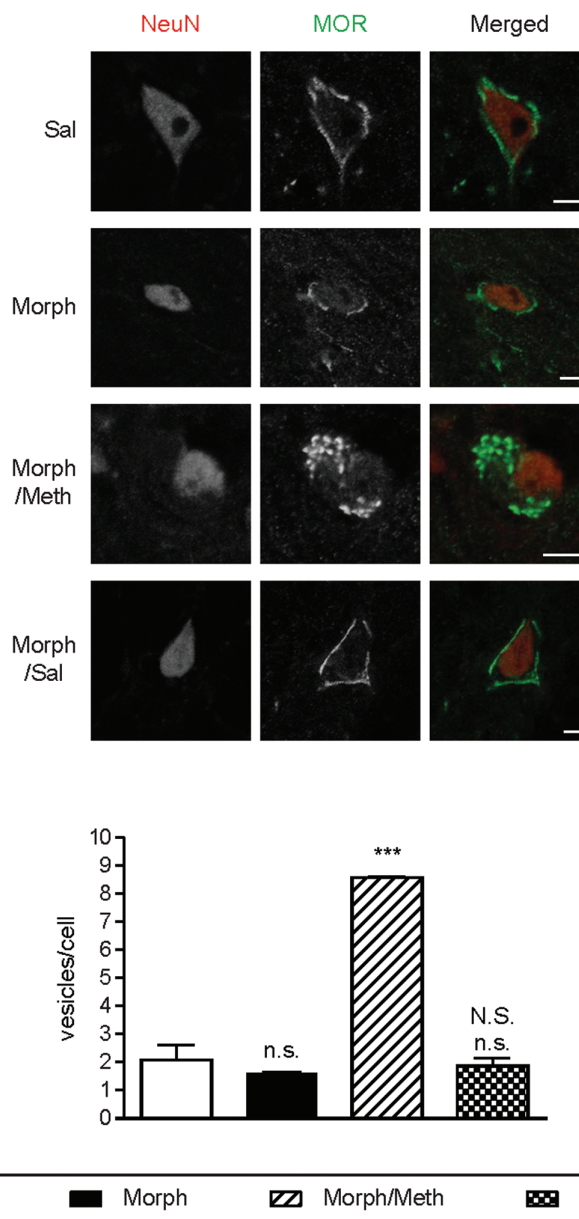
**Table 1.** Intrinsic Efficacies ( $E_{max}$ ) and Potencies ( $\log EC_{50}$ ) of DAMGO-Induced [<sup>35</sup>S]-GTPγS Binding in PAG Membrane

	$-\log EC_{50} \pm SEM$ (M)	$E_{max} \pm SEM$ (%)
Sal	$7.21 \pm 0.14$	$199 \pm 5$
Morph	$7.48 \pm 0.23$	$189.7 \pm 5.6$ (n.s.)
Morph/Meth	$7.17 \pm 0.28$	$205.6 \pm 7.5$ (n.s.)
Morph/Sal	$7.06 \pm 0.22$	$176 \pm 5.2$ (n.s.; N.S.)

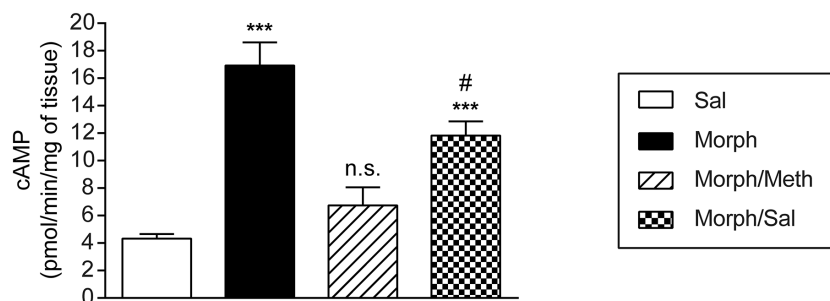
Obtained from Figure 2 (1-way ANOVA for  $E_{max}$ ,  $F_{3,11} = 4.776$ ,  $P < .05$ . n.s. (not significant) vs Sal group; N.S. (not significant) vs Morph group. One-way ANOVA for  $-\log EC_{50}$ ,  $F_{3,11} = 0.5313$ ,  $P = .67$ ;  $n = 3$  to 4 determinants/group.



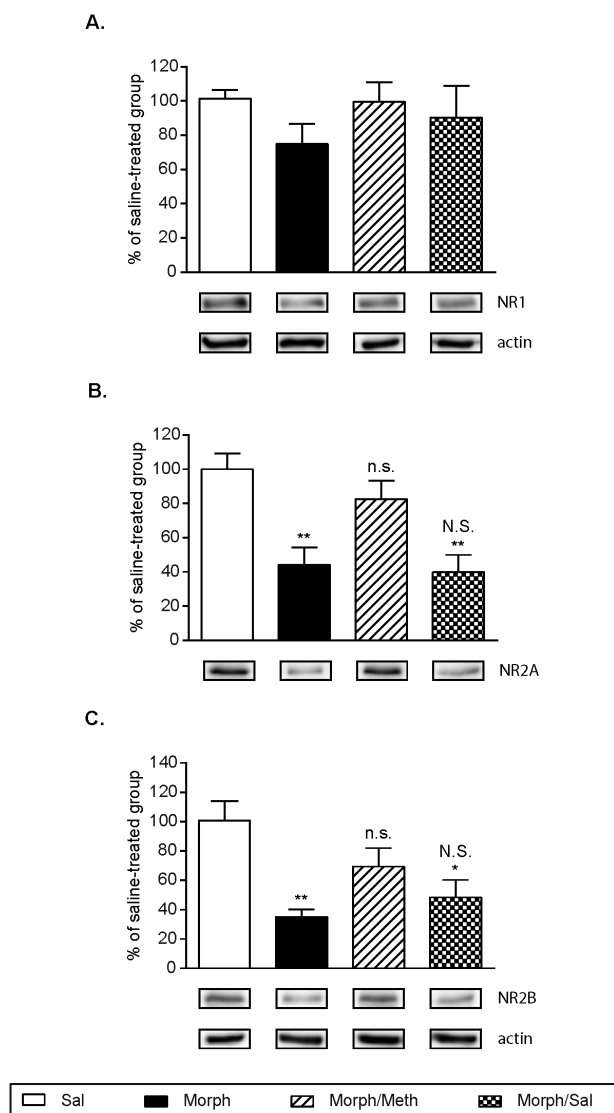
**Figure 2.** Effects of methadone on mu opioid receptor (MOPr) coupling in morphine-treated mice. Animals were treated with i.p. injection, twice daily, for 4 days with morphine (10mg/kg) followed by 1 day of treatment with saline (Morph/Sal), morphine (Morph), methadone (2.5mg/kg, Morph/Meth), or for 5 days with saline (Sal). On the sixth day, periaqueductal gray matter (PAG) was extracted and [<sup>35</sup>S]-GTPγS binding was measured in the presence of increasing concentration of D-Ala<sup>2</sup>,N-Me-Phe<sup>4</sup>,Gly-ol<sup>5</sup>enkephalin (DAMGO). Plotted values represent percentage of basal [<sup>35</sup>S]-GTPγS binding (without agonist), as mean  $\pm$  SEM,  $n = 3$  to 4 determinants/group.



**Figure 3.** Mu opioid receptor (MOPr) localization in periaqueductal gray matter (PAG) after opiate treatment. Animals were treated with i.p. injection, twice daily, for 4 days with morphine (10 mg/kg) followed by 1 day of treatment with saline (Morph/Sal), morphine (Morph), methadone (2.5 mg/kg, Morph/Meth), or for 5 days with saline (Sal). One hour after the last injection, brains were fixed and PAG-containing slices were immunostained with anti-MOPr (green) and anti-NeuN (red) antibodies (scale bar, 5  $\mu$ m). For quantification, slides were encoded and vesicles counted by a second party from at least 10 cells from 3 animals/group. Results are expressed as vesicles/cell (mean  $\pm$  SEM). One-way ANOVA,  $F_{3,8} = 124.5$ ,  $P < .0001$ . \*\*\* $P < .001$ , n.s. (not significant) vs Sal group; N.S. (not significant) vs Morph group. Bonferroni posthoc test.



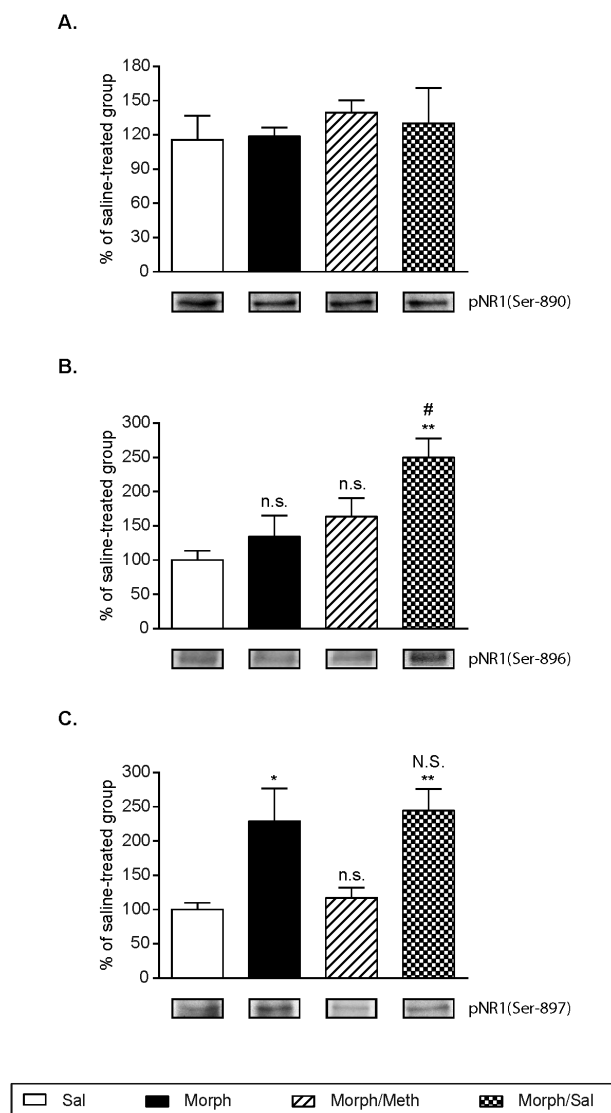
**Figure 4.** Adenylyl cyclase activity in periaqueductal gray matter (PAG) from opiate-treated animals. Mice were treated with i.p. injection, twice daily, for 4 days with morphine (10 mg/kg) followed by 1 day of treatment with saline (Morph/Sal), morphine (Morph), methadone (2.5 mg/kg, Morph/Meth), or for 5 days with saline (Sal). On the sixth day, adenylyl cyclase activity was measured in PAG and expressed in pmol of cAMP/min/mg of tissue (mean  $\pm$  SEM). One-way ANOVA,  $F_{3,24} = 22.95$ ,  $P < .0001$ . \*\*\* $P < .001$ , n.s. (not significant) vs Sal group; # $P < .05$  vs Morph group. Bonferroni posthoc test,  $n = 5$  (Morph), 7 (Morph/Sal), or 8 (Sal, Morph/Meth) determinants/group.



**Figure 5.** Quantitative variation of NMDA receptor subunit in PAG after opiate treatment. Mice were treated with i.p. injection, twice daily, for 4 days with morphine (10 mg/kg) followed by one day of treatment with saline (Morph/Sal), morphine (Morph), methadone (2.5 mg/kg, Morph/Meth), or for 5 days with saline (Sal). The 6<sup>th</sup> day, NR1, NR2A and NR2B were quantified with western-blotting in PAG. Bars are the means  $\pm$  SEM of subunit immunoreactivity expressed in percentage of saline-treated group. One-way ANOVA,  $F_{3,24} = 0.7198$ ,  $p = 0.55$  (A). One-way ANOVA,  $F_{3,17} = 8.995$ ,  $p < 0.0001$  (B). One-way ANOVA,  $F_{3,18} = 5.219$ ,  $p < 0.01$  (C). \* $p < 0.05$ , \*\* $p < 0.01$ , n.s. (not significant) vs Sal group; N.S. (not significant) vs Morph group. Bonferroni post-hoc test,  $n = 5$  to 8 determinants/group.

## Discussion

Understanding mechanisms of opiate tolerance is a great challenge to find new strategies to limit analgesic tolerance. Recently, a strategy for reducing opiate tolerance consisting of coadministering morphine with a MOPr internalizing agonist has been successfully validated in rodents (He et al., 2002; He and Whistler, 2005). However, whereas opiate coadministration in clinics is commonly used for breakthrough pain, it is not to reduce tolerance. The classical way to reduce analgesic tolerance is a sequential use of opiate called opioid rotation, consisting of switching from one opiate to another (Portenoy and Ahmed, 2014). We therefore investigated the effects of a treatment with



**Figure 6.** NR1 subunit phosphorylation in periaqueductal gray matter (PAG) from morphine- and methadone-treated animals. Mice were treated with i.p. injection, twice daily, for 4 days with morphine (10 mg/kg) followed by 1 day of treatment with saline (Morph/Sal), morphine (Morph), methadone (2.5 mg/kg, Morph/Meth), or for 5 days with saline (Sal). On the sixth day, PAG were extracted and phosphorylation of NR1 at Ser-890, Ser-896, and Ser-897 residues was quantified. Bars are the means  $\pm$  SEM of subunit immunoreactivity expressed in percentage of saline-treated group. One-way ANOVA,  $F_{3,15} = 0.3058$ ,  $p = 0.8208$  (A). One-way ANOVA,  $F_{3,20} = 5.878$ ,  $P < .01$  (B). One-way ANOVA,  $F_{3,14} = 7.583$ ,  $P < .01$  (C). \* $P < .05$ , \*\* $P < .01$ , n.s. (not significant) vs Sal group; N.S. (not significant) vs Morph group. Bonferroni posthoc test,  $n = 4$  to 7 determinants/group.

methadone, a MOPr internalizing agonist (Borgland et al., 2003; Celver et al., 2004), in mice tolerant to the analgesic effect of morphine. To the best of our knowledge, this is the first demonstration that a treatment with methadone was able to reverse analgesic tolerance to morphine in morphine-tolerant animals. A cross-tolerance to analgesic effects is rather observed between opiates following repeated treatment (Craft and Dykstra, 1990; Allen and Dykstra, 2000), but in this case, the tolerance was measured for the second opiate used.

Methadone is a racemic compound composed of R- and S-methadone. R-methadone has a higher affinity toward MOPr (Pert and Snyder, 1973) and is more potent than S-methadone (Scott et al., 1948; Wallisch et al., 2007). S-methadone has been

demonstrated to block NMDA receptor (Ebert et al., 1998), an effect that could contribute to inhibit morphine tolerance (Davis and Inturrisi, 1999). We found that S-methadone was devoid of any effect on morphine tolerance, whereas R-methadone was able to reverse the analgesic tolerance to morphine in the same way as the racemic. This demonstrates that the effect of methadone on analgesic tolerance to morphine was mediated by MOPr and not by activation of other receptors according to previous data, suggesting that methadone concentration measured after systemic injection was too low to act on NMDA receptor (Kreek, 2000). Methadone has a short half-life in rodents (2 hours in mice [LeVier et al., 1995] and <2 hours in rats [Ling et al., 1981]), so the analgesia we observed following the morphine challenge is not due to the remaining presence of methadone, as more than 18 hours separated the last methadone injection and the hot plate test. This period is greater than the 7 half-lives necessary to wash-out 99% of methadone.

To explain this tolerance reversal by methadone in morphine-tolerant mice, we investigated several cellular adaptations usually observed following chronic opiate treatment. MOPr involved in nociception are located both centrally and peripherally. As we choose to measure the jumping latencies in the hot plate, which is a centrally integrated response, we decided to focus our biochemical study in PAG, a MOPr-enriched structure (Mansour et al., 1994) and the first descending center of pain control involved in supraspinal response (Flores et al., 2004). Nevertheless, our results did not exclude changes in other MOPr-expressing structures involved in nociception such as thalamus or locus coeruleus. After a sustained activation by an agonist, MOPr usually undergoes a functional uncoupling that promotes desensitization (Noble and Cox, 1996; Bagley et al., 2005) and could contribute to tolerance (Williams et al., 2013; Allouche et al., 2014). In our protocol, we were unable to find any MOPr uncoupling following morphine treatment, suggesting that MOPr uncoupling is not mandatory for morphine-induced tolerance. Our results might be unexpected. Indeed, in beta-arrestin-2 knockout mice, a lower cellular tolerance was measured on voltage-gated calcium channel currents in PAG neurons after a chronic morphine treatment (Connor et al., 2015). In this later study, the authors did not investigate tolerance to analgesia contrary to Bohn's study (Bohn et al., 2000), where a lower tolerance to morphine-induced analgesia was associated with a lack of receptor uncoupling. However, these authors measured receptor coupling in brainstem, a brain structure unrelated to nociception. It seems that the modifications in receptor signaling are region specific. Indeed, in mice tolerant to analgesic effects of morphine, Sim-Selley and co-workers (2007) detected a significant decrease of MOPr-stimulated [<sup>35</sup>S]-GTPγS in spinal cord but not in PAG. Treatment duration might also be another factor. So, Garzon and co-workers (2005) showed MOPr uncoupling in PAG but after an acute intracerebroventricular morphine injection (Garzon et al., 2005).

In the majority of the studies, morphine was unable to promote MOPr endocytosis (Arden et al., 1995; Keith et al., 1996; Finn and Whistler, 2001; Borgland et al., 2003). Our data are in line with this observation, as no MOPr internalization was measured in PAG following morphine exposure. Interestingly, the morphine treatment did not affect the ability of methadone to promote MOPr endocytosis. These data seem to be in contradiction with recent studies showing that prolonged morphine treatment impairs the ability of full opioid agonists to promote receptor endocytosis. However, in this case, MOPr was continuously exposed to morphine (Eisinger et al., 2002; Quillinan et al., 2011) compared with our protocol with intermittent exposure.

Our data suggest that MOPr endocytosis is necessary to recover from morphine tolerance as evidenced by the ability of fentanyl (a MOPr internalizing agonist [Minnis et al., 2003]) to reverse morphine tolerance, conversely to oxycodone (a mu receptor agonist unable to promote receptor internalization [Koch et al., 2009]) and by the lack of tolerance reversal in the morphine/saline group. Some authors suggested that the inability of morphine to promote receptor endocytosis would contribute to recruit signaling pathways involved in tolerance by maintaining active MOPr/morphine complex at the plasma membrane (Kieffer and Evans, 2002; Martini and Whistler, 2007). Among the cellular hallmark of tolerance, ACase superactivation has been observed following chronic opiate treatment (Avidor-Reiss et al., 1995; Mohammed et al., 2013), and PKA inhibitors were shown to block morphine-induced tolerance (Javed et al., 2004). We found that morphine treatment promoted ACase superactivation that disappeared after methadone treatment, suggesting that removing MOPr activated by morphine from cell surface ends this phenomenon (Finn and Whistler, 2001).

Increasing amounts of data accumulate to demonstrate that chronic morphine induced NMDA receptor-dependent behavioral and neurochemical plasticity (Inoue et al., 2003; He et al., 2009; Rodriguez-Munoz et al., 2012; Zhao et al., 2012) and that NMDA antagonists blocked morphine tolerance to analgesia (Trujillo and Akil, 1991; Ko et al., 2008). We found, like He and co-workers (2009), that NR2A and NR2B were downregulated in PAG following morphine treatment. This downregulation was blocked with the methadone exposure, suggesting the involvement of NR2A and NR2B in morphine tolerance (Inoue et al., 2003; Ko et al., 2008). We did not find any significant changes in NR1 expression in morphine or morphine/methadone group according to previous studies where no regulation of NR1 mRNA was detected in PAG after chronic morphine treatment (Zhu et al., 1999). As the morphine treatment did not modify NR1 expression, we wondered if posttranslational modifications might occur. NR1 has 3 phosphorylation sites in its intracellular carboxyl-terminus tail: Ser-890 and Ser-896 phosphorylated by PKC, and Ser-897 phosphorylated by PKA (Tingley et al., 1997; Sanchez-Perez and Felipe, 2005). We found an increase of Ser-897 phosphorylation following morphine treatment that was reversed with the methadone exposure. This result agrees with the data obtained on the cAMP overshoot. Indeed, following morphine treatment, the cAMP overshoot would activate PKA that will in turn phosphorylate Ser-897. Whereas PKC is considered to be a downstream effector in MOPr signaling pathway (Allouche et al., 2014), we surprisingly did not find phosphorylation of NR1(Ser-890) or NR1(Ser-896) following morphine treatment. This lack of phosphorylation could be due to a transient phosphorylation (Caudle et al., 2005) or the absence of the PKC isoform that phosphorylate NR1(Ser-890) and NR1(Ser-896) in the brain structure studied. Indeed, NR1(Ser-890) and NR1(Ser-896) are preferentially phosphorylated by PKC gamma and PKC alpha, respectively (Sanchez-Perez and Felipe, 2005), which are expressed at a very low level in PAG (Lin et al., 2012). It is noteworthy that in the morphine/saline group, we measured a significant increase of pNR1(Ser-896) that could be triggered by an upregulation of PKC alpha after withdrawal (Ventayol et al., 1997).

In conclusion, we demonstrated that methadone exposure in mice pretreated with morphine was able to reverse morphine tolerance by blocking several cellular hallmarks of tolerance such as cAMP overshoot or NMDA receptor regulation. Moreover, this effect involves the ability of methadone to promote MOPr endocytosis. Our results were obtained with intermittent opiate treatment, but it would be interesting to validate our data



in a situation of continuous opiate administration in chronic pain models, a situation closer to clinics. Our data might lead to rational strategies to tackle opiate tolerance in the frame of opiate rotation protocol where a MOPr internalizing agonist would be used between morphine administrations.

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## Statement of Interest

None.

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