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Implication of DOP2 but not DOP1 in development of morphine analgesic tolerance in a rat model of chronic inflammatory pain

H. Beaudry[1,2], **L. Gendron**[2,3,*], and **Jose A. Morón**[1,*]

[1]Department of Anesthesiology, College of Physicians and Surgeons, Columbia University, New York, NY, USA

[2]Département de physiologie et biophysique, Faculté de médecine et des sciences de la santé, Université de Sherbrooke, Sherbrooke, Québec, Canada

[3]Institut de pharmacologie de Sherbrooke, Faculté de médecine et des sciences de la santé and Centre de recherche du CHUS, Université de Sherbrooke, Sherbrooke, Québec, Canada

Abstract

Opioids are well known for their robust analgesic effects. Chronic activation of mu opioid receptors (MOPs) is however accompanied by various unwanted effects such as analgesic tolerance. Among other mechanisms, interactions between MOP and delta opioid receptor (DOP) are thought to play an important role in morphine-induced behavioral adaptations. Interestingly, certain conditions such as inflammation enhance the function of the DOP through a MOP-dependent mechanism. Here, we investigated the role of DOP during the development of morphine-tolerance in an animal model of chronic inflammatory pain. Using behavioral approaches we first established that repeated systemic morphine treatment induces morphine analgesic tolerance in rats coping with chronic inflammatory pain. We then observed that blockade of DOP with subcutaneous naltrindole (NTI), a selective DOP antagonist, significantly attenuates the development of morphine tolerance in a dose-dependent manner. We confirmed that this effect was DOP-mediated by showing that an acute injection of NTI had no effect on morphine-induced analgesia in naïve animals. Previous pharmacological characterizations revealed the existence of DOP1 and DOP2 subtypes. As opposed to NTI, 7-benzylidenenaltrexone (BNTX) and naltriben (NTB) were reported to be selective DOP1 and DOP2 antagonists, respectively. Interestingly, NTB but not BNTX was able to attenuate the development of morphine analgesic tolerance in inflamed rats. Altogether, our results suggest that targeting of DOP2 with antagonists provides a valuable strategy to attenuate the analgesic tolerance that develops after repeated morphine administration in the setting of chronic inflammatory pain.

Keywords

hyperalgesia; opioid; heteromer; inflammation; antagonist

Corresponding authors: Jose A Morón, Ph.D., Department of Anesthesiology, Columbia University Medical Center, P&S Box 46, 630 West 168th Street, New York, NY 10032, USA, Tel: +1 212 342 0023, Fax: +1 212 305 0777, jm3473@cumc.columbia.edu. Louis Gendron, Ph.D., Department of Physiology and Biophysics, Faculty of Medicine and Health Sciences, Université de Sherbrooke, 3001, 12th Avenue North, Sherbrooke, Québec, J1H 5H4, Canada, Tel: (819) 820-6868, Ext: 12760, Fax: (819) 820-6887, Louis.Gendron@USherbrooke.ca.

*These authors contributed equally

INTRODUCTION

Opioids are the most commonly used drugs for severe pain management. However, a significant reduction in the analgesic properties of opioids as well as the development of physical dependence resulting from prolonged use negatively impact the clinical benefits of these drugs. Although the exact mechanisms of opioid tolerance remain unknown, a putative role for a crosstalk between delta (DOP) and mu (MOP) opioid receptors has recently emerged (for a review see (Costantino *et al.*, 2012)).

We and others have shown that the blockade of DOPs or a lack of functional DOPs is associated with a reduction in the rewarding properties (Chefer & Shippenberg, 2009; Shippenberg *et al.*, 2009; Billa *et al.*, 2010; Moron *et al.*, 2010; Le Merrer *et al.*, 2011), and in the withdrawal symptoms of morphine (Crain & Shen, 1995; Fundytus *et al.*, 1995; Hepburn *et al.*, 1997; Nitsche *et al.*, 2002). Interestingly, the selective blockade of DOPs was deemed sufficient to prevent morphine analgesic tolerance in rodents (Abdelhamid *et al.*, 1991; Crain & Shen, 1995; Fundytus *et al.*, 1995; Hepburn *et al.*, 1997; Roy *et al.*, 2005; Abul-Husn *et al.*, 2007; McNaull *et al.*, 2007). Similar prevention of morphine analgesic tolerance was observed in mice treated with antisense oligonucleotide to knock-down DOP or in DOP-KO mice (Kest *et al.*, 1996; Zhu *et al.*, 1999; Nitsche *et al.*, 2002). While two DOP subtypes (DOP1 and DOP2) have been identified (Jiang *et al.*, 1991; Mattia *et al.*, 1991; Sofuoglu *et al.*, 1991), pharmacological studies using selective antagonists have shown that blockade of DOP2, and not DOP1, is implicated in the modulation of morphine-induced behaviors (Miyamoto *et al.*, 1993; Shippenberg *et al.*, 2009; Billa *et al.*, 2010). Altogether, these results suggest a mechanism by which DOP can regulate MOP functions. Interestingly, the use of bivalent opioid ligands has brought new insights on DOP-MOP interactions in opioid analgesic tolerance. Indeed, single molecules combining a MOP agonist and a DOP antagonist, MDANs (MOP-agonist-DOP-antagonist), were shown to produce less analgesic tolerance and dependence than classical opioids (Daniels *et al.*, 2005; Lenard *et al.*, 2007).

As opposed to most GPCRs, the DOP is mainly localized intracellularly. However, under specific conditions such as inflammation and chronic morphine treatment, the targeting of DOP to the plasma membrane is enhanced (for a review see (Gendron *et al.*, 2014)). Interestingly, we and others have shown that MOP is essential for the functional emergence of DOP (Morinville *et al.*, 2003; Morinville *et al.*, 2004a; Gendron *et al.*, 2007b), suggesting functional interactions between these receptors.

As stated above, DOPs have been shown to mediate morphine analgesic tolerance in acute pain models but surprisingly, giving the fact that inflammatory pain enhances DOP functions, there are no studies investigating the role of DOP in the development of morphine-induced analgesic tolerance in the setting of chronic pain. In the present study, we sought to investigate the role of DOP in morphine analgesic tolerance in an inflammatory pain model. Interestingly, we show that the selective blockade of DOP2, but not DOP1, prevented morphine tolerance in Complete Freund Adjuvant's (CFA)-inflamed rats.

EXPERIMENTAL PROCEDURES

Animals

Experiments were carried out in adult male Sprague-Dawley rats weighting 225–250 g (Charles River, St-Constant, QC, Canada) maintained on a 12 h light/dark cycle (06:00–18:00 h). Laboratory chow and water were available *ad libitum.* Behavioral tests were conducted between 07:00 and 11:30 (light cycle). All experiments were approved by the animal care committee of the Université de Sherbrooke (Protocol #242-10B) and all procedures conformed to the directives of the Canadian Council on Animal Care and guidelines of the International Association for the Study of Pain. All animal experiments were designed to minimize the number of animals used and their suffering.

Induction of inflammation and morphine tolerance

Unilateral inflammation of the hind limb and development of hyperalgesia was induced by a single injection of 100 μL emulsified complete Freund's adjuvant 50 μg/100 μL (CFA; Calbiochem, San Diego, CA, USA) in the plantar surface of the left hind paw of rats under brief isoflurane anesthesia. Inflammation was used to enhance cell surface availability of DOP (Cahill *et al.*, 2003; Morinville *et al.*, 2004b; Gendron *et al.*, 2006; Gendron *et al.*, 2007a; Gendron *et al.*, 2007b). Hargreaves tests (heat hyperalgesia) were carried out 72 h after CFA injection as described below. Morphine analgesic tolerance was induced as previously showed (Beaudry *et al.*, 2009). Morphine sulfate was injected subcutaneously every 12 hours for 72 h (5 injections of 10 mg/kg), starting 12h after CFA injection. Hargreaves tests were carried out 72 h after CFA injection (see Figure 1 for timeline representation).

Drugs

Morphine sulfate (lots BK8689 and CC0630; Sandoz, Montréal, QC, Canada and lot 43156/C; Medisca, Montréal, QC, Canada; an additional lot was obtained from the National Institute on Drug Abuse Drug Program) was diluted in sterile saline solution (0,9 % NaCl) to concentrations of 0.3, 1, 3 and 10 mg/ml and stored at room temperature protected from light. Control rats received equivalent volume of sterile saline. As demonstrated in pharmacological studies, different DOP subtypes selective antagonists are available: the DOP 1/2 antagonist (naltrindole; NTI) (Portoghese *et al.*, 1988), the DOP2 antagonist (naltriben; NTB) (Sofuoglu *et al.*, 1991), and the DOP1 antagonist (7-benzylidenenaltrexone; BNTX) (Portoghese *et al.*, 1992). NTI (Tocris Bioscience, Minneapolis MN, USA) was dissolved in DMSO at 100 mM and stored in aliquots at −20 °C until use. For experiments, NTI was diluted in sterile saline to 0.003 to 0.03 mg/ml and control rats received equivalent volume of sterile saline. NTB (Tocris Bioscience, Minneapolis MN, USA) was dissolved in a sterile saline solution at 5 mg/ml and stored in aliquots at −20 °C until use. For experiments, NTB was diluted in sterile saline to 0.1 mg/ml and control rats received equivalent volume of sterile saline. BNTX (Tocris; Tocris Bioscience, Minneapolis MN, USA) was dissolved in a sterile saline solution at 50 mg/ml and stored in aliquots at −20 °C until use. For experiments, BNTX was diluted in sterile saline to 1 mg/ml and control rats received equivalent volume of sterile saline. NTI, NTB and BNTX doses have been used elsewhere (Shippenberg *et al.*, 2009) and are known to

fully block DOP agonists effects (Suzuki *et al.*, 1997; Schultz *et al.*, 1998; Broom *et al.*, 2002; Maslov *et al.*, 2010; Zeng *et al.*, 2011; Maslov *et al.*, 2014). All drugs and vehicles were administered subcutaneously.

Hargreaves test

Response to noxious heat stimulus was evaluated using the Hargreaves test in hyperalgesic conditions (induced by CFA intraplantar injection) to determine antihyperalgesic effects of drugs. Animals were acclimatized 30 min to the Hargreaves test environment and were placed in Plexiglas boxes positioned on a glass surface (IITC Life Science Inc., Woodland Hills, CA, USA), 24 h prior to baseline measurements. The following day, corresponding to −72 h, the heat source was positioned under the plantar surface of the hind paw after a 15 min habituation period and the latency for each hind paw withdrawal in response to radiant heat was measured three times in alternation. Subsequently, CFA was injected in the left hind paw as described above. Seventy-two hours after injection of CFA, baseline withdrawal latencies (identified as 0 min) of each hind paw were measured two times in alternation preceding subcutaneous injection of a challenging dose of morphine. Afterward, latencies to paw withdrawal were recorded every 15 min for 60 min. To prevent tissue damage, a cut-off time of 20 s was imposed. If an animal reached the cut-off, the light beam was automatically turned off and the animal was assigned the maximum score. Area under curve was calculated with Prism 6.0 on the curve obtained between 0 and 60 min after morphine challenge dose (Y baseline set for each animal according to its latency to paw withdrawal after inflammation).

Data Analysis

Calculations were done with Excel (2010), graphs with SigmaPlot11.0, and statistical analysis with Prism GraphPad 6. Data are expressed as the mean ± SEM. *P*-values are presented in figure legends.

RESULTS

Effect of acute naltrindole treatment on morphine analgesic efficacy

Naltrindole (NTI) is a selective DOP antagonist that does not interfere with morphine analgesic properties in naïve animals (Abdelhamid *et al.*, 1991; Abul-Husn *et al.*, 2007). However, its selectivity has not been tested in models known to upregulate DOP such as CFA-induced inflammation (Cahill *et al.*, 2003; Gendron *et al.*, 2006; Gendron *et al.*, 2007a). In this first set of experiments we verified the effect of an acute injection of NTI on morphine analgesic efficacy in a model of chronic inflammatory pain. As it can be seen in Figure 2, CFA-induced inflammation triggered heat hyperalgesia. In inflamed rats receiving saline for 15 min before morphine, 3 mg/kg morphine induced a robust time-dependent alleviation of CFA-induced thermal hyperalgesia with maximal effect at 45 min (11.8 ± 1.6 sec compared to 4,7 ± 0.2 sec for *0 min* and *45 min* respectively). When s.c. NTI 0.03 mg/kg was given 15 min before morphine, morphine antihyperalgesic effect was similar that in *Saline s.c. 15 min* group rats (p=0.9594 using Two-way ANOVA). Moreover, NTI pretreatment did not modify CFA-induced hyperalgesia (5.3 ± 0.8 sec compared to 4.6 ± 0.3 sec at *0* for *NTI 0.03 mg/kg s.c. 15 min* and *Saline s.c. 15 min* respectively). These results

indicate that NTI 0.03 mg/kg does not interfere with morphine antihyperalgesic effect in CFA-inflamed rats.

Effect of chronic naltrindole treatment on morphine analgesic efficacy in inflamed rats

In the next set of experiments, we sought to determine the effect of chronic NTI on morphine analgesic tolerance in CFA-inflamed rats. We first examined the dose of NTI needed to prevent morphine analgesic tolerance in inflamed rats. As it can be seen in Figure 3, morphine has a robust analgesic effect in control inflamed rats, reaching an AUC of 27.3 ± 5.4 (Fig. 3; *Saline/Saline*). Following chronic morphine pretreatment, the analgesic effect of morphine was significantly reduced compared to saline-treated rats (Fig. 3; AUC of 9.0 ± 1.1 compared to 27.3 ± 5.4 for *Saline/Morphine* and *Saline/Saline* respectively; $p<0.001$ using One-way ANOVA with Bonferroni's multiple comparisons test). Interestingly, when NTI was administered 15 min before every morphine administration, we observed a dose-dependent prevention of morphine analgesic tolerance. This effect was significant with a dose of 0.03 mg/kg NTI (Fig. 3; AUC of 22.0 ± 2.8 compared to 9.0 ± 1.1 for *NTI 0.03mg/kg/Morphine* and *Saline/Morphine* respectively; $p<0.05$ using One-way ANOVA with Bonferroni's multiple comparisons test). Moreover, the analgesic effect of morphine in NTI 0.03 mg/kg pretreated rats was not different from analgesia measured in Saline/Saline rats. These results indicate that NTI pretreatment completely prevented morphine analgesic tolerance (Fig. 3; AUC of 22.0 ± 2.8 compared to 27.3 ± 5.4 for *NTI 0.03mg/kg/Morphine* and *Saline/Saline* respectively; $p>0.05$ using One-way ANOVA with Bonferroni's multiple comparisons test).

We next compared the analgesic effect of different doses of the morphine challenge in inflamed rats pretreated with a combination of saline/morphine or NTI 0.03 mg/kg/morphine. As it is shown in Figure 4A, NTI 0.03 mg/kg prevented morphine analgesic tolerance which resulted in a significant increase in the analgesic effect of morphine when the dose of the challenge was 3 mg/kg (Fig. 4A; AUC of 22.1 ± 3.8 compared to 11.6 ± 1.9 for *NTI 0.03 mg/kg/Morphine* and *Saline/Morphine* respectively; $p<0.05$ using Two-way ANOVA with Bonferroni's multiple comparisons test) or 10 mg/kg (Fig. 4A; AUC of 44.5 ± 1.7 compared to 30.4 ± 4.0 for *NTI 0.03 mg/kg/Morphine* and *Saline/Morphine* respectively; $p<0.0001$ using Two-way ANOVA with Bonferroni's multiple comparisons test). In Figure 4B, results are expressed as latency to paw withdrawal in function of time after morphine injection. Chronic pretreatment with NTI did not affect CFA-induced thermal hyperalgesia (Fig. 4B; 5.5 ± 0.7 sec compared to 9.9 ± 0.6 sec for *0 min* and *−72h* respectively; $p<0.05$ using Two-way ANOVA with Bonferroni's multiple comparisons test). Moreover, NTI and saline pretreated groups developed similar inflammation-induced thermal hyperalgesia (5.6 ± 0.8 sec compared to 4.7 ± 0.3 sec for *NTI 0.03 mg/kg/Morphine* and *Saline/Morphine* respectively) but significantly increased the analgesic effect of morphine at 15 min (Fig. 4B; 15.1 ± 1.6 sec compared to 8.7 ± 1.0 sec for *NTI 0.03 mg/kg/Morphine* and *Saline/Morphine* respectively; $p<0.05$ using Two-way ANOVA with Bonferroni's multiple comparisons test) and 45 min (Fig. 4B; 11.5 ± 1.7 sec compared to 6.3 ± 0.6 sec for *NTI 0.03 mg/kg/Morphine* and *Saline/Morphine* respectively; $p<0.01$ using Two-way ANOVA with Bonferroni's multiple comparisons test). Taken together, these results show that NTI pretreatment is sufficient to prevent morphine analgesic tolerance in inflamed rats.

Effect of chronic 7-benzylidenenaltrexone and naltriben treatment on morphine analgesic tolerance in inflamed rats

In order to assess the role of DOP1 and DOP2 in morphine analgesic tolerance in inflamed rats, we used BNTX or NTB selective antagonists for DOP1 and DOP2, respectively. As shown in Figure 5A, pretreatment with BNTX or NTB did not affect CFA-induced thermal hyperalgesia (Fig. 4B *BNTX 1 mg/kg/Morphine*; 4.4 ± 0.1 sec compared to 11.6 ± 1.0 sec for *0 min* and *−72h* respectively; $p<0.01$ using Two-way ANOVA with Bonferroni's multiple comparisons test) (Fig. 4B *NTB 0.1 mg/kg/Morphine*; 4.8 ± 0.3 sec compared to 10.2 ± 0.1 sec for *0 min* and *-72h* respectively; $p<0.05$ using Two-way ANOVA with Bonferroni's multiple comparisons test). Moreover, BNTX, NTB and saline pretreated groups developed similar inflammation-induced thermal hyperalgesia (4.7 ± 0.3 sec compared to 4.8 ± 0.4 sec and 4.8 ± 0.3 sec for *Saline/Morphine*, *BNTX 1 mg/kg/Morphine*, and *NTB 0.1 mg/kg/Morphine* respectively). Interestingly, inflamed rats pretreated with 0.1 mg/kg NTB had a significantly increased morphine analgesic effect at 15 min (12.2.5 ± 2.2 compared to 8.7 ± 1.0 for *NTB 0.1 mg/kg/Morphine* and *Saline/Morphine* respectively; $p<0.05$ using Two-way ANOVA with Bonferroni's multiple comparisons test) and 45 min compared to tolerant rats (11.5 ± 2.0 compared to 6.3 ± 0.6 for *NTB 0.1 mg/kg/Morphine* and *Saline/Morphine* respectively; $p<0.0001$ using Two-way ANOVA with Bonferroni's multiple comparisons test). Surprisingly, BNTX-pretreated rats showed similar paw withdrawal latencies than saline-pretreated rats after morphine challenge indicating that morphine analgesic tolerance still develop after DOP blockade with BNTX. Comparing the AUC for the pretreatment with each DOP antagonist we did not observe an effect on morphine analgesic efficacy following BNTX pretreatment compared to tolerant rats (Fig. 5B; AUC of 10.5 ± 2.5 compared to 11.0 ± 1.5 for *BNTX 1 mg/kg/Morphine* and *Saline/Morphine* respectively). In contrast, NTI and NTB pretreatment induced a significant increase in morphine analgesic efficacy (AUC of 22.1 ± 3.8 and 26.0 ± 5.6 for *NTI 0.03 mg/kg/Morphine* and *NTB 0.1 mg/kg/Morphine* respectively) and this effect was similar to the morphine analgesic effect obtained in naïve inflamed-rats (Fig. 5B; dashed line corresponding to AUC of 27.8 obtained in *Saline/Saline* group as illustrated in Fig. 3). Taken together, our results show that DOP inhibition with NTI (a non-selective DOP antagonist) or NTB (a selective DOP2 antagonist) but not BNTX (a selective DOP1 antagonist), is sufficient to prevent morphine analgesic tolerance in inflamed rats.

DISCUSSION

Opioid tolerance has been shown to be an adaptive cellular process that involves modulation of MOP function, but growing evidence suggest that DOP is also implicated (Zhang *et al.*, 2006). Indeed, DOP has been shown to prevent morphine analgesic tolerance in acute pain models but surprisingly, there are no studies investigating the role of DOP in the development of morphine-induced analgesic tolerance in the setting of chronic pain. Several studies have demonstrated that DOP function is increased in the presence of inflammatory pain (Hylden *et al.*, 1991; Hurley & Hammond, 2000; Cahill *et al.*, 2003; Gendron *et al.*, 2006; Gendron *et al.*, 2007a), suggesting a role for DOP in morphine tolerance in the presence of chronic pain. In the present study, we investigated the role of DOP in morphine analgesic tolerance in an inflammatory pain model known to upregulate DOP (Cahill *et al.*,

2003). CFA-inflamed rats were treated repeatedly with systemic morphine to induce analgesic tolerance and this morphine regimen induced a robust decrease in morphine analgesic efficacy. In morphine tolerant and inflamed rats, we compared the ability of NTI (non-selective DOP antagonist), NTB (selective DOP2 antagonist) or BNTX (selective DOP1 antagonist) to prevent morphine analgesic tolerance. Interestingly, our results show that NTI and NTB, but not BNTX, prevented morphine analgesic tolerance. These results indicate that the role of DOP in morphine analgesic tolerance is mainly mediated by DOP2.

In this study we examined the effect of DOP inhibition during the onset of morphine analgesic tolerance in an inflammatory pain model. To achieve this goal, DOP selective antagonists were administered twice a day, before each morphine injection. As it was previously reported that the genetic ablation of DOP slightly increases the CFA-induced heat hyperalgesia compared to wildtype animals (Gaveriaux-Ruff *et al.*, 2008), one could argue that chronic DOP blockade may also affect the level of hyperalgesia induced by CFA. However, in the present study the CFA-induced heat hyperalgesia was developed similarly among all groups, independently of the pretreatment. These discrepancies could be explained by the time frame in which our experiments were done. In the study conducted by Gaveriaux-Ruff and coworkers, CFA-induced heat hyperalgesia was different between DOP −/− and wildtype mice only after 5 days whereas our study was conducted only up to 3 days following CFA injection. Therefore, we cannot rule out the possibility that repeated use of DOP antagonists would not affect heat hyperalgesia over a longer period of time.

In naïve animals, acute DOP inhibition has been shown to increase morphine analgesic efficacy (Gomes *et al.*, 2004; Abul-Husn *et al.*, 2007; He *et al.*, 2011). However, our results showed no difference in morphine analgesic efficacy between acute saline- and acute NTI-treated rats. This discrepancy could be due to the fact that our experiments were conducted in inflamed rats. Indeed, we have shown that CFA-induced DOP upregulation is dependent on MOP (Morinville *et al.*, 2004b; Gendron *et al.*, 2007b) suggesting that opioid receptor interactions may be involved in a complex regulatory mechanism during inflammation. Interestingly, prolonged morphine treatment is also implicated in DOP regulation (Cahill *et al.*, 2001; Morinville *et al.*, 2003; Morinville *et al.*, 2004a) as well as in formation of MOP/DOP dimer (Gupta *et al.*, 2010). Actually, no data are available regarding MOP/DOP heteromerization under inflammatory conditions, but one can speculate that inflammation induces changes in MOP/DOP interactions. Therefore, the effect of acute DOP blockade on morphine analgesic efficacy in inflamed rats would be different than in naïve rats, as seen in the present study. Altogether, these data suggest that inflammation induces changes in opioid receptor function that impacts the effect of acute DOP inhibition on MOP analgesic effect.

On the other hand, our results show that repeated DOP inhibition prevented morphine analgesic tolerance during the onset of inflammatory pain, as it has been reported in acute pain models (Abdelhamid *et al.*, 1991; Zhu *et al.*, 1999; Abul-Husn *et al.*, 2007; He *et al.*, 2011). The effect of repeated DOP blockade on MOP function is likely due to events that take place over time and not a direct effect on MOP, since morphine analgesic efficacy was not affected by acute DOP antagonist pretreatment. Interestingly, we show that NTI and NTB, but not BNTX, prevented morphine analgesic tolerance in inflamed-rats, indicating

that this effect is mediated by DOP2. Similarly, morphine sensitization has been shown to be prevented by NTI and NTB but not BNTX (Shippenberg *et al.*, 2009). Pharmacological evidence revealed that the DOP2 subtype might correspond to a MOP/DOP complex (Porreca *et al.*, 1992; Xu *et al.*, 1993) whereas DOP1 would be a DOP/KOP complex (Portoghese & Lunzer, 2003; Bhushan *et al.*, 2004; Xie *et al.*, 2005). It has been proposed that chronic morphine treatment may increase MOP/DOP heteromer formation with morphine acting as a pharmaco-chaperone bringing the dimer to the cell surface (Costantino *et al.*, 2012) and that the presence of the MOP/DOP heteromer would favor morphine tolerance. Interestingly, a strategy using a $TM1^{MOP}$ mimicking peptide to selectively disrupt the MOP-DOP heteromer prevented morphine analgesic tolerance (He *et al.*, 2011). Taken together, these results together with ours suggest that NTI and NTB block DOP which then disrupts the MOP/DOP dimer, leading to prevention of morphine analgesic tolerance.

To our knowledge, we are the first to compare the effect of DOP1 and DOP2 blockade on morphine analgesic tolerance in a chronic inflammatory pain model. Altogether, results shown in this study provide more support to the idea that the selective blockade of DOP2 in combination with MOP agonists is a promising approach to treat chronic pain conditions without unwanted side effects such as analgesic tolerance.

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ABBREVIATIONS

DOP	delta opioid receptor
MOP	mu opioid receptor
KO	knock-out
DOP1	delta opioid receptor subtype 1
DOP2	delta opioid receptor subtype 2
MDAN	MOP-agonist-DOP-antagonist
GPCR	G protein coupled receptor
CFA	complete Freund’s Adjuvant
NTI	naltrindole
NTB	naltriben
BNTX	7-benzylidenenaltrexone
DMSO	dimethyl sulfoxyde
AUC	area under curve
KOP	kappa opioid receptor

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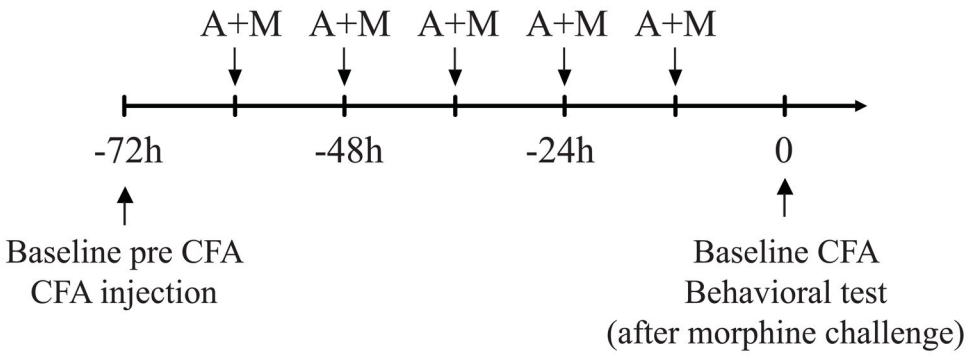

Figure 1. Timeline representation of drug administration and behavioral measurements

Basal latencies to paw withdrawal were measured before CFA administration (Baseline pre CFA) and CFA was injected in the left hindpaw. Twelve hours later, DOP antagonist or vehicle (A) was administered s.c. 15 min before morphine 10 mg/kg or vehicle (M) every 12h for 5 consecutive injections. Twelve hours after the last A+M treatment, thermal latencies to paw withdrawal were measured (Baseline CFA) and Hargreaves test was performed as described in the text.

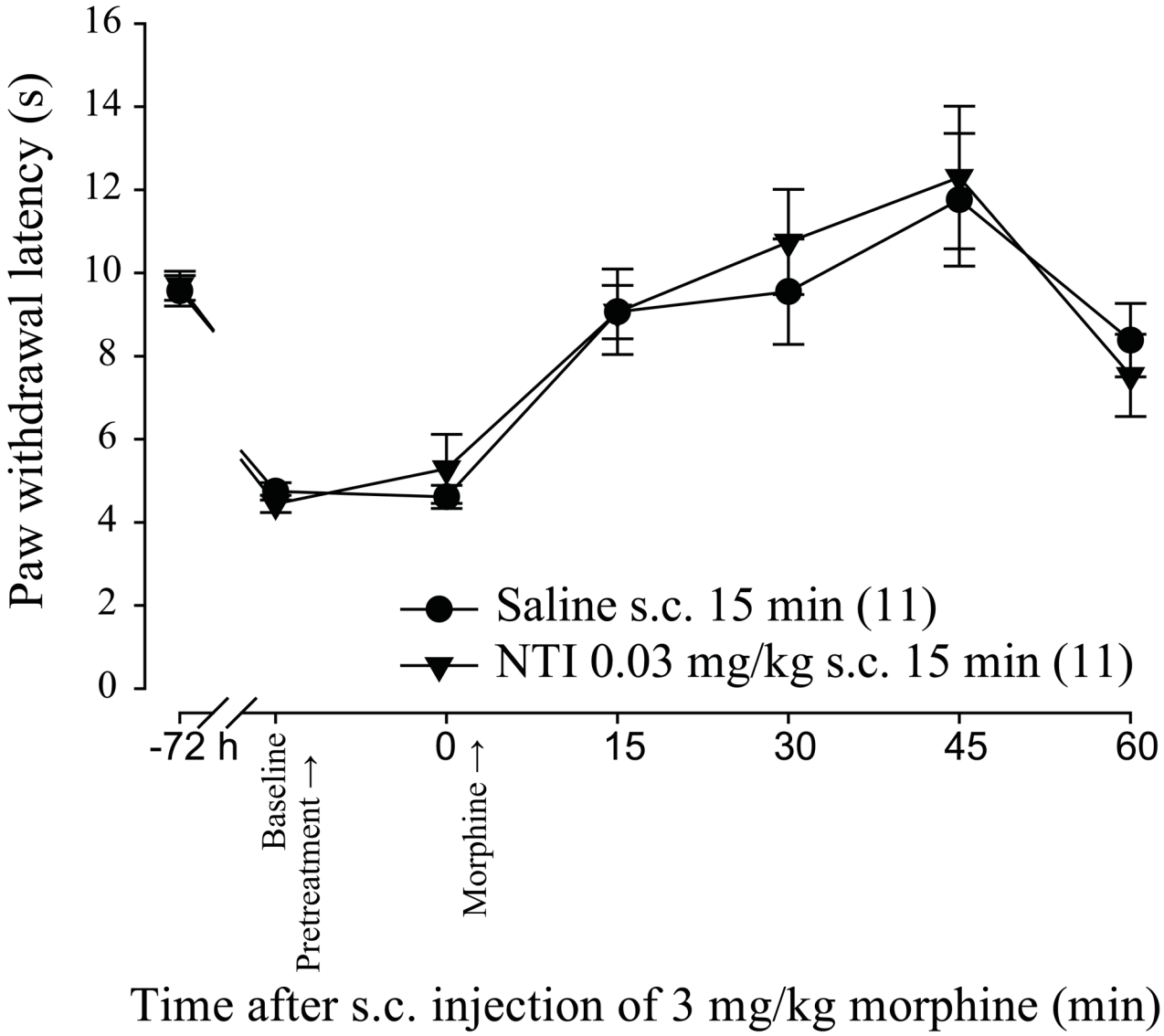

Figure 2. Effect of acute NTI injection on morphine antihyperalgesic effect in inflamed rats

Sprague–Dawley rats were injected with CFA in the plantar surface of the hind paw. Seventy-two hours after CFA injection, the latency to paw withdrawal (in sec) was tested before (Baseline) and after (0) a pretreatment with NTI or saline (s.c.; 15 min) using the Hargreaves test. Morphine 3 mg/kg (Morphine) was administered in both groups to compare morphine's antihyperalgesic effect every 15 min (from 15 to 60 min). Number given in the legend inset represents the number of animals per group. Acute NTI did not modify morphine antihyperalgesic effect.

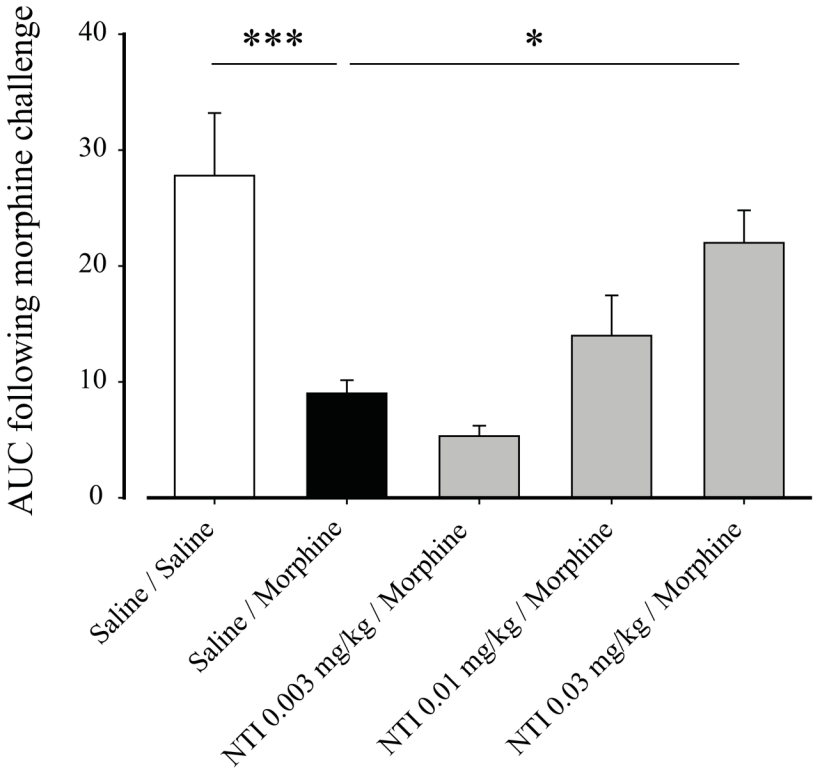

Figure 3. Determination of NTI dose necessary to prevent morphine analgesic tolerance in inflamed rats

CFA inflamed rats were pretreated as illustrated in Fig. 1 with NTI (0.003 to 0.3 mg/kg) as a DOP antagonist. Twelve hours after the last pretreatment injection (saline followed 15 min later by saline (□), saline followed 15 min later by morphine (■) or three different doses of NTI (■)), the analgesic effect of a challenging dose of morphine (3 mg/kg) was measured using the Hargreaves test. Results are expressed as area under curve obtained between 0 and 60 min after morphine challenge dose (Y baseline set for each animal according to its latency to paw withdrawal after inflammation). (N = 9–15 rats), *, $p<0.05$ and ***, $p<0.001$ when groups were compared to Saline/Morphine group. NTI at 0.03 mg/kg is sufficient to prevent morphine analgesic tolerance.

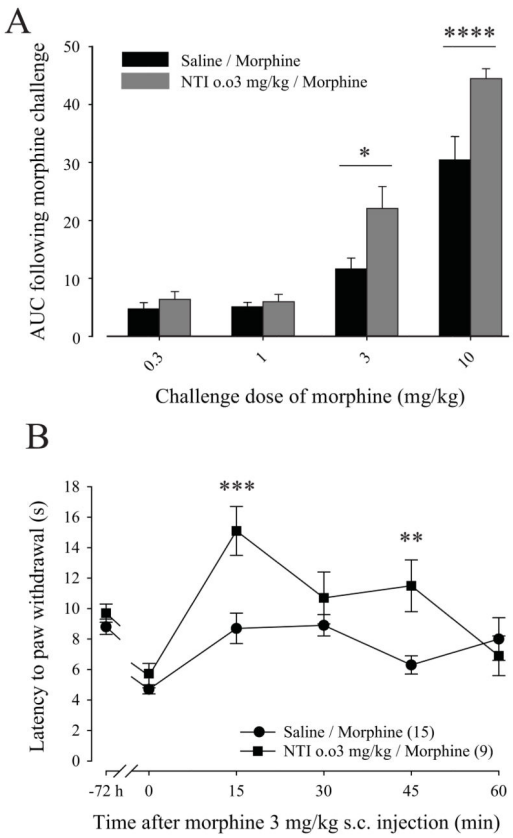

Figure 4. Effect of NTI pretreatment on morphine analgesic tolerance in inflamed rats

(A) CFA inflamed rats were pretreated as illustrated in Fig. 1 with NTI 0.3 mg/kg as a DOP antagonist. Twelve hours after the last treatment, the analgesic effect of a challenging dose of morphine (0.3 to 10 mg/kg) was measured using the Hargreaves test. Results are expressed as area under curve obtained between 0 and 60 min after morphine challenge dose (Y baseline set for each animal according to its latency to paw withdrawal after inflammation). (N = 8–13 rats), *, $p<0.05$ and ****, $p<0.0001$ when groups were compared together within a similar morphine challenge dose. (B) CFA inflamed rats were pretreated as illustrated in Fig. 1 with NTI 0.3 mg/kg as a DOP antagonist. Twelve hours after the last treatment, the analgesic effect of a challenging dose of morphine (3 mg/kg) was measured using the Hargreaves test. The latency to paw withdrawal (in sec) was tested every 15 min (from 0 to 60 min) after morphine injection. Number given in the legend inset represents the number of animals per group. **, $p<0.01$ and ***, $p<0.001$ when groups were compared together. NTI pretreatment prevented morphine analgesic tolerance in inflamed rats.

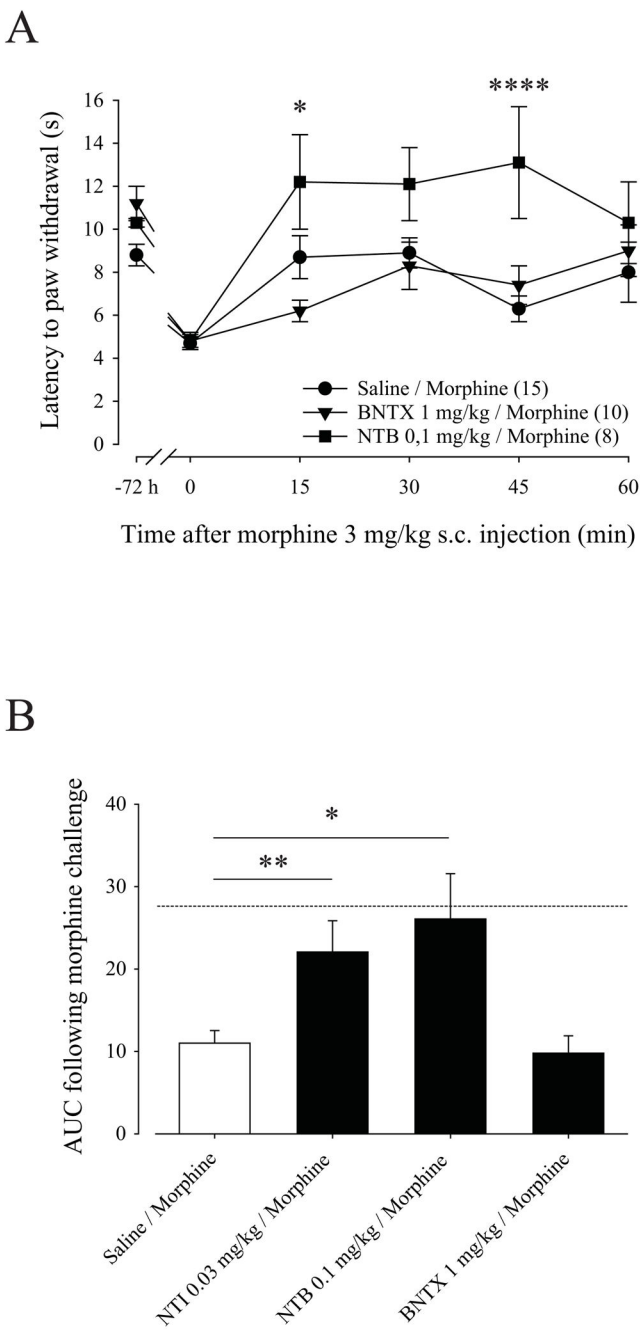

Figure 5. Effect of BNTX and NTB pretreatment on morphine analgesic tolerance in inflamed rats

(A) CFA inflamed rats were pretreated as illustrated in Fig. 1 with BNTX 1 mg/kg or NTB 0.1 mg/kg as DOP antagonists. Twelve hours after the last treatment, the analgesic effect of a challenging dose of morphine (3 mg/kg) was measured using the Hargreaves test. The latency to paw withdrawal (in sec) was tested every 15 min (from 0 to 60 min) after morphine injection. Number given in the legend inset represents the number of animals per group. *, $p<0.05$ and ****, $p<0.0001$ when groups were compared to Saline/Morphine group. (B) Results obtained with the three DOP selective antagonists are expressed as area

under curve obtained between 0 and 60 min after morphine challenge dose (Y baseline set for each animal according to its latency to paw withdrawal after inflammation). Dashed lined represent AUC obtained for control Saline/Saline group. (N = 8–13 rats). *, $p<0.05$ and **, $p<0.01$ when groups were compared to Saline/Morphine group. Chronic injections of NTI and NTB, but not BNTX, prevented morphine analgesic tolerance in inflamed-rats and the morphine analgesic efficacy in NTI- or NTB-pretreated rats reached the efficacy obtained in control rats.