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### Authors

de Guglielmo, Giordano  
Kallupi, Marsida  
Scuppa, Giulia  
[et al.](#)

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## RESEARCH PAPER

# Analgesic tolerance to morphine is regulated by PPAR $\gamma$

Giordano de Guglielmo<sup>1</sup>, Marsida Kallupi<sup>1</sup>, Giulia Scuppa<sup>1</sup>, Serena Stopponi<sup>1</sup>, Gregory Demopulos<sup>2</sup>, George Gaitanaris<sup>2</sup> and Roberto Ciccocioppo<sup>1</sup>

<sup>1</sup>School of Pharmacy, Pharmacology Unit, University of Camerino, Camerino, Italy, and <sup>2</sup>Omeros Corporation, Seattle, WA, USA

### Correspondence

Professor Roberto Ciccocioppo,  
School of Pharmacy,  
Pharmacology Unit, University of  
Camerino, via Madonna delle  
Carceri, 62032 Camerino, MC,  
Italy. E-mail:  
roberto.ciccocioppo@unicam.it

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## BACKGROUND AND PURPOSE

Opioid drugs are potent analgesics. However, their chronic use leads to the rapid development of tolerance to their analgesic effects and subsequent increase of significant side effects, including drug dependence and addiction. Here, we investigated the role of PPAR $\gamma$  in the development of analgesic tolerance to morphine in mice.

## EXPERIMENTAL APPROACH

We monitored analgesia on alternate days using the tail immersion test.

## KEY RESULTS

Daily administration of morphine (30 mg·kg<sup>-1</sup>, bid) resulted in the rapid development of tolerance to thermal analgesia. Co-administration of pioglitazone (10 and 30 mg·kg<sup>-1</sup>, bid) significantly attenuated the development and expression of tolerance. However, pretreatment with GW-9662 (5 mg·kg<sup>-1</sup>, bid), a selective PPAR $\gamma$  antagonist, completely abolished this effect. Injection of GW-9662 and a lower dose of morphine (15 mg·kg<sup>-1</sup>, bid) accelerated the development of tolerance to its antinociceptive effect. Subsequently, we found that conditional neuronal PPAR $\gamma$  knockout (KO) mice develop a more rapid and pronounced tolerance to morphine antinociception compared with wild-type (WT) controls. Moreover, in PPAR $\gamma$  KO mice, pioglitazone was no longer able to prevent the development of morphine tolerance.

## CONCLUSIONS AND IMPLICATIONS

Overall, our results demonstrate that PPAR $\gamma$  plays a tonic role in the modulation of morphine tolerance, and its pharmacological activation may help to reduce its development. These findings provide new information about the role of neuronal PPAR $\gamma$  and suggest that combining PPAR $\gamma$  agonists with opioid analgesics may reduce the development of tolerance and possibly attenuate the potential for opioid abuse.

## Abbreviations

KO, knockout; MOP receptor,  $\mu$ -opioid receptor; WT, wild type

## Table of Links

TARGETS	LIGANDS
$\mu$ opioid (MOP) receptor	IL-1 $\beta$
PPAR $\alpha$	IL-6
PPAR $\delta$	GW-9662
PPAR $\gamma$	Morphine
TNF $\alpha$	Pioglitazone

This Table lists key protein targets and ligands in this document, which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a,b).

## Introduction

Opioids are essential medications in the management of pain and are widely used to treat acute severe pain following trauma, extensive burns or surgery, as well as chronic pain. One of the major problems associated with chronic opioid use is the significant risk of developing drug dependence and possibly addiction (Kreek, 2001; Inturrisi, 2002). The rapid development of tolerance to the analgesic effect of opioids, which requires dose escalation to maintain adequate analgesia, clearly contributes to this risk. Moreover, increased opioid dosing is associated with side effects, such as severe constipation, nausea and urinary retention, which are highly disliked by patients. Identification of innovative strategies to attenuate opioid tolerance development could help reduce the risks of opiate abuse associated with their chronic use, thus offering important benefits in the management of chronic pain.

Opioid tolerance is a complex phenomenon and several mechanisms may be responsible for it. Chronic exposure to opioid agonists can cause opioid receptor down-regulation, internalization, uncoupling from G-proteins (Bailey and Connor, 2005; Martini and Whistler, 2007; Koch and Hollt, 2008) or desensitization mediated by PKC-dependent mechanism (Bailey *et al.*, 2009). For instance, repeated morphine injections produce a marked decrease in brain  $\mu$ -opioid receptor (MOP receptor) density (Davis *et al.*, 1979; Tao *et al.*, 1987; Diaz *et al.*, 2000), down-regulation of the high-affinity MOP receptor site in rats and reduction of MOP receptor signalling in sensory neurons and brainstem nuclei (Sim *et al.*, 1996; Johnson *et al.*, 2006). Changes in transcription factor activation following chronic opioid treatment have also been proposed to play a relevant role in opioid tolerance and addiction (Carlezon *et al.*, 2005; Zachariou *et al.*, 2006). Finally, emerging evidence suggests that opioid hyperalgesia and tolerance are influenced by the activation of the central immune system. For instance, it has been shown that the activation of glial cells, induction of the NF- $\kappa$ B signalling pathway and up-regulation of the transcription, translation and release of pro-inflammatory cytokines, such as IL-1  $\beta$ , IL-6 and TNF- $\alpha$ , may all contribute to the attenuation of opioid analgesia and tolerance (Hutchinson *et al.*, 2011). In contrast, blockade of these pro-inflammatory mechanisms

via inhibition of the cytokines IL-1 $\beta$ , IL-6 and TNF- $\alpha$  (Raghavendra *et al.*, 2002; Shavit *et al.*, 2005; Hutchinson *et al.*, 2007; 2008a) and inhibition of microglia activation following anti-inflammatory treatments have been shown to be effective in reducing the development of morphine tolerance (Hutchinson *et al.*, 2007; 2008b; 2011). Of particular interest, inhibitors of glial activation, such as ibudilast and minocycline, have also been shown to reduce the expression of opioid withdrawal and reward, indicating the anti-abuse potential of these agents (Bland *et al.*, 2009; Hutchinson *et al.*, 2009).

PPARs are a group of nuclear receptor proteins that primarily regulate gene expression through their role as ligand-activated transcription factors (Michalik *et al.*, 2006). PPAR $\gamma$  is one of the three distinct isoforms identified (the other two are PPAR $\alpha$  and PPAR $\delta$ ) and is predominantly expressed in adipose tissue and macrophages. PPAR $\gamma$  regulates adipocyte differentiation and is involved in sugar and lipid homeostasis and in the control of inflammatory responses (Landreth and Heneka, 2001; Berger and Wagner, 2002; Kapadia *et al.*, 2008). Recent studies, however, have shown that PPAR $\gamma$  is also expressed in the CNS, where it has been found not only in neurons but also in astrocytes and microglia (Moreno *et al.*, 2004; Gofflot *et al.*, 2007; Sarruf *et al.*, 2009). Importantly, the activation of PPAR $\gamma$  results in a marked neuroprotective and anti-inflammatory response in the CNS (Landreth and Heneka, 2001; Berger and Wagner, 2002; Kapadia *et al.*, 2008; Tontonoz and Spiegelman, 2008). These effects have been attributed to the ability of PPAR $\gamma$  agonists to act as inhibitors of glial activation and to their subsequent ability to reduce NF- $\kappa$ B and pro-inflammatory cytokine expression (Tureyen *et al.*, 2007; Xing *et al.*, 2007). This background led us to hypothesize that inhibition of pro-inflammatory mechanisms following activation of PPAR $\gamma$  might result in the reduction of opioid tolerance and possibly an increase in their analgesic effect. To test this hypothesis, we studied the effect of pioglitazone, a brain-penetrating PPAR $\gamma$  agonist with modest effects on PPAR $\alpha$  (Gillies and Dunn, 2000; Smith, 2001), on the development of analgesic tolerance following chronic morphine administration. Using GW-9662, a selective PPAR $\gamma$  antagonist, we also examined the effect of receptor blockade on the development of morphine tolerance. Finally, using conditional neuronal PPAR $\gamma$  knockout (KO) mice, we

explored the effect of cell-specific deletion of PPAR $\gamma$  on morphine tolerance and the effects of pioglitazone.

## Methods

### Animals

Experiments were performed on male C57 mice (Harlan, Correzzana, Italy) and on conditional neuronal PPAR $\gamma$  KO mice and their wild-type (WT) counterparts (bred at the School of Pharmacy, University of Camerino, Italy). Mice weighed 28–30 g at the beginning of the experiment. KO mice had neuron-specific PPAR $\gamma$  deletion generated using nestin Cre-LoxP technology. Neural deletion of PPAR $\gamma$  was achieved by crossing mice with a floxed PPAR $\gamma$  allele [TgH(PPAR $\gamma$  lox)1Mgn, TgH(PPAR $\gamma$  del)2Mgn] with Nestin-Cre mice [B6.Cg-Tg(Nes-cre)1Kln/J]. All mice were fully backcrossed to a C57/BL6 genetic background (Jones *et al.*, 2002; Sarruf *et al.*, 2009). Animals were housed in ventilated plastic common cages (five animals per cage) in rooms with constant temperature (20–22°C) and humidity (45–55%) under a normal day/night cycle. During the experiments, animals were offered free access to tap water and food pellets (4RF18, Mucedola, Settimo Milanese, Milan, Italy). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). A total of 269 C57 male mice and 60 male PPAR $\gamma$  KO mice were used in the experiments. Experiments were approved by the ethical committee (Comitato Etico di Ateneo per la Protezione Animale) of the University of Camerino and are in adherence with the European Community Council Directive for Care and Use of Laboratory Animals.

### Drugs

Pioglitazone was prepared from Actos® 30 mg tablets (Takeda Pharmaceuticals, Tokyo, Japan). It was dissolved in sterile water and given p.o. via gavage in a volume of 10 mL·kg<sup>-1</sup> at doses of 10 or 30 mg·kg<sup>-1</sup> (Stopponi *et al.*, 2011).

GW-9662 was purchased from Sigma-Aldrich (Milan, Italy). It was dissolved in 5% DMSO, 5% cremophor and 90% distilled water, and injected i.p. in a volume of 10 mL·kg<sup>-1</sup> at the doses of 2.5 or 5 mg·kg<sup>-1</sup> (Ciccocioppo *et al.*, 2012).

Morphine hydrochloride was purchased from Salars (Milan, Italy). It was dissolved in 0.9% NaCl and injected i.p. in a volume of 10 mL·kg<sup>-1</sup>.

### Experimental procedure

To induce tolerance to morphine-induced antinociception, we injected morphine twice daily (between 0900 h and 1000 h and 1700 h and 1800 h) for 9 days (Contet *et al.*, 2008). On the first and last day of the experiment, we reduced the dose of morphine injected (15 mg·kg<sup>-1</sup>) to better show the development of tolerance. For the other 7 days of the experiment, the morphine dose was 30 mg·kg<sup>-1</sup> (in Exp. 1, 2 and 4) or 15 mg·kg<sup>-1</sup> (in Exp. 3 and 5). In certain experiments, mice received pioglitazone (0, 10 or 30 mg·kg<sup>-1</sup>) and/or GW-9662 (0, 2.5 or 5 mg·kg<sup>-1</sup>) before the morphine injections. For the evaluation of the effects of pioglitazone on reversal of morphine tolerance, animals received pioglitazone only in the evening of day 8 and on day 9, before the morphine injections. Analgesia was monitored on alternate days using the

tail immersion test 45 min after the evening injection. The tail immersion test is based on thermal (heat) noxious stimulus. This test was chosen because it involves a spinally mediated reflex response and can be repeated several times on the same animal (Mogil *et al.*, 2000; Le Bars *et al.*, 2001). Briefly, each mouse was restrained in a soft tissue pocket, and the distal half of the tail was dipped into a water bath set at 52°C. The latency to withdrawal of the tail from the water bath was measured. Two tail-withdrawal measures (separated by 30 s) were recorded and averaged. A 10 s cut-off time was applied to avoid tail burns. The tail immersion test was performed on alternate days 45 min after the evening morphine injection.

### Locomotor activity test and assessment of body temperature

Tolerance to morphine antinociception was induced in 32 male C57 mice with the same procedure described earlier. Body temperature was measured with a digital thermometer and a thermistor probe manufactured by Physitemp Instruments, Inc. (Clifton, NJ, USA). The body temperature was measured 45 min after the last injection of morphine by inserting the lubricated probe 2 cm into the rectum for 30 s.

Automated locomotor activity boxes (Med Associate, St. Albans, VT, USA) were used to quantify behavioural activity. Each animal was placed in the activity box, a square plastic box measuring 43 × 43 × 30 cm, and spontaneous locomotor activity parameters were monitored. Activity was recorded for 15 min, starting 5 min after placing the animal in the test cage. Locomotor activity of each mouse was automatically recorded by interruption of two orthogonal light beams, which were connected to an automatic software. The behavioural parameter observed was locomotion (as reflected by the number of beam breaks). Between each test session, the apparatus was cleaned with alcohol (70%) and dried with a clean cloth. Locomotor activity test was performed on day 9, 10 min after the assessment of body temperature.

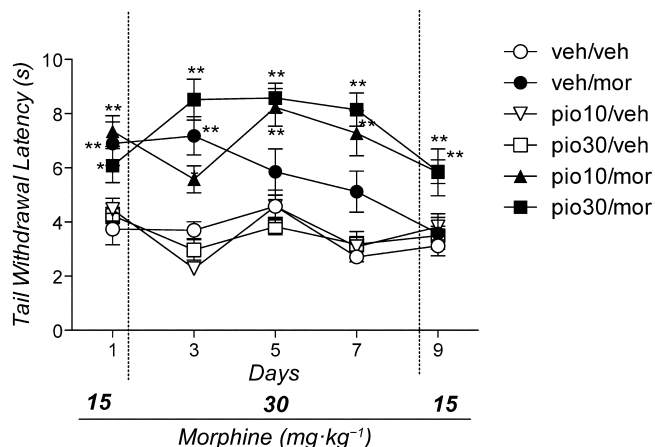
### Statistical analysis

The effect of pioglitazone, of GW-9662, or their combination on the development of morphine tolerance or in the reversal of its expression was analysed by mixed-factorial ANOVA with treatments (pioglitazone, morphine or their vehicles) as the between-subject factor and time (days) as the within-subject factor. Differences in the development of morphine tolerance in PPAR $\gamma$  KO mice and their WT counterparts were assessed by mixed-factorial ANOVA with strains (KO and WT) and treatment (morphine or saline) as the between-subject factors and time (days) as the within-subject factor. Where appropriate, the Newman-Keuls *post hoc* test was performed. Statistical significance was set at  $P < 0.05$ .

## Results

### Tolerance to the antinociceptive effect of morphine is attenuated by co-administration of the PPAR $\gamma$ agonist pioglitazone

C57 mice ( $n = 59$ ) were divided into six groups. Group 1 ( $n = 10$ ) received drug vehicles (veh/veh); group 2 ( $n = 10$ ) received pioglitazone vehicle plus morphine (veh/mor) at 15 (day 1



**Figure 1**

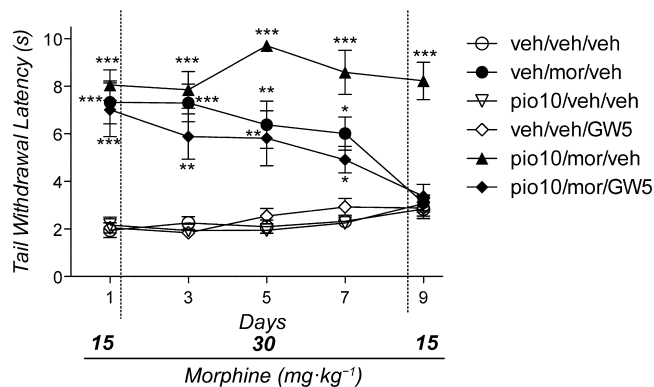
Effect of morphine, pioglitazone or their combination on tail immersion test. Mice ( $n = 59$ ) were divided into six groups: group 1 ( $n = 10$ ) received drug vehicles (veh/veh); group 2 ( $n = 10$ ) received pioglitazone and GW-9662 vehicles and morphine (veh/veh/mor); group 3 ( $n = 10$ ) and group 4 ( $n = 10$ ) received 10 or 30 mg·kg<sup>-1</sup> of pioglitazone followed by morphine vehicle (pio10/veh and pio30/veh); group 5 ( $n = 9$ ) and group 6 ( $n = 10$ ) received 10 or 30 mg·kg<sup>-1</sup> of pioglitazone followed by morphine (pio10/mor and pio30/mor). Significantly different from controls (veh/veh): \*\* $P < 0.01$  and \* $P < 0.05$ .

and 9) or 30 mg·kg<sup>-1</sup> (day 2–8); group 3 ( $n = 10$ ) and group 4 ( $n = 10$ ) received pioglitazone at 10 (pio10/veh) or 30 mg·kg<sup>-1</sup> plus morphine vehicle (pio30/veh); group 5 ( $n = 9$ ) and group 6 ( $n = 10$ ) received pioglitazone at 10 (pio10/mor) or 30 mg·kg<sup>-1</sup> (pio30/mor) plus morphine at 15 (day 1 and 9) or 30 mg·kg<sup>-1</sup> (day 2–8).

The ANOVA demonstrated a significant effect of treatment [ $F_{(5,53)} = 25.443$ ;  $P < 0.0001$ ], time [ $F_{(4,53)} = 9.78$ ;  $P < 0.0001$ ] and the treatment  $\times$  time interaction [ $F_{(20,212)} = 9.056$ ;  $P < 0.0001$ ]. As shown in Figure 1, the Newman–Keuls test revealed that, compared with the vehicle, morphine retained a significant antinociceptive effect on day 1, 3 ( $P < 0.01$ ) and 7 ( $P < 0.05$ ). Its antinociceptive efficacy progressively decreased, and on test day 9 no difference from controls was found. Treatment with pioglitazone attenuated the development of morphine tolerance, and, as shown in Figure 1, in the mice that received morphine and pioglitazone (10 and 30 mg·kg<sup>-1</sup>), opioid analgesia was maintained for the entire treatment period ( $P < 0.01$ ). Treatment with pioglitazone did not result in analgesic effects.

### *Treatment with the PPAR $\gamma$ antagonist GW-9662 blocked the effect of pioglitazone in the development of tolerance to the antinociceptive effect of morphine*

Mice ( $n = 59$ ) were divided into six groups. Group 1 ( $n = 10$ ) received drug vehicles (veh/veh/veh); group 2 ( $n = 10$ ) received GW-9662 and pioglitazone vehicles followed by morphine (veh/veh/mor); group 3 ( $n = 10$ ) received pioglitazone (10 mg·kg<sup>-1</sup>) plus morphine and GW-9662 vehicles (pio/veh/veh); group 4 ( $n = 10$ ) received GW-9662 (5 mg·kg<sup>-1</sup>) followed by pioglitazone and morphine vehicles (veh/GW5/



**Figure 2**

Effect of morphine, pioglitazone, GW-9662 or their combination on tail immersion test. Mice ( $n = 59$ ) were divided into six groups: group 1 ( $n = 10$ ) received drug vehicles (veh/veh/veh); group 2 ( $n = 10$ ) received pioglitazone and GW-9662 vehicles and morphine (veh/veh/mor); group 3 ( $n = 10$ ) received pioglitazone (10 mg·kg<sup>-1</sup>) and morphine and GW-9662 vehicles (pio10/veh/veh); group 4 ( $n = 10$ ) received pioglitazone and morphine vehicles and GW-9662 (5 mg·kg<sup>-1</sup>) (veh/veh/GW5); group 5 ( $n = 10$ ) received pioglitazone (10 mg·kg<sup>-1</sup>), morphine and GW-9662 vehicle (pio10/veh/mor); group 6 ( $n = 9$ ) received pioglitazone, morphine and GW-9662 (5 mg·kg<sup>-1</sup>) (pio10/mor/GW5). Significantly different from controls (veh/veh/veh): \*\*\* $P < 0.001$ , \*\* $P < 0.01$  and \* $P < 0.05$ .

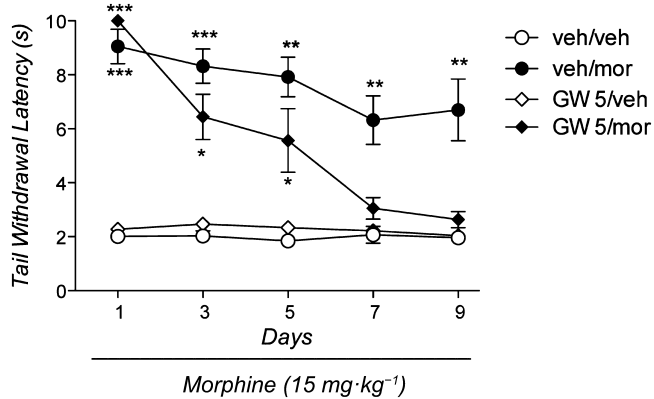
veh); group 5 ( $n = 10$ ) received pioglitazone 10 mg·kg<sup>-1</sup>, morphine and GW-9662 vehicle (pio/veh/mor); group 6 ( $n = 9$ ) received pioglitazone, morphine and GW-9662 (5 mg·kg<sup>-1</sup>) (pio/GW5/mor).

The ANOVA revealed a significant effect of treatment [ $F_{(5,41)} = 62.79$ ;  $P < 0.001$ ], time [ $F_{(4,41)} = 2.41$ ;  $P < 0.05$ ] and the treatment  $\times$  time interaction [ $F_{(20,164)} = 3.54$ ;  $P < 0.001$ ]. As shown in Figure 2, the Newman–Keuls test revealed that morphine significantly increased the latency of tail withdrawal ( $P < 0.01$ ). However, its effect progressively decayed, and on day 9 it was no longer evident. This result confirmed the previous results, in that the co-administration of pioglitazone (10 mg·kg<sup>-1</sup>) attenuated the development of morphine tolerance, and the analgesic effect was maintained throughout the treatment period ( $P < 0.01$ ). Of note, pretreatment with GW-9662 (5 mg·kg<sup>-1</sup>) completely antagonized the effect of pioglitazone (10 mg·kg<sup>-1</sup>), and the analgesic curve with this treatment was indistinguishable from that generated by morphine alone.

Treatment with pioglitazone or GW-9662 did not provide analgesic effects.

### *Blockade of PPAR $\gamma$ accelerates the development of tolerance to the antinociceptive effect of morphine*

In this experiment, a lower dose (15 mg·kg<sup>-1</sup>) of morphine was used for the duration of treatment to elicit mild tolerance. Mice ( $n = 39$ ) were divided into four groups. Group 1 ( $n = 10$ ) received drug vehicles (veh/veh); group 2 ( $n = 10$ ) received GW-9662 vehicle and morphine (veh/mor); group 3 ( $n = 10$ ) received GW-9662 (5 mg·kg<sup>-1</sup>) and morphine vehicle (GW5/veh); group 4 ( $n = 9$ ) received GW-9662 (5 mg·kg<sup>-1</sup>) and morphine (GW5/mor).



**Figure 3**

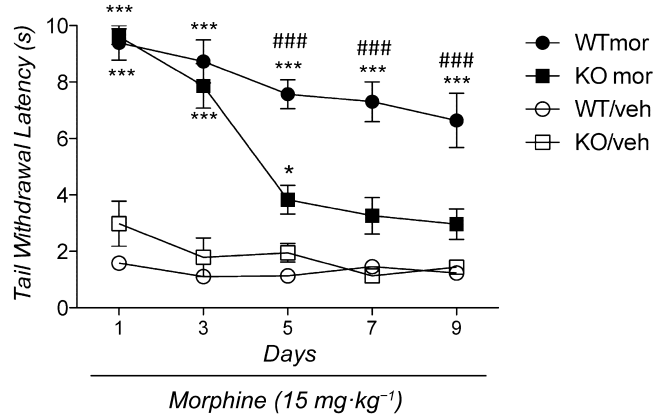
Effect of morphine, GW-9662 or their combination on tail immersion test. Mice ( $n = 39$ ) were divided into four groups: group 1 ( $n = 10$ ) received drug vehicles (veh/veh); group 2 ( $n = 10$ ) received GW-9662 vehicle and morphine (veh/mor); group 3 ( $n = 10$ ) received GW-9662 ( $5 \text{ mg}\cdot\text{kg}^{-1}$ ) and morphine vehicle (GW5/veh); group 4 ( $n = 9$ ) received GW-9662 ( $5 \text{ mg}\cdot\text{kg}^{-1}$ ) and morphine (GW5/mor). Significantly different from controls (veh/veh): \*\*\* $P < 0.001$ , \*\* $P < 0.01$  and \* $P < 0.05$ .

The ANOVA revealed a significant effect of treatment [ $F_{(3,35)} = 28.84$ ;  $P < 0.0001$ ], time [ $F_{(3,35)} = 17.16$ ;  $P < 0.001$ ] and the treatment  $\times$  time interaction [ $F_{(9,105)} = 3.01$ ;  $P < 0.01$ ]. As shown in Figure 3, in morphine-treated animals, no overt signs of tolerance were observed and the Newman–Keuls test revealed a significant antinociceptive effect of morphine throughout the treatment period ( $P < 0.01$ ). However, in the group treated with  $5.0 \text{ mg}\cdot\text{kg}^{-1}$  GW-9662 plus morphine, the antinociceptive effect of the opioid progressively decreased and the difference from vehicle-treated mice was no longer significant at days 7–9.

### Development of tolerance to the antinociceptive effect of morphine is more pronounced in conditional neuronal PPAR $\gamma$ KO mice than in WT controls

As in the previous experiment,  $15 \text{ mg}\cdot\text{kg}^{-1}$  of morphine was used for the duration of treatment to elicit mild tolerance. PPAR $\gamma$  KO mice ( $n = 20$ ) and WT controls ( $n = 20$ ) were divided into two groups each ( $n = 10/\text{group}$ ) and were treated with  $15 \text{ mg}\cdot\text{kg}^{-1}$  of morphine (morph) or with its vehicle (veh).

The ANOVA revealed a significant effect of treatment [ $F_{(3,36)} = 251.41$ ;  $P < 0.0001$ ], time [ $F_{(4,36)} = 20.37$ ;  $P < 0.0001$ ] and the treatment  $\times$  time interaction [ $F_{(12,144)} = 20.37$ ;  $P < 0.0001$ ]. Significant effects of strain [ $F_{(1,36)} = 11.11$ ;  $P < 0.01$ ], strain  $\times$  treatment interaction [ $F_{(1,36)} = 28.5$ ;  $P < 0.0001$ ], time  $\times$  strain interaction [ $F_{(4,44)} = 6.09$ ;  $P < 0.001$ ] and time  $\times$  strain  $\times$  treatment interaction [ $F_{(4,144)} = 2.23$ ;  $P < 0.05$ ] were also observed. As shown in Figure 4, the Newman–Keuls *post hoc* analysis demonstrated that morphine elicited a significant antinociceptive effect in both PPAR $\gamma$  KO mice and WT controls. However, over the course of the treatment, the antinociceptive effect of morphine decreased faster in KO mice than in WT controls. This phenomenon was confirmed by the statistical analysis, which showed a significant effect of morphine



**Figure 4**

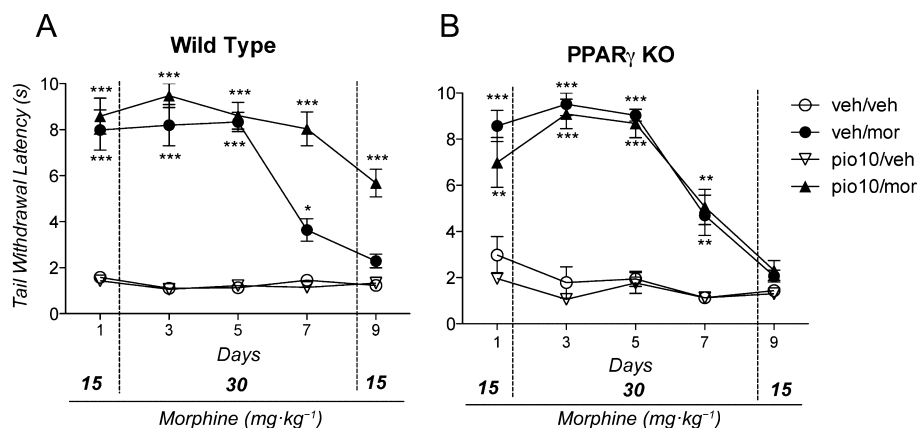
Effect of morphine on tail immersion test in PPAR $\gamma$  knockout (KO) and wild-type (WT) mice. Each line was divided into two groups containing ( $n = 10/\text{group}$ ): groups 1 (WT/veh) and 2 (KO/veh) received morphine vehicle; groups 3 (WT mor) and 4 (KO mor) received morphine at the dose of  $15 \text{ mg}\cdot\text{kg}^{-1}$ . Significantly different from respective control vehicle-treated mice: \* $P < 0.05$  and \*\*\* $P < 0.0001$ . Significant difference between KO (mor) and WT (mor) mice: ### $P < 0.0001$ .

throughout the treatment period in WT mice ( $P < 0.001$ ), whereas in KO mice the effect remained significant until day 5 and disappeared on day 7.

### Treatment with pioglitazone attenuated the development of tolerance to the antinociceptive effect of morphine in WT but not in PPAR $\gamma$ KO mice

PPAR $\gamma$  KO ( $n = 40$ ) and WT ( $n = 40$ ) mice were both divided into four groups of 10 animals each. Group 1 received drug vehicles (veh/veh); group 2 received morphine ( $15 \text{ mg}\cdot\text{kg}^{-1}$  on day 1 and 9;  $30 \text{ mg}\cdot\text{kg}^{-1}$  on days 2–8) plus pioglitazone vehicle (veh/mor); group 3 received  $10 \text{ mg}\cdot\text{kg}^{-1}$  of pioglitazone and morphine vehicle (pio10/veh); group 4 received morphine ( $15 \text{ mg}\cdot\text{kg}^{-1}$  on day 1 and 9;  $30 \text{ mg}\cdot\text{kg}^{-1}$  on days 2–8) plus  $10 \text{ mg}\cdot\text{kg}^{-1}$  of pioglitazone (pio10/mor).

As shown in Figure 5A, the ANOVA revealed a significant effect of the treatment [ $F_{(3,36)} = 251.41$ ;  $P < 0.0001$ ], time [ $F_{(4,36)} = 20.37$ ;  $P < 0.0001$ ] and treatment  $\times$  time interaction [ $F_{(12,144)} = 20.37$ ;  $P < 0.0001$ ] in WT mice. *Post hoc* Newman–Keuls analysis showed that morphine significantly increased the latency of tail withdrawal on days 1, 3 and 5 ( $P < 0.001$ ). The antinociceptive effect of morphine progressively decreased over the course of the treatment, and on day 9 it was no longer significant compared with vehicle-treated animals. Pretreatment with pioglitazone markedly attenuated the development of tolerance. Compared with the vehicle, the antinociceptive effect of morphine remained significant for the entire treatment period ( $P < 0.001$ ). Pioglitazone alone had no effect on the tail immersion test. As shown in Figure 5B, the ANOVA revealed a significant effect of treatment [ $F_{(3,36)} = 58.73$ ;  $P < 0.0001$ ], time [ $F_{(4,36)} = 56.95$ ;  $P < 0.0001$ ] and treatment  $\times$  time interaction [ $F_{(12,144)} = 14.46$ ;  $P < 0.0001$ ] in PPAR $\gamma$  KO mice. The Newman–Keuls test showed that morphine significantly increased the latency of tail withdrawal



**Figure 5**

Effect of morphine, pioglitazone or their combination on tail immersion test in: (A) wild-type (WT) mice that were divided into four groups ( $n = 10$ /group): group 1 (veh/veh) received drug vehicles; group 2 (veh/mor) received pioglitazone vehicle and morphine; group 3 (pio10/veh) received  $10 \text{ mg}\cdot\text{kg}^{-1}$  of pioglitazone plus morphine vehicle; group 4 (pio10/mor) received  $10 \text{ mg}\cdot\text{kg}^{-1}$  of pioglitazone and morphine; (B) PPAR $\gamma$  knockout (KO) mice that were divided into four groups + ( $n = 10$ /group): group 1 (veh/veh) received drug vehicles; group 2 (veh/mor) received pioglitazone vehicle and morphine; group 3 (pio10/veh) received  $10 \text{ mg}\cdot\text{kg}^{-1}$  of pioglitazone plus morphine vehicle; group 4 (pio10/morph) received  $10 \text{ mg}\cdot\text{kg}^{-1}$  of pioglitazone and morphine. Significantly different from controls (veh/veh): \*\*\* $P < 0.0001$ , \*\* $P < 0.01$  and \* $P < 0.05$ .

on days 1–5 ( $P < 0.001$ ) and on day 7 ( $P < 0.05$ ). However, the antinociceptive effect of morphine progressively decreased over the treatment period, and on day 9 it was no longer different from vehicle-treated animals. Pretreatment with pioglitazone did not attenuate the development of tolerance to the antinociceptive effect of morphine, indicating its ineffectiveness in mice lacking neuronal PPAR $\gamma$  receptors.

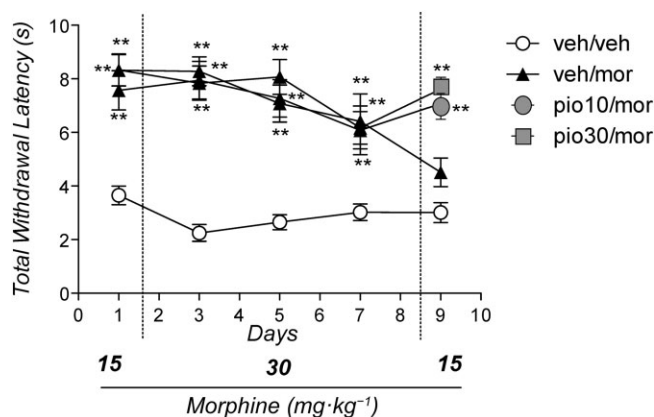
### Reversal of tolerance to the antinociceptive effect of morphine, by administration of the PPAR $\gamma$ agonist pioglitazone

C57 mice ( $n = 40$ ) were divided into four groups. Group 1 ( $n = 10$ ) received drug vehicles (veh/veh); group 2 ( $n = 10$ ) received pioglitazone vehicle (evening day 8 and day 9) plus morphine (veh/mor) at  $15 \text{ mg}\cdot\text{kg}^{-1}$  (day 1 and 9) or  $30 \text{ mg}\cdot\text{kg}^{-1}$  (day 2–8); group 3 ( $n = 10$ ) and group 4 ( $n = 10$ ) received  $10 \text{ mg}\cdot\text{kg}^{-1}$  (pio10/mor) or  $30 \text{ mg}\cdot\text{kg}^{-1}$  (pio30/mor) of pioglitazone (evening day 8 and day 9) plus morphine at  $15 \text{ mg}\cdot\text{kg}^{-1}$  (day 1 and 9) or  $30 \text{ mg}\cdot\text{kg}^{-1}$  (day 2–8).

The ANOVA demonstrated a significant effect of treatment [ $F_{(3,36)} = 31.995$ ;  $P < 0.0001$ ], time [ $F_{(4,36)} = 5.7775$ ;  $P < 0.001$ ] and the treatment  $\times$  time interaction [ $F_{(12,144)} = 2.412$ ;  $P < 0.01$ ]. As shown in Figure 6, the Newman–Keuls test revealed that, compared with the vehicle, morphine retained a significant antinociceptive effect on day 1, 3, 5 and 7 ( $P < 0.01$ ). Its antinociceptive efficacy progressively decreased, and on test day 9 no difference from controls was found. Treatment with pioglitazone (on day 8 and 9) completely reversed the expression of morphine tolerance, and, as shown in Figure 6, in the mice that received morphine and pioglitazone ( $10$  and  $30 \text{ mg}\cdot\text{kg}^{-1}$ ), opioid analgesia was restored on day 9 ( $P < 0.01$ ).

### Effect of pioglitazone on locomotor activity and body temperature

C57 mice ( $n = 32$ ) were divided into four groups. Group 1 ( $n = 8$ ) received drug vehicles (veh/veh); group 2 ( $n = 8$ ) received



**Figure 6**

Effect of pioglitazone on reversal of morphine tolerance on tail immersion test. Mice ( $n = 40$ ) were divided into four groups. Group 1 ( $n = 10$ ) received drug vehicles (veh/veh); group 2 ( $n = 10$ ) received pioglitazone vehicle (evening day 8 and day 9) plus morphine (veh/mor) at  $15 \text{ mg}\cdot\text{kg}^{-1}$  (day 1 and 9) or  $30 \text{ mg}\cdot\text{kg}^{-1}$  (day 2–8); group 3 ( $n = 10$ ) and group 4 ( $n = 10$ ) received  $10 \text{ mg}\cdot\text{kg}^{-1}$  (pio10/mor) or  $30 \text{ mg}\cdot\text{kg}^{-1}$  (pio30/mor) of pioglitazone (evening day 8 and day 9) plus morphine at  $15 \text{ mg}\cdot\text{kg}^{-1}$  (day 1 and 9) or  $30 \text{ mg}\cdot\text{kg}^{-1}$  (day 2–8). Significantly different from controls (veh/veh): \*\* $P < 0.01$ .

pioglitazone vehicle plus morphine (veh/mor) at  $15 \text{ mg}\cdot\text{kg}^{-1}$  (day 1 and 9) or  $30 \text{ mg}\cdot\text{kg}^{-1}$  (day 2–8); group 3 ( $n = 8$ ) received pioglitazone  $30 \text{ mg}\cdot\text{kg}^{-1}$  plus morphine vehicle (pio30/veh). Group 4 ( $n = 8$ ) received  $30 \text{ mg}\cdot\text{kg}^{-1}$  (pio30/mor) of pioglitazone plus morphine at  $15 \text{ mg}\cdot\text{kg}^{-1}$  (day 1 and 9) or  $30 \text{ mg}\cdot\text{kg}^{-1}$  (day 2–8). Results showed no significant differences between groups in terms of body temperature [ $F_{(3,28)} = 2.316$ ;  $P$  not significant] (data not shown). In the locomotor activity test, ANOVA demonstrated a significant effect of the treatment [ $F_{(3,28)} = 26.27$ ;  $P < 0.001$ ]. The total distance travelled was

2106.7  $\pm$  175.9 cm for the group veh/veh, 2362.8  $\pm$  177.7 cm for the group pio30/veh, 3712.0  $\pm$  579.7 cm for the group veh/mor and 6066.8  $\pm$  660.3 cm for the group pio30/mor. Newman-Keuls *post hoc* test showed that locomotor activity was increased in animals treated with morphine ( $P < 0.05$ ). Treatment with pioglitazone was able to potentiate the morphine-induced increase of locomotor activity in mice ( $P < 0.001$ ).

## Discussion and conclusions

The results showed that repeated daily administration of morphine lead to a rapid development of tolerance to its antinociceptive effect. Concomitant administration of pioglitazone markedly reduced the development of tolerance. In addition, we demonstrated that acute treatment with pioglitazone was able to reverse an established morphine tolerance. Pioglitazone alone did not show any effect in response to noxious thermal stimuli, suggesting that this compound lacks antinociceptive properties in our experimental model. Hence, the observed effect on morphine tolerance cannot be secondary to changes in the pain threshold following pioglitazone administration. This finding is particularly relevant because previous studies have shown that activation of PPAR $\gamma$  elicits a pronounced attenuation of neuropathic pain following partial nerve ligation in rats (Churi *et al.*, 2008; Iwai *et al.*, 2008; Fehrenbacher *et al.*, 2009; Takahashi *et al.*, 2011; Morgenweck *et al.*, 2013). Importantly, pioglitazone did not alter body temperature neither reduced locomotor activity, ruling out unspecific or sedative effects that might have influenced animals' reactivity in the tail withdrawal test. To confirm that the effect of pioglitazone was mediated by PPAR $\gamma$ , in a subsequent set of experiments, we administered the selective receptor antagonist GW-9662 prior to pioglitazone. As expected, co-administration of the PPAR $\gamma$  blocker completely abolished the effect of pioglitazone on morphine tolerance. Confirming the lack of a role of PPAR $\gamma$  in thermal analgesia, GW-9662 alone did not show effects in the tail immersion test. Intriguingly, when animals were treated with GW-9662, we observed a tendency towards the facilitation of analgesic tolerance to morphine. We explored this possibility by treating animals with a lower dose of morphine (15 mg·kg<sup>-1</sup>), which, when given for the entire treatment period (9 days), led to a modest non-significant analgesic tolerance. Confirming our initial observations, co-administration of GW-9662 significantly accelerated the development of tolerance, and the analgesic effect of morphine started to decrease from treatment day 3. This is a major finding that indicates a role of PPAR $\gamma$  in the regulation of the endogenous mechanisms controlling the development of tolerance to the antinociceptive effect of morphine. An intriguing hypothesis, therefore, is that conditions associated with reduced tonic activity or down-regulation of PPAR $\gamma$  may facilitate opioid tolerance. Some evidence may support this view; for example, it has been shown that injections of the bacterial endotoxin lipopolysaccharide accelerate the development of analgesic tolerance to morphine, but they also reduce the expression of the PPAR $\gamma$  transcript (Johnston and Westbrook, 2005; Necela *et al.*, 2008). Conversely, physical stress that has been associated with increased PPAR $\gamma$  expres-

sion (Garcia-Bueno *et al.*, 2005), among other effects on analgesia, also reduces the development of morphine tolerance (MacRae and Siegel, 1987; Takahashi *et al.*, 1988; Yamashiro *et al.*, 1990). A growing body of evidence indicates that opioids activate glial cells and modulate the immune response and that these effects may have a major impact in opioid tolerance and possibly also addiction (Narita *et al.*, 2006; Hutchinson *et al.*, 2007; 2008a). Hence, one potential mechanism through which PPAR $\gamma$  agonists may reduce tolerance to the antinociceptive effects of morphine is through their ability to act as a glial inhibitors and to reduce the expression of pro-inflammatory factors such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$  and NF- $\kappa$ B (Bernardo and Minghetti, 2006). However, we found that the effect of pioglitazone is not present in conditional KO mice lacking the PPAR $\gamma$  receptor in neurons. We also observed facilitation of the development of analgesic tolerance to morphine in these mice, which further confirms the role of neuronal PPAR $\gamma$  in the regulation of this phenomenon. Based on these findings, it seems, therefore, that neuron-mediated mechanisms are responsible for the effects of pioglitazone observed here. However, an indirect role of glia-mediated mechanisms that could be under the control of neuronal PPAR $\gamma$  cannot be excluded. Further studies are needed in order to clarify this point.

Like for opioids, chronic administration of cannabinoid agonists results in the development of tolerance to most of their acute pharmacological effects (Maldonado, 2002). Importantly, the interaction between cannabinoids and opioids on the development of tolerance is supported by cross-modulation of several pharmacological responses induced by these compounds, such as antinociception, hypolocomotion, catalepsy and hypothermia (Pertwee *et al.*, 1993; Fan *et al.*, 1994; Vigano *et al.*, 2005; Maguma and Taylor, 2011). Based on these functional similarities between the opioid and the cannabinoid systems and given that PPAR $\gamma$  mediates some of the cannabinoid effects on neuroinflammation (O'Sullivan *et al.*, 2006; Sun *et al.*, 2006), we speculate that treatment with pioglitazone could also affect the development of cannabinoid tolerance. However, this aspect needs to be clarified with further studies.

Recently, important effects mediated by brain PPAR $\gamma$  in relation to drugs of abuse have been described. For instance, our laboratory has shown that administration of pioglitazone attenuates alcohol self-administration and relapse to alcohol seeking in rats (Stopponi *et al.*, 2011; 2013). More importantly, we also found that it reduced intravenous heroin self-administration and morphine-induced reward, possibly by inhibiting the opioid stimulation of mesolimbic dopamine transmission (Ciccocioppo *et al.*, 2012). Altogether, these results suggest that the combination of PPAR $\gamma$  agonists with opioid agents may reduce the development of analgesic tolerance and possibly reduce the potential for abuse of these compounds. It is noteworthy that patients who have become physically dependent on opioids following chronic pain treatment may persist with drug use once the pain has dissipated to avoid withdrawal symptoms (Savage *et al.*, 2008). Delayed development of tolerance should reduce the requirement for escalating its dosage to maintain adequate chronic analgesia. This, in turn, may diminish the development of physical dependence and therefore withdrawal when the treatment is discontinued. Moreover, reduced escalation of



the opioid dose may also favourably impact the side effects associated with the use of high doses of these drugs such as sedation, respiratory depression, constipation, nausea and urinary retention. PPAR $\gamma$  agonists are also known to have intrinsic anti-inflammatory properties and to reduce neuropathic pain (Churi *et al.*, 2008; Iwai *et al.*, 2008; Napimoga *et al.*, 2008; Fehrenbacher *et al.*, 2009; Takahashi *et al.*, 2011; Morgenweck *et al.*, 2013). This effect, combined with the ability of PPAR $\gamma$  agonists to attenuate opioid tolerance and addiction, suggests the attractive possibility of combining them with opioids to achieve an enhanced analgesic effect and reduced potential for abuse. This approach could have a relevant clinical impact because management of chronic pain (including neuropathic pain) remains an important medical need that is limited by all the problems linked to protracted opioid use. Another major clinical problem is the use of opioid analgesics in patients at a high risk of developing dependence, such as individuals with a positive history of substance misuse (i.e., alcohol, benzodiazepines etc.). Currently, these patients are less likely to receive effective pain treatment due to concerns of opioid misuse (Rupp and Delaney, 2004). The combination of PPAR $\gamma$  agonists and opioids may help to address the ethical issue related to the appropriateness of the use of opioid analgesics in those patients. In summary, our results indicate that PPAR $\gamma$  plays an important role in the modulation of tolerance to morphine, and pioglitazone may potentially be used as an add-on medication to attenuate the development of opioid tolerance when these drugs are used for chronic pain. Clinical trials to test this possibility should be relatively easy to conduct because pioglitazone is already used clinically and its pharmacological and toxicological profile has been well documented.

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## Author contributions

G. d. G. and R. C. were responsible for the study concept and design. G. d. G. and G. S. contributed to the acquisition of animal data and drafted the manuscript. M. K. and S. S. assisted with interpretation of findings and contributed with data collection. G. D. and G. G. provided critical revision of the manuscript for important intellectual content. All authors critically reviewed the content and approved the final version for publication.

## Conflict of interest

G. D. is Chairman and CEO and G. G. is Chief Scientific Officer of Omeros Corporation. Omeros exclusively controls

the intellectual property rights from the University of Camerino. R. C. is the inventor on a number of patent applications, which have been assigned to Omeros, relating to the therapeutic use of PPAR $\gamma$  agonists in addiction. He is entitled to receive payments and royalties from Omeros under such licensing arrangement. The other authors have no conflict of interest.

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