Psychopharmacology (2017) 234:223–234
DOI 10.1007/s00213-016-4452-1

ORIGINAL INVESTIGATION

Pioglitazone attenuates the opioid withdrawal and vulnerability to relapse to heroin seeking in rodents

Giordano de Guglielmo [1,2] · Marsida Kallupi [1,2] · Giulia Scuppa [1] · Gregory Demopulos [3] · George Gaitanaris [3] · Roberto Ciccocioppo [1]

Received: 30 June 2016 / Accepted: 26 September 2016 / Published online: 6 October 2016

Abstract
Rationale Relapse to opioids is often driven by the avoidance of the aversive states of opioid withdrawal. We recently demonstrated that activation of peroxisome proliferator-activated receptor gamma (PPARγ) by pioglitazone reduces the motivation for heroin and attenuates its rewarding properties. However, the role of PPARγ in withdrawal and other forms of relapse to heroin is unknown.
Objectives To further address this issue, we investigated the role of PPARγ on the development and expression of morphine withdrawal in mice and the effect of pioglitazone on several forms of heroin relapse in rats.
Methods We induced physical dependence to morphine in mice by injecting morphine twice daily for 6 days. Withdrawal syndrome was precipitated on day 6 with an injection of naloxone. In addition, different groups of rats were trained to self-administer heroin and, after the extinction, the relapse was elicited by cues, priming, or stress. The effect of different doses of pioglitazone was tested on these different paradigms.
Results Data show that chronic and acute administration of pioglitazone attenuates morphine withdrawal symptoms, and these effects are mediated by activation of PPARγ receptors. Activation of PPARγ by pioglitazone also abolishes yohimbine-induced reinstatement of heroin seeking and reduces heroin-induced reinstatement, while it does not affect cue-induced relapse.
Conclusions These findings provide new insights on the role of PPARγ on opioid dependence and suggest that pioglitazone may be useful for the treatment of opioid withdrawal in opioid-addicted individuals.

Keywords Addiction · Heroin · Pioglitazone · Withdrawal · Relapse · Yohimbine · Cues · Priming

✉ Giordano de Guglielmo
giordano@scripps.edu

✉ Roberto Ciccocioppo
roberto.ciccocioppo@unicam.it

[1] School of Pharmacy, Pharmacology Unit, University of Camerino, via Madonna delle Carceri, 62032 Camerino, MC, Italy

[2] Committee on the Neurobiology of Addictive Disorders, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

[3] Omeros Corporation, 201 Elliot Avenue West, Seattle, WA 98119, USA

Introduction

Opioid dependence is emerging as a major health problem caused by consumption of illicit compounds such as heroin or inappropriate use of prescription narcotics (UNODC 2012). Following chronic exposure to opioid agonists, tolerance and dependence develop. An abrupt suspension of drug use is often associated with the expression of severe withdrawal syndrome, characterized by somatic signs (e.g., stomach cramps, diarrhea, rhinorrhea, sweating, elevated heart rate, and increased blood pressure), and negative affects including dysphoria, anxiety, and depression (O'Brien 1996). Several studies have shown that in dependent individuals, the seeking and consumption of opioids is often driven by negative reinforcement mechanisms to avoid the extremely aversive withdrawal symptoms associated with the cessation of use rather than being motivated by the anticipation of heroin reward itself. In addition, when patients reach abstinence following detoxification, relapse to drug use often results from two factors, environmental conditioning and stress (Weiss et al. 2001).

Currently, the most successful pharmacotherapy used to treat opioid dependence involves opioid substitution therapy (OST) (Stotts et al. 2009). Approved medications used for this purpose are methadone and buprenorphine (alone or in combination with naloxone). Administration under medical supervision of these compounds has been shown to be effective in treating withdrawal symptoms and drug craving without eliciting marked euphoric response (Gonzalez et al. 2004; Kreek and Vocci 2002). Nonetheless, although OST substantially improves the management of opiate withdrawal and dependence, relapse to opioid seeking often occurs when the dose of the medication is tapered or the therapy is suspended (Kreek and Vocci 2002). In addition, these medications are characterized by substantial toxicity linked to their abuse potential (Fugelstad et al. 2007; Strang et al. 2010). Development of novel therapeutic approaches that are able to reduce opioid withdrawal and relapse without intrinsic addictive properties and the associated high-level requisite of medical supervision remains a major goal in addiction medicine.

Recent findings from our laboratory have shown that peroxisome proliferator-activated receptor-gamma (PPARγ), one of the three members of the nuclear PPAR receptor family, is a potential target for the treatment of opioid dependence. Activation of PPARγ by the selective agonist pioglitazone is able to reduce heroin self-administration, blunting the ability of heroin in activating the mesolimbic dopamine reward system (de Guglielmo et al. 2015). PPARγ also plays a tonic role in the modulation of morphine tolerance, and its pharmacological activation reduces the development of analgesic tolerance (de Guglielmo et al. 2014). These findings prompted us to test whether activation of PPARγ affects negative reinforcement mechanisms associated with opioid withdrawal and abstinence. To test this hypothesis, we studied the effect of pioglitazone on the expression and development of morphine withdrawal following chronic morphine administration. Using GW-9662, a selective PPARγ antagonist, we also examined the effect of receptor blockade on the expression of morphine withdrawal. Finally, we evaluated the efficacy of pioglitazone in preventing relapse to drug seeking using three classical drug reinstatement models in rats: stress-, cue-, and heroin-induced reinstatement.

In stress-induced reinstatement, the resumption of drug seeking was obtained following the injection of the pharmacological stressor yohimbine, while in cue-induced reinstatement, the relapse was induced by reexposure to the previous paired drug discriminative cues (Ciccocioppo et al. 2001; Shaham et al. 2003; Stewart 2003). Heroin-induced reinstatement (drug priming) refers to noncontingent injections of heroin prior to the reinstatement test (de Wit and Stewart 1983).

Materials and methods

Animals

Male Wistar rats (Charles River, Calco, Italy) weighing 150–200 g at the beginning of the experiments and male CD1 mice (Harlan, Italy) weighing 20–25 g at the beginning of the experiments were used. Pairs of rats were housed in a room with artificial 12:12-h light/dark cycle (lights off at 9 a.m.). Mice were housed in ventilated plastic common cages (five animals per cage) under a normal day/night cycle. Animals were housed in rooms with constant temperature (20–22 °C) and humidity (45–55 %). Animals were given ad libitum access to food and water throughout except during experimental test sessions. All procedures were conducted in adherence with the European Community Council Directive for Care and Use of Laboratory Animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Intravenous catheterization

Rats were anesthetized by intramuscular injection of 100–150 μl of a solution containing tiletamine chloridrate (58.17 mg/ml) and zolazepam chloridrate (57.5 mg/ml). For intravenous catheterization (IV) surgery, incisions were made to expose the right jugular vein and a catheter made from silicon tubing (I.D. = 0.020 in., O.D. = 0.037 in.) was subcutaneously positioned. After insertion into the vein, the proximal end of the catheter was anchored to the muscles underlying the vein with surgical silk. The distal end of the catheter was attached to a stainless-steel cannula bent at a 90° angle. The cannula was inserted in a support made by dental cement on the scull of the animals, fixed with screws, and covered with a plastic cap. For 1 week after surgery, rats were daily treated with 0.2 ml of the antibiotic sodium cefotaxime (262 mg/ml). For the duration of the experiments, catheters were daily flushed with 0.2–0.3 ml of heparinized saline solution. Body weights were monitored every day, and catheter patency was confirmed approximately every week with an injection of 0.2–0.3 ml of thiopental sodium (250 mg/ml) solution. Patency of the catheter was assumed if there was an immediate loss of reflexes. Self-administration experiments began 1 week after surgery.

Drugs

Pioglitazone was prepared from Actos® (30 mg) tablets (Takeda). The powder was transferred into 20-ml test tubes and was suspended in distilled water (10, 30, and 60 mg/ml according to the drug dose used). For mice, the volume of injection was 10 ml/kg. The test tubes were vortexed before filling a 1-ml syringe for oral injection. Drug vehicle consisted of distilled water that was given per os (PO), which is the same

route of administration of the active agent. Pioglitazone was given twice a day (12 hours and 1 hour) prior to tests. GW-9662 was purchased from Sigma-Aldrich (Milano, Italy). It was dissolved in 5 % DMSO, 5 % cremophor, and 90 % distilled water, and injected intraperitoneally (IP) in a volume of 10 ml/kg at the doses of 2.5 or 5 mg/kg. Morphine hydrochloride was purchased from Salars (Milano, Italy). It was dissolved in 0.9 % NaCl and injected IP at the dose of 10 mg/kg in a volume of 10 ml/kg. Naloxone was purchased from Sigma-Aldrich (Milano, Italy). It was dissolved in NaCl 0.9 % and was injected subcutaneously (SC) at the dose of 1 mg/kg in a volume of 10 ml/kg. Heroin (SALARS, Como, Italy) was dissolved in 0.9 % NaCl and self-administered intravenously (20 μg/0.1 ml/infusion). Yohimbine (Sigma-Aldrich) was dissolved in distilled water and injected IP at the concentration of 1.25 mg/kg.

Operant training

The self-administration stations consisted of operant conditioning chambers (Med Associate Inc.) enclosed in light, sound-attenuating, ventilated environmental cubicles. The front door and the back wall of the chamber were made of transparent plastic, and the other walls were opaque metal. Each chamber was equipped with two retractable levers located in the front panel of the chamber. Heroin was delivered through a plastic tube that was connected to the catheter before the beginning of the session. An infusion pump was activated by responses on the right "active" lever, while responses on the left "inactive" lever were recorded but did not result in any programmed consequences. Activation of the pump resulted in a delivery of 0.1 ml of fluid. An IBM-compatible computer controlled the delivery of fluids and recording of the behavioral data.

Induction of morphine dependence and withdrawal in mice

Physical dependence on morphine in mice was induced by twice-daily (IP) injections of morphine (10 mg/kg) for five consecutive days. On the morning of day 6, mice were additionally treated with morphine. Withdrawal syndrome was precipitated 2 h later by a SC injection of naloxone (1 mg/kg). Mice were then immediately placed inside a transparent cylinder (50 cm high × 30 cm diameter) and observed for 30 min. The following somatic withdrawal signs were monitored and counted: total number of jumps, paw tremors, teeth chattering, and wet dog shakes. Occurrence of ptosis and piloerection were scored, giving a value to each withdrawal sign from 0 to 3. In order to summarize the results obtained from the different observations, a global withdrawal syndrome score of all the animals was calculated from each withdrawal sign.

Effect of pioglitazone administration on the expression of morphine withdrawal

Mice (n = 50) were divided into five groups: the first one (n = 10) received distilled water and saline (the vehicles of pioglitazone and of morphine); the second group (n = 10) received distilled water and morphine (10 mg/kg); the third (n = 10) received pioglitazone (30 mg/kg) and saline; the fourth group (n = 10) was injected with pioglitazone (10 mg/kg) and morphine (10 mg/kg); and the fifth group received pioglitazone (30 mg/kg) and morphine (10 mg/kg). Mice were treated twice daily for five consecutive days with IP administration of morphine or saline. On the evening of day 5 and on the morning of day 6, animals were pretreated with pioglitazone 1 h before morphine injection. Withdrawal was precipitated 2 h after the last morphine injection with naloxone (1.0 mg/kg).

Effect of pioglitazone on the development of morphine withdrawal

Mice (n = 50) were divided into four groups: the first one (n = 12) received the vehicles of pioglitazone and morphine; the second group (n = 13) received the vehicle of pioglitazone and morphine (10 mg/kg); the third group (n = 13) received pioglitazone (30 mg/kg) and saline (the vehicle of morphine); and the fourth group (n = 13) was treated with pioglitazone (30 mg/kg) and morphine (10 mg/kg).

Mice were treated twice daily for five consecutive days with pioglitazone or its vehicle. One hour after pioglitazone's administration, animals were subjected to morphine or vehicle administrations. On the morning of day 6, animals were pretreated with pioglitazone 1 h before morphine injection. Withdrawal was precipitated 2 h after the last morphine injection with naloxone (1.0 mg/kg).

Effect of pioglitazone, GW-9662, or their combination on the expression of morphine withdrawal

Mice (n = 40) were divided into four groups: the first group (n = 8) received the vehicles of pioglitazone, GW-9662, and morphine; the second group (n = 8) was injected with the vehicle of pioglitazone, the vehicle of GW-966, and morphine (10 mg/kg); the third group (n = 8) received pioglitazone (30 mg/kg), the vehicle of GW-9662, and morphine (10 mg/kg); the fourth group (n = 8) was administered with pioglitazone (10 mg/kg), GW-9662 (5 mg/kg), and morphine (10 mg/kg); and the last group (n = 8) received the vehicle of pioglitazone, GW-9662 (5 mg/kg), and morphine 10 mg/kg.

Mice were treated twice daily for five consecutive days with morphine or saline. On the evening of the fifth day, animals were treated with GW-9662 or its vehicle 30 min prior to pioglitazone which was followed by morphine or its vehicle

1 h later. On the morning of day 6, mice were again treated with GW-9662, followed by pioglitazone and morphine or their respective vehicles.

Effect of pioglitazone on yohimbine-induced reinstatement of heroin seeking

Rats ($n = 7$) were trained to self-administer heroin under a FR-1 schedule of reinforcement; each active lever pressing resulted in the delivery of one dose of heroin (20 μg/0.1 ml infusion) for two consecutive hours. Following each heroin infusion, a 20-s time-out (TO) period occurred, during which the active lever pressings did not lead to any programmed consequences. TO was accompanied by illumination of a cue light located above the active lever to signal delivery of the positive reinforcement, while an intermittent tone was sounded throughout the 2-h session. Following acquisition of a stable baseline of IV heroin self-administration (variation less than 10 % over the last three sessions), rats were subjected to the extinction phase. During extinction, lever presses were no longer contingently associated with heroin delivery; hence, operant responding rapidly decreased. This phase lasted 13 days, until the mean number of reinforced responses was five times lower than the training-reinforced responses. The day after the last extinction session, rats were subjected to the reinstatement test. To evaluate the effect of pioglitazone on yohimbine-induced reinstatement, rats were administered pioglitazone (10 or 30 mg/kg) or its vehicle in a counterbalanced Latin square design, 12 and 1 h (9:00 pm and 8:00 am) before the reinstatement test, respectively. To elicit reinstatement, yohimbine (1.25 mg/kg) was given to all animals 30 min after the second pioglitazone administration, which corresponds to 30 min prior to the initiation of the reinstatement session. The yohimbine dose, time of injection, and experimental design were as described in previous studies (de Guglielmo et al. 2013; Marinelli et al. 2007; Stopponi et al. 2011).

Reinstatement experiments and drug treatments were performed every third day. Between tests, rats were subjected to extinction sessions.

Effect of pioglitazone on cue-induced reinstatement of heroin seeking

This experiment consisted of the following the phases:

Discrimination training phase The purpose of this procedure was to train the rats to self-administer intravenous heroin while simultaneously establishing discriminative stimuli (SD) associated with heroin availability or nonavailability. Once rats ($n = 7$) developed stable levels of 2-h daily heroin intake (training phase), they were subjected to a discrimination-learning regimen as follows. In three daily 1-h sessions, either heroin or saline was available as the only infusion solution. Each training day included two 1-h heroin sessions and one 1-h saline session in a random order. Between sessions, animals were removed from the operant boxes for at least 1 h. The sessions were initiated by extension of the levers into the chambers and concurrent onset of the respective SD, which remained present until termination of the session by retraction of the levers. The SD associated with the heroin availability (S+) consisted of an intermittent tone (7 kHz, 70 dB), whereas the SD predictive of the saline vehicle solution (S− or no reward) consisted of continuous illumination of the self-administration chamber's house light. To prevent accidental overdosing, drug infusions were followed by a 20-s TO period, which was signaled by illumination of a white cue light while the lever remained inactive. Saline infusions produced by lever presses during the S− sessions were followed by a 20-s TO period and signaled by a white noise (70 dB). Two levers were always present in both conditions (S+) and (S–). Only the right lever was active and produced an IV infusion of the respective solution when pressed. The left lever was inactive, and responses at this lever were recorded as a measure of nonspecific activation.

Extinction phase Responses at the previously active lever activated the syringe pump motor only but had no other programmed consequences (neither heroin nor saline was administered, and no cues were presented). These sessions lasted 1 h and were conducted once daily until extinction of response was reached (less than ten total responses over the last three sessions).

Reinstatement test Reinstatement test began the day after the last extinction day. To evaluate the effect of pioglitazone on cue-induced heroin seeking, rats were treated with pioglitazone (10, 30, or 60 mg/kg) or its vehicle, in a Latin square counterbalanced order, 12 and 1 h before the reinstatement test. This test lasted 1 h under conditions identical to those in the discrimination phase except that heroin was not available. Pioglitazone was given in the S+ condition and an interval of 3 days was allowed between tests.

Effect of pioglitazone on priming-induced reinstatement of heroin seeking

Following acquisition of a stable baseline of IV heroin self-administration (15 infusions), rats ($n = 10$) were subjected to the extinction phase. During extinction, lever presses were no longer contingently associated with heroin delivery; hence, operant responding rapidly decreased. This phase lasted 15 days until extinction of response was reached (less than ten total responses over the last three sessions).

The day after the last extinction session, rats were subjected to the reinstatement test. To evaluate the effect of

pioglitazone on priming-induced reinstatement of heroin seeking, rats were administered pioglitazone (10, 30, or 60 mg/kg) or its vehicle, in counterbalanced order (Latin square), 12 and 1 h (9:00 p.m. and 8:00 a.m.) before the beginning of the test, respectively. All rats were exposed to a heroin priming injection (0.25 mg/kg SC) just before the test sessions. This test lasted 1 h under conditions identical to those in the training phase except that heroin was not available. Reinstatement experiments and drug treatments were performed every third day.

Statistical analysis

Withdrawal experiments Total number of jumps, paw tremors, teeth chattering episodes, and wet dog shakes were analyzed with a one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc tests when appropriate. Ptosis and pilo-erection were analyzed by the nonparametric Kruskal-Wallis test followed by the Dunn's multiple comparison test when appropriate.

Reinstatement experiments Differences in responding between the mean value of the last three extinction sessions and the reinstatement test under vehicle conditions were analyzed by paired *t* test. The effects of pioglitazone on different reinstatements were analyzed by one-way ANOVA followed by Newman-Keuls post hoc tests when appropriate. Statistical significance was set at $p < 0.05$.

Results

Effect of pioglitazone on the expression of morphine withdrawal

ANOVA revealed a significant [$F(4,45) = 13.75$; $p < 0.0001$] overall effect of pioglitazone on total withdrawal score calculated as the sum of jumps, paw tremors episodes, teeth chattering, and wet dog shakes. As shown in Fig. 1a, post hoc analysis revealed that administration of morphine (10 mg/kg), twice daily for a period of 5 days followed by a single injection of naloxone (1 mg/kg, SC), precipitated a significant ($p < 0.01$) withdrawal syndrome in mice. Treatment with pioglitazone, 10 and 30 mg/kg, significantly attenuated the morphine withdrawal syndrome ($p < 0.05$ and $p < 0.01$, respectively).

When the Kruskal-Wallis test was used to evaluate piloerection and ptosis scores, the analysis revealed an overall significant effect of treatment [$df = 4$; $p < 0.001$]. As shown in Fig. 1b, Dunn's multiple comparison tests revealed a significant increase in piloerection and ptosis in the morphine group compared to the vehicle-treated one ($p < 0.01$). Pioglitazone at the dose of 30 mg/kg significantly attenuated piloerection and ptosis elicited by morphine withdrawal ($p < 0.05$). No significant effect was observed at 10 mg/kg of pioglitazone.

Effect of pioglitazone on the development of morphine withdrawal

ANOVA revealed a significant [$F(3,45) = 15.94$; $p < 0.0001$] overall effect of pioglitazone on total withdrawal score calculated as the sum of jumps, paw tremors episodes, teeth chattering, and wet dog shakes.

As shown in Fig. 2a, post hoc analysis revealed that administration of morphine (10 mg/kg), twice daily for a period of 5 days, followed by a single injection of naloxone (1 mg/kg, SC), precipitated a significant ($p < 0.001$) withdrawal syndrome in mice. Treatment with pioglitazone 30 mg/kg significantly attenuated morphine withdrawal syndrome ($p < 0.01$).

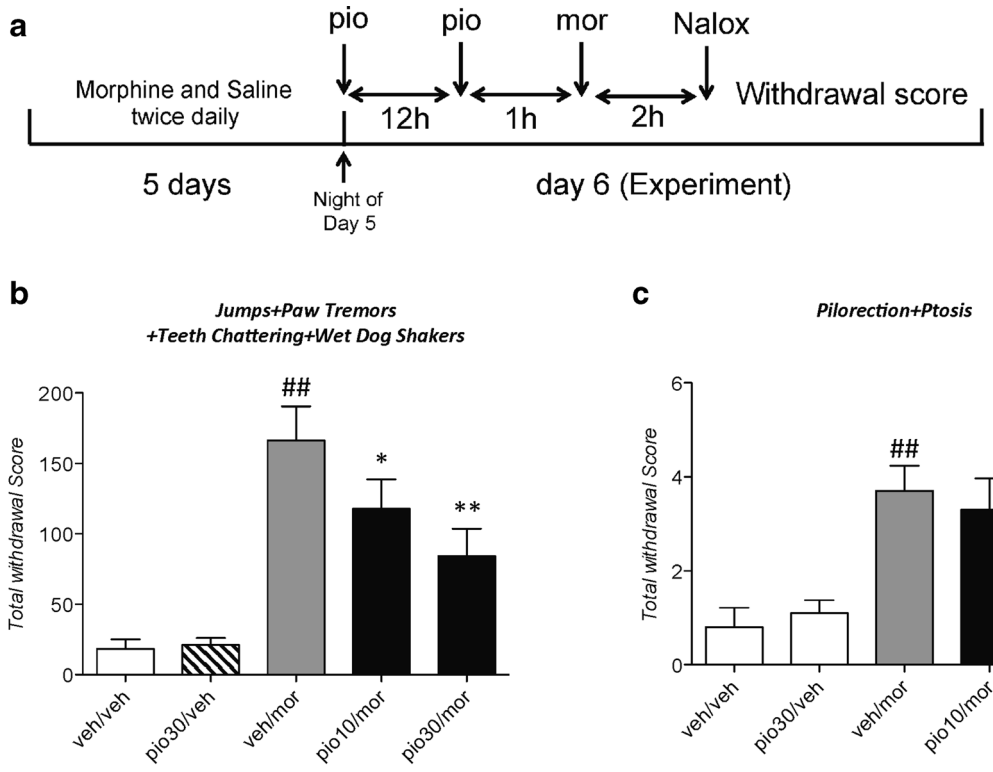
When the Kruskal-Wallis test was used to evaluate piloerection and ptosis scores, the analysis revealed an overall significant effect of treatment [$df = 3$; $p < 0.0001$]. As shown in Fig. 2b, Dunn's multiple comparison tests revealed a significant increase in piloerection and ptosis in the morphine group compared to the vehicle-treated one ($p < 0.01$). Pioglitazone at the dose of 30 mg/kg significantly attenuated piloerection and ptosis elicited by morphine withdrawal ($p < 0.05$).

Effect of pioglitazone, GW-9662, or their combination on the expression of morphine withdrawal

To confirm that the effect of pioglitazone on morphine withdrawal was mediated by activation of PPARγ, the selective PPARγ antagonist GW-9662 was used. One-way ANOVA revealed a significant [$F(4,37) = 16.79$; $p < 0.0001$] overall effect of treatment on total withdrawal score calculated as the sum of jumps, paw tremor episodes, teeth chattering, and wet dog shakes.

As shown in Fig. 3a, post hoc analysis revealed that administration of morphine (10 mg/kg), twice daily for a period of 5 days, followed by a single injection of naloxone (1 mg/kg, SC), precipitated a significant ($p < 0.001$) withdrawal syndrome in mice. Treatment with pioglitazone 30 mg/kg significantly prevented morphine withdrawal ($p < 0.001$). Administration of GW-9662 (5 mg/kg) completely abolished the protective effect of pioglitazone.

When the Kruskal-Wallis test was used to evaluate piloerection and ptosis scores, the analysis revealed an overall significant effect of treatment [$df = 4$; $p < 0.0001$]. As shown in Fig. 3b, Dunn's multiple comparison tests revealed a significant increase in piloerection and ptosis in the morphine group compared to the vehicle-treated one ($p < 0.01$). Pioglitazone at the dose of 30 mg/kg significantly attenuated piloerection and ptosis elicited by morphine withdrawal ($p < 0.05$). This effect was fully blocked by coadministration of GW-9662 (5 mg/kg).



d

MEAN (± S.E.M)	JUMPS	PAW TREMORS	TEETH CHATTERING	WET DOG SHAKERS	PILO ERECTION	PTOSIS
VEH/VEH	3,8 ± 0,5	9,2 ± 3,7	3,8 ± 1,5	1,4 ± 0,4	0,5 ± 0,3	0,3 ± 0,2
PIO30/VEH	0,7 ± 0,5	12,4 ± 4,4	6,4 ± 1,3	1,5 ± 0,5	0,5 ± 0,2	0,6 ± 0,2
VEH/MOR	23,4 ± 9,5	104 ± 23	29,7 ± 5,5	9,3 ± 1,2	1,8 ± 0,3	1,9 ± 0,3
PIO10/MOR	13,1 ± 5,4	73,5 ± 18,8	23,4 ± 4,8	7,8 ± 2,8	1,7 ± 0,4	1,6 ± 0,3
PIO30/MOR	7,3 ± 3,5	57 ± 17,4	16,2 ± 3,3	3,5 ± 1,3	1 ± 0,4	0,8 ± 0,3

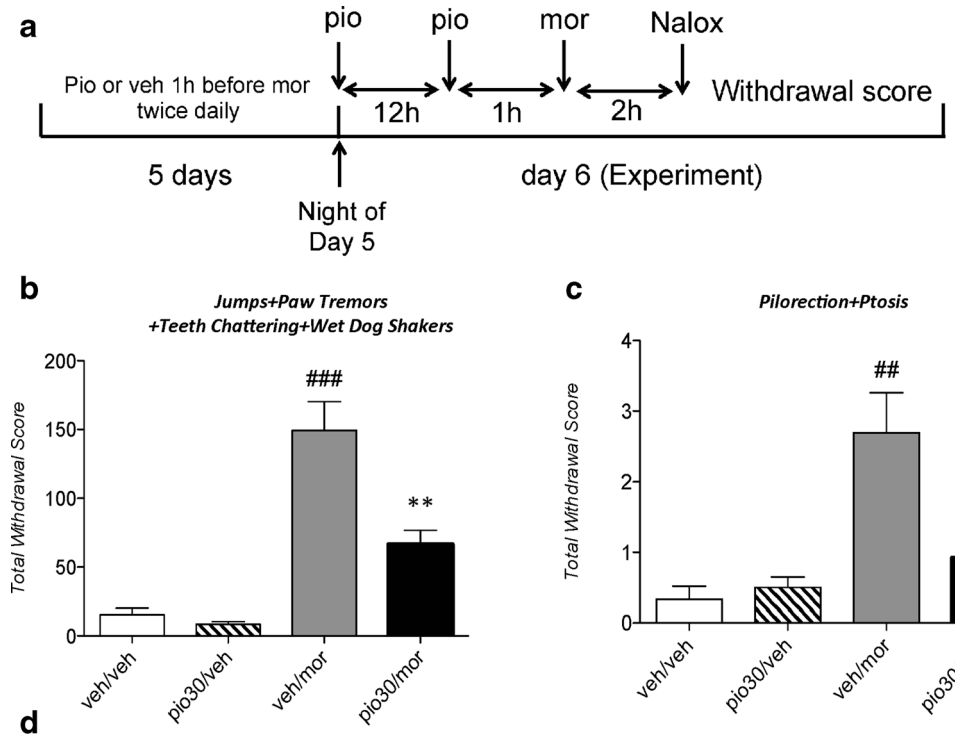
Fig. 1 Effect of pioglitazone on the expression of morphine withdrawal. **a** Visual timeline of the experiment. **b** Mean ± SEM of the total withdrawal score (jumps + paw tremors + teeth chattering + wet dog shakes). **c** Mean ± SEM of piloerection + ptosis. **d** Table summarizing the mean ± SEM of all the withdrawal symptoms. $^{\#\#}p < 0.01$ vs veh/veh, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs veh/mor (*veh* vehicle, *mor* morphine 10 mg/kg, *pio10* 10 mg/kg pioglitazone, *pio30* 30 mg/kg pioglitazone)

Effect of pioglitazone on yohimbine-induced reinstatement of heroin seeking

A stable baseline of responses to heroin was established in 16 days. During this training period, the reinforced responses for heroin reached a value of 14.4 ± 3.8. Following heroin self-administration training, the extinction phase started. Responses on the active lever progressively decreased from 45.2 ± 5.6 of the first day to 10.9 ± 2.7 on the last day. As shown in Fig. 4, administration of yohimbine significantly increased responding on the previously active lever [$t(6) = 2.787$; $p < 0.05$]. In contrast, inactive lever responding was not modified by yohimbine [$t(6) = 1.7605$; p = NS]. When the efficacy of pioglitazone (30 mg/kg) was evaluated through a one-way ANOVA, results revealed an overall effect of treatment [$F(2,6) = 3.71$; $p < 0.05$]. Post hoc analysis showed a significant reduction ($p < 0.05$) of yohimbine-induced relapse following 30 mg/kg of pioglitazone. Responses on the inactive lever were not affected by the treatments [$F(2,6) = 1.35$; p = NS].

Effect of pioglitazone on discriminative cue-induced reinstatement of heroin seeking

Throughout the conditioning phase, during which rats learned to discriminate between heroin and saline, they exhibited a stronger preference for heroin. On the last day of the discrimination period (day 10), animals reached a reinforced response for heroin of 9.6 ± 1.6 in the first session and of 6.6 ± 1.3 in the second session, while the response for saline was 3.6 ± 0.6.



d

MEAN (± S.E.M)	JUMPS	PAW TREMORS	TEETH CHATTERING	WET DOG SHAKERS	PILO ERECTION	PTOSIS
VEH/VEH	6 ± 3.49	6 ± 2.71	2.3 ± 0.91	0.7 ± 0.36	0.3 ± 0.13	0.1 ± 0.08
PIO30/VEH	6.5 ± 2.5	7.3 ± 1.8	1.9 ± 1	0.3 ± 0.45	0.2 ± 0.12	0.2 ± 0.05
VEH/MOR	25.6 ± 8.5	98.5 ± 20.44	19.3 ± 5.17	5.08 ± 1.09	1.6 ± 0.33	1.08 ± 0.35
PIO30/MOR	20.5 ±5.03	34.2 ± 7.42	9.1 ± 2.04	2.7 ± 0.72	0.9 ± 0.27	0.1 ± 0.09

Fig. 2 Effect of pioglitazone on the development of morphine withdrawal. **a** Visual timeline of the experiment. **b** Mean ± SEM of the total withdrawal score (jumps + paw tremors + teeth chattering + wet dog shakes). **c** Mean ± SEM of piloerection + ptosis. **d** Table summarizing the mean ± SEM of all the withdrawal symptoms. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs veh/veh; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs veh/mor

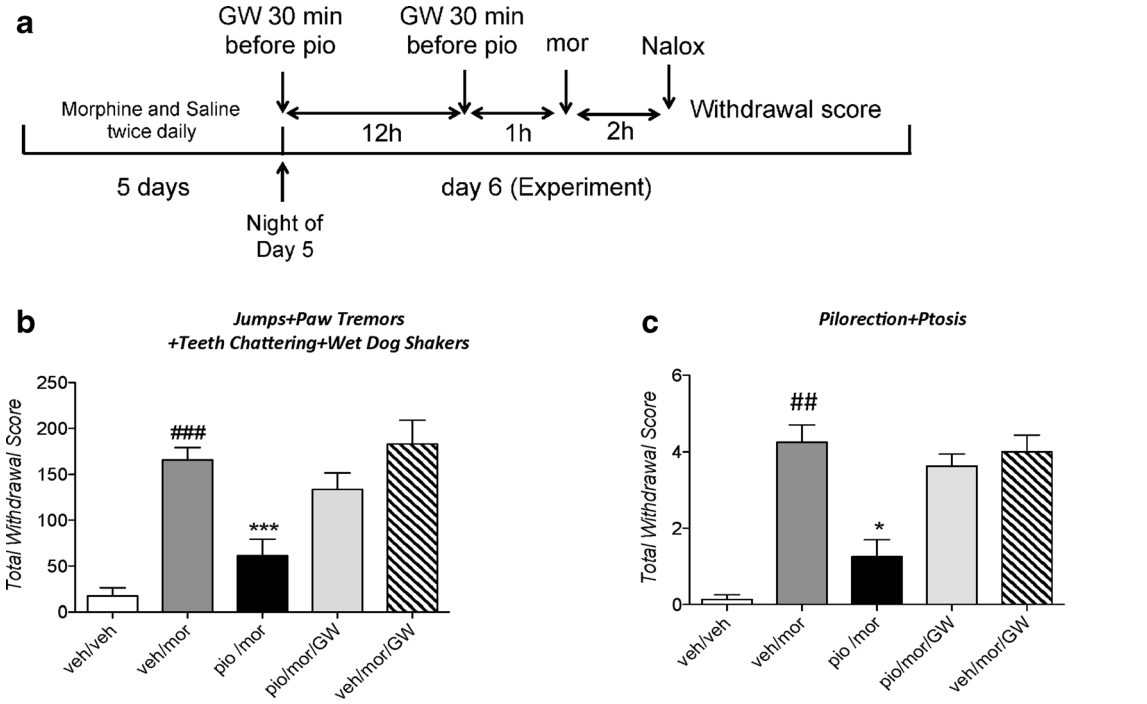
During extinction, lever presses progressively decreased from 49.6 ± 6.2 of the first extinction day to 8.4 ± 2.3 of the last extinction day. As shown in the Fig. 5, reintroduction of heroin (S+) but not saline-associated cues (S−) significantly increased active lever responding [$F(2,6) = 14.59$, $p < 0.001$] in the reinstatement test. Inactive lever presses were not modified by presentation of the cues [$F(2,6) = 0.075$, NS]. Moreover, one-way ANOVA demonstrated a lack of effect of pioglitazone (10, 30, or 60 mg/kg) on cue-induced reinstatement of heroin seeking [$F(3,6) = 0.34$, p = NS].

Effect of pioglitazone on priming-induced reinstatement of heroin seeking

As shown in the Fig. 6, priming injection of heroin in animals in the extinction phase just before the test sessions significantly reinstated the operant response for the active lever [$t(9) = 3.076$; $p < 0.05$]. In contrast, inactive lever was not modified by heroin priming injection [$t(9) = 1441$; p = NS]. One-way ANOVA showed an overall effect of treatment with pioglitazone [$F(3,9) = 4.07$; $p < 0.05$]. The Newman-Keuls post hoc test demonstrated a significant inhibition of reinstatement following administration of both 30 and 60 mg/kg of pioglitazone ($p < 0.05$). Inactive lever responding was not modified by the treatment with pioglitazone [$F(3,9) = 1.11$; p = NS].

Discussion

Results showed that treatment with pioglitazone reduced the development and expression of naloxone-induced morphine withdrawal in mice. Pioglitazone, per se, did not show any effect on nondependent mice treated with naloxone.



d

MEAN (± S.E.M)	JUMPS	PAW TREMORS	TEETH CHATTERING	WET DOG SHAKERS	PILO ERECTION	PTOSIS
VEH/VEH	13,9 ± 8,4	3,0 ± 0,7	0,1 ± 0,1	0,4 ± 0,2	0,1 ± 0,1	0,0 ± 0,0
VEH/MOR	53,0 ± 4,7	98,8 ± 11,3	8,1 ± 3,2	6,0 ± 1,3	2,0 ± 0,3	2,3 ± 0,3
PIO10/MOR	21,6 ± 10,8	33,4 ± 6,8	29,7 ± 5,5	1,9 ± 0,7	0,9 ± 0,1	0,6 ± 0,4
PIO10/MOR/GW5	36,4 ± 8,2	80,1 ± 8,5	14,4 ± 8,2	8,6 ± 3,5	1,7 ± 0,2	2,1 ± 0,2
VEH/MOR/GW5	56,3 ± 21,6	112,6 ± 23,1	8,1 ± 3,3	6,1 ± 1,4	2,0 ± 0,3	2,0 ± 0,2

Fig. 3 Effect of pioglitazone, GW-9662, or their combination on the expression of morphine withdrawal. **a** Visual timeline of the experiment. **b** Mean ± SEM of the total withdrawal score (jumps + paw tremors + teeth chattering + wet dog shakes). **c** Mean ± SEM of piloerection + ptosis. **d** Table summarizing the mean ± SEM of all the withdrawal symptoms. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs veh/veh; $^{*}p < 0.05$ and $^{***}p < 0.001$ vs veh/mor (*GW* 5 mg/kg GW-9662)

Pretreatment with the selective PPARγ receptor antagonist GW-9662 prevented the effects of pioglitazone, demonstrating that pioglitazone's effects are mediated by the PPARγ receptor. GW-9662 alone did not affect morphine withdrawal. Since pioglitazone does not alter locomotor activity in animals chronically treated with morphine (de Guglielmo et al. 2014), it is unlikely that the effects observed here are due to nonspecific sedative effects that could have attenuated the behavioral responses associated with morphine withdrawal.

Reinstatement of opioid-related behaviors is partly motivated by the negative affective states of opioid withdrawal. Therefore, the observed reduction of withdrawal symptoms after pioglitazone suggests that pioglitazone may also reduce relapse.

Stress plays an important role in the relapse to opioid addiction (Shaham et al. 2000) and the pharmacological stressor yohimbine has been shown to increase opioid withdrawal symptoms and opioid seeking behavior in heroin-dependent individuals (Greenwald et al. 2013). In line with human data, it has been shown that yohimbine is able to reinstate drug seeking in rats with a history of chronic drug self-administration (Le et al. 2005; Marinelli et al. 2007; Stine et al. 2002; Umhau et al. 2011). We used yohimbine to investigate the effect of pioglitazone on stress-induced reinstatement of heroin seeking. We found that yohimbine significantly increased reinstatement of heroin seeking and that pretreatment with pioglitazone prevented yohimbine-induced heroin relapse in a dose-dependent manner. Responses on the inactive lever were not affected by yohimbine or pioglitazone, excluding nonspecific actions of these compounds. These data are in accordance with our previous work demonstrating that pioglitazone attenuates yohimbine-induced relapse in rats trained to self-administer alcohol (Stopponi et al. 2011). Immunohistochemistry and gene expression analysis revealed

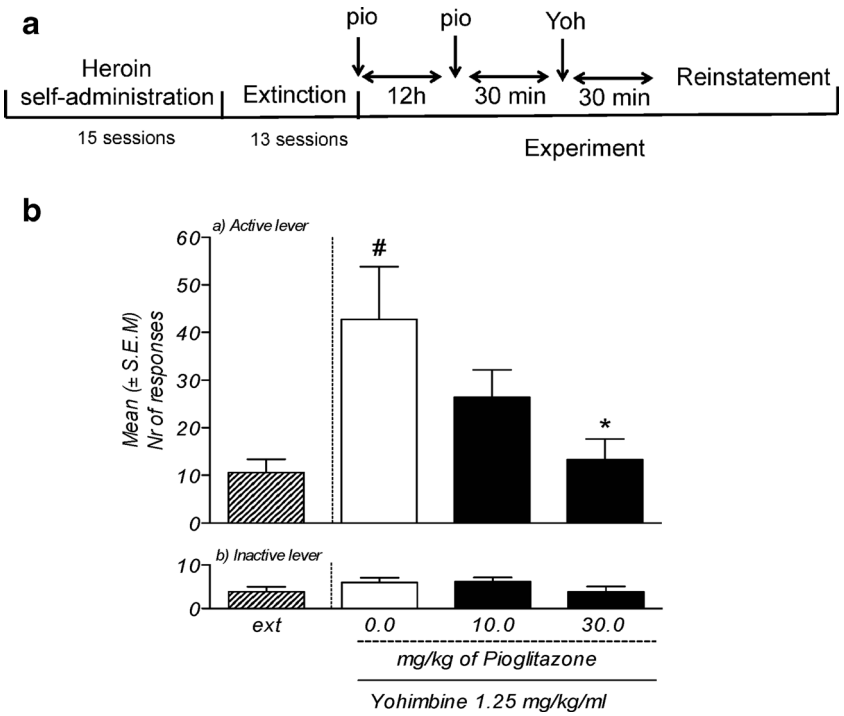


Fig. 4 **a** Visual timeline of the experiment. **b** Effect of pioglitazone on yohimbine-induced reinstatement of heroin seeking. During the training phase, the animals reached a stable baseline of heroin responding, while during extinction sessions the number of responses progressively decreased. Compared to extinction (Ext), yohimbine (1.25 mg/kg IP) elicited a significant reinstatement of response that was significantly reduced following treatment with pioglitazone (0.0, 10.0, and 30.0 mg/kg). Values represent the mean (±SEM) number of responses at the **a** active lever and **b** the inactive lever. Significant difference from extinction (Ext) $^{\#}p < 0.05$ and from controls (0.0) $^{*}p < 0.05$

that the PPARγ receptor is largely expressed in the lateral hypothalamus (LH), in the arcuate nucleus (ARC), in the ventromedial nucleus (VMN), in the ventral tegmental area (VTA), and in the paraventricular nucleus (PVN) (Sarruf et al. 2009). Of note, it has been shown that activation of PPARγ in the PVN attenuates the expression of corticotropin-releasing factor (CRF), which is one of the main stress hormones in mammals (Festuccia et al. 2008). Because CRF also plays a key role in stress-induced drug relapse and the effects of yohimbine are prevented by CRF1 receptor antagonists (Ayanwuyi et al. 2013; Marinelli et al. 2007), it is tempting to hypothesize that the effects of pioglitazone on yohimbine may be mediated by the inhibition of CRF neurotransmission through PPARγ activation.

Environmental conditioning factors are also known to play a pivotal role in heroin seeking (O'Brien et al. 1992). To evaluate the effect of pioglitazone on relapse elicited by discriminative cue predictive of drug heroin availability, we

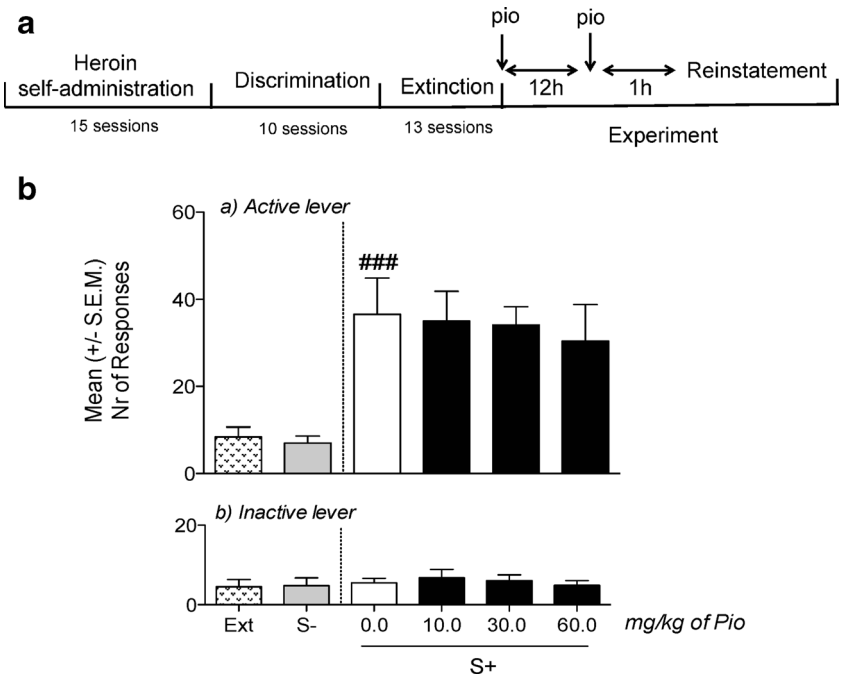


Fig. 5 **a** Visual timeline of the experiment. **b** Effect of pioglitazone on discriminative-cue-induced reinstatement of heroin seeking. During the discrimination-training phase, rats learned to press the lever for heroin in the presence of S+ and for saline (nonreward) in the presence of S−. During extinction (Ext), lever responding progressively decreased. Ext value represents the mean value of the last three extinction days. Values represent the mean (±SEM) number of responses at the **a** active lever and **b** the inactive lever. Significant difference from extinction (Ext) and from S− $^{\#\#\#}p < 0.001$

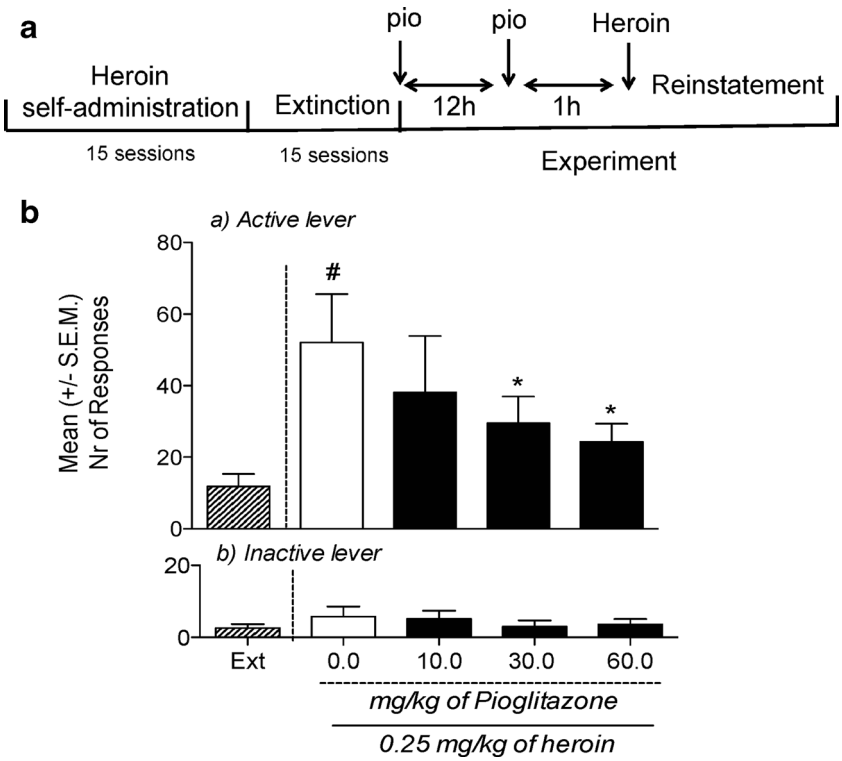


Fig. 6 **a** Visual timeline of the experiment. **b** Effect of pioglitazone (10.0, 30.0, and 60.0 mg/kg) on priming-induced reinstatement of heroin seeking. Extinction (Ext) value represents the mean value of the last three extinction days. Values represent the mean (±SEM) number of responses at the **a** active lever and **b** the inactive lever. Significant difference from extinction (Ext) #$p < 0.05$ and from controls (0.0) *$p < 0.05$

trained rats on an extinction reinstatement procedure. According to this procedure, reexposure of the animals to environmental stimuli predictive of the drug elicited a robust reinstatement of lever responding (Kallupi et al. 2013). Contrary to yohimbine-induced reinstatement, pretreatment with pioglitazone did not show any effects on cue-induced relapse. This finding replicates the results of a previous study in which PPARγ agonists did not prevent cue-induced relapse to alcohol seeking (Stopponi et al. 2011).

Activation of μ opioid (MOP) receptors is critical for heroin reinforcement (Mello and Negus 1996) and activation of these receptors by heroin priming is an important trigger of relapse. For example, priming injections of morphine, which preferentially activates MOP receptors, mimic the effect of heroin and lead to reinstatement (Stewart and Wise 1992). We found that administration of pioglitazone at the doses of 30 and 60 mg/kg markedly reduced lever pressing following a priming injection of heroin. This effect was selective because the treatment with pioglitazone did not modify operant response on the inactive lever. Previous work in our laboratory demonstrated that activation of PPARγ results in a marked inhibition of mesolimbic dopamine transmission elicited by opioids (de Guglielmo et al. 2015). Considering that reinstatement of drug seeking elicited by heroin priming requires recruitment of the mesolimbic DAergic system (Shaham and Stewart 1996), it is possible that the effect of pioglitazone may depend upon its ability to attenuate heroin-induced release of dopamine in the nucleus accumbens shell (de Guglielmo et al. 2015).

As PPARγ is a nuclear receptor, changes in gene expression are generally considered to be the mechanism through which pioglitazone exerts its effects. However, recently nongenomic effects mediated by PPARγ activation have been also reported (Griggs et al. 2015). In our experiments, multiple injections of pioglitazone were given prior to behavioral tests. Hence, the effects of pioglitazone reported here are most likely mediated by post-transcriptional mechanisms. Nevertheless, the possibility that non-genomic mechanisms can also be involved cannot be excluded based on present data.

Collectively, these results suggest that activation of PPARγ, through its ability to attenuate opioid withdrawal symptoms and to prevent relapse, may represent a novel treatment for opioid addiction. Previous studies have shown 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). Activation of PPARγ has been shown to attenuate the development of tolerance to the analgesic effect of morphine and to alleviate neuropatic pain (de Guglielmo et al. 2014; Morgenweck et al. 2013; Park et al. 2007). Pioglitazone has been in clinical use for several years for the treatment of type 2 diabetes, and its tolerability has been largely demonstrated. The ability of pioglitazone to prevent relapse, to attenuate development of tolerance and physical dependence following chronic opioids opens the prospect for immediate clinical investigation in opioid addicted patients or in individuals taking narcotics to treat chronic pain. An important consideration in this regard is to whether pioglitazone

given at clinically approved dosages may exert these effects. In our preclinical studies, the drug was given at relatively higher doses compared to those needed to attenuate insulin resistance and hepatic damages (Enomoto et al. 2003; Ikeda et al. 1990). This is likely due to the modest brain penetrating properties of the compound (Maeshiba et al. 1997). On the other hand, as shown in several rodent studies, to exert CNS effects, pioglitazone has to be given at doses comparable to those used here (Barbiero et al. 2014; Griggs et al. 2015; Salehi-Sadaghiani et al. 2012; Stopponi et al. 2011). Ideally, PPARγ agonist with improved brain penetrating properties would offer significant advantages compared to classical thiazolidinediones.

Acknowledgments This study was supported by the University of Camerino (to RC).

Authors contribution GdG and RC were responsible for the study concept and design. GdG, MK, and GS contributed to the acquisition of animal data and drafted the manuscript. GD and GG provided critical revision of the manuscript for important intellectual content. All authors critically reviewed the content and approved the final version for publication.

Compliance with ethical standards All procedures were conducted in adherence with the European Community Council Directive for Care and Use of Laboratory Animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals

Conflict of interest Dr. Demopulos is the chairman and CEO and Dr. Gaitanaris is the chief scientific officer of Omeros Corporation. Omeros exclusively controls the intellectual property rights from the University of Camerino. Dr. Ciccocioppo is the inventor on a number of patent applications, which have been assigned to Omeros, relating to the therapeutic use of PPARγ 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.

References

Ayanwuyi LO, Carvajal F, Lerma-Cabrera JM, Domi E, Bjork K, Ubaldi M, Heilig M, Roberto M, Ciccocioppo R, Cippitelli A (2013) Role of a genetic polymorphism in the corticotropin-releasing factor receptor 1 gene in alcohol drinking and seeking behaviors of marchigian sardinian alcohol-preferring rats. Front Psychiatry 4:23

Barbiero JK, Santiago RM, Persike DS, da Silva Fernandes MJ, Tonin FS, da Cunha C, Lucio Boschen S, Lima MM, Vital MA (2014) Neuroprotective effects of peroxisome proliferator-activated receptor alpha and gamma agonists in model of parkinsonism induced by intranigral 1-methyl-4-phenyl-1,2,3,6-tetrahyropyridine. Behav Brain Res 274:390–399

Ciccocioppo R, Sanna PP, Weiss F (2001) Cocaine-predictive stimulus induces drug-seeking behavior and neural activation in limbic brain regions after multiple months of abstinence: reversal by D(1) antagonists. Proc Natl Acad Sci U S A 98:1976–1981

de Guglielmo G, Cippitelli A, Somaini L, Gerra G, Li H, Stopponi S, Ubaldi M, Kallupi M, Ciccocioppo R (2013) Pregabalin reduces cocaine self-administration and relapse to cocaine seeking in the rat. Addict Biol 18:644–653

de Guglielmo G, Kallupi M, Scuppa G, Stopponi S, Demopulos G, Gaitanaris G, Ciccocioppo R (2014) Analgesic tolerance to morphine is regulated by PPARgamma. Br J Pharmacol 171:5407–5416

de Guglielmo G, Melis M, De Luca MA, Kallupi M, Li HW, Niswender K, Giordano A, Senzacqua M, Somaini L, Cippitelli A, Gaitanaris G, Demopulos G, Damadzic R, Tapocik J, Heilig M, Ciccocioppo R (2015) PPARgamma activation attenuates opioid consumption and modulates mesolimbic dopamine transmission. Neuropsychopharmacology 40:927–937

de Wit H, Stewart J (1983) Drug reinstatement of heroin-reinforced responding in the rat. Psychopharmacology 79:29–31

Enomoto N, Takei Y, Hirose M, Konno A, Shibuya T, Matsuyama S, Suzuki S, Kitamura KI, Sato N (2003) Prevention of ethanol-induced liver injury in rats by an agonist of peroxisome proliferator-activated receptor-gamma, pioglitazone. J Pharmacol Exp Ther 306:846–854

Festuccia WT, Oztezcan S, Laplante M, Berthiaume M, Michel C, Dohgu S, Denis RG, Brito MN, Brito NA, Miller DS, Banks WA, Bartness TJ, Richard D, Deshaies Y (2008) Peroxisome proliferator-activated receptor-gamma-mediated positive energy balance in the rat is associated with reduced sympathetic drive to adipose tissues and thyroid status. Endocrinology 149:2121–2130

Fugelstad A, Stenbacka M, Leifman A, Nylander M, Thiblin I (2007) Methadone maintenance treatment: the balance between life-saving treatment and fatal poisonings. Addiction 102:406–412

Gonzalez G, Oliveto A, Kosten TR (2004) Combating opiate dependence: a comparison among the available pharmacological options. Expert Opin Pharmacother 5:713–725

Greenwald MK, Lundahl LH, Steinmiller CL (2013) Yohimbine increases opioid-seeking behavior in heroin-dependent, buprenorphine-maintained individuals. Psychopharmacology 225:811–824

Griggs RB, Donahue RR, Morgenweck J, Grace PM, Sutton A, Watkins LR, Taylor BK (2015) Pioglitazone rapidly reduces neuropathic pain through astrocyte and nongenomic PPARgamma mechanisms. Pain 156:469–482

Ikeda H, Taketomi S, Sugiyama Y, Shimura Y, Sohda T, Meguro K, Fujita T (1990) Effects of pioglitazone on glucose and lipid metabolism in normal and insulin resistant animals. Arzneimittelforschung 40:156–162

Kallupi M, de Guglielmo G, Cannella N, Li HW, Calo G, Guerrini R, Ubaldi M, Renger JJ, Uebele VN, Ciccocioppo R (2013) Hypothalamic neuropeptide S receptor blockade decreases discriminative cueinduced reinstatement of cocaine seeking in the rat. Psychopharmacology (Berl) 226:347–55.

Kreek MJ, Vocci FJ (2002) History and current status of opioid maintenance treatments: blending conference session. J Subst Abus Treat 23:93–105

Le AD, Harding S, Juzytsch W, Funk D, Shaham Y (2005) Role of alpha-2 adrenoceptors in stress-induced reinstatement of alcohol seeking and alcohol self-administration in rats. Psychopharmacology 179:366–373

Maeshiba Y, Kiyota Y, Yamashita K, Yoshimura Y, Motohashi M, Tanayama S (1997) Disposition of the new antidiabetic agent pioglitazone in rats, dogs, and monkeys. Arzneimittelforschung 47:29–35

Marinelli PW, Funk D, Juzytsch W, Harding S, Rice KC, Shaham Y, Le AD (2007) The CRF1 receptor antagonist antalarmin attenuates yohimbine-induced increases in operant alcohol self-administration and reinstatement of alcohol seeking in rats. Psychopharmacology 195:345–355

Mello NK, Negus SS (1996) Preclinical evaluation of pharmacotherapies for treatment of cocaine and opioid abuse using drug self-administration procedures. Neuropsychopharmacology : Official

Publication of the American College of Neuropsychopharmacology 14:375–424

Morgenweck J, Griggs RB, Donahue RR, Zadina JE, Taylor BK (2013) PPARgamma activation blocks development and reduces established neuropathic pain in rats. Neuropharmacology 70: 236–246

O'Brien CP (1996) Recent developments in the pharmacotherapy of substance abuse. J Consult Clin Psychol 64:677–686

O'Brien C, Childress AR, Ehrman R, Robbins S, McLellan AT (1992) Conditioning mechanisms in drug dependence. Clin Neuropharmacol 15(Suppl 1 Pt A):66A–67A

Park SW, Yi JH, Miranpuri G, Satriotomo I, Bowen K, Resnick DK, Vemuganti R (2007) Thiazolidinedione class of peroxisome proliferator-activated receptor gamma agonists prevents neuronal damage, motor dysfunction, myelin loss, neuropathic pain, and inflammation after spinal cord injury in adult rats. J Pharmacol Exp Ther 320:1002–1012

Salehi-Sadaghiani M, Javadi-Paydar M, Gharedaghi MH, Zandieh A, Heydarpour P, Yousefzadeh-Fard Y, Dehpour AR (2012) NMDA receptor involvement in antidepressant-like effect of pioglitazone in the forced swimming test in mice. Psychopharmacology 223: 345–355

Sarruf DA, Yu F, Nguyen HT, Williams DL, Printz RL, Niswender KD, Schwartz MW (2009) Expression of peroxisome proliferator-activated receptor-gamma in key neuronal subsets regulating glucose metabolism and energy homeostasis. Endocrinology 150: 707–712

Savage SR, Kirsh KL, Passik SD (2008) Challenges in using opioids to treat pain in persons with substance use disorders. Addiction science & clinical practice 4:4–25

Shaham Y, Stewart J (1996) Effects of opioid and dopamine receptor antagonists on relapse induced by stress and re-exposure to heroin in rats. Psychopharmacology 125:385–391

Shaham Y, Erb S, Stewart J (2000) Stress-induced relapse to heroin and cocaine seeking in rats: a review. Brain Res Brain Res Rev 33:13–33

Shaham Y, Shalev U, Lu L, De Wit H, Stewart J (2003) The reinstatement model of drug relapse: history, methodology and major findings. Psychopharmacology 168:3–20

Stewart J (2003) Stress and relapse to drug seeking: studies in laboratory animals shed light on mechanisms and sources of long-term vulnerability. The American journal on addictions / American Academy of Psychiatrists in Alcoholism and Addictions 12:1–17

Stewart J, Wise RA (1992) Reinstatement of heroin self-administration habits: morphine prompts and naltrexone discourages renewed responding after extinction. Psychopharmacology 108:79–84

Stine SM, Southwick SM, Petrakis IL, Kosten TR, Charney DS, Krystal JH (2002) Yohimbine-induced withdrawal and anxiety symptoms in opioid-dependent patients. Biol Psychiatry 51:642–651

Stopponi S, Somaini L, Cippitelli A, Cannella N, Braconi S, Kallupi M, Ruggeri B, Heilig M, Demopulos G, Gaitanaris G, Massi M, Ciccocioppo R (2011) Activation of nuclear PPARgamma receptors by the antidiabetic agent pioglitazone suppresses alcohol drinking and relapse to alcohol seeking. Biol Psychiatry 69:642–649

Stotts AL, Dodrill CL, Kosten TR (2009) Opioid dependence treatment: options in pharmacotherapy. Expert Opin Pharmacother 10: 1727–1740

Strang J, Hall W, Hickman M, Bird SM (2010) Impact of supervision of methadone consumption on deaths related to methadone overdose (1993–2008): analyses using OD4 index in England and Scotland. BMJ 341:c4851

Umhau JC, Schwandt ML, Usala J, Geyer C, Singley E, George DT, Heilig M (2011) Pharmacologically induced alcohol craving in treatment seeking alcoholics correlates with alcoholism severity, but is insensitive to acamprosate. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 36: 1178–1186

UNODC (2012) World Drug Report. http://www.unodc.org/unodc/en/data-and-analysis/WDR-2012.html

Weiss F, Ciccocioppo R, Parsons LH, Katner S, Liu X, Zorrilla EP, Valdez GR, Ben-Shahar O, Angeletti S, Richter RR (2001) Compulsive drug-seeking behavior and relapse. Neuroadaptation, stress, and conditioning factors. Ann N Y Acad Sci 937:1–26