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## Regular Research Article

# Activation of the Mu-Delta Opioid Receptor Heteromers Blocks Morphine Rewarding Effects

Ariadna Requana Aradas, Youssra Djaboub, Isabelle McCort-Tranchepain,  Zuzana Hajasova, Loïc Clémenceau, Corinne Canestrelli, Anika Mann, Stefan Schulz, Angélique Delaval, Francine Acher, Dominique Massotte, Florence Noble, Nicolas Marie

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## Abstract

**Background:** Evidence has accumulated demonstrating the existence of opioid receptor heteromers, and recent data suggest that targeting these heteromers could reduce opioid side effects while retaining therapeutic effects. Indeed, CYM51010 characterized as a MOR (mu opioid receptor)/DOR (delta opioid receptor) heteromer–preferring agonist promoted antinociception comparable with morphine but with less tolerance. In the perspective of developing these new classes of pharmacological agents, data on their putative side effects are mandatory.

**Methods:** Therefore, in this study, we investigated the effects of CYM51010 in different models related to drug addiction in mice, including behavioral sensitization, conditioned place preference and withdrawal.

**Results:** We found that, like morphine, CYM51010 promoted acute locomotor activity as well as psychomotor sensitization and rewarding effect. However, it induced less physical dependence than morphine. We also investigated the ability of CYM51010 to modulate some morphine-induced behavior. Whereas CYM51010 was unable to block morphine-induced physical dependence, it blocked reinstatement of an extinguished morphine induced-conditioned place preference.

**Conclusions:** Altogether, our results reveal that targeting MOR-DOR heteromers could represent a promising strategy to block morphine reward.

**Keywords:** CYM51010, morphine, MOR-DOR heteromers, reward, withdrawal, behavioral sensitization

## Significance Statement

Targeting mu-delta opioid receptor heteromers might represent an interesting strategy as it might promote opiate-associated therapeutic effects such as antinociception but with less tolerance. However, very few data are available regarding possible side effects when activating these heteromers. Therefore, we investigated the effects of CYM51010, a mu-delta opioid receptor heteromer–preferring agonist in different models related to addiction in mice. We found that it promoted, like morphine, behavioral sensitization, rewarding effects and physical dependence. Interestingly, we also found that it was able to block morphine-induced reinstatement of an extinguished rewarding effect. In conclusion, our results suggest that targeting mu-delta receptor heteromers could be interesting to use after morphine exposure to reduce its rewarding effects but not in naive individuals at the risk of inducing the same side effects as opioids, particularly addiction.

## INTRODUCTION

Opioid receptors (OR) are G protein-coupled receptors involved in many physiological functions, including nociception, reward, emotional response and respiratory function. There are 3 main

types of OR: mu (MOR), delta (DOR), and kappa (KOR). For many years, some researchers suggested the existence of OR heteromers (Jordan and Devi, 1999). Many combinations exist and confer to these new molecular entities novel properties in terms of ligand binding, signaling, and trafficking (Gaborit and Massotte,

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2023). The use of double-fluorescent knock-in mouse coexpressing MOR and DOR fused to fluorescent proteins allowed to map neuronal coexpression of both ORs in the nervous system and physical proximity was established in the hippocampus (Erbs et al., 2015), spinal cord (He et al., 2011), and dorsal root ganglions (DRGs) (Xie et al., 2009). Modifications in the signaling and trafficking of these endogenous heteromers have also been reported. For instance, whereas MOR is preferentially recycled back to plasma membrane after endocytosis (Trafton et al., 2000) and DOR is targeted to lysosomes for degradation (Whistler et al., 2002), coexpression of MOR and DOR results in ligand-selective cotargeting to lysosomal compartments in primary hippocampal neurons (Derouiche et al., 2020). To investigate the physiopathological role of these heteromers, ligands that could selectively target these molecular entities are required. Using a high-throughput screening approach, Gomes and coworkers discovered CYM51010, a MOR-DOR heteromer-preferring agonist. Compared with morphine, this compound promoted a comparable antinociception but with a lower tolerance (Gomes et al., 2013). More recently, Tiwari and coworkers found that CYM51010 could induce analgesic responses in a model of neuropathic pain generated by sciatic nerve ligation in rat (Tiwari et al., 2020), a type of pain generally resistant to classical opioids like morphine. It also exerted a mechanical antiallodynic effect in morphine-tolerant animals (Tiwari et al., 2020). In addition to tolerance, opioid prescription is restricted as a protracted use because they may promote dependence. In the original paper describing CYM51010, Gomes and coworkers investigated physical dependence and found that a 9-day treatment promoted less naloxone-precipitated withdrawal signs (diarrhea and body weight loss) compared with morphine (Gomes et al., 2013). All these data suggest that CYM51010 could present a benefit compared with classical opioids, as it could be efficient in pain model where these molecules have a low efficacy and with less side effects following repeated administration. However, data on the effects of CYM51010 in other models of drug dependence are mandatory in the perspective of developing compounds targeting OR heteromers for therapeutics. Therefore, in this study, we investigated the effects of CYM51010 in different models related to addiction including behavioral sensitization, conditioned place preference (CPP), and withdrawal. Moreover, the ability of CYM51010 to modulate morphine-induced behavior was also investigated.

## MATERIALS AND METHODS

### Animals

Male C57Bl/6Rj (Janvier Labs, France), 7 weeks at the beginning of the experiments, were housed 6 per cage on a 12 hour-light/dark cycle (light 8:00 AM-8:00 PM) in a temperature-controlled room (21±2°C) with food and water available ad libitum. Animal experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) as well as French law, with the standard ethical guidelines and under the control of the Ethical Committee of the university (APAFIS #28489-202007201332471 v4). Upon arrival, animals were given a week to acclimatize before any manipulation. Every effort was made to minimize animal numbers and discomfort.

### CYM51010 Synthesis

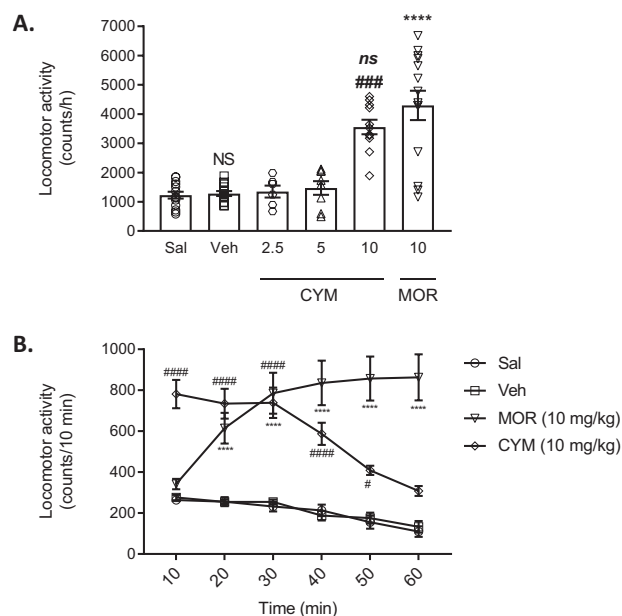
All the reagents were from Sigma-Aldrich and the solvents were distilled prior to use. All the anhydrous reactions were carried out under argon atmosphere and were monitored by thin-layer

chromatography with Merck 60F-254 precoated silica (0.2 mm) on glass. Each product was purified by flash chromatography using Biotage Isolera Prime system with detectors at 254 and 280 nm, liquid deposition on Grace Resolv silica cartridge. Purity of each product was checked by spectroscopy methods: <sup>1</sup>H NMR (500 MHz) and <sup>13</sup>C NMR (126 MHz) spectra were recorded on a Bruker AVANCEII-500 spectrometer and mass spectra on a Thermo Finnigan LCD Advantage spectrometer.

CYM51010 was synthesized according to the procedure described by Pinello and coworkers (Pinello et al., 2010) but with some modifications. Ethyl *N*-Boc-piperidine-4-carboxylate and *N*-[4-(chloromethyl)phenyl]acetamide were each synthesized in 2 steps from inexpensive commercially available isonipecotic acid and 4-acetamidobenzaldehyde, respectively, with 90% and 89% yields. The major improvement was for the crucial alkylation step to create the quaternary carbon. Indeed, by using the described conditions, in our hands, from 2-bromoethyl benzene, the 4-phenethyl-piperidine-1,4-dicarboxylic acid 1-*tert*-butyl ester 4-ethyl ester was obtained in a very low yield instead of a 35% yield. On the other hand, by using freshly prepared lithium diisopropylamide (1 M in hexane-Tetrahydrofuran) and the more reactive 2-iodoethyl benzene, the expected product was obtained in a 44% yield. CYM51010 (see supplementary Figure 1 for purity analysis) was then obtained in 2 steps as a white solid according to the described procedure.

### Drug Treatment

Morphine hydrochloride (Francopia, France), naloxone hydrochloride (Sigma-Aldrich, France), and naltrindole hydrochloride (Tocris Bioscience, UK) were dissolved in saline (0.9% (w/v) NaCl).



**Figure 1. CYM51010 increases locomotor activity.** Mice were injected with saline (Sal), CYM51010 vehicle (Veh), morphine (10 mg/kg, MOR), or CYM51010 (2.5, 5 or 10 mg/kg, CYM), and locomotor activity was recorded for 60 minutes and expressed in counts per hour (means ± SEM) for total locomotor activity (A) or in counts per 10 minutes (means ± SEM) for the kinetics (B) (n=6–14 animals per group). (A) \*\*\*\*P < .0001, NS (not significant) vs Sal group; ns (not significant) vs MOR group; \*\*\*P < .001 vs Veh group; Kruskal-Wallis test with Dunn multiple comparisons test. (B) \*\*\*\*P < .0001 vs Sal group; \*\*\*\*P < .0001 vs Veh group; 2-way ANOVA with repeated measures with Tukey multiple comparisons test.

Cyprodime hydrochloride (Tocris Bioscience) was prepared in 1 % (v:v) ethanol/ 0.9% (w:v) NaCl. SNC80 (a DOR selective agonist; [Bilsky et al., 1995](#)), Tocris Bioscience, UK) was dissolved in 5.8 mM HCl/ 0.9% (w:v) NaCl. CYM51010 was dissolved in vehicle (0.4 % (v:v) dimethyl sulfoxide/ 1% (v:v) Tween-80). All compounds were injected via the i.p. route (0.1 mL/10 g bodyweight).

### Locomotor Activity and Behavioral Sensitization

Locomotor activity of mice was measured in an actimeter (Imetronic, France) composed of 8 cages (19×11×14 cm) under low illumination (<5 lux). One mouse was placed in each box to record its movements, and displacements were measured by photocell beams located across the long axis and above the floor. Horizontal locomotor activity was recorded for 1 hour and expressed in counts per hour as the total number of interruptions of the photocell beams.

For behavioral sensitization, mice were treated (1 injection per day) for 5 days with morphine (10 mg/kg) or CYM51010 (10 mg/kg). Immediately after injection, mice were placed in an actimeter for 1 hour. After 5 days of withdrawal in their home cage, animals were challenged with morphine (5 mg/kg) or CYM51010 (2.5 or 5 mg/kg), and locomotor activity was measured for 1 hour.

### Conditioned Place Preference

Drug rewarding effects were evaluated using an unbiased CPP protocol ([Tzschentke, 2007](#); [Hajasova et al., 2018](#)). The CPP apparatus (Imetronic, France) consisted of 4 identical boxes. Each one included 2 lateral chambers (15×15×20 cm) connected by a central alley (5×15×20 cm) and 2 sliding doors to separate the chambers from the alley. In each chamber, 2 Plexiglas prisms with triangular bases (5×7×19 cm) were placed to form different patterns and cover the same surface of the chamber. They were used as conditioned stimuli along with 2 different types of embossed Plexiglas floors (one gridded and one striped).

The protocol consisted of 3 phases: (1) preconditioning phase (1 day): drug-naïve animals had free access to both compartments for 18 minutes, and the time spent in each compartment was recorded; (2) conditioning phase (4 days): in the morning of the first conditioning day, animals were treated with saline or vehicle and placed individually in one of the conditioning environments for 18 minutes. In the afternoon, the animals received drug (CYM51010 at various doses or 10 mg/kg morphine) in the opposite compartment and this sequence alternated during the next 3 days. Control animals received saline or vehicle twice a day and were submitted to an alternated sequence between the 2 compartments; (3) test phase (1 day): this phase took place 1 day after the final conditioning session and was carried out exactly as the preconditioning phase. Results were expressed in scores (in seconds) calculated as the difference between the time spent in the drug-paired compartment during the test phase minus the time spent in the same compartment during the preconditioning phase.

**Extinction of CPP**—To extinguish the acquired place preference, mice were subjected to the same alternation between the 2 conditioning chambers but without any injection ([Lu et al., 2011](#); [Hajasova et al., 2018](#)). After 4 days, extinction of morphine CPP was measured: animals were again placed in the center and left to freely explore the apparatus for 18 minutes.

**Reinstatement of CPP**—After place preference was extinguished, all animals received an acute injection of morphine (5 mg/kg) and

they were placed in the CPP apparatus immediately after for CPP test for 18 minutes ([Lu et al., 2011](#); [Hajasova et al., 2018](#)).

### Physical Dependence and Withdrawal

Mice were treated for 5 days with the drug (morphine or CYM51010 at 10 mg/kg, 1 injection per day). On the fifth day, 2 hours after the last injection of agonist, withdrawal was precipitated with an acute injection of naloxone (1 mg/kg). Jumps, as the main withdrawal sign, were measured for 20 minutes.

### Statistical Analysis

All data (expressed as the mean±SEM) were analysed with GraphPad Prism 7. If data followed a normal distribution and variances were similar (determined by the Brown-Forsythe test), data were analyzed by a Student t test (2 groups) or 1- or 2-way ANOVA followed by an appropriate post hoc test for multiple comparisons (more than 2 groups). In case of unequal variances or non-normal data distribution, data were analyzed by a Mann-Whitney test (2 groups) or Kruskal-Wallis test followed by an appropriate post hoc test for multiple comparisons (more than 2 groups).

## RESULTS

### CYM51010 Induces Hyperactivity

In a first set of experiments, we characterized the profile of CYM51010 in the locomotor activity test to find a dose equi-active to that of morphine, the opioid of reference. The dose of 10 mg/kg of morphine was chosen because it is widely used and active in many behavioral tests ([Dockstader and Kooy, 2001](#); [Leite-Morris et al., 2004](#)). We tested different doses of CYM51010. Kruskal-Wallis test showed a significant treatment effect ( $H_{(6)} = 37.35$ ,  $P < .0001$ ; [Figure 1A](#)), with the CYM51010 vehicle devoid of any effect on locomotor activity ( $P > .999$  SAL vs Veh). Only the dose of 10 mg/kg of CYM51010 promoted hyperactivity ( $P = .0007$  Veh vs CYM 10) at a level not significantly different from 10 mg/kg morphine ( $P > .999$  CYM 10 vs MOR). Interestingly, CYM51010 induced a different profile of locomotor activity compared with morphine. Analysis of locomotor activity kinetics revealed effects of treatment ( $F_{(3, 49)} = 29.21$ ,  $P < .0001$ ), time ( $F_{(5, 245)} = 10.71$ ,  $P < .0001$ ), and an interaction between time and treatment ( $F_{(15, 245)} = 23.62$ ,  $P < .0001$ ). CYM51010 induced a fast-onset hyperactivity, with the maximum reached 10 minutes after the injection and lasting for 30 minutes, followed by a rapid decrease (level returned to vehicle treated animals in the last 10 minutes:  $P = .16$  CYM vs Veh), whereas the increase of locomotor activity induced by morphine was slower and reached a plateau after 20–30 minutes ([Figure 1B](#)).

### Role of MOR and DOR in CYM51010 Locomotor Effects

In a second set of experiments, we sought to determine the implication of MOR-DOR heteromers in CYM51010-induced hyperactivity using the MOR antagonist cyprodime and the DOR antagonist naltrindole ([Portoghese et al., 1988](#); [Márki et al., 1999](#)) alone or in combination. We used cyprodime at 10 mg/kg and naltrindole at 5 mg/kg, doses known to selectively block MOR and DOR ([Broccardo et al., 1998](#); [Robinson et al., 2017](#)) (supplementary [Figure 2](#)), respectively. Accordingly, cyprodime blocked morphine-induced hyperlocomotion (supplementary [Figure 2A](#)) and naltrindole blocked SNC80-induced hyperlocomotion (supplementary [Figure 2B](#)). Statistical analysis revealed a treatment

effect in the 3 conditions: cyprodime ( $H_{(3)}=18.38$ ,  $P<.001$ ; Figure 2A) or naltrindole ( $H_{(3)}=18.95$ ,  $P<.0001$ ; Figure 2B) alone or in combination ( $F_{(2, 31)}=20.56$ ,  $P<.0001$ ; Figure 2C). Whereas CYM51010 at 10 mg/kg induced locomotor hyperactivity ( $P<.0001$  vs Cont group; Fig. 2A and C;  $P<.001$  vs Cont group; Figure 2B), cyprodime and naltrindole were slightly able to reduce CYM51010-induced locomotor activity although not significantly ( $P=.0178$  CYM vs Cyp +CYM group;  $P>.99$  CYM vs NTI + CYM group; Figure 2). When the 2 opioid antagonists were combined, only a weak, but not significant, reduction of CYM51010-induced locomotor

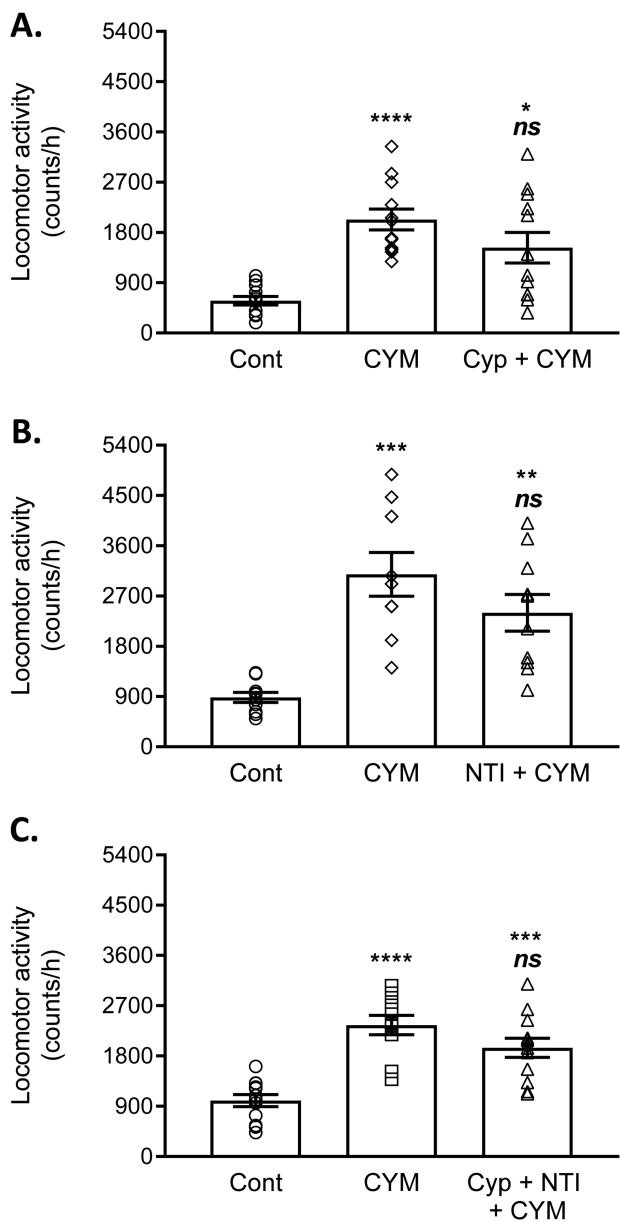
activity was observed ( $P=.17$  CYP + NTI + CYM vs CYM group; Figure 2C).

## Effect of CYM51010 on Behaviors Related to Addiction

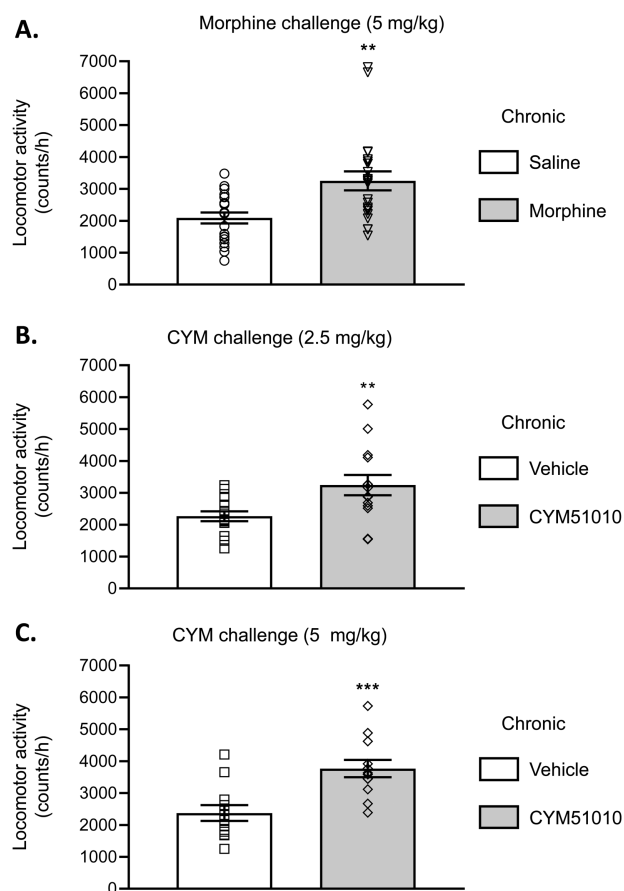
Because CYM51010 targets opioid receptors, we sought to determine if this molecule could be active in some common preclinical models related to addiction: locomotor sensitization (to evaluate dopaminergic system sensitization), CPP (to measure rewarding effect), and antagonist-precipitated withdrawal (to evaluate physical dependence).

### Behavioral Sensitization—

To induce locomotor sensitization, mice were treated for 5 days in the actimeter. After 5 days of withdrawal, they received an agonist challenge dose before locomotor activity measurement. As depicted in Figure 3A, mice that were subjected to repeated morphine exposure at 10 mg/kg showed a significant increase of their locomotor activity compared with saline-treated mice ( $U=99$ ,  $P=.0019$ ). When mice were repeatedly treated with CYM51010, a CYM51010 challenge after 5 days of withdrawal increased locomotor activity, whatever the



**Figure 2. Effects of MOR and DOR antagonists on CYM51010-induced locomotor activity.** Mice were injected with CYM51010 (CYM, 10 mg/kg) and locomotor activity (expressed as the means  $\pm$  SEM) was measured for 60 minutes. Mice received a cyprodime (Cyp, 10 mg/kg) or naltrindole (NTI, 5 mg/kg) injection 1 hour or 15 minutes before CYM51010 treatment, respectively. Control (Cont) mice received saline and the corresponding vehicles of cyprodime or naltrindole ( $n=12$  [A], 9–10 [B], and 10–12 [C] animals per group). \* $P<.05$ , \*\* $P<.01$ , \*\*\* $P<.001$ , \*\*\*\* $P<.0001$  vs Cont group; ns (not significant) vs CYM group; Kruskal-Wallis test (A, B) or 1-way ANOVA (C) followed by Dunn (A, B) or Tukey (C) multiple comparisons test.



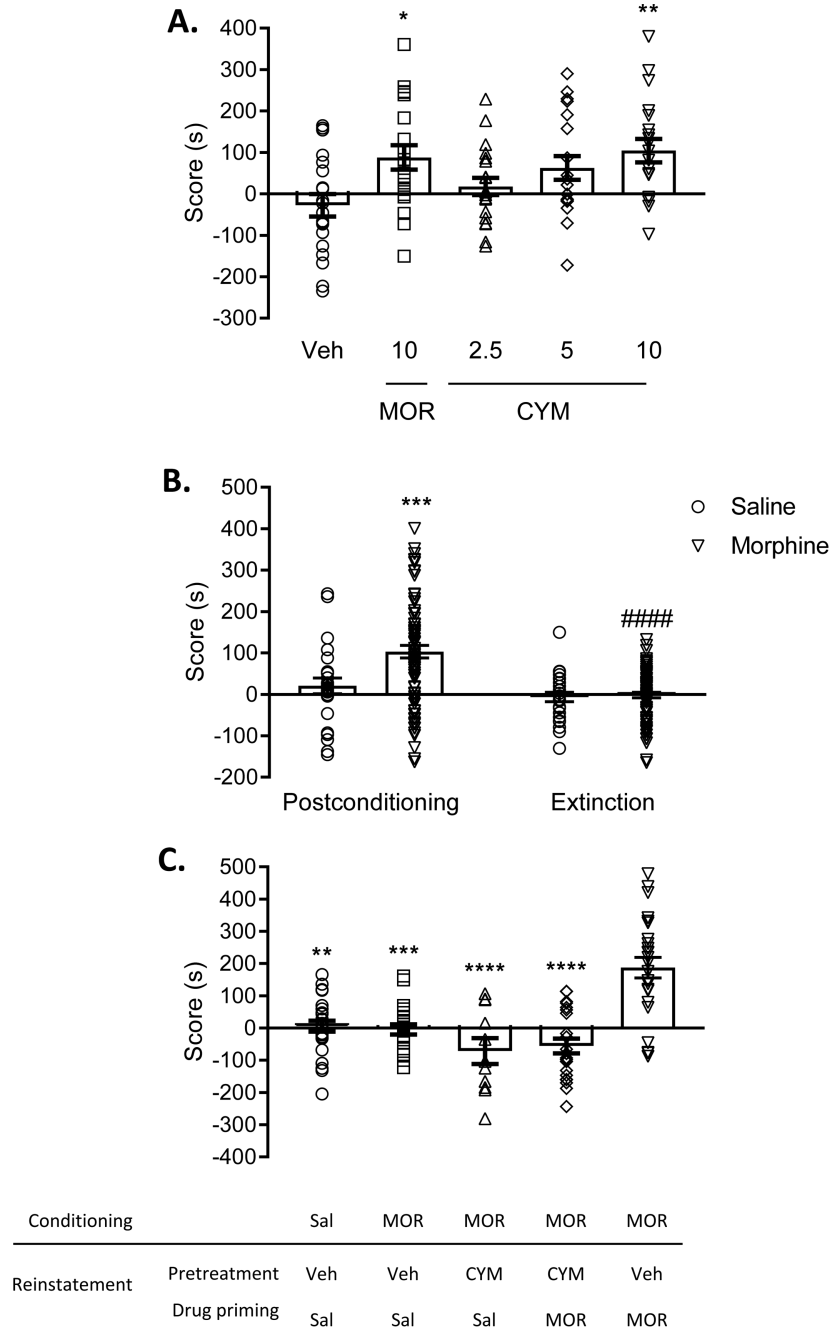
**Figure 3. CYM51010 induces behavioral sensitization.** Mice were treated with saline (Saline, A), CYM51010 vehicle (Vehicle, B and C), morphine (10 mg/kg, A), or CYM51010 (10 mg/kg, B and C) for 5 days (1 injection per day). On the fifth day of withdrawal, all animals received a morphine (5 mg/kg, A) or CYM51010 (2.5 mg/kg [B] or 5 mg/kg [C]) challenge and locomotor activity (expressed as means  $\pm$  SEM) was recorded for 60 minutes ( $n=12$  [A, C] or 14 [B] animals per group). \* $P<.01$ , \*\*\* $P<.001$  vs Saline or Vehicle group; Student t (A, B) or Mann-Whitney (C) test.



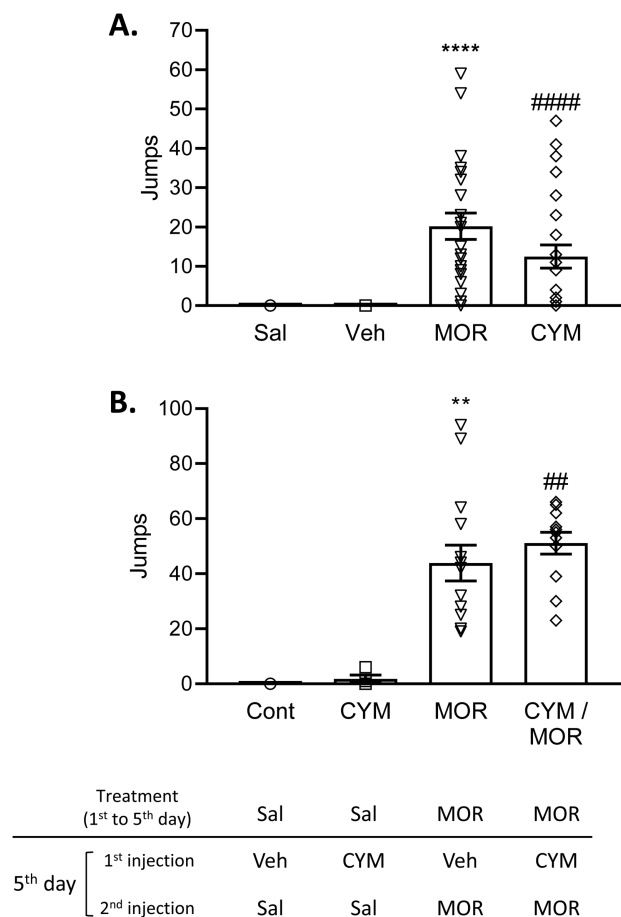
challenging dose used: 2.5 mg/kg ( $U=40, P=.0067$ ; Figure 3B) or 5 mg/kg ( $t_{(22)}=3.802, P=.001$ ; Figure 3C).

**Rewarding Effects**—CPP was used to measure putative reinforcing effects of CYM51010 at different doses (Figure 4A). Statistical analysis revealed a treatment effect ( $F_{(4,94)}=4.044, P<.01$ ) with the dose of 10 mg/kg CYM51010 inducing a significant CPP ( $P<.01$  CYM 10 mg/kg vs Veh group), not different from morphine ( $P=.9995$  CYM 10 mg/kg vs MOR group).

**Physical Dependence**—Physical dependence was measured using naloxone-precipitated withdrawal signs paradigm (see Materials and Methods for details). Only jumps were measured because it is the most reliable sign of withdrawal when a short period treatment is used (El-kadi and Sharif, 1994). Statistical analysis revealed an effect of treatment ( $H_{(4)}=61.61, P<.001$ ; Figure 5A) with 10 mg/kg morphine promoting naloxone-precipitated withdrawal signs ( $P<.0001$  vs Sal group). Interestingly, CYM51010 at 10 mg/kg also promoted naloxone-precipitated withdrawal



**Figure 4. Effects of CYM51010 on reward and on morphine-primed reinstatement of an extinguished conditioned place preference (CPP).** Rewarding effects of CYM51010 (A): CPP was induced with vehicle (Veh), morphine (10 mg/kg, MOR), or different doses of CYM51010 (2.5, 5 and 10 mg/kg, CYM) ( $n=19$  to 20 animals per group). (B) Effects of CYM51010 on morphine-primed reinstatement of an extinguished CPP: CPP was induced with morphine (MOR) or saline (Sal) and then extinguished. After extinction, CPP was reinstated with an acute injection of morphine (MOR) at 5 mg/kg or saline (Sal) in absence (Veh) or presence of CYM51010 (CYM) at 10 mg/kg injected 2 hours before MOR priming (C) ( $n=11-26$  animals/group). CPP scores (in seconds) were expressed as the means  $\pm$  SEM. (A)  $P<.05, P<.01$  vs Veh group; 1-way ANOVA and Sidak multiple comparisons test. (B)  $^{***}P<.001$  vs saline/postconditioning,  $^{####}P<.0001$  vs morphine/postconditioning; 2-way ANOVA with repeated measures followed by Sidak multiple comparisons test. (C)  $^{**}P<.01, ^{***}P<.001, ^{****}P<.0001$  vs MOR/Veh/MOR group; 1-way ANOVA and Dunn multiple comparisons test.



**Figure 5. Effect of CYM on physical dependence.** Induction of physical dependence with opioid agonists (A): mice were treated for 5 days (1 injection per day) with saline (Sal), Vehicle (Veh), morphine (MOR), or CYM51010 (CYM) at 10 mg/kg. On the fifth day, mice received naloxone (1 mg/kg) 2 hours after the saline, vehicle, or agonist injection and jumps (means  $\pm$  SEM) were quantified for 20 minutes ( $n=19$  to 25 animals/group). (B) Effects of CYM51010 on morphine-induced physical dependence: mice received saline (Cont, CYM) or morphine (MOR, CYM/MOR) for 5 days. On the fifth day, 2 hours before the saline or morphine injection, they were given CYM51010 vehicle (Cont, MOR) or CYM51010 (CYM, CYM/MOR) at 10 mg/kg. Then 2 hours after saline or morphine injection, they received naloxone (1 mg/kg), and jumps (means  $\pm$  SEM) were quantified for 20 minutes ( $n=4-5$  animals per group for Cont and CYM groups and 12–14 for MOR and CYM/MOR groups). (A) \*\*\*\* $P < .0001$  vs Sal group, #### $P < .0001$  vs Veh group; (B) \*\* $P < .01$  vs Cont group, ## $P < .01$  vs CYM group; Kruskal-Wallis test followed by a Dunn multiple comparisons test.

signs ( $P < .0001$  vs Veh group), but nearly one-half as important as the morphine (20.17 jumps for morphine vs 12.48 jumps for CYM51010).

### Effects of CYM51010 on Morphine-Induced Rewarding Effects and Physical Dependence

Previous studies showed that morphine treatment increased MOR-DOR neuronal coexpression and heteromerization (Gupta et al., 2010; Pierre et al., 2019), suggesting that targeting heteromers could affect morphine effects. We first tested the effect of CYM51010 on morphine-induced reinstatement of an extinguished CPP. Figure 4B shows the CPP scores after morphine conditioning (postconditioning) and after the extinction procedure with an effect of morphine treatment ( $F_{(1, 105)} = 6.069$ ,  $P = .015$ ),

conditioning (postconditioning vs extinction) ( $F_{(1, 105)} = 22.02$ ,  $P < .0001$ ), an interaction between treatment and conditioning ( $F_{(1, 105)} = 7.685$ ,  $P = .0066$ ), and a subject effect ( $F_{(105, 105)} = 1.6112$ ,  $P = .007$ ). Post hoc analysis revealed that morphine induced CPP ( $P < .001$  saline/postconditioning vs morphine/postconditioning). The CPP score of morphine-treated animals after extinction was at the same level as the saline-conditioned mice ( $P = .969$  saline/extinction vs morphine/extinction), and the score of the saline group was not affected by the extinction procedure ( $P = .47$  saline/postconditioning vs saline/extinction) (Figure 4B). To determine the effects of CYM51010 on reinstatement, CPP was reinstated after an injection of a low dose of morphine (5 mg/kg) (Hajasova et al., 2018) in the presence or absence of CYM51010. The Kruskal-Wallis ANOVA revealed a significant effect of treatment ( $H_{(5)} = 35.6$ ,  $P < .0001$ ; Figure 4C). Post hoc analysis revealed that challenging with morphine, but not saline, promoted reinstatement of an extinguished CPP ( $P < .001$  MOR/Veh/MOR vs MOR/Veh/Sal). Reinstatement was no more observed in the presence of CYM51010 ( $P < .0001$  MOR/CYM/MOR vs MOR/Veh/MOR) with a score not significantly different from saline- or morphine-conditioned and saline-primed mice ( $P = .889$  MOR/CYM/MOR vs Sal/Veh/Sal;  $P > .999$  MOR/CYM/MOR vs MOR/Veh/Sal) (Figure 4C).

Finally, we determined the effects of CYM51010 on morphine-induced physical dependence. The statistical analysis showed a significant effect of treatment ( $H_{(4)} = 20.78$ ,  $P < .001$ ; Figure 5B). Whereas the 5-day treatment with morphine was able to induce an increase of withdrawal signs after naloxone injection ( $P < 0.01$  MOR vs Cont), administration of CYM51010 2 hours before morphine injection did not modify the number of withdrawal signs precipitated by the antagonist ( $P > .99$  CYM/MOR vs Cont) (Figure 5B).

## DISCUSSION

In recent years, evidence accumulated in favor of the existence of opioid receptor heteromers (Massotte, 2015). However, their role in opioid signaling has yet to be understood. Indeed, very few tools are available to investigate their function. By screening a small compound library, Devi's group developed the CYM51010, a preferring MOR-DOR heteromer agonist. This compound demonstrated an analgesic effect with less propensity to induce tolerance and withdrawal; however, no data were available regarding other opioid behaviors related to addiction (Gomes et al., 2013).

It is well known that activation of either MOR or DOR increases locomotor activity (Andrew Mickleley et al., 1990). Our data showed that CYM51010 induced hyperactivity to the same extent as morphine. Interestingly, both MOR and DOR antagonists had only a partial effect on CYM51010-induced locomotor activity. It is unlikely that this partial blockade was due to the use of a subactive dose of cyprodime or naltrindole. Indeed, the doses we used blocked hyperlocomotion induced by DOR or MOR selective agonists (Broccardo et al., 1998; Robinson et al., 2017) (supplementary Figure 2). As previously reported, CYM51010 was not only able to activate MOR-DOR heteromers but also non-associated MOR (Tiwari et al., 2020) or DOR (Derouiche et al., 2020) receptors. If locomotor activity was resulting from the activation of the non-associated MOR and DOR, co-administration of the DOR and MOR selective antagonists should prevent it. However, concomitant administration of CYP and NTI was unable to block CYM51010-induced locomotor activity. This suggests that the main effect of CYM51010 was not mediated through non-associated MOR and DOR but rather by MOR-DOR heteromers. Moreover, naloxone

(a nonselective opioid receptor antagonist) was able to block CYM51010-induced locomotor activity (supplementary Figure 3A), whereas nor-BNI (a KOR selective antagonist) was not (supplementary Figure 3B). This effect of naloxone combined with the observation that this antagonist seems capable of destabilizing opioid receptor dimers (Möller et al., 2020) reinforces the idea that CYM51010 acts on MOR-DOR heteromers. Our data are in accordance with the pharmacological characteristics of CYM51010 described as a preferred MOR-DOR ligand with a potency ratio of 1 (MOR)/4(DOR)/6(MOR/DOR) (Gomes et al., 2013). The data are also in agreement with MOR-DOR neuronal coexpression in the motor pathway (Erbs et al., 2015). Regarding locomotor activity kinetics, morphine and CYM51010 behaved differently. CYM51010 acted very rapidly, with the maximum effect reached 10 minutes after injection, whereas 30 minutes was necessary for morphine to reach its maximum effect. This might be explained by high  $\text{miLogP}$  (3.99) for CYM51010 compared with 1.1 for morphine ( $\text{miLogP}$  calculated with <https://www.molinspiration.com>), which would confer a higher propensity to cross the blood-brain barrier.

In rodents, repeated exposure to opiates could lead to “behavioral sensitization,” defined as an increase of behavioral responses following repeated drug administration (Robinson and Berridge, 1993). Our data clearly showed that repeated treatment with CYM51010 induced behavioral locomotor sensitization. This result is of particular importance. Indeed, behavioral sensitization is considered a good marker of neurochemical changes that underlie addiction (Robinson and Berridge, 1993, 2003; Vanderschuren and Kalivas, 2000; Vezina and Leyton, 2009) because it involves brain structures known to play a role in reward processes (Wise and Bozarth, 1987). One of the easiest and most robust paradigms to measure the rewarding effects of a drug is CPP (Tzschentke, 2007). Our data clearly showed that CYM51010 had dose-dependent rewarding effects, similarly to morphine (Fig. 4A). In a recent study, Tiwari and coworkers were able to induce CPP with CYM51010 in rats following sciatic nerve ligation (Tiwari et al., 2020). Our results clearly demonstrated that CYM51010 had reinforcing effects even in naive animals. Besides rewarding effects, chronic opioid use could lead to physical dependence, evidenced by some behavioral signs following the agonist withdrawal or by the injection of an opioid antagonist (Glass, 2010). Our data showed that a short treatment with CYM51010 induced a physical dependence but to a lower level than morphine. These data are in agreement with previous findings by Gomes and coworkers indicating that repeated treatment with CYM51010 promoted less severe signs of withdrawal compared with morphine (Gomes et al., 2013), which might be due to its partial agonist activity at MOR (Faouzi et al., 2020). Altogether, these data showed that the preferential MOR-DOR agonist CYM51010 behaved like a regular MOR agonist in some models related to addiction.

Chronic morphine treatment increases MOR-DOR neuronal coexpression and heteromerization in circuits related to drug reward, motor activity, visceral control, and emotional processing underlying withdrawal (Gupta et al., 2010; Pierre et al., 2019), suggesting that targeting heteromers could modulate the behavioral effects of morphine. Importantly, the increase in MOR-DOR expression is maintained after 4 weeks of abstinence in brain structures involved in CPP reinstatement such the nucleus accumbens (Hearing et al., 2016) and for which no heteromers were detected in basal state (Pierre et al., 2019). In this case, we might expect an increase of the MOR-DOR proportion vs MOR after the extinction of CPP. So, when CYM51010 is applied before the reinstatement with morphine, it could lead to

both MOR internalization (dragged with DOR in the MOR-DOR heteromers) (Derouiche et al., 2020) and phosphorylation (supplementary Figure 5) leading to MOR desensitization. Altogether, it will prevent morphine action on MOR and thus precludes CPP reinstatement as observed. However, this hypothesis could be in apparent contradiction with the results obtained in a physical dependence experiment, where CYM51010 was unable to prevent naloxone-induced morphine withdrawal. This suggests a brain region-dependent regulation of MOR-DOR heteromers. The increase in MOR-DOR heteromerization following morphine treatment may be lower in regions involved in withdrawal, such as the motor circuit, compared with the reward circuit. Hence, the contribution MOR-DOR heteromers to morphine dependent behavior would be lower in this circuit compared with the contribution of non-associated MORs. Thus, when CYM51010 was administered 2 hours before the morphine injection, the higher proportion of non-associated MOR would be responsible for the increased locomotor activity in response to morphine administration (supplementary Fig. 4). Because naloxone is not a selective opioid receptor ligand (Raynor et al., 1994), an alternative explanation would be the involvement of other opioid receptors such as KOR in the expression of withdrawal symptoms. Indeed, KOR knockout mice showed reduced morphine withdrawal symptoms (Simonin et al., 1998), and nor-BNI (Norbinaltorphimine) is able to precipitate withdrawal syndrome after a morphine treatment (Maldonado et al., 1992).

## Conclusions

Taken together, we found that although CYM51010 shares many properties with classical MOR agonists in models related to addiction, this ligand has interesting pharmacological properties. Indeed, it promotes behavioral sensitization, reinforcing effect and withdrawal symptoms. However, our results confirm lower physical dependence compared with morphine. Moreover, the ability of CYM51010 to block the reinstatement of an extinguished morphine-induced CPP suggests that targeting MOR-DOR heteromers could be useful to reduce craving and subsequent relapse.

## Supplementary Materials

Supplementary data are available at *International Journal of Neuropsychopharmacology* (IJNPPY) online.

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## Interest Statement

None of the authors report potential conflicts of interest.

## Data Availability

The data underlying this article will be shared on reasonable request to the author.



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