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ORIGINAL ARTICLE

Involvement of the nociceptin opioid peptide receptor in morphine-induced antinociception, tolerance and physical dependence in female mice

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Abstract

Nociceptin opioid peptide (NOP) receptor modulates pain transmission and is considered a prospective target for pain management. Under acute pain conditions in rodents, however, no definitive conclusions about effects of systemically intervening NOP receptors on nociception, classical opioid-induced antinociception, tolerance and physical dependence have been drawn. Given that opioid analgesia has sex differences, and females experience greater pain and consume more opioids, clarifying these issues in females will help develop novel analgesics. To clarify the role of NOP receptors on the pharmacological profiles of μ-opioid receptor agonists, in this study, a selective agonist (SCH221510) and antagonist (SB612111) of the NOP receptor were subcutaneously administered in female mice in multiple animal models. In hot-plate test, neither SCH221510 (3 and 10 mg/kg, sc) nor SB612111 (10 mg/kg, sc) produced significant antinociception. SCH221510 (3 mg/kg, sc) attenuated but SB612111 (10 mg/kg, sc) enhanced morphine-induced antinociception, with rightward and leftward shift of morphine dose-response curves, respectively. SCH221510 (3 mg/kg, sc) combined with morphine (10 mg/kg, sc) accelerated the development of morphine antinociceptive tolerance. Conversely, SB612111 (10 mg/kg, sc) delayed morphine tolerance development. Neither SCH221510 (3 mg/kg, sc) nor SB612111 (10 mg/kg, sc) statistically significantly altered the development of morphine-induced physical dependence. Therefore, systemic activation of NOP receptors attenuated morphine antinociception to acute thermal stimuli, facilitated morphine-induced antinociceptive tolerance but did not robustly alter physical dependence in female mice. Systemic blockade of NOP receptors produced opposite actions. These findings demonstrate that N/OFQ-NOP receptor system plays diverse roles in modulating pharmacological profiles of μ-opioid receptor agonists.

Keywords Nociceptin opioid peptide receptor · Acute pain · Morphine · Antinociception · Tolerance · Physical dependence

Introduction

Opioid receptors, especially the μ subtype, are essential pain management targets. μ-Opioid receptor agonists (e.g., morphine and fentanyl) are commonly used in the clinical setting to relieve severe pain. However, the clinical utility of μ-opioid agonists is severely limited by several undesirable effects, including respiratory depression and sedation. Long-term administration of μ-opioid agonists also produces analgesia tolerance, physical dependence, and addiction liability.

Nociceptin opioid peptide receptor (NOP receptor), also named opioid receptor-like receptor 1, is the fourth member of the opioid receptor family, whose endogenous ligand is the nociceptin/orphanin FQ (N/OFQ) opioid peptide (Toll et al. 2016). Like μ-, δ-, and κ-opioid receptors, NOP receptors couple with pertussis toxin-sensitive $G_{i/o}$ protein; however, most ligands that bind to μ-, δ-, and κ- subtypes display a low affinity for the NOP receptor (Toll et al. 2016). NOP receptors enrich in both ascending and descending pain pathways, including the periaqueductal gray matter (PAG), thalamic nuclei, somatosensory cortex, rostral ventral medulla (RVM), lateral parabrachial nucleus, spinal cord, and dorsal root ganglia (DRGs), suggesting modulation of

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nociceptive processing (Neal et al. 1999; Florin et al. 2000). NOP receptors are also widely distributed in the mesolimbic pathways associated with reward and motivation (Neal et al. 1999). Because activation of NOP receptors not only lacks μ-opioid receptor-associated adverse effects such as respiratory depression, rewarding and reinforcement but also attenuates μ-opioid agonist-induced rewarding and reinforcing effects in conditioned place preference and self-administration models in rodents (Murphy and Maidment 1999; Ciccocioppo et al. 2000; Rutten et al. 2010; Zaveri 2011; Sukhtankar et al. 2014), the NOP receptor system may function as a useful μ-opioid receptor agonist adjunct. Recently, the mixed μ/NOP receptor agonist cebranopadol has displayed potential as a novel analgesic in several clinical trials (Calo and Lambert 2018).

However, the modulation of NOP receptors for pain transmission as well as μ-opioid receptor-induced analgesia is complex, depending on the species (rodents vs. non-human primates), pain states (acute pain vs. chronic pain), and routes of drug administration (the systemic, intrathecal, or intracerebral route). There are contradictory reports regarding the acute pain of rodents even with the same administration routes, especially with systemic administration. For example, systemic combinations of Ro 64-6198, a selective NOP receptor agonist, and a μ-opioid agonist produced additive antinociception in the mouse hot-plate test (Reiss et al. 2008). Conversely, SB612111, a selective high-affinity NOP receptor antagonist, potentiated antinociception induced by buprenorphine and the other mixed μ/NOP receptor agonists in the mouse tail-flick assay (Khroyan et al. 2009), suggesting that systemic activation of NOP receptors attenuating μ-opioid receptor-mediated antinociception. Similarly, no definite conclusions about the role of systemic modulating NOP receptor in the development of morphine analgesic tolerance and physical dependence have been drawn until now.

Furthermore, sex-related difference in pain and opioids is another important issue. Although women experience greater clinical pain, lower pain threshold and tolerance, more sensitivity and distress to experimentally induced pain compared with men, sex differences in response to opioid treatment revealed inconsistent results (Nasser and Afify 2019). Similarly, contradictory results have been shown in different animal pain models (Mogil et al. 2000; Cicero et al. 1996; Kest et al. 1999). Male rats show more sensitivity to the analgesic effects of morphine than females in the hot-plate, abdominal-constriction and tail-flick tests (Cicero et al. 1996); however, in CBA/J mice, females exhibit a 5-fold increase in sensitivity to morphine compared with males (Kest et al. 1999). The sex differences can be attributed to neuroanatomical factors such as distinct μ-opioid receptor expression and activation in PAG and hormonal aspects (Nasser and Afify 2019). Moreover, previous research has reported sex differences in the N/OFQ-NOP receptor system in rat spinal cord following chronic morphine treatment (Zhang et al. 2012). Considering that male animals are usually used in the previous research, it is of great importance to investigate the role of NOP receptors in pain modulation and opioid-induced antinociception in females.

Since systemic administration is the standard analgesic delivery route in the clinic, it is essential to clarify the effects of systemically activating or blocking NOP receptors on the pharmacological profiles of μ-opioid receptor agonists in the same experimental system, which is necessary to develop novel analgesic medications targeting NOP and μ-opioid receptors. By systemic administration of a selective agonist (SCH221510) and antagonist (SB612111) of the NOP receptor, we investigated NOP receptor modulation of morphine-induced antinociception in an acute pain model and the antinociceptive tolerance and physical dependence of morphine in female mice.

Materials and methods

Animals

Female C57BL/6J mice, 8 weeks old, weighing 18–20 g at the start of the experiment, were purchased from SPF (Beijing) Laboratory Animal Technology Co. Ltd. (Beijing, China). Animals were housed under a 12-h light/dark cycle (lights on at 6:00 a.m. and lights off at 6:00 p.m.) at 22–24 °C and 45–75 % humidity, with food and water available *ad libitum*. All experimental protocols were approved by the Institutional Review Committee for the Use of Animals (IACUC of AMMS-06-2019-002) and were conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals. All experiments were conducted during the light cycle. On behavioral test days, animals were transported to the testing room and acclimated to the environment for 1 h. After behavioral test, mice were sacrificed by overdose of pentobarbital sodium. The sample size was determined by PASS software calculation ($\alpha = 0.05$, $\beta = 0.1$) and our pilot experiment, which was also in accordance with 3R principle of animal experiment.

Reagents

Morphine sulfate (Qinghai Pharmaceutic Factory, Xining, China) and naloxone hydrochloride (SigmaAldrich, St. Louis, MO, USA) were dissolved in normal saline. SCH221510 (Tocris Bioscience, Bristol, UK) was dissolved in 2 % dimethyl sulfoxide (DMSO), 2 % Tween-80, and normal saline. SB612111 was dissolved in 2 % DMSO and normal saline. All drugs except naloxone were administered via subcutaneous (sc) injection at a volume of 0.1 ml/10 g;

naloxone was administered via intraperitoneal (ip) injection at a volume of 0.1 ml/10 g.

Hot-plate test

Mice were individually placed on a hot-plate apparatus (BIO-CHP, Bioseb, France) maintained at 55 ± 0.1 °C, and the time until licking the hind paws was recorded as the latency period. The cut-off time was 60 s to avoid tissue damage. Before drug administration, the baseline latency of hind paw licking was examined. Animals with baseline latencies lower than 10 s or above 25 s were excluded. After drug administration, the hind paw licking latency was tested again. Antinociception was expressed as the maximum possible effect (MPE): MPE% = (latency after drug administration-baseline latency) / (60-baseline latency) × 100.

Antinociceptive action

To observe the antinociception of SCH221510 (3 and 10 mg/kg, sc) or SB612111 (10 mg/kg, sc), the hind paw licking latency was tested at 30, 60, and 120 min after drug administration in each case (n = 12 per group). The doses of SCH221510 and SB612111 were chosen according to previous studies (Fichna et al. 2014; Khroyan et al. 2009, 2011; Sobczak et al. 2014).

To determine the effect of activating or blocking NOP receptors on morphine-induced antinociception, SCH221510 (3 mg/kg, sc) or SB612111 (10 mg/kg, sc) was administered 15 min before morphine injection (n = 10 per group). The hind paw licking latencies were measured for the time-response curve at 30, 60, and 120 min after morphine (5.6 mg/kg, sc) injection. For the dose-response curve, latencies were tested 30 min after morphine (3.2–10 mg/kg, sc) injection, and the ED_{50} value was calculated by the linear regression method.

Morphine-induced antinociceptive tolerance

To induce antinociceptive tolerance, the mice received a constant dose of morphine (10 mg/kg, sc) twice daily for six consecutive days (Lutfy et al. 2001). Morphine antinociception was tested every day. At 8:00 a.m., the baseline latency of hind paw licking was tested, and morphine (10 mg/kg, sc) was administered. Thirty minutes after morphine injection, the latency was tested again, and then SCH221510 (3 mg/kg, sc) or SB612111 (10 mg/kg, sc) was administered immediately. For another drug administration at 8:00 p.m., SCH221510 (3 mg/kg, sc) or SB612111 (10 mg/kg, sc) was administered 15 min prior to morphine (10 mg/kg, sc) injection (n = 10 per group). On day 7, morphine antinociception (10 mg/kg, sc) was tested.

Morphine-induced physical dependence and naloxone-precipitated withdrawal

To induce physical dependence, mice received ascending morphine doses (10, 20, 30, 40 and 50 mg/kg) twice daily for five consecutive days, which was in accordance with previous report (Kest et al. 2001). Four hours after the last morphine injection, naloxone (5 mg/kg, ip) was injected and the number of jumps was counted within 30 min. To determine the effect of NOP receptor on the development of morphine-induced physical dependence, SCH221510 (3 mg/kg, sc) or SB612111 (10 mg/kg, sc) pretreatment was administered 15 min before each morphine injection (n = 10 per group).

Locomotion test

Locomotor activity was assessed in a locomotor chamber (Anilab, Ningbo, China) and monitored by a video tracking system. Before drug treatment, mice were acclimatized to the locomotor chambers for 30 min per day for 2 days. The average locomotor activities during acclimatization were considered baseline, and the mice were grouped according to their basal locomotion. On the test day, mice received the SCH221510 (3 or 10 mg/kg, sc) or SB612111 (10 mg/kg, sc) injection, and their locomotion was measured immediately for 60 min (n = 10 per group).

Rotarod test

To determine the effects on motor coordination, the rotarod test was performed using a constant speed device (YLS-4 C, China). One day before the test, mice were trained on the rotarod at a constant speed of 25 rpm in three trials with 3 min/trial. Only those mice that could stay on the rod for 180 s in two consecutive trials were used for the following tests. On the testing day, the fall latency was recorded for each mouse at 30, 60, and 90 min after SCH221510 (3 or 10 mg/kg, sc) or SB612111 (10 mg/kg, sc) administration in each case (n = 10 per group). A cut-off time of 180 s was chosen.

Statistical analyses

All data are presented as the mean ± standard deviation (S.D.) for parametric and median and interquartile range for nonparametric data. Data was statistically analyzed using SPSS 26.0 and plotted with GraphPad Prism 7.04. Shapiro-Wilk and Levene tests were used for normality and homogeneity analysis, respectively. One-way analysis of variance (ANOVA) followed by *Tukey* test, nonparametric test or two-way ANOVA with one repeated measurement (*Greenhouse-Geisser* correction) followed by *Tukey* test was used to compare the differences among multiple groups. Statistical

significance was defined as $P<0.05$. The ED_{50} values were calculated from lg dose-response curves by linear regression analysis. The statistical difference between the ED_{50} values was analyzed using a *t*-test.

Results

Systemic administration of NOP receptor agonist attenuated but antagonist enhanced morphine-induced antinociception in the acute pain model of female mice

Whether systemic administration of SCH221510 or SB612111 produce antinociception, sedation or discoordination was tested firstly. Figure 1a shows the time-antinociceptive response curves of systemic administration of SCH221510 and SB612111 in the mouse hot-plate test, a thermal-stimulated acute pain model. Two-way ANOVA with one repeated measurement revealed no statistical significance in the treatment ($F_{(3,44)}=0.176$, $P=0.912$), time ($F_{(3.1,136.8)}=1.379$, $P=0.251$) or interaction ($F_{(9.3,136.8)}=1.108$, $P=0.361$). This finding indicated that neither SCH221510 (3 and 10 mg/kg, sc) nor SB612111 (10 mg/kg, sc) produced significant antinociceptive action. Meanwhile, both SCH221510 (3 and 10 mg/kg, sc) and SB612111 (10 mg/kg, sc) failed to alter the locomotor activity in the locomotion test ($F_{(3,36)}=0.614$, $P=0.611$; one-way ANOVA; Fig. 1b) and the latency to fall in the rotarod test (treatment: $F_{(3,36)}=0.525$, $P=0.668$; time: $F_{(1.4,49.8)}=1.704$, $P=0.198$; interaction: $F_{(4.2,49.8)}=0.765$, $P=0.557$; two-way ANOVA with one repeated measurement; Fig. 1c), suggesting no sedation or motor coordination dysfunction in mice.

Then the effect of systemic administration of SCH221510 or SB612111 on morphine-induced antinociception was investigated. When NOP receptor ligands in combination with morphine (5.6 mg/kg, sc), SCH221510 (3 mg/kg, sc) decreased the antinociceptive response compared with that in the morphine group (treatment: $F_{(3,36)}=9.079$, $P<0.001$; time: $F_{(1.9,68.6)}=85.942$, $P<0.001$; interaction: $F_{(5.7,68.6)}=6.185$, $P<0.001$; two-way ANOVA with one repeated measurement; Fig. 2a). Furthermore, morphine (3.2–10 mg/kg, sc) produced a dose-dependent antinociceptive effect. When SCH221510 (3 mg/kg, sc) was concurrently administered with morphine, the dose-response curve of morphine antinociception shifted rightward (Fig. 2b). In contrast, SB612111 (10 mg/kg, sc) caused a leftward shift in the dose-response curve of morphine antinociception (Fig. 2b). Additionally, the ED_{50} value of SCH221510 + morphine group was higher than that of the morphine group ($t' = 7.526$, $T=1.96$, $t' > T$, $P<0.05$), whereas the ED_{50} value of the SB612111 + morphine group was lower than that of the morphine group ($t' = 5.758$, $T=1.96$, $t' > T$, $P<0.05$) (the ED_{50} values are shown

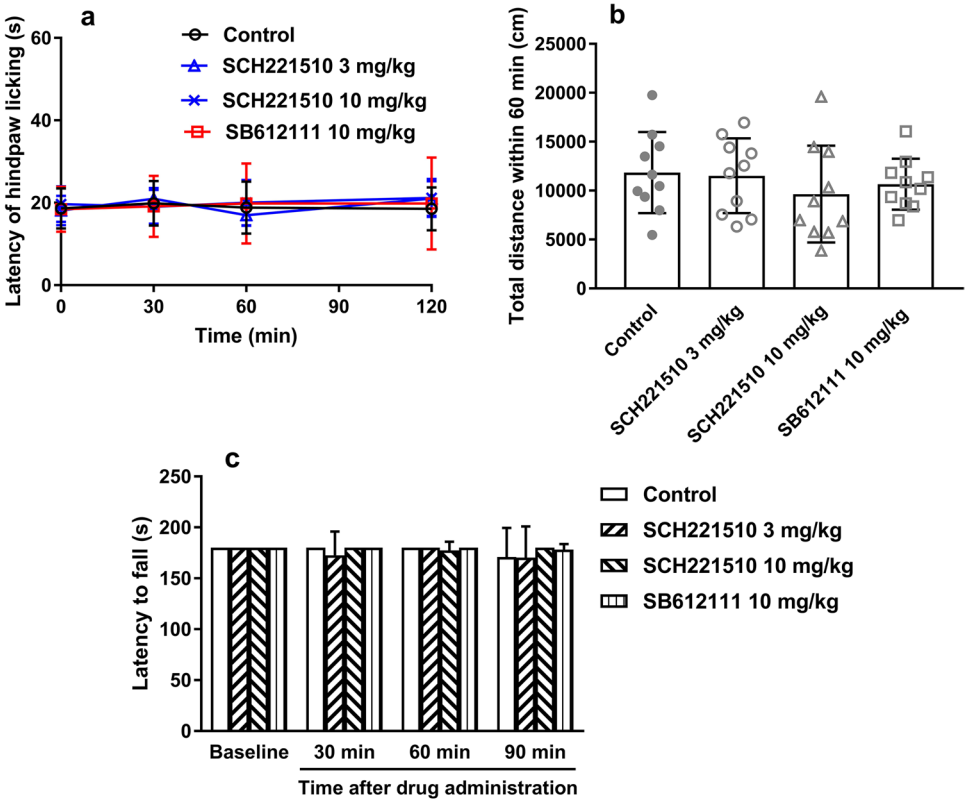

Fig. 1 Effects of systemic administration of SCH221510 and SB612111 on nociceptive responses, locomotor activity and motor coordination in female mice. (**a**) Hot-plate test; $n=12$, mean ± S.D. (**b**) Locomotion test; $n=10$, mean ± S.D. (**c**) Rotarod test; $n=10$, mean ± S.D

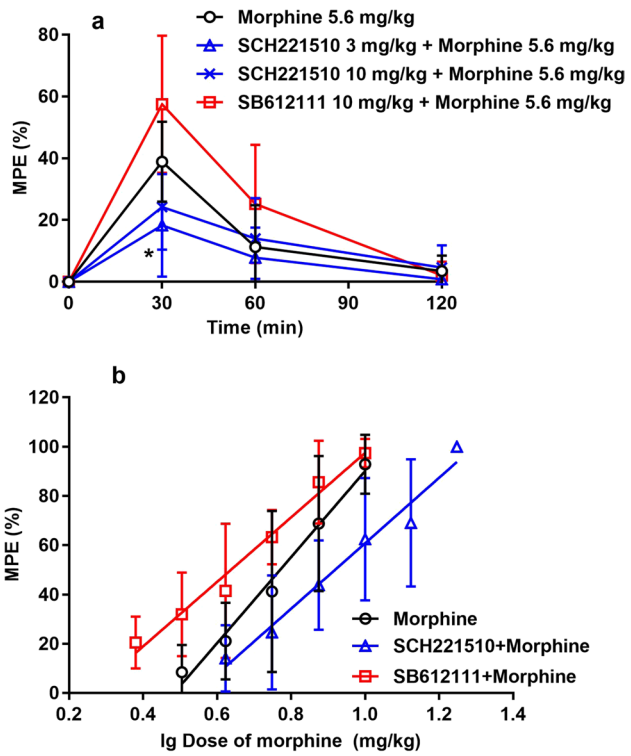

Fig. 2 Effects of systemic administration of SCH221510 and SB612111 on morphine antinociception in hot-plate test of female mice. The NOP receptor agonist SCH221510 (3 or 10 mg/kg) or antagonist SB612111 (10 mg/kg) was administered subcutaneously (sc) 15 min before morphine injection. $n = 10$, mean ± S.D. (**a**) The time-response curves for morphine (5.6 mg/kg, sc) antinociception. $^*P = 0.044$, the SCH221510 3 mg/kg + morphine 5.6 mg/kg group vs. the morphine 5.6 mg/kg group; $P = 0.081$, the SB612111 10 mg/kg + Morphine 5.6 mg/kg group vs. the Morphine 5.6 mg/kg group; two-way ANOVA with one repeated measurement followed by *Tukey* test. (**b**) Dose-response curves of morphine antinociception

Table 1 ED_{50} values of morphine antinociception in the mouse hot-plate test

	ED_{50} (mg/kg)	95 % Confidence intervals (mg/kg)
Morphine	5.81	5.43, 6.21
SCH221510 + Morphine	8.39	7.82, 9.03
SB612111 + Morphine	4.47	4.19, 4.76

in Table 1). These results indicated that systemic administration of SCH221510 attenuated but SB612111 potentiated morphine-induced antinociception in the mouse hot-plate test.

Systemic administration of NOP receptor agonist facilitated but antagonist reduced the development of morphine-induced antinociceptive tolerance in female mice

The role of NOP receptor in the development of chronic morphine-induced antinociceptive tolerance was observed, and the procedure was shown in Fig. 3a. The above results showed that SCH221510 and SB612111 affected the antinociceptive action of morphine; therefore, drugs were administered immediately after each test for morphine antinociception (Fig. 3a). Two-way ANOVA with one repeated measurement revealed statistically significant differences in treatment ($F_{(2,27)} = 11.757$, $P < 0.001$), time ($F_{(4.0,109.2)} = 152.976$, $P < 0.001$) and interaction ($F_{(8.1,109.2)} = 3.685$, $P = 0.001$). As shown in Fig. 3b, chronic administration of morphine (10 mg/kg, sc, twice daily) for 6 days induced tolerance to antinociception, showing that the antinociception of morphine (10 mg/kg, sc) declined in a time-dependent manner (Fig. 3b). SCH221510 (3 mg/kg, sc) combined with morphine (10 mg/kg, sc) caused the time-response curve of morphine antinociception to shift leftward and downward, suggesting that SCH221510 systemic injection accelerated the development of morphine tolerance. However, SB612111 (10 mg/kg, sc) accompanied with morphine (10 mg/kg, sc) caused the time-response curve of morphine antinociception to shift rightward and upward (Fig. 3b), suggesting SB612111 systemic injection delayed the development of morphine tolerance.

Systemic administration of NOP receptor agonist and antagonist did not significantly alter the development of morphine-induced physical dependence in female mice

Finally, we investigated whether or not NOP receptor participates in the development of morphine physical dependence. Chronic morphine treatment in ascending doses (10–50 mg/kg, sc; twice daily for 5 days) resulted in physical dependence, in which naloxone (5 mg/kg, ip) precipitation evoked robust withdrawal jumping ($P = 0.001$, morphine group vs. saline group, nonparametric test; Fig. 4). When SCH221510 (3 mg/kg, sc) was combined with morphine treatment, the median of jumping number induced by naloxone precipitation was lower than that of the morphine group (57.5 vs. 110.5) but was not statistically significant (Fig. 4). SB612111 (10 mg/kg, sc) combined with morphine slightly increased naloxone-precipitated jumping compared with that in the morphine group (121 vs. 110.5 of the median), without statistical significance (Fig. 4).

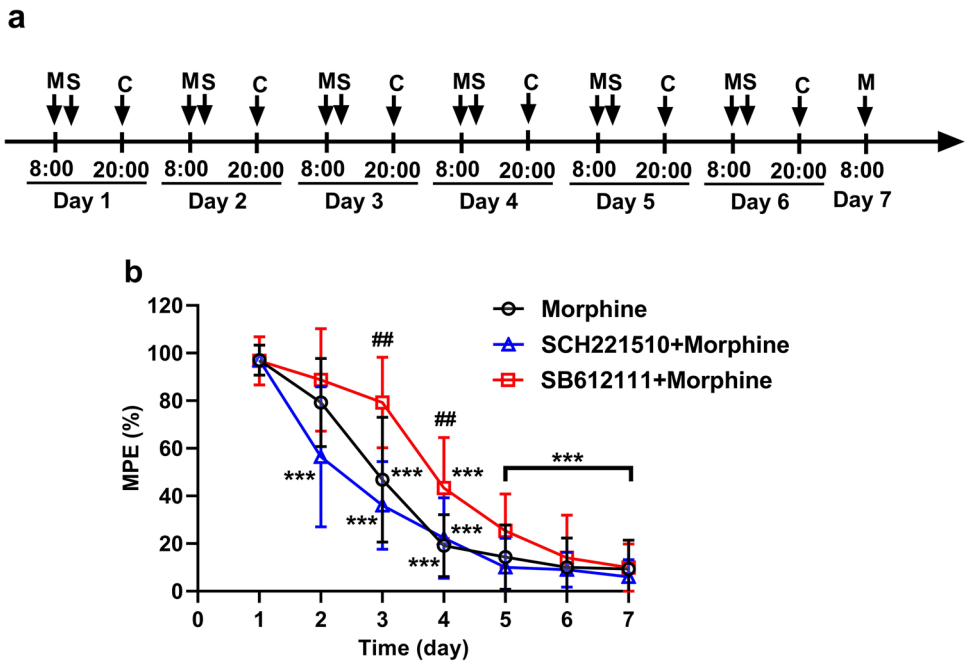

Fig. 3 Effects of systemic administration of SCH221510 or SB612111 on the development of morphine-induced antinociceptive tolerance in female mice. (**a**) Schedule of morphine tolerance induction and testing. M: morphine (10 mg/kg, sc) injection 30 min prior to the antinociception test; S: SCH221510 (10 mg/kg, sc) or SB612111 (3 mg/kg, sc) injection immediately after the antinociception test; C: SCH221510 (10 mg/kg, sc) or SB612111 (3 mg/kg, sc) administration 15 min prior to morphine (10 mg/kg, sc) injection without the antinociception test. (**b**) Time curves of the development of tolerance to morphine antinociception. $n = 10$, mean ± S.D. $^{***}P < 0.001$ vs. Day 1 of each group; $^{\#\#}P = 0.007$ at Day 3 and $^{\#\#}P = 0.005$ at Day 4, the SB612111 + Morphine group vs. the Morphine group; two-way ANOVA with one repeated measurement followed by *Tukey* test

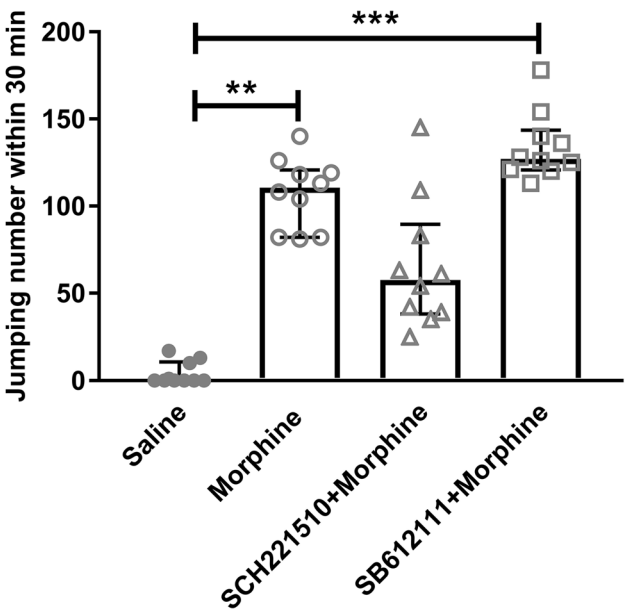

Fig. 4 Effects of systemic administration of SCH221510 and SB612111 on the development of morphine-induced physical dependence in female mice. $n = 10$, median and interquartile range. $^{**}P = 0.001$ and $^{***}P < 0.001$, vs. Saline group; nonparametric test

Discussion

NOP receptors are present in almost all central nervous system areas and contribute to the modulation of a host of biological functions. The present study found that systemic activation or blockade of NOP receptor failed to produce antinociception in hot-plate test of female mice, but it could modulate morphine's actions, including morphine antinociception and tolerance. Since opioid analgesia has sex difference, the role of NOP receptor in morphine's actions in male mice is another study.

Many non-peptide compounds that penetrate the brain to act on NOP receptors have been synthesized. SCH221510 and SB612111 function as an agonist and antagonist, respectively, of the NOP receptor with high affinity and selectivity (Varty et al. 2008; Toll et al. 2016). Moreover, SCH221510 mimics but SB612111 blocks N/OFQ actions in various *in vivo* behavioral assays (Varty et al. 2008; Jenck et al. 2000; Sobczak et al. 2014; Sukhtankar et al. 2014; Wu and Liu 2018; Toll et al. 2016). Thus, these two compounds are suitable tools for examining the biological functions and functional interactions between NOP and other receptors in the N/OFQ-NOP receptor system.

Several studies have indicated that activation of NOP receptors modulates nociceptive transmission in a

site-specific manner in rodents, with antinociception when peripheral and spinal activation and pronociception when supraspinal activation (Schröder et al. 2014). The net effect of systemically intervening NOP receptors on nociception depends on the relative contribution of peripheral, spinal and supraspinal sites, as well as experimental conditions. Our data showed that systemic administration of the selective NOP receptor agonist SCH221510 or the antagonist SB612111 failed to produce significant antinociception in the mouse hot-plate test. Considering that both spinal and superspinal sites exert effects on nociceptive processing triggered by acute noxious thermal stimuli in the hot-plate test, our results indicates that systemic administration may act on NOP receptors both spinally and superspinally, thus counteracting the effect of each other. This result was consistent with the effects of other μ/NOP receptor-mixed agonists (such as BU08028, SR16435 and cebranopadol), in which antinociception was not attributed to systemic activation of NOP receptor in acute pain models of rodents (Khroyan et al. 2011, 2007; Linz et al. 2014).

However, Reiss et al. have reported that systemic administration of Ro64-6198, another selective non-peptide NOP receptor agonist, exerted antinociceptive effects in the mouse hot-plate test in male mice (Reiss et al. 2008). The discrepancy may be due to the differences in the sexes (male vs. female), the intensity of noxious stimuli (hot-plate temperature of 55 °C vs. 52 °C) and the measurement indicator (the latency of hind paw licking vs. the latency of forepaw licking and jumping off). In agreement with our results, other studies have reported that Ro64-6198 did not produce an antinociceptive effect in the rat tail flick test or the mouse tail immersion test after systemic administration (Jenck et al. 2000; Dautzenberg et al. 2001; Kotlinska et al. 2003). Therefore, in rodents, systemic activation of the NOP receptor is largely ineffective against acute thermal nociception.

NOP receptors could interact with μ-opioid receptors to modulate pain transmission, and we found that systemic administration of the NOP receptor agonist SCH221510 attenuated morphine-induced antinociception in the mouse hot-plate test. Thus, when combined with a μ-opioid receptor agonist, systemic activation of NOP receptors displayed anti-opioid action under the present experimental conditions in rodents. Indeed, NOP receptors were co-localized with μ-opioid receptors in DRG neurons and RVM ON (or secondary) cells of rodents, whereas only NOP receptors, rather than μ-opioid receptors, were expressed in RVM OFF (or primary) cells (Heinricher, McGaraughty, and Grandy 1997). This site-specific pattern of NOP and μ-opioid receptors contributes to spinal antinociceptive and supraspinal pronociceptive actions of N/OFQ in rodents (Heinricher, McGaraughty, and Grandy 1997). Direct inhibition of OFF cell firing by NOP receptor activation would effectively block the disinhibition of this subset of cells produced by μ receptor agonists. In the cells co-expressing NOP and μ receptors, the NOP receptor formed heterodimers with the μ receptor (Pan et al. 2002; Wang et al. 2005; Evans et al. 2010), and the heterodimer was found to impair μ receptor-activated signals (Mandyam et al. 2003; Wang et al. 2005). These two aspects may provide the cellular and neural circuitry bases of anti-morphine antinociception mediated by systemic activation of NOP receptors in mice.

One of the clinical drawbacks of opioid analgesia is the development of tolerance, which results in a dose escalation and increased overdose risk. Modulation of NOP receptors can influence the development and the expression of morphine tolerance in rodents; however, the conclusions differ widely due to their methodologies (morphine regimen to induce tolerance, route of drug administration, pain model to assess tolerance, among others). To distinguish the role of NOP receptor in the development from its role in the expression of morphine tolerance, NOP receptor ligands were administered after each morphine antinociceptive test. In our mouse hot-plate test, we found that systemic activation of the NOP receptor by SCH221510 accelerated the development of constant morphine-induced antinociceptive tolerance. However, blockade of the NOP receptor by SB612111 reduced the development of morphine tolerance, similar to the results obtained for J-113,397 (Ueda et al. 1997; Chung et al. 2006), another selective antagonist of the NOP receptor. Although there is some controversy in the literature (Kest et al. 2001; Mamiya et al. 2001), morphine tolerance was significantly reduced in mice in whom either the NOP receptor or ppN/OFQ has been knocked out (Ueda et al. 1997; Chung et al. 2006).

Some evidence suggests that the brain area relevant for the endogenous N/OFQ-NOP receptor system action on opioid tolerance may be the ventrolateral PAG (Scoto et al. 2010). Another study has suggested that chronic morphine treatment resulted in the upregulation of the NOP system in the brain, which attenuated morphine analgesia and, in turn, can be reversed by an NOP receptor antagonist. At the cellular level, (Evans et al. 2010) have found that prolonged exposure to selective agonists of either μ or NOP receptors caused the internalization of both receptors as a μ/NOP receptor/Cav2.2 complex, which may be the molecular basis underlying the activation of NOP receptor-facilitated morphine tolerance to antinociception.

Considering the localization of NOP receptors in noradrenergic and dopaminergic nuclei including the locus coeruleus and the ventral tegmental area (Neal et al. 1999), NOP receptors may have the potential to modulate opioid-induced physical dependence and addiction liability. Clearly, NOP receptor agonists inhibit morphine-induced dopamine release in the mesolimbic pathway (Murphy et al. 1996; Murphy et al. 1999) and suppress the rewarding and reinforcing effects induced by classical opioids (particularly

μ-opioid receptor agonists) in CPP and self-administration procedures in rodents (Murphy and Maidment 1999; Ciccocioppo et al. 2000; Rutten et al. 2010; Sukhtankar et al. 2014). This finding suggests that the activation of NOP receptors could reduce the abuse potential of opioid analgesics.

In the context of physical dependence, previous study has shown that the NOP receptor agonist N/OFQ suppressed naloxone-precipitated withdrawal after morphine treatment in rats (Kotlińska et al. 2000). Because an increase in noradrenergic neurons firing in the locus coeruleus is crucial to the expression of opioid-type physical dependence, it is reasonable to presume that the inhibition of NOP receptors in noradrenergic neurotransmission may contribute to the suppression of the expression of physical dependence. However, administration of the NOP receptor agonist Ro646198 during the dependence induction phase did not prevent the development of morphine dependence in mice (Kotlinska et al. 2003). In the present study, we found that systemic administration of a selective NOP receptor agonist (SCH221510) in combination with morphine did not show a statistically significant reduction to the development of opioid-type physical dependence in mice, though a lower naloxone-precipitated withdrawal jumping number than that in the morphine alone group. Furthermore, chronic systemic treatment with cebranopadol, a combined μ/NOP receptor agonist, produced reduced development of physical dependence compared with morphine treatment in mice and rats (Tzschentke et al. 2018), which attributed to the NOP receptor by using NOP receptor knockout mice (Ruzza et al. 2019). Thus, whether or not NOP receptors robustly modulate the development of physical dependence induced by μ-opioid receptor agonists still needs to be clarified.

It is widely known that opioid analgesia has sex difference, thus the role of NOP in modulating morphine analgesia, tolerance and physical dependence should be clarified in males and females, respectively. Considering that women experience greater pain and consume more opioids in clinical, it is essential to pay more attention to females. Actually, previous research has revealed sex differences in N/OFQ-NOP receptor expression and NOP receptor activity following chronic morphine treatment in rats (Zhang et al. 2012). While male rats were more tolerant to the antinociceptive actions of morphine than females, N/OFQ levels in the spinal cord was higher in females than in males, and chronic morphine treatment further pronounced the differences between males and females. In addition, N/OFQ content in cerebrospinal fluid was reduced more in male than in female rats with chronic morphine exposure, but increased in PAG of both sexes. Moreover, chronic morphine treatment increased NOP receptor levels more in males than in females, while decreasing affinity in both. Consistently, the efficacy of N/OFQ-stimulated [^{35}S]GTPγS binding to spinal cord membranes from male rats increased after chronic morphine treatment (Zhang et al. 2012). However, there is no reports about sex differences in the effects of NOP agonists and antagonists until now due to limited research in females. In the present study, we systematically revealed that activation of NOP receptors attenuated morphine antinociception to acute thermal stimuli and facilitated the development of morphine-induced antinociceptive tolerance in female mice. Indeed, further research in males is essential, which is being carried on as another study.

Given that the hormonal factors contribute to the sex differences in the pain modulation, the extent of opioid analgesia seems to be greatly influenced by the hormonal level in different phases of the estrus cycle. However, contradictory results have been reported about whether estrogen and progesterone levels affect morphine-induced analgesia in rodents (Stoffel et al. 2003; Kepler et al. 1989; Loyd et al. 2008; Mogil et al. 2000). The association between the hormonal menstrual cycle phases and the opioid analgesic responses has also been reported in clinical studies (Ribeiro-Dasilva et al. 2011; Olofsson et al. 1996), while inconsistent results demonstrating no significant difference in the overall tramadol or morphine consumption between women in luteal and follicular phases following total abdominal hysterectomy (Ahmed et al. 2012). Therefore, there is no definite conclusion about whether the hormonal menstrual cycles affect morphine analgesia in females to date. In this study, we did not distinct whether all female mice start the same menstruation cycle, which was a limitation.

It is noteworthy that the systemic activation of NOP receptors translates into the very complex pharmacology of pain modulation, depending on the animal species and the pain state. In acute pain models, most studies (including our study) have shown that systemic administration of NOP receptor agonists fails to elicit robust antinociception but decreases classical opioid-induced antinociception in rodents, whereas it exerts efficacious antinociception and synergistic action to opioid antinociception in non-human primates (Ko et al. 2009; Podlesnik et al. 2011; Cremeans et al. 2012). Conversely, in chronic inflammatory pain and neuropathic pain models, systemic administration of NOP receptor agonists to both rodents and non-human primates produces significant anti-hypernociceptive effects and enhances opioid-induced anti-hypernociception (Schröder et al. 2014). The exact reason for this functional difference is still unclear. This may be due to the specific expression patterns of NOP receptors in the supraspinal nociceptive regions of rodents and non-human primates and the functional plasticity of NOP receptors under chronic pain conditions (Schröder et al. 2014). Given that rodents are the common species used in preclinical studies of analgesic agents, caution must be exerted when translating conclusions regarding NOP receptor ligands derived from rodent

acute pain models to clinical study. Thus, the subtle differences in NOP receptor locations in the characterized cells (such as ON and OFF cells of the RVM) between rodents and primates should be elucidated in the future.

Taken together, this study found that systemic activation of NOP receptors attenuated morphine antinociception to acute thermal stimuli, facilitated the development of morphine-induced antinociceptive tolerance and did not robustly alter the morphine-induced physical dependence in female mice. Systemic blockade of NOP receptors produced opposite actions. These results demonstrate that the endogenous N/OFQ-NOP receptor system plays diverse roles in modulating the pharmacological profiles of μ-opioid receptor agonists.

Authors' contributions NW and JL designed the study. XQH, ZYW and JMC performed the experiments. NW, XQH, ZYW and JMC analyzed the data. NW, XQH and JL wrote the manuscript.

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Data availability Datasets from this study are available from the corresponding authors on reasonable request.

Code availability SPSS 26.0 and GraphPad Prism 7.04.

Declarations

Conflicts of interest All authors state that there is no conflict of interest.

Ethical approval All animal procedures were approved by the Institutional Review Committee for the Use of Animals (Beijing Institute of Pharmacology and Toxicology, China; Ethical approval number: IACUC of AMMS-06-2019-002) and were performed in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80 – 23). None of the authors performed any human experiments as part of this research.

Consent to participate All authors have given their consent to participate the study.

Consent for publication All authors have read the manuscript and given their consent for publication.

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