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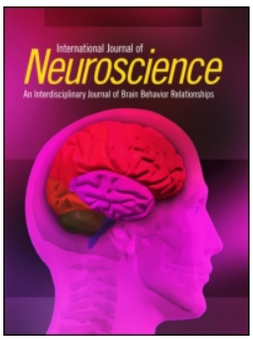

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ORIGINAL ARTICLE

Persistent effects of the orexin-1 receptor antagonist SB-334867 on naloxone precipitated morphine withdrawal symptoms and nociceptive behaviors in morphine dependent rats

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ABSTRACT

Aim of the study: In this study, we investigated the effect of long-term administration of orexin receptor 1 (OXR1) antagonist on naloxone-precipitated morphine withdrawal symptoms and nociceptive behaviors in morphine-dependent rats.

Materials and methods: Wistar rats received subcutaneous (s.c.) injections of morphine (6, 16, 26, 36, 46, 56, and 66 mg/kg, 2 ml/kg) at an interval of 24 h for 7 days. In chronic groups, the OXR1 antagonist, SB-334867 (20 mg/kg, i.p.), or its vehicle, was injected repetitively from postnatal day 1 (PND1)-PND23 and then for the following seven days before each morphine injection. Meanwhile, in acute groups, SB-334867, or its vehicle, was administered before each morphine injection. In groups of rats that were designated for withdrawal experiments, naloxone (2.5 mg/kg, i.p.) was administered after the last injection of morphine. In the formalin-induced pain, the effect of OXR1 inhibition on the antinociceptive effects of morphine was measured by injecting formalin after the final morphine injection.

Results: Animals that received long-term SB-334867 administration before morphine injection demonstrated a significant reduction in chewing, defecation, diarrhea, grooming, teeth chattering, wet-dog shake, and writhing. Inhibiting OXR1 for a long time increased formalin-induced nociceptive behaviors in interphase and phase II of the formalin-induced pain.

Conclusions: Our results indicated that the inhibition of OXR1 significantly reduces the development of morphine dependence and behavioral signs elicited by the administration of naloxone in morphine-dependent rats. Furthermore, the prolonged blockade of OXR1 might be involved in formalin-induced nociceptive behaviors.

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Orexin receptor 1; morphine; withdrawal; nociceptive behaviors; formalin-induced pain

Introduction

Orexins are neurotransmitters with an important role in drug addiction [1–3]. Orexin A is a well-known peptide in the lateral hypothalamus (LH). It is involved in feeding, wake cycle [4,5], and reward-related processes, including drug abuse and addiction [4–14]. Orexin neuropeptides activate OXR1 and orexin type 2 (OXR2) receptors, which are G-protein coupled receptors [15]. OXR1 has a higher affinity to orexin-A than orexin-B, while OXR2 has the same affinity for both peptides [15]. In one-week old animals, only a small subset of neurons in the LH are orexin-A positive [16]. Based on previous studies, translation of orexin mRNA can be detected at very low levels on the day of birth, followed by a rise to the maximum at PND20 [17]. Furthermore, glucosensitivity [18] of LH neurons and their response to sensory afferents [19] mature during PND0 to 3. Therefore, orexin might play an important role in the response and reaction of LH during development [17].

Orexinergic neurons are typically located in the lateral hypothalamus, dorsomedial hypothalamus, and perifornical area. They provide extensive afferents to numerous brain areas [4,12,15]. In another study, it was shown that orexin was involved in the development of morphine dependence and reward by activating OXR1 in the ventral tegmental area, nucleus accumbens, the locus coeruleus (LC), and also the paragigantocellularis nucleus (PGi), which principally

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provides glutamatergic projections to LC [2,12,20–22]. Both LC and PGi express OXR1 and are involved in opiate dependence and tolerance [23,24, 25]. In our previous study on the visual cortex, chronic blockade of OXR1 attenuated phospholipase C expression in all six layers of visual cortex [26,27].

A study [22] demonstrated that orexin cells are activated after chronic morphine consumption and withdrawal. The same study similarly displayed that orexin knockout mice developed attenuated physical signs of morphine dependence compared to wild type controls. Most studies have revealed that orexin exerts an excitatory effect on some neurons of the central nervous system. Naloxone-precipitated morphine withdrawal increases c-Fos expression in orexinergic neurons of the hypothalamus [3]. Moreover, orexinergic neurons initiate excitation in naloxone-precipitated withdrawal syndrome [2]. It has been demonstrated that central and systemic administration of SB-334867, reduced naloxone precipitated withdrawal signs [3,28]. In another study, it was revealed that blocking OXR1 in LC diminished the development of morphine dependency in rats [24,29].

Orexin-A also has analgesic effects. The analgesic efficacy of orexin-A is similar to morphine, as assessed by the hot-plate test. Some studies showed that blocking OXR1 attenuated morphine tolerance and physical dependence in rats [30]. Several studies have revealed that orexinergic neurons and their receptors are expressed in some parts of the descending pain modulation network [31].

In this respect, it was essential to clarify the effect of persistent postnatal inhibition of orexin function on the development of morphine dependency in morphine-dependent rats. We also made an attempt to reveal the effect of postnatal blockade of OXR1 on nociceptive behaviors in morphine-dependent rats.

Materials and methods

Animals

Wistar rats (PND1 to PND30, $n = 96$) were obtained from Pasteur Institute of Tehran, Tehran, Iran, and placed in plexiglass cages with their mothers. Temperature ($22 \pm 2\,^{\circ}C$) and humidity ($54 \pm 2\%$) were controlled. Rats were given 12-h light/dark cycles (light at 07:00 am) and had free access to food and water. This study was performed in accordance with the ethical guidelines of Iran University of Medical Sciences Ethics Committee, Tehran, Iran, which is based on the NIH Guide for the Care and Use of Laboratory Animals. The animals were handled gently every day by an experimenter blind to the nature of the drugs that were used.

Induction of morphine dependence and withdrawal syndrome

To induce morphine dependence, morphine was injected (6, 16, 26, 36, 46, 56, and 66 mg/kg, 2 ml/kg) for 7 days. After 24 h following the final morphine injection, naloxone (2.5 mg/kg, i.p.) was administered in order to assess whether withdrawal signs precipitate. Afterward, animals were placed in a clear plexiglass cylinder test chamber (dimensions: 30 cm in diameter and 50 cm height) for 25 min, where somatic withdrawal signs (chewing, diarrhea, defecation, grooming, teeth chattering, wet-dog shake, and writhing) were monitored with reference to our previous report [25]. Behavioral responses were analyzed by an experimenter who was blind to the treatments. Injections were carried out at a fixed time during the whole period of study.

OXR1 antagonist, SB-334867, was dissolved in aCSF (pH 7.4), comprising of 1% DMSO. The solution was divided into portions and frozen in $-20\,^{\circ}C$. SB-334867 (20 mg/kg, i.p.) and its vehicle were injected prior to each morphine administration [25].

Experiment 1: Monitoring opiate withdrawal symptoms

Rats ($n = 48$) were divided into six experimental groups ($n = 8$ for each group) as follows: Group 1 (intact), animals received saline for 7 days and then naloxone (Saline.Nal); Group 2, animals were treated with subcutaneous administration of morphine for 7 days from PND24 to PND30 and then received naloxone (Mor.Nal); Group 3 and 4, animals received OXR1 antagonist, SB-334867 (20 mg/kg, i.p.; SBa-Mor.Nal) or its vehicle (Veha-Mor.Nal), respectively, for 7 days before each morphine injection from PND24 to PND30 [24]; Group 5 and 6, animals received OXR1 antagonist, SB-334867 (20 mg/kg, i.p.; SBc-Mor.Nal) or its vehicle (Vehc-Mor.Nal), respectively, for 23 days from PND1 to PND24 and also from PND24 to PND30 before each morphine injection.

In withdrawal experiments, all six groups of rats received naloxone (2.5 mg/kg, i.p.) 24 h after the final morphine injection, in order to assess whether withdrawal signs precipitate.

Experiment 2: Nociceptive behaviors in morphine dependent rats

This experiment was designed to examine the effect of i.p. injection of SB-334867 on morphine antinociception in the formalin-induced nociception. For this

purpose, six separate groups of rats ($n = 48$), similar to withdrawal groups, received formalin but not naloxone. Formalin-induced pain tests were performed in a plexiglass chamber ($30 \times 30 \times 30$ cm) with a mirror placed underneath at a 45° angle to allow an unimpeded view of the paws of the animal.

In the present study, rats were first acclimatized for 30 min in an acrylic observation chamber. 10 to 20 min after the final morphine injection, rats were subcutaneously injected with formalin (50 µl; 2%) in the plantar surface of their right hind paw with a 25 gauge needle. Formalin injection produced a typical biphasic nociceptive response, including an early phase (minutes 0–5), quiescent interphase (minutes 5–20), and a second long-lasting phase (minutes 20–60), all of which are active phases. To ensure stable scores from formalin, it was necessary to make sure that the needle was inserted through the skin and went through 5 mm under the skin. Subsequently, each rat was immediately returned to the observation box and behavioral recording was commenced. Pain behaviors were scored as follows; 0: the injected paw was not favored; 1: the injected paw had little or no weight placed on it; 2: the injected paw was elevated and not in contact with any surface; and 3: the injected paw was licked or bitten. Recording of nociceptive behaviors began immediately after formalin injection (minute 0) and continued for 60 min.

The duration of licking/biting the formalin-injected hind paw was measured as an indicator of nociceptive response in each phase with a digital time-out stopwatch. In all groups, the behavioral reaction of rats throughout the first phase, interphase, and the second phase were discretely assessed. We used each animal just once, i.e. the formalin was never injected into the same animal twice [31,32].

In order to examine whether repeated injections of SB-334867 after birth caused stress-induced hyperalgesia, we compared pain scores from rats that received long-term injections of SB-vehicle with those that did short-term injections. In other words, animals that received SB-vehicle after birth, from PND1 to PND30, were compared with those that received the vehicle from PND24 to PND30 (Vehc-Mor.Nal/Veha-Mor.Nal). We observed no significant difference between these two groups (data not shown). Furthermore, SBc-treated rats were compared to Vehc-Mor.Nal, both of which were injected with the same time-scale from PND1 to PND30. Therefore, these protocols would rule out the effect of any possible stress-induced hyperalgesia through repeated injections. Finally, rats were handled and acclimatized throughout the experiments. Handling of rats is required to minimize stress induced by injection.

Data analysis

Data has been expressed as mean ± SEM and analyzed using unpaired two-tailed Student's t-test and one-way analysis of variance (ANOVA) for comparison of two or more groups, respectively. The defined level of statistical significance was $p < 0.05$.

Drugs

The following drugs were used in this study: Morphine sulfate (Temad, Tehran, Iran), naloxone hydrochloride (Sigma–Aldrich, St. Louis, USA), SB-334867 which is a selective OXR1 antagonist (Tocris, Bristol, UK), DMSO (Sigma–Aldrich, Germany), and 2% formalin which was prepared by diluting 37% formaldehyde (Temad, Tehran, Iran) using sterile saline.

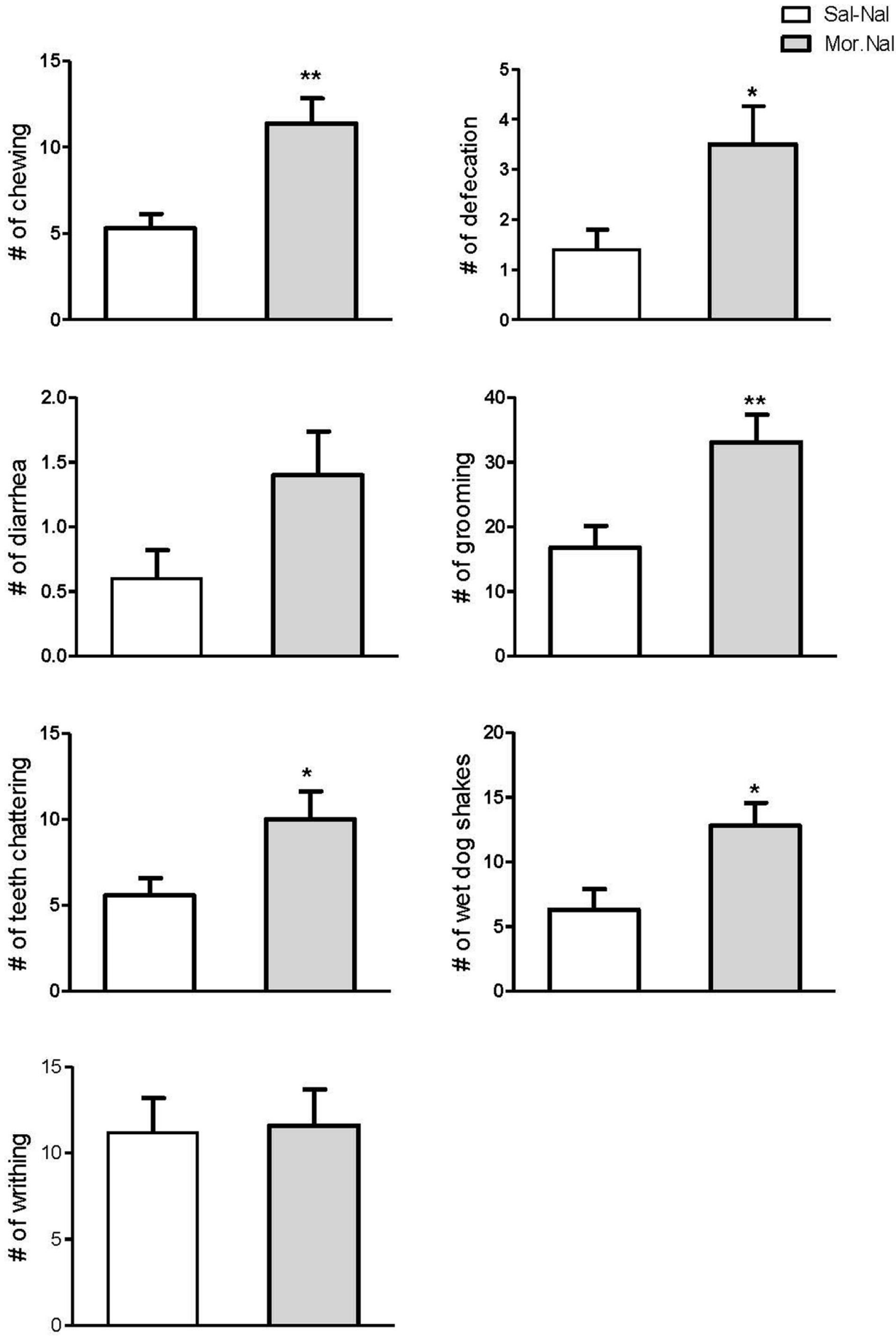
Results

Effects of SB-334867 on naloxone-induced withdrawal behaviors

Animals were monitored for the effect of naloxone-induced morphine withdrawal signs, such as teeth chattering, grooming, chewing, diarrhea, defecation, and wet-dog shake, as described in the materials and methods section.

While administration of naloxone hydrochloride did not induce withdrawal signs in saline-treated group ($n = 8$), morphine-treated rats ($n = 8$) exhibited the characteristics of morphine withdrawal signs such as chewing, defecation, grooming, teeth chattering, and wet-dog shake ($p < 0.05$, analyzed by unpaired t-test). Other signs, such as diarrhea and writhing, did not change (Figure 1).

Next, we investigated whether administration of SB-334867 before morphine injection modulated naloxone-precipitated responses in morphine-treated animals. We found that acute SB-334867 administration significantly reduced teeth chattering ($p < 0.001$), wet-dog shake ($p < 0.01$), writhing ($p < 0.01$), grooming ($p < 0.001$), chewing ($p < 0.001$), diarrhea ($p < 0.01$), and defecation ($p < 0.05$) compared to animals receiving SB-vehicle prior to morphine injection (analyzed by ANOVA, Figure 2). Other symptoms, such as head tremor, jumping, rearing, scratching, and sniffing, did not change. Chronic SB-334867 administration significantly reduced chewing ($p < 0.001$), teeth chattering ($p < 0.001$), wet-dog shake ($p < 0.001$), writhing ($p < 0.01$), grooming ($p < 0.001$), diarrhea ($p < 0.001$),


Sal-Nal
Mor.Nal
# of chewing
15
10
5
0
**
# of defecation
5
4
3
2
1
0
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# of diarrhea
2.0
1.5
1.0
0.5
0.0
# of grooming
40
30
20
10
0
**
# of teeth chattering
15
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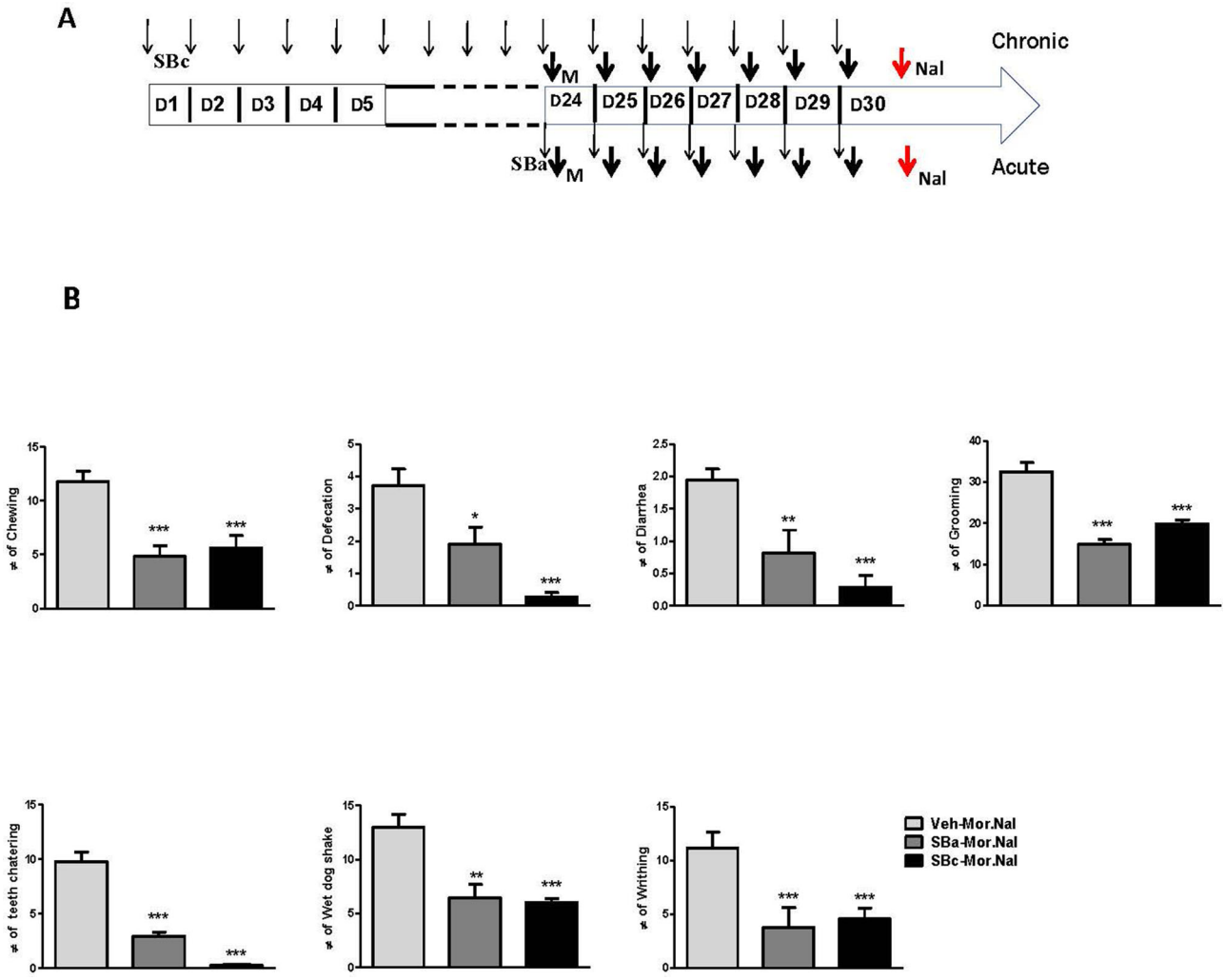

Figure 2. The effect of acute and chronic injection of SB-334867 on naloxone-precipitated (2.5 mg/kg, i.p.) morphine withdrawal in morphine-dependent rats, including teeth chattering, grooming, chewing, wet-dog shake, diarrhea, and defecation. A. Schematic design of experimental protocols used for measuring withdrawal signs following acute (SBa-Mor.Nal) and chronic (SBc-Mor.Nal) injection of SB-334867 in morphine-dependent rats. B. Bar charts demonstrate the withdrawal behaviors of rats acutely and chronically pretreated with SB-334867 prior to morphine, compared to ones pretreated with SB-vehicle (Veh-Mor.Nal). Data was analyzed by ANOVA and expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $n = 8$ per group, # frequency of behavior.

and defecation ($p < 0.001$) compared to animals receiving SB-vehicle prior to morphine injection (analyzed by ANOVA, Figure 2).

In withdrawal experiments, we investigated whether acute administration of SB-334867 could produce the same effect as a chronic treatment. The results revealed that chronic inhibition of OXR1 caused a higher reduction in some withdrawal behaviors, including wet-dog shake, diarrhea, and defecation. Other symptoms, such as chewing, grooming, teeth chattering, and writhing, did not show any significant difference.

Effects of SB-334867 on morphine antinociception

We used the formalin-induced pain in order to investigate the effect of morphine tolerance. The nociceptive score was measured in each phase of the pain

Figure 1. Naloxone-induced morphine withdrawal signs in morphine-dependent rats. Naloxone administration (2.5 mg/kg, s.c.) induced morphine withdrawal signs including chewing, defecation, diarrhea, grooming, teeth chattering, wet-dog shake, and writhing in morphine-treated rats. The graph displays the behaviors of rats receiving morphine (Mor.Nal) compared to those treated with saline (Sal.Nal). Data was statistically analyzed by unpaired two-tailed student's t-test and expressed as mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, $n = 8$ per group, # frequency of the behavior.

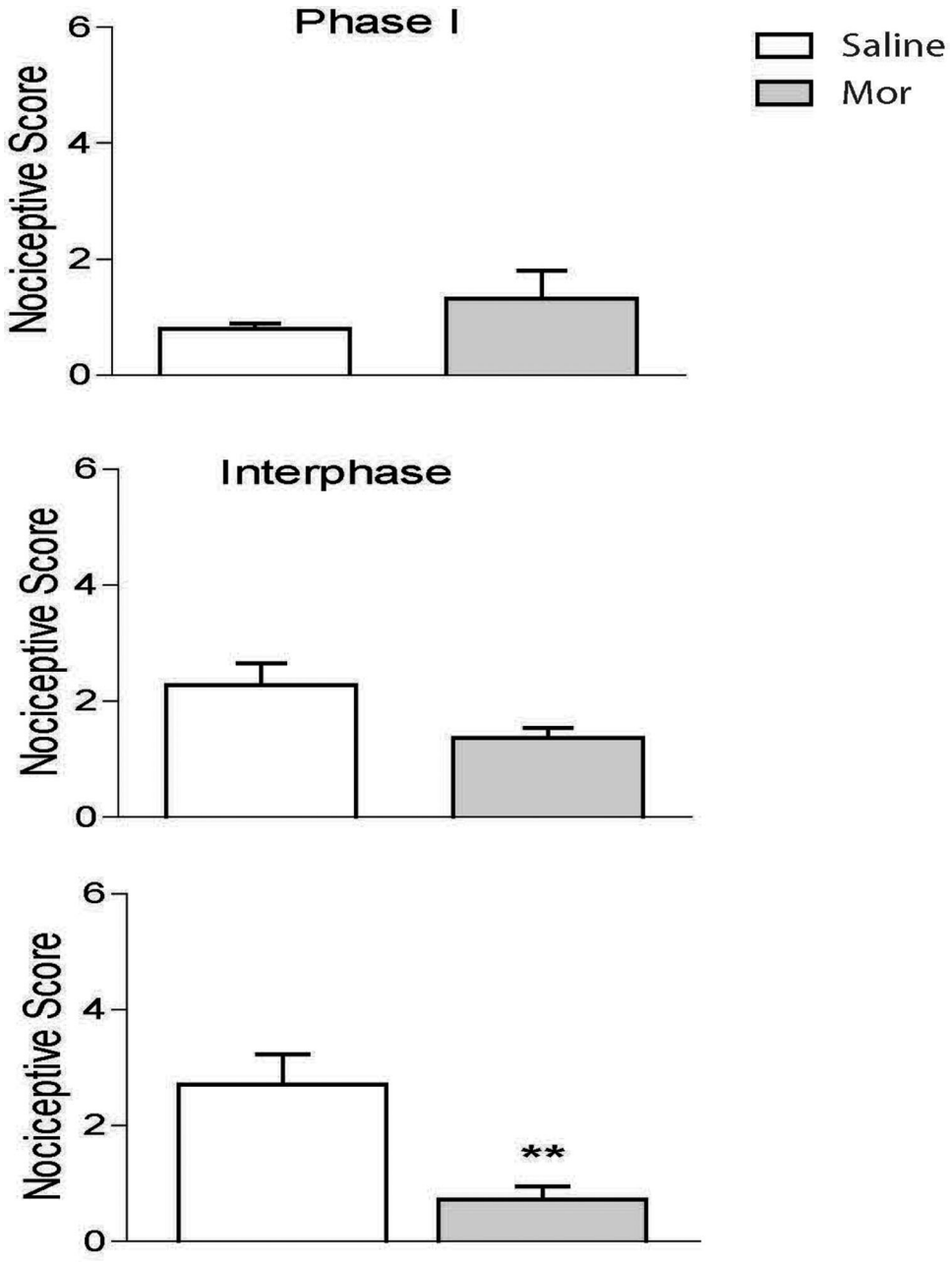

Figure 3. Formalin-induced nociceptive behaviors following the infusion of morphine. Bar chart for injection of morphine (Mor) in the formalin-induced pain represents mean of the nociceptive score in each phase: phase 1 (minutes 0–5), interphase (minutes 5–20), and phase 2 (minutes 20–60). Recording of nociceptive behaviors began immediately for 60 min after formalin injection (5% in saline, 50 µl, s.c.) into the hind paw (minute 0). Data is expressed as mean ± SEM. $^{*}p < 0.05$ in comparison with Saline group, $n = 8$ per group.

induced by formalin (phase I, interphase, and II). Morphine administration decreased pain behaviors induced by formalin in phase II ($p < 0.01$), but not phase I or interphase (analyzed by unpaired t-test, Figure 3).

We found that chronic administration of SB-334867 increased pain behaviors induced by formalin in morphine-treated rats in interphase ($p < 0.05$) and phase II ($p < 0.05$), but not phase I (analyzed by ANOVA, Figure 4). However, acute administration of SB-334867 failed to change pain behaviors induced by formalin in morphine-treated rats in any phase (analyzed by ANOVA, Figure 4).

Discussion

Numerous approaches have been taken to produce morphine dependence in rats, such as dissolving morphine in drinking water [33], subcutaneous implantation of pellets [34], and i.p. injection [35]. In the

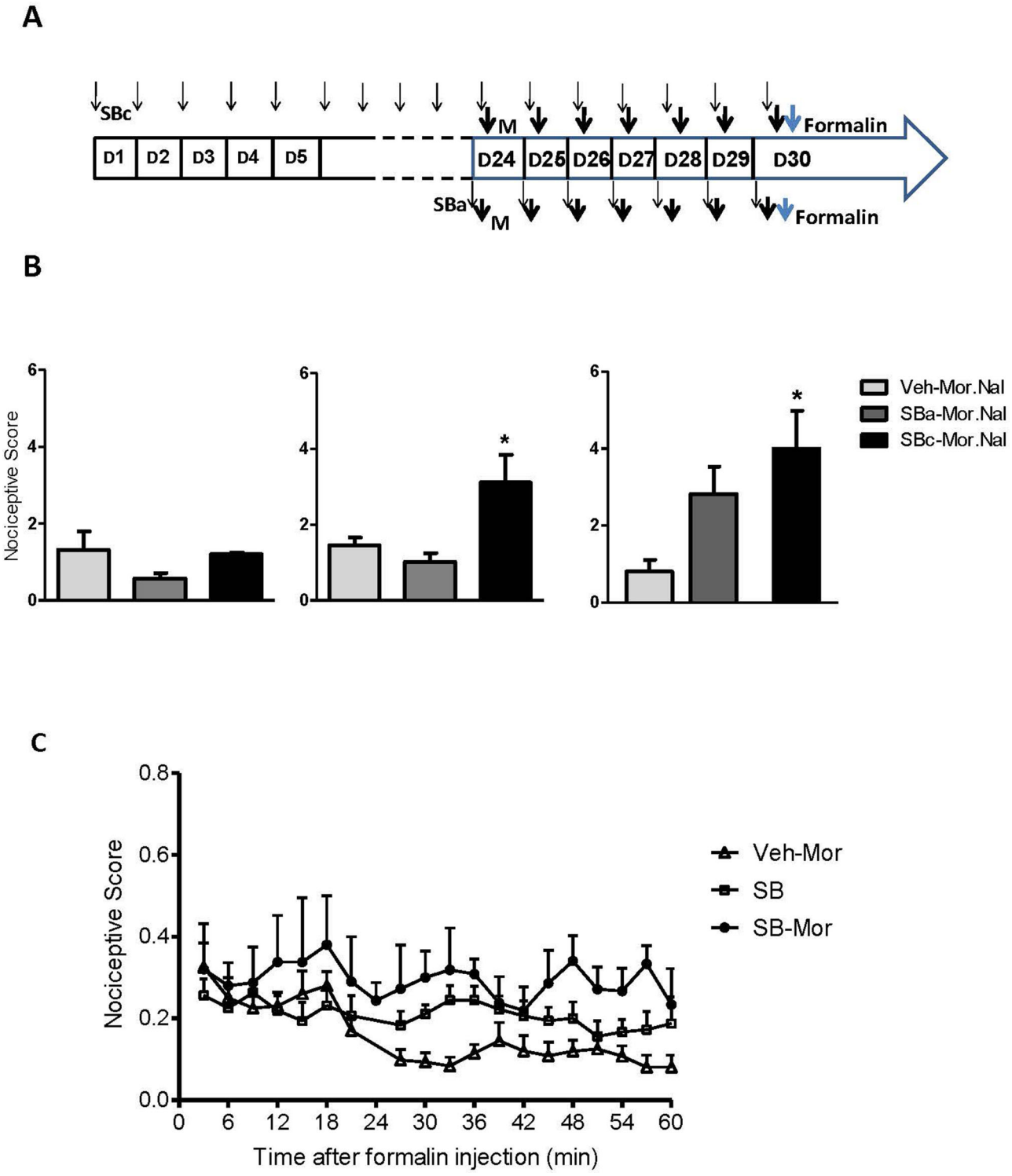

Figure 4. The effect of acute and chronic infusion of SB-334867 on formalin-induced nociceptive behaviors. A. Schematic design of experimental protocols used for measuring nociceptive behaviors following acute and chronic injection of SB-334867 in morphine-dependent rats. B. Bar chart for acute (SBa-Mor) and chronic (SBc-Mor) injection of SB-334867 in the formalin-induced pain represents mean nociceptive score of each phase: phase 1 (minutes 0–5), interphase (minutes 5–20), and phase 2 (minutes 20–60) compared to Veh-Mor group. Recording of the nociceptive behaviors began immediately for 60 min after formalin injection (5% in saline, 50 μl, s.c.) into the hind paw (minute 0). C. Time scores of formalin-induced nociceptive behaviors following formalin injection were measured every 3 min. Data is expressed as mean ± SEM. $^{*}p < 0.05$ in comparison with SB-vehicle (Veh-Mor), $n = 8$ per group.

present study, we chose i.p. injection, since it allows accurate dosage administration. In addition, previous studies have shown that various brain areas are involved in withdrawal signs [36], therefore, we selected i.p. injection of SB-334867 in order to inhibit the effect of orexin in all brain areas involved in withdrawal.

In the current research, SB-334867 was injected before each morphine administration in order to evaluate its effect on the development of morphine dependence. It was established that administrating OXR1 antagonist before each morphine injection diminished the development of morphine dependence in rats. These results showed that repeated injection of SB-334867 significantly reduced some behavioral signs of naloxone-induced morphine withdrawal syndrome, such as chewing, defecation, grooming, teeth chattering, and wet dog shake. However, no significant reduction was observed in naloxone-precipitated withdrawal diarrhea and writhing. One reasonable explanation for the failure of SB-334867 to subdue these behaviors may arise from the high deviation of these somatic signs.

Our results demonstrate that chronic OXR1 inhibition has a higher efficiency than acute inhibition in reducing some withdrawal behaviors such as defecation, diarrhea, and wet dog shake. As an alternative interpretation of the data, orexin might activate intracellular pathways involved in the reward system during postnatal development. Our findings are consistent with the research by Georgescu et al. in orexin knockout mice that showed diminished morphine withdrawal signs [2]. Whereas, normal rats pretreated with SB-334867 in current study, exhibited similar attenuation in withdrawal behaviors compared to orexin-knockout animal, there are some interesting differences between them. For instance, orexin-knockout animal displayed a considerable decrease in jumping. In addition, orexin-knockout animal did not display any significant diminution in naloxone-precipitated withdrawal wet-dog shake, while SB-treated rats displayed a significant reduction in the behavior. Since SB-334867 is selective antagonist for OXR1, the above differences might underlie the involvement of OXR2 in morphine withdrawal responses. Moreover, strain differences (rats versus mice), SB-334867 injection method (systemic versus intracerebroventricular), and different methodological approaches may each play a role in the differences between the two studies.

In agreement with our study, it was established that inhibiting OXR1 in LC, reduced morphine withdrawal symptoms in rats [28]. It was shown that systemic inhibition of OXR1 by i.p. injection of SB-334867 diminished precipitated withdrawal symptoms. Although prolonged morphine application failed to change the levels of c-Fos or orexin mRNA expression in orexinergic neurons, administrating naloxone triggered the expression of c-Fos induced by morphine withdrawal in these neurons [2,3,37,38].

We found no significant difference in withdrawal behavioral symptoms by administrating SB-vehicle, which may imply that SB-vehicle was unable to change withdrawal symptoms. Therefore, it can be concluded that the observed changes can only be attributed to the pharmacological effects of SB-334867.

Our results demonstrated that pain behavior in rats that received repeated morphine administration had no significant difference with saline-treated rats in phase I and interphase. This absence of analgesia may be due to the tolerance induced by long-term morphine administration. Abbott et al., who had found a mostly antinociceptive role of morphine using the formalin-induced pain test, suggested that a high dose morphine can cause tolerance, but not necessarily low doses [39]. In this study the challenge dose of morphine for inducing full tolerance was higher than than the dose they used to induce a partial tolerance.

We also studied the effect of acute SB-334867 treatment along with morphine injection, which failed to change nociceptive behaviors. This finding is in line with previous studies where tolerance to morphine antinociception was prevented by co-administrating SB-334867 [40,41].

Long-term administration of OXR1 antagonist, SB-334867, prior to morphine injection, significantly increased morphine tolerance at inter- or late-phase of the formalin-induced pain in this study. Most orexin neurons express morphine receptors and produce c-Fos in response to morphine. Additionally, some studies have revealed that intra-periaqueductal gray administration (a supraspinal site) of orexin-A may produce antinociceptive effects [31]. Other studies have reported that SB-334867 inhibits morphine- [30,42] and stress-induced antinociception [43]. Based on the evidence above, it seems that orexin may demonstrate an intrinsic antinociceptive effect by itself. Therefore, the antinociceptive effect of morphine in the formalin-induced pain might be due to regulation of orexinergic cells or modulation of descending inhibitory pathways by orexin-A [30,42]. Thus, orexin can reduce pain similar to morphine. Furthermore, some elements related to antinociception are mediated by the orexinergic system through the activation of OXR1. Moreover, acute injection of SB-334867 combined with morphine did not produce hyperalgesia. Therefore, put together, our finding may suggest that long-term injection of SB-334867 along with morphine during neural development might have a

synergistic effect on morphine tolerance, and hence lead to hyperalgesia. Nucleus raphe magnus, which is a thermoregulatory center, possesses high densities of not only opiate receptors but also orexin receptors [12,44,45]. Raphe magnus serotonergic neurons tonically modify nociceptive transmission [46]. Therefore, it may be postulated that SB-334867 may induce hyperalgesia by changing the modulatory effect of raphe magnus on nociception. Nucleus tractus solitarius as a center of cardiovascular regulation also is involved in hyperalgesia [47,48]. Hence, SB-induced hyperalgesia may result from activation of this nucleus.

While SB-334867 produced hyperalgesia at inter- or late-phase of formalin-induced pain in morphine-dependent animals, it failed to change neurogenic nociception during phase I. The transient early phase I, which is acute pain, reflects nociceptive sensory C-fiber activation by formalin [37]. Therefore, it seems that although long-term injection of SB-334867 did not impact nociceptor transduction and transformation through afferent C fibers of pain, but it did modulate the inter- or late-phase of the formalin-induced pain.

In conclusion, based on the current findings, it appears that orexin might either act directly or indirectly on the signaling pathways related to morphine to provide a pathway for the development of morphine dependence, making it a novel potential therapeutic target in the treatment of both withdrawal syndrome and pain. Nonetheless, further *in vitro* and *in vivo* studies are necessary to clarify the role of the orexinergic system in pain modulation and morphine dependance.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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