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Original Research

Orexinergic–Opioidergic Interactions Within the Nucleus Accumbens in the Modulation of Acute Pain: Evidence From the Tail-Flick Test in Rats

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Abstract

Background: The nucleus accumbens (NAc) plays a central role in integrating motivational, reward-related, and nociceptive inputs. Opioid and orexin systems within the NAc are both implicated in pain modulation, yet their functional interaction in acute nociception is not fully understood. This study examined the interplay between opioidergic and orexinergic signaling in the NAc during acute pain processing. **Methods:** Adult male Wistar rats underwent unilateral intra-NAc administration of morphine (5, 10, 25, and 50 mmol) to assess dose-dependent antinociception using the tail-flick test. Orexinergic involvement was evaluated by intra-NAc injection of orexin-A (0.25, 0.5, 1, and 2 nmol), the orexin receptor type 1 (OX1R) antagonist SB334867 (3, 10, 30, and 100 nmol), or the orexin receptor type 2 (OX2R) antagonist TCS OX2 29 (3, 10, 30, and 100 nmol). To assess functional interactions, naloxone was administered prior to orexin-A, and SB334867 or TCS OX2 29 were given before morphine. **Results:** Intra-NAc injections of morphine and orexin-A produced significant and dose-dependent antinociceptive effects. Pre-treatment with SB334867 or TCS OX2 29 markedly reduced the analgesic responses induced by both morphine and orexin-A. Likewise, naloxone pre-treatment significantly attenuated orexin-A-evoked antinociception. **Conclusions:** These findings indicate a bidirectional interaction between opioidergic and orexinergic systems within the NAc in regulating acute nociception. The demonstrated functional crosstalk suggests that targeting orexinergic pathways may enhance endogenous analgesic mechanisms and could potentially reduce opioid requirements in clinical pain management.

Keywords: nociception; orexin receptor antagonists; opioid receptor; nucleus accumbens; rats; tail-flick test

1. Introduction

Pain perception and suppression abilities are both essential to survive life-threatening situations. Neural circuits involving in these processes are implicated in recuperative behaviors such as nociceptive avoidance, defensive responses, and survival-promoting actions [1]. Conserved evolutionary neurobiological processes are involved in pain processing, making it possible for the fauna to exhibit the best survival response [2]. Every alteration in the neural circuits responsible for the balance of pain perception and suppression may lead to the pathological condition of chronic pain, affecting nearly 20% of the general population, with a devastating economic burden on healthcare systems [3], necessitating a better understanding of pain modulation mechanisms to develop new nonaddictive analgesic drugs.

Although many investigations have been made to elucidate the exact mechanism behind pain perception and modulation, the sophisticated nature of neural circuits involved in pain makes it enigmatic [2]. As a highly individualized subjective sensation, pain perception is altered not only by painful stimuli but also by cognition and motivations [4]. Furthermore, the aversive reaction to painful experiences is rewarding and contributes to learning and memory formation to avoid pain [2]. These findings implied the role of reward, learning, and motivation neural circuits in pain processing, and the nucleus accumbens (NAc), as a major part of the mesolimbic system, plays a significant role in pain modulation [5].

Recent advances have expanded the classical gate control theory by emphasizing dynamic interactions between ascending nociceptive inputs and descending modulatory pathways, rather than a purely spinal gating mechanism. This updated framework highlights how higher brain regions, including limbic structures, actively regulate pain through bidirectional signaling across the neuroaxis [6]. Such insights further support the involvement of integra-

tive centers, such as the NAc, in coordinating sensory and affective components of pain. The NAc, a multifunctional part of the ventral striatum, plays a major role in pleasure, reward, addiction, and pain modulation [2]. The involvement of the NAc in pain processing is implied by the explicit neural connections of the NAc to the pain-processing brain structures such as the prefrontal cortex, thalamus, anterior cingulate cortex, habenula, and periaqueductal gray [7,8]. Further investigations revealed higher neural activity within the NAc region in response to painful stimuli [9]. Furthermore, NAc could be considered a therapeutic target for pain control with deep-brain stimulation [10]. Several neurotransmitters have been identified in pain modulation within the NAc, most importantly endogenous opiates, dopamine, calcitonin gene-related peptide, glutamate, γ-aminobutyric acid (GABA), substance P, and orexin [11,12,13,14,15,16,17].

High production of the endogenous opiate peptides and concentration of the opiate receptors within the NAc demonstrate the involvement of opiate transmission in pain modulation in this region [18,19]. Furthermore, studies involving microinjections into the NAc have demonstrated that activation of μ-opioid receptors elevates the pain threshold, whereas their blockade reduces it [20]. Parenthetically, the afferent lateral hypothalamus orexinergic neurons collaborate with NAc neural cells on pain modulation [21]. Several investigations have shown pain-modulatory properties of the orexin molecule at spinal and supraspinal levels. Moreover, the efficacy of orexinergic transmission in pain modulation has been demonstrated in multiple pain models including thermal, mechanical, and chemical-induced analgesia [22]. Recent findings suggest that orexinergic transmission may interact with the opioidergic system, especially on addiction-related behavior [23]. Although both opioidergic and orexinergic systems have been implicated in pain modulation, the extent of their functional interaction within the NAc remains unclear. Given the NAc's integrative role in reward and nociceptive processing, understanding how these systems interact may uncover new mechanisms of endogenous analgesia. Therefore, this study aimed to examine the bidirectional relationship between opioidergic and orexinergic signaling in the NAc during acute pain modulation using receptor-selective pharmacological manipulations. The findings may provide mechanistic insight into how these systems cooperate in pain regulation and highlight potential targets for developing safer, non-addictive analgesics.

2. Materials and Methods

2.1 Animals

Two hundred and thirty-seven male Wistar albino rats (Pasteur Institute, Tehran, Iran), weighing 220–250 g, were enrolled in the study. All animals were acclimated to the facility for at least seven days before the study protocol commenced. During this adaptation period, the rats were housed in groups of three in plexiglass cages (38 × 33 × 17 cm) under standard conditions: a temperature of 22 ± 1 °C and a 12-hour light/dark cycle (lights on at 7:00 AM). Each rat was handled daily for 5 minutes by a researcher. Food and water were available ad libitum. Animals were randomly assigned to experimental groups (6–8 rats per group) using a random number table. The sample size was determined based on previous studies with similar experimental designs, with a minimum of six animals per group to ensure adequate statistical power. The experimenter performing the behavioral assessments and data analysis was blinded to the treatment groups. Animals were excluded from the study if they exhibited signs of illness, injury, or abnormal behavior before the initiation of the experiments. All experiments were performed in accordance with the ARRIVE guidelines 2.0 (for further details, see **Supplementary Material**).

2.2 Drugs

Morphine hydrochloride (Temad, Tehran, Iran), as a μ-opioid receptors agonist was prepared in 5, 10, 25, and 50 mmol concentrations. Naloxone (Tocris Bioscience, Bristol, UK; Cat. No. 1419), as a μ-opioid receptor antagonist was utilized in 1.5, 5, 15, and 45 mmol concentrations. Orexin-A agonist peptide (Tocris Bioscience; Cat. No. 1455) was prepared in 0.25, 0.5, 1, and 2 nmol concentrations. SB334867 (Tocris Bioscience; Cat. No. 1960), as an orexin receptor type 1 (OX1R) antagonist, and TCS OX2 29 (Tocris Bioscience; Cat. No. 3371), as an orexin receptor type 2 (OX2R) antagonist, were administered in 3, 10, 30, and 100 nmol dosages.

The sterile saline solution was used as a solvent for morphine, naloxone, and orexin-A peptide. SB334867 and TCS OX2 29 were dissolved in dimethyl sulfoxide DMSO 12% (Sigma Aldrich, Taufkirchen, Germany; CAS Number: 67-68-5). All solutions and drugs were prepared fresh on the day of the experiments.

2.3 Stereotaxic Surgery

Rats were deeply anesthetized with intraperitoneal injections of ketamine (100 mg/kg; Alfasan Woerden, Holland) and xylazine (10 mg/kg; Alfasan Woerden, Holland). Surgical procedures were performed using a stereotaxic apparatus (Stoelting Co., Wood Dale, IL, USA). The animals' heads were secured in the stereotaxic frame, and a midline incision was carefully made along the scalp to expose the skull. The skin, underlying soft tissues, and periosteum were gently retracted to allow access to the cranial surface. A stainless-steel guide cannula (23-gauge, 11 mm in length) was then unilaterally implanted 1 mm above the coordinates of the NAc (anteroposterior: +1.6 ± 0.15 mm, mediolateral: ±1.6 mm, dorsoventral: −7.8 mm), according to the rat brain atlas [24]. After completing the surgical procedure, a local anesthetic solution containing lidocaine combined with epinephrine (0.2 mL/L) was administered sub-

cutaneously around the incision site. This treatment was applied to reduce local bleeding and attenuate nociceptive responses associated with the surgical manipulation, thereby contributing to postoperative analgesia. In addition, enrofloxacin 10% was administered subcutaneously at a dose of 2.5 mg/kg to minimize the risk of postoperative infection. The cannula was secured in place using dental acrylic cement and two stainless-steel screws anchored to the skull. During the postoperative recovery period, a stainless-steel stylet was inserted into the guide cannula to prevent occlusion. A recovery period of one week was allowed before initiating the tail-flick test experiments.

2.4 Drug Administration

A total volume of 0.5 µlL of the drug solution was administered into the target region of the NAc via microinjection. Injections were performed using a 30-gauge stainless steel injector needle (12 mm in length) connected to a 1-µlL Hamilton syringe (Hamilton Company, Reno, NV, USA) by polyethylene tubing (PE-20). The solution was infused slowly over 60 seconds to ensure controlled distribution within the target site. Following the infusion, the injector remained in the guide cannula for an additional 60 seconds to prevent backflow of the administered solution.

2.5 Tail-Flick Test

The D'Amour and Smith method and tail-flick apparatus (Harvard Apparatus, Holliston, MA, USA) were used to perform a tail-flick test [25]. The heat was applied to the upper surface of the rats 3, 5, and 7 cm apart from the tail tip. The test was run after 30 minutes of adaptation and 5 minutes after the end of the drug injections. The tail flick latency (TFL) was measured by an automated sensor (Harvard Apparatus) that measured tail withdrawal time. At each time point (before, 5, 15, 30, 45, and 60 minutes after drug injection), the TFL was measured as the mean of two consecutive tests. The maximum heat source intensity was set at 45% and a cut-off time of 10 seconds was assumed to prevent heat injuries to the animals. The maximum possible effect (MPE) was measured according to the following formula:

$$\begin{aligned}\text{MPE}\ (\%) = [&\text{Post drug latency}\ (\text{sec}) \\ -&\text{Baseline latency}\ (\text{sec})]/[\text{Cut off point value}\ (\text{sec}) \\ -&\text{Baseline latency}\ (\text{sec})] * 100\end{aligned}$$

2.6 Locomotor Activity

The locomotor activity of the intact group of animals, the sham-operated animals, and following the injection of the maximum dose of the drugs (morphine: 50 mmol, Naloxone: 45 mmol, Orexin-A: 2 nmol, SB334867: 100 nmol, TCS OX2 29: 100 nmol) used in this study were recorded. The locomotor activities were analyzed to ensure that the injections did not significantly affect the results of the TFLs. An open field (60 × 60 cm) and a 3CCD camera (Panasonic Inc., Osaka, Japan)two meters apart from the open field were used to record the traveled distance. The EthoVision video tracking system (Version 3.1, Noldus Information Technology, Wageningen, the Netherlands)were utilized to analyze the results.

2.7 Experimental Design

Fig. 1 presents the timeline of the procedures. Three distinct investigation steps were used to elucidate the possible interaction between opioidergic and orexinergic neural circuits in pain modulation in the NAc. Each group of rats consists of 7–8 animals. The total volume of injections for each rat was set at 0.5 µlL. Using the dose-response curve, this study found the optimum drug concentration of the microinjections.

Step 1: In the first phase of the experiment, the impact of unilateral intra-accumbal administration of various morphine doses on nociceptive behavior was assessed (Fig. 1A). The vehicle group (n = 7) received 0.5 µlL of saline, whereas experimental groups (n = 7–8 per dose) were administered morphine at dosages of 5, 10, 25, and 50 mmol (Table 1).

Step 2: To assess the role of orexinergic signaling within the NAc in pain modulation, several experimental groups were established (Table 1). The vehicle group received a saline solution, while four groups were administered orexin-A at doses of 0.25, 0.5, 1, and 2 nmol (Fig. 1B). To further examine receptor-specific mechanisms, one group received DMSO (vehicle) plus orexin-A (1 nmol). Additional groups were pretreated with either the OX1R antagonist SB334867 (3, 10, and 30 nmol; Fig. 1C) or the OX2R antagonist TCS OX2 29 (3, 10, 30, and 100 nmol; Fig. 1D), followed by orexin-A (1 nmol) microinjection. All animals were subsequently tested for nociceptive response using the tail-flick assay.

Step 3: To investigate the interaction between the opioidergic and orexinergic systems within the NAc in pain modulation, several combination treatments were administered (Table 1). Four groups received intra-NAc co-administration of naloxone (1.5, 5, 15, or 45 mmol) followed by orexin-A (1 nmol) to assess the effect of µ-opioid receptor blockade on orexin-induced analgesia (Fig. 1E). Another four groups were pretreated with the SB334867 (3, 10, 30, and 100 nmol) prior to morphine (10 mmol) administration (Fig. 1F), and three groups received the TCS OX2 29 (3, 10, and 30 nmol) before morphine (10 mmol) injection (Fig. 1G). Following the microinjections, nociceptive thresholds were assessed using the tail-flick test to evaluate the functional crosstalk between these neurotransmitter systems.

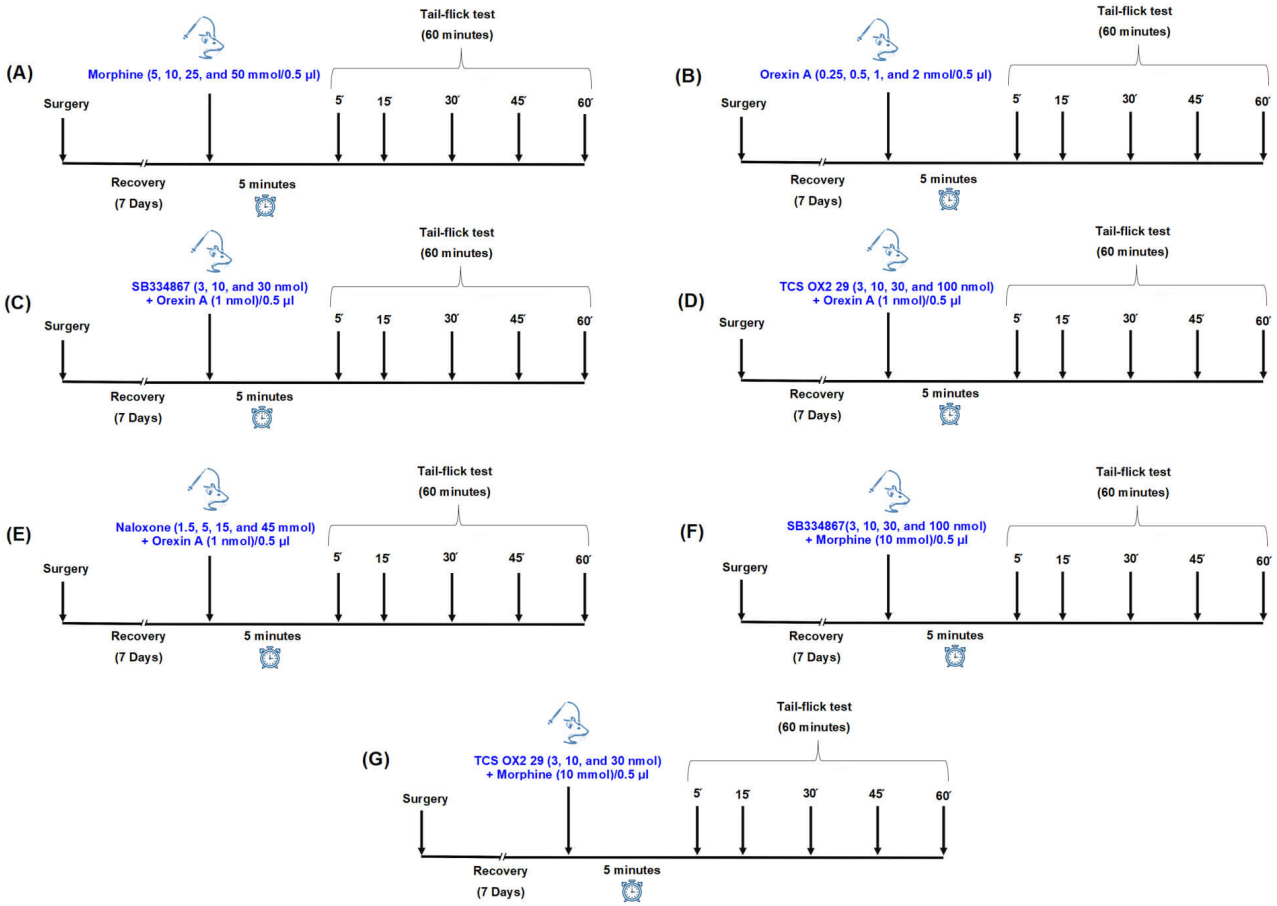

Fig. 1. The schematic representation of the procedure timeline. Injection of the vehicles, agonists, or antagonists was performed according to the experimental group protocol. (A) The experimental protocol for investigating the effect of morphine, as a µ-opioid receptors agonist, microinjection in the nucleus accumbens (NAc) on pain-related behavior. (B) The experimental protocol for investigating the effect of orexin-A microinjection in the NAc on pain-related behavior. (C) The experimental protocol for investigating the effect of intra-NAc injection of SB334867, as an orexin receptor type 1 (OX1R) antagonist, on Orexin-induced antinociceptive responses. (D) The experimental protocol for investigating the effect of TCS OX2 29, as an orexin receptor type 2 (OX2R) antagonist, on orexin-induced antinociceptive responses. (E) The experimental protocol for investigating the effect of intra-NAc injection of naloxone, as a µ-opioid receptor antagonist, on orexin-A-induced antinociceptive responses. (F) The experimental protocol for investigating the effect of intra-NAc injection of SB334867 on morphine-induced antinociceptive responses. (G) The experimental protocol for investigating the effect of intra-NAc injection of TCS OX2 29 on morphine-induced antinociceptive responses.

2.8 Histologic Verification

At the conclusion of the behavioral procedures, rats were euthanized by intraperitoneal administration of an overdose of ketamine (200 mg/kg) and xylazine (20 mg/kg). The brain was extracted with minimal damage and kept in a 10% formalin solution for three days. The tissue was then dissected into 50 µm coronal sections to confirm the location of the microinjections. Only data from the animals with approved guide cannula locations were entered for the analysis (17 animal data were excluded due to misplacement). The Paxinos atlas was used as a reference to verify the anatomical site of the cannula (Fig. 2).

2.9 Statistics

The present study report data values by mean ± SEM. A *p*-value cutoff of less than 0.05 is considered statistically significant. The repeated measure two-way analysis of variance (ANOVA) followed by the Bonferroni post-*hoc* test was utilized for MPE values of the tail-flick test analysis. Furthermore, the repeated measures one-way ANOVA followed by Dunnett's multiple comparison tests was used to compare the calculated area under the curve (AUC) obtained from MPE (%) values. In addition, the effect size (η^2) of morphine, Orexin, SB334867 + morphine, TCS OX2 29 + morphine, and naloxone + Orexin was calculated by dividing the mean difference between the experimental groups by the standard deviation of the population from which the different treatment groups were selected.

Table 1. Control and experimental groups.

	Group name	Microinjections (0.5 µL)	#of rats
Opioid (Step 1)	Vehicle	Saline	7
	Morphine	5 mmol	7
		10 mmol	8
		25 mmol	8
		50 mmol	8
	Vehicle + morphine	DMSO + 10 mmol morphine	7
Orexin (Step 2)	Vehicle	Saline	8
	Orexin-A	0.25 nmol	8
		0.5 nmol	7
		1 nmol	8
		2 nmol	8
	Vehicle + Orexin-A	DMSO + 1 nmol Orexin	8
	SB334867 + Orexin-A	3 nmol SB + 1 nmol Orexin	8
		10 nmol SB + 1 nmol Orexin	8
		30 nmol SB + 1 nmol Orexin	7
	TCS OX2 29 + Orexin-A	3 nmol TCS + 1 nmol Orexin	8
		10 nmol TCS + 1 nmol Orexin	7
		30 nmol TCS + 1 nmol Orexin	7
		100 nmol TCS + 1 nmol Orexin	6
Interaction (Step 3)	Naloxone + Orexin-A	1.5 mmol Naloxone + 1 nmol Orexin	8
		5 mmol Naloxone + 1 nmol Orexin	7
		15 mmol Naloxone + 1 nmol Orexin	7
		45 mmol Naloxone + 1 nmol Orexin	6
	SB334867 + morphine	3 nmol SB + 10 mmol morphine	7
		10 nmol SB + 10 mmol morphine	7
		30 nmol SB + 10 mmol morphine	7
		100 nmol SB + 10 mmol morphine	7
	TCS OX2 29 + morphine	3 nmol TCS + 10 mmol morphine	7
		10 nmol TCS + 10 mmol morphine	7
		30 nmol TCS + 10 mmol morphine	7
Total	Included	220	237
	Excluded	17	

# indicates the number of animals per group.

3. Results

3.1 Effects of Intra-Accumbal Morphine Microinjection on Acute Pain Response

As the data passed the normality test in the Kolmogorov-Smirnov test (p-Value $>$ 0.10), the Two-way repeated measure ANOVA followed by the Bonferroni post-*hoc* test was utilized to evaluate the between-group differences. Results demonstrate that the intra-accumbal microinjections of morphine can significantly increase the pain response threshold in the acute pain model of the tail-flick test compared to the saline as a vehicle (treatment effect: $F [4, 33] = 20.44$, $p < 0.0001$; time effect: $F [4, 132] = 14.14$, $p < 0.0001$; time × treatment: $F [16, 132] = 1.303$, $p = 0.2045$; Fig. 3A). The observed analgesic effect started 5 minutes after injection and lasted up to the 45 min of experiment. To compare the AUC values, a one-way ANOVA followed by Dunnett's post hoc test was used to determine the optimal morphine dose administered into the NAc. The results suggest that the morphine microinjections with a dose of 10, 25, and 50 mmol can significantly reduce the pain sensitivity in the tail-flick test ($F [4, 33] = 13.48$, $p < 0.0001$; Eta-square effect size (η^2) = 0.62; Fig. 3B).

3.2 Effects of Intra-NAc Orexin-A Administration on Behaviors Associated With Acute Pain

As the data passed the normality test in the Kolmogorov Smirnov test (p-Value $>$ 0.10), the Two-way repeated measure ANOVA followed by the Bonferroni post-*hoc* test was utilized to evaluate the between-group differences. The results demonstrate that the intra-NAc microinjections of orexin-A can significantly increase the pain response threshold in the acute pain model of the tail-flick test compared to the saline as a vehicle group (treatment effect: $F [4, 34] = 12.5$, $p < 0.0001$; time effect: $F [4, 136] = 12.75$, $p < 0.0001$; time × treatment: $F [16, 136] = 1.95$, p

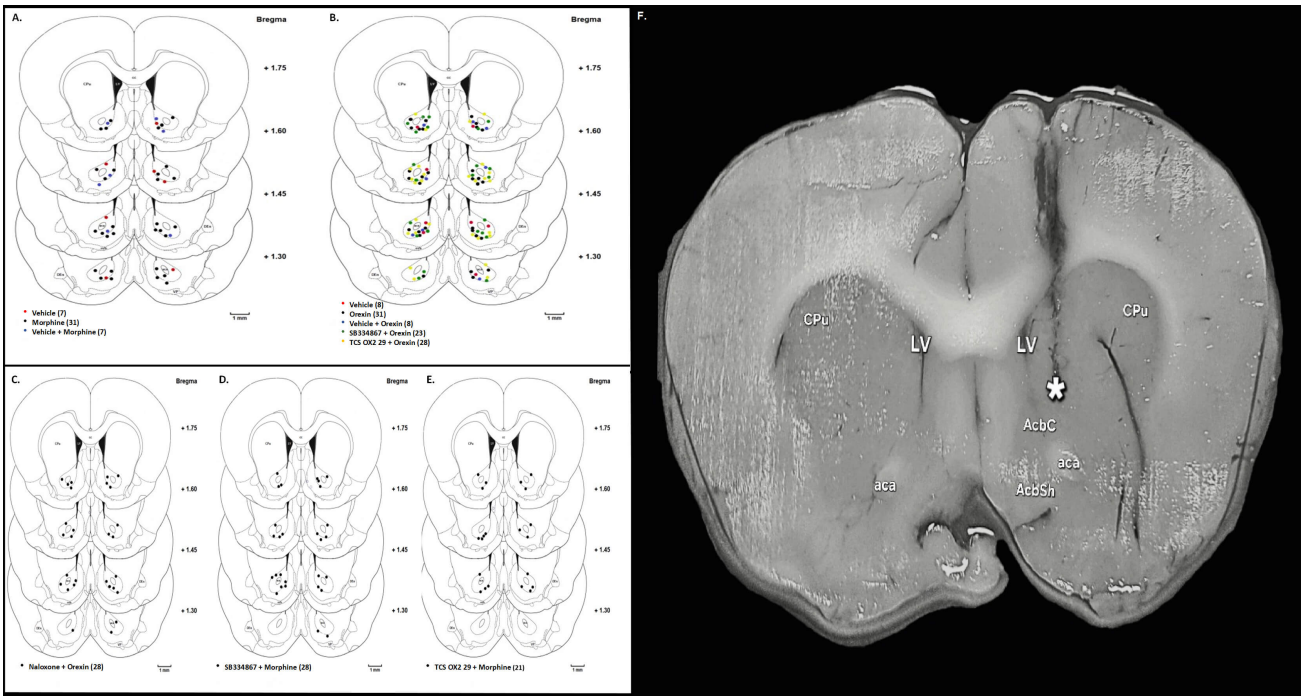

Fig. 2. Histologic validation of the microinjections. The diagram represents the location of unilateral microinjections placed within the NAc territory of the rat brain coronal sections. (A) Opioid microinjection groups. (B) Orexin microinjection groups. (C) Naloxone + Orexin groups. (D) SB334867 + morphine groups. (E) TCS OX2 29 + morphine groups. Scale bar = 1 mm. (F) Photomicrograph of a representative coronal brain section showing the microinjection site and cannula placement in the NAc. The asterisk (*) indicates the tip of the injection needle. CPu, caudate putamen; LV, lateral ventricle; AcbC, accumbens nucleus (core); AcbSh, accumbens nucleus (shell); aca, anterior commissure anterior part. n = 6, 7 or 8 rats per group, as specified in Table 1.

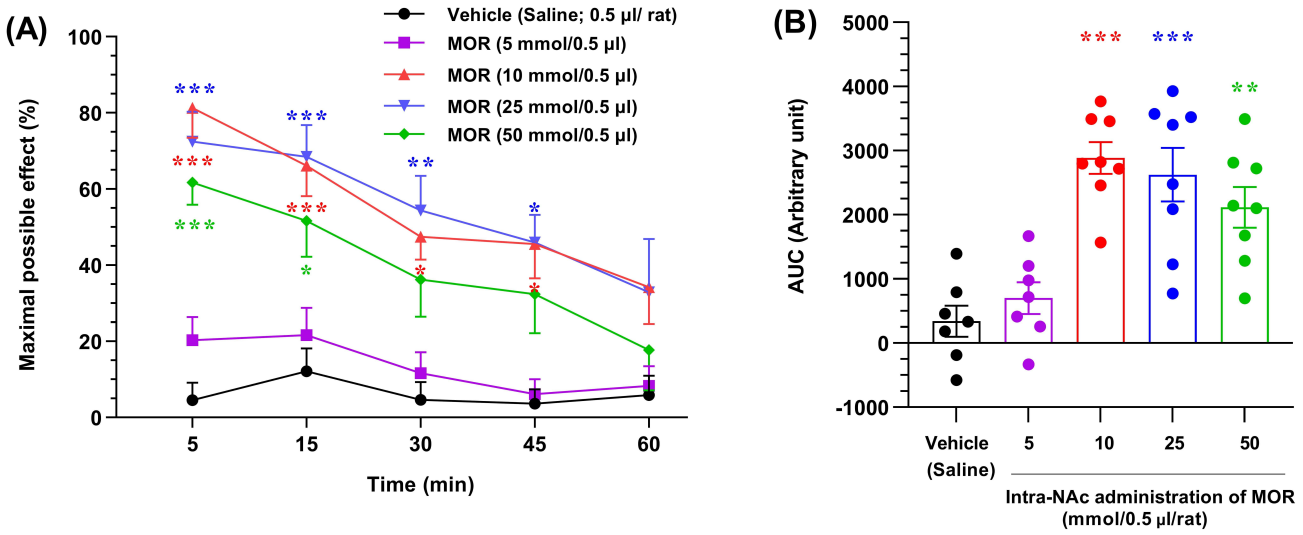

Fig. 3. The effect of intra-NAc microinjection of morphine in acute pain-related behavior model. (A) The two-way repeated-measures analysis of variance (ANOVA) results of maximum possible effect (MPE) (%) following morphine (MOR) microinjections with 5, 10, 25, and 50 mmol vs saline. (B) One-way ANOVA results for comparison of the calculated area under the curve (AUC) for morphine and saline microinjections. The charts represent Mean ± SEM for experimental groups. n = 7 or 8 rats per group. *p-value < 0.05, **p-value < 0.01, and ***p-value < 0.001 are demarcated to represent significant differences between drug and vehicle microinjections.

= 0.0208; Fig. 4A). The observed analgesic effect started 5 minutes after injection and last up to the end of experiment.

To compare the AUC values, a one-way ANOVA followed by Dunnett's post hoc test was used to determine the op-

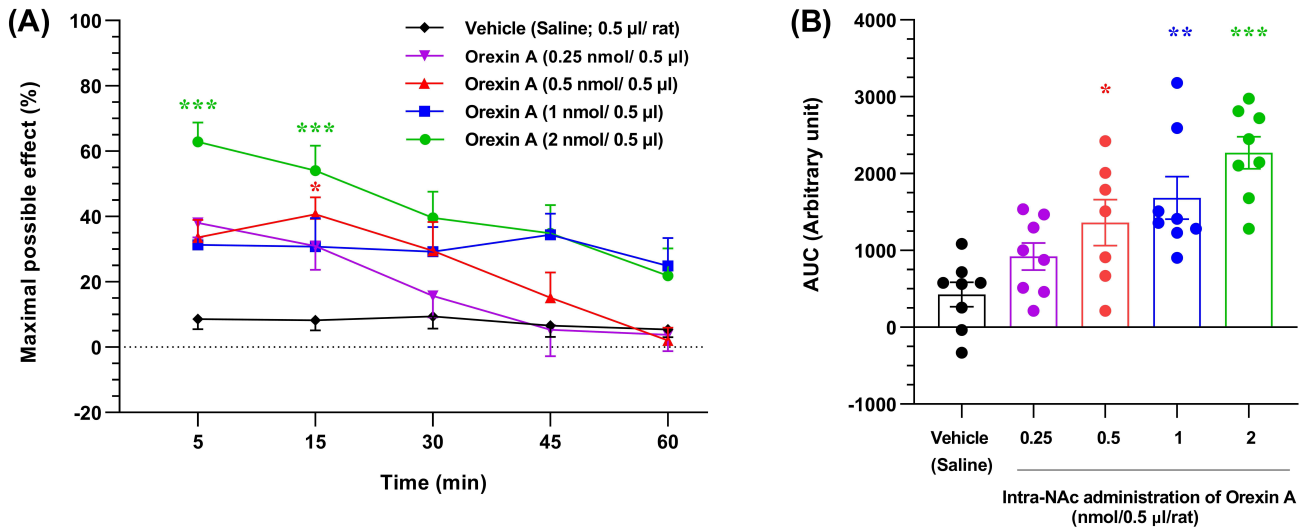

Fig. 4. The effect of localized orexin-A administration into the NAc on acute pain-related behavior. (A) The two-way repeated-measures ANOVA results of MPE (%) following orexin-A microinjections with 0.25, 0.5, 1, and 2 nmol vs saline. (B) One-way ANOVA results for comparison of the calculated AUC for orexin-A and saline microinjections. The charts represent Mean ± SEM for experimental groups. n = 7 or 8 rats per group. **p*-value < 0.05, ***p*-value < 0.01, and ****p*-value < 0.001 are demarcated to represent significant differences between drug and vehicle microinjections.

timal orexin dose administered into the NAc. The results suggest that the orexin-A microinjections with a dose of 1 and 2 nmol can significantly reduce the pain sensitivity in the tail-flick test ($F [4, 34] = 10.05$, $p < 0.01$; $\eta^2 = 0.54$; Fig. 4B).

Investigation of the role of OX1R blockade in attenuating orexin-mediated analgesia within the NAc showed that pre-administration of SB334867 significantly inhibited the analgesic effect of orexin-A microinjection (K–S $p > 0.10$; two-way repeated-measures ANOVA, treatment effect: $F [3, 27] = 12.09$, $p < 0.0001$; time effect: $F [4, 108] = 13.98$, $p < 0.0001$; time × treatment: $F [12, 108] = 0.75$, $p = 0.8080$; Fig. 5A). Comparison of AUC values across SB334867 doses indicated that 10 and 30 nmol significantly reduced the orexin-induced analgesic response in the tail-flick test ($F [3, 27] = 13.35$, $p < 0.001$; $\eta^2 = 0.54$; Fig. 5B).

Similarly, blockade of OX2R revealed that pre-treatment with TCS OX2 29 markedly suppressed the orexin-induced analgesic effect (K–S $p > 0.10$; two-way repeated-measures ANOVA, treatment effect: $F [4, 31] = 11.43$, $p < 0.0001$; time effect: $F [4, 124] = 13.29$, $p < 0.0001$; time × treatment: $F [16, 124] = 1.075$, $p = 0.3837$; Fig. 6A). AUC analysis demonstrated that 30 and 100 nmol of TCS OX2 29 significantly diminished the analgesic response produced by orexin-A in the tail-flick test ($F [4, 31] = 9.152$, $p < 0.0001$; $\eta^2 = 0.54$; Fig. 6B).

3.3 Assessment of the Orexinergic and Opioidergic System's Interaction in Acute Pain Modulation Within the NAc

The two-way repeated-measures ANOVA with multiple comparisons revealed significant group differences in the MPE across various naloxone doses compared with saline when pre-administered before an effective dose of orexin-A within the NAc (K–S $p > 0.10$; treatment effect: $F [4, 31] = 9.808$, $p < 0.0001$; time effect: $F [4, 124] = 13.87$, $p < 0.0001$; time × treatment: $F [16, 124] = 1.121$, $p = 0.3432$; Fig. 7A). These findings indicate that naloxone pretreatment markedly suppressed the analgesic effect produced by accumbal orexin administration. AUC analysis confirmed that naloxone at doses of 1.5, 5, 15, and 45 mmol significantly reduced orexin-induced antinociception in the tail-flick test ($F [4, 31] = 13.04$, $p < 0.0001$; $\eta^2 = 0.63$; Fig. 7B).

Similarly, analysis of the interaction between opioidergic and orexinergic systems showed that pretreatment with the OX1R antagonist produced a dose-dependent inhibition of morphine-induced analgesia within the NAc. The two-way repeated-measures ANOVA indicated significant treatment effects across SB334867 doses compared with DMSO controls (K–S $p > 0.10$; treatment effect: $F [4, 30] = 13.39$, $p < 0.0001$; time effect: $F [4, 120] = 13.73$, $p < 0.0001$; time × treatment: $F [16, 120] = 1.093$, $p = 0.3690$; Fig. 8A). AUC comparisons demonstrated that 10, 30, and 100 nmol of SB334867 significantly attenuated morphine-induced antinociception in the tail-flick test ($F [4, 31] = 11.13$, $p < 0.0001$; $\eta^2 = 0.59$; Fig. 8B).

In another set of experiments, blocking OX2R with TCS OX2 29 also reduced morphine-mediated analgesia. Significant between-group differences were observed across TCS OX2 29 doses compared with DMSO (K–S $p > 0.10$; treatment effect: $F [3, 24] = 13.78$, $p < 0.0001$; time effect: $F [4, 96] = 18.75$, $p < 0.0001$; time × treatment: $F [12, 96] = 0.579$, $p = 0.854$; Fig. 9A). AUC analysis re-

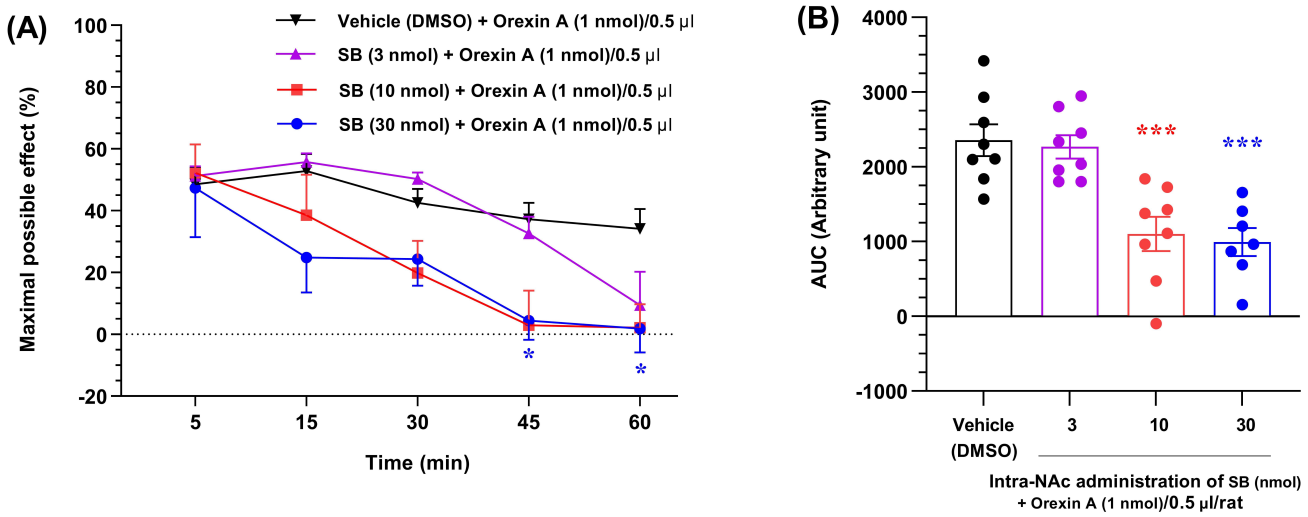

Fig. 5. The influence of SB334867 (SB) pretreatment within the NAc on the analgesic effect of orexin-A (1 nmol) in the acute pain model. (A) The two-way repeated-measures ANOVA results of MPE (%) following intra-NAc microinjections of SB334867 (3, 10, and 30 nmol) versus vehicle (DMSO) in rats receiving orexin-A (1 nmol). (B) One-way ANOVA results for comparison of the calculated AUC for SB334867 and vehicle (DMSO) + orexin-A (1 nmol) microinjections. The charts represent Mean ± SEM for experimental groups. n = 7 or 8 rats per group. **p*-value < 0.05 and ****p*-value < 0.001 are demarcated to represent significant differences between drug and vehicle (DMSO) + orexin-A (1 nmol) microinjections.

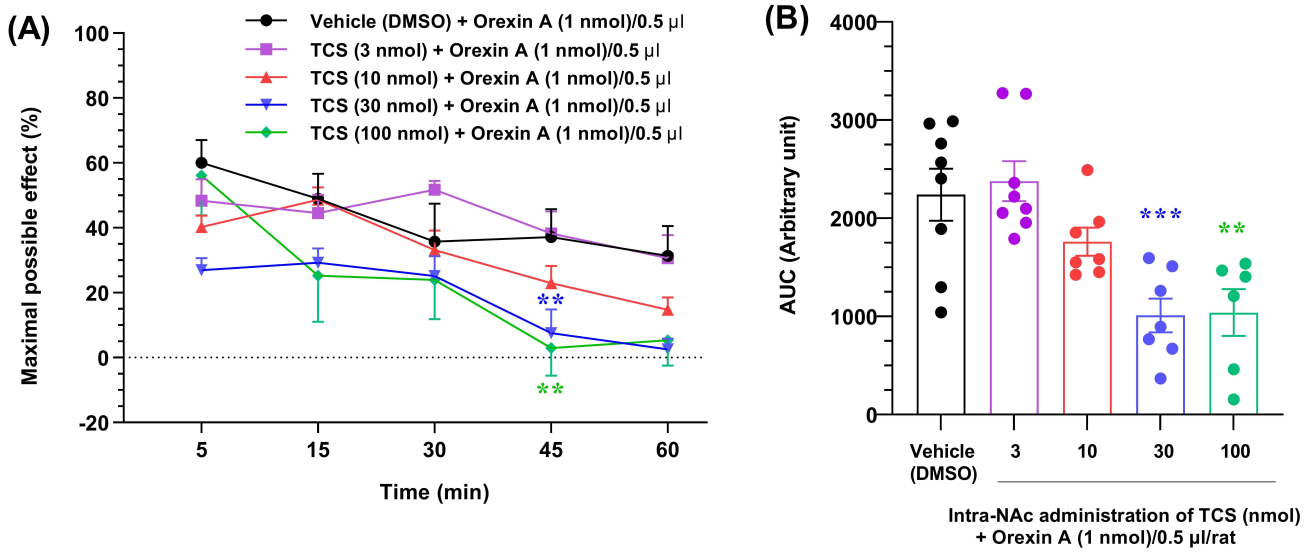

Fig. 6. The impact of TCS OX2 29 (TCS) administration in the NAc region on the orexin-A (1 nmol)–induced antinociceptive response in the acute pain paradigm. (A) The two-way repeated-measures ANOVA results of MPE (%) following intra-NAc microinjections of TCS OX2 29 (3, 10, 30, and 100 nmol) versus vehicle (DMSO) in rats receiving orexin-A (1 nmol). (B) One-way ANOVA results for comparison of the calculated AUC for TCS OX2 29 and vehicle (DMSO) + orexin-A (1 nmol) microinjections. The charts represent Mean ± SEM for experimental groups. n = 6, 7 or 8 rats per group. ***p*-value < 0.01 and ****p*-value < 0.001 are demarcated to represent significant differences between drug and vehicle (DMSO) + orexin-A (1 nmol) microinjections.

vealed that 10 and 30 nmol of TCS OX2 29 significantly diminished the antinociceptive effect of morphine in the tail-flick test ($F [3, 24] = 14.16$, $p < 0.001$; $\eta^2 = 0.64$; Fig. 9B).

3.4 Locomotor Activity

One-way ANOVA followed by a Tukey's multiple-comparison test was performed to find the variations in locomotor activity among the intact, sham, vehicle, morphine: 50 mmol, naloxone: 45 mmol, Orexin-A: 2 nmol, SB334867: 100 nmol, TCS OX2 29: 100 nmol groups. The

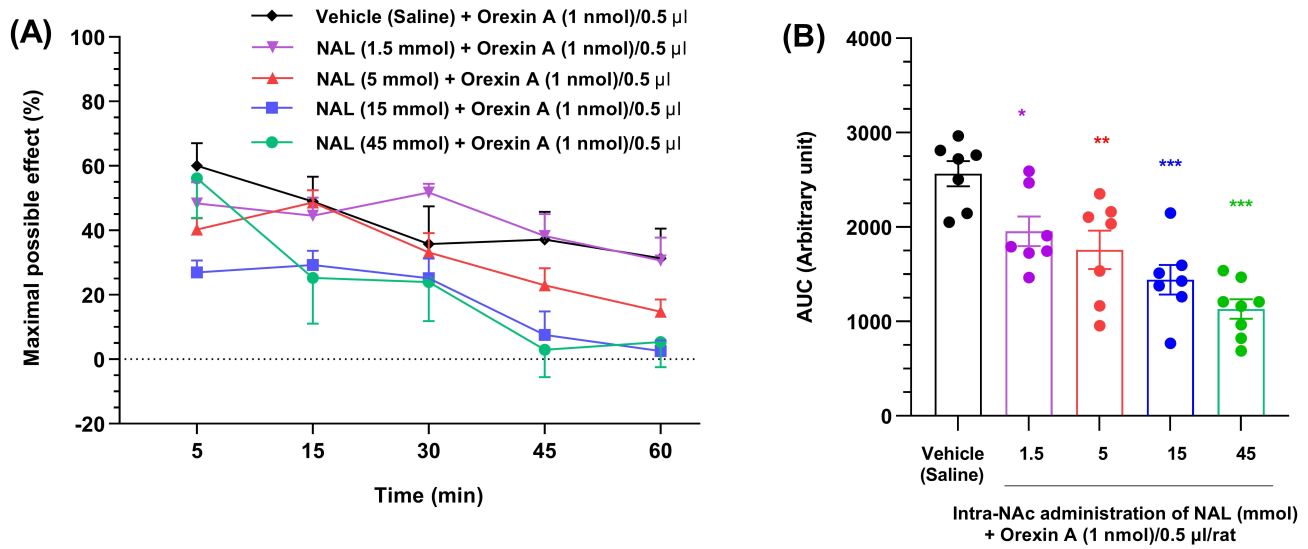

Fig. 7. Evaluation of the interaction between opioidergic and orexinergic transmission on acute pain-related behavior. (A) The two-way repeated-measures ANOVA results of MPE (%) following intra-NAc microinjections of naloxone (NAL) (1.5, 5, 15, and 45 mmol) versus vehicle (saline) in rats receiving orexin-A (1 nmol). (B) One-way ANOVA results comparing the calculated AUC for naloxone and vehicle (saline) + orexin-A (1 nmol) microinjection. The charts represent Mean ± SEM for experimental groups. n = 7 or 8 rats per group. **p*-value < 0.05, ***p*-value < 0.01, and ****p*-value < 0.001 are demarcated to represent significant differences between drug and vehicle (saline) + orexin-A (1 nmol) microinjections.

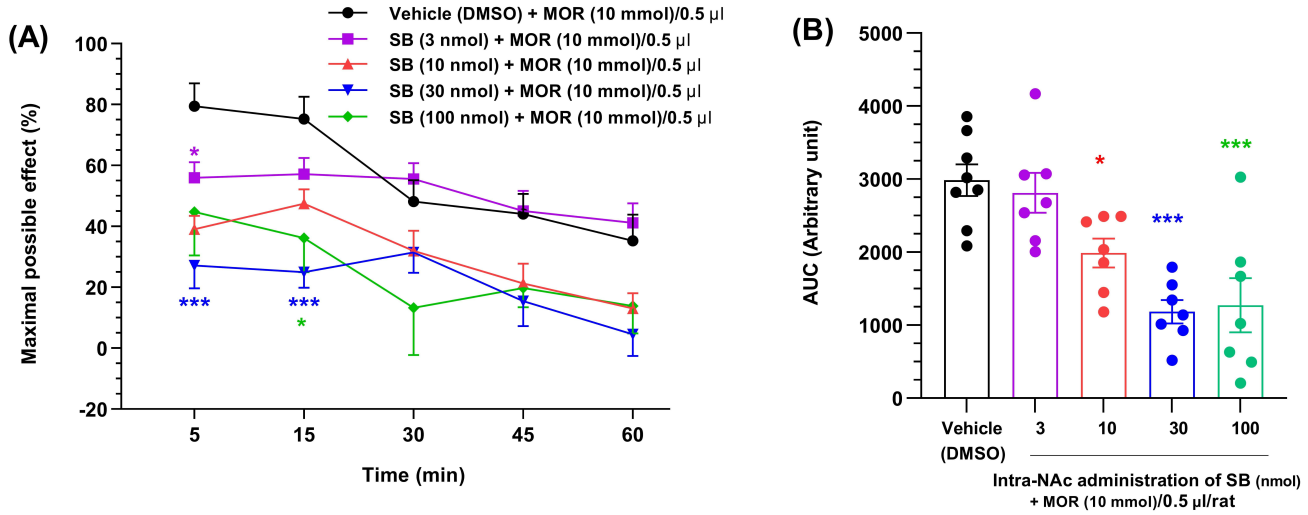

Fig. 8. Evaluation of the interaction between opioidergic and orexinergic transmission on acute pain-related behavior. (A) The two-way repeated-measures ANOVA results of MPE (%) following intra-NAc microinjections of SB334867 (3, 10, 30, and 100 nmol) versus vehicle (DMSO) in rats receiving morphine (10 mmol). (B) One-way ANOVA results for comparison of the calculated AUC for SB334867 and vehicle (DMSO) + morphine (10 mmol) microinjection. The charts represent Mean ± SEM for experimental groups. n = 7 or 8 rats per group. **p*-value < 0.05 and ****p*-value < 0.001 are demarcated to represent significant differences between drug and vehicle (DMSO) + morphine (10 mmol) microinjections.

results (F = 0.4917, $p = 0.8364$; Fig. 10) showed no statistically significant differences in locomotor activity between these groups.

4. Discussion

The present study observed that the intra-NAc administration of morphine and orexin-A induced significant, dose-dependent elevations in the pain threshold, as measured by the tail-flick assay. The analgesic effects of both compounds were evident approximately 5 minutes post-

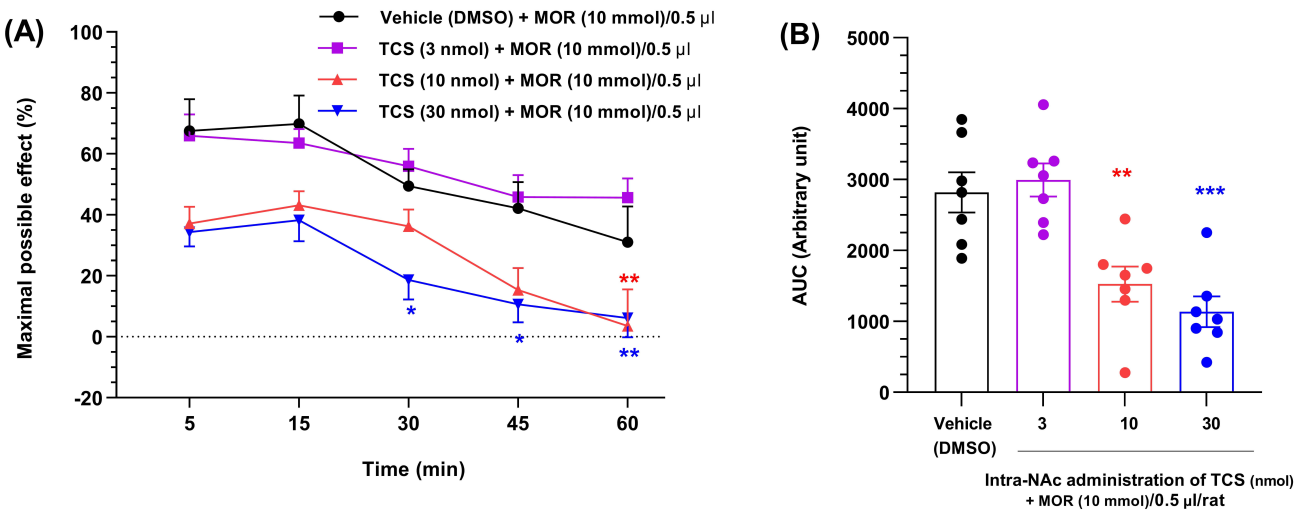

Fig. 9. Evaluation of the interaction between opioidergic and orexinergic transmission on acute pain-related behavior. (A) The two-way repeated-measures ANOVA results of MPE (%) following intra-NAc microinjections of TCS OX2 29 (3, 10, and 30 nmol) versus vehicle (DMSO) in rats receiving morphine (10 mmol). (B) One-way ANOVA results for comparison of the calculated AUC for TCS OX2 29 and vehicle (DMSO) + morphine (10 mmol) microinjection. The charts represent Mean ± SEM for experimental groups. n = 7 rats per group. **p*-value < 0.05, ***p*-value < 0.01, and ****p*-value < 0.001 are demarcated to represent significant differences between drug and vehicle (DMSO) + morphine (10 mmol) microinjections.

injection and persisted for up to 60 minutes, indicating that activation of local opioidergic and orexinergic circuits within the NAc significantly contributes to acute pain suppression. Consistent with the established role of this region in integrating reward and nociceptive signaling, orexin-A elicited a robust antinociceptive response, while morphine exhibited the most pronounced analgesic efficacy at concentrations of 10 and 25 mmol.

Pharmacological blockade experiments further elucidated the receptor-specific mechanisms mediating these effects. Pretreatment with the OX1R antagonist SB334867 (≥10 nmol) or the OX2R antagonist TCS OX2 29 (≥30 nmol) significantly and dose-dependently attenuated orexin-induced analgesia, confirming the involvement of both orexin receptor subtypes in NAc-mediated pain modulation.

Dose-response analysis revealed that the antinociceptive attenuation was consistent across the effective dose range. For SB334867, significant inhibitory effects were noted starting at 10 nmol and persisting through 30 nmol, suggesting a stable inhibitory window. For TCS OX2 29, significant attenuation was established at 30 nmol, with the 100 nmol dose maintaining this effect. This suggests that the inhibitory efficacy reaches a plateau at these concentrations within the current experimental design. Collectively, the present findings demonstrate that both OX1R and OX2R antagonism dose-dependently inhibit NAc-mediated analgesia. The observation that higher doses (100 nmol) did not result in a significant increase in inhibition compared to intermediate doses (30 nmol) suggests that the underlying orexinergic contribution to this pathway reaches a functional saturation point at these concentrations, ensuring robust but manageable modulation of the opioidergic system.

Similarly, pretreatment with the µ-opioid receptor antagonist naloxone (1.5, 5, 15, and 45 mmol) markedly reduced orexin-evoked analgesia, corroborating the functional role of opioid receptors in local nociceptive control. Notably, the current interaction experiments revealed a bidirectional crosstalk between these two neurochemical systems.

The original gate control theory proposed a spinal mechanism for pain modulation [26]. Subsequent investigations led to the description of the descending pain modulatory system, particularly the periaqueductal gray–rostral ventromedial medulla pathway, as a major neural circuit involved in pain control [27]. A recent study has also suggested an ascending nociceptive control pathway, in which pain-related signals engage mesolimbic structures such as the NAc to produce robust and sustained analgesia through opioid- and dopamine-dependent mechanisms [28]. These findings support functional cooperation between ascending and descending pain modulatory systems and suggest a role for mesolimbic circuits in broader pain regulation [2,28].

The NAc is widely recognized for its role in reward and motivation; however, it is also implicated in pain perception, affective processing, addiction, and limbic-motor integration [2]. Its connections with the prefrontal cortex, thalamus, anterior cingulate cortex, habenula, and periaqueductal gray support its participation in nociceptive modulation [7,8,29]. Electrophysiological studies have shown increased NAc activity in response to painful stim-

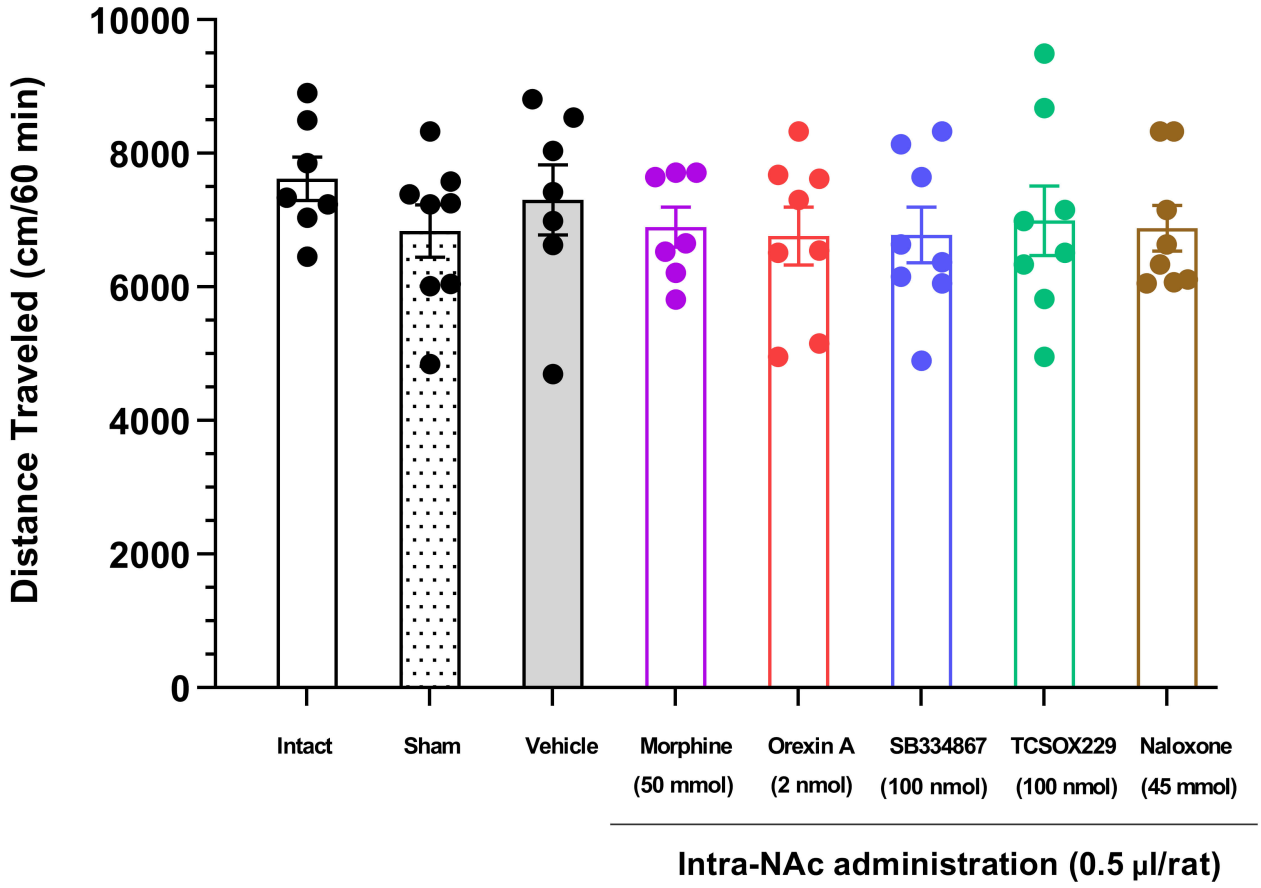

Fig. 10. The effect of microinjections of drugs within the NAc on the motor activity of rats is represented as the mean ± SEM for each experimental group. A one-way ANOVA test followed by the Tukey's post hoc test was equipped to compare the difference in the distance traveled during the open-field test. The analysis showed nonsignificant between-group differences in the distance traveled during the open-field test. n = 7 or 8 rats per group.

uli [9], and deep-brain stimulation studies suggest that the NAc may represent a potential target for pain control [10]. Several neurotransmitters, including endogenous opioids, dopamine, calcitonin gene-related peptide, glutamate, GABA, substance P, and orexin, have been implicated in pain modulation within the NAc [11,12,13,14,15,16,17].

The dense expression of opioid receptors and endogenous opioid peptides in the NAc supports the involvement of opioidergic transmission in local pain modulation [18,19]. In the central nervous system, µ-opioid receptors are particularly significant in pain processing [30]. Previous studies have shown that intra-NAc morphine produces antinociception and this effect is reversed by naloxone, supporting a primary role for µ-opioid receptor-mediated signaling in this region [2,16]. In addition, orexinergic projections from the lateral hypothalamus innervate the NAc, and both OX1R and OX2R are expressed in NAc neurons. Orexin-A has been shown to exert antinociceptive effects in several pain models, including the tail-flick test [21,31,32]. In the present study, both OX1R and OX2R contributed to analgesia within the NAc, although OX2R appeared to have a slightly greater effect than OX1R. This difference may reflect receptor expression patterns and model-dependent variation in orexin receptor function, rather than a hierarchy of receptor potency [33,34].

The present findings also suggest that dopamine may serve as a convergent neuromodulator linking orexinergic and opioidergic signaling within the NAc. Previous studies have shown that orexin can regulate dopamine release in mesolimbic circuits [35,36], and opioid signaling can also influence dopaminergic transmission in reward- and pain-related pathways [5,37]. Thus, dopamine may help shape the downstream effect of both systems on NAc neuronal output, thereby contributing to their functional crosstalk.

In addition, µ-opioid receptors and orexin receptors may converge on common intracellular signaling cascades, including the extracellular regulated protein kinases (ERK)/cAMP-response element binding protein (CREB) pathway. Activation of this pathway has been implicated in pain modulation, synaptic plasticity, and analgesic responses [38,39]. Therefore, ERK/CREB signaling may

represent one possible intracellular mechanism underlying the interaction between opioidergic and orexinergic systems in the NAc.

The robust effect sizes observed across these experiments (η^2 = 0.47–0.64) underscore the physiological significance of orexinergic-opioidergic interactions within the NAc. The obtained findings establish the NAc-centered orexinergic system as a critical "gatekeeper" of morphine-induced antinociception; the observation that OX1R and OX2R antagonists significantly attenuate opioid efficacy implies that endogenous orexin signaling is essential for maintaining optimal analgesic potency.

From a translational perspective, these results provide a compelling preclinical basis for targeting NAc orexin receptors to address the clinical challenges of opioid-based pain management. Given the urgent need for opioid-sparing strategies to mitigate dose-dependent side effects—such as respiratory depression, sedation, hyperalgesia, and addiction—pharmacological activation of orexin receptors offers a promising therapeutic avenue. Specifically, orexin receptor agonists could be utilized as adjuvants to enhance the analgesic efficacy of opioids, potentially enabling the use of lower, sub-therapeutic doses to achieve comparable clinical relief while improving patient safety and outcomes.

5. Conclusions

This study demonstrates that opioidergic and orexinergic signaling within the NAc cooperatively modulate acute nociceptive responses through a bidirectional functional crosstalk. The present pharmacological evidence confirms that both OX1R and OX2R are essential for maintaining morphine-induced antinociception, while endogenous opioid signaling is critical for orexin-mediated pain relief. The robust effect sizes observed highlight the physiological significance of these interactions within mesolimbic circuitry. Translating these findings to clinical practice, the present data suggest that the orexinergic system acts as a key gatekeeper for opioid efficacy. Consequently, targeting orexin receptors within the NAc represents a promising strategy for opioid-sparing therapeutic protocols, potentially enabling effective pain management with lower opioid doses to mitigate dose-dependent side effects and reduce the risk of dependence.

6. Study Limitations

Several limitations should be considered when interpreting the present findings. First, the experiments were conducted only in male rats; therefore, potential sex-dependent differences in orexinergic and opioidergic interactions were not evaluated. Second, the study focused on acute nociception using the tail-flick test, which may not fully represent more complex or chronic pain conditions. Third, although pharmacological antagonists were used to investigate receptor involvement, the precise cellular and circuit-level mechanisms underlying the interaction between orexinergic and opioidergic signaling within the NAc remain unclear. In addition, OX1R and OX2R antagonists were administered separately; therefore, the potential synergistic or cooperative interaction between these receptor subtypes was not examined. Finally, while the present findings provide clear behavioral evidence of bidirectional orexinergic-opioidergic crosstalk within the NAc, this study does not provide direct electrophysiological or molecular evidence of the neural mechanisms involved. The current behavioral pharmacology approach establishes the functional relevance of these interactions; however, it remains to be determined how orexin and opioid signaling converge at the synaptic level to regulate NAc neuronal firing. Future research, including the combined blockade of orexin receptors and the use of *in vivo* electrophysiological recording, optogenetic circuit manipulation, or calcium imaging, is warranted to delineate the specific synaptic mechanisms and neuronal populations subserving these interactions.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

AH was responsible for the concept and design of the study. MSJ and MM actively participated in data gathering and performing laboratory tasks. AH, RM, and MSJ performed data analysis and interpretation. RM wrote the manuscript. RM and AH provided critical revision of the manuscript content. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication, 8th edition, revised 2011) and were approved by the local ethics committee (Shahid Beheshti University of Medical Sciences, Tehran, Iran, IR.SBMU.PHNS.REC.1403.059). All experiments were performed in accordance with the ARRIVE guidelines 2.0.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/JIN50757.

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