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Deep brain stimulation of the anterior cingulate cortex reduces opioid addiction in preclinical studies

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Substance Use Disorder (SUD) is a medical condition where an individual compulsively misuses drugs or alcohol despite knowing the negative consequences. The anterior cingulate cortex (ACC) has been implicated in various types of SUDs, including nicotine, heroin, and alcohol use disorders. Our research aimed to investigate the effects of deep brain stimulation (DBS) in the ACC as a potential therapeutic approach for morphine use disorder. Additionally, we measured c-Fos protein expression as an indicator of neural activity in the nucleus accumbens (NAc) and prefrontal cortex (PFC). Our findings indicate that high-frequency (130 Hz) DBS at different amperages, 150 µA and 200 µA in the ACC during the acquisition phase of morphine conditioned place preference (CPP) inhibited the rewarding properties of morphine. Furthermore, DBS at these intensities during the extinction phase facilitated the extinction and mitigated the reinstatement of morphine CPP triggered by drug priming. Morphine conditioning was associated with impaired novel object conditioning (NOR) and locomotor activity. While DBS in the acquisition and extinction phases at both intensities restored NOR memory, only DBS at 200 µA recovered locomotor activity in the open field test. Treatment with DBS at 200 µA decreased c-Fos protein expression in the NAc and PFC (compared to morphine-only group). In conclusion, our data indicate an intensity-dependent effect of ACC DBS on the acquisition, extinction, and reinstatement of morphine-induced CPP in rats. These findings suggest that ACC DBS could be a potential intervention for the treatment of morphine use disorder.

Keywords Addiction, Deep brain stimulation, Morphine, Anterior cingulate cortex

Substance Use Disorder (SUD) is associated with an ongoing and uncontrolled drive to search for and use substances to achieve a desired positive effect or to alleviate the physical and mental distress that arises after the cessation of substance use. SUD is characterized by a high rate of recurrence and a cycle of dependence, cessation, and relapse. Indeed, despite the existence of different withdrawal treatments such as Marlatt's intervention, self-help intervention, and cue exposure therapy, approximately 85% of individuals with SUD experience relapse within one year of treatment[1,2]. Three primary causes of relapse include craving[3,4], substance cues, and stress[5], and there is a higher likelihood of relapse prevention by tackling these factors.

The ACC is implicated in decision-making processes, particularly in evaluating the salience of stimuli and in regulating emotional responses[6]. It is linked to the posterior cingulate cortex and orbitofrontal cortex via the cingulum bundle[7], to the amygdala and nucleus accumbens (NAc) via the uncinate fasciculus, and to various regions of the insula via the extreme capsule[8]. Along with other brain regions such as the ventral striatum, insula, amygdala, thalamus, and ventromedial prefrontal cortex (PFC), the ACC plays a crucial role in reward processing[9,10] and the attribution of incentive salience to drugs and cues associated with drug use[11,12].

Studies have shown that the ACC is involved in various types of SUDs, including nicotine, heroin and alcohol use disorders[13–15]. Increased neural activity in the ACC is associated with both craving and impulsive behavior[16].

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Studies have discovered abnormal values of white matter fractional anisotropy in the ACC concerning compulsive internet addiction disorder[17], alcohol use disorder[18] and heroin dependence[19]. Additionally, there is evidence of structural hyperconnectivity between the thalamus, orbitofrontal cortex, and ACC, which is associated with impulsive and compulsive behaviors[20].

In a recent meta-analysis involving 433 individuals with alcohol use disorder and 498 healthy controls, a notable reduction in gray matter volume was observed in the ACC of the patient group when compared to the healthy control group[21]. This finding aligns with a previous study that reported gray matter atrophy in the dorsal dACC and anterior insula among individuals with SUD[22]. In individuals with SUDs, the pregenual anterior cingulate cortex (pgACC) has been found to exhibit heightened activity[16]. This overactivity in the pgACC is associated with a reduced utilization of alternative strategies and, consequently, contributes to impulsive behaviors[23].

Deep Brain Stimulation (DBS) is a neurosurgical therapy that involves the permanent placement of electrodes deep within the brain. These electrodes are used to modulate and regulate maladaptive brain networks, aiming to improve the functioning of these networks and alleviate symptoms associated with various neurological and psychiatric conditions[24,25]. The potential use of DBS in the treatment of SUD was initially indicated by a series of case reports. These studies unexpectedly found that patients with DBS electrodes implanted in the NAc experienced a reduction in comorbid alcohol dependency[26] and nicotine dependency[27]. These findings suggested the potential of DBS as a therapeutic approach for addressing addictive behaviors.

The c-Fos gene expression has been employed as a crucial marker of neuronal activity in morphine addiction. By being induced in response to morphine exposure and withdrawal, c-Fos immunoreactivity reveals various brain regions involved in drug dependence and reward[28–30].

Studies have shown that c-Fos expression in the NAc is necessary for the acquisition of morphine-conditioned behaviors, highlighting its importance in drug craving and addiction[29]. Additionally, c-Fos induction in the striatum and NAc by morphine is similar to that observed with other drugs of abuse like cocaine and amphetamine, indicating the involvement of these brain regions in mediating drug abuse[31]. The expression of c-Fos in crucial brain regions is closely associated with morphine dependence, emphasizing its importance in understanding the neural mechanisms behind drug dependence and withdrawal.

Given the role of the ACC in addiction, it has been suggested that targeting this region of the brain may be a promising approach for SUD treatment. Furthermore, implants in the anterior cingulate cortex, involving surgically placed electrodes for continuous neuromodulation, have been used for alcohol use disorder, with promising results[15].

Deep brain stimulation allows direct neuromodulation of dysregulated brain circuits and has shown promise as a potential therapeutic approach for SUD. However, there is limited data on using ACC DBS specifically for treating opioid use disorder. Therefore, the present study aimed to investigate the effects of high-frequency (130 Hz) DBS at different amperages (150 μA and 200 μA) of the ACC on morphine reward and addiction-related behaviors in rats. We evaluated the ability of ACC DBS to disrupt the acquisition, extinction, and reinstatement of morphine CPP. Additionally, we measured c-Fos protein expression in the NAc and PFC as a marker of DBS-induced neuronal changes in these key addiction neurocircuits. This work provides insight into the therapeutic potential of ACC neuromodulation for opioid use disorder.

Materials and methods

Animals

A total of 108 male Wistar rats, weighing 230–270 g at the beginning of experiments, were included in the study. Male Wistar rats were obtained from the Pasteur Institute of Iran. The rats were housed in a standard environment with a controlled temperature range of 23 °C to 29 °C and a 12-hour light-dark cycle (lights on from 7:00 AM to 7:00 PM). They had unrestricted access to food and water over the entire course of experiments unless during the two hours before the behavioral tests to increase their motivation for task performance. Before the surgical procedure, the rats were given one week to acclimate to the laboratory environment. The behavioral experiments were conducted during the light phase under semidarkness conditions. All experiments were performed in accordance with relevant guidelines and regulations. All research protocols were approved by the Institutional Review Board at Tehran University of Medical Sciences (Code: IR.TUMS.AEC.1400.002). Also, this study is reported in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

Implanting the stimulating electrodes

The rats were anesthetized with intraperitoneal (i.p) injection of ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg) and placed in a stereotaxic apparatus (Stoelting, IL). Two stainless steel bipolar electrodes (127-μm bare diameter; A-M Systems, WA) were bilaterally implanted into the ACC at the following coordinates relative to the bregma: AP + 2 mm, ML ± 0.6 mm, and DV -3 mm. The location of the implanted electrodes can be seen in Fig. 1. The free ends of the electrodes were attached into a plastic connector and the whole assembly was secured to the skull using two anchoring screws and dental acrylic. Following the surgery, the rats were individually housed and given 5 to 6 days for recovery before the behavioral experiments. During the recovery period, each rat was handled by the experimenter for approximately 10–15 min per day to reduce stress and improve acclimatization to the experimental conditions, particularly in preparation for behavioral tests.

Measuring morphine reward

Morphine reward was assessed with unbiased CPP apparatuses using a biased experimental design[32], which consisted five consecutive phases: preconditioning, conditioning, postconditioning, extinction, and reinstatement. The experimental setup included three Plexiglas CPP boxes each with two side compartments of

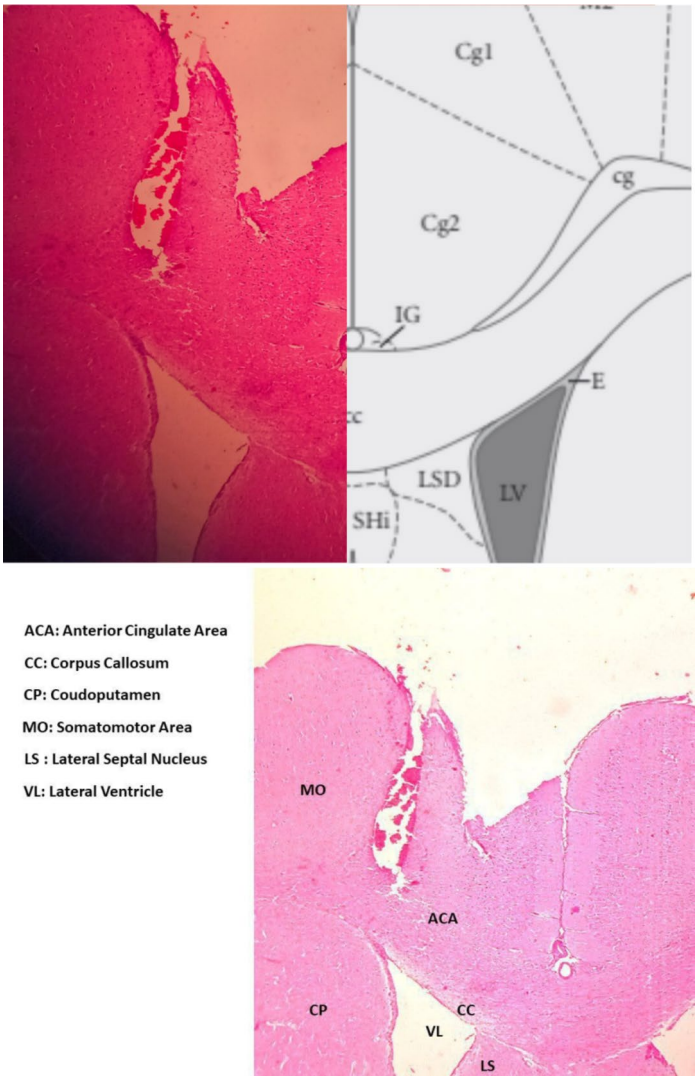

Fig. 1. Histological verification of electrode placement in the anterior cingulate cortex (ACC). This coronal brain section illustrates the precise placement of the electrode in the anterior cingulate area (ACA) of a rat. The electrode track is clearly observed within the ACA, a region critical for the study. Key anatomical landmarks, including the Corpus Callosum (CC), caudoputamen (CP), somatomotor area (MO), lateral septal nucleus (LS), and lateral ventricle (VL), are labeled to provide context. This histological evidence ensures the accuracy of electrode targeting, validating the subsequent data obtained from the deep brain stimulation (DBS) experiments.

equal size but different patterns (30 × 30 × 40 cm) and a neutral connecting compartment separable from the side compartment by a sliding guillotine door.

Before the tests, the rats were accustomed to the experimenter's handling, subcutaneous injection of saline, attachment of the DBS cables to the head mount, and the CPP arena. In all groups, the tether was attached to the electrode assembly on all days of the experiment, regardless of whether the rats received DBS or not. During the preconditioning test (day 1), the rats were allowed to freely explore the whole arena for 20 min to determine their initial side preference. The time spent by individual rats in each compartment was recorded using a video-tracking system (MazeRouter, Iran). Rats that exhibited a significant initial preference for any compartment (>65% for a side compartment or >45% for the neutral compartment) were excluded from the study. The conditioning phase occurred over three days (days 2–4) each with two training sessions in the morning (8:00–12:00) and afternoon (15:00–18:00). The rats received subcutaneous injection of morphine (3, 5, and 7 mg/kg, SC on days 2–4, respectively) and were confined to their nonpreferred compartment for 40 min in one session and received saline (equal volume, SC) and confined to their preferred compartment for 40 min in the next session. The order of morphine and saline sessions was reversed each day to minimize any potential confounding effects. This reversal was counterbalanced across subjects within each group. In the groups treated with saline, rats received saline injections in both compartments with the initial nonpreferred side as the reference context. In the postconditioning test (day 5), the rats were given unrestricted access to all compartments for 20 min. Side preference, an index of morphine reward, was measured as the percentage of time spent in the morphine-

paired compartment to the time spent in both the morphine- and saline-paired compartments. In the extinction phase (from day 6 onwards), the rats were exposed to the entire arena in a drug-free state daily similar to the postconditioning test (20 min/day), and the side preference was measured each day until there was no longer any preference for the drug-paired compartment. The reinstatement test was performed four days after the complete extinction of morphine CPP in each rat. The rats were injected with a priming dose of morphine (2 mg/kg, SC) and immediately tested for the renewal of side preference for 20 min. The interval between the extinction and reinstatement was planned for performing behavioral tests of locomotion and memory (see below).

Deep brain stimulation

Monophasic current pulses (150 μA or 200 μA, 100 μs, and 130 Hz) were generated by a current-based stimulator (ePulse, ScienceBeam, Iran) and continuously delivered deep inside the ACC. DBS was applied when the rats were in the CPP box during morphine-pairing trials (40 min, once daily on conditioning days) or during extinction training sessions (20 min, once daily on extinction days).

Experimental groups

Rats were assigned to six groups including (1) a saline group ($n = 6$) that were anesthetized, their skull were exposed with no electrode implantation, and received saline during the conditioning; (2) a sham-DBS group ($n = 14$; DBS-0) that were implanted with stimulating electrodes and received morphine during the conditioning, but did not receive DBS; (3, 4) two DBS groups that were implanted with electrodes, received morphine during the conditioning, and underwent DBS at 150 μA during the conditioning ($n = 5$; DBS-150 C) or extinction period ($n = 5$; DBS-150E); and (5, 6) two DBS groups that were implanted with electrodes, received morphine during the conditioning, and underwent DBS at 200 μA in conditioning ($n = 10$; DBS-200 C) or extinction period ($n = 10$; DBS-200E).

Measuring locomotion and memory

Spontaneous locomotor activity and novel object recognition (NOR) were measured in an open-field box ($60 \times 60 \times 40$ cm) without DBS application. Briefly, one day after the complete extinction of morphine CPP, the rats were individually placed in the box for 5 min to habituate with it. On the following day, their horizontal movement in the arena was recorded using the video-tracking system (MazeRouter, Iran) for 5 min. NOR was measured on the next day in two stages. In the familiarization stage, the rats were presented with two identical objects placed in opposite corners of the box approximately 10 cm away from the walls and allowed to explore them for 5 min. Afterward, they were returned to the homecage and left undisturbed for 4 h, which served as the retention interval. In the choice stage, the rats were reintroduced to the box and presented with one familiar object and one novel object located at the same point of the box as in the familiarization stage. The locations of the familiar and novel objects were counterbalanced across the subjects. The time spent exploring each object was carefully recorded for 5 min. Exploration was defined as sniffing, nose poking, or simply directing the nose toward the object within a proximity of approximately 2 cm. Locomotor activity was calculated as the total distance traveled in the box in centimeters, and the recognition index was calculated as the time spent exploring the novel object to the time spent exploring both the novel and familiar objects.

Tissue sampling and c-Fos quantification

Since peak Fos protein expression occurs at 90–120 min after application of a stimulus[33], 1.5–2 h after the reinstatement test, the rats were euthanized. The process was carried out under deep anesthesia induced by an intraperitoneal (IP) injection of an overdose of chemical anesthetics. Specifically, 2–3 times the standard anesthetic dose was administered, consisting of ketamine (100 mg/kg) and xylazine (10 mg/kg). Once deep anesthesia was confirmed, the rats were decapitated using a rodent guillotine. All euthanasia procedures were performed by appropriately trained personnel in accordance with institutional guidelines and ethical standards for animal research. The brains were immediately removed and immersed in ice-cold PBS solution. The PFC and NAc were excised and snap-frozen in Nitrogen tank. The samples were transferred to -70 °C after 48 h. For measuring c-Fos protein levels, tissue samples were centrifuged at 4000 rpm for 10 min to extracted serum, rinsed with 1X PBS to remove the excess blood, homogenized in 20 mL of 1X PBS, and stored overnight at ≤ -20 °C. After performing two freeze-thaw cycles to break the cell membranes, the homogenates were centrifuged at 5000 g for 5 min. Supernatant was collected and c-Fos protein was measured using ELISA Kit (ABIN5592913, antibodies, Germany) according to the manufacturers' instruction. The optical density (O.D.) of each sample was determined using a microplate reader (Stat Fax 4200, Awareness Technologies Inc.) at a wavelength of 450 nm. The concentration of c-Fos in the samples was determined by comparing the O.D. of the samples to the standard curve.

Statistical analyses

Data is expressed as mean ± standard error of the mean (SEM). Two-way repeated measures analysis of variance (ANOVA) and Bonferroni follow-up tests were used for finding statistical difference in side preference. Kaplan-Meier test was employed for measuring time to extinction of morphine CPP. Between-group difference in locomotor activity and NOR was assessed by ordinary one-way ANOVA followed by Tukey's or Fisher's LSD tests. c-Fos expression data was analyzed by Kruskal-Wallis and Dunn's tests. A two-tailed $p < 0.05$ was considered statistically significant. Statistical assessment and plotting of the graphs were carried out in GraphPad Prism 9 (GraphPad Software, CA).

Results

DBS prevented the development of morphine CPP

Comparing the morphine- and saline-treated groups, a two-way repeated measures ANOVA revealed that there were significant main and interaction effects of time (preconditioning vs. postconditioning) and treatment (morphine vs. saline) on side preference [Time: F (1, 19) = 27.3, $p < 0.0001$; Treatment: F (1, 19) = 6.5, $p = 0.02$; Interaction: F (1, 19) = 12.5, $p = 0.002$]. In other words, morphine (3, 5, and 7 mg/kg, administered once daily for three days), but not saline, injection in the conditioning phase caused a significant strong preference for the drug-paired context (Fig. 2A). To assess whether DBS of the ACC suppresses the reinforcing properties of morphine, electrical stimulation was applied at 150 µA or 200 µA during the conditioning trials after morphine injection. Figure 2B depicts changes in side preference in sham-DBS and DBS groups over the entire behavioral course. In this part of the study, the post-conditioning test was repeated four times (once daily on days 5–8) to ensure a long-lasting effect of DBS. As shown in the figure, morphine administration to the sham-DBS rats resulted in a remarkable and sustained side preference. However, DBS at both 150 µA and 200 µA blocked the development of morphine CPP. To rule out the lack of side preference was resulted from state-dependent memory, a priming morphine injection (2 mg/kg) was given on day 10 immediately before the final CPP test. Although CPP was not expressed in 200 µA DBS group, it did express in sham-DBS and 150 µA DBS groups. Figure 2C depicts the mean side preference in each phase across different groups and provides statistical evidence for data in Fig. 2B. Two-way ANOVA showed significant main and interaction effects of time and treatment on side preference [Time: F (2, 50) = 24.07, $p < 0.0001$; Treatment: F (2, 26) = 7.53, $p = 0.0026$; Interaction: F (4, 50) = 7.79, $p < 0.0001$]. Pairwise-comparison with Bonferroni test revealed significant differences in the side preference between pre- and post-conditioning phases in sham-DBS group ($p < 0.0001$) and between pre-conditioning and reinstatement in sham-DBS ($p < 0.0001$) and DBS-150 ($p = 0.02$) groups (Fig. 1C).

DBS accelerated the extinction and blocked the reinstatement of morphine CPP

To examine whether DBS in the ACC also affects a previously established morphine CPP, it was applied in the extinction phase during the drug-free CPP trials (20 min, once daily). Figure 3A illustrates changes in side preference in sham-DBS and DBS groups over the entire behavioral course. As it is shown, morphine administration in the sham-DBS rats resulted in a remarkable and sustained side preference that lasted for 12 days. However, DBS at 150 µA and 200 µA shortened the extinction of morphine CPP by 8–10 days. To test the effect of DBS applied in the extinction phase on the subsequent reinstatement of morphine CPP, a priming morphine injection (2 mg/kg) was given four days after the last extinction session immediately before the final CPP test. Although CPP was reinstated in sham-DBS group, it was not reinstated in 150 µA and 200 µA DBS groups. Figure 3B depicts the mean side preference in each phase across different groups and provides statistical evidence for data in Fig. 3A. ANOVA test showed significant main and interaction effects of time and treatment on side preference [Time: F (2, 52) = 29.9, $p < 0.0001$; Treatment: F (2, 26) = 7.0, $p = 0.003$; Interaction: F (4, 52) = 3.54, $p = 0.01$]. Bonferroni test revealed significant differences in the side preference between the post-conditioning and extinction phases ($p < 0.0001$) and between the extinction and the reinstatement phases in sham-DBS group ($p < 0.001$). There were also significant differences in side preference between the post-conditioning and the extinction phases in DBS-150 ($p = 0.009$) and DBS-200 ($p < 0.0001$) groups. However, no significant difference was found between the extinction and reinstatement in the DBS-150 ($p = 0.3$) and DBS-200 ($p = 0.9$) groups (Fig. 2B), suggesting that ACC DBS effectively blocked the reinstatement of morphine CPP. A Kaplan-Meier test demonstrated that the DBS-150 and DBS-200 groups had significantly shorter survivals of morphine CPP (less time to full extinction) compared to the sham-DBS group (median survival: 2, 2.5, and 11 days, respectively; log-rank test: Chi square 23.59, $p < 0.0001$; Fig. 3C).

DBS restored locomotor activity and memory

To check if there was any adverse effect of DBS, we also measured novel object recognition (NOR) memory and locomotor activity and in a DBS-off state (after DBS treatment phase and before reinstatement test). Figure 4A and B show exploration time for familiar and novel objects in the familiarization and choice phases of the NOR test. Two-way repeated measures ANOVA and Bonferroni tests showed significant differences in the exploration time of the familiar and novel objects in all but sham-DBS group (Fig. 4B). An ordinary one-way ANOVA also showed a significant difference in NOR memory among the groups [F (5, 36) = 9.25, $p < 0.0001$]. Tukey's test indicated that morphine conditioning in sham-DBS group caused a significant reduction in recognition index compared to the saline group ($p < 0.0001$). However, all DBS groups had significantly increased recognition index compared to the sham-DBS group ($p < 0.0001$), indicating complete recovery of NOR memory (Fig. 4C).

In addition, there was an overall significant difference in distance traveled in the open field arena among the groups [ANOVA: F (5, 36) = 4.15, $p = 0.004$]. Fisher's LSD test revealed that locomotion was significantly decreased in sham-DBS ($p = 0.03$), DBS-150 C ($p = 0.03$) and DBS-150E ($p = 0.003$) groups compared to the saline group; however, it was restored to normal in DBS-200 C and DBS-200E groups ($p = 0.04$ and $p = 0.03$, respectively, compared to sham-DBS; Fig. 4D). It is worth mentioning that locomotor activity measured during the CPP test in the DBS-on state in either the conditioning or the extinction phase did not show any significant differences among the groups, indicating lack of potential interference of DBS with morphine reward (data not shown).

DBS reduced c-FOS expression in NAc and PFC

Since DBS at each intensity (150 µA and 200 µA) was effective in both the acquisition and the extinction phase of morphine conditioning, we pooled samples from both phases for c-Fos protein measurement. Kruskal-Wallis test showed that c-Fos protein expression was changed in the NAc ($p = 0.04$) and the PFC ($p = 0.003$). Dunn's test revealed that although there was no significant change in c-Fos expression in sham-DBS group compared

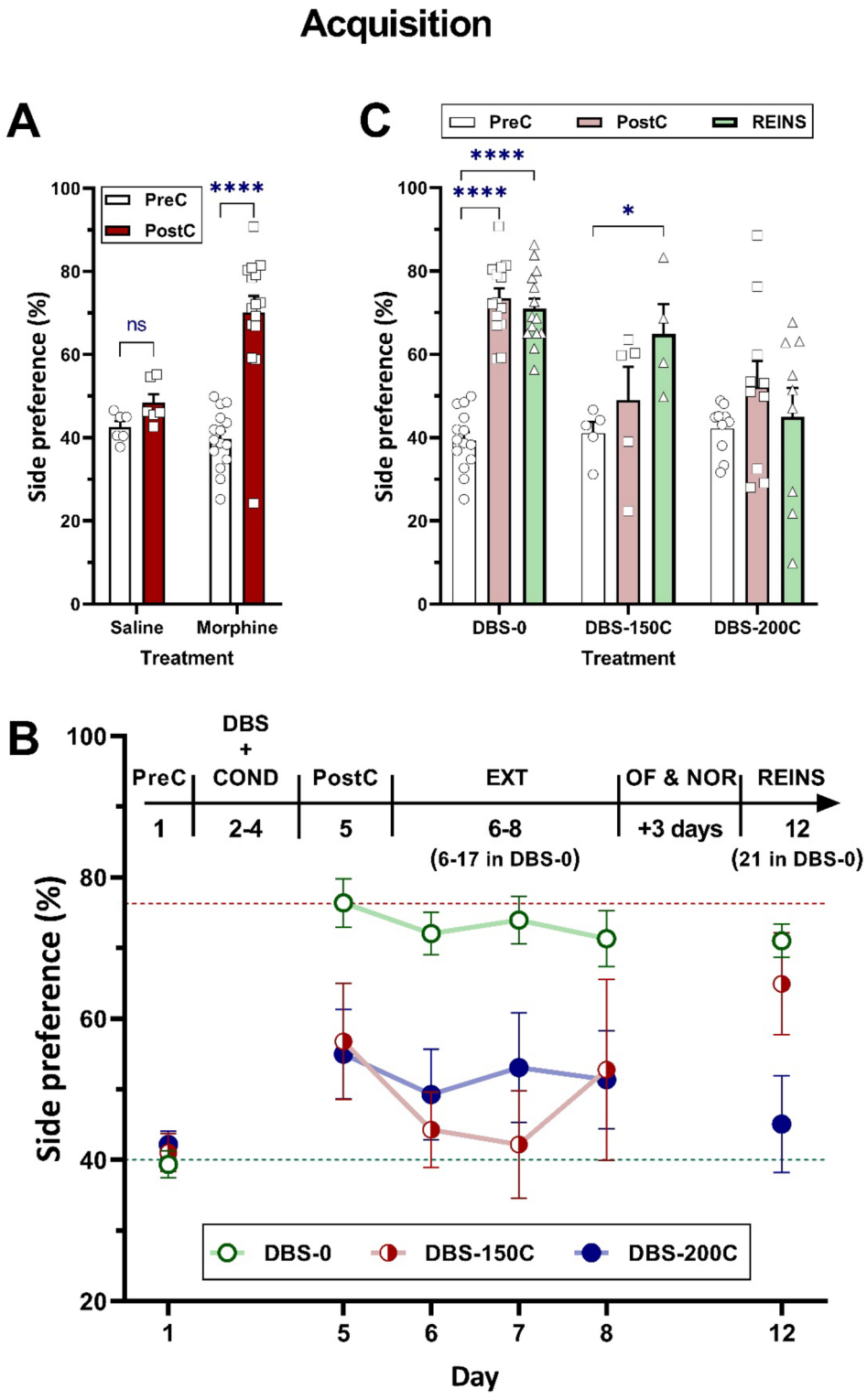

Fig. 2. The acquisition phase of CPP. (**A**) Administration of morphine (in sham-DBS group), but not saline, during the conditioning (COND) period resulted in a greater preference for the drug-paired chamber. (**B**) The alterations in the side preference observed in morphine-conditioned groups over the whole behavioral session. Administering morphine to rats that did not receive DBS (DBS-0) resulted in a noticeable and long-lasting preference for the drug-paired side. Nevertheless, DBS at both 150 µA and 200 µA effectively inhibited the formation of morphine CPP. Reinstatement (REINS) was tested on day 12 in DBS-150 C and DBS-200 C groups and on day 21 in DBS-0 group (see Fig. 3B; data in DBS-0 group are from the homonymous group illustrated in Fig. 3B) after three days of performing open field (OF) and novel object recognition (NOR) tests. (**C**) The average side preference in each phase across the groups that present statistical proof (2-way repeated measures ANOVA and Bonferroni's test) for the data shown in (**B**). Results are shown as mean ± SEM ($n = 6$ rats in Saline group, 14 in Morphine/DBS-0, 5 in DBS-150 C, and 10 in DBS-200 C). *$p < 0.05$, ****$p < 0.0001$.

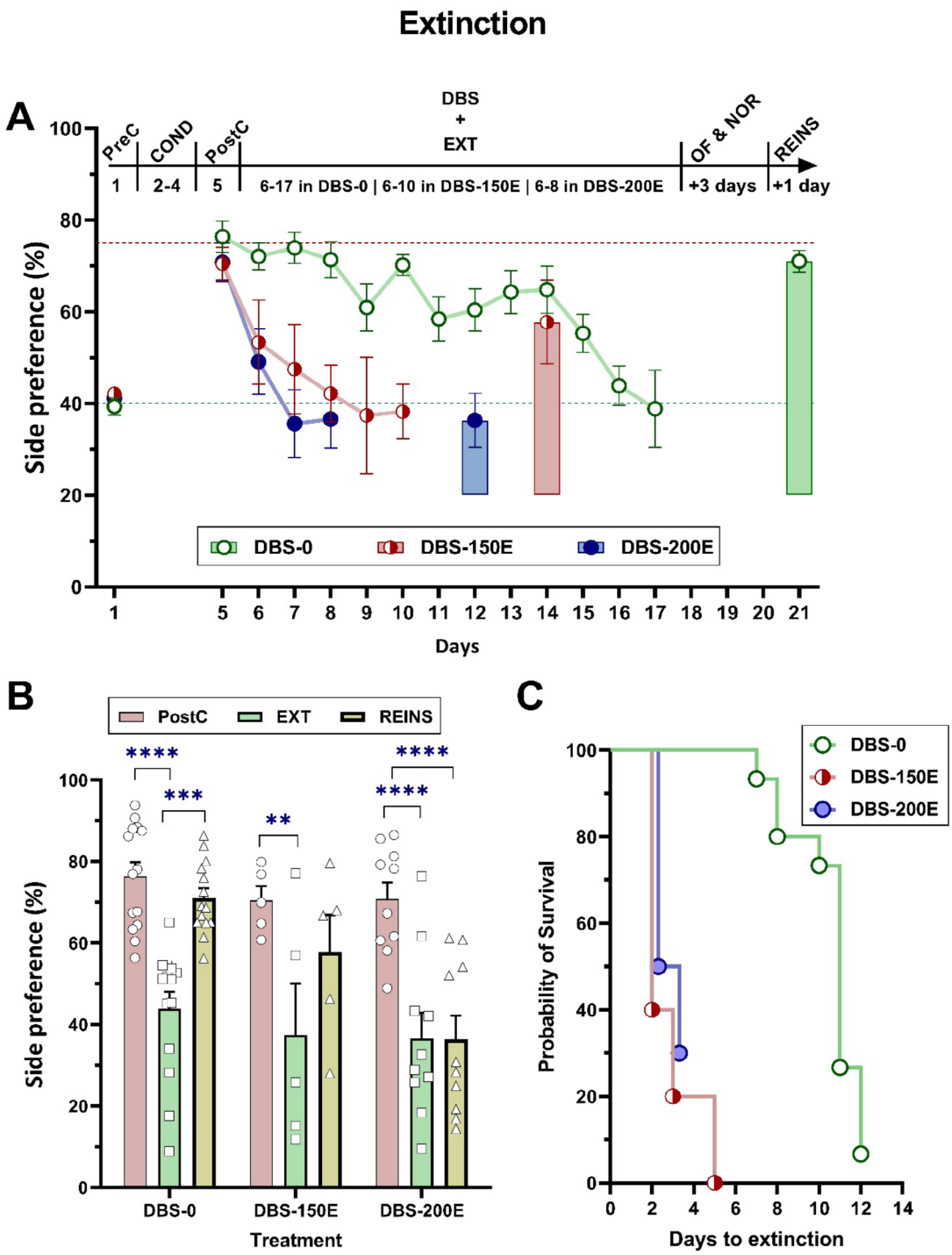

Fig. 3. The extinction phase of CPP. (**A**) Changes in side preference along the whole behavioral course. Bars represent reinstatement (REINS) test performed four days after the complete extinction (EXT) of morphine CPP. (**B**) The average side preference in each phase of CPP across the groups that present statistical proof (2-way repeated measures ANOVA and Bonferroni's test) for the data shown in (**A**). The bars for the extinction phase represent the last day of the phase when complete extinction occurred. The findings demonstrated significant differences in side preference between the post-conditioning (PostC) and the extinction phases in all groups, indicating complete extinction of morphine CPP. There was a significant difference in side preference between the extinction and reinstatement phases in the sham-DBS (DBS-0) group, but not in DBS-150E and DBS-200E groups, suggesting that ACC DBS successfully impeded the reinstatement of morphine CPP. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. (**C**) The Kaplan-Meier test demonstrated that the DBS-150E and DBS-200E groups had markedly reduced survival rates for morphine CPP (time to complete extinction) compared to the sham-DBS group (median survival: 2, 2.5, and 11 days, respectively, log-rank). Results are shown as mean ± SEM ($n = 14$ rats in DBS-0 group, 5 in DBS-150E, and 10 in DBS-200E).

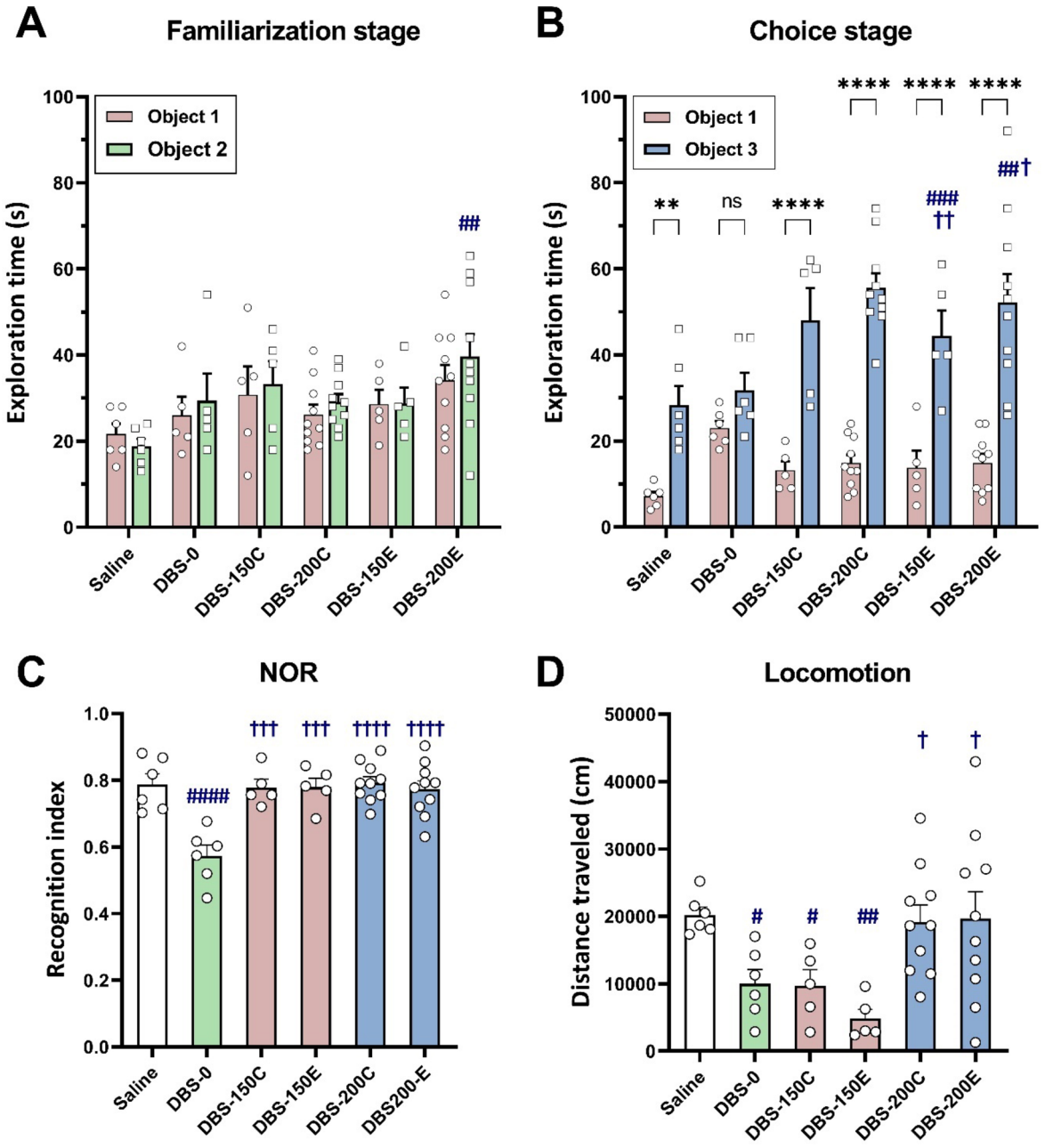

Fig. 4. Novel object recognition (NOR) and locomotor activity tests. (**A**) Exploration time of two identical objects (familiar objects 1 and 2) in the familiarization stage of the NOR test. (**B**) Exploration time of a familiar (Object 1) and a novel object (Object 3) in the choice stage of the NOR test. (**C**) Ordinary 1-way ANOVA and Tukey's tests showed that morphine conditioning in the sham-DBS group resulted in a significant reduction in the recognition index as compared to the saline group. Nevertheless, all DBS groups showed a significant improvement in the recognition index when compared to the sham-DBS group. This suggests that the DBS treatment led to a complete restoration of the NOR memory. (**D**) 1-way ANOVA and Fisher's LSD tests demonstrated significant decreases in spontaneous movement in the DBS-0, DBS-150 C, and DBS-150E groups when compared to the saline group. Nevertheless, DBS-200 C and DBS-200E groups had locomotor activity similar to that of saline group. Results are shown as mean ± SEM ($n = 6$ in Saline and DBS-0 groups, 5 in DBS-150c and DBS-150E groups, and 10 in DBS-200 C and DBS-200E groups). $^{**}p < 0.01$, $^{****}p < 0.0001$ within-group comparison; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ compared to the saline group; $^{\dagger}p < 0.05$, $^{\dagger\dagger\dagger}p < 0.001$, $^{\dagger\dagger\dagger\dagger}p < 0.0001$ compared to DBS-0 group.

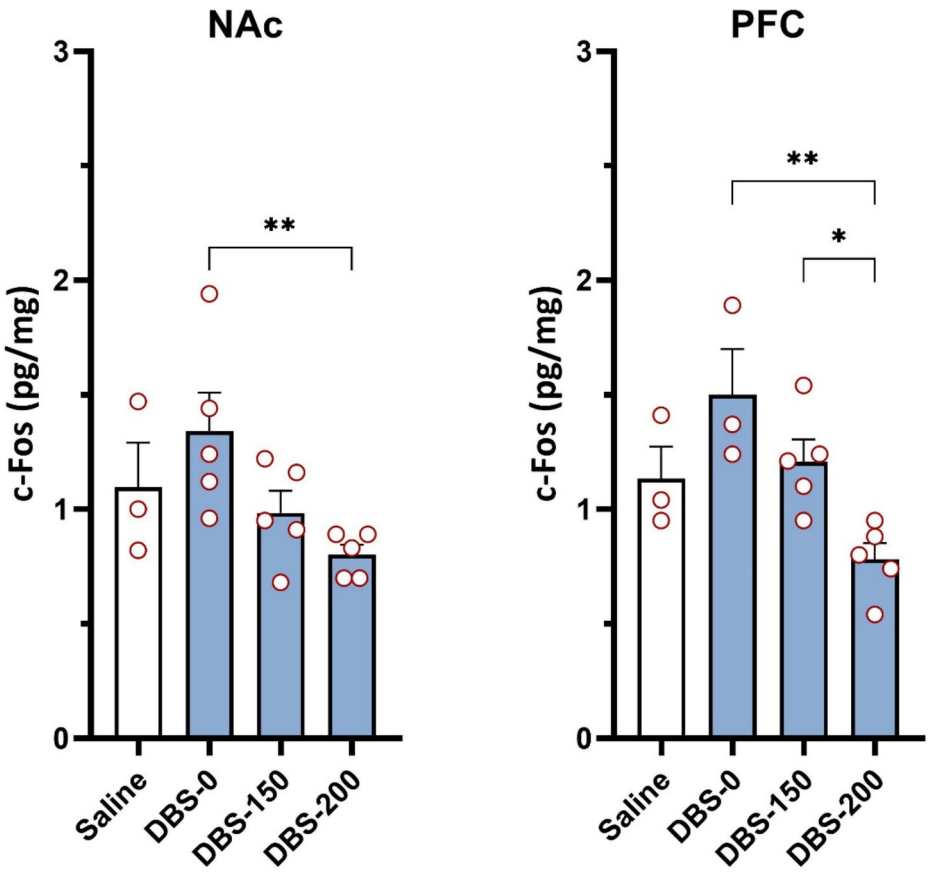

Fig. 5. ELISA analysis of c-Fos in the NAc and PFC. The findings indicated that there was an alteration in the expression of c-Fos protein in the NAc (**A**) and PFC (**B**). The results of Dunn's test indicated that there was no statistically significant alteration in c-Fos expression between the sham-DBS group and the saline group. However, the DBS-200 group exhibited a substantial reduction in c-Fos levels compared to the sham-DBS group. Results are shown as mean ± SEM ($n = 3–5$/group).

to saline group ($p = 0.36$ for NAc and $p = 0.26$ for PFC), DBS-200 led to significant decreases in c-Fos levels compared to the sham-DBS group ($p = 0.004$ for the NAc and $p = 0.005$ for the PFC). However, DBS-150 did not change c-Fos protein levels ($p = 0.12$ for NAc and $p = 0.37$ for PFC; Fig. 5). Taken together, our results also showed that ACC DBS significantly decreased c-Fos protein content in the NAc and the PFC compared to the morphine-conditioned rats that had not been treated with DBS.

Discussion

Here we demonstrate that high-frequency DBS)130 Hz (applied at different amperages, 150 µA and 200 µA in the ACC during the acquisition of morphine CPP blocked morphine reward as it compromised learning to associate subjective morphine effect with the context where morphine was administered. In addition, DBS at 150 µA and 200 µA applied in the extinction phase facilitated the extinction and (particularly at 200 µA) prevented subsequent drug priming-induced reinstatement of morphine CPP. Morphine conditioning was associated with impaired NOR memory and locomotor activity. While DBS at both intensities restored NOR memory, only DBS at 200 µA recovered locomotor activity. DBS at 200 µA also significantly reduced c-Fos protein levels in both the NAc and PFC.

The ACC plays a crucial role in addiction, as it is involved in motivation, attention, and error detection[34]. It is also implicated in emotional self-control, problem-solving, and adaptive responses to changing conditions[35]. All of these functions are implicated in the development and maintenance of addictive behaviors. Specifically, studies in SUD patients have shown that craving for cocaine was positively correlated with neural activity in, among other regions, the ACC in cocaine abusers[36]. A meta-analysis study showed that the ACC is abnormally hyperactivated by cue-induced craving for nicotine and cocaine and that activity in this region is correlated with self-reported craving in alcohol, nicotine, and cocaine-dependent patients[37]. Further, ACC activity in alcohol-dependent patients was higher in individuals who successfully abstain compared to those who relapse[16,38]. Animal studies have also implicated the ACC in drug addiction. For example, bilateral ACC lesions in rats resulted in a decrease in both the acquisition and retention of a learned preference for morphine solution[39]. Another study showed that bilateral laser destruction of the ACC reduced sensitivity to the positive reinforcing effects of morphine, delayed the development of dependence, and alleviated symptoms of the abstinence

syndrome in morphine-dependent animals, suggesting that this structure plays a key role in the development of physical dependence on morphine and abstinence syndrome[40]. Furthermore, both acute and chronic morphine treatment decreased the extracellular concentration of glutamate in the ACC, with the chronic morphine effect being reversible by naloxone, suggesting that glutamate neurotransmission in the ACC is involved in the central actions of morphine[41]. In addition, it has been shown that nonspecific inhibition of ACC neurons or selective inhibition of VTA-projecting neurons decreased oxycodone CPP, drug seeking, and spontaneous opioid withdrawal[42]. On the other hand, specific chemical lesions of the ACC failed to block morphine, cocaine, or amphetamine CPP, but did compromise CPP induced by CGP37849, an NMDA glutamate receptor antagonist[43].

Therefore, the ACC has been considered as both a central hub in addiction-related neural networks of cognitive functions and a potential therapeutic target for substance use disorder[16]. With the advent of DBS and other modes of neuromodulation, it is possible to effectively treat some intractable, severely addicted patients. Nevertheless, the available data for ACC DBS in the treatment of drug addiction is extremely rare.

The present study aimed to elucidate whether high-frequency (130 Hz) DBS at different amperages (150 μA and 200 μA) of the ACC may mitigate morphine addiction-like behavior in a rat model. CPP is a behavioral model widely used to study the association of an unconditioned rewarding stimulus with a specific context. This model has been particularly useful in studying the role of context associations in reward-related behaviors including drug use[44]. It has also been used to study relapse to opiates, with re-exposure to the drug producing the same preference even after the extinction of conditioning[45].

In this study, we selected 150 μA and 200 μA stimulation intensities for deep brain stimulation (DBS) based on evidence indicating that varying DBS intensities can differentially modulate neural circuits. High-frequency DBS in the range of 130 Hz has shown efficacy in modulating neural activity associated with addiction-related behaviors, while different current intensities may recruit distinct neural populations and produce variable therapeutic outcomes. Previous studies have suggested that higher stimulation intensities, such as 200 μA, often exert a greater effect on both neural circuits and behavior compared to lower intensities[46,47]. This intensity-dependent effect may explain why DBS at 200 μA produced more pronounced behavioral recovery in our study, specifically in locomotor activity and resistance to morphine reinstatement. Testing both 150 μA and 200 μA allowed us to evaluate the potential range of DBS effects, which is crucial for optimizing DBS parameters for addiction treatment. Our results demonstrate the intensity-dependent effects of DBS across different phases of the addiction cycle. At 150 μA in the acquisition phase, stimulation significantly inhibited the development of morphine CPP, as it was not expressed in the postconditioning tests (evidenced by a diminished time spent in the morphine-paired environment). In this set of experiments, the postconditioning test was repeated four times on different days to ensure the durability of the treatment. The results further confirmed lack of side preference over time and this also rules out a rebound effect of DBS or any latent morphine reward. Interestingly, however, side preference did emerge in the reinstatement test after priming morphine injection, indicating morphine reward had been registered in the brain during the conditioning but remained quiescent by DBS 150 μA and reactivated by morphine priming. On the other hand, at 200 μA in the acquisition phase, DBS blocked morphine CPP generation strongly enough to also prevent its reactivation by morphine priming.

Taken together, we have shown that DBS at 150 μA and 200 μA in the extinction phase accelerated the process as evidenced by a remarkably shorter time to full extinction of morphine CPP (2–3 days by DBS versus 11 days normally). In addition, DBS especially at 200 μA abolished drug priming-induced reinstatement of CPP.

Previous studies have shown that bilateral DBS of the ACC in human subjects suffering from refractory neuropathic pain resulted in mild to moderate improvements, in particular, in the affective component of pain with no major adverse effects[48]. There are also a few studies that targeted the ACC as a stimulation site to treat SUD. For example, in a case study, a double cone transcranial magnetic stimulation (TMS) coil was located over the medial PFC to stimulate the dorsal ACC in a 48-year-old woman. Results have shown that alcohol craving was dramatically decreased for three months after TMS sessions[49]. A similar protocol was then used in a larger study on 18 patients with alcohol use disorder that caused a significant decrease of self-reported alcohol craving and blood alcohol volume lasted for one-month post-stimulation[50]. In another study, 18 patients with cocaine use disorder were stimulated by TMS coil over the medial PFC and the ACC for three weeks. They were allowed to choose between cocaine self-administration and money at baseline (pre-TMS), after 4 days of TMS, and after 13 days of TMS. The results showed that the choices for cocaine significantly decreased by high frequency TMS[51].

There is only one study testing the effect of DBS of the ACC in alcohol use disorder. This feasibility study has shown that ACC implants may be effective in suppressing alcohol craving in individuals with severe alcohol use disorder[15]. In addition, there is one animal study with negative results about the ACC DBS. Guercio et al. have shown that DBS of the ACC delivered at 160 Hz, 150 μA, and 60 μs during the reinstatement session had no influence on cocaine reinstatement in a cocaine self-administration model[52]. Therefore, our study adds to the current data on the potential use of ACC DBS in the treatment of SUDs.

In another part of our study, we have shown that morphine conditioning reduced horizontal movement in the open field test, whereas DBS at 200 μA (but not at 150 μA) restored locomotor activity to normal levels, suggesting that higher-intensity stimulation may be more effective in improving locomotor activity. A recent study showed that a large population of ACC neurons exhibit phasic firing at the start or at the end of running on a spherical treadmill to drink water[53]. Therefore, by modulating this locomotor-associated activity in ACC neurons, DBS may improve locomotion deficits in morphine-treated rats. Additionally, morphine conditioning impaired recognition memory in NOR test, whereas DBS at both 150 μA and 200 μA fully restored it. Previous studies indicated ACC involvement in acquisition and expression of contextual fear memory[54] as well as retrieval of NOR memory[55]. Another study showed that parvalbumin interneurons in the ACC are critical for NOR memory[56]. Therefore, DBS may improve recognition memory deficits in morphine-treated rats by changing the activity of these neurons or overall functioning of the ACC area. Despite the observed improvements in memory recognition and locomotor activity with DBS, it is important to acknowledge a limitation of our study that is the

lack of a saline + DBS control group. This makes it unclear whether the observed improvements are specifically due to the reversal of morphine-induced deficits or represent a general effect of ACC DBS on locomotor activity and recognition memory even in animals with no impairment.

Our study also found that DBS significantly reduced c-Fos expression in the NAc and PFC, regions critical for reward processing. Previous studies have shown that morphine inducted c-Fos and JunB in neurons of the NAc[30]. The protein products of these immediate-early genes form an AP-1 complex that cause persistent changes in the expression of downstream genes[57,58]. c-Fos expression in the NAc appears to be necessary for the acquisition of morphine CPP[29]. In addition, extinction of morphine CPP has been shown to be associated with a decrease in c-Fos expression in the NAc core[59]. Therefore, our finding that c-Fos content in the NAc was decreased after the extinction of morphine CPP by DBS is consistent with previous results. It has been shown that disconnection of the ACC and the NAc core in rats disrupted learning to associate between a conditioned stimulus and food reward[60]. The ACC provides major glutamatergic input to the NAc and inhibition of these projection was associated with CPP induced by pain-relief[61]. In addition, glutamate NMDA receptor antagonists that block the development of a morphine CPP[62,63] also block c-Fos induction in the NAc[30]. Therefore, DBS-driven decreases in the NAc c-Fos may reflect an accelerated inhibition of neural activity in the ACC that diminished glutamate release in the NAc and promoted extinction of morphine reward.

Previous studies have shown that although a single morphine administration did not change c-Fos level in the PFC of rats[64], morphine CPP was associated with an increase in c-Fos expression in the PFC[65] including the cingulate subregion[66]. Our results suggest that, by decreasing the PFC c-Fos content, DBS have impaired CPP induction after morphine conditioning.

Conclusion

In conclusion, our data indicates an intensity-dependent effect of ACC DBS on the acquisition, extinction, and reinstatement of morphine-induced CPP in rats. These findings provide further insight into DBS modulation of addiction-related neural circuits. Further investigation of optimal stimulation parameters in different animal models may increase the potential for translation of preclinical DBS studies for treating substance use disorder in human patients.

Data availability

The data generated and/or analyzed during the current study are not publicly available for legal/ethical reasons but are available from the corresponding author on reasonable request.

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Author contributions
E.R., G.H., and M.F. (Maryam Farahmandfar) developed the idea and designed the experiments. M.F. (Mahdi Fatemizadeh) and F.R. conducted the experiments. M.F. (Mahdi Fatemizadeh) and A.T.B. analyzed the data. M.F. (Mahdi Fatemizadeh) wrote the first draft. All authors confirmed the final manuscript before the submission and agreed to the published version of the manuscript.

Declarations

Competing interests
The authors declare no competing interests.

Additional information
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