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Linalool restores post-morphine hippocampal-striatal PI3K/Akt and CREB/ΔFosB mRNA expression and LFP power in adult male rats

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ABSTRACT

Objective(s): Opioid use disorder (OUD) is a chronic, relapsing condition characterized by compulsive drug seeking and profound abstinence-related disturbances. Opioid receptors are distributed across reward-related regions, including the ventral tegmental area (VTA), the ventral striatum (VS), and the hippocampus. Key mediators—PI3K-Akt in the hippocampus and CREB/ΔFosB in the striatum—modulate opioid addiction along the hippocampal–VS axis.

Materials and Methods: Rats underwent conditioned place preference (CPP) with three phases: pre-conditioning, conditioning, and post-conditioning. During conditioning, animals received morphine (10 mg/kg, IP) for three days, with or without linalool (50 mg/kg, IP, 30 min prior to morphine). This three-day protocol models reward learning rather than chronic dependence. Groups: saline, morphine, and morphine+linalool. Behavioral tests assessed CPP score, passive avoidance memory, anxiety- and depression-like behaviors, and locomotion. Molecular analyses measured PI3K and Akt mRNA expression (real-time PCR) in the hippocampus and CREB/ΔFosB in the VS. Local field potentials (LFPs) were recorded directly from the hippocampal CA1 and the nucleus accumbens (NAc, part of the VS) via implanted electrodes.

Results: Linalool significantly attenuated morphine-induced CPP, improved passive avoidance memory, and reduced anxiety/depressive-like behaviors. Linalool decreased morphine-induced up-regulation of CREB/ΔFosB in VS and PI3K/Akt mRNA in the hippocampus. LFP power in CA1 and NAc was reduced by morphine and restored by linalool.

Conclusion: Linalool attenuated morphine-induced reward, memory deficits, and affective disturbances, which are key features of opioid abstinence-induced behavioral disturbances, and normalizes molecular and electrophysiological alterations in the hippocampal-striatal circuit. Although direct causality is not established, linalool is a promising natural compound for managing opioid-associated behavioral disturbances.

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Introduction

The reward network is a complex system that plays an important role in reward processing, motivation, and the reinforcement of behaviors. Dysregulation of this network is associated with addiction, anxiety, depression, and other psychological disorders. The core reward circuitry primarily comprises the ventral tegmental area (VTA) and its projections to the nucleus accumbens (NAc) within the ventral striatum (VS). However, this network extensively interacts with other brain regions—including the hippocampus, prefrontal cortex (PFC), hypothalamus, and amygdala—which modulate reward processing by providing contextual, emotional, and cognitive inputs. The ventral striatum, including the NAc, is rich in gamma-aminobutyric acid (GABA) medium spiny neurons (approximately 95%), which receive dopaminergic projections from the VTA and glutamatergic afferents from the hippocampus, PFC, and amygdala. It is important to clarify that, while VTA dopaminergic neurons can co-release GABA in certain contexts, broadly characterizing them as "inhibitory" mischaracterizes their primary function; their main effect in the NAc is to modulate glutamatergic inputs and influence postsynaptic excitability (1, 2). A prominent excitatory glutamatergic input to the ventral striatum originates from the CA1/subiculum of the hippocampus through the hippocampal–ventral striatal circuits, which are involved in the modulation of reward-related behavior, particularly in the context of contextual memory and reinforcement (1, 3, 4). Opioid drugs, such as morphine, exert their effects through modulation of the reward network via several neurotransmitter systems, including GABA, glutamate, and dopamine (5, 6). Morphine predominantly acts through μ-opioid receptors, which are widely distributed in the

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NAc, VTA, and hippocampus (6). In the VTA, activation of μ-opioid receptors on GABAergic interneurons inhibits GABA release, thereby disinhibiting dopaminergic neurons. This disinhibition allows these neurons to fire, leading to dopamine release in both the NAc (which mediates reward-related behaviors) and the hippocampus (which facilitates the consolidation of memory related to drug-associated contexts) (7, 8). It is well established that the ventral striatum plays a key role in the reinforcing effects of addictive drugs (9).

The transition from voluntary, recreational drug use to compulsive substance abuse is a hallmark of addiction. This process gradually reduces an individual's voluntary control over drug use, transforming it into a habit through stimulus–response (S–R) mechanisms, ultimately leading to compulsive usage that cannot be easily relinquished (9, 10). In animals, the conditioned place preference (CPP) test is a common behavioral paradigm that evaluates the rewarding properties of drugs (such as opiates) by assessing an animal's preference for an environment previously paired with drug administration (3). Several mediators and signaling pathways are involved in CPP reinforcement within the reward network. The hippocampus is considered a primary structure for learning and memory acquisition in the morphine-induced CPP model. In this way, μ-opioid receptors in the hippocampus directly mediate morphine CPP through several downstream targets, such as the phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinases (MAPK) signaling pathway (6). In the present study, PI3K and Akt mRNA expression levels were measured in hippocampal tissue using real-time PCR (as described in Methods). Phosphatidylinositol 3-kinase (PI3K) and its downstream target, Akt, are critical molecules involved in the learning and memory of morphine CPP through their roles in cell growth, synaptic plasticity, and long-term potentiation (LTP) in the hippocampus (6). A well-established molecular correlate of drug-induced neuroplasticity is the induction of transcription factors. Fos-family proteins (c-Fos, FosB, Fra1, and Fra2) are rapidly and transiently induced in the NAc after acute drug exposure. In contrast, ΔFosB, a highly stable splice variant, accumulates in the NAc after chronic drug exposure, but not after acute exposure. Therefore, ΔFosB is believed to mediate many of the long-lasting synaptic plasticity changes and adaptations that persist even after weeks of withdrawal (11, 12). Two transcription factors, cAMP response element-binding protein (CREB) and serum response factor (SRF), are necessary upstream regulators for ΔFosB induction, as they bind to the fosB gene promoter in the striatum or NAc (13-15). In fact, ΔFosB is hypothesized to act as a "molecular switch" that contributes to the transition from voluntary drug use to compulsive use (13). Consistent with this, chronic exposure to virtually all drugs of abuse, including opiates, induces ΔFosB in the NAc (16).

Cessation of chronic opiate use precipitates a withdrawal syndrome, characterized by both physical and affective symptoms, including lowered mood, anhedonia, decreased motivation, anxiety, and depression (17, 18). Both spontaneous and naloxone-precipitated withdrawal produce anxiety- and depressive-like behaviors in animal models. However, the present study focused on opioid-associated behavioral disturbances during early abstinence following a brief conditioning period, rather than on a full withdrawal syndrome induced by chronic dependence (see Discussion). These negative affective symptoms are key drivers of relapse, as they motivate individuals to resume opiate use to alleviate this negative state (19, 20). It has been found that approximately 11 proteins are differentially expressed in the hippocampus during morphine withdrawal, with roles in synaptic transmission, signal transduction, chaperone function, energy metabolism, and cytoskeletal structure (17).

Regarding pharmacological therapy for opioid dependence, recent research has highlighted the potential efficacy of natural products in managing withdrawal symptoms and reducing drug-seeking behavior (21). Among these, linalool (3,7-dimethyl-1,6-octadien-3-ol) is an acyclic monoterpene abundant in lavender essential oils (above 94%) and found in numerous plant families. Linalool exhibits a wide range of pharmacological properties, including anxiolytic, antidepressant, anticonvulsant, and analgesic effects (22-24). —all of which are particularly relevant to treating the negative affective symptoms of opioid withdrawal. These therapeutic effects are mediated through modulation of several neurotransmitter systems (including GABA, glutamate, and nitric oxide) and signaling pathways such as ERK, Nrf-2, PI3K/Akt/mTOR, and HMGB1/TLR4/NF-κB. Pharmacokinetically, linalool has a short half-life (approximately 13 min in the aqueous phase and 3 hr in the lipid phase) and poor bioavailability, with intraperitoneal LD50 values in rodents ranging from 200 mg/kg to 2864 mg/kg. These properties highlight the need for novel delivery systems to enhance their therapeutic potential (25-27).

Given linalool's modulatory effects on neurotransmitter systems implicated in addiction—particularly its interaction with GABA and glutamate signaling in brain regions such as the hippocampus and NAc—it represents a promising candidate for further investigation in opioid use disorder models.

Materials and Methods

Chemicals

Forty adult male Wistar rats (220 ± 20 g) were kept under standard conditions (12-hour light/dark cycle, room temperature of 23 ± 2 °C, humidity at 60%, and ad libitum access to food and tap water) in accordance with the guidelines of the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, located in Ahvaz, Iran (ethics code: IR.AJUMS.ABHC.REC.1401.071). Chemicals, including morphine and linalool, were purchased from Sigma-Aldrich Co. (Sigma-Aldrich, St. Louis, MO, USA).

Animals

The rats were randomly divided into three groups: Sham group: The sham group received the vehicles for both morphine and linalool over a period of three days; (2) Morphine-exposed group (Morphine): This group received morphine at a sub-lethal dose (10 mg/kg/1 ml, S.C., vehicle: saline, for 3 days); and (3) Pre-treatment group: The morphine-exposed group was pretreated with linalool at a sub-sedative dose (50 mg/kg/10 ml, IP, vehicle: polyethylene glycol/saline (1:1), for 3 days) with an interval of 30 min (21, 28, 29).

Following the CPP test on day 5, animals were randomly divided into two cohorts. The first cohort (molecular assessment group, n=5) was immediately euthanized on day 5; hippocampal and ventral striatal tissues were collected and

stored at -80°C for subsequent gene expression analysis by real-time PCR. This time point was selected to capture acute molecular changes immediately after morphine withdrawal, prior to any behavioral testing that could confound gene expression profiles. This time point was selected to capture acute molecular changes immediately after the last morphine injection (during early abstinence), prior to any behavioral testing that could confound gene expression profiles. As the study used a three-day conditioning protocol, it does not model chronic opioid dependence or a classic withdrawal syndrome.

Days 610: The second cohort underwent extinction training (free access to all chambers without drug injection). Behavioral tests (anxiety, depression, locomotion, n=6) were performed on days 68, and electrophysiological recordings (LFPs) on days 910 during ongoing extinction. LFPs were recorded in the hippocampal CA1 and nucleus accumbens (NAc) region of the ventral striatum. After recordings, these animals were also euthanized, and tissues were collected to confirm the persistence of molecular changes during early abstinence (Figure 1).

Conditioned place preference test (CPP)

To perform the CPP test, rats were placed in a special CPP box in three stages: pre-conditioning, conditioning, and post-conditioning. The CPP box consists of three separate sensory chambers. The conditioning chambers are distinguished by the color of the walls and the material of the floor (visual and tactile stimuli). One side chamber has a black-and-white horizontal-striped interior wall and a shiny mesh floor. The opposite-side chamber features a white interior wall and a black fine-mesh iron floor. The central gray chamber contains a black iron plate. Sliding doors separate each chamber from the central chamber.

In the pre-conditioning phase (first day), rats were placed in the central gray chamber and allowed free access to all three chambers for 15 min. The time spent in each chamber to assess biased design was recorded with a stopwatch, after which the animals were returned to their cages. Any rat showing a strong innate place preference (more than 9 min) was removed from the experiment (exclusion criteria).

In the conditioning phase (from the second to the fourth day), the rats were injected with morphine and saline in the morning and evening. Specifically, the rats that received morphine in one chamber in the morning were injected with normal saline in the opposite chamber in the evening and were kept in that chamber for 30 min. On the next day, saline was injected in the morning and morphine in the evening. The order of morphine and saline injections was balanced among the groups.

In the post-conditioning phase (fifth day), the CPP test was performed by placing each animal in the central gray chamber and allowing free access to all three chambers. The time spent in each chamber was recorded with a camera. The CPP score was defined as the difference in time spent in the reward-paired chamber between the post-conditioning and pre-conditioning phases (post minus pre) (30).

Behavioral test

After the post-conditioning phase (Days 6-8), groups were tested for behavioral assessments in the following order to minimize the influence of each other's results, including:

- Spontaneous locomotor activity test

The open-field (OF) apparatus is a black Plexiglas box divided into 25 squares on the floor. Locomotion indices, including total visit count (zone), distance traveled (cm), and speed (cm/s), were evaluated for each rat during a 5-minute observation period. Spontaneous locomotor activity is measured after a habituation session to reduce novelty-induced bias.

- Elevated plus maze test of anxiety (EPM)

This apparatus consists of a central platform elevated above the floor, with two open arms and two closed arms. For anxiety assessment, rats were placed in the central portion facing the open arm and observed for 5 min. Indices such as %OAT (percentage of open arm time) and %OAE (percentage of open arm entries) were measured.

- Passive avoidance test

Initially, rats were placed in the illuminated chamber of a shuttle box apparatus and allowed to move freely between the chambers for 10 min without any electrical shock. The next day, as on the previous day, upon entering the dark chamber, the sliding door closed, and a single electrical shock (50 Hz, 0.2 mA, 3 s) was delivered through the stainless-steel bars on the floor. Rats were kept in the dark chamber for 2 min for a consolidation period. On the third day, the delay in

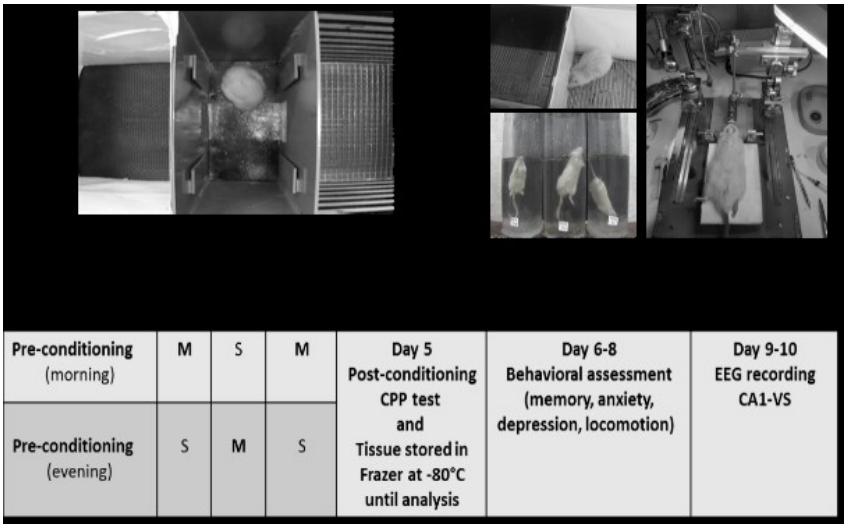

Figure 1. Experimental design timeline for morphine preconditioning, conditioning, and post-conditioning protocol with behavioral and molecular assessments

transitioning from the illuminated chamber to the dark chamber was considered the step-through latency, with a maximum of 300 sec.

- Forced swimming test of depression (FST)

A glass cylindrical swim tank (45 cm × 20 cm) filled with tap water was used to assess depressive behaviors. On the first day, rats were allowed to swim for 10 min. On the test day, the total immobility time (floating on the water) was recorded for 6 min as an indicator of depressive state (31, 32).

Real-time PCR

On day 5, after euthanasia, tissues (hippocampus and ventral striatum) were dissociated and stored in a Frazer -80°C freezer until gene expression analysis via real-time PCR. Briefly, the optical absorption ratio of the extracted RNA (5 microliters) at 260/280 nm was measured using a Nanodrop device (Pishro Pajoohesh spectrometer, Iran) to assess RNA quality. Following this, cDNA synthesis was initiated from the RNA using Takara's cDNA synthesis kit according to the manufacturer's instructions.

In the next step, real-time PCR was used to convert cDNA into mRNA. For this purpose, duplicate final volumes of 12.5 µl of amplicons were prepared, consisting of: 0.5 µl specific primers (Table 1), 6.25 µl SYBR Green qPCR Master Mix (2x), 3 µl template cDNA, 1 µl ROX dye, 2.75 µl RNase-free water. Amplicons were placed in a RunMei Q200 (China) real-time PCR machine for a total of 50 cycles, which included initial heat activation (5 min at 94 °C), denaturation (15 sec at 94 °C), annealing (15 sec at 60 °C), and extension (30 sec at 72 °C). Melting curve analysis was performed to ensure the specificity of the PCR products. Relative quantification of gene expression was performed using the comparative threshold cycle (Ct) method with the RunMei QC3.2 software and the 2-ΔΔCt formula (33).

Electrophysiology (Local field potential recording)

For the recording of local field potentials (LFPs) from the CA1 region of the hippocampus and the NAc region of the ventral striatum, rats were anesthetized with ketamine/xylazine (90/10 mg/kg), and their skulls were fixed in a stereotaxic apparatus. In the next step, a stainless-steel bipolar metal wire electrode (stainless steel, Teflon-coated, 0.005" bare, 0.008" coated; A-M Systems, Inc., WA, USA) was implanted in holes with a diameter of 1.0 mm in the skull above the targeted regions, based on the stereotaxic map established by Paxinos and Watson. The electrode was fixed with dental cement and two small steel screws.

The coordinates for the hippocampus CA1 region were: anterior-posterior (AP): -3.4 mm, mediolateral: ±1.5 mm, and dorso-vertical (DV): 4.4 mm. For the ventral striatum NAc region, the coordinates were: AP = +1.6 mm, anterior to bregma, ML = 2.0 mm, and DV = −4.5 mm, relative to the surface of the skull bone. After a recovery period, LFPs from the CA1 and NAc of anesthetized rats were recorded using a 4-channel PowerLab system, LabChart software (version 7), and an ML-135 bioamplifier (AD Instruments, 1Australia) with an amplification of 1 mV. Sample recordings were conducted with band-pass filtration set to 0.3-70 Hz for 5 sec. The raw LFP signal (0.3-70 Hz) and its specific frequency band power changes—gamma (30-70 Hz), beta (12–30 Hz), alpha (8–12 Hz), theta (4–8 Hz), and delta (0.5–4 Hz)—were measured in $\mu V^2/Hz$ during three 5-second periods (4, 34).

Statistical analysis

Data were analyzed using a parametric one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test (mean±SEM), with a significance level set at $P<0.05$ in GraphPad Prism 8 software (San Diego, USA). A Kolmogorov-Smirnov normality test was conducted to assess the data's normality.

Results

Conditioned place preference test (CPP)

A comparison of the CPP score revealed a significant increase in the morphine exposed group compared to the control group ($P<0.001$). In this context, pretreatment with linalool significantly reduced morphine-induced CPP in male rats ($P<0.0$) (Figure 2).

Behavioral test

Passive avoidance memory: Figure 3A illustrates the protective effects of linalool pretreatment on step-through latency (STL) in rats injected with morphine. The time taken to enter the dark box (in seconds) significantly decreased in the morphine-exposed group compared to the

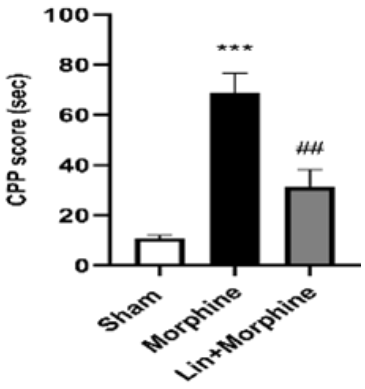

Figure 2. Effect of linalool on the conditioned place preference (CPP) test in the 3 main rat groups:
Control (Sham), morphine, and linalool (Lin: 50 mg/kg/10 ml, IP, 3 days), which was injected 30 min before the administration of morphine (10 mg/kg/1ml, SC, 3 days). One-way ANOVA, followed by Bonferroni's *post hoc* test for multiple comparisons, was used for the analysis. Data is presented as mean±SEM (n=6); *$P<0.05$, **$P<0.01$,

Table 1. Forward and reverse primers for Real-Time PCR (RT-PCR)

GENE	Forward primer	Reverse primer	GeneBank Accession NO
GAPDH	CAGCCTCAAGATCATCAGCAATG	CATGAGTCCTTCCACGATACCA	NM_002046.7
PI3K	GAATGCAACTGCCTTGCACA	CTTTTTCCCACCACGACTTGAT	NM_053481.2
AKT	TTCAGACTGTGGCTGATGGA	AAACTCGTTCATGGTCACACG	NM_033230.3
CREB	CATACCAGATTCGCACAGCA	TCTTGCTGCTTCCCTGTTCT	NR_003141.4
ΔFosB	AGGCAGAGCTGGAGTCGGAGAT	GCCGAGGACTTGAACTTCACTCG	NM_001256509.1

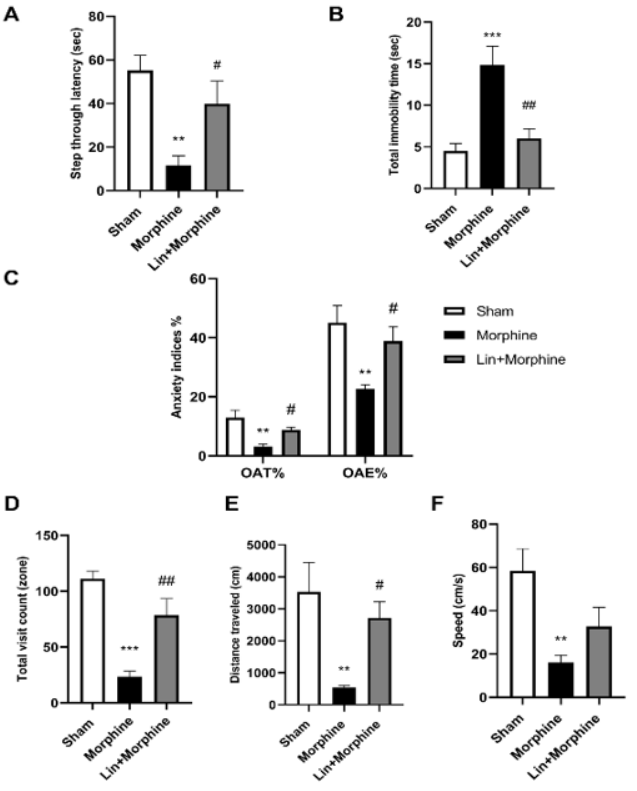

Figure 3. Effect of linalool on step through latency in the rat shuttle box test (A), total immobility time in the FST (B), anxiety indices in the EPM (C), and locomotor activity in the open field (D-F), in the 3 main groups: control (Sham), morphine and linalool (Lin: 50 mg/kg/10 ml, IP, 3 days), which was injected 30 min before the administration of morphine (10 mg/kg/ml, SC, 3 days)

One-way ANOVA, followed by Bonferroni's *post hoc* test for multiple comparisons, was used for the analysis. Data is presented as mean±SEM (n=6-8); **P*<0.05, ***P*<0.01, ****P*<0.001 vs sham and #*P*<0.05, ##*P*<0.01, ###*P*<0.001 vs morphine group.

FST: Forced swim test; EPM: Elevated plus maze; SC: Subcutaneous injection

sham group (*P*<0.01). Administration of linalool (50 mg/kg) significantly increased the STL parameter compared to the morphine-exposed group (*P*<0.05).

Depression: Figure 3B shows that morphine injection significantly increased immobility time in the forced swim test (FST) compared to the sham group (*P*<0.001). In contrast, pretreatment with linalool significantly decreased depressive behavior in the FST when compared to the morphine-exposed group (*P*<0.01).

Anxiety: As illustrated in Figure 3C, anxiety indices, including OAT% and OAE%, were significantly decreased in the morphine-exposed group (*P*<0.01). In contrast, linalool pretreatment significantly increased these anxiety indices compared to the morphine-exposed group (*P*<0.05).

Locomotor Activity: Locomotor activity (Figure 3D-F) was also significantly reduced in morphine-exposed rats during the extinction phase, which included total zone visit count (*P*<0.001), distance traveled (*P*<0.01), and speed (*P*<0.01). However, pretreatment with linalool increased locomotor indices, with significant improvements in zone visit count (*P*<0.01) and distance traveled (*P*<0.05) (Figure 3).

Real-time PCR (hippocampus)

One-way ANOVA revealed a significant increase in the fold change in PI3K and Akt mRNA expression in the hippocampus of morphine-exposed rats compared to the sham group (*P*<0.001 and *P*<0.01, respectively). As shown in Figure 4, pretreatment with linalool (50 mg/kg) significantly decreased the mRNA expression of PI3K and Akt in comparison to the morphine-injected rats (*P*<0.01, *P*<0.05, respectively) (see Figure 4).3.4. Real-time PCR (ventral striatum)

The mRNA expression levels of the CREB and ΔFosB genes in the ventral striatum tissue showed a significant increase in the morphine-exposed group compared to the control group (*P*<0.001 and *P*<0.01, respectively). According to one-way ANOVA analysis, pretreatment with linalool significantly reduced the mRNA expression levels of CREB and ΔFosB genes compared to the morphine-exposed group (*P*<0.05) (Figure 5).

Electrophysiology (CA1 region from hippocampus)

As shown in Figure 6 A-F, the total LFP power in the CA1 region significantly decreased in the morphine-exposed group compared to the sham group (*P*<0.001). Linalool pretreatment significantly increased LFP power compared to the morphine-exposed group (*P*<0.01). In the analysis of the separated frequency bands, all wave types in the morphine-exposed group showed a significant

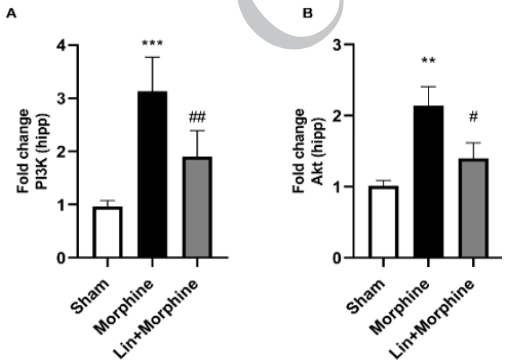

Figure 4. Effect of linalool on the mRNA expression of PI3k (A) and Akt (B) genes in hippocampus in the 3 main rat groups: control (Sham), morphine and linalool (Lin: 50 mg/kg/10 ml, IP, 3 days), which was injected 30 min before the administration of morphine (10 mg/kg/ml, SC, 3 days)

One-way ANOVA, followed by Bonferroni's *post hoc* test for multiple comparisons, was used for the analysis. Data is presented as mean±SEM (n=5); **P*<0.05, ***P*<0.01, ****P*<0.001 vs sham and #*P*<0.05, ##*P*<0.01, ###*P*<0.001 vs morphine group.

SC: Subcutaneous injection

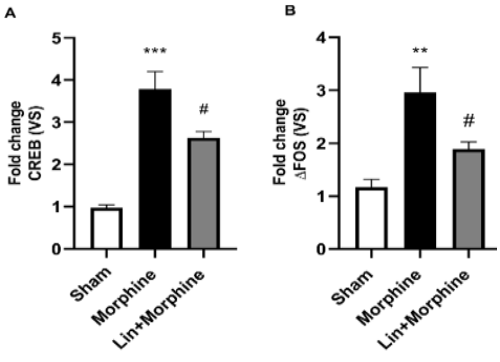

Figure 5. Effect of linalool on the expression of CREB (A) and ΔFosB (B) genes in the ventral striatum in the 3 main groups: control (Sham), morphine and linalool (Lin: 50 mg/kg/10 ml, IP, 3 days) which was injected 30 min before the administration of morphine (10 mg/kg/ml, SC, 3 days).

One-way ANOVA, followed by Bonferroni's *post hoc* test for multiple comparisons, was used for the analysis.

Data is presented as mean±SEM (n=5); **P*<0.05, ***P*<0.01, ****P*<0.001 vs sham and #*P*<0.05, ##*P*<0.01, ###*P*<0.001 vs morphine group.

CREB: cAMP response element-binding protein; SC: Subcutaneous injection

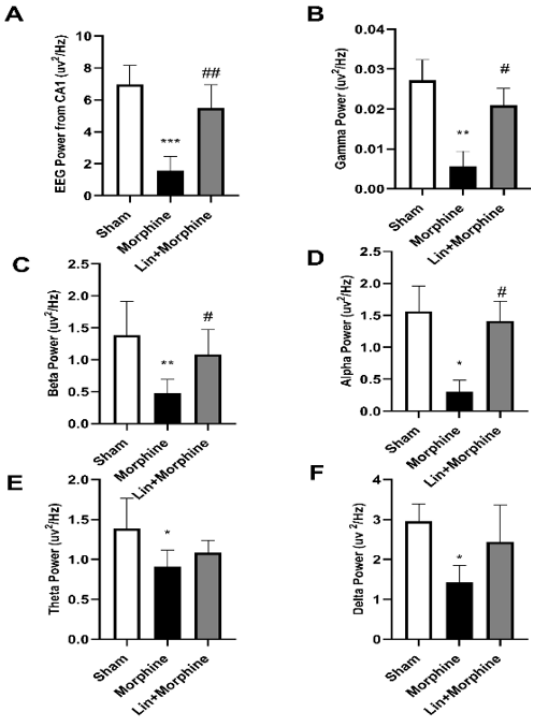

Figure 6. Effect of linalool on electrical waves from CA1 region of hippocampus including total LFP power (A), gamma (B), beta (C), alpha (D), theta (E) and delta (F) in the 3 main rat groups: control (Sham), morphine and linalool (Lin: 50 mg/kg/10 ml, IP, 3 days) which was injected 30 min before the administration of morphine (10 mg/kg/ml, SC, 3 days).
One-way ANOVA, followed by Bonferroni's *post hoc* test for multiple comparisons, was used for the analysis. Data is presented as mean±SEM (n=6); **P*<0.05, ***P*<0.01, ****P*<0.001 vs sham and #*P*<0.05, ##*P*<0.01, ###*P*<0.001 vs morphine group.
IP: Intraperitoneal injection; SC: Subcutaneous injection

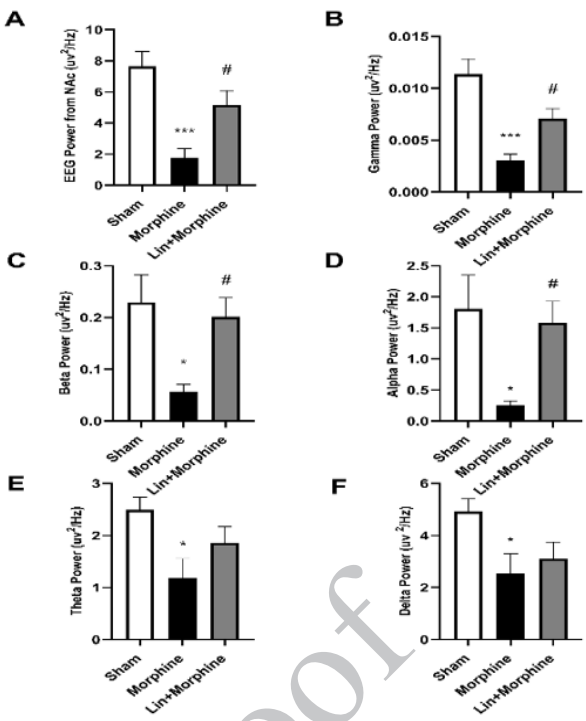

Figure 7. Effect of linalool on electrical waves from the nucleus accumbens region of ventral striatum (NAc) including total LFP power (A), gamma (B), beta (C), alpha (D), theta (E) and delta (F) in the 3 main rat groups: control (Sham), morphine and linalool (Lin: 50 mg/kg/10 ml, IP, 3 days) which was injected 30 min before the administration of morphine (10 mg/kg/ml, SC, 3 days)
One-way ANOVA, followed by Bonferroni's *post hoc* test for multiple comparisons, was used for the analysis. Data is presented as mean±SEM (n= 6); **P*<0.05, ***P*<0.01, ****P*<0.001 vs sham and #*P*<0.05, ##*P*<0.01, ###*P*<0.001 vs morphine group.
IP: Intraperitoneal injection; SC: Subcutaneous injection; LFP: Local field potentials

decrease relative to the control group (*P*<0.01 for gamma and beta waves; *P*<0.05 for alpha, theta, and delta waves). Linalool (50 mg/kg) significantly increased the power of gamma, beta, and alpha waves compared to the morphine-exposed group (*P*<0.05). However, this increase was not significant in other frequency bands (Figure 6).

Electrophysiology (NAc region from ventral striatum)

As graphed in Figure 7 A-F, the total LFP power from the NAc region significantly decreased in the morphine-exposed group (*P*<0.001), with a partial recovery observed following linalool administration (*P*<0.05). Local LFP recordings from the ventral striatum indicated that all wave types in the morphine-exposed group exhibited a significant decrease compared to the control group (*P*<0.001 for gamma and *P*<0.05 for beta, alpha, theta, and delta). Treatment with linalool (50 mg/kg) significantly increased the power of gamma, beta, and alpha waves (*P*<0.05). In contrast, no significant increases were observed in other frequency bands compared to the morphine-exposed group (Figure 7).

Discussion

Behavioral Findings: Several studies have investigated the role of natural compounds in modulating morphine dependence. Among these, linalool at an optimal dose of 50 mg/kg (IP) significantly reduced the time spent in the opiate-paired chamber during the post-conditioning phase (for craving assessment), extinction (for withdrawal), and reinstatement (for relapse) compared to the opiate group. This indicates that linalool could reduce CPP and reinstatement, and accelerate extinction, at a sub-sedative dose (21, 29). Consistent with these reports, our findings demonstrate that linalool at the same dose (50 mg/kg) effectively attenuated morphine-induced CPP and reversed opioid-associated behavioral disturbances (including anxiety- and depressive-like behaviors, memory deficits, and locomotor changes) as well as molecular and electrophysiological alterations, further supporting its therapeutic potential in opioid use disorder. It should be noted that our study employed a three-day morphine conditioning protocol, which is acceptable for CPP induction but may not fully model chronic opioid dependence. Therefore, our findings primarily inform reward learning and addiction-related behaviors rather than long-term dependence or severe withdrawal syndromes.

The injection of morphine into the VTA, NAc, or hippocampus induces CPP in rodents (35). Consistent with these reports, our findings demonstrate that morphine (10 mg/kg) effectively induced CPP, and this effect was significantly attenuated by linalool pretreatment. Based on Do Couto's findings in 2005, administration of morphine at doses of 3 mg/kg and higher induced CPP. The CPP induced at 3, 5, 10, and 40 mg/kg persisted for 1, 2, 4, and 4 weeks after the post-conditioning phase, respectively, before eventually disappearing. This suggests that addiction as a chronic and recurrent disorder is associated with long-lasting changes in the brain (36). Our observation that linalool reduced CPP expression supports the possibility that it interferes with the establishment of these long-

lasting changes during conditioning. In agreement, it has been shown that during the post-conditioning phase, the percentage of CPP scores in morphine-received mice was significantly higher than in saline-received mice (37). Memory impairment is also one of the manifestations of opiate addiction, resulting from hippocampal degeneration that occurs due to apoptosis or long-term potentiation (LTP) deficits (38). Several studies have shown that chronic opiate administration correlates with reduced hippocampal LTP (39). In line with these reports, our data revealed passive avoidance memory deficits in morphine-treated rats, which were significantly improved by linalool pretreatment. Previous studies indicated that one day of withdrawal after chronic opiate administration is associated with anxiety and depression, along with a decline in locomotor activity. This effect may be due to the dysregulation of the dopaminergic, serotonergic, and adrenergic systems, as well as the hypothalamic-pituitary-adrenal (HPA) axis, alongside c-Fos accumulation in reward-related brain areas (20, 40). Our behavioral assessments during early withdrawal (days 6-10) similarly demonstrated anxiety- and depressive-like behaviors, as well as reduced locomotor activity, all of which were reversed by linalool. During conditioning, repeated morphine injections are accompanied by sensitization of locomotor velocity, likely due to glutamatergic and dopaminergic neurotransmission in the striatum, which is primarily involved in movement control (35). According to Bobzean's findings in 2019, the first symptoms of spontaneous somatic withdrawal syndrome were observed 12 hr after the last chronic morphine injection, peaking at 36 hr, and declining by 72 hr. The withdrawal symptoms may correlate with an enhancement of GABA release in the VTA (7). In the brainstem, dopamine and GABA undergo significant changes, decreasing and increasing, respectively, one hour following narcotic withdrawal. However, the most significant changes in neurotransmitter systems (dopaminergic, noradrenergic, and serotonergic) were observed at 36 hr and one week after morphine withdrawal (41). This time course is consistent with our observations of behavioral deficits during early withdrawal (days 9-10), which fall within the one-week window reported by these studies. It has been reported that morphine withdrawal-induced conditioned place aversion (CPA) up-regulates kappa opioid receptors and their endogenous ligand dynorphin in the dorsal hippocampus, but not in the amygdala, NAc, or PFC. Therefore, the dorsal hippocampus plays a more critical role in consolidating aversive memory following withdrawal (42). Kappa opioid receptors and dynorphin also play important roles in aversive emotions such as anxiety and depression (43-45). Although we did not directly measure CPA, the anxiety- and depressive-like behaviors observed in our study may be related to these kappa opioid receptor-mediated mechanisms, and the ability of linalool to attenuate these behaviors raises the possibility that it may modulate this system. It seems that the amygdala and the dorsal hippocampus (CA1) may be more critical than the NAc in morphine withdrawal signs (17). During abuse or withdrawal periods, alterations in opioid or dopamine receptors may contribute to impaired memory, anxiety, and depression (39, 40, 46). Morphine withdrawal, through a decrease in dopamine levels in the VTA, NAc, and PFC, as well as a decrease in the expression of tyrosine hydroxylase (the rate-limiting enzyme in dopamine synthesis), could induce anxiety and depression (46). Our findings that linalool reversed these behavioral deficits suggest it may help normalize dopaminergic function during withdrawal. However, direct measurements of dopamine or tyrosine hydroxylase are needed to confirm this hypothesis.

Molecular Findings- based on findings from Cui in 2010, the activation of the PI3K-Akt signaling pathway is strongly involved in morphine-induced CPP in the CA3 region of the hippocampus, but not in the NAc, VTA, or CA1. Specifically, microinjection of a PI3K inhibitor into the CA3 or blockage of μ-opioid receptors abolished the acquisition of morphine CPP (6). Consistent with these reports, our findings revealed that morphine administration significantly increased PI3K and Akt mRNA expression in the hippocampus on day 5 (acute withdrawal), and this up-regulation was reversed by linalool pretreatment. We acknowledge that our measurement reflects mRNA expression levels rather than protein levels or phosphorylation status; therefore, we cannot directly infer pathway activity from these data. This suggests that linalool may interfere with the PI3K-Akt signaling pathway in the hippocampus, thereby attenuating morphine reward acquisition. According to Guo's findings (2015), a PI3K inhibitor reduces Akt phosphorylation in the hippocampal dentate gyrus (DG), a key structure for learning and drug relapse, and prevents morphine CPP (47). Our results extend these observations by demonstrating that a natural compound, linalool, can similarly modulate this pathway.

According to Kaplan, repeated morphine exposure induced ΔFosB overexpression in the prefrontal cortex, striatum, and amygdala, which is correlated with the executive, motivational, motor, and cognitive functions associated with addictive dependence or relapse of opiates (11). In agreement, our molecular data showed that morphine significantly increased CREB and ΔFosB expression in the ventral striatum, and this effect was attenuated by linalool. Given that ΔFosB accumulation in the striatum is hypothesized to act as a "molecular switch" converting voluntary drug use into compulsive use, the ability of linalool to reduce ΔFosB levels suggests it may interfere with this transition. In addition, it has been reported that chronic administration of tramadol significantly increased μ-opioid receptors, ΔFosB, and CREB in the NAc, which is related to synaptic adaptations to drug tolerance (48). Our findings with morphine are consistent with this pattern, supporting the role of CREB and ΔFosB in opioid-induced neuroadaptations. Another experiment indicated that cannabinoid-opioid interactions could regulate c-Fos and CREB levels during or before extinction in the NAc or VTA. For example, intra-NAc injection of a cannabinoid (CB1) antagonist could reduce the pCREB/CREB ratio in the NAc and VTA during extinction and diminish the rewarding effects of morphine (16). While we did not investigate cannabinoid interactions, our results demonstrate that linalool similarly reduces CREB expression in the ventral striatum, raising the possibility that it interacts with the endocannabinoid system—a hypothesis warranting further investigation.

Taken together, our molecular findings suggest that linalool modulates two key pathways involved in opioid addiction: the PI3K-Akt pathway in the hippocampus (associated with drug-context memory) and the CREB-ΔFosB pathway in the ventral striatum (associated with reward neuroadaptations). This dual modulation may

underlie the behavioral improvements observed in CPP, memory, and affective symptoms.

Electrophysiological findings: Local field potentials (LFPs), rather than conventional scalp EEG, were recorded directly from the hippocampal CA1 and nucleus accumbens using implanted electrodes (as described in Methods). Our electrophysiological recordings revealed pronounced neuronal hypoactivity in both the hippocampal CA1 and NAc regions during early withdrawal (days 9-10), as evidenced by a significant decrease in total EEG power and a widespread reduction in oscillatory activity across all frequency bands. This finding aligns with previous studies reporting a 35-70% decline in EEG voltage output during morphine abstinence and prolonged hypofunction in multiple brain regions, reflecting a state of hypofunction in reward-related circuits (49). Linalool pretreatment significantly reversed this hypoactivity, restoring high-frequency oscillations (β, γ, and α bands) to levels comparable to those of the control group. Given the association of high-frequency oscillations with consciousness, arousal, and cognitive processing, this restoration suggests that linalool may facilitate neural recovery during withdrawal. While the precise mechanisms remain to be elucidated, the normalization of neuronal activity by linalool likely involves its modulation of GABAergic and glutamatergic neurotransmission, as well as its antioxidant and neuroprotective properties. A key strength of our study is the temporal dissociation between molecular and electrophysiological assessments. Molecular analyses were performed immediately after the CPP test on day 5 (acute withdrawal) to capture peak expression of addiction-related mediators, including CREB, ΔFosB, PI3K, and Akt. In contrast, behavioral and electrophysiological evaluations were conducted during early withdrawal (days 6-10), allowing us to assess the persistence of withdrawal-induced alterations. This design reveals that while molecular changes may peak early, their functional consequences—manifested as neuronal hypoactivity and behavioral deficits—persist for at least 10 days after morphine cessation.

The interaction between hippocampal and ventral striatal activity during reward processing has been well documented. A study by Van Der Meer *et al.* in 2011 found that the theta rhythm of the hippocampal (CA1)-VS formation exhibited precise and non-independent synchronization with reward-related behavior in the Y-maze. Despite the presence of theta oscillations in both regions, the average theta rhythm did not differ. Consequently, separating these two regions resulted in the absence of place preference. In fact, the theta firing phase increases as the animal approaches the reward location. Ventral striatal neurons are involved in place-reward associations, while hippocampal neurons play a role in rapid memory encoding, both contributing to the modulation of motivated behavior (4). Our finding that both regions exhibited parallel hypoactivity during withdrawal further supports the functional interdependence of the hippocampal-striatal circuit in reward-related behaviors.

In another experiment, Reakkamnuan *et al.* reported that local field potential (LFP) current during the morphine conditioning phase increased significantly in the hippocampus (CA1)/NAc pairs, but not in the hippocampus (CA1)/VTA pairs. It was also shown that alpha and beta waves increased in the post-conditioning phase only in the NAc, which may explain morphine-dependent craving and relapse in this region. Striatal LFP spectrograms exhibited relatively stable activity during pre-conditioning, conditioning, and post-conditioning of CPP (35). But the question is, what is the situation like during withdrawal? Our study addresses this gap by demonstrating that, during withdrawal, neuronal activity in both regions is significantly depressed, unlike during conditioning or post-conditioning. During withdrawal, GABAergic neurons in the tail of the VTA (tVTA) inhibit VTA dopaminergic neurons. The tVTA loses its inhibition after re-exposure to opiates, which correlates with long-term negative emotions (18). Withdrawal actually increases GABA inhibitory postsynaptic currents (IPSCs) and blocks dopaminergic terminals. In contrast, opiates inhibit GABA IPSCs and promote dopamine transmission (46, 50). During opiate exposure, IPSCs undergo inhibition of 46% from the tVTA, 18% from the NAc, and only 11% from VTA interneurons (50). This GABAergic disinhibition mechanism may explain the neuronal hypoactivity observed in our study, as increased GABAergic tone during withdrawal would suppress neuronal firing in target regions such as the hippocampus and ventral striatum. The ability of linalool to reverse this hypoactivity suggests it may modulate GABAergic transmission, consistent with its known effects on the GABA system.

In 2021, Ma *et al.* reported that both acute and chronic morphine injection and its withdrawal (especially at 3 days) impaired spatial memory, reduced EEG amplitude, and increased locomotor activity in a dose/state-dependent manner (39). Our findings at days 9-10 extend this observation by showing that EEG amplitude reductions persist beyond the 3-day time point, and that linalool can reverse this effect. Based on Chunchun's findings in 2022, analysis of the EEG power spectrum showed that naloxone injection could increase the total power of the EEG and its multiple power spectrum components in the hippocampus, especially the high-frequency fast wave power (β and γ) in saline-extinct mice but not in morphine-extinct mice. This phenomenon explains the role of naloxone in promoting consciousness and arousal behavior. However, it is noteworthy that morphine dependence has significant effects on the hippocampus, which were not reversed by extinction or naloxone treatment (37). In contrast to naloxone's limited effect in morphine-dependent animals, our results demonstrate that linalool pretreatment restored EEG power, suggesting a different mechanism of action that may involve neuroprotection rather than acute receptor antagonism. So, more research on this topic needs to be undertaken to understand better the association between local EEG activity and morphine in different brain areas within withdrawal. Our study provides a foundation for such investigations by identifying the hippocampal-striatal circuit as a key locus of withdrawal-induced dysfunction and a potential target for therapeutic intervention.

Future directions and limitations – As noted above, the following limitations should be considered: (1) The three-day conditioning protocol models reward learning rather than chronic dependence; (2) PI3K/Akt measurements reflect mRNA levels, not total protein levels; (3) The observed correlations do not establish direct causality. Future studies incorporating pathway-specific inhibitors (e.g., LY294002 for PI3K signaling, or dominant-negative CREB constructs) would substantially strengthen mechanistic conclusions. Additionally, direct assessment of spontaneous versus naloxone-precipitated withdrawal in a chronic dependence model (e.g., 14-day morphine

exposure) would clarify whether linalool's effects extend to full withdrawal syndrome.

Conclusion

As the main input nucleus of the basal ganglia, the ventral striatum (including the nucleus accumbens, NAc) receives dopaminergic inputs from the VTA and glutamatergic inputs from the hippocampus. This convergence underlies reward-motivated behavior and is critically involved in addiction and opioid-associated behavioral disturbances. In line with this, linalool pretreatment attenuated these behavioral disturbances by down-regulating key addiction-related mediators, including PI3K/Akt mRNA expression in the hippocampus and the CREB/ΔFosB pathway in the ventral striatum. Additionally, it enhanced local field potential (LFP) power in both the CA1 hippocampal region and the NAc, suggesting functional restoration of these interconnected centers. While these findings are promising, direct mechanistic causality remains to be established. Future studies using pathway-specific inhibitors and chronic dependence models are warranted to validate linalool as a potential therapeutic agent for opioid use disorder.

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Ethical Approval

The study was conducted in accordance with the guidelines of the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences in Ahvaz, Iran (ethics code: IR. AJUMS.ABHC.REC.1401.071).

Data Availability

The data that support the findings of this study are available from the corresponding author, [SP.N], upon reasonable request.

Authors' Contributions

M KP conducted the experiment and analyzed the data. Y F and A S conceptualized the experiment. N Y programmed the research, while SPN designed it and wrote the main manuscript. All authors reviewed the manuscript.

Conflicts of Interest

The authors disclosed no potential conflicts of interest.

Declaration

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