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RESEARCH

Alpha-pinene modulates hippocampal MAPKs/c-Fos pathways during morphine dependence and withdrawal in rats

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Abstract

Rationale A potential strategy for managing morphine addiction is preventing mitogen-activated protein kinases (MAPKs) activation and neuroinflammation. The molecular basis of inflammation involves inflammatory cytokines, whose production and activity are modulated by the MAPKs signaling pathways.

Objectives This study investigated how alpha-pinene (APN), an anti-inflammatory monoterpene, influences hippocampal MAPKs levels during chronic morphine exposure and the subsequent prolonged withdrawal phase.

Methods Three experimental groups as dependent model groups intraperitoneally received 10 days of saline + DMSO, morphine (10 mg/kg) + DMSO, and morphine + APN (20 mg/kg). Three additional groups were designated as withdrawal models, treating them with either saline (group 1) or morphine (groups 2 and 3) for 10 days before subjecting them to a 30-day withdrawal period. During the withdrawal, the first and second groups were administered daily 5% DMSO injections, whereas the third group received daily APN (20 mg/kg) treatment. *Results*: The results revealed that Hippocampal expression of MAPKs, including p38, ERK1/2, and JNK remained largely unchanged subsequent to both the chronic morphine exposure and the 30-day morphine wash-out phase. However, hippocampal levels of the phosphorylated form of the MAPKs significantly increased after both the morphine exposure and withdrawal phase. Interestingly, APN treatment during morphine exposure or over the 30-day of withdrawal phase significantly restored hippocampal levels of the phosphorylated MAPKs and decreased c-Fos expression.

Conclusions It can be concluded that APN treatment may contribute to preventing hippocampal MAPK overactivation and decreasing c-Fos expression resulting from chronic morphine exposure and the subsequent withdrawal phase.

Keywords Morphine · Opioid Use Disorders · Anti-nociceptive effect · Alpha-pinene · Anti-inflammatory effect · MAPK · C-Fos · Hippocampus

Introduction

Opioids, such as morphine, continue to be essential potent painkillers in clinical practice. However, chronic opioid exposure eventually results in opioid use disorder (OUD) (Strang et al. 2020; Volkow and Blanco 2021). A main outcome of OUD in clinical use of morphine is developing tolerance to its antinociceptive effects, a state in which its effectiveness decreases with prolonged use (Dydyk et al. 2025). The antinociceptive tolerance necessitates higher opioid doses for equivalent pain relief, heightening the risk of adverse effects, including addiction (Benyamin et al. 2008). Furthermore, individuals may encounter withdrawal symptoms upon abrupt cessation or substantial reduction of morphine use (Monroe and Radke 2023). The withdrawal

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symptoms can lead to physical and psychological discomfort and cravings, which in turn increase risk of relapse (Ali et al. 2017; Pergolizzi et al. 2020; Wang 2019). Given the data mentioned above, gaining a deeper understanding of the molecular mechanisms underpinning outcomes of OUD is of significant interest for mitigating its adverse side effects.

Research shows that the outcomes of OUD and memory processes share overlapping brain regions, similar intracellular signaling cascades, and changes in synaptic plasticity (Hyman et al. 2006; Kelley 2004; Kutlu and Gould 2016). As opioid addiction develops, the brain's reward system produces connections between substance use and its enjoyable effects, strengthening the recall of the drug's pleasurable consequences (Christie 2008; Kutlu and Gould 2016). Furthermore, research indicates that withdrawal symptoms reinforce the brain's association between drug use and the alleviation of discomfort, further solidifying these memory connections (Dai et al. 2022; Fournier et al. 2023; Frenois et al. 2005). Thus, drug addiction and withdrawal symptoms may stem from dysfunctional learning and memory processes, driven by maladaptive adaptations in the relevant brain areas (Baidoo et al. 2020; Hyman et al. 2006; Torregrossa et al. 2011). Accumulating data suggests that the hippocampus, a key area for learning and memory processing, also plays a significant role in the development of OUD (Dai et al. 2022; Goodman and Packard 2016; Koob and Volkow 2010; Wei et al. 2013). Numerous investigations have demonstrated that prolonged morphine use impairs hippocampal long-term potentiation (LTP), a well-established cellular process critical for memory consolidation (Kutlu and Gould 2016; Salmanzadeh et al. 2003). Evidence also suggests that synaptic remodeling and neuroinflammation are significant pathophysiological mechanisms contributing to OUD in humans (Butelman et al. 2023; Seney et al. 2021). Accumulating data shows that neuroinflammation in a vicious cycle exaggerates other undesirable effects of OUD like dependence and withdrawal (Osmanlıoğlu et al. 2020; Toloff and Woodcock 2022; Zhou et al. 2021). However, the mechanisms underlying these effects of morphine are poorly understood.

The mitogen-activated protein kinases (MAPKs) are a group of protein kinases that operate on serine/threonine residues located in target proteins, and are crucial in cellular signaling pathways (Kyosseva 2004). They are divided into three distinct groups: p38 kinases, extracellular signal-regulated kinases (ERKs), and c-Jun N-terminal kinases (JNKs) (Badmi et al. 2018). Upon activation by extracellular signals such as growth factors, cytokines, and stress, MAPKs phosphorylate downstream targets (e.g., transcription factors and other kinases) to mediate key cellular processes including proliferation, differentiation, motility, inflammation, and apoptosis (Johnson and Lapadat 2002; Morrison 2012). There is substantial evidence indicating that MAPKs can be activated by chronic morphine treatment and that application of MAPK inhibitors reduces morphine tolerance, dependence, and withdrawal (Cao et al. 2006; Chen and Sommer 2009; Cui et al. 2006; El Rawas et al. 2020; Gutstein et al. 1997). The activation of central immune mechanisms, mediated by activation of toll-like receptor 4 (TLR4), play crucial roles in opioid pharmacology. This TLR4 signaling results in the production of proinflammatory mediators via activation of the adaptor protein MyD88 and downstream molecules including MAPKs and NF-κB (Butelman et al. 2023; Hutchinson et al. 2008; Mustafa et al. 2023). Therefore, a potential strategy for managing OUD could be preventing MAPK activation and neuroinflammation.

Alpha-pinene (APN), a bicyclic monoterpene commonly found in essential oils from plants like pine, rosemary, eucalyptus, and wild pistachios, exhibits neuroprotective and anti-inflammatory effects (Ahmed 2017). The precise mechanism of action of APN remains intricate and incompletely comprehended within the academic literature. The supposed mechanism of action involves the modulation of various pathways, such as those related to neurotransmitter systems, signaling pathways implicated in inflammation, oxidative stress, and neuroprotection in the cortex and hippocampus in different rodent models (Hashemi and Ahmadi 2023a; Khoshnazar et al. 2020; Rahimi et al. 2023). Research also shows that APN regulates key signaling pathways, notably the cAMP-PKA-CREB and MAPKs cascades (Kim et al. 2015; Zhang et al. 2020). We have previously established that APN treatment exerts a protective effect against morphine-induced neuroinflammation and cognitive decline. Specifically, we found that APN prevents the rise in hippocampal proinflammatory cytokines and the associated impairment of spatial working memory in morphine-dependent and withdrawing rats (Ahmadi et al. 2026). We have also demonstrated that APN treatment, whether co-administered with morphine or given during a 30-day withdrawal, modulates the hippocampal TLRs/MyD88/PI3K/AKT1B pathways (Ahmadi et al. 2025a). Given the established involvement of MAPK signaling in inflammation, morphine dependence, and withdrawal, in the present study we extended our previous molecular investigations to determine the possible contribution of MAPK signaling to the behavioral improvements previously reported to be induced by APN during morphine dependence induction and prolonged withdrawal in the rat hippocampus.

Methods

Animals

In adherence to the reduction principle of the 3Rs, this study analyzed hippocampal tissues from animals used in our prior investigation (Ahmadi et al. 2026). Male Wistar

rats when reached an average weight of 250 g were examined. Rats were kept in an animal house under standard conditions, including appropriate temperature (22 ± 2 °C), humidity (40–60%), and 12 h light/dark cycle (lights on at 7:00 A.M.). Rats had unrestricted admittance to food and water, and every effort was taken to minimize animal use and their distress. The University of Kurdistan Research Ethics Committee (REC) approved the experimental protocols (IR.UOK.REC.1402.010), which followed the National Academy of Sciences Institute for Laboratory Animal Research's Guide for the Care and Use of Laboratory Animals (2011).

Drugs

Morphine sulfate, a white crystalline powder, was purchased from Active Pharmaceutical Ingredients manufacturer (Temad Co., Tehran, Iran). Each 10 milligrams (mg) of morphine powder were dissolved in 1 mL of physiological saline. APN is a natural monoterpene found in the essential oils of numerous plants, such as conifers, rosemary, sage, and wild pistachios (Ahmed 2017; Hosseini et al. 2022). It is a colorless, organic liquid that is insoluble in water but soluble in oil and ethanol (Rahman 2018). The APN utilized in this study was prepared by Van Company (Van Co., Sanandaj, Iran). They extracted APN from *Pistacia atlantica* L. subsp. *kurdica* (wild pistachio tree) with 97% purity. A 5% dimethyl sulfoxide (DMSO) solution was used to dilute APN to the desired concentration and also served as its vehicle in the respective control groups. This concentration of APN has been previously employed in rat studies and no toxic effect was observed (Ahmadi et al. 2025a).

Experimental groups and treatments

In our previous experiments, based on a power analysis (effect size = 0.75, α = 0.05, power = 0.85) for one independent variable across three groups, a sample size of eight rats per group was determined. Morphine sulfate (10 mg/kg) and physiologic saline (1 mL/kg) were injected subcutaneously. APN (20 mg/kg) and 5% DMSO (1 mL/kg) were injected intraperitoneally. The three groups of morphine-dependent model received 10 days of treatments as follows. The control group received physiological saline twice daily at six-hour intervals. Additionally, they received a single daily injection of 5% DMSO. The second group received a combination of morphine (10 mg/kg, twice daily) and an injection of 5% DMSO (once daily), while the third group received twice daily morphine injection (10 mg/kg,) plus APN (20 mg/kg, once daily). After the 10-day course of repeated drug injections, the rat brains were removed, and the bilateral hippocampi were dissected for molecular analysis. The second set of additional three groups received the same 10-day regimen of saline or morphine as the first set, followed by a 30-day withdrawal phase. Throughout the 30-day withdrawal phase, the first and second groups were administered daily injections of 5% DMSO (1 mL/kg). Simultaneously, the third group received daily injections of APN (20 mg/kg). On the 30th day of the withdrawal, rats were sacrificed, the rat brains were extracted, and the bilateral hippocampi were dissected for molecular analysis.

Hippocampal dissection

For brain tissue extraction, each rat was completely anesthetized by using a mixture of ketamine and xylazine (100 and 10 mg/kg, respectively). Once anesthetized, each rat sacrificed by using a guillotine, followed by the cutting of the skull bone with scissors. The brain was carefully extracted from the skull by using a curved spatula. Immediately after extraction, the brain was rinsed with cold physiological saline, and the bilateral hippocampi were dissected on a clean surface on ice. The hippocampal tissues from each rat were then powdered and thoroughly mixed in liquid nitrogen. Following tissue separation, the tissue samples were stored in a freezer until further analyses.

Western blot

In our previous work (Ahmadi et al. 2026), eight rats were allocated per group. Due to constraints in the number of wells on the SDS-PAGE gel, four hippocampal samples from these eight rats per group were randomly selected for western blot analysis. This random selection was not influenced by any prior behavioral or molecular results from that study. Western blot procedures followed established protocols from our earlier publications (Ahmadi et al. 2025a, 2026). The data for MAPK and c-Fos presented in this manuscript are novel and have not been published elsewhere. By utilizing an ultrasonic homogenizer (Iranian Pajohesh Nasir, Iran), fifty mg of the hippocampi was harvested in radioimmunoprecipitation assay buffer (RIPA buffer, Abcam, U.S.A.). To separate the tissue proteins from the insoluble fraction, the homogenates were centrifuged at 12,000 RCF for 10 min at 4 °C. The protein samples were separated on SDS-PAGE and transferred onto a PVDF (Polyvinylidene fluoride) membrane through a semi-dry transfer system (Bio-Rad Laboratories, CA, USA). Then, a 5% skimmed milk solution in TBST was prepared and applied for one hour at 4 °C to block the membrane. Next, the membrane was washed three times with TBST and then incubated in a primary antibody solution for 24 h at 4 °C (Ahmadi and Khaledi 2020; Hashemi and Ahmadi 2023b). The primary antibodies were as follow: beta-actin (sc-517582), c-FOS (sc-166940), p38 (sc-535), phospho-p38 (p-p38, sc-17852-R), JNK (sc-7345),

phospho-JNK (p-JNK, sc-6254), ERK1/2 (sc-374239), and phospho-ERK (p-ERK, MAB1018).

The membrane was then washed three times for 5 min each with TBST. Subsequently, it was incubated with the appropriate secondary antibody for one hour at room temperature. The following secondary antibodies were used: anti-rabbit (sc-2357) or anti-mouse (sc-516102) polyclonal HRP-conjugated antibodies. All of the antibodies were products of Santa Cruz biotechnology (USA) except for p-ERK, which was a product of biotechne company (USA). Following initial probing, the membrane was stripped with a mild glycine-HCl/SDS buffer (pH 2.2) to remove antibodies before reprobing. The protein bands were visualized using ECL reagents (Santa Cruz), and images were captured with a Western blot imaging system (Day Tadjhiz Aryan, Iran). Finally, band intensities were converted to quantitative data using ImageJ software.

Statistical analysis

Band densities for p38, ERK1/2, JNK, and c-Fos were normalized to β-actin (loading control). Additionally, the phosphorylated MAPK levels were normalized to their respective total MAPK levels. GraphPad Prism version 8 software was employed for data analysis and graph generation. The normality of the data was assessed using Q-Q plots. Then, to determine the significance of differences in protein expression among the experimental groups, one-way analysis of variance (ANOVA) was conducted. If the ANOVA results were significant, post hoc pairwise assessments were performed using Tukey's test to detect between groups statistical differences. Where effects were significant, effect sizes were calculated as partial eta-squared. These were interpreted based on Cohen's (1988) conventions, with values greater than 0.4 denoting a large effect size (Cohen 1988). Data are available upon reasonable request from the corresponding author.

Results

APN treatment reversed the withdrawal-induced increase in hippocampal p38 expression, which was not affected by morphine dependence alone

The results of one-way ANOVA showed that hippocampal p38 expression was unaltered between experimental groups after 10 days of repeated injections [$F(2, 9) = 0.73$, $P > 0.05$] (Fig. 1A). Subsequent to the withdrawal phase, one-way ANOVA showed that hippocampal p38 expression differed significantly across the experimental groups [$F(2, 9) = 19.18$, $P < 0.001$; partial eta-squared = 0.81]. Post-hoc analysis showed that hippocampal p38 expression was significantly higher in the morphine-withdrawal group than in the saline-treated control group ($P < 0.001$). However, 30-day APN treatment during the withdrawal phase partially restored the elevated p38 expression to a level comparable to that in the control rats (Fig. 1B).

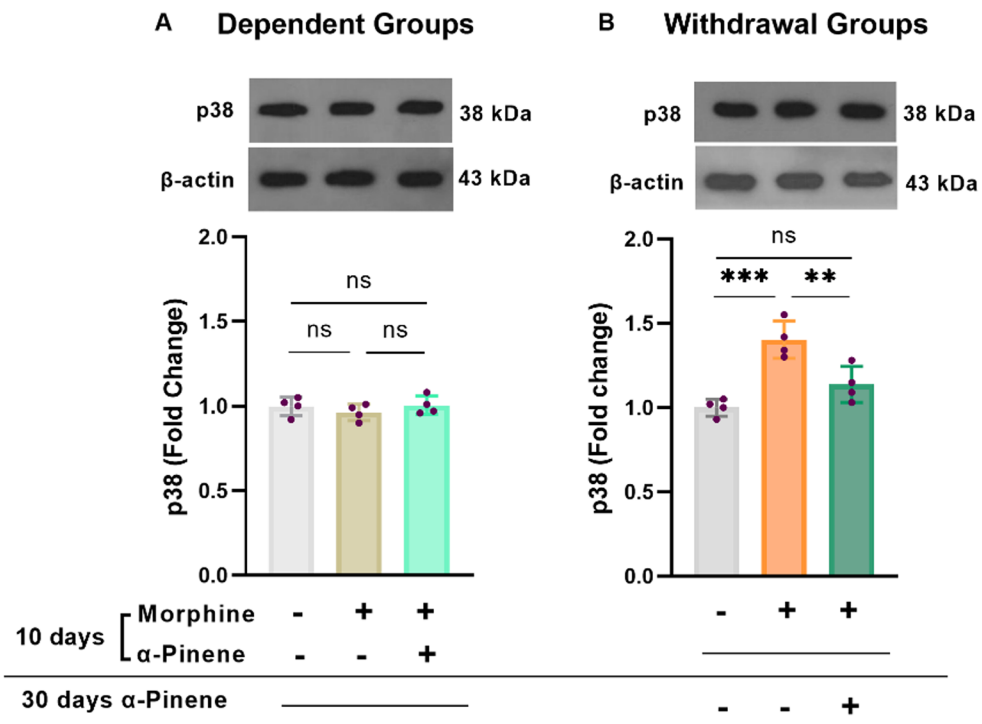

Fig. 1 (**A**) Hippocampal p38 expression in three experimental groups of morphine-dependent model. (**B**) Hippocampal p38 expression in three experimental groups following 30 days of withdrawal. Each bar designates the mean ± SD of 4 rats in each group. The upper panels represent western blot images of hippocampal β-actin expression and p38 (as a protein of interest) in three experimental groups of either dependent or withdrawal categories. "ns" denotes no significant difference. ** $P < 0.01$ and *** $P < 0.001$

The elevated hippocampal p-p38 levels resulting from chronic morphine exposure and a prolonged withdrawal phase were reversed by APN treatment

Hippocampal phospho-p38 expression differed significantly between the experimental groups after 10 days of repeated injections [One-way ANOVA, $F(2, 9) = 33.06$, $P < 0.001$; partial eta-squared = 0.88]. Hippocampal phospho-p38 levels in morphine-dependent group notably increased compared to the control rats ($P < 0.001$). Co-treatment with APN during the 10-day drug regimen partly prevented the increase in phospho-p38 levels. However, APN-treated rats still showed higher p-p38 levels than controls (Fig. 2A). Analysis of the results in withdrawal model groups also exhibited that hippocampal p-p38 levels altered between experimental groups [$F(2, 9) = 24.6$, $P < 0.01$; partial eta-squared = 0.85]. The morphine-withdrawal group exhibited a significant increase in hippocampal p-p38 expression compared to the saline-treated control group. Nevertheless, APN treatment during the 30-days withdrawal phase completely restored the elevated p-p38 levels toward its control levels detected in the saline-treated controls (Fig. 2B).

The hippocampal ERK1/2 expression remained unchanged after both chronic morphine exposure and following a prolonged withdrawal phase

No significant alteration between experimental groups was detected in hippocampal ERK1/2 expression after chronic morphine exposure [One-way ANOVA, $F(2, 9) = 0.1$, $P > 0.05$] (Fig. 3A). Similarly, the results of hippocampal ERK1/2 expression subsequent to the 30-day withdrawal phase also indicated no notable alteration between experimental groups [$F(2, 9) = 3.9$, $P > 0.05$] (Fig. 3B).

APN treatment restored the elevated hippocampal p-ERK levels after withdrawal but had no effect on its levels following morphine dependence

A significant alteration within experimental groups was detected in hippocampal p-ERK levels on day 10 of repeated injections [One-way ANOVA, $F(2, 9) = 9.6$, $P < 0.01$; partial eta-squared = 0.68]. Morphine-dependent group had significant increases in hippocampal p-ERK levels compared to the saline-treated control group. APN treatment over 10 days of morphine treatment did not restore the elevation in hippocampal p-ERK levels (Fig. 4A). Furthermore, hippocampal p-ERK levels significantly altered among withdrawal experimental model groups [$F(2, 9) = 24.23$, $P < 0.001$; partial eta-squared = 0.84]. Morphine-withdrawal group showed a sharp increase in hippocampal p-ERK levels compared to the saline-treated control rats ($P < 0.001$). Interestingly, APN treatment during the withdrawal phase completely restored hippocampal p-ERK levels to those of the saline-treated control group (Fig. 4B).

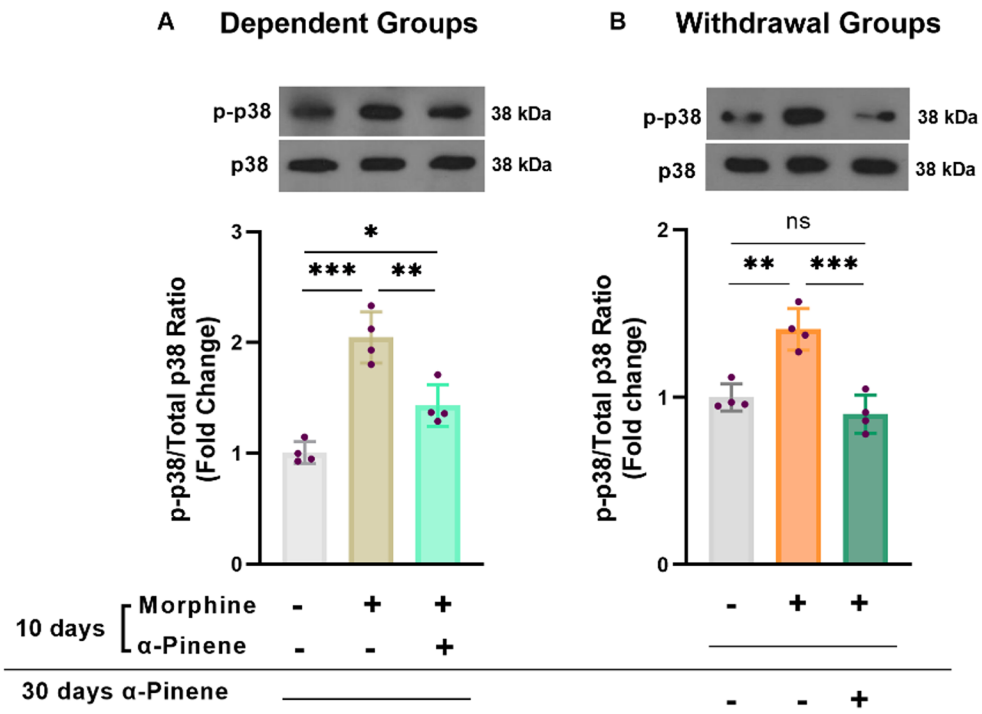

Fig. 2 (**A**) Hippocampal p-p38 levels in three experimental groups of morphine-dependent model. (**B**) Hippocampal p-p38 levels in three experimental groups subsequent to the 30-day withdrawal phase. The upper panels represent western blot images of hippocampal expression of p-p38 and total p38 in three experimental groups of either dependent or withdrawal categories. The bar graphs show the quantification of protein expression in which data are presented as the mean ± SD ($n = 4$ per group). "ns" designates no group difference. ** $P < 0.01$, and *** $P < 0.001$

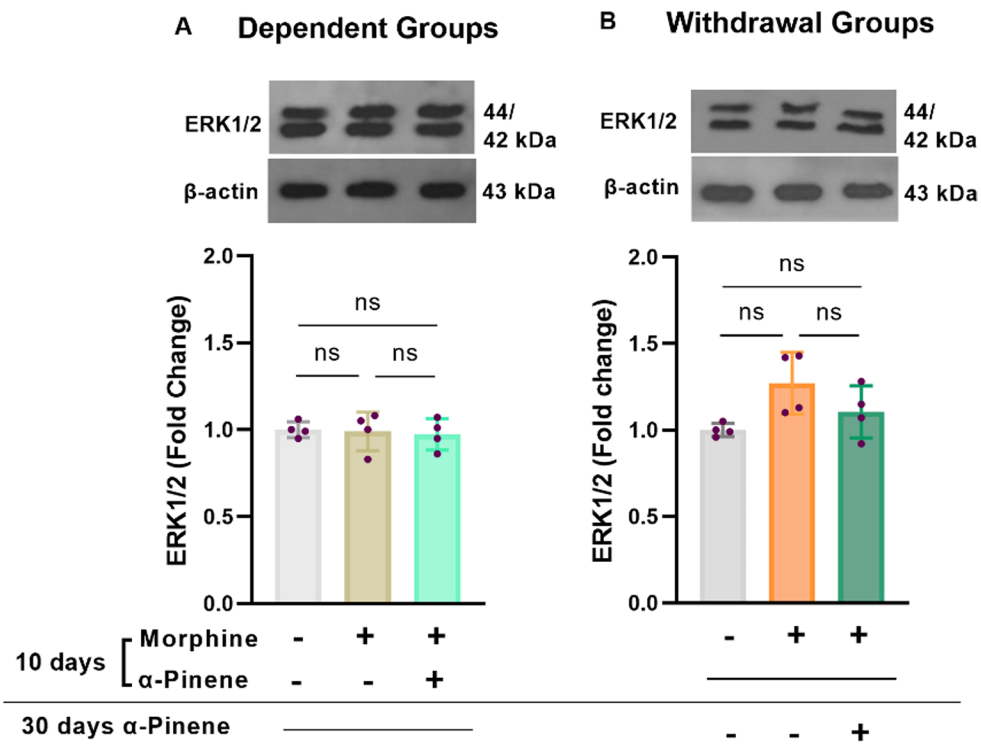

Fig. 3 (**A**) Hippocampal ERK1/2 expression following 10 days of the repeated injections. (**B**) Hippocampal ERK1/2 expression subsequent to 30 days of withdrawal. The upper panels represent western blot images of hippocampal expression of β-actin and ERK1/2 in three experimental groups of either dependent or withdrawal categories. The bars represent the mean and SD of data related to four rats in each experimental group. "ns" symbolizes the contracted form of no significant group difference

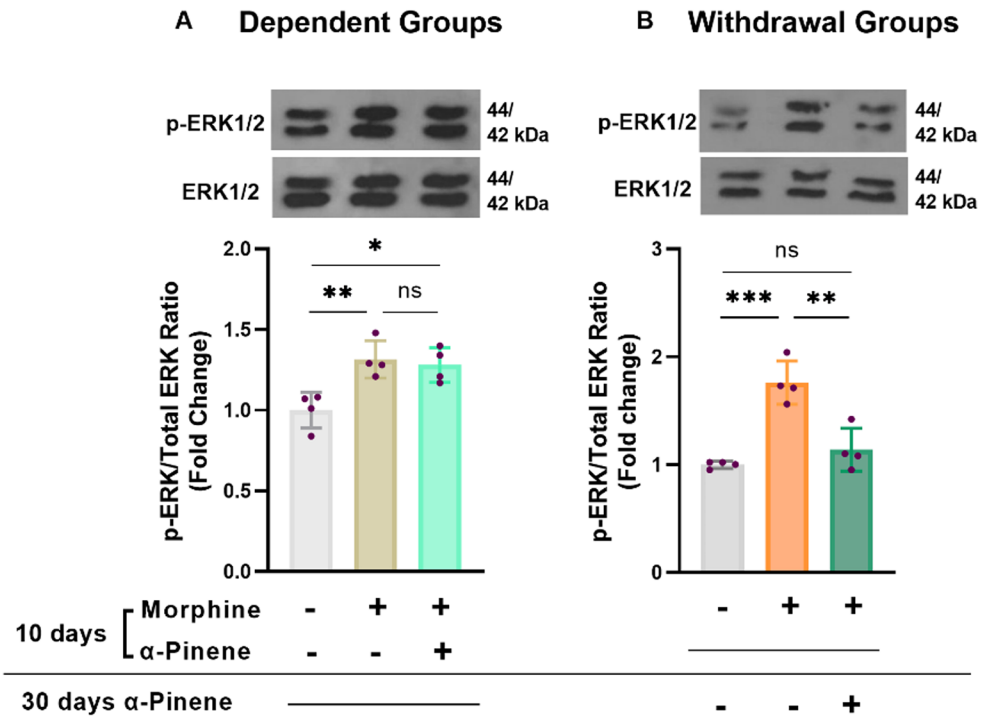

Fig. 4 (**A**) Hippocampal p-ERK levels subsequent to 10 days of the frequent injections. (**B**) Hippocampal p-ERK levels following withdrawal phase. The images in the upper panels represent the western blot results for hippocampal expression of p-ERK and total ERK in either dependent (**A**) or withdrawal (**B**) groups. The bar graphs show the quantification of protein expression in which data are presented as the mean ± SD ($n=4$ per group). ns: not significant, *, **, and *** symbolize $P<0.05$, $P<0.01$, and $P<0.001$, respectively

The hippocampal expression of JNK remained unchanged subsequent to both dependence to morphine and a prolonged withdrawal phase

One-way ANOVA revealed no significant difference in hippocampal JNK expression among the experimental groups after 10 days of repeated injections [F (2, 9) = 0.65, $P > 0.05$] (Fig. 5A). Similarly, one-way ANOVA indicated that hippocampal JNK expression remained unchanged across the experimental groups after the withdrawal phase [F (2, 9) = 1.9, $P > 0.05$] (Fig. 5B).

APN treatment over withdrawal phase but not during frequent morphine exposure restored the increased hippocampal p-JNK levels

The one-way ANOVA evaluations indicated a notable elevation in hippocampal p-JNK levels among the experimental groups that received 10 days of repeated injections [F (2, 9) = 8.7, $P < 0.01$; partial eta-squared = 0.66]. The morphine-dependent group exhibited significantly elevated hippocampal p-JNK levels relative to the saline-treated controls, as revealed by pairwise analysis. Moreover, APN administration during the 10-day morphine treatment did not significantly attenuate the elevated p-JNK levels (Fig. 6A). Following withdrawal, statistically significant differences in hippocampal p-JNK levels among experimental groups was detected [F (2, 9) = 37.68, $P < 0.001$; partial eta-squared = 0.89]. The morphine withdrawal group showed significantly elevated hippocampal p-JNK levels compared to its respective control group. However, APN treatment over the withdrawal phase significantly reversed the increased hippocampal p-JNK levels (Fig. 6B).

APN treatment, administered during both the repeated injections and the withdrawal phase, mitigated the increased expression of c-Fos in the hippocampus

Hippocampal c-Fos expression significantly altered among the experimental groups that received 10 days of repeated injections [One-way ANOVA, F (2, 9) = 54.56, $P < 0.001$; partial eta-squared = 0.92]. The morphine-dependent group markedly showed an increase in hippocampal c-Fos expression compared to the saline-treated controls. Although not completely reversing the effect, APN administration during the 10-day morphine treatment significantly attenuated morphine-induced elevations in the c-Fos expression (Fig. 7A). The results also showed a significant alteration in hippocampal c-Fos expression among the experimental groups following withdrawal [F (2, 9) = 59.08, $P < 0.001$; partial eta-squared = 0.93]. The morphine withdrawal group exhibited significantly elevated hippocampal c-Fos expression compared to saline-treated controls. However, APN treatment during the 30-day withdrawal period almost completely normalized hippocampal c-Fos expression levels (Fig. 7B).

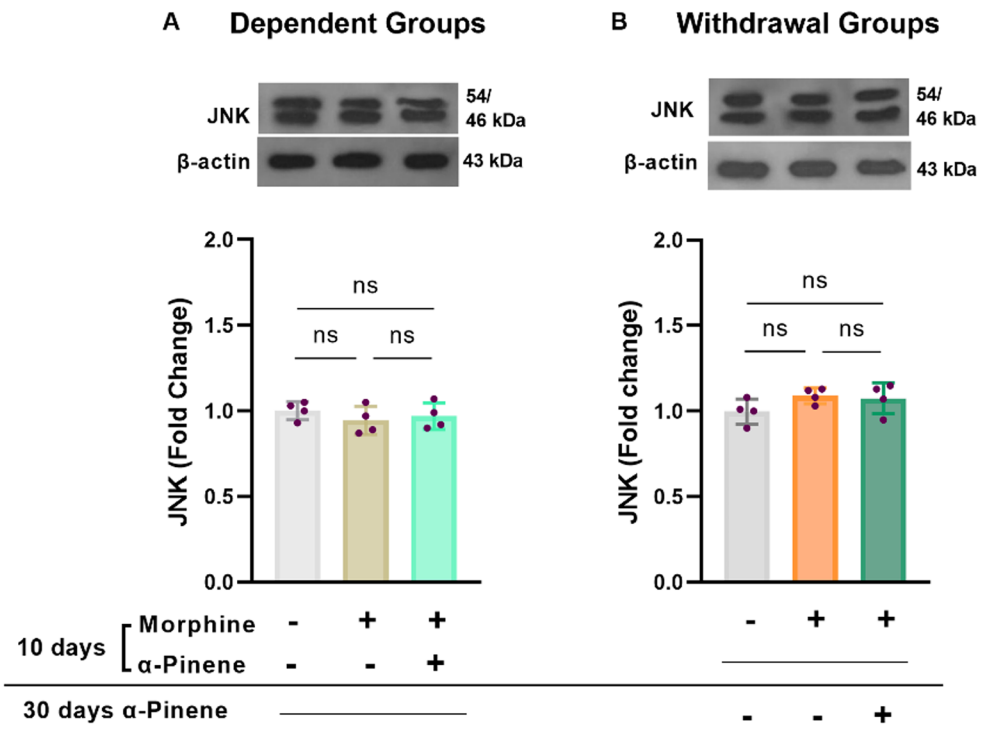

Fig. 5 (**A**) Hippocampal expression of JNK in three experimental groups that received 10 days of repeated injections. (**B**) Hippocampal JNK expression across the withdrawal group models. The images in the upper panels represent the western blot images for hippocampal expression of β-actin and JNK in either dependent (**A**) or withdrawal (**B**) groups. Data are presented as the mean ± SD related to four rats in each group. "ns" symbolizes no significant statistical group difference

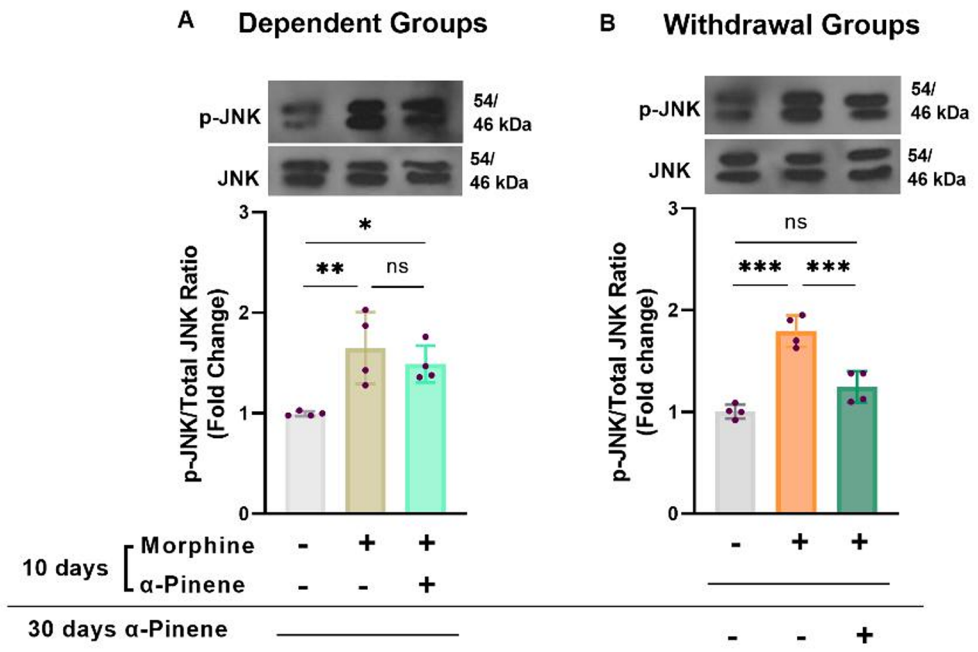

Fig. 6 (**A**) Hippocampal p-JNK levels in three experimental groups that received 10 days of repeated injections. (**B**) Hippocampus p-JNK levels in three experimental group models of withdrawal. The upper panels represent western blot images for p-JNK and total JNK proteins in the hippocampus in three experimental groups of dependent (**A**) or withdrawal (**B**) models. The bar graphs show the quantification of protein expression in which data are presented as the mean ± SD ($n = 4$ per group). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ between the specified groups. "ns": designates no significant statistical group difference

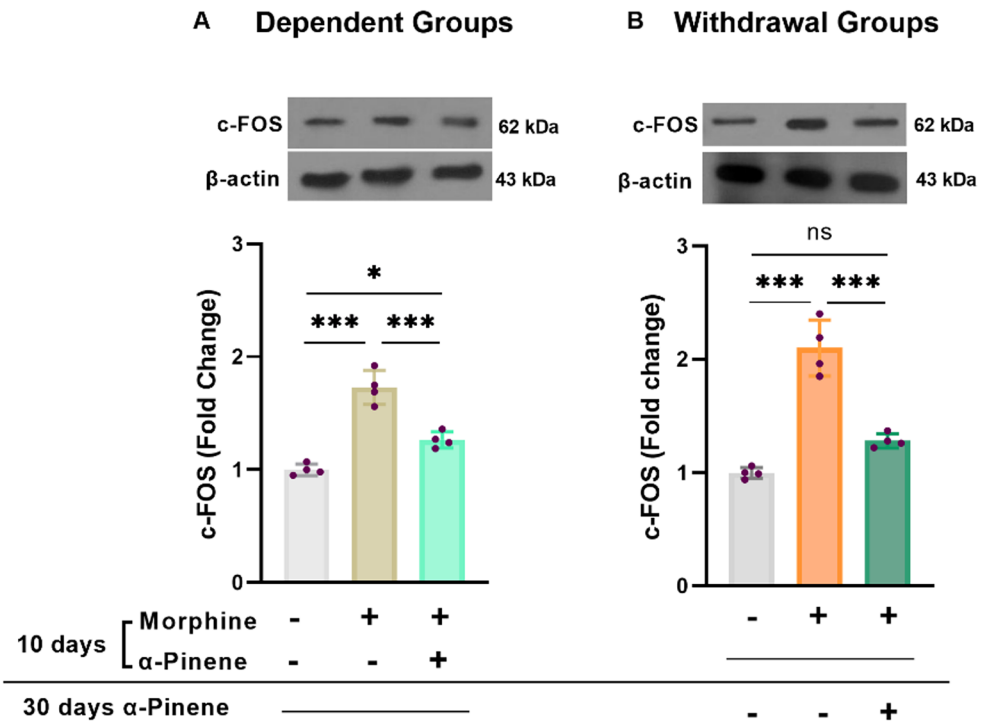

Fig. 7 (**A**) Hippocampal c-Fos expression in three experimental groups that received 10 days of repeated injections. (**B**) Hippocampal c-Fos expression in three experimental group models of withdrawal. The upper panels represent western blots of β-actin (a housekeeping protein) and c-Fos (as a protein of interest) in the hippocampus in three experimental groups. The bar graphs show mean ± SD of the quantification of protein expression data ($n = 4$ per group). * $P < 0.05$, *** $P < 0.001$, and "ns" designates no significant group difference

Discussion

We have previously shown that repeated morphine exposure for 8–10 days induces tolerance to its antinociceptive effect and drug dependence, as evidenced by a hotplate test of analgesia and a naloxone-precipitated withdrawal syndrome, respectively (Ahmadi et al. 2021, 2023). Repeated morphine administration over 10 days attenuated its anti-nociceptive effect and impaired cognitive functions, specifically spatial working memory, as measured by the Y-maze test (Ahmadi et al. 2025b). Interestingly, a 30-day withdrawal phase partially, but not completely, reversed the morphine-induced attenuation of anti-nociception and impairment of spatial working memory (Ahmadi et al. 2024a, b). Accumulating

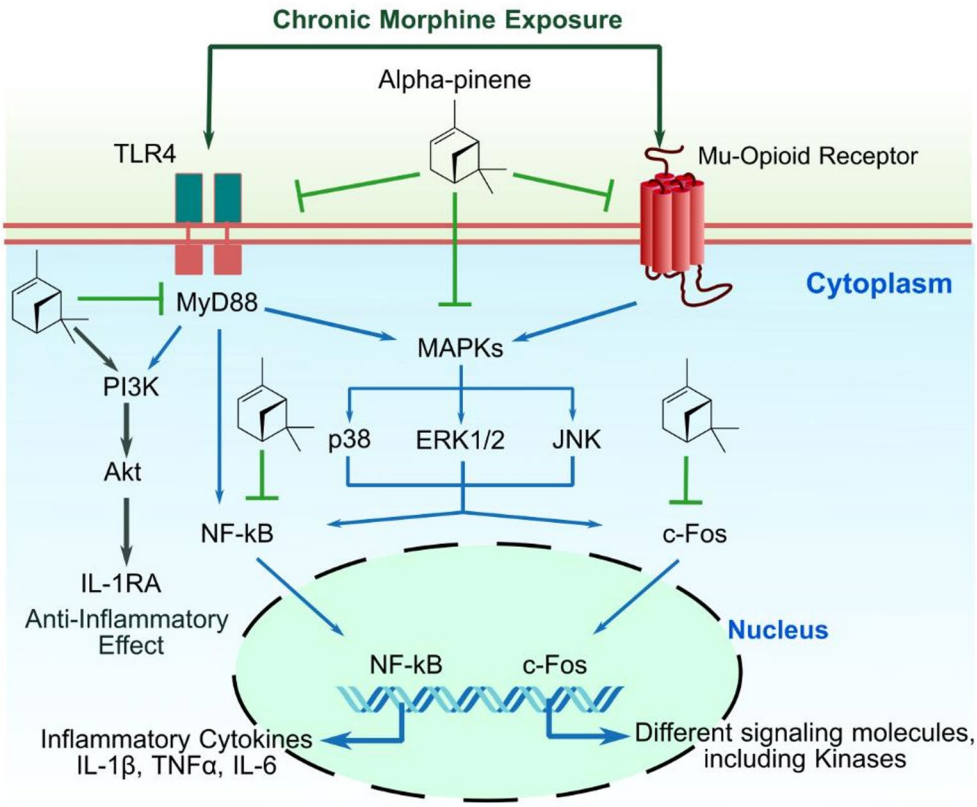

Fig. 8 Possible effects of APN on inflammatory signaling pathways involved in chronic CNS effects of morphine. Chronic morphine exposure shifts mu-opioid receptor signaling towards the activation of MAPKs, ultimately leading to the activation of transcription factors such as NF-κB and c-Fos. This affects gene expression patterns linked to neuroinflammation and cognitive decline. Furthermore, morphine can activate MyD88-dependent signaling pathways by binding to TLR4, which through NF-κB activation drives the transcription of inflammatory cytokines. MyD88 can also activate the PI3K/Akt pathway, leading to the recruitment of anti-inflammatory effects via IL-1RA in special situations (Ahmadi et al. 2025a). APN, as a lipophilic molecule, can affect neurons by modulating both transmembrane receptors and intracellular signaling pathways. APN likely inhibits the activation of various kinases and transcription factors to induce anti-inflammatory effects in the hippocampus

data propose that the activation of glial cells and subsequent neuroinflammation induced by chronic morphine exposure underlies, at least partly, the neuroadaptations associated with OUDs (Hutchinson et al. 2008; Osmanlıoğlu et al. 2020; Zhang et al. 2024; Zhou et al. 2021). There is a growing body of evidence in favor of anti-inflammatory effects of APN in animal models of epilepsy, stroke, etc. (Bakhtazad et al. 2024; Hashemi et al. 2025; Khoshnazar et al. 2020). We have recently found that APN prevents increases in proinflammatory cytokines and improves spatial working memory impairment in morphine-dependent and withdrawing rats (Ahmadi et al. 2026). APN treatment, whether co-administered with morphine or given during a 30-day withdrawal, affected hippocampal TLRs/MyD88/PI3K/AKT1B pathways (Ahmadi et al. 2025a). However, the effects of APN on morphine-induced changes in hippocampal MAPK signaling, both during chronic exposure and after prolonged withdrawal, remain unknown. In line with our previous reports, in the present experiments we examined changes in hippocampal MAPKs expression and phosphorylation as well as c-Fos expression after either the 10 days of initial morphine exposure or following the 30-day withdrawal phase, with or without APN co-treatment. Figure 8 integrates these findings and outlines how APN may mitigate the inflammatory signaling pathways associated with chronic morphine exposure in the CNS.

The current results revealed no significant alterations in hippocampal p38 MAPK expression in the morphine-dependent group. APN co-treatment during the 10-day morphine regimen had no significant effect on p38 expression. However, the results indicated that p38 expression increased after the withdrawal phase, but was almost fully restored to baseline levels by APN treatment during that phase. The present findings also indicated significant increases in hippocampal p-p38 levels both after dependence to morphine and following the drug withdrawal. Although the combination treatment of APN with morphine over the 10 days of repeated injections significantly decreased the elevated hippocampal p-p38 levels, they remained higher than control levels. Nevertheless, in the group that received APN during the 30-day withdrawal period, p-p38 levels returned completely to control levels.

The present findings also demonstrated that there were no significant changes in protein expression of ERK1/2 and JNK in the hippocampus both after chronic exposure to morphine and following the prolonged withdrawal. However, repeated morphine exposure led to markedly elevated hippocampal p-ERK1/2 and p-JNK levels, and this effect persisted despite APN administration during the 10-day repeated injection protocol. Interestingly, compared to the morphine withdrawal group, APN treatment for 30 days

during withdrawal led to an almost complete reversal of the heightened hippocampal p-ERK1/2 and p-JNK levels. The current experimental data also revealed upregulation of c-Fos protein in the hippocampus under conditions of both chronic morphine exposure and subsequent to the 30-day withdrawal period. However, when APN was administered either concurrently with morphine over the 10-day repeated injection phase or during the 30-day withdrawal period, it nearly restored the heightened hippocampal c-Fos expression to levels comparable to those in groups not treated with APN.

The challenges of morphine dependence and withdrawal highlight the urgent need to develop new treatments that can alleviate their adverse consequences (Benyamin et al. 2008; Listos et al. 2019). The exact molecular mechanisms underlying neuroadaptation associated with morphine dependence and withdrawal remain elusive. Some research has shown changes in expression of mu-opioid receptors and downstream signaling pathways in both phenomena (Badshah et al. 2023; Zhou et al. 2021). Consequently, the primary approved pharmacotherapies for OUD and management of relapse are opioid agonists, such as methadone and buprenorphine (Oakley et al. 2021; Saxon et al. 2013). Furthermore, accumulating data suggests that chronic morphine exposure activates MAPKs, including p38, ERK1/2, and JNK, in both the peripheral nervous systems (PNS) and CNS (Chen and Sommer 2009). Previous studies indicate that chronic morphine exposure increases the phosphorylation of MAPKs in dorsal root ganglion neurons, contributing to the development of tolerance to opioid-induced anti-nociceptive effects (Ma et al. 2001). While the exact mechanism is not fully understood, recent evidence suggests that MAPK activation induced by long-term morphine exposure may contribute to morphine's side effects by influencing proinflammatory cytokine production (Yang et al. 2023). Research indicates that preventing neuroinflammation and MAPK activation is a promising therapeutic strategy for morphine addiction, as demonstrated by studies in which MAPK inhibitors attenuated morphine tolerance and dependence in rodent models (Chen and Sommer 2009; de Freitas et al. 2019). Given the well-documented anti-inflammatory effects of APN in various experimental models, it is a potential candidate for preventing morphine addiction and withdrawal. However, the role of APN in preventing MAPK activation in rodent models of addiction remains poorly understood. Our findings therefore represent the first report to demonstrate this effect.

Studies have shown that APN suppresses MAPKs and the nuclear factor-kappa B (NF-κB) pathway to exert its anti-inflammatory effects in mouse peritoneal macrophages (Kim et al. 2015). Additionally, studies have reported that a cannabis oil extract containing monoterpenes such as APN inhibited the phosphorylation of MAPKs in a rat model of formalin-induced paw edema (Shebaby et al. 2021). Research indicates that the activation of p38 and JNK MAPKs was blocked by APN in IEC-6 cell line (Bouzenna et al. 2017). We have recently shown that APN treatment during either chronic morphine exposure or over a 30-day withdrawal period restores the elevated levels of proinflammatory cytokines and NF-κB expression in the hippocampus (Ahmadi et al. 2026). Our previous findings further indicate that APN acts via toll-like receptors and their downstream signaling pathways, including MyD88 and PI3K/Akt (Ahmadi et al. 2025a). The present findings confirms that APN affects signaling cascades that ultimately results in preventing activation of MAPK signaling pathways. Furthermore, long-term morphine use is known to increase the expression of the transcription factor c-Fos, which regulates numerous genes implicated in addiction (Crews et al. 2011). We therefore propose a cascade whereby elevated c-Fos expression, induced by chronic morphine exposure and subsequent withdrawal, leads to increased activity in kinase pathways in the hippocampus, subsequently promoting MAPK phosphorylation. Our results suggest that APN suppresses this hippocampal MAPK phosphorylation, at least in part, by inhibiting c-Fos, an effect that is particularly evident following a 30-day withdrawal period. Some limitations of this study warrant more attention for future researches.

Alongside the novel effects of APN on MAPK and c-Fos expression—whether co-administered with morphine or given during withdrawal—some study limitations should be noted. First, the study was conducted exclusively on male rats, so the results may not fully reflect sex-specific responses. Second, the sample size limits the robustness of normality testing and ANOVA assumptions. Third, the molecular effects of long-term APN exposure without morphine were not assessed; therefore, APN-specific baseline effects cannot be excluded. Although behavioral neutrality was previously observed in APN-treated animals (Ahmadi et al. 2026), this does not guarantee molecular neutrality. Consequently, investigating APN-specific molecular effects in the hippocampus of morphine-dependent and withdrawing rats remains a key objective for future research.

Conclusion

Increases in the phosphorylation of hippocampal MAPKs (p38, ERK1/2, and JNK) and c-Fos expression following repeated morphine exposure and a subsequent 30-day withdrawal period may underlie negative outcomes of OUD. Treatment with APN during morphine exposure, and particularly during the withdrawal phase, partly attenuated these molecular changes. These results indicate that APN may contribute to the modulation of MAPK and c-Fos signaling in the hippocampus of morphine-dependent and withdrawn rats. By integrating our

present and previous findings with the existing literature, we propose a model wherein APN may prevent neuroinflammation by concurrently modulating multiple pathways, including TLRs, NF-κB, MAPK, c-Fos, and proinflammatory cytokines. The potential involvement of additional signaling cascades cannot be excluded and warrants further investigation. These conclusions, however, should be interpreted in light of the study's limitations.

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Data availability The corresponding author can be contacted with a legitimate request for the data.

Declarations

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