Repeated use of morphine induces anxiety via affecting a proinflammatory cytokine signaling pathway in the prefrontal cortex in rats

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Research Article

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Abstract

We examined the involvement of toll-like receptors (TLRs) and proinflammatory cytokine signaling pathways in the prefrontal cortex (PFC) in anxiety-like behaviors after repeated use of morphine. Morphine dependence in male Wistar rats was induced via twice-daily morphine injection (10 mg/kg) for eight days. On day 8, opioid dependence was confirmed by measuring morphine withdrawal signs precipitated with naloxone. On days 1 and 8, anxiety-like behaviors were evaluated using a light/dark box test. On day 8, TLR1 and 4, proinflammatory cytokines, and some of the downstream signaling molecules were evaluated at mRNA and protein levels in the PFC. The results revealed that morphine caused anxiolytic-like effects on day 1, which significantly decreased after eight days of the repeated injection. On day 8, a significant decrease in TLR1 expression in the PFC was detected in morphine-dependent rats but TLR4 remained unaffected. Repeated morphine injection significantly increased the IL-1β, TNFα, and IL6 expression but decreased IL1R and TNFR at mRNA and protein levels except for IL6R at the protein level in the PFC. The p38α mitogen-activated protein (MAP) kinase expression significantly increased but the JNK3 expression decreased in the PFC of morphine-dependent rats. The NF-κB expression also increased significantly in the PFC after repeated injection of morphine. Significant increases in Let-7c, mir-133b, and mir-365 were also detected in the PFC in morphine-dependent rats. We conclude that TLR1 and proinflammatory cytokines in the PFC via a MAP kinase/NF-κB pathway may partly underlie the decrease in the anxiolytic-like effect of morphine in dependent rats.

Introduction

Opioids, such as morphine are mainly used for pain control but their prolonged use not only increases the risk of analgesic tolerance but also induces dependence [1, 2]. Although acute morphine administration has anxiolytic-like effects [3] chronic use of the drug induces anxiety [4]. Neuronal alteration and adaptations at both cellular and molecular levels in neurons and synapses at different levels of the nervous system may underlie tolerance, dependence, and anxiety induced by morphine [5–7]. It is well-known that the rewarding properties of morphine leading to drug dependence are mediated through disinhibition of the mesolimbic dopaminergic pathway originating from the ventral tegmental area (VTA) projecting to some forebrain areas, including the nucleus accumbens (NAc), the prefrontal cortex (PFC), and the hippocampus [8, 9]. The PFC includes the anterior portion of the frontal lobe that has executive functions such as planning, problem-solving, and social control [10, 11]. The PFC via descending excitatory glutamatergic projections modulates the processing of reward stimuli in the VTA and the NAc [12, 13]. The PFC not only is important in executive functions but also has a prominent role in pain processing [10].

Numerous studies have also shown that the PFC via having interconnections with structures including the amygdala, the bed nucleus of the stria terminalis, and the ventral hippocampus involved in information processing related to anxiety [14, 15]. Neural circuitry and molecular profiles of the PFC can be changed by experiences, which influence its functions [16]. In particular, the PFC is directly and
indirectly affected by repeated use of morphine (Koob and Volkow, 2010; Quirion et al., 1983), which may in turn underlie cognitive and behavioral side effects of morphine, including anxiety.

The actions of morphine on neurons mainly are mediated by mu-opioid receptors [17, 18]. However, a growing body of evidence shows that morphine also activates glial cells, including microglia and astrocytes [19–21]. Morphine affects glial cells by binding to immune cells’ toll-like receptors (TLRs) especially TLR4 leading to the release of proinflammatory cytokines, which in turn affect the neuronal transmission and plasticity involved in opioid functions [21, 22]. Based on the evidence in the literature of the past two decades, immune signaling is a significant contributor to the negative consequences of opioid therapy including hyperalgesia, tolerance, dependence, addiction, and withdrawal [19–23]. Liu et al. (2019) have reviewed the reciprocal relationship between abnormal expression of proinflammatory cytokines and induction of analgesic tolerance followed by chronic administration of morphine [24]. However, little is known about the role of proinflammatory cytokines and immune signaling in different brain areas especially in the PFC in anxiety induced by prolonged use of morphine.

We aimed to examine the expression of TLRs, proinflammatory cytokines, their receptors, and downstream signaling molecules in the PFC to assess their involvement in neural adaptation in the PFC underlying anxiety-like behaviors after repeated morphine injection in rats. Different microRNAs (miRNAs) have also been identified to be affected by chronic use of morphine [25, 26]. Based on target gene prediction for miRNAs, we also examined the expression of pre-miRNAs for let7-C1, mir-133b, and mir-365, which have a putative role to modulate the expression of inflammatory cytokine receptors.

Materials & Methods

Subjects

Thirty-two male Wistar rats (Rattus norvegicus) weighing 280 ± 20 g were used. Rats were kept in a colony room under constant temperature (22 ± 2 °C) and 12-12 h light/dark cycle (light off at 19:00). The animals had free access to food and water except for during the behavioral experiments. All efforts were made to minimize the number of animals used and their suffering. All procedures were done according to the Guide for the Care and Use of Laboratory Animals (2011) prepared by the National Academy of Sciences’ Institute for Laboratory Animal Research. The Research Ethics Committee (REC) at the University of Kurdistan approved the study protocol (IR.UOK.REC.1398.021).

Drug treatment

Every sixteen rats were randomly assigned to either a control saline-treated group or a morphine-treated group. Morphine sulfate (Temad, Daroopakhsh Co., Tehran, Iran) was dissolved in physiologic saline. The morphine-treated group received a subcutaneous injection of morphine (10 mg/ml/kg) twice a day for eight consecutive days. The control group received saline (1 ml/kg) instead of morphine during the eight days of the repeated injection.
Naloxone-precipitated withdrawal test

We assessed dependence to morphine by measuring the behavioral expression of morphine withdrawal precipitated with naloxone on day 8 of the injection. In two independent groups of animals, naloxone hydrochloride (1 mg/kg, i.p.) was injected 4 h after the injection of saline or morphine in the morning session on day 8 [27]. After the naloxone injection, each rat was immediately moved to a square clear Plexiglas chamber (40×40×40 cm high) and was videotaped for 15 min with two video cameras (Sony, Japan). Two classes of signs, including graded and checked signs, were evaluated and scored as described elsewhere (Table 1) [27, 28]. The somatic signs of withdrawal were recorded as the number of events observed during 15 min of the recorded experiment.

Table 1 Scoring of naloxone-precipitated withdrawal signs, including graded and checked signs

<table>
<thead>
<tr>
<th>Type of Signs</th>
<th>Symptoms</th>
<th>No.</th>
<th>Weighing Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graded Signs</td>
<td>Rearing</td>
<td>&lt;30</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30-40</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥ 40</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Jumping, Writhing, and Rubbing</td>
<td>1-4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5-9</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥ 10</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Wet-dog shake</td>
<td>1-2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥ 3</td>
<td>3</td>
</tr>
<tr>
<td>Checked Signs</td>
<td>Ptosis</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Diarrhea</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Chattering</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Genital grooming</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Irritability</td>
<td>+</td>
<td>3</td>
</tr>
</tbody>
</table>

Rearing: Lifting the forepaws off the ground and standing on the hind paws; Jumping: Attempt to escape out of the chamber; Writhing: Abdominal stretching; Rubbing: Moving the jaw or the torso on the ground; Wet-dog shake: Shaking of the whole body; Ptosis: Squinting of the eyes; Diarrhea: Watery or unshaped feces; Chattering: Teeth grinding or rapidly opening-closing of jaws; Genital grooming: Evidence of licking of the genital; Irritability: Attack or vocalization when touched at the end of the period.
Light and dark box test of anxiety

On days 1 and 8 of the injections, anxiety-like behaviors were examined with a light/dark box test of anxiety in two other experimental groups based on our previous report [29]. The apparatus consisted of a light chamber (30*30*24 cm) and a dark chamber (20*30*24 cm) made of white and dark Plexiglas, respectively. The floor of the chambers was marked off in nine and six squares (10 x 10 cm), respectively. A white light source (6 W) lit the light chamber, whereas the dark chamber was illuminated by a dim red-light source (3 W), which both of them placed 40 cm above the floor of the chambers. The dividing wall between the two chambers had also a 40 cm height to prevent the emission of the light sources into the opposite chamber. A connecting middle door (10 x 10 cm) on the floor level of the dividing wall permits voluntary transitions of the animals between the chambers. The floor and wall of both chambers were cleaned between trials with an ethanol solution (10% v/v) and dried fully before the testing. Thirty min after the drug treatments at the morning session on days 1 and 8, the light/dark test was performed. The test started when each rat was placed in the center of the white arena facing away from the middle door. Then, each rat was allowed for 5 min freely explore the apparatus, which simultaneously was videotaped through a video camera fixed above the apparatus (100 cm from the floor of the apparatus). Finally, the videos were analyzed by a researcher blinded to experimental groups and different parameters including 1) lightbox time spent, 2) the number of squares crossing as an index of locomotor activity, 3) the number of rearing behavior in the white chamber, and 4) the total crossing or transitions between the two chambers were evaluated [29-32].

Brain dissections

Two h after the performing light/dark box test on day 8, each rat was decapitated, the whole brain was quickly removed from the skull, and the bilateral PFCs were immediately dissected on an ice-chilled sterile surface [33-35]. Tissues were immediately frozen in liquid nitrogen and stored in a -80 freezer until used for molecular analysis.

Real-time polymerase chain reaction

Total RNA was extracted by using a commercial kit according to the manufacturer's supplied protocol (High Pure miRNA isolation kit, Roche, Germany). The total RNAs were reverse transcribed to complementary DNA (cDNA) by using a two-step cDNA synthesis kit according to the manual provided by the manufacturer (First Strand cDNA Synthesis Kit, Thermo Fisher Scientific, USA). Real-time PCR was performed using LightCycler 96 system thermal cycler (Roche, Germany). Each PCR reaction of 20 µl volume consisted of 10 µl YTA SYBR Green qPCR MasterMix (Yekta Tajhiz Azma Co., Tehran, Iran), 8 µl cDNA (5 ng/µl), 2 µl mix of forward and reverse primers (5 µM). Triplicate technical repeats were determined for each biological sample. Thermal cycling was initiated with a pre-incubation step (95°C for 30 Sec), followed by 40 cycles of two-step amplification (95°C for 5 Sec and 60°C for 30 Sec), followed by melting (95°C for 5 Sec, 60°C for 60 Sec, and 95°C for 1 Sec), and finalized by cooling at 50°C for 30 Sec. The $2^{-\Delta\Delta CT}$ method was used to assess relative gene expression [36]. Glyceraldehyde 3-phosphate
dehydrogenase (\textit{Gapdh}) was selected as a housekeeping gene and was used for normalization. The sequences of PCR primers and the respective amplicon sizes have been summarized in Table 2.

\textbf{Table 2} Primers used for real-time PCR
<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences (5′-3′)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
</table>
| Gapdh  | F: AGTGCCAGCCTCGTCTCATA  
R: GTAACCAGGCGTCCGATAC                                                               | 77                |
| Il1    | F: CAGGATGAGGACCCAAGC  
R: TCATCCCACGAGTCACAGAGG                                                             | 74                |
| Tnfa   | F: AAATGGGCTCCCTCTCATCAGTTTC  
R: TCTGCTTTGGTGTTTGGCTACGAC                                                            | 111               |
| Il6    | F: AGCGATGAGGACTGCTAGA  
R: GGAACTCCAGAAGACCAGAGC                                                              | 127               |
| Il1r1  | F: ACAGGGACTCTCTGCTCTGAT  
R: TCCCTCTCCGTAGGTCTTGG                                                             | 95                |
| Tnfrs1a| F: TACGGATCCCTCAACCTGTG  
R: CCACAGCATACAGCATGCGAG                                                            | 115               |
| Il6r   | F: CCATCAGGGTCCCATAACAGC  
R: TTGCTGTTGTCATTAGGGCAC                                                              | 95                |
| Tlr1   | F: CTGCTTTGGGGAGCAACAAC  
R: AACACTAGTGCCACAGTCGG                                                               | 130               |
| Tlr4   | F: CCCTGCCACCATTTACAGTTCG  
R: GAGTCCCAGCCAGTCAAGAG                                                              | 96                |
| p38α   | F: TGACGAAATGACCGGCTAC  
R: AGCCCACGGAACCAATATC                                                              | 104               |
| Jnk3   | F: GCTACAAGGAGAAGGCTTGAG    
R: ACGGAGCTCAGCTGCTCTA                                                               | 132               |
| Nfkb1  | F: TTACGGGAGATGTGAAGATG  
R: ATGATGGCTAAGTGAGGAC                                                              | 94                |
| Let7c1 | F: GTGCATCCGCGGTGGTAGT  
R: GCTCCAAGGAAAGCTAGAAGGT                                                             | 86                |
| mir-133| F: CTGCTCTGCTGCTAAACG  
R: CTGCTGTAGCTGGTGAGGG                                                             | 73                |
| mir-365| F: ACAGCAAGAAAAATGAGGGAC                                                          | 72                |
R: GGATTTTTAGGGGCATTATGAC

Gapdh: Glyceraldehyde-3-phosphate Dehydrogenase; Il1: Interleukin 1; Tnfa: Tumor necrosis factor α; Il1r1: Interleukin 1 receptor type 1; Tnfrs1a: TNF receptor superfamily member 1A; Tlr1: toll-like receptor 1; Tlr4: Toll-like receptor 4; p38α: p38α MAP kinase (MAPK 14); Jnk3: c-Jun N-terminal kinase 3 (MAPK 10); Nfkb: Nuclear factor kappa B subunit 1; mir-133b: pre-miRNA133b; mir-365: pre-miRNA-365.

Enzyme-linked immunosorbent assay (ELISA)

Ten mg of the dissected PFC from each rat in 100 µl phosphate buffer saline (PBS) containing protease inhibitor cocktail (Sigma-Aldrich, USA) was homogenized using an ultrasonic homogenizer (FAPAN 300UPS Ultrasonic Homogenizer, Fanavari Iranian Pajohesh Nassir, Iran). Then, the homogenate was centrifuged at 13000 g for 40 min, and the supernatant phase was transferred to a new tube. ELISA kits for interleukin 1-β (IL1-β), tumor necrosis factor-α (TNF-α), IL6, Phospho-JNK, and Phospho-P38α were used for the protein level assessments according to the manufacturer's instructions (DuoSet ELISA kits, R&D Systems, USA). Three technical repeats were assessed for eight biological samples in each experimental group. After plate preparation, 100 µl of the sample was added per well and incubated at room temperature for 2 h. The plates were then washed three times using a wash buffer (0.05% Tween 20 in PBS). Next, 100 µl of the detection antibody diluted in 1% bovine serum albumin (BSA) in PBS was added to each well and incubated at room temperature for 2 h followed by three times washing. Then, 100 µl of Streptavidin-HRP was added to each well and incubated for 20 min at room temperature followed by three times washing steps. After that, 100 µl of substrate solution was added to each well and incubated for 20 min at room temperature. Finally, reactions were stopped by adding 50 µl of a stop solution to each well. The optical density of each well immediately was determined using a microplate reader at 450 and 540 nm. Reading at 540 nm was subtracted from the reading at 450 nm to correct the optical imperfections in the plate. A standard curve was prepared using the standard solutions for each protein provided in the related kits, and the concentration of each protein in the samples was calculated according to the respective standard curve.

Western Blotting

The western blotting protocol was almost the same as a previous report from our lab [29]. In brief, twenty mg of the PFC from each rat was added to 200 µl RIPA lysis buffer (Abcam, U.S.A) and was homogenized to extract tissue total protein (FAPAN 300UPS Ultrasonic Homogenizer, Fanavari Iranian Pajohesh Nassir, Iran). The homogenate was centrifuged at 10000 g for 10 min, subsequently, the aqueous supernatant phase was moved to a new tube, and was stored in a -80°C freezer until further analysis. The protein concentration of the samples was evaluated using Bradford technique and a standard curve for BSA. Equal amounts of samples (20 µg/15 µl per lane) were subjected to SDS-PAGE electrophoresis (12% gel), then the blots were transferred to a nitrocellulose membrane on a semi-dry apparatus (Trans-Blot SD semi-dry transfer cell, BIORAD, USA). Next, blots were blocked in a 5% nonfat dry milk dissolved in Tris-Buffered Saline (TBS) containing 0.1% Tween 20 (TBST) for one h at 4°C. Primary mouse monoclonal antibodies for beta-actin, TLR1, TLR4, IL1R, TNFR, IL6R, and NF-κB (Santa Cruz Biotechnology, USA) were
diluted (1:1000) and were separately used on the independent membranes incubated at 4°C overnight. Then, the membrane was washed three times in TBST, and the second antibody (anti-mouse polyclonal HRP-conjugated antibody, 1:2000 dilution) was added to the membrane and incubated for one h at room temperature. Next, enhanced chemiluminescence (ECL) reagents (Santa Cruz Biotechnology, USA) were mixed and added to the membrane for 1 min followed by image capturing using a western blotting imaging system (Day Tadjhiz Aryan, Tehran, Iran). Finally, the bands’ densities were quantified using Image J software.

**Statistical analysis**

Normality of the data was evaluated with the Shapiro–Wilk test and equality of variance was assessed with the Brown-Forsythe test. All data passed normality and equal variance tests. A mixed between-within subject’s ANOVA with two factors, including “the drug” with two levels (saline and morphine) and “days of testing” with two levels (days 1 and 8) was performed for analyzing anxiety-like behaviors. Post hoc Tukey's test was used for pairwise comparisons. An unpaired two-tailed Student t-test was used to analyze naloxone precipitated withdrawal behavioral signs and gene expression as well as protein levels data. \(P < 0.05\) was considered a statistically significant level. Data analysis and plotting data were done using GraphPad Prism version 9.0. The corresponding author is responsible for data sharing upon reasonable request.

**Results**

_Naloxone precipitated withdrawal signs revealed induction of morphine dependence after 8 days of repeated administration of the drug_

On day 8 of the repeated injection, naloxone-precipitated withdrawal symptoms were assessed to confirm dependence to morphine in two independent experimental groups. The results of student t-test revealed significant increases in the number of all withdrawal graded signs, including locomotor activity \([ t (14) = 5.1, P < 0.001]\), rearing \([ t (14) = 9.6, P < 0.001]\), jumping \([ t (14) = 6.2, P < 0.001]\), writhing \([ t (14) = 9.8, P < 0.001]\), rubbing \([ t (14) = 11.3, P < 0.001]\), and shaking \([ t (14) = 5.6, P < 0.001]\), as well as the total withdrawal score \([ t (14) = 9.9, P < 0.001]\) in morphine-treated group compared with saline-treated control group (Fig. 1).

_Morphine induced anxiolytic-like effects on day 1, which were significantly decreased on day 8 of the repeated injection_

The light/dark box test is based on an approach-avoidance conflict between exploration of novel environments and avoidance of a brightly lit open space. In this test, a higher time spent in the lightbox is interpreted as lower anxiety in animals. The results revealed a significant interaction between morphine treatment and days of testing on the total time spent in the lightbox \([ F (1, 14) = 66, P < 0.001]\). The main effects for both factors on total time spent in the lightbox were also significant; for drug \([ F (1, 14) = 88, P < 0.001]\), and for days of testing \([ F (1, 14) = 139, P < 0.001]\). According to the post hoc Tukey’s test,
morphine-induced anxiolytic-like effects compared with the saline-treated group (P < 0.001) on the first day of the injection. However, repeated injection of morphine induced anxiety as revealed by a significant decrease in lightbox time spent compared with the saline-treated control group on day 8 (P < 0.001).

The results also revealed a significant interaction between both factors, including the drug and days of testing on the number of locomotor activity in the lightbox [F (1, 14) = 57, P < 0.001]. The main effects for both factors on locomotion in the light box were also significant; for drug [F (1, 14) = 91, P < 0.001], and for days of testing [F (1, 14) = 18, P < 0.001]. The results revealed no significant interaction between both factors on rearing behavior in the lightbox [F (1, 14) = 0.7, P > 0.05]. The main effect of days of testing on rearing behavior in the light box was also not significant [F (1, 14) = 4.4, P > 0.05], but the main effect of the drug on rearing behavior in the light box was significant [F (1, 14) = 14, P < 0.01]. The results also indicated a significant interaction between the morphine treatment and days of testing on crossing between light and dark boxes [F (1, 14) = 39, P < 0.001]. The main effects for both factors on crossing or transitions between both boxes were also significant; for drug [F (1, 14) = 20, P < 0.001], and for days of testing [F (1, 14) = 58, P < 0.001] (Fig. 2).

Repeated injection of morphine decreased TLR1 expression but had no significant effect on the TLR4 expression in the PFC

We examined the expression of TLR1 and TLR4 at mRNA and protein levels in the PFC after 8 days of the repeated injection of morphine. Analysis of the qPCR results revealed that the Tlr1 gene expression significantly decreased [t (14) = 3.8, P < 0.01] but no significant alteration was detected for Tlr4 gene expression [t (14) = 0.4, P > 0.05] in the PFC in morphine-treated group compared with saline-treated control group. Interestingly, the western blotting results revealed a significant decrease in TLR1 protein level [t (6) = 6.66, P < 0.001] but no significant alteration was detected for TLR4 protein level [t (6) = 0.3, P > 0.05] (Fig. 3).

Repeated injection of morphine increased IL1-β, TNF-α, and IL6 expression in the PFC

We examined expression of proinflammatory cytokines, including Il1, Tnfa, and Il6 in the PFC after 8 days of the repeated injection of morphine. Analysis of the qPCR data revealed significant increases in the gene expression of Il1 [t (14) = 13.04, P < 0.001], Tnfa [t (14) = 5.4, P < 0.001], and Il6 [t (14) = 5.3, P < 0.001] compared with the saline-treated control group. The ELISA results assessing protein levels of the proinflammatory cytokines also revealed significant increases in protein levels of IL1-β [F (1, 14) = 13.2, P < 0.001], TNF-α [F (1, 14) = 9.3, P < 0.001], and IL6 [F (1, 14) = 7.1, P < 0.001] in the PFC in morphine-dependent rats compared with the saline-treated control group (Fig. 4).

Repeated injection of morphine decreased IL1R, TNFR, and IL6R expression in the PFC

The qPCR results revealed significant decreases in the gene expression of Il1r [t (14) = 6.2, P < 0.001] and Tnfr [t (14) = 5.5, P < 0.001]; however, the Il6r gene expression significantly increased [t (14) = 4.7, P < 0.001] in morphine-treated group compared with the saline-treated control group. In addition, the western
blotting results revealed that IL1R \( t (6) = 7.8, P < 0.001 \) and TNFR \( t (6) = 4.45, P < 0.001 \) protein levels significantly decreased but no significant alteration detected for protein level of IL6R \( t (6) = 0.66, P > 0.05 \) in the PFC in morphine-dependent rats compared with the saline-treated control group (Fig. 5).

**p38-α MAP kinase expression increased but the JNK3 expression decreased in the PFC after repeated injection of morphine**

Protein kinases are considered key modulators of the cellular adaptations associated with opioid tolerance and dependence [37]. We examined the expression of p38α and JNK3 MAP kinases in the PFC after 8 days of repeated administration of morphine. Analysis of the qPCR results revealed a significant increase in the \( p38\alpha \) gene expression \( t (14) = 13.5, P < 0.001 \) and a significant downregulation in the \( Jnk3 \) gene expression \( t (14) = 6.2, P < 0.001 \) in rats treated with morphine compared with saline-treated group. In line with the gene expression results, the results of ELISA also revealed a significant increase in Phospho-p38-α \( t (14) = 5.8, P < 0.001 \) and a significant decrease in phospho-JNK protein levels \( t (14) = 8.88, P < 0.001 \) in the PFC in morphine-dependent rats compared with the saline-treated control group (Fig. 6).

**Repeated injection of morphine increased the nuclear factor- kappa B (NF-κB) expression in the PFC**

A growing body of research shows that the effects of chronic morphine treatment on gene expression are mediated by alterations in transcription factors [38, 39]. We assessed the expression of NF-κB in the PFC after 8 days of repeated injection of morphine. The results revealed significant upregulation in the \( Nfkb \) gene expression \( t (14) = 6.3, P < 0.001 \) and protein level of NF-κB \( t (6) = 5.8, P < 0.001 \) in the PFC after repeated injection of morphine compared with the saline-treated group (Fig. 6).

**Expression of Let-7c1, mir-133b, and mir365 increased in the PFC after repeated injections of morphine**

Chronic morphine treatment affects miRNAs expression, which in turn can influence tolerance and addiction to morphine. We assessed the expression of three pre-miRNAs, including \( \text{Let-7c1, mir-133b, and mir-365} \) in the PFC on day 8 of the repeated injection. The qPCR results revealed significant increases in the expression of \( \text{Let-7c1} \) \( t (14) = 10.4, P < 0.001 \), \( \text{mir-133b} \) \( t (14) = 10.1, P < 0.001 \), and \( \text{mir-365} \) \( t (14) = 10.9, P < 0.001 \) in the PFC after repeated injection of morphine compared with the saline-treated group (Fig. 7).

**Discussion**

The present results indicated that repeated administration of morphine induced drug dependence as revealed by significant withdrawal signs induced by naloxone precipitation on day 8 of the repeated injections of morphine. Morphine is still the first choice for controlling chronic pain in the clinic. Unfortunately, besides tolerance to the analgesic effect of morphine and dependence to the drug, anxiety has been described as an important comorbidity in patients suffering from chronic pain [40]. However, the exact mechanisms of the anxiety induced by prolong morphine treatment remain elusive. The present
results revealed that morphine induces anxiolytic-like behaviors on day 1 of the repeated injection as revealed by a significant increase in lightbox time spent compared with the saline-treated rats. On day 1, consistent with the increased lightbox time spent, the locomotor activity of the animals in the lightbox significantly increased compared with the saline-treated control group. This significant increase in locomotion in the lightbox on day 1 relatively discards the possibility of freezing behavior in the lightbox and further supports the anxiolytic-like effects of morphine on day 1. However, rearing behavior in the lightbox and crossing between two chambers showed a tendency to increase in morphine-treated animals compared with the saline-treated animals but no significant alteration was detected on day 1. On contrary, repeated morphine injection induced anxiety-like behaviors on day 8 of the treatment as revealed by significant decreases in lightbox time spent and locomotion in the light chamber compared with the saline-treated animals. However, on day 8, the number of crossings between light and dark chambers in the morphine-treated group was significantly higher than its respected number on day 1, which may also reflect an increase in anxiety or a decrease in the anxiolytic-like effects after 8 days of repeated injection of morphine due to approach-avoidance conflict between exploration of both chambers.

According to research, morphine induces anxiolytic-like properties during initial exposure [41]. However, other investigators in line with the present results have reported anxiety-like behaviors after chronic administration of morphine [42, 43]. Therefore, besides depression and cognitive dysfunctions, anxiety-like behaviors can be considered among undesired outcomes of dependence to morphine [44–46]. The amygdala functions to detect the threat and generate emotions such as fear and anxiety, whereas the PFC and hippocampus function to downregulate the amygdala [47]. Exposure to drugs of abuse may cause a reorganization of molecular patterns in the PFC that alter the responsiveness of the PFC to reward inputs and also the firing of neurons projected from the PFC to the other limbic structures such as the amygdala [48, 49]. Therefore, further analyses of molecular pathways in the PFC are important to understand the mechanisms resulting in the development of morphine dependence and the decrease in its anxiolytic-like effects after prolonged use of the drug. Activation of glia by morphine via binding to TLRs has attracted considerable attention to explain, at least partly, non-neuronal mechanisms of the induction of tolerance and dependence to morphine [20–22, 50]. However, little is known about the involvement of neuroinflammation pathways upon activation of TLRs and production of inflammatory cytokines in anxiety-like behaviors after repeated injection of morphine.

The involvement of TLR2 and TLR4 in the development of morphine tolerance and dependence has been previously reported [21, 51]. Zhang et al. (2011) reported that lacking TLR2 in mice inhibited morphine-induced microglial activation and the levels of proinflammatory cytokines [51]. However, there are not considerable reports on the involvement of TLR1 mediating morphine functions. The present results indicated significant downregulation of TLR1 in the PFC after prolonged morphine injection for 8 days. However, no significant alteration was detected for the TLR4 expression at mRNA and protein levels after repeated injection of morphine. Initiation of TLR's signaling involves a series of protein conformational changes initiated by dimerization of their extracellular domains [52]. There is considerable data that TLR2 forms heterodimers with other TLRs such as TLR1 and upon stimulation by the specific ligand, these heterodimers are recruited [53]. Therefore, one possible explanation for the alteration in TLR1 in the PFC
is its heterodimerization with TLR2 to initiate intracellular signals leading to the production of proinflammatory cytokines. However, we did not examine TLR2 levels, and its association with the effects of chronic morphine treatment in the PFC needs further investigation. We suggest that the downregulation of TLR1 is a homeostatic response to the chronic morphine treatment. Taken together, the present results may support the involvement of TLR1 in neuroadaptation in the PFC after repeated morphine treatment, which may underlie subsequent behavioral outcomes in morphine-dependent rats.

The expression results also revealed significant increases in IL1-β, TNF-α, and IL6 expression at mRNA and protein levels in the PFC after repeated morphine treatment compared with the saline-treated group. On contrary, IL1R and TNFR were downregulated at both mRNA and protein levels but IL6R was upregulated at mRNA level but remained unchanged at the protein level in the PFC in morphine-dependent rats compared with the saline-treated group. It has been shown that prolonged administration of morphine is followed by less effective opioid actions [54]. Accumulating data shows that the ineffectiveness of morphine results from adaptation within opioid receptor-expressing neurons in the reward pathways. However, this adaptation is not only restricted to neurons but also to activation of glial cells by morphine, and subsequent production of proinflammatory cytokines is considered as an alternate process affecting morphine actions. Therefore, the role of glia and activation of TLRs in the development of morphine tolerance and addiction has been postulated during the past two decades and has been also recently reviewed [20, 22]. Based on the present results, it is logical to propose that repeated morphine treatment activates glial cells in the PFC as revealed by upregulation of the proinflammatory cytokines. On the other hand, downregulation of IL1R and TNFR may serve as homeostatic adaptations in response to the increased level of the respective cytokine ligands. The central immune signaling profoundly affects behavioral responses in the brain [50]. Therefore, the anxiety-like behaviors after repeated use of morphine may result, at least partly, from the altered cytokine levels and their contribution to neuronal excitability in the PFC. In support of this suggestion, it has been reported that IL-1β can, directly and indirectly, increase NMDA receptor activity, which may also contribute to neuroexcitation [50].

A growing body of evidence shows that chronic morphine administration influences different protein kinases, including MAP kinases [37]. We examined the expression of p38α and JNK3 MAP kinases in the PFC after repeated injection of morphine. The results revealed that p38α expression significantly increased but JNK3 expression significantly decreased at both mRNA and protein levels in the PFC in morphine-treated animals. The present data support the involvement of p38α and JNK3 MAP kinases in neuronal adaptations after repeated morphine treatment in the PFC [55]. Chronic exposure to morphine leads to phosphorylation and binding of arrestin protein to the opioid receptors [56]. Phosphorylated arrestin-bound opioid receptors in neurons recruit alternate downstream signaling cascades, including the MAP kinase cascade [57, 58], which may regulate transcription factors, ion channels, and neurotransmitter release [59]. A clear role for JNK signaling pathways in morphine tolerance has been previously reported [60]. Inhibition of the JNK signaling pathway delays tolerance to the antinociceptive effects of chronic morphine in diverse pain models [61]. Therefore, chronic administration of morphine via a MAP kinase cascade may alter the expression of proinflammatory cytokines, which in turn via
affecting neurotransmission and firing of the PFC outputs may affect anxiety-like behaviors in morphine-dependent animals.

There is some evidence that p38 and JNK are involved in delayed hyperalgesia induced by the opioid [62]. There is not enough report on the role of JNK3 in the PFC in behavioral alteration after morphine treatment. We have reported recently the downregulation of Jnk3 in the PFC in a rat model of hepatic encephalopathy [63]. Since the role of neuroinflammation has been verified in hepatic encephalopathy, the decreased JNK3 expression in the PFC in the present study may indirectly reflect induction of neuroinflammation after repeated injection of morphine. Considering the increased expression of inflammatory cytokines in the present study, the downregulation of JNK3 may play as a compensatory response to prevent further neuroexcitation induced by cytokines.

Activation of p38 MAP kinase in the spinal microglia mediates morphine antinociceptive tolerance [64], and its inhibition prevents the antinociceptive tolerance induced by the drug [65]. p38 MAP kinase signaling pathway in the NAc is also involved in the induction of conditioned place preference [66]. However, little is known about the role of p38α MAP kinase in the PFC in anxiety-like behaviors after prolonged treatment of morphine. It is possible that inhibition of cytokine signaling and especially the p38 MAP kinase pathway also be useful in the prevention of dependence to morphine and alleviating anxiety-like behaviors after repeated use of the drug.

The present results also revealed that NF-κB expression in the PFC increased after repeated injections of morphine. Activation of the p38/NF-κB signaling pathway in the NAc plays a critical role in morphine-conditioned place preference [67]. The expression of proinflammatory cytokines genes is dependent on the activation of NF-κB. Therefore, it is logical to propose that the increase in the expression of NF-κB is in line with the increased level of proinflammatory cytokines seen in the present experiments.

miRNAs have been reported to be involved in morphine analgesic tolerance and addiction [68, 69]. The present results also revealed that expression of pre-miRNAs, including Let-7c1, mir-133b, and mir-365 were significantly upregulated in the PFC after induction of morphine dependence. The Let-7 family of miRNAs has a critical regulatory role in opioid tolerance [70]. Consistent with the present results, He et. al. (2010) reported that morphine significantly upregulated let-7 expression in SH-SY5Y cells and a mouse model of opioid tolerance [71]. Mir-133b via affecting dopaminergic neurons may be a regulatory mechanism for the development of addiction to morphine [72, 73]. However, there is no report on mir-133b expression in the PFC after repeated use of morphine. Considering the putative targets of mir-133b and mir-365 such as IL1R and TNFR family receptors based on TargetScan target gene prediction, we suggest that the increased level of mir-133b and mir-365, at least partly, be involved in the downregulation of these receptors in the PFC. However, further experiments may require clarifying the exact effects of these miRNAs on the expression of the proposed targets, and also affecting other target genes cannot be excluded, which needs further experiments in the future.

Conclusions
The present data revealed that prolonged morphine injection not only induced dependence to the drug but also caused anxiety. An increase in proinflammatory cytokines via a MAP kinases/NF-κB signaling pathway in the PFC leads to a shift in the PFC's normal functions. A top-down neural system originating from the PFC projecting to some other areas such as the amygdala and the ventral hippocampus may in turn affect emotional processing such as anxiety in morphine-dependent animals.

**Declarations**

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**Competing interests**

The authors have no relevant financial or non-financial interests to disclose.

**Authors contribution**

**Shamseddin Ahmadi**: Conceptualization and design of the work, Writing- Original Draft Preparation, Writing- Reviewing and Editing, Supervision, Project Administration, and Funding Acquisition. **Mohammad Zobeiri, Shiva Mohammadi Talvar, Kayvan Masoudi**: Acquisition, analysis, interpretation of data, and Writing- Original Draft Preparation. All authors approved the final version for publication.

**Data availability**

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval**

All procedures were done according to the Guide for the Care and Use of Laboratory Animals (2011) prepared by the National Academy of Sciences' Institute for Laboratory Animal Research. The Research Ethics Committee (REC) at the University of Kurdistan approved the study protocol (IR.UOK.REC.1398.021).

**Consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

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**Figures**

Figure 1

Repeated injection of morphine for eight days induced dependence to the drug. On day 8 of the repeated injection, naloxone precipitated withdrawal symptoms were assessed immediately after injection of naloxone (1 mg/kg, i.p.). Naloxone was injected 4 h after the injection of saline or morphine in the morning session. Each bar represents the mean ± SD of the specified sign of withdrawal in each group. Circle dots and triangles on the bars represent the individual data in each group. *** $P < 0.001$ compared with the saline-treated control group.
Repeated injection of morphine induced anxiety-like behaviors. Two experimental groups (n = 8) received either saline (1 ml/kg) or morphine (10 mg/kg) twice a day for eight consecutive days. Anxiety-like behaviors using a light/dark box test, including lightbox time spent, locomotor activity, rearing, and crossing or transitions between the two light and dark chambers were assessed on days 1 and 8 of the injections. Each bar represents the mean ± SD of the specified behavior in each group on a respective day. Dots and triangles on the bars represent the individual data in each group. ** $P < 0.01$ and *** $P < 0.001$ compared with the specified group.
Figure 3

Expression of Toll-like receptors in the PFC after the induction of morphine dependence. Expression of TLRs at mRNA and protein levels were examined in the PFC on day 8 of the repeated injection. Each bar in the upper graphs represents the mean ± SD of the gene expression data (n= 8) in each experimental group. The middle panel shows western blotting images for evaluating TLR1 and TLR4 protein levels in both experimental groups. The graphs in the lower panel represent the densitometries data of protein levels (n = 4) in both experimental groups. Circle dots and diamond shapes on the bars represent the individual data in each group. ** \( P < 0.01 \) and *** \( P < 0.001 \) compared with the control group, and ns: non-significant.
Figure 4

Expression of proinflammatory cytokines in the PFC after morphine dependence. The graphs in the upper panel show the gene expression results but the graphs in the lower panel represent the ELISA results of proinflammatory cytokines levels in the PFC. Each bar represents the mean ± SD of the expression data (n = 8) in each experimental group. Circle dots and diamond shapes on the bars represent the individual data in each group. *** $P < 0.001$ compared with the control group.
Figure 5

Expression of cytokine receptors in the PFC after morphine dependence. The graphs in the upper panel show the gene expression results but the graphs in the lower panel represent the western blotting data of cytokine receptors in the PFC. The right panel represents western blotting images of cytokine receptor protein levels in both experimental groups. Each bar in the graphs represents the mean ± SD of either the gene expression data (n = 8) or protein level (n = 4) in each experimental group. Circle dots and diamond shapes on the bars represent the individual data in each group. ** P < 0.01 and *** P < 0.001 compared with the control group, and ns: non-significant.
Figure 6

Expression of p38α and JNK3 MAP kinases, and NF-κB in the PFC after the induction of morphine dependence. The graphs in the upper panel show the gene expression results but the graphs in the lower panel represent either the ELISA results for phospho-P38α and phosphor-JNK or the western blotting data of NF-κB in the PFC. The right panel represents western blotting images of NF-κB protein levels in both experimental groups. Each bar in the graphs represents the mean ± SD of either the gene expression data (n = 8) in the upper panel. Each bar in the graphs of the lower panel represents the mean ± SD of either the ELISA results (n = 8) or the western blotting result (n = 4). Circle dots and diamond shapes on the bars represent the individual data in each group. NS indicates no significant statistical group difference. ** $P < 0.01$ and *** $P < 0.001$ compared with the control group.
Figure 7

Effect of repeated morphine injection on the expression of Let7c1, mir-133b, and mir-365 in the PFC. The expression of three pre-miRNAs, including let-7c, mir-133b, and mir-365 were examined in the PFC on day 8 of the repeated injection. Each bar represents the mean ± SD of the specified pre-miRNA expression in each experimental group. Circle dots and diamond shapes on the bars represent the individual data in each group. *** $P < 0.001$ compared with the control group.