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ORIGINAL PAPER

NGF, BDNF and Arc mRNA Expression in the Hippocampus of Rats After Administration of Morphine

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Abstract

Morphine can influence immediate early genes (IEG) of activity-regulated cytoskeletal-associated protein (Arc) and brain-derived neurotrophic factor (BDNF) which are activated in response to physiological stimuli during learning, as well as the nerve growth factor (NGF) gene which increases the expression of several IEGs for memory formation. The purpose of the current study was first to evaluate the effect of acute (1-day) and subchronic (15-days) morphine administration on memory retrieval of rats and second to determine the hippocampal expression of NGF, BDNF and Arc genes as potential contributors in the observed effects in each setting. The effects of morphine (intraperitoneal, 10, 15 and 20 mg/kg) on memory function and gene expression were assessed using inhibitory avoidance test and real-time polymerase chain reaction, respectively. We found that a single dose of morphine at the highest dose of 20 mg/kg decreases the post-training step-through-latency, while repeated administration of the same dose for 15 successive days increases this indicator of memory retrieval. We did not detect a significant change in the hippocampal expression of Arc, BDNF or NGF genes after a single episode of morphine treatment. However, subchronic morphine administration (15 and 20 mg/kg) increased the expression of Arc and BDNF genes in a dose dependent manner. A higher mRNA expression for the NGF was observed at the higher dose of 20 mg/kg. We hypothesize that the subchronic effects were morphine-induced behavioral sensitization which may have been enhanced through increased hippocampal Arc expression.

Keywords Morphine · NGF gene · BDNF gene · Arc gene · Rat · Memory

Introduction

Opioids have key roles in several physiologic functions including pain, perception, respiration, immune system, food intake and reward [1, 2]. Three types of opioid receptors, mu, delta, and kappa, are expressed in various areas of the brain such as the hippocampus [3, 4]. Mu receptors have a critical role in the rewarding effect of opioids [5] while delta receptors mediate neuronal plasticity and adaptive responses in drug addiction and drug sensitization [6]. Morphine is a highly abused substance which affects the neurobiology of major psychiatric diseases such as mood disorders and addiction. Several studies have demonstrated that morphine facilities memory [7, 8], and its repeated administration induces adaptive responses and learning [9]. Moreover, previous use of addictive drugs has been shown to increase the sensitivity to repeated administration effects [10]. On the contrary, some studies showed that morphine impairs the learning and memory in both human and animals [11]. These conflicting results depend on the dosing regimen and arise from various phenomena such as tolerance, dependence, withdrawal, and sensitization for which the understood underlying mechanisms are poorly understood.

Immediate early genes (IEG) are referred to those genes that are rapidly activated in response to an extracellular stimulus and play a key role in long-term potentiation (LTP) which is considered as the cellular correlate of learning and memory [12–14]. The activity-regulated cytoskeletal-associated protein (Arc) and neurotrophin brain-derived neurotrophic factor (BDNF) genes belong to a subgroup of IEGs, both with proven roles in memory formation [15].

ARC has a specific synaptic function in both LTP and memory, and overexpresses following learning experience and induction of LTP in rodent forebrain [16–18]. Injection of ARC antisense oligodeoxynucleotides has been

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shown to impair the maintenance of LTP and hence the learning and memory. Following morphine administration, ARC protein levels have incremented in the nucleus accumbens [19]. BDNF, a member of the neurotrophin family of neurotrophic factors, is one of the important regulators of the memory process and is critical for opiate-induced plasticity of neurons during memory and learning [20–23]. The BDNF protein has been shown to facilitate the behavior of cocaine addiction and to increase in the ventral tegmental area following chronic opioid administration [21, 24]. However, the effect of morphine exposure on the expression of these genes in the hippocampus is still unclear. Nerve growth factor (NGF) is essential for the development and maintenance of the central and peripheral nervous systems and is expressed during hypothermia, memory task, traumatic brain injury, transient and cerebral ischemia, and early maternal separation [25]. Moreover, it can lead to an increase in the expression of several IEGs critical for memory formation and LTP [17]. NGF up-regulation increases sensitivity to opioid reward [26].

The aim of the current study was initially to evaluate of the effect of acute and subchronic administration of morphine on learning ability of rats. Later on, given the known role of the IEGs in memory formation, the hippocampal expression of the NGF, BDNF and ARC genes was quantified as a potential contributor for the observed effects.

Materials and Methods

Experimental Groups

The animal experiments were approved by the Parand university local ethics committee. Adult male Wistar rats weighing 200–250 g were housed in a standard animal house with temperature of 22 ± 3 °C and 12 h dark/light cycles, and fed rodent chow and water. After two weeks of adaptation to the new environment, they were randomly allocated into four sets. The cognitive effects of acute and subchronic morphine administration were assessed in the first and second sets after 1 and 15 consecutive days of morphine administration, respectively. The other two sets received acute and subchronic morphine treatment followed by gene expression evaluation. Each set contained four groups of eight rats each: a control and three experimental groups. The control group received saline (morphine's vehicle) while the animals in the three groups were injected intraperitoneally with 10, 15 and 20 mg/kg body weight of morphine sulfate (Sigma Chemical Co. St. Louis, MO, USA) daily in a final volume of 1 cc for each dose.

Cognitive Behavioral Study

Animals' learning and memory were tested using an inhibitory avoidance task [22]. The test apparatus was consisted of two bright and dark compartments of the same size ($20 \times 20 \times 30$ cm^3), separated by a guillotine door. The floor of the dark compartment was made of a stainless steel grid through which intermittent electric shocks (50 Hz, 3 s, 1.5 mA) were delivered. For both acute and subchronic sets, the training session was carried out before the last injection. After allowing to habituate in the experimental room for at least 30 min, the animal was placed in the bright room. Five seconds later, the guillotine door was opened and the animal was allowed to enter the dark room. The latency for the cross into the dark compartment, known as step-through latency (STL), was recorded. After the entrance into the dark room, the guillotine door was closed and the animal was immediately withdrawn from the dark compartment. Those animals that waited for more than 100 s to enter the dark compartment were excluded from the study. After 30 min, a similar training trial was repeated whereas this time a foot shock was delivered upon animal's entrance into the dark compartment. After 20 s, the rat was immediately withdrawn from the apparatus and placed into its home cage. Two minutes later, the step through procedure followed by foot-shock upon re-entrance into the dark compartment was repeated. Training cycles were repeated until the animal remained in the light compartment for 120 s consecutively.

Memory retrieval test was performed one day after the training session during which the animal was placed in the light compartment of apparatus, the guillotine door was opened after 5 s, and the step-through latency (sec) was measured in absence of electric foot shocks, as an indicator of inhibitory avoidance behavior. A cutoff of 300 s was set for the STL during memory retrieval test.

Gene Expression Study

One day after the administration of the last dose of saline/morphine, animals were deeply anesthetized and rapidly sacrificed by decapitation. The brains were dissected, placed on an ice-cold cutting board, and then hippocampus was extracted and stored at – 70 °C until further tests [27].

RNA Extraction and Complementary DNA Synthesis

Total RNA of the hippocampus was isolated using the RNX-TM plus (CinnaGen Inc., Tehran, Iran). The quantity and purity of the extracted RNA was examined by spectrophotometer (Nano- Drop ND-2000, NanoDrop Technologies; Wilmington, Delaware, US), and only the extracted RNAs

with an A260/A280 ratio ranging from 1.8 to 2.0 were used for complementary DNA (cDNA) synthesis. Based on the manufacturer's instructions, 1 µg of RNA and a first strand cDNA synthesis kit (Fermentas; Thermo Scientific, Waltham, MA, USA) were used for Real-time transcription.

Real-Time Quantitative PCR Using SYBER Green

The quantitative mRNA expression of NGF, BDNF, and ARC genes with GAPDH as an internal control was determined using Real-time PCR. The relative quantification was done by determining increased fluorescence light as a result of SYBR Green bonding using an Illumina real-time PCR system Illumina, Inc. (San Diego, California, USA). Amplification was achieved by a combination of 1 µl of cDNA, 12.5 µl of SYBR Green Master Mix (Master mix Green-No Rox, Ampliqon, Denmark), 5 pmol of each complementary primer and 10.5 µl of deionized water in a final volume of 25 µl. BLAST tool (NCBI, www.ncbi.nlm.nih.gov/blast) was used to design and undergo primers. Sequences of NGF, BDNF, ARC and GAPDH primers and annealing temperature used for real-time PCR are presented in Table 1.

The amplification conditions were optimized as follows: predenaturation 95 °C for 5 min followed by 32 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. Comparative cycle threshold (ΔΔCT) method using GAPDH as an internal control was used to analyze quantitative gene expression [28]. The relative fold increase (RFI) was calculated using the following equation: $RFI = 2^{-\Delta\Delta CT}$.

Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) test in the SPSS statistical software environment (SPSS, Inc., Chicago, IL, USA). When required, Tukey test was used for post-hoc comparison of means between multiple groups. A p-value of less than 0.05 was considered statistically significant.

Results

Effect of Acute and Subchronic Morphine Administration on Cognitive Behavior

Figures 1 and 2, respectively, show the effect of acute and subchronic intraperitoneal morphine administration (10, 15, and 20 mg/kg) on step-through latency. With acute administration, we found no significant difference between the control and morphine-treated groups in the STL in the pre-training session [One-way ANOVA, $F(3, 28) = 0.706$, $p > 0.05$], while in the test session, morphine altered the STL compared to the control group [One-way ANOVA, F

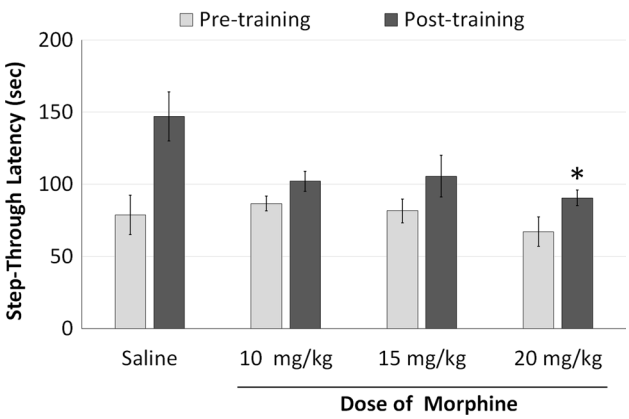

Fig. 1 Effect of intraperitoneal acute (1 day) injection of different doses of the morphine (10, 15 and 20 mg/kg) on step-through latency (STL). Data are expressed as mean ± SEM (n = 8 in each group). We found no significant difference between the control and morphine-treated groups in STL in the pre-training session ($p > 0.05$). Morphine at 20 mg/kg significantly decreased the STL in the post-training session versus control (*$p < 0.05$) (Tukey's post hoc test)

Table 1 Primer sequences used for real-time PCR

Gene	Primers	Product size (bp)	Annealing (°C)
NGF	Forward: 5′-AATCAACTCCTGCTTGGC-3′	349	55
	Reverse: 5′-GTATTTAGCCCCTCCTCC-3′		
BDNF	Forward: 5′-CCGGTATCCAAAGGCCAACT-3′	80	55
	Reverse: 5′-CTGCAGCCTTCCTTGGTGTA-3′		
Arc	Forward: 5′-CTCCAGGGTCTCCCTAGTCC-3′	133	55
	Reverse: 5′-TGAGACCAGTTCCACTGCTG-3′		
GAPDH	Forward: 5′-TGC CACTCAGAA GAC TGTGG-3′	85	53
	Reverse: 5′- GGA TGC AGG GAT GAT GTT CT-3′		

NGF nerve growth factor, *BDNF* brain-derived neurotrophic factor, *Arc* activity-regulated cytoskeleton-associated protein, *GAPDH* glyceraldehyde3-phosphate dehydrogenase, *PCR* polymerase chain reaction

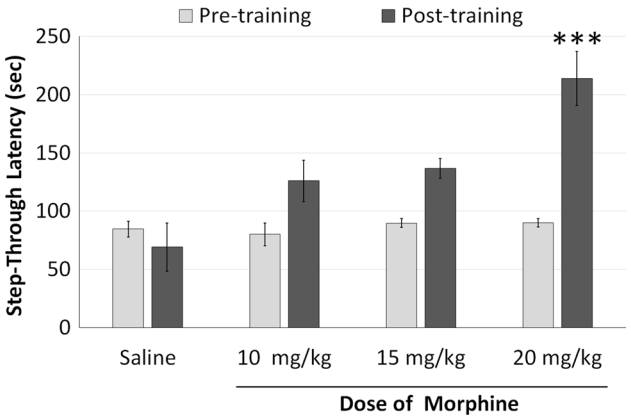

Fig. 2 Effect of intraperitoneal subchronic (15 days) administration of different levels of the morphine (10, 15 and 20 mg/kg) on step-through latency (STL). Data are expressed as mean ± SEM (n = 8 in each group). We found no significant difference between the control and morphine-treated groups in STL in the pre-training session ($p > 0.05$). Morphine at 20 mg/kg significantly increased the STL in the post-training session versus control (***$p < 0.001$) (Tukey's post hoc test)

(3, 28) = 4.231, $p < 0.01$]. Post-hoc analysis indicated that the higher dose of morphine (20 mg/kg) impaired memory retrieval (Fig. 1).

As seen in Fig. 2, with subchronic morphine treatment, no significant difference was observed in the STL between the control and morphine-treated groups in the pre-training session [One-way ANOVA, F (3, 28) = 0.518, $p > 0.05$]. In the test session, however, intraperitoneal injection of the morphine altered the STL compared to the control group [One-way ANOVA, F (3, 28) = 10.475, $p < 0.001$]. Post-hoc analysis indicated that the higher dose of morphine (20 mg/kg) increased memory retrieval.

Expression of the ARC Gene After Acute and Subchronic Morphine Administration

The effect of morphine administration on the mRNA expression of the ARC gene is illustrated in Fig. 3. While acute morphine administration did not significantly alter the Arc mRNA levels [One-way ANOVA, F (3, 28) = 1.730, $p > 0.05$], subchronic morphine treatment at doses of 15 and 20 mg/kg respectively increased ARC mRNA levels 3.83 ($p < 0.01$) and 8.97($p < 0.001$) times the control group [One-way ANOVA, F (3, 28) = 36.256, $p < 0.001$].

Expression of the BDNF Gene After Acute and Subchronic Morphine Administration

Figure 4 shows the effect of morphine administration on the mRNA expression of the BDNF gene. Accordingly, acute morphine treatment failed to alter mRNA levels of BDNF in the hippocampal cells [One-way ANOVA, F (3, 28) = 1.905, $p > 0.05$]. However, subchronic morphine administration at doses of 10, 15, 20 mg/kg increased the BDNF mRNA levels by factors of 1.22 ($p < 0.05$), 1.20 ($p < 0.05$) and 1.25 ($p < 0.01$), compared to the control group, respectively [One-way ANOVA, F (3, 28) = 6.262, $p < 0.01$].

Expression of the NGF Gene After Acute and Subchronic Morphine Administration

The effect of morphine administration on the mRNA expression of the NGF gene in the rat hippocampus is shown in Fig. 5. Analysis of the data on acute morphine administration revealed no significant change in the NGF gene mRNA levels between the morphine treated and control groups [One-way ANOVA, F (3, 28) = 2.301, $p > 0.05$]. With subchronic morphine administration, post-hoc analysis indicated that morphine only at 20 mg/kg significantly increases

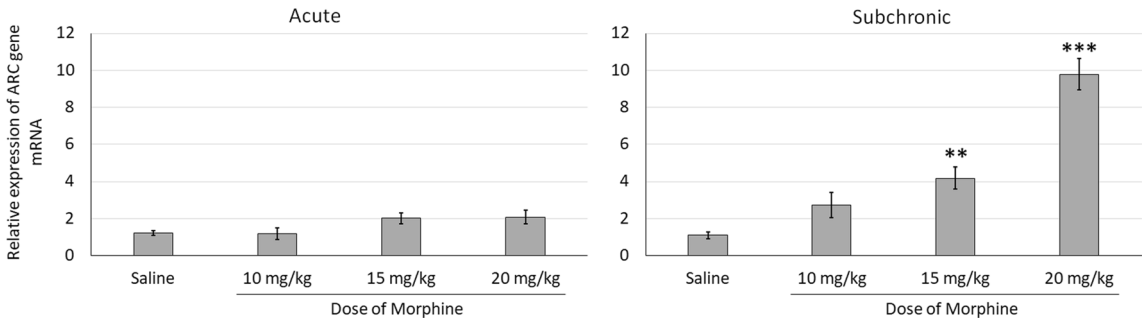

Fig. 3 Effect of intraperitoneal injection of different levels of the morphine (10, 15 and 20 mg/kg) on the expression of Arc in the rat hippocampus. GAPDH was amplified as a housekeeping gene and showed no changes during the experiment. Data are expressed as mean ± SEM of the ratio of the treated rats to sham controls (n = 8 in each group). No statistically significant alteration was seen with acute morphine administration (left). There was a significant increase in Arc mRNA levels with subchronic morphine administration at 15 (**$p < 0.01$) and 20 (***$p < 0.001$) mg/kg versus control (Tukey's post hoc test) (right). *Arc* activity-regulated cytoskeleton-associated protein, *GADPH* glyceraldehyde 3-phosphate dehydrogenase

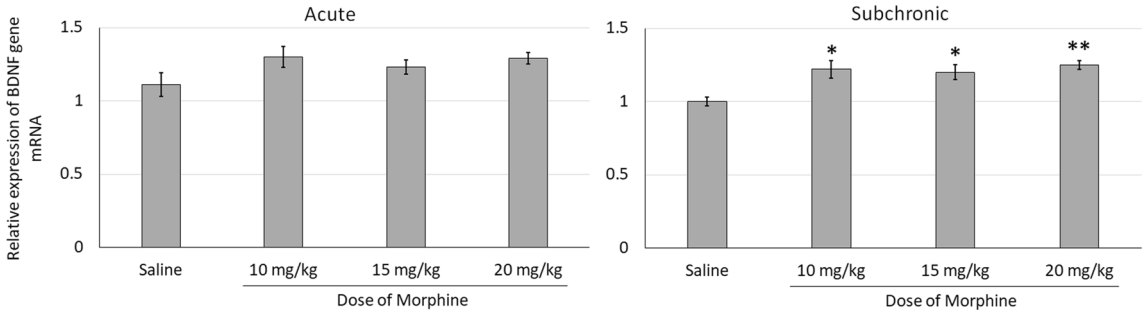

Fig. 4 Effect of intraperitoneal injection of different levels of the morphine (10, 15 and 20 mg/kg) on mRNA expression of BDNF in the rat hippocampus. GAPDH was amplified as a housekeeping gene and showed no changes during the experiment. Data are expressed as mean ± SEM of the ratio of the treated rats to sham controls (n = 8 in each group). No statistically significant alteration was seen with acute morphine administration (left). There was a significant increase in BDNF mRNA levels with subchronic morphine administration at doses 10 (*p < 0.05), 15 (*p < 0.05) and 20 mg/kg (**p < 0.01) versus control (Tukey's post hoc test) (right). *BDNF* brain-derived neurotrophic factor, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase

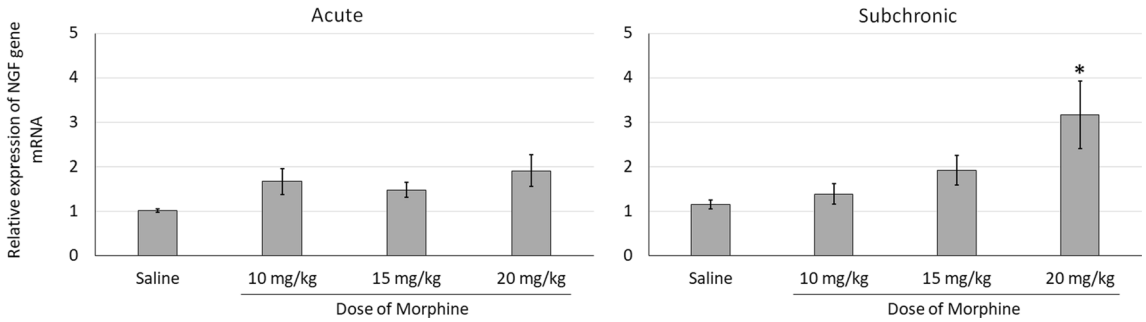

Fig. 5 Effect of intraperitoneal injection of different levels of the morphine (10, 15 and 20 mg/kg) on mRNA expression of NGF in the rat hippocampus. GAPDH was amplified as a housekeeping gene and showed no changes during the experiment. Data are expressed as mean ± SEM of the ratio of the treated rats to sham controls (n = 8 in each group). No statistically significant alteration was seen with acute morphine administration (left). There was a significant increase in NGF mRNA levels with subchronic morphine administration at dose 20 mg/kg (*p < 0.05) versus control (Tukey's post hoc test) (right). *NGF* nerve growth factor, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase

NGF mRNA levels (2.75 times the control group) [One-way ANOVA, $F(3, 28) = 4.292$, $p < 0.05$] (Fig. 3).

Discussion

Despite multiple prior studies, the role of morphine in learning and memory formation and its underlying neurochemical mechanisms are not well known. The purpose of the current study was first to evaluate the effect of acute and subchronic administration of morphine on memory retrieval of rats and second to determine the hippocampal expression of NGF, BDNF and Arc genes as potential contributors to morphine-induced memory formation. An inhibitory avoidance task was used to test memory retrieval. We found that a single dose of morphine at the highest dose of 20 mg/kg decreases the post-training step-through-latency, while repeated administration of the same dose for 15 successive days increases this measure of memory retrieval. We did not detect a significant change in the hippocampal expression of Arc, BDNF or NGF genes after a single episode of morphine treatment. However, subchronic morphine administration (15 and 20 mg/kg) for 15 consecutive days increased the expression of Arc and BDNF genes in a dose dependent manner. A higher mRNA expression for the NGF was observed at the higher dose of 20 mg/kg.

Morphine causes neuroadaptations in the CNS which lead to tolerance, dependence, and addiction [29, 30]. It is also known to modulate memory formation and retrieval, although having dual effects depending on the dose and timing of exposure [31–33]. The effect can become more complicated in different states of opioid dependency, tolerance, withdrawal and sensitization. The bulk of scientific literature supports that morphine impairs memory function. This has been shown with both acute and chronic exposures [34, 35]. Same adverse effect has been observed in early withdrawal with complete or partial improvement in memory tasks over time [36]. Counterintuitively, repeated administration of

morphine in some other studies, has proved enhancement of the behavioral effects and even reverse the morphine-induced amnesia [37, 38]. This latter phenomenon, identified as behavioral sensitization, has been hypothesized to contribute to the development of opiate related addiction and relapse presumably through mesolimbic dopaminergic pathway [39, 40]. While we observed impairment of memory retrieval in acute morphine exposure, our results in the subchronic setting are suggestive of memory improvement.

Both NGF and BDNF have diverse trophic effects on the functional plasticity of the central synapses in the brain [26]. NGF can induce mRNA expression of several immediate early gene such as Arc which have known links to memory consolidation [17]. BDNF plays a crucial role in the pathogenesis of neurological behaviors and mediates cognitive function in rats [41, 42], likely through enhanced synthesis of Arc in synaptoneurosomes [43]. Arc is one of the most characterized genes involved in learning which is regulated by natural stimuli in the hippocampus [44]. It has tight experience-dependent regulation and high dendritic mRNA transport, and codes for a synaptic protein [45]. Arc acts as one of the effectors of the dopaminergic, glutamatergic, serotonin and BDNF signaling [46].

Alteration in the expression of immediate early genes following treatment with opiates has been studied extensively, the results varying on the area of the brain and the drug exposure regimen. It has been shown that chronic exposure to abuse drugs increases BDNF mRNA levels in the ventral tegmental area neurons [47, 48]. One study showed increased Arc expression in the mice striatum with acute morphine administration but no change with chronic injection [49]. Naloxone-precipitated morphine withdrawal using a single-dose morphine showed elevated Arc expression in the rat prefrontal and sensory cortices, striatum, and amygdala, suggesting that these areas are involved in the negative withdrawal memory formation, but no significant change was observed in two of the most important regions in memory formation, nucleus accumbens and hippocampus [50]. A single session of cocaine administration, however, increased Arc levels in the medial prefrontal cortex [51].

Direct intracellular mechanism of action of morphine on ARC gene expression is not fully understood, but it seems that morphine leads to activation of *N*-methyl-D-aspartate receptors and Ca^{2+} channels. This increases second-messenger cAMP and intracellular Ca^{2+} levels, and leads to activation of protein kinase A and Ca^{2+}/calmodulin-dependent protein kinase IV. Subsequent activation of cAMP response element-binding protein (CREB) and interaction with CREB-binding protein (CBP), causes binding of CREB to cAMP response elements in the target genes and increases expression of IEGs such as BDNF and ARC [52].

Our observation of memory impairment following a single dose of morphine administration is in alignment with previous studies in which such effect has been shown to be mediated via mu receptors [22]. We hypothesize that the improved memory retrieval in our subchronic experiments follows a behavioral sensitization pattern which is enhanced through increased Arc mRNA levels. The role of dopamine receptors in morphine-induced behavioral sensitization has been shown before where the amnesia induced by pre-training morphine was significantly decreased in the animals that previously received dopamine agonists [40]. Arc gene, on the other hand, has a bidirectional interaction with dopamine. Arc-deleted animal models have shown a dysregulation in the dopaminergic system with elevated dopamine levels in the striatum and decreased levels in the cortex which was accompanied with cognitive deficits along with positive and negative symptoms of schizophrenia [53]. These findings are in alignment with our suggested role of Arc expression in morphine-induced behavioral sensitization. Investigating the hippocampal dopamine levels during morphine behavioral sensitization can further clarify this possible role.

Besides behavioral sensitization, tolerance to amnestic effects of morphine may also explain our observation of memory improvement in the subchronic setting. Our study was limited in this regard, as it did not explicitly differentiate behavioral sensitization from tolerance to amnestic effects of morphine as the underlying mechanism.

Conclusion

In conclusion, we showed that acute morphine administration can impair memory without significant change in the hippocampal expression of NGF, BDNF and Arc genes. Subchronic administration of morphine, on the other hand, improved memory retrieval with a dose-dependent increase in the expression of BDNF and Arc genes. We hypothesize that the subchronic effects were morphine-induced behavioral sensitization which may have been enhanced through increased hippocampal Arc expression, although tolerance to amnestic effects of morphine may result in similar observation.

Compliance with Ethical Standards

Conflict of interest There are no conflicts of interest associated with the present study.

Ethical Approval All protocols for animal experiments were approved by the institutional animal Ethical Committee, Parand branch, Islamic Azad University, Parand, Iran.

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