Research Report

Morphine could increase apoptotic factors in the nucleus accumbens and prefrontal cortex of rat brain's reward circuitry

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Abstract

The nucleus accumbens (NAc) and prefrontal cortex (PFC) are two parts of neuronal reward circuit involved in motivated and goal-directed behaviors. Some data suggest that morphine is toxic to neurons and induces apoptosis, while other evidence shows that morphine could have beneficial effects against cell death. This study was designed to evaluate the effect of morphine on apoptosis by measuring the expression of apoptotic proteins in two important regions, the NAc and PFC, in the rat brain’s reward circuitry. Morphine subchronic administration in different doses (0.5, 5 and 10 mg/kg) in conditioned place preference (CPP) paradigm (3 times in 3 days, for each dose in each group of rats) was used to induce its rewarding effect. Then, the expression of four apoptotic factors; Bax, Bcl2, caspase3 and PARP, in the NAc and PFC were assessed using the Western blot technique. All of morphine-treated groups showed increase of apoptotic factors in these regions. In the NAc, morphine significantly increased the Bax/Bcl-2 ratio, caspase3 and PARP in the lowest dose (0.5 mg/kg) but in the PFC considerable increase was seen in dose of 5 mg/kg. Elevation of apoptotic factors in the NAc and PFC implies that morphine can affect the molecular mechanisms which interfere with apoptosis through different receptors. Our findings suggest that the NAc and PFC may have a different distribution of receptors which become active in different doses of morphine.

1. Introduction

Morphine, well known extract of opium poppy, has become one of the most potent pain relievers. Morphine also activates the brain’s reward circuitry. The ability of morphine to strongly activate brain reward mechanisms and its ability to chemically alter the normal functioning of these systems can produce morphine dependency (Zhang and QCaL, 2008).

Nucleus accumbens (NAc) is a critical element of the mesocorticolimbic system, a brain circuit implicated in reward and motivation. This basal forebrain structure receives glutamatergic inputs from regions including prefrontal cortex (PFC), amygdala (AMG), and hippocampus (HIP) (Carlezon and Thomas, 2009), and receives the dopaminergic inputs from the ventral tegmental area (VTA) of the midbrain. Lots of evidences support the involvement of VTA dopaminergic...
system in drug induced reward as well as natural rewards (Nestler and Carlezon, 2006). Morphine can modulate cellular functions through modulation of molecular mechanisms. Until recently, little was known about the changes in molecular mechanisms underlying morphine effects.

Morphine may act as the modulator of cell proliferation and cell death. It has been shown that opioids can protect astrocytes from apoptosis triggered by apoptosis promoting agents (Kim et al., 2001), delay neuronal death in the avian ciliary ganglion (Meriney and Gray, 2001), and promote the growth of tumor cells (Gupta et al., 2002; Ishikawa et al., 1993; Moon, 1988). On the other hand, opioids have also been demonstrated to induce apoptosis of immunocytes (Singhal et al., 1997, 1998), cancer cells (Hatzoglou et al., 1996; Tegeder et al., 2003), neuroblastoma cells such as SK-N-SH, NG108-15 and PC12 cells (Kugawa et al., 1998; Oliveira et al., 2003; Yin et al., 1997) and neuronal cells (Boronat et al., 2001; Mao et al., 2002), as well as human microglia (Hu et al., 2002). Accumulating evidence has demonstrated that opiates can cause apoptosis and cell injury of neuronal cells and other cells such as immunocytes (Singhal et al., 2001; Wang et al., 2001) and cancer cells (Yoshida et al., 2000).

In the molecular mechanisms of apoptosis various key proteins are involved in the regulation of programmed cell death (Kinloch et al., 1999; Sastry and Rao, 2000). Some members of the Bcl-2 family of proteins, such as Bcl-2 and Bcl-xl, suppress apoptosis, while the expression of others, such as the homologs Bax and Bak, are pro-apoptotic (Adams and Cory, 2007). Hoehenberg et al. (1990) showed that the Bcl-2 oncoprotein, localized mainly to the mitochondrial membranes and prevents the release of cytochrome c (induced by Bax) play an important role in protecting neurons from apoptosis. Signals from either intrinsic or extrinsic agents can trigger apoptotic cascade; activation of caspases which degrade their cellular targets until cell dies (Garcia-Fuster et al., 2007; Hatzoglou et al., 1996; Singhal et al., 1999; Yin et al., 1997). The activation of effector caspases such as caspase-3 leads to downstream cleavage of various cytoplasmic or nuclear substrates including PARP. These downstream cleavage events mark many of the morphological features of apoptotic cell death (Huppertz et al., 1999). Other studies show that different addictive drugs, such as heroin, can induce apoptosis in several cultured cell lines. Heroin induces mitochondrial malfunction, caspase activation, poly-ADP ribose polymerase (PARP) cleavage, and DNA fragmentation in PC-12 cells (Meriney and Gray (2001)).

According to the above mentioned studies, we set out to determine whether morphine would affect apoptosis in the nucleus accumbens and the prefrontal cortex by assessing the apoptotic proteins involved in the regulation of programmed cell death Bcl-2 (anti-apoptotic), Bax (pro-apoptotic), Caspase-3 and PARP in rats.

### 2. Results

#### 2.1. Behavioral test

In conditional place preference paradigm as a behavioral test, our results showed that administration of morphine in conditioning phase could induce place preference (Fig. 1). One-way ANOVA followed by Dunnett's multiple comparison test \( F(3,23) = 15.33, P < 0.0001 \) indicated that there were significant differences in conditioning scores between saline and morphine-treated groups. Each point shows the mean ± SEM for 6–8 rats. *P < 0.05, **P < 0.01 different from the saline or pre-test group.

![Fig. 1 – Effects of different doses of morphine on conditioning scores (place preference) in rats. One-way ANOVA followed by Dunnett's multiple comparison test \( F(3,23) = 15.33, P < 0.0001 \) indicated that there were significant differences in conditioning scores between saline and morphine-treated groups. Each point shows the mean ± SEM for 6–8 rats. *P < 0.05, **P < 0.01 different from the saline or pre-test group.](image)

#### 2.2. Western blot analysis

In molecular experiments, we examined the modifications of apoptotic factors, Bax, Bcl-2 and their ratio, caspase-3 and PARP, after behavioral test in saline- and morphine-treated animals.

##### 2.2.1. Change in Bax/Bcl2 ratio in the NAc and PFC after conditioned place preference paradigm

In this set of experiments, the level of pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins was measured by the Western blot analysis in the presence of different doses of morphine (0.5, 5 and 10 mg/kg) after performance of the CPP paradigm. As shown in Fig. 2, Bax/Bcl-2 ratio increased in all doses of morphine in a reverse dose-dependent manner compared with saline group in the NAc. The most significant increase in this ratio has been appeared in animals that received the lowest dose of morphine (0.5 mg/kg; \( P < 0.01 \)) during conditioning phase. This increasing was about 4 folds. Additionally, in the PFC (Fig. 3), Kruskal–Wallis non-parametric test revealed that there were significant differences in Bax/Bcl-2 ratio between experimental and saline control group in this region. The ratio of Bax/Bcl-2 increased by these doses of morphine (0.5 and 5 mg/kg) about 2.58 (\( P < 0.05 \)) and 3.40 folds (\( P < 0.01 \)) as compared to the saline group, respectively.
2.2.2. Change in the level of active form of caspase-3 in the NAc and PFC after conditioned place preference paradigm

To determine whether morphine has anti- or pro-apoptotic effect in the NAc and PFC, we measured the level of cleaved caspase-3 in each morphine-treated group. Kruskal–Wallis followed by Dunn’s multiple comparison test revealed that there were significant differences in amount of caspase-3 between experimental and saline control group in the NAc. As shown in Fig. 4, the level of cleaved caspase-3 in the NAc increased significantly in 0.5 mg/kg morphine-treated group by 4.16 folds (P<0.01). Increase in the level of cleaved caspase-3 at 5 and 10 mg/kg doses of morphine was not as much as that at the dose of 0.5 mg/kg. Furthermore, this set of experiments in the PFC showed that there were significant differences in the level of cleaved caspase-3 between morphine- and saline-treated groups in CPP paradigm. This level increased in all doses of morphine similar to results in previous section (Bax/Bcl-2 ratio) in the PFC (Fig. 5). The expression of cleaved caspase-3 significantly increased in the presence of 0.5 and 5 mg/kg of morphine by 3.25 (P<0.05) and 4 folds (P<0.01), respectively. The most effective dose of morphine in increasing the level of cleaved caspase-3 in the PFC region was 5 mg/kg of morphine.

2.2.3. Change in cleaved PARP levels in the NAc and PFC after conditioned place preference paradigm

We examined the level of PARP cleavage by the Western blot analysis. As shown in Fig. 6, in rats injected with 0.5 mg/kg of morphine, accumulation of the 89-kDa and 24-kDa PARP cleaved fragments is evident, confirming the activation of caspase-3 as the main protease, responsible for PARP cleavage. Kruskal–Wallis test revealed that PARP degradation (amounts of 89-kDa and 24-kDa PARP cleavage fragments) increased significantly in all morphine-treated animals compared with the saline control group in the NAc during conditioning phase. The most significant increase in amounts of 89-kDa (P<0.01) and 24-kDa (P<0.01) PARP cleavage fragments was observed in animals that received 0.5 mg/kg morphine during conditioning phase (Fig. 6) while increase in the cleaved PARP was lesser than that in rats received 5 and 10 mg/kg doses compared to 0.5 mg/kg dose of morphine. On the other hand, these experiments in the PFC showed that there were significant differences in the amounts of 89-kDa and 24-kDa PARP cleavage fragments between morphine- and saline-treated groups. Furthermore, in the PFC of rats receiving 0.5 and 5 mg/kg of doses of morphine, accumulation of
the 89-kDa and 24-kDa PARP cleaved fragments was more than that in rats receiving 10 mg/kg dose of morphine (Fig. 7).

3. Discussion

The present study demonstrated that administration of morphine dose-dependently induces reward and changes the level of apoptotic factors in the brain structures involved in drug reward. The major findings of this study were (i) significant increase in the amount of apoptotic factors in the NAc and PFC after application of morphine during conditioned place preference model and (ii) the most effective doses of morphine on alterations of Bax/Bcl-2 ratio, caspase-3 and PARP were 0.5 and 5 mg/kg in the NAc and PFC, respectively. There are data indicating that morphine induces cytotoxicity in various systems. For example, it has been suggested that the chronic morphine administration results in immunosuppression (Alexander et al., 2005). Morphine also modulates apoptosis of splenocytes and has anticancer activity mediated by cancer cell apoptosis (Gralow, 2002; Singhal et al., 1997). Our results are generally consistent with previous studies and demonstrated that morphine induces apoptosis in neuronal systems. In previous studies, chronic application of morphine triggered apoptosis in rat spinal, cortical, and hippocampal areas (Atici et al., 2004). Morphine also increases neurotoxicity in human-cultured neurons (Turchan-Cholewo et al., 2006).

In our study, apoptotic factors (Bax/Bcl-2 ratio, caspase-3 and PARP) were increased after administration of morphine and it was in agreement with other studies which have shown that morphine induces up-regulation of Bax and caspase-3 (pro-apoptotic factors) and down-regulation of Bcl-2 (anti-apoptotic factor) in rat brain (Boronat et al., 2001). However, some previous studies have shown that morphine can have beneficial effects against cell death, for example, protecting astrocytes from apoptosis triggered by some agents (Kim et al., 2001) and delay neuronal death in the avian ciliary ganglion (Menney and Gray, 2001). Morphine has been found to be protective against microglia-mediated, lipopolysaccharide (LPS)-, or 1-methyl-4-phenylpyridinium-induced dopaminergic neurotoxicity in rat primary mesencephalic neuron-glia cultures. In other study, morphine was...
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found to induce heat shock protein 70 and 27 mRNAs in rat brains but not in HEK293 cells expressing μ-opioid receptors (Zhang and QCAL, 2008). We suggest that decreased apoptotic factors in the highest dose of morphine relate to its neuroprotective effects via opioid receptors with the low affinity to morphine such as δ- and κ-opioid receptors. It has been shown that opiate drugs (and specifically the δ-agonists) could promote survival signals in the brain through inhibition of Fas-associated death domain (FADD), which in turn depends on the activation of anti-apoptotic ERK1/2 signaling pathway (Garcia-Fuster, 2007). In vitro studies have shown that morphine and DAMGO, a specific μ-opioid receptor agonist, induce apoptosis in T lymphocytes and/or Jurkat cells, through mechanisms associated with a decrease in expression of Bcl-2 and an enhancement in that of Bax (Singhal et al., 1999). Of the three major types of opioid receptors (μ, δ, κ) in the nervous system, μ-opioid receptor shows the highest affinity for morphine which mediates its nociceptive, autonomic, and psychological effects (Ding et al., 1996). Based on these evidences, we suppose that our sub-chronic application of morphine might be activated predominantly by μ-opioid receptors within the NAc and PFC that can lead to an increase in the apoptotic factors. However, further investigations are required to fully establish this hypothesis.

According to this study different distributions and affinity of opioid receptors in the NAc and PFC may promote inconsistent changes in the expression of pro or anti-apoptotic factors in different morphine treated groups. Probably, μ-opioid receptors with high affinity to morphine (Le Merrer et al., 2009) were activated in low dose in the NAc and increased pro-apoptotic factors while in higher doses (5 and 10 mg/kg) other opioid receptors such as δ- and κ-opioid receptors with lower affinity were also activated and triggered survival molecular pathways and caused a decrease in apoptotic factors. In the other word, significant difference in enhancement of apoptotic factors in low and high doses of morphine can be related to possibility of induction of survival pathways in high dose, for example the activation of

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**Fig. 6** – Increase in cleavage of PARP in the nucleus accumbens in morphine-induced conditioned place preference’s rats. *Upper panels* are the representative immunoblots of 116-kDa, 89-kDa and 24-kDa PARP proteins in this area. Bottom graphs show the mean 116-kDa, 89-kDa and 24-kDa PARPs calculated from densitometric quantification of the corresponding bands. Each point shows the mean ± SEM for 3–4 rats. *P<0.05, **P<0.01 significantly different from saline group.*

**Fig. 7** – Increase in cleavage of PARP in the prefrontal cortex in morphine-induced conditioned place preference’s rats. *Upper panels* are the representative immunoblots of 116-kDa, 89-kDa and 24-kDa PARP proteins in this area. Bottom graphs show the mean 116-kDa, 89-kDa and 24-kDa PARPs calculated from densitometric quantification of the corresponding bands. Each point shows the mean ± SEM for 3–4 rats. *P<0.05, **P<0.01 significantly different from saline group.*
anti-apoptotic ERK1/2 signaling pathway (Garcia-Fuster, 2007), and leads to decrease in pro-apoptotic factors which needs more investigations. Therefore, it seems that difference in the most effective dose of morphine on alterations of apoptotic factors in these both areas, NAc and PFC, may be due to the affinity and distribution of opioid receptors in these regions that needs more investigations. Also, it can be concluded that despite the lack of drug effect in behavioral model in the low dose, in the molecular test, it was able to cause apoptosis in the NAc and PFC. We conclude that morphine-induced expression of apoptotic factors seems to be a complex phenomenon involving dosage, distribution of receptors and region interactions with no coincidence with behavioral rewarding effect. Thus, it remains to evaluate morphine-induced apoptosis histologically and determine type and quantity of opioid receptors involved in apoptotic versus rewarding effects.

4. Experimental procedure

4.1. Animals

Thirty-two adult male Wistar rats, (Pasteur Institute, Tehran, Iran) weighing 220–320 g, were used in these experiments. Animals were housed in groups of three per cage in a 12/12 h light/dark cycle (lights on between 7:00 AM and 7:00 PM) with free access to chow and tap water. The animals were randomly allocated to different experimental groups. Rats were habituated to their new environment and handled for 1 week before the experimental procedure was started. All experiments were executed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication no. 80-23, revised 1996) and were approved by the Research and Ethics Committee of Shahid Beheshti University of Medical Sciences.

4.2. Drugs

The following agents were used in the present study: morphine sulfate (Temad, Tehran, Iran) dissolved in normal saline. Antibodies directed against Caspase-3, Bax, Bcl-2, poly (ADP-ribose) polymerase-1 (PARP-1) and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA) and Electrochemiluminescence (ECL) kit was provided from Amersham Bioscience (Piscataway, USA).

4.3. Experimental protocol

4.3.1. Behavioral test

4.3.1.1. Conditioning place preference paradigm. The testing apparatus consisted of three wooden compartments. Two compartments were identical in size (30 cm × 30 cm × 40 cm) but differed in shading and texture. Compartment A was white with black horizontal stripes 2 cm wide on walls and also had a textured floor. Compartment B was black with vertical white stripes 2 cm wide and also had a smooth floor. The third compartment C was a red tunnel (30 cm × 15 cm × 40 cm). It protruded from the rear of two large compartments and connected the entrances to them. Conditioned place preference consisted of a 5-day schedule with three distinct phases: pre-conditioning, conditioning and post-conditioning. This method (unbiased design) was similar to that used in previous studies (Haghparsad et al., 2011; Taslimi et al., 2012; Zarepour et al., 2013).

4.3.1.2. Pre-conditioning phase. During this phase (day 1), each animal was placed in compartment C with the guillotine door removed to allow access to entire apparatus for 10 min. Each animal displacement was recorded.

4.3.1.3. Conditioning phase. This phase started 1 day after pre-conditioning phase. It consisted of six, 30-min sessions (three saline and three drug pairing) in a 3-day schedule. These sessions were conducted twice each day (from day 2 to day 4) with a 6-h interval. On each day, separate groups of animals received a conditioning session with morphine and another with saline. During 30-min session intervals for morphine/saline, the animals were confined to one compartment by closing the removable wall. Treatment compartment and order of presentation of morphine/saline were counter-balanced for either group.

4.3.1.4. Post-conditioning phase. On the fifth day (test day), the partition was removed, and the rats could access the entire apparatus. The mean time spent for each rat in both compartments was recorded. Conditioning score (CPP score) represents the time spent in the reward-paired compartment minus the time spent in the same compartment on the test day (post-conditioning phase) during a 10 min period.

4.3.1.5. Locomotion tracking apparatus. Animal displacement was recorded using a 3CCD camera (Panasonic Inc., Japan) placed 2 m above the CPP boxes and locomotion tracking was measured by Ethovision software (Version 3.1), a video tracking system for automation of behavioral experiments (Noldus Information Technology, the Netherlands). In these experiments, total distance traveled (cm) was measured on the test day, during a 10 min period, in control and experimental groups (Azizi et al., 2009; Haghparsad et al., 2011).

4.3.2. Molecular protocol

4.3.2.1. Western blot analysis. Following CPP test (after post-conditioning phase), the rats were immediately sacrificed. Their brains were removed and put onto a glass plate of ice. Nucleus accumbens and prefrontal cortex were then dissected out according to rat brain atlas (Paxinos and Watson, 2005). The NAc and PFC were homogenized in lysis buffer containing protease inhibitor cocktail. The total proteins were then electrophoresed in 12% SDS-PAGE gels, transferred to polyvinylidine fluoride membranes and probed with specific antibodies. Immunoreactive polypeptides were detected by chemiluminescence using enhanced ECL reagents and subsequent autoradiography. Quantification of the results was performed by densitometric scan of films. Data analysis was done by Image J, measuring integrated density of bands after background subtraction. Protein concentrations were determined according to Bradford’s method (Bradford, 1976). Standard plot was generated using bovine serum albumin.
4.3.3. Experimental design
In the present study, we have four groups of animals in conditioned place preference paradigm. Three doses of morphine (0.5, 5 and 10 mg/kg; sc) were tested for producing place preference. A separate group of animals received saline (1 ml/kg; sc) instead of morphine as a control saline group.

4.4. Statistics
In behavioral study, all data are expressed as mean ± SEM (standard error of mean). Data were analyzed by GraphPad Prism® (version 5.0) software. In order to compare the conditioning scores and distance traveled obtained in control and experimental groups, one-way analysis of variance (ANOVA) followed by post-hoc Dunnett’s test was used. In molecular section, Western blot analysis, the optical densitometric data were expressed as mean ± SEM (standard deviation) and were analyzed by Kruskal–Wallis followed by Dunn’s multiple comparison test, and Mann–Whitney test, as needed. P-values less than 0.05 (P < 0.05) were considered to be statistically significant.

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