Research report

Effects of voluntary and treadmill exercise on spontaneous withdrawal signs, cognitive deficits and alterations in apoptosis-associated proteins in morphine-dependent rats

Amin Mokhtari-Zaer a, Shahrbanoos Ghodrati-Jaldbakhan a, Abbas Ali Vafaei a, Hossein Miladi-Gorji a, Maziar M. Akhavan b, Ahmad Reza Bandegi a,b, Ali Rashidy-Pour b,c ∗

a Laboratory of Learning and Memory, Research Center and Department of Physiology, School of Medicine, Semnan University of Medical Sciences, Semnan, Iran
b Skin Research Center, Laboratory of Protein and Enzyme, Shahid Beheshti University (M.C.), Shahada-e Tajrish Hospital, Shahrdari St., 1989934148 Tehran, Iran

HIGHLIGHTS

• Chronic morphine leads to apoptosis and impairment of cognitive functions.
• Voluntary and treadmill exercise enhance cognitive functions.
• Exercise inhibits chronic morphine-induced apoptosis.
• Exercise alleviates memory impairment induced by chronic morphine.
• Exercise could be a potential method to ameliorate the adverse effects of opiate abuse.

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ABSTRACT

Chronic exposure to morphine results in cognitive deficits and alterations of apoptotic proteins in favor of cell death in the hippocampus, a brain region critically involved in learning and memory. Physical activity has been shown to have beneficial effects on brain health. In the current work, we examined the effects of voluntary and treadmill exercise on spontaneous withdrawal signs, the associated cognitive defects, and changes of apoptotic proteins in morphine-dependent rats. Morphine dependence was induced through bi-daily administrations of morphine (10 mg/kg) for 10 days. Then, the rats were trained under two different exercise protocols: mild treadmill exercise or voluntary wheel exercise for 10 days. After exercise training, their spatial learning and memory and aversive memory were examined by a water maze and by an inhibitory avoidance task, respectively. The expression of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 in the hippocampus were determined by immunoblotting. We found that chronic exposure to morphine impaired spatial and aversive memory and remarkably suppressed the expression of Bcl-2, but Bax expression remained constant. Both voluntary and treadmill exercise alleviated memory impairment, increased the expression of Bcl-2 protein, and only the later suppressed the expression of Bax protein in morphine-dependent animals. Moreover, both exercise protocols diminished the occurrence of spontaneous morphine withdrawal signs. Our findings showed that exercise reduces the spontaneous morphine-withdrawal signs, blocks the associated impairment of cognitive performance, and overcomes morphine-induced alterations in apoptotic proteins in favor of cell death. Thus, exercise may be a useful therapeutic strategy for cognitive and behavioral deficits in addict individuals.

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1. Introduction

Chronic exposure to opiates can induce cognitive deficits in humans, and experimental animals [1–8], and also leads to changes in spine density, neurogenesis and synaptic transmission in the hippocampus of rats [9,10]. Prenatal morphine exposure also impairs
learning and memory in juvenile rats [11]. Chronic morphine impairs spatial memory and hippocampal long-term potentiation, a form of synaptic plasticity that may be a cellular substrate of learning and memory [12–14], via accumulation of extracellular adenosine acting on adenosine A1 receptors [13].

Apoptosis or programmed cell death is a physiological form of cell death that occurs in embryonic development and aging [15,16]. Also, neuronal loss by an inappropriate or excessive apoptosis has been implicated in many pathological conditions [17]. Several key proteins including Bcl-2 family proteins play a pivotal role in the regulation of apoptosis. The Bcl-2 family is classified into anti-apoptotic proteins (Bcl-2, Bcl-XL, etc.) and pro-apoptotic proteins (BH3 only family, and Bax family), and play a crucial role in the regulation of the intrinsic (mitochondrial) apoptotic pathway. This pathway involves the release of cytochrome c from the mitochondria into cytosol and the subsequent activation of specific proteases termed caspases, which promotes apoptosis. Bcl-2 preserves mitochondrial membrane integrity, whereas the Bax family contributes to the permeabilization of the outer mitochondrial membrane, allowing efflux of cytochrome c [18–20]. Some studies on experimental animals have demonstrated that chronic exposure to opioid drugs can produce apoptosis in the central nervous system (CNS) [21,22]. This apoptotic effect may lead to learning and memory impairment following chronic morphine [23].

Human studies suggest that exercise could have benefits for overall health and cognitive functions [24]. In rodents, both voluntary and forced exercise can increase neurogenesis, and improve learning and memory in a variety of spatial and non-spatial tasks [25–28]. Physical activity increases central BDNF in rodents [29]. Changes in BDNF are apparently necessary for the effects of exercise on cognition in rodents, as blocking BDNF signaling also prevents the enhancement of cognitive function following physical training [7,29,30].

We recently found that concurrent of physical exercise with morphine administration reduced the occurrence of naloxone-potentiated withdrawal signs and blocked the ability of chronic morphine to impair spatial memory retention in rats via the BDNF-TrkB mechanism [7]. However, when extrapolating to the human conditions, it is less likely that individuals that suffer from drug addiction will engage in physical exercise while they are under the influence of drugs of abuse. Thus, an important question is whether exercise could blunt the deleterious effects of drugs of abuse after the exposure to these substances or after long periods of abstinence.

In this study, we investigated whether both voluntary and mild treadmill exercise would reduce spontaneous withdrawal signs, ameliorate the associated cognitive deficits, and return the changes of hippocampal expression of apoptotic proteins (Bcl-2 and Bax) in favor of cell survival following chronic morphine. We used both voluntary and forced exercise because different kinds of physical activity induce differential effects on neuronal adaptations in different brain regions, and cognitive functions [31,32].

2. Materials and methods

2.1. Animals

Adult male Wistar rats (220 ± 10 g) were individually housed in cages (50 cm × 26 cm × 25 cm) in a 12-h light/dark cycle at 22–24 °C, with food and water ad libitum. The experimental protocol was approved by the Ethical Review Board of Semnan University of Medical Sciences (Iran). All of the experimental trials were conducted in agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Induction of morphine dependence

Morphine sulphate (Temad Company, Tehran, Iran) was dissolved (10 mg/ml) in 0.9% saline and chronically administered via subcutaneous injections at a volume of 1 ml/kg. These injections were given twice per day at 12 h intervals for 10 days. This morphine administration protocol is sufficient to induce dependence [7,13]. The control rats were treated similarly, except with injections of saline replacing morphine.

2.3. Voluntary exercise paradigm

A detail of description of the voluntary running wheel has been given in our previous reports [7,33]. Briefly, each of the exercising rats was given all day/night access to a cage equipped with a running wheel (diameter = 34.5 cm, width = 9.5 cm) (Novidan.Tab, Iran) that was freely rotated against a resistance of 100 g. The sedentary rats were confined to similar cages with no access to a wheel. The exercising groups were exposed to exercise following the development of dependence on morphine, which took 10 days before the start of the biochemical or behavioral experiments [7].

2.4. Treadmill exercise paradigm

The rats in the treadmill exercise group were forced to run on a motorized treadmill (Borjisanat, Iran) for 30 min once a day for 10 days. The exercise load consisted of running at a speed of 5 m/min for the first 5 min, 8 m/min for the next 5 min, and 10 m/min for the last 20 min, with no incline. This exercise regimen is a light-intensity exercise [34].

2.5. Spontaneous withdrawal responses

Spontaneous withdrawal responses of the exercising rats and their sedentary control were measured during exercise once a day for 10 days. Each rat was placed in a transparent cylinder (35 cm diameter, 50 cm height) for 30 min, and their behavior was monitored by a rater blinded to the rats’ treatment. Withdrawal signs were recorded and scored according to a modified version of the Gellert–Holtzman scale [35]. Briefly, on the Gellert and Holtzman scale graded signs are assigned a weighting factor 1–4 based on frequency of appearance, and checked signs receive values of 2–3 depending upon the particular withdrawal sign noted, but regardless of frequency of appearance. Graded signs including jumps, wet dog shakes, and abdominal contractions were counted as the number of events occurring during the total test time. Checked signs including diarrhea, ptosis, erection or genital grooming, teeth chattering, writhing, and irritability were counted as positive if the sign occurred at any time during the observation period. After completion of the observation session, the overall withdrawal severity was calculated by summing the proper weighting factor of somatic signs [7,35].

2.6. Water maze

A detailed description of the apparatus and the tracking system has been given in our previous reports [7,33]. In brief, the water maze (WM) was a black circular pool (140 cm in diameter and 60 cm high) of 25 cm depth filled with 20 °C water.

2.6.1. Training

The WM protocol was a stringent protocol consisting of four trials per day for 5 days. During each trial, the rat was placed into the water from one of the four cardinal points of the compass (N, E, S, and W), which varied from trial to trial in aqua randomly order. The rat had to swim until it climbed onto the escape platform. The rats...
were guided by hand to the platform if they failed to locate it within 60 s. The rat was allowed to stay on the platform for 20 s during the inter-trial interval. After the last trial, the animal was towel dried and returned to its home cage with no access to a running wheel.

2.7.2. Probe test

A spatial probe test was performed 2 days after the last acquisition trial, during which the platform was removed. The rats were allowed to swim for 60 s, during which, the latency to reach precise platform coordinates (platform location latency), and the time spent swimming within a zone, which had a 20 cm radius that was centered either on the original training location (target zone) or on an equivalent location in other quadrants (opposite, left and right adjacent quadrants) were recorded. The velocity of each animal was also calculated. The analysis of the time spent within a specified radius (zone) is a sensitive measure of the WM probe test performance, in terms of detecting group differences [36,37].

2.7. Inhibitory avoidance task

The experimental apparatus was a shuttle box (UgoBasile, Spain) divided into dark and light compartments. Both compartments had a grid floor (2 mm stainless steel rods spaced at 6 mm) connected to a shock generator. An automated apparatus registered the latency of passage from the light to the dark side of the box. The apparatus was located in the sound attenuated room.

2.7.1. Training

All experimental rats were first habituated to the apparatus. The rat was placed in the illuminated compartment and the guillotine door was raised 7 s later. Upon entering the dark compartment, the door was closed and the rat was taken from the dark compartment into the home cage. The acquisition trial was done 30 min later during which the door was closed and a 50 Hz, 1 mA constant current shock was applied for 2 s immediately after the rat had entered the dark compartment. The rat was removed from the dark compartment about 10 s after receiving the shock and returned to his home cage.

2.7.2. Memory testing

Avoidance memory of all animals was assessed by a 9 min extinction trial 48 h later, during which shock was never delivered. The rat was placed in the illuminated chamber, as in the training session, and the latency of entering the dark chamber (step-through latency, STL) and time spent in the light chamber (TLC) were recorded. Longer STL and TLC were interpreted as indicating better memory retention.

2.8. Protein measurement and Western blotting

Rats were sacrificed, and the hippocampal tissues were collected, and were then immediately frozen at −70 °C. Hippocampal samples were homogenized and prepared in lysis buffer (137 mM NaCl, 20 mM Tris–HCl pH 8.0, 1% NP-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 1 µg/ml leupeptin, and 0.5 mM sodium vanadate). Tissue extracts were centrifuged to remove insoluble materials (12,500 × g for 20 min at 4 °C) and total protein concentration was determined according to the Micro BCA procedure (Pierce, Rockford, IL, USA). Equal amounts (25 µg) of protein from each sample were loaded on 15% polyacrylamide gels and separated by a standard SDS-PAGE. Protein bands were transferred to PVDF membranes and then blocked using 5% skim milk and 0.1% Tween-20 in Tris-buffered saline. Membranes were incubated with primary antibodies overnight at 4 °C [anti-Bax, 1:500 (sc-493); anti Bcl-2, 1:200 (sc-492); anti-actin (sc-13065)] and then with a secondary antibody [goat anti-rabbit IgG horseradish peroxidase conjugated antibody, 1:2000 (sc-2004)] for 1 h at room temperature. Immunocomplexes were visualized by chemiluminescence using the ECL kit (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions. The results for target proteins, Bax, and Bcl-2 were quantified by densitometric analysis (using Gel-Pro analyzer imaging software). The results for the Bax-to-Bcl-2 ratio were also calculated.

2.9. Measurement of serum corticosterone levels

Immediately after the last exercise session (between 9 and 11 am), the rats were decapitated, trunk blood was collected in tubes with EDTA and centrifuged (3000 × g, 20 min) and the plasma was stored at −70 °C until used for the corticosterone assay. Serum corticosterone levels were determined in duplicates using enzyme-linked immunosorbent assay (ELISA) kit (Abcam, England) following the manufacturer’s instructions.

2.10. Statistical analysis

The withdrawal data were not distributed normally and were analyzed using non-parametric Kruskal–Wallis analysis followed by the Mann–Whitney test. Other data exhibited a normal distribution and were analyzed by mixed- and between factor analyses of variance (ANOVARs) followed by the Tukey’s test. Differences were considered significant if the P value was less than 0.05.

3. Results

3.1. Effects of voluntary and treadmill exercise on spontaneous withdrawal signs in rats

To determine the effects of voluntary and forced exercise on the severity of morphine dependence, the global severity of the spontaneous withdrawal responses was measured daily in the sedentary and exercising morphine-treated rats during 10 days of physical activity. Rats (n = 7–8 rats per group) were divided into the saline-sedentary (Sal/Sed), the morphine-sedentary (Mor/Sed), the morphine-voluntary exercise (Mor/VE), and the morphine-treadmill exercise (Mor/TE) groups. The global severity of the morphine withdrawal responses was measured as previously described (Fig. 1, Experiment 1).

Fig. 2 shows the running distance for the voluntary exercise groups. A two-way ANOVA with repeated measure (day) for the average running distance (m) during 10 days of voluntary exercise revealed the absence of a significant effect of groups (F1,12 = 2.19, P = 0.14), a significant effect of days (F12,120 = 14.88, P = 0.0001), and no significant interaction between both factors (F12,120 = 0.59, P = 0.80). In general, the running distance is increased significantly in both groups as exercise days progressed. Moreover, morphine dependence did not affect voluntary running distance.

The results of the spontaneous withdrawal responses are shown in Fig. 3. Analysis of data indicated significant differences among groups in day 1 (H2 = 17.94, P = 0.0001), day 2 (H2 = 15.82, P = 0.001), day 3 (H2 = 16.74, P = 0.001), day 4 (H2 = 20.584, P = 0.0001), day 5 (H2 = 14.96, P = 0.002), and day 6 (H2 = 15.00, P = 0.0002). The two-tailed Mann–Whitney U non-parametric test indicated a significant difference between the Sal/Sed group with other three groups on days 1–6 (all, P < 0.05). There was a significant difference between the Mor/Sed and Mor/VE groups on days 4–6 (all, P < 0.05), and between the Mor/Sed and Mor/TE groups on day 6 (P < 0.05).

3.2. Effects of voluntary and forced treadmill exercise on memory deficits in morphine-dependent rats

To determine the effect of voluntary and forced exercise on memory deficits induced by chronic morphine, rats (n = 10 rats per
group) were divided into the Sal/Sed, the Sal/VE, the Sal/TE, the Mor/Sed, the Mor/VE, the Mor/TE groups. The learning and memory capabilities of all groups were tested as previously described (Fig. 1, Experiment 2).

3.3. Spatial learning

The pattern of voluntary running for voluntary exercise groups was similar to that shown in Fig. 2 (data not shown). An ANOVA carried out on swim speed revealed no differences between groups. Moreover, data related to the distance swam to reach the platform and latency to reach the platform followed similar patterns; thus, we only present latency data. Latency data during the 5 days of training in the WM are illustrated in Fig. 4. A three-way ANOVA (morphine treatment × exercise × training days) were used to analyze the escape latencies during training. All groups learned to locate the platform during the five successive days of training, as indicated by decreasing escape latencies as training progressed ($F_{4,270} = 44.62, P < 0.0001$). The effect of exercise was significant ($F_{2,270} = 16.81, P < 0.0001$): the exercising groups exhibited significantly shorter escape latencies on days 3 and 4 the WM training than those of the sedentary control groups ($Ps$ ranging from $<0.05$ to $<0.0001$). The main effect of morphine treatment was not significant ($F_{1,270} = 1.48, P = 0.222$). There were no significant interaction

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**Fig. 1.** Timelines of experiments (see Section 2 for details).
between morphine treatment and exercise and days ($F_{8,270} = 0.756$, $P = 0.642$). These findings indicate that both voluntary and treadmill exercise enhanced the learning rate in both control and chronic morphine groups.

3.4. Spatial memory

The data for the memory retention test are shown in Fig. 5. A two-way ANOVA on the platform location latency (Fig. 5A) showed significant effects of exercise ($F_{2,54} = 5.59$, $P < 0.01$), and of morphine treatment ($F_{1,54} = 4.02$, $P < 0.05$), and a significant interaction between both factors ($F_{2,54} = 3.47$, $P < 0.05$). The between group comparisons indicated that the platform location latency of the Mor/Sed group was significantly longer than that of the Sal/Sed group ($P < 0.05$, Fig. 5A), indicating that chronic morphine impairs memory retention. The platform location latency of the Sal/VE and Sal/TE groups was significantly shorter than that of the Sal/Sed group ($P < 0.01$ and $P < 0.05$, respectively). The platform location latency of the Mor/VE and Mor/TE groups was significantly shorter than that of the Mor/Sed group ($P < 0.05$ and $P < 0.01$, respectively).

A three-way ANOVA with quadrant (Fig. 5B) as repeated measures showed a significant interaction between morphine treatment and exercise and zones ($F_{3,216} = 134.52$, $P < 0.0001$). The between-group comparisons indicated that both the Sal/VE and Sal/TE groups spent significantly more time in the target zone than the Sal/Sed ($P < 0.01$) and Mor/Sed groups ($P < 0.001$). The Mor/Sed group spent more time in the opposite zone than the Sal/Sed group ($P < 0.05$).

To control for differences in WM performance, we also recorded each animal’s swimming speed. We found no difference ($F_{5,54} = 0.6$, $P = 0.55$) in the swimming speeds of all six groups: the Sal/Sed group (33.33 cm/s), the Sal/VE group (33.17 cm/s), the Sal/TE (30.91 cm/s), the Mor/Sed group (34.22 cm/s), and the Mor/VE group (35.89 cm/s), and the Mor/TE group (35.76 cm/s).

3.5. Retention memory in an inhibitory avoidance task

Memory retention data of inhibitory avoidance task are illustrated in Fig. 6. A two-way ANOVA on STL data (Fig. 6A) indicated a significant effect of exercise ($F_{2,54} = 14.75$, $P < 0.0001$), a significant effect of chronic morphine ($F_{2,54} = 2.79$, $P = 0.1$) and no significant interaction between both factors ($F_{2,54} = 0.21$, $P = 0.80$). Post hoc comparisons showed that STL of the Sal/VE and Sal/TE groups was significantly longer than the Sal/Sed (both, $P < 0.01$). The STL of the Mor/Sed group was significantly shorter than the Sal/Sed (both, $P < 0.05$). The STL of Mor/VE, and Mor/TE groups was significantly longer than the Mor/Sed (both, $P < 0.01$).

ANOVA on TLC data (Fig. 6B) also showed a significant effect of exercise ($F_{2,54} = 14.25$, $P < 0.0001$), a significant effect of chronic morphine ($F_{2,54} = 9.24$, $P < 0.01$) and no significant interaction between both factors ($F_{2,56} = 0.98$, $P = 0.33$). Post hoc comparisons

![Figure 2](image-url) Fig. 2. The average of running distance (expressed in meter per day) in the voluntary exercising groups.

![Figure 3](image-url) Fig. 3. Effect of voluntary and treadmill exercise on the spontaneous withdrawal signs in morphine-dependent rats. The Gellert–Holtzman score of the overall withdrawal severity was calculated using the proper weighting factor. Both voluntary and treadmill exercise significantly decreased the occurrence of spontaneous withdrawal signs. Data are expressed as the median ± inter-quartile range. * $P < 0.05$ compared with the Sal/Sed group; † $P < 0.05$ compared with the Mor/Sed group. Mor: morphine, VE: voluntary exercise, TE: treadmill exercise.
showed that the TLC of the Sal/VE and Sal/TE groups was significantly longer than the Sal/Sed (both, \( P < 0.001 \)). The TLC of the Mor/Sed group was significantly shorter than the Sal/Sed (\( P < 0.05 \)). The TLC of the Mor/VE, and Mor/TE groups was significantly longer than the Mor/Sed group (both, \( P < 0.001 \)).

### 3.6. Effects of voluntary and forced exercise on hippocampal apoptosis proteins and serum corticosterone levels in morphine-dependent rats

To determine the effect of voluntary and forced exercise on serum corticosterone levels and hippocampal expression of apoptotic-associated proteins induced by chronic morphine, rats (\( n = 7 \) rats per group) were divided into the Sal/Sed, the Sal/VE, the Sal/TE, the Mor/Sed, the Mor/VE, and the Mor/TE groups (Fig. 1, Experiment 3). To verify apoptosis induced by chronic morphine, we determined the relative expression of Bax, and Bcl-2 proteins (Fig. 7A). The ratio of Bax to Bcl-2 was also calculated.

The pattern of voluntary running for voluntary exercise groups was similar to that shown in Fig. 2 (data not shown). When the level of Bax protein in the Sal/Sed group was set at 100, the level of Bax was 94.16 \( \pm \) 0.51 in the Sal/VE group, 93.7 \( \pm \) 0.27 in the Sal/TE group, 99.43 \( \pm \) 0.43 in the Mor/Sed group, 97.12 \( \pm \) 0.4 in the Mor/VE group, and 96.92 \( \pm \) 0.32 in the Mor/TE group (Fig. 7B). A two-way ANOVA on Bax data showed significant effects of treatment (\( F_{1,36} = 67.20, P < 0.0001 \)), no significant effects of exercise (\( F_{2,36} = 1.78, P = 0.18 \)), but a significant interaction between both factors (\( F_{2,36} = 13.57, P < 0.0001 \)). The between-group comparisons indicated that chronic morphine did not change the expression of Bax protein. Both VE and TE significantly (\( P < 0.01 \)) decreased the expression of Bax protein in saline-treated rats. Only TE decreased the expression of Bax in morphine treated groups (\( P < 0.05 \)).

When the level of Bcl-2 protein in the Sal/Sed group was set at 100, the level of Bcl-2 was 117.96 \( \pm \) 0.97 in the Sal/VE group, 116.1 \( \pm \) 1.12 in the Sal/TE group, 82.45 \( \pm \) 3.83 in the Mor/Sed group, 100.64 \( \pm \) 3.83 in the Mor/VE group, and 91.62 \( \pm \) 3.94 in the Mor/TE group (Fig. 7C). A two-way ANOVA on Bcl-2 data showed significant effects of treatment (\( F_{1,36} = 52.34, P < 0.0001 \)), exercise (\( F_{2,36} = 15.36, P < 0.0001 \)), and no significant interaction between both factors (\( F_{2,36} = 0.73, P = 0.49 \)). The between-group comparisons indicated that chronic morphine decreased the expression of Bcl-2 protein (\( P < 0.01 \)). Both VE and TE significantly (\( P < 0.01 \)) increased the expression of Bcl-2 protein in both the saline and morphine treated groups.

When the level of Bax to Bcl-2 ratio in the Sal/Sed group was set at 100, this level was 78.84 \( \pm \) 1.11 in the Sal/VE group, 79.75 \( \pm \) 0.84 in the Sal/TE group, 120.20 \( \pm \) 4.97 in the Mor/Sed group, 96.12 \( \pm \) 3.53 in the Mor/VE group, and 105.54 \( \pm \) 4.15 in the Mor/TE group (Fig. 7D). A two-way ANOVA on Bax to Bcl-2 ratio data showed significant effects of treatment (\( F_{1,36} = 24.38, P < 0.0001 \)), exercise (\( F_{2,36} = 12.79, P < 0.0001 \)), and no significant interaction between both factors (\( F_{2,36} = 1.47, P = 0.49 \)). Chronic morphine increased significantly the Bax to Bcl-2 ratio as compared with control group (\( P < 0.01 \)). Both voluntary and treadmill exercise
Exercise 4.1. **Fig. 166**

significantly ($P < 0.01$) decreased the Bax to Bcl-2 ratio in both the saline and morphine treated groups.

**Discussion**

The main findings of the present study are that exercise reduces spontaneous morphine-withdrawal and blocks the associated impairment of cognitive performance. In parallel, exercise prevents the hippocampal increase of the ratio Bax/Bcl-2 induced by morphine dependence. These findings suggest that exercise could be a useful therapy to prevent cognitive deficits and neuronal apoptosis in addicts individuals.

4.1. **Voluntary and treadmill exercise reduce spontaneous withdrawal signs**

We found that both voluntary and forced exercise after chronic morphine exposure decrease the occurrence of spontaneous withdrawal signs in morphine-dependent rats. Interestingly, the effects of voluntary exercise was appeared 4 days, while that of treadmill was seen 6 days following exercise, indicating a more effective effect of voluntary exercise than forced exercise. These findings are in accordance with our previous studies showing that voluntary exercise during morphine dependence development reduces dependence severity [7]. Additionally, it is important to note that exercise exerts a mild effect in reducing withdrawal signs, while the abstinence period is the main factor responsible for the withdrawal score reduction in all groups. Presently, it is not clear how exercise during the abstinence period can reduce the occurrence of spontaneous withdrawal signs. Exercise has been shown to reduce self-administration of morphine [38], the craving for morphine [39], and the potency of morphine [40]. Additionally, long-term exercise (6 weeks) decreases sensitivity of the anti-nociceptive effects of morphine and other mu opioids, which is mediated by the chronic release of endogenous opioid peptides and functional changes in the opioid receptor system [40,41]. Finally, voluntary exercise can induce a rewarding state that continues following exercise cession, and cause plastic changes in the dopaminergic reward pathway of the mesolimbic system [42]. Consequently, these changes in potency and sensitivity of morphine and neu-ropaum modifications in the reward pathway may contribute to the attenuation of spontaneous withdrawal signs following exercise.
4.2. Chronic morphine impairs memory retention and induces apoptosis

We found that chronic morphine impairs memory retention in both spatial and aversive tasks. These findings are consistent with previous studies showing prenatal as well as postnatal administration of morphine can impair cognitive functions [3,6–8,11,20,23,37]. This deficit is unlikely because of spontaneous withdrawal, given that no significant difference was found in signs of withdrawal between the Sal/Sed and Mor/Sed groups when somatic signs were assessed 7 days after the last morphine injection (Fig. 3). This suggests that morphine withdrawal is not still going at the time of training and testing. Thus, the cognitive deficits observed in the dependent rats are related to past exposure to either opiates and/or opiate withdrawal but not to direct opiate withdrawal. Although the mechanism that underlies the impairing effects of morphine remains unknown, long-term opiate use may induce maladaptive plasticity in brain structures involved in learning and memory, such as the hippocampus. Chronic exposure to morphine can interact with hippocampal synaptic transmission, and cause cognitive deficits in several hippocampal-dependent tasks [4,8,11,43].

Past studies have shown that chronic morphine induces up-regulation of the pro-apoptotic Fasl, Fas, and Bad and the active fragments of caspases-8 and 3 in mice and rat brains [21]. The enhanced apoptosis by chronic contact to opioid drugs may interfere with memory and learning [23]. Prenatal morphine exposure has been also demonstrated to enhance the level of neuroblast apoptosis and delays the neural tube closure in developing CNS [44]. This prenatal imbalanced apoptosis may continue even after delivery, as in a recent study the memory deficits resulted from prenatal morphine exposure is attributed to the increased neuronal apoptosis in the hippocampus of young offspring [43]. We found that chronic exposure to morphine induced down-regulation of the anti-apoptotic protein Bcl-2 in the hippocampus, but Bax expression remained constant, resulting in a high Bax/Bcl-2 ratio. These alterations, in turn, shifted the balance between pro-apoptotic and anti-apoptotic factors in favor of cell death. A previous study has shown that chronic-morphine treated mice and abstinence mice (one day after the last morphine injection) exhibited scattered apoptotic neurons and astrocytes in the brain. This neurotoxic effect was accompanied by up-regulation of the pro-apoptotic proteins and the active fragments of caspases in cortical and hippocampal lysates. Abstinence from chronic morphine also resulted in a reduced expression of the anti-apoptotic protein Bcl-2 in mice [45]. This finding is in agreement with the present results showing similar changes in Bcl-2 expression in the abstinence rats (11 days after the last injection of morphine). Chronic morphine may enhance apoptosis through the sustained activation of opioid receptors as the opioid receptor antagonist naloxone completely prevented morphine-induced changes in the expression of apoptosis-associated protein in the brain [21]. Thus, the
induction of deviant apoptosis in specific regions of brain such as hippocampus may be a major consequence of the neuronal damage induced by opiate drugs after long-term exposures [46]. This neuronal damage may lead to cognitive deficits and anxiety and mood disorders that seen in drug abusers [1,2]. In the present study, we did not identify/quantify apoptotic cells in the hippocampus. Thus, further study such as the quantification of neuronal apoptosis following chronic morphine will be required to determine the overall significance of morphine induced reduction of Bcl-2 expression. Although these data from animal studies may indicate the abnormal activation of apoptotic pathways following chronic exposure to opiates, a study performed on postmortem human brain from heroin or methadone abusers revealed that the extrinsic and intrinsic apoptotic pathways are not abnormally activated in the prefrontal cortex, suggesting the differential effects of long-term use of opiates on apoptotic pathways in humans and experimental animals [47].

4.3. Voluntary and treadmill exercise alleviate cognitive deficits and the enhanced-apoptosis induced by chronic morphine

We found that both voluntary and treadmill exercise enhance cognitive functions in spatial and aversive tasks, confirming and extending previous studies showing the beneficial effects of different types of exercise on learning and memory in a variety of tasks [3,5,7,18,19]. These beneficial effects of exercise on memory and neuroplasticity are mediated by the BDNF-TrkB pathway in distinct brain regions such as the hippocampus, as blockade of this
pathway abolished the enhancing effects of exercise on learning and memory [7,29,48,49].

We found that both types of exercise enhanced the up-regulation of the anti-apoptotic protein Bcl-2 and down-regulation of the pro-apoptotic protein Bak in the hippocampus, which shifted the balance between pro-apoptotic and anti-apoptotic factors in favor of cell survival. These effects were seen in both the saline and morphine-treated animals, suggesting that exercise has a positive influence on cell survival in normal and patho-physiological conditions. In accordance with these findings, other studies have shown that both types of exercise suppressed neuronal apoptotic cell death in the hippocampus probably via BDNF- and NGF-mediated mechanisms and subsequently, improve learning and memory [28,49–51].

Previous studies have demonstrated that different types of exercise induce neuroplastic changes and neuronal adaptations in different brain regions, and thus exert differential effects on various forms of learning and memory [31,32]. The exercise-induced different intensity of stress and hence glucocorticoid levels could be an underlying modulator for their differential effects on brain functions. We found that both exercise did not significantly increased serum corticosterone levels, but exerted similar effects on cognitive functions and apoptosis-related proteins. This suggests that both types of exercises did not induce a significant stress response which may interfere with their beneficial effects on brain.

In conclusion, we observed that both voluntary and treadmill exercise increased cognitive functions and shifted the alterations in apoptosis-related proteins in favor of cell survival in normal conditions. Chronic exposure to morphine impaired cognitive functions and remarkably suppressed the expression of Bcl-2 with no significant changes in Bak expression, shifting the balance of pro-apoptotic and anti-apoptotic proteins in favor of cell death in the hippocampus and consequently, impairment of learning and memory. Conversely, both voluntary and treadmill exercise were able to alleviate memory impairment and return the changes of apoptotic proteins in favor of cell survival in morphine-dependent rats. Although specific extrapolation of the results of rodent experiments to the human condition appears difficult, our results could suggest the application of exercise as a useful therapeutic strategy for cognitive and behavioral deficits in individuated individuals.

Conflict of interest statement

We attested that we have herein disclosed any and all financial or other relationships that could be construed as a conflict of interest and that all sources of financial support for this study have been disclosed.

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