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Deleterious effects of prenatal exposure to morphine on the spatial learning and hippocampal BDNF and long-term potentiation in juvenile rats: Beneficial influences of postnatal treadmill exercise and enriched environment

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ABSTRACT

Prenatal morphine exposure causes a variety of neurobehavioral alterations observed in later life. The present study investigated the effects of postnatal exercise and enriched environment (EE) on alterations in water maze learning and hippocampal long-term potentiation (LTP) and brain derived neurotrophic factor (BDNF) levels induced by exposure to morphine during prenatal period in rats. On gestation days 11–18, pregnant rats were injected twice daily with saline or morphine. Offspring were subjected to postnatal exercise and EE for 30 days and afterward, spatial learning and hippocampal LTP and BDNF levels were investigated. Prenatal morphine-exposure impaired the spatial learning and hippocampal LTP in both male and female offspring. Interestingly, postnatal exercise and EE increased performance in the water maze and improved LTP in both prenatally saline and morphine-exposed male and female rats. Prenatal morphine exposure also caused a reduction in the hippocampal BDNF levels in the female, but not male rats, and postnatal exercise and EE alleviated this deficit. Our results demonstrate that postnatal exercise and EE can improve deficits in water maze learning and hippocampal LTP and BDNF levels caused by prenatal morphine exposure.

1. Introduction

Exposure to the drug of abuse can influence individuals throughout life, initiating in prenatal life. Exposure to opioids during pregnancy can predispose individuals to the expansion of physiological and affective deficits that continue through adulthood. In opioid addiction, children born to morphine – or heroin – addicted mothers have been shown to have greater mortality and deficit in the central nerve system (CNS) (Ostrea, Ostrea, & Simpson, 1997; Yanai et al., 2003).

Opioids are used routinely and effectively for the treatment of pains and their effects are caused by binding to classical opioid receptors and/or Toll-like receptors existing at CNS (Chaves, Remião, Cisterninoa, & Declèves, 2017). Exposure to opioids during prenatal life can change opioid receptor distribution and density, this, in turn, could affect the development of neural connections by accelerating or delaying neural outgrowth during fetal and/or postnatal periods (Vathy, 1995).

Prenatal contact to inflammatory or infectious insults can increase the risk of developing neuropsychiatric disorder in later life, including schizophrenia, bipolar disorder, and autism and prenatal immune activation induces adult onset of presynaptic hippocampal deficits (Giovanoli, Weber-Stadlbauer, Schedlowski, Meyer, & Engler, 2016).

One of the most broadly abused substances is morphine, and its influences on prenatal development and infant consequences of addicted mothers have been well investigated. The effects of morphine on spatial learning and memory are controversial. Different studies indicate negative or positive effects of morphine on learning and memory processes in rodents (Classen & Mondadori, 1984; McNamara &

Abbreviations: EE, enriched environment; PND, postnatal day; GD, gestation day; SA, saline; MO, morphine; EX, exercise; SE, standard environment; WM, water maze; NE, northeast; SE, southeast; SW, southwest; NW, northwest; BDNF, brain derived neurotrophic factor; LTP, long-term potentiation

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Skelton, 1991; Mondadori & Waser, 1979; Shiigi & Kaneto, 1990). Animal investigations also have been shown that contact to morphine through pregnancy is related to a collection of adverse neurobehavioral outcomes for the offspring. In rodents, the adverse effects of prenatal morphine exposure consist of various deficiencies in their offspring, including brain development degree and body weight (Davis & Lin, 1972; Eriksson & Rönnbäck, 1989), sensitivity in analgesia induced by morphine (Kilby, DeRossett, & Holtzman, 1982), play behavior and locomotive activity (Hol, Niesink, van Ree, & Spruijt, 1996), and newborn survival rate (Davis & Lin, 1972). It has also been shown that prenatal morphine exposure causes impairment in the spatial learning of early steps of acquisition stage in the water maze but did not influence the memory phase (probe trial) of this task (S. N. Yang et al., 2003, 2006). One of the major hypotheses about the neurobehavioral deficits seen following prenatal morphine exposure is that declines in synaptic plasticity can be an essential brain mechanism underlying such deficits in learning and memory. Hippocampal long-term potentiation (LTP) is a demonstration of synaptic plasticity, which is a potential neural substrate for learning and memory (Artola & Singer, 1987; Bliss & Collingridge, 1993a). It has been demonstrated that prenatal morphine contact decreased hippocampal LTP in adult anesthetized rats (Sarkaki, Assaei, Motamedi, Badavi, & Pajouhi, 2008), or in freely moving rats (Villarreal, Derrick, & Vathy, 2008). In addition, in the hippocampal CA1 area of adult rats, prenatal morphine exposure depressed LTP of the excitatory postsynaptic potential (EPSP) in vitro (Velísek, Stanton, Moshé, & Vathy, 2000a). Also, it has been shown that prenatal morphine exposure impairs spatial memory and in vivo LTP in the dentate gyrus (DG) area of juvenile rats prenatally exposed to morphine (Niu, Cao, Zhu, Mei, & Wang, 2009).

There is a growing body of information about the advantageous effects of exercise on cognitive functions in humans and experimental animals (Kramer, Erickson, & Colcombe, 2006). The effectiveness of exercise in drug addiction recovery and rehabilitation is demonstrated. For instance, we have demonstrated that running exercise regimens could blunt the damaging effects of drugs of abuse (Mokhtari-Zaer et al., 2014) and chronic morphine-induced anxiogenesis and memory impairment in female rats (Ghodrati-Jaldbakhan et al., 2017). On the other hand, different deficits seen in animal models of prenatal morphine contact are almost the opposite of the advantages that are seen subsequent to exercise. For example, exercise is advantageous for brain health and function, resulting in enhancement of hippocampal neurogenesis (Marlatt, Potter, Lucassen, & van Praag, 2012; van Praag, 2008), performance in the different mazes (Alomari, Khabour, Alzoubi, & Alzubi, 2013; Trejo, 2008) and LTP (Farmer et al., 2004) in rodents. Consequently, many investigations have studied the influences (commonly advantageous) of exercise along with animal models of various prenatal insults in rats offspring, such as prenatal exposure to glucocorticoids (Liu et al., 2012), alcohol (Sim et al., 2008), ethanol (Christie et al., 2005), stress (Daniels & Russell, 2009) or morphine (Ahmadalipour & Rashidy-Pour, 2015). These investigations provide support for the capacity of exercise to modify changes seen after prenatal insults.

In addition to exercise, enriched environment (EE) has been demonstrated to increase the number of hippocampal synapses and spine densities (Moser, Trommald, & Andersen, 1994), improve synaptic plasticity in the hippocampus and increase spatial learning and memory (Duffy, Craddock, Abel, & Nguyen, 2001; Falkenberg et al., 1992; van Praag, Kempermann, & Gage, 2000). EE has been shown to counteract cognitive deficits induced by developmental lead exposure (Guilarte, Toscano, McGlothan, & Weaver, 2003), improve cognitive performance after early-life status epilepticus (Wang et al., 2007), reverse the cognitive deficit induced by prenatal stress (Koo et al., 2003; Yang et al., 2007), reduce the behavioral deficits of prenatal cocaine exposure on play behaviors (Neugebauer et al., 2004), reverse age-related changes in LTP and alleviate some of behavioral deficits induced by prenatal morphine exposure (Ahmadalipour et al., 2015).

Brain derived neurotrophic factor (BDNF) is one of the most important neurotrophic factors that links exercise and EE with improvements in cognitive function (Bekinschtein, Oomen, Saksida, & Bussey, 2011; Vaynman, Ying, & Gomez-pinilla, 2004). BDNF is extensively expressed in the hippocampus and is implicated in neuroplasticity for learning and memory (Vaynman et al., 2004). BDNF increases LTP induction at hippocampal CA1 synapses (Callaghan, Ohle, & Kelly, 2007; Diógenes et al., 2011). BDNF can promote morphological alterations and in confirmation of this, it has been demonstrated that increased brain BDNF levels in exercise and EE changes neural morphology and synaptic plasticity (Cao et al., 2014; Vaynman et al., 2004). Conversely, prenatal insults cause reduction of the BDNF levels of offspring (Boersma et al., 2013; Yeh, Huang, & Hsu, 2012), and it has also been demonstrated that prenatal opiate contact reduces the levels of BDNF protein and its precursor in the hippocampus (Schrott, Franklin, & Serrano, 2008).

As young adulthood and adolescence represent critical periods for postnatal brain maturation (Schneider, 2013), during which external factors and environmental conditions may have a great impact on immune, hormonal and behavioral mechanisms (Laviola et al., 2004), the current study investigated the effects of treadmill exercise and EE during adolescence on water maze learning performance and hippocampal LTP and BDNF levels of rat offspring born to the morphine injected mothers during pregnancy. Because a large number of rodent and human studies show that there are sex differences in drug addiction and underlying neurobiological mechanisms (Zhou, Zhao, Zhou, & Li, 2016), all experiments were performed in juvenile male and female offspring to study the probable sex differences as well.

2. Experimental procedures

2.1. Prenatal treatment

30 adult Wistar virgin female rats (250–275 g) were mated with age-matched male rats. Observation of vaginal plug and a great rate of body weight gain in the next 10 days were utilized as the indices for pregnancy. The pregnant rats were randomly assigned to one of two groups: (1) a morphine sulfate (MO) injected group, or (2) a saline (SA) injected control group. Rats of the MO group were injected twice on gestation day (GD) 11, once in the morning (07:00hr) and once in the afternoon (18:00hr) with 5 mg/kg morphine (Temad Company, Tehran, Iran) subcutaneously. On GD12 the animals were injected with 5 mg/kg morphine in the morning and 10 mg/kg in the afternoon. On GD13 and afterward through GD 18 the animals received two daily injections of 10 mg/kg morphine (Biochemistry & Method, 1985). The control SA group of female rats received two daily injections of physiological saline on GD 11–18. These injection times (mid to late gestational days) are chosen based on previous studies (Niu et al., 2009; Schindler, Czy, Hnactzuk, Riley, & Vathy, 2004; Vathy, Etgen, & Barfield, 1985) to coincide with regarding the fetal expression of opioid receptors as all three major opioid receptors are present in the rat brain from day 14 of gestation (Clendeninn, Petraitis, & Simon, 1976), and the fact that late gestation is a time frame critical for hippocampus and cortical development (Bayer, Altman, & Raymond, 1993). To mimic addiction process in rats that is usually long period in human subjects, injection starts 3 days before GD14 (GD 11–18).

2.2. Postnatal treatment

From GD 20, the pregnant rats were observed for deliveries twice a day. The day of delivery was chosen as postnatal day (PND 0). At this time, the pups were sexed and weighed. The injection pattern did not alter body weight of the pups or the number of pups per litter as described in previous studies (Vathy, Etgen, Rabii, & Barfield, 1983). Within 12–36 h of parturition, pups from both morphine- and saline-treated dams were fostered and culled to 10 per litter. Cross-fostering

procedure was performed (Calatayud, Coubard, & Belzung, 2004) in order to control for possible variations in maternal behavior, such that every mother raised half of her own and half of the adopted pups receiving the different prenatal treatment. Offspring were weaned on PND 21 and dispersed into 12 experimental groups; 6 groups of male and 6 groups of female offspring (n = 8 per group in Experiment 1, n = 5–8 per group in Experiment 2, and n = 6–7 per group in Experiment 3): *Saline/Standard* (SA/SE): offspring from saline exposed dam and housed in standard environment (SE) from PND 21 to PND 50; *Morphine/Standard* (MO/SE): offspring from morphine-exposed dams and housed in SE from PND 21 to PND 50; *Saline/EE*(SA/EE): offspring from saline exposed dam and housed in EE from PND21 to PND 50; *Morphine/EE*(MO/EE): offspring from morphine-exposed dams and housed in EE from PND 21 to PND 50; *Saline/Exercise* (SA/EX): offspring from dams prenatally exposed to saline and did running exercise from PND 21 to PND 50 and *Morphine/Exercise* (MO/EX): offspring from dams prenatally exposed to morphine as previously described and did running exercise from PND 21 to PND 50.

Offspring in exercise groups at first were habituated to a motor-driven treadmill at a speed of 2 m/min for 10 min/day during first and second days of exercise. Then, they were made to run on the treadmill for 30 min once a day for 4 consecutive weeks (PND 21 to PND 50), 6 days per week. Exercise load used for exercise groups consisted of running at a speed of 2 m/min for the first 5 min, 5 m/min for the next 5 min and then at a speed of 8 m/min for the last20 min, at a 0° inclination (Kim et al., 2010). The EE group was housed in a plexiglas cages (100 cm × 100 cm × 50 cm) (12 animals per cage) having a raised platform, a running wheel, a collection of plastic tunnels, steel chains, different size plastic balls and toys changed every 5–6 days. The SE group was housed in standard plexiglas cages (60 cm × 40 cm × 20 cm) (four animals per cage). In order to prevent probably mating male and female rats were separately located in standard or EE.

Three experiments were performed to investigate behavioral, electrophysiological and biochemical changes induced by prenatal morphine exposure or postnatal exercise or EE. In experiment 1 we investigated spatial learning (PND 51–55) (Fig. 1A). In experiment 2 we investigated changes in the hippocampal LTP (PND 51–57) (Fig. 1B). In experiment 3 we investigated changes in the hippocampal BDNF levels (PND 51) (Fig. 1C). In each experiment, 12 experimental groups (6 males and 6 female groups) were used as described in the above.

2.3. Water maze

Experiment 1 was done using Morris water maze (WM) to investigate spatial learning. A detailed description of the apparatus and the tracking system has been given in our previous reports (Miladi, Rashidy-pour, & Fathollahi, 2008; Mokhtari-Zaer et al., 2014). In brief, the WM was a black circular pool (140 cm in diameter and 60 cm high) of 25 cm depth filled with 22 ± 1 °C water. 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 aquasi-random order. The rats were allowed to search for the escape platform until they climbed onto the platform and 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. As it has been demonstrated that prenatal morphine exposure causes impairment in the performance of early phase of acquisition stage of water maze but did not affect memory phase (probe trail) (Yang et al., 2003, 2006), thus in the current study, we investigated only the acquisition phase (learning phase) of water maze performance. The parameters escape latency (i.e., time to reach the platform, in seconds), path length (cm), and thigmotaxis (i.e., the portion of the total distance that the rats swam in the outer 10 cm of the pool) were analyzed for all 5 days.

2.4. Electrophysiology experiment

In Experiment 2, offspring were deeply anesthetized with urethane (1.2 g/kg, i.p.) on PND 50–57. For electrophysiological recording, stimulating and recording electrodes were prepared by gluing together a couple of twisted Teflon-coated 90% platinum and 10% iridium wires (135 μm). The animals were anesthetized with urethane (1–1.2 g/kg, i.p.) and stimulating electrode was implanted in the medial PP (coordinates: Anterior-posterior (AP), 7 mm; medial–lateral (ML), 4 mm; dorsal–ventral (DV), 3–3.3 mm, from skull surface) and a recording electrode was implanted in the DG granule cell layer (coordinates: AP, 3; ML, 2; DV, 2.7–3.2 from skull surface).

Stimulation and recording. Constant current rectangular stimulus pulses (200 μs, 0.1 Hz) were delivered during a 10 min period after electrode placement (stabilization period). To achieve this, it was at times necessary to reposition the stimulating and/or recording electrodes until the highest potential could be obtained. When the variation in the population spike (PS) amplitude was less than ± 10% for 10 min, the baseline recording was considered stable. An input/output (I/O) profile was established by increasing the stimulus intensity and measuring the PS amplitude.

The stimulus intensity that evoked a PS or field excitatory post-synaptic potentials (fEPSP) of 50–60% of the baseline maximum response was chosen for subsequent train stimuli. The evoked response was amplified, filtered (bandpass: 1 Hz–3 kHz) and sampled at a rate of 20 kHz and stored on the hard disk. All recording and stimulation were performed using an on-line computerized oscilloscope–stimulator and data analysis interface system by Data Acquisition D3111 set up and NeuroTrace Software (www.ScienceBeam.com). After baseline synaptic responses had been stable for at least 20 min, the theta pattern primed bursts (PBs) tetanic stimulation was used for LTP induction which was consisted of eight PBs at intervals of 10 s. Each PB consisted of a single priming pulse followed 170 ms later by a burst of 10 pulses delivered at 100 Hz. Theta PB stimulation is an effective protocol for inducing robust and persistent LTP, which is based on the physiology of hippocampus. After tetanus delivery, responses were recorded 5, 15, 30, 45, 60, 75, 90, 105, and 120 min after train stimuli. The magnitude of potentiation was evaluated as the percentage change in the PS amplitude and the slope of fEPSP relative to the pre-tetanus test value. The acceptable level of a defined LTP was more than a 30% increase in the PS amplitude and/or EPSP slope.

2.5. BDNF measurements

In experiment 3, mating, MO/SA injection and random grouping assignments until PND 21 were similar to those of experiment 1. Pups were weaned on PND 21 and were housed in SE or EE (Fig. 1B). On PND 51, rats (n = 6–7 per group) were sacrificed, and the hippocampi were dissected and were then immediately frozen at −70 °C. The BDNF protein levels were assessed using Rat BDNF ELISA kits (Biorbyt, UK) according to the manufacturer's recommendations. The hippocampal extracts were prepared in lysis buffer and the supernatants obtained after centrifugation at 12,000*g* for 20 min at 4 °C were used for BDNF assay. Rat BDNF specific polyclonal antibodies were precoated onto 96-well plates. The rat specific detection monoclonal antibodies were biotinylated. We added the test samples and biotinylated detection antibodies to the wells subsequently and then washed with PBS or TBS buffer. Then we added Avidin-Biotin-Peroxidase Complex and washed unbound conjugates away with PBS or TBS buffer. We used horseradish peroxidase (HRP) substrate TMB to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to yield a blue color product that altered into yellow after adding acidic stop solution. The density of yellow is proportional to the rat BDNF amount of sample captured in plate. The sensitivity of the assay was < 2 pg/ml. The level of total protein in

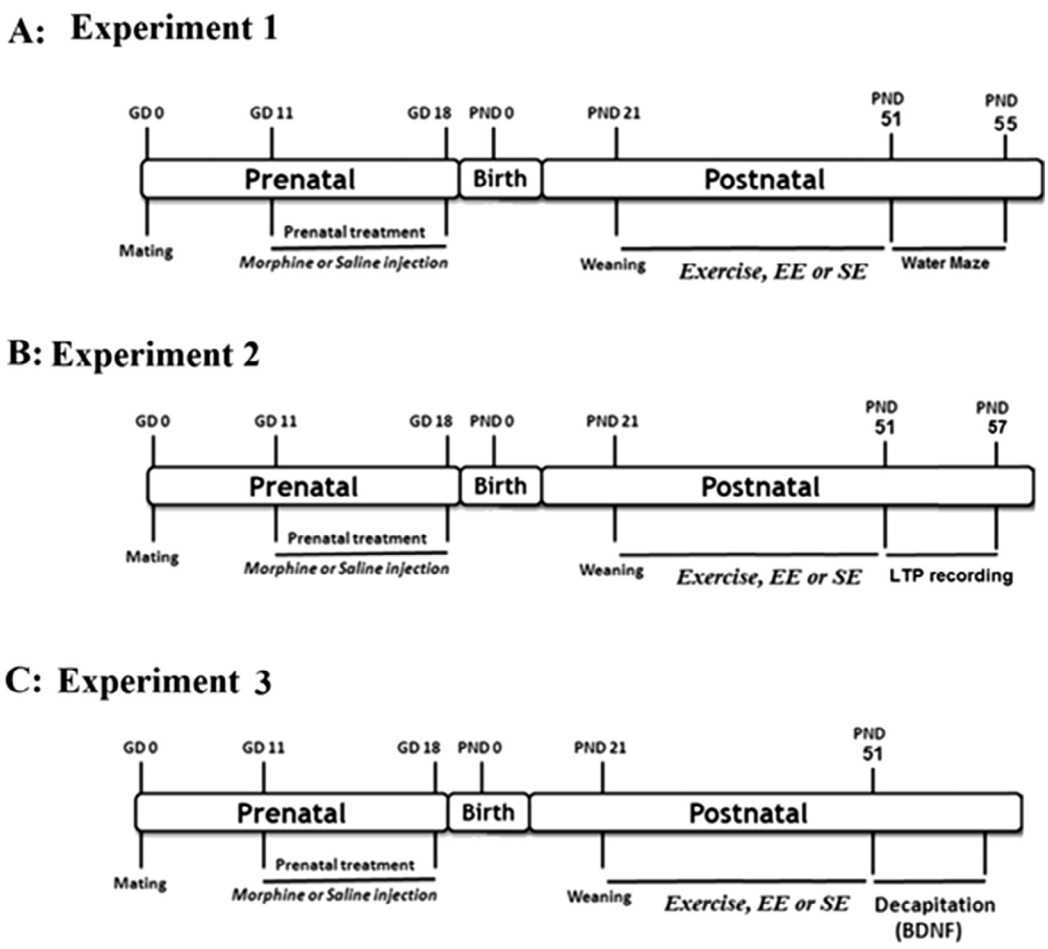

Fig. 1. Timeline of prenatal treatment, postnatal enriched environment and exercise and spatial water maze testing (Experiment 1), LTP measurements (Experiment 2) and BDNF measurement (Experiment 3). See Experimental procedures for details. EE: Enriched environment; PND: Postnatal day; GD: Gestation day; EX: Exercise; SE: Standard Environment.

supernatants was determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

2.6. Statistical analysis

The significant interactions between prenatal morphine treatment and postnatal exercise and EE on the measured BDNF levels and LTP were determined using an overall two-way analysis of variance (ANOVA) with the fixed factors being prenatal treatments (SA and MO) and postnatal groups (EX, EE and SE) and followed by the Tukey's test and Bonferroni adjustment. For the WM test, mixed repeated measures ANOVAs were used to analyze the effects of prenatal treatment, postnatal groups and training day followed by the Tukey's test. The significant level was set at $p \leq .05$. Results are expressed as mean ± S.E.M.

3. Results

3.1. Effects of prenatal morphine exposure and postnatal exercise and EE on spatial learning of offspring

3.1.1. Escape latency of water maze in male rats

The acquisition data of the experimental groups during the 5 days of water maze test in male rats are illustrated in Fig. 2A. Mixed repeated measure ANOVA on latency to find the platform indicated that all rats learned the task during the five successive days of training ($F_{4, 144} = 46.09$, $P = .0005$).There were significant interactions between days and groups (EE, SE and EX)($F_{4, 144} = 3.591$, $P = .008$) and between days and treatments (prenatal MO and SA) ($F_{8, 144} = 3.155$, $P = .003$) but the interaction between days, groups and treatments was not significant ($F_{8, 144} = 1.269$, $P = .264$). Between group comparison revealed that the escape latency of the MO/SE group was significantly more than those of the SA/SE group on day 1($P < .05$). The MO/EX group exhibited significantly shorter escape latencies on day 1 than those of the MO/SE group ($P < .05$). The SA/EE group exhibited significantly shorter escape latencies on day 2 than those of the SA/SE group ($P < .05$). The SA/EX group exhibited significantly shorter escape latencies on days1 and 3 than those of the SA/SE group ($P < .05$). Also, analysis of escape latencies of the Sal/SE and MO/SE groups in the four trials on day 1 indicated a significant difference in trials 2 ($P = .003$), 3 ($P = .03$) and 4 ($P = .042$), suggesting differences in learning rate between two groups.

3.1.2. Swimming distance of water maze in male rats

The data of the distance traveled to reach the platform in male rats was parallel to the pattern as the latency (Fig. 2B). All experimental groups traveled shorter distances to locate the platform during the five successive days of training ($F_{4, 144} = 36.334$, $P = .0001$). There was significant interaction between days and prenatal treatments (MO and SA) ($F_{8, 144} = 8.059$, $P = .05$) but the interactions between days and postnatal groups (EE, SE and EX) ($F_{4, 144} = 1.123$, $P = .348$) and between days, postnatal groups and prenatal treatments ($F_{8, 144} = 0.52$, $P = .84$) were not significant. Between groups comparison revealed that the MO/SE group significantly traveled longer distances than those of the SA/SE group on days 1 and 2 ($P < .05$), indicating that prenatal morphine exposure impairs water maze learning in offspring. The SA/EX group significantly traveled shorter distances on day 1 than those of the SA/SE group ($P < .05$). The MO/EX group significantly traveled shorter distances on day 1 than those of the MO/SE group ($P < .01$). The MO/EE group significantly traveled shorter distances on days 1 and

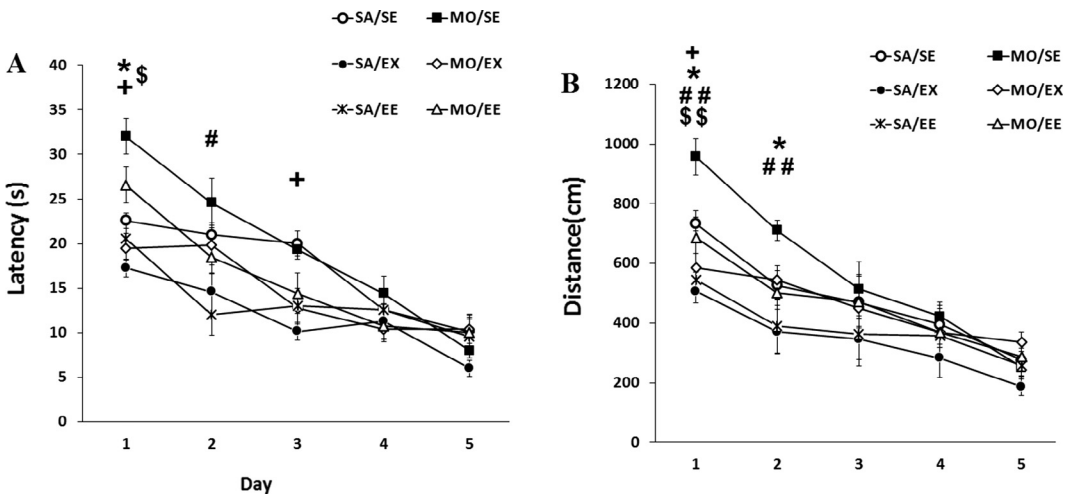

Fig. 2. Effects of prenatal treatment with morphine and postnatal EE and exercise on spatial learning in the water maze task in male rat offspring. Each bar represents mean (± S.E.M.) of the escape latency (A) in and swim distance (B). Significant difference; In A: $^{*}P < .05$the MO/SE group vs. SA/SE group on day 1.$^{\$}P < .05$ the MO/EX group vs. MO/SE group on day 1. $^{+}P < .05$ the SA/EX group vs. SA/SE group on days 1 and 3. $^{\#}P < .05$ the SA/EE group vs. SA/SE group on day 2. In B: $^{*}P < .05$ the MO/SE group vs. SA/SE group on days 1 and 2. $^{+}P < .05$ the SA/EX group vs. SA/SE group on day 1. $^{\$\$}P < .01$ the MO/EX group vs. the MO/SE group on day 1. $^{\#\#}P < .01$ the MO/EE group vs. MO/SE group on days 1 and 2.MO: Morphine; SA: Saline, SE: Standard environment, EE: Enriched environment, EX: Exercise.

2 than those of the MO/SE group ($P < .01$).

Thigmotaxic distance declined across days for all groups, indicating that male pups habituated to the stress of the task across time (data not shown). Three-way ANOVA (day × prenatal treatments × postnatal interventions) on the distance traveled in the thigmotaxic zone revealed a significant decline in swim distance across training days ($F_{4, 144} = 26.3$, $P = .0001$), but no effect of prenatal or postnatal interventions, or interaction. This suggests that the impairing effect of morphine on spatial learning is not simply due to any non-specific effects of chronic morphine, but results from its specific effect on learning process.

3.1.3. Escape latency of water maze in female rats

The data of the experimental groups during the 5 days of water maze test in female rats are illustrated in Fig. 3A. Mixed repeated measure ANOVA on latency to find the platform indicated that all rats learned the task during the five successive days of training ($F_{4, 144} = 41.277$, $P = .0005$). There were significant interactions between days and postnatal groups (EE, SE and EX) ($F_{8, 144} = 3.997$, $P = .004$) and between days and prenatal treatments (MO and SA) ($F_{4, 144} = 2.313$, $P = .023$) but the interaction between days, postnatal groups and prenatal treatments was not significant ($F_{8, 144} = 1.283$, $P = .257$). Between groups comparison revealed that the escape latency of the prenatal MO/SE group was significantly more than those of the SA/SE group on day 2 ($P < .01$). The MO/EX group exhibited significantly shorter escape latencies on day 1 than those of the MO/SE group ($P < .01$). The MO/EE group exhibited significantly shorter escape latencies on day 1 than those of the MO/SE group ($P < .01$). Also, analysis of escape latencies of the Sal/SE and MO/SE groups in the four trials on day 2 indicated a significant difference in trials 2 ($P = .002$), 3 ($P = .036$) and 4 ($P = .038$), suggesting differences in learning rate between saline and morphine-treated groups.

3.1.4. Swimming distance of water maze in female rats

The data of the distance traveled to reach the platform in female rats was also parallel to the pattern of the latency (Fig. 3B). All experimental groups traveled shorter distances to locate the platform during the five successive days of training ($F_{4,144} = 21.517$, $P = .0005$). There was significant interaction between days and prenatal treatments (MO and SA) ($F_{4,144} = 2.355$, $P = .045$) but the interactions between days and

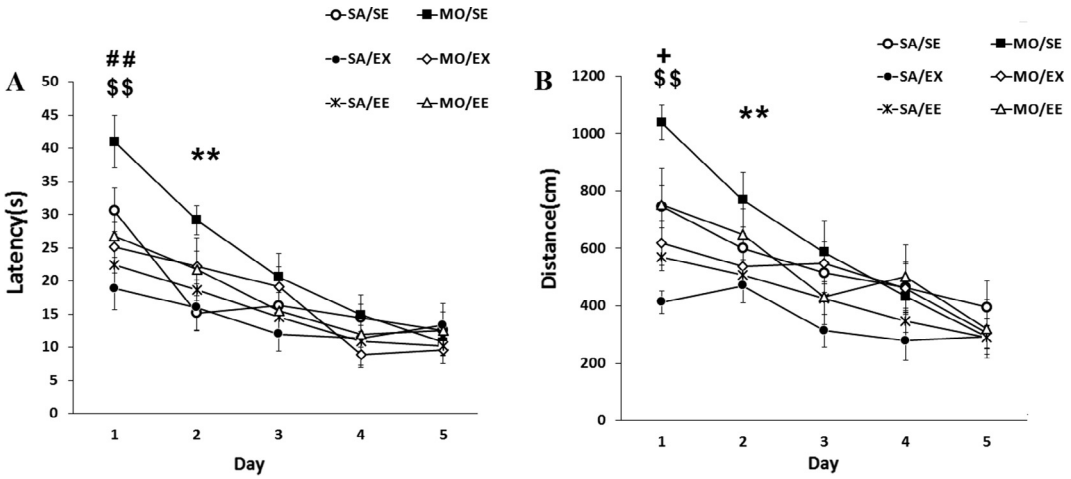

Fig. 3. Effects of prenatal treatment with morphine and postnatal EE and exercise on spatial learning in the water maze task in female rat offspring. Each bar represents mean (± S.E.M.) of the escape latency (A) in and swim distance (B). In A: $^{**}P < .01$ the MO/SE group vs. SA/SE group on day 2. $^{\$\$}P < .01$ the MO/EX group vs. MO/SE group on day 1. $^{\#\#}P < .01$ the MO/EE group vs. MO/SE group on day 1.In B: $^{**}P < .01$ the MO/SE group vs. SA/SE group on day 2. $^{+}P < .05$ the SA/EX group vs. SA/SE group on day 1. $^{\$\$}P < .01$ the MO/EX group vs. MO/SE group on day 1. MO: Morphine; SA: Saline, SE: Standard environment, EE: Enriched environment, EX: Exercise.

postnatal groups (EE, SE and EX) ($F_{8,144}$ = 1.717, P = .099) and between days, groups and treatments were not significant ($F_{8,144}$ = 1.2, P = .303). Between groups comparison revealed that the MO/SE group significantly traveled longer distances than those of the SA/SE group on day 2 (P < .01). The SA/EX group significantly traveled shorter distances on day 1 than those of the SA/SE group (P < .05). The MO/EX group significantly traveled shorter distances on day 1 than those of the MO/SE group (P < .01).

Thigmotaxic distance declined across days for all groups, indicating the female pups habituated to the stress of the task across time (data not shown). Three-way ANOVA (day × prenatal treatments × postnatal interventions) on the distance traveled in the thigmotaxic zone revealed a significant decline in swim distance across training days ($F_{4, 144}$ = 13.26, P = .0005), but no effect of prenatal or postnatal interventions, or interaction.

3.2. Effects of postnatal EE and exercise on hippocampal LTP induction in prenatally morphine-exposed offspring

3.2.1. PS amplitude in male offspring

The results of the experiment in which LTP was elicited with theta PB stimulation in the vehicle- control or morphine-treated male offspring are summarized in Fig. 4(A and B). A two-way ANOVAs for the overall comparison of the of PS amplitude during 120-min recording revealed significant postnatal group effect ($F_{1,291}$ = 25.428, P = .0005), significant prenatal treatment effect ($F_{2, 291}$ = 29.184, P = .0005) and no interaction between both factors ($F_{2,291}$ = 2.612, P = .075) (Fig. 4B). Additionally, between-group post-hoc comparisons indicated that PS amplitude in the MO/SE group was significantly lower than the SA/SE group (P = .0005) indicating that the LTP induction impaired in rats prenatally exposed to morphine. The results also indicated that PS amplitudes in the MO/EX and the MO/EE groups were significantly higher than the MO/SE group (both, P = .0005), indicating that postnatal EE resulted in the enhancement of LTP in hippocampal DG region in rats prenatally exposed to morphine. PS amplitude in the SA/EE group was also significantly higher than the SA/SE group (P = .005) showing the beneficial effects of EE in the saline group as well.

3.2.2. EPSP slope in male offspring

A two-way ANOVA for the overall comparison of the of EPSP slope during 120-min recording for male offspring revealed significant postnatal group effect ($F_{1, 237}$ = 27.783, P = .0005), significant prenatal treatment effect ($F_{2, 237}$ = 5.809, P = .003) and significant interaction between both factors ($F_{2, 237}$ = 2.941, P = .05) (Fig. 5B). Between-group post-hoc comparisons indicated that EPSP slope was significantly different only between the SA/SE and the SA/EE groups (P = .007) and the differences between other groups were not significant (P > .05).

3.2.3. PS amplitude in female offspring

The results of the LTP experiment in control or morphine-treated female offspring are summarized in Fig. 6. A two-way ANOVA for the overall comparison of the of PS amplitude during 120-min recording revealed significant postnatal group effect ($F_{1, 209}$ = 47.628, P = .0005), significant prenatal treatment effect ($F_{2, 291}$ = 23.802, P = .0005), and no interaction between both factors ($F_{2, 291}$ = 1.504, P = .216) (Fig. 5A). Additionally, between-group post-hoc comparisons indicated that PS amplitude in the MO/SE group was significantly lower than the SA/SE group (P = .0005), indicating that the LTP induction decreased in rats prenatally exposed to morphine. The results also

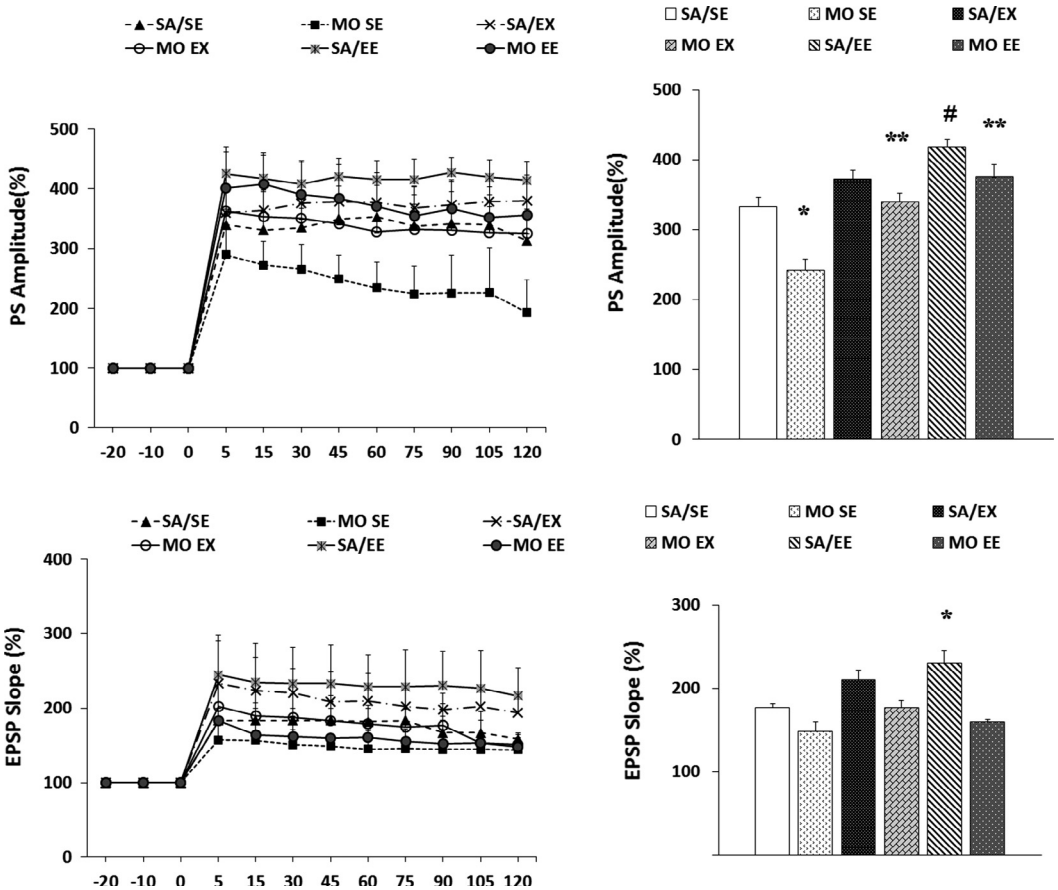

Fig. 4. Effects of prenatal treatment with morphine and postnatal EE and exercise on LTP in male rat offspring. The data are expressed as the mean ± S.E.M. (Insets) Representative superimposed PS amplitude (A) and fEPSPs (B) at baseline and during 120 min after PBs tetanus delivery. In A: *P = .0005 the MO/SE group vs. the SA/SE group; $^{\#}$P = .005 the SA/EE group vs. SA/SE; **P = .0005 the MO/EE and MO/EX groups vs. the MO/SE group. In B: *P = .007; the SA/EE group vs. the SA/SE.

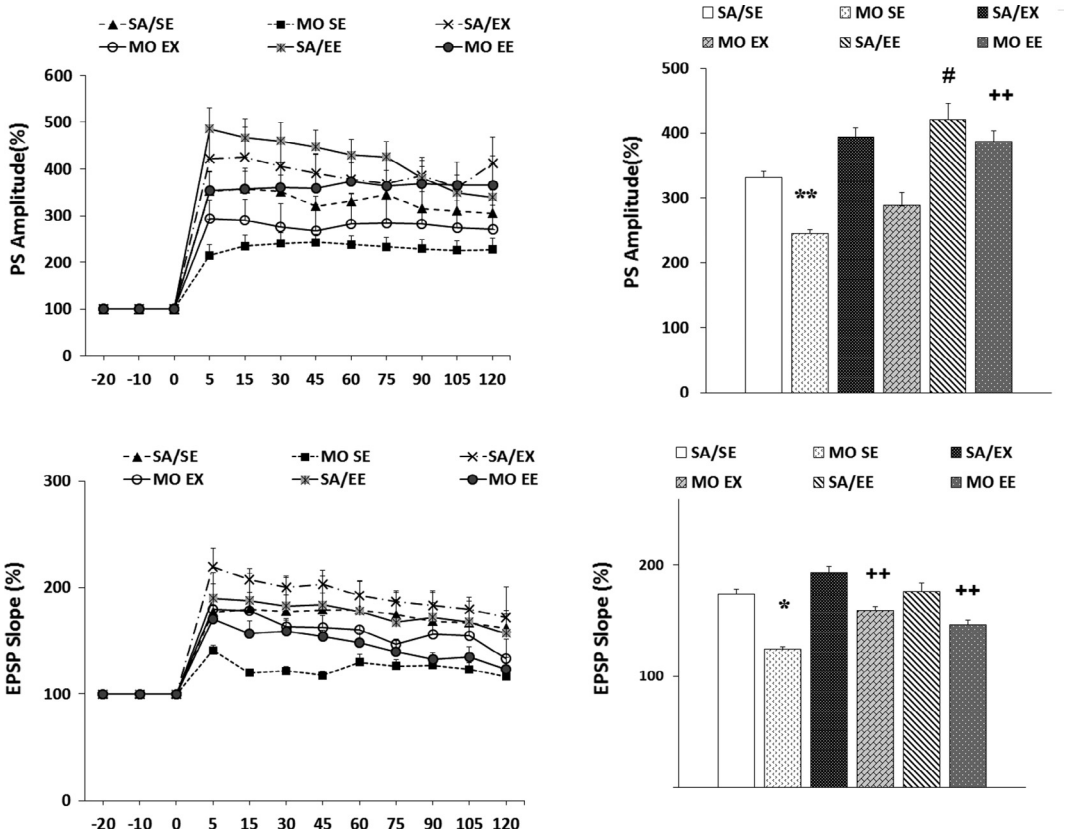

Fig. 5. Effects of prenatal treatment with morphine and postnatal EE and exercise onLTP in female rat offspring. The data are expressed as the mean ± S.E.M. (Insets) Representative superimposed PS amplitude (A) and fEPSPs (B) at baseline and during 120 min after PBs tetanus delivery. In A: **P = .0005 the MO/SE vs the SA/SE, $^{\#}$P = .0005 the SA/EE vs. the SA/SE; $^{++}$P = .0005 he MO/EE group vs. the MO/SE group. In B: *P = .0005 the MO/SE vs. the SA/SE; $^{++}$P = .0005 the MO/EE or MO/EX groups vs. the MO/SE.

Fig. 6. Effects of prenatal treatment with morphine and postnatal housing in an EE (EE) or Exercise on BDNF levels in the hippocampus: *P < .05 the MO/SE group vs. SA/SE group. **P < .05 the SA/EE group vs. SA/SE group. $^{\#}$P < .05 the MO/EX group vs. MO/SE group. $^{+}$ P < .05 the MO/EE group vs. MO/SE group. MO: Morphine; SA: Saline, SE: Standard environment, EE: Enriched environment, EX: Exercise. BDNF: Brain derived neurotrophic factor.

indicated that PS amplitudes in the MO/EE group were significantly higher than the MO/SE group (P = .0005), indicating that postnatal EE resulted in the enhancement of LTP in hippocampal DG region in rats prenatally exposed to morphine. PS amplitudes in the SA/EE group were also significantly higher than the SA/SE group, showing the beneficial effects of EE in the saline group as well.

3.2.4. EPSP slope in female offspring

A two-way ANOVA for the overall comparison of the of EPSP slope during 120-min recording for female offspring revealed significant postnatal group effect ($F_{1,228}$ = 95.754, P = .0005), significant prenatal treatment effect ($F_{2,228}$ = 16.72, P = .0005) and no significant interaction between both factors ($F_{2,228}$ = 2.066, P = .129) (Fig. 5B). Between-group post-hoc comparisons indicated that EPSP slope in the MO/SE was significantly lower than the SA/SE group (P = .0005), indicating that the LTP induction decreased in rats prenatally exposed to morphine. The results also indicated that EPSP slope in the MO/EE and MO/EX groups was significantly higher than the MO/SE group (both, P = .0005), indicating that postnatal EE and exercise resulted in the enhancement of LTP in hippocampal DG region in rats prenatally exposed to morphine.

3.3. Effects of prenatal morphine exposure and postnatal exercise and EE on hippocampal BDNF levels

Using analysis of variance, differences in BDNF levels of hippocampus between experimental groups were analyzed (Fig. 6). In male rats, a two-way ANOVA revealed significant effects of postnatal groups ($F_{2,\ 31}$ = 5.675, P = .008), and prenatal treatments ($F_{1,\ 31}$ = 4.324, P = .043) but no significant interaction between both factors ($F_{2,\ 31}$ = 0.365, P = .697) (Fig. 4). Post-hoc analysis revealed that the BDNF protein level was significantly higher than the MO/SE group only in the MO/EE group (P = .028). In female rats, a two-way ANOVA revealed significant effects of prenatal treatments ($F_{1,\ 33}$ = 18.5, P = .001), significant effects of postnatal groups ($F_{2,33}$ = 7.90, P = .002) and a significant interaction between both factors ($F_{2,\ 33}$ = 6.13, P = .005). Post-hoc analysis revealed that the BDNF protein level of the MO/SE group was significantly lower than those of the SA/SE (P = .0001). The BDNF levels in the MO/EX (P = .001) and MO/EE groups (P = .007) were significantly higher than the MO/SE group.

Correlation analysis revealed that BDNF levels (Pearson's correlation; r = 0.6) in male rats were positively correlated with female offspring. This positive correlation allowed us to combine data for male

and female offspring. For male + female rats, one-way ANOVA indicated that there are significant differences between groups ($F_{5,65}$ = 8.069, P = .0005). Post-hoc analysis revealed that the BDNF protein level of the MO/SE group was significantly lower than those of the SA/SE (P = .023), MO/EX (P = .028), and MO/EE (P = .0005). BDNF protein level of the SA/EE group was significantly higher than those of the SA/SE (P = .04).

4. Discussion

To verify whether the postnatal treadmill running and EE modify learning in prenatally morphine or saline exposed rats, the learning performance in Morris water maze task was assessed in offspring (PND 51–55). The results of the water maze performance of prenatally morphine-exposed rats are consistent with those of Yang and colleagues who found a significant difference in the performance of the morphine group in the first day of acquisition phase (Yang et al., 2003) or first and second days (Yang et al., 2006) in the acquisition phase of water maze. It is also demonstrated that in the radial maze, morphine-exposed males started out with a deficit in time to complete regular trials but at the end of the regular trials, morphine-exposed males caught up to the level of control males in the time required to complete the task (Slamberová et al., 2001).

We also found that postnatal exercise or EE increased the performance in the water maze, as compared to both saline and morphine age-matching sedentary-control offspring. In addition, there is no study reporting impairment of the probe trial performance in prenatally morphine-exposed offspring. In fact, a relationship between prenatal morphine exposure and impairment in spatial memory (retention phase) has not been reported yet. Yang et al. (2003) reported that no difference existed between prenatally morphine or saline exposed offspring in the probe trial performance (Yang et al., 2003). They also demonstrated that the difference in acquisition phase existed only on the first day of water maze training. In another study, Yang and his colleagues demonstrated that differences between morphine and saline exposed offspring in the learning performance existed in the first and second days, but not in the next consecutive two days of water maze training and also there was no report in that study about the differences between groups in the probe trial (Yang et al., 2006). This may propose that the spatial learning (acquisition phase) is impaired rather than spatial memory (probe trial). In addition, a recent study found that rats prenatally exposed to morphine showed impaired acquisition but enhanced maintenance of contextual fear memory compared with control animals that were prenatally exposed to saline (Tan et al., 2015).

The mechanisms underlying the neurobehavioral changes such as the impairment of water maze performance in the offspring born to morphine-exposed mothers remain to be determined. One possibility might be the neurotrophic factors changes caused by prenatal morphine. Others and our results showed that prenatal morphine exposure reduces BDNF or its precursor expression in the hippocampus of offspring (Ahmadalipour et al., 2015; Nasiraei-moghadam & Sherafat, 2012), specifically in the female rats. BDNF is a very important molecule in the several processes in the nervous system. Another potential mechanism whereby prenatal morphine can influence the postnatal behaviors is through the alteration of plasticity in the hippocampus of offspring; as it has been shown that prenatal morphine exposure impairs LTP in offspring (Sarkaki et al., 2008; Yang et al., 2003). LTP is thought to reflect the synaptic plasticity in the central nervous system (Bliss & Collingridge, 1993b).

The immersion of the animals into the water and the feeling of being trapped in it may cause significant stress. Decreased time of escape latency during training days indicates the tendencies of the animals to escape from aversive surroundings (Akirav, Sandi, & Richter-Levin, 2002; Harrison, Hosseini, & McDonald, 2009). It can be supposed that although morphine-exposed rats started out with a deficit in time to find the platform in the acquisition phase, they were able to overcome this with experience at the end of this phase and caught up to the control males level in the time needed to find the platform. On the other hand, postnatal exercise and EE increased performance in the water maze in prenatally morphine and saline exposed male and female rats. Likewise, we have shown in our previous studies that exercise and EE seem to be two important non-drug therapeutic strategies in the reduction of behavioral deficits of offspring born to morphine injected mothers (Ahmadalipour & Rashidy-Pour, 2015; Ahmadalipour et al., 2015); we found that the juvenile behavioral profile of prenatal morphine-exposed rats in anxiety tests and avoidance memory is different from control animals and, interestingly, EE and treadmill running exercise could be protective against such altered behavioral responses.

As a secondary observation, the present results showed impaired in vivo synaptic plasticity in the DG area of hippocampus in juvenile male and female rats prenatally exposed to morphine. The hippocampal LTP has been well known as the principal mechanism underlying the acquisition of new memories (Neves, Cooke, & Bliss, 2008). It is reported that in adult rats, prenatal contact to morphine weakens LTP induction in Schaffer collateral-CA1 synapses in vitro (Velísek, Stanton, Moshé, & Vathy, 2000b) and late-LTP in the lateral perforant pathway in vivo (Villarreal et al., 2008).

The mechanisms underlying the changes of LTP from prenatal morphine exposure in the hippocampus in offspring remain to be determined. Prenatal exposure to morphine changes kinetic properties of N-methyl-D-aspartate (NMDA) subtype of glutamate receptors-mediated synaptic currents in the hippocampus of rat offspring(Yang et al., 2000). NMDA receptors (NMDARs) are dynamically connected with the postsynaptic density 95 (PSD-95) protein, a submembranous cytoskeletal specialization to form a synaptic complex in postsynaptic neurons (Migaud et al., 1998). This complex serves chief neurobiological functions, including mammalian learning and memory (Lin et al., 2009). Prenatal morphine contact results in significant decreases in mRNA and whole levels of the PSD-95 and three NMDAR subunits (NR1, NR2A, and NR2B) in offspring (Lin et al., 2009).

In the hippocampus, LTP is composed of two independent elements: a synaptic component (population spike; PS) and an EPSP-to-spike coupling component (Taube & Schwartzkroin, 1988). It is reported that LTP of the PS amplitude in the DG area was reduced due to prenatal morphine exposure in juvenile rats but LTP of the EPSP slope did not depress (Niu, Cao, Zhu, Mei, Wang, et al., 2009). These results are consistent with our findings suggesting that prenatal morphine exposure impaired EPSP-spike potentiation.

We found that in the female morphine group, both PS and EPSP LTP was depressed whereas, in the male morphine group, only PS LTP was impaired significantly, suggesting that PS LTP is more vulnerable and sensitive to prenatal morphine exposure which is consistent with previous findings (Niu, Cao, Zhu, Mei, Wang, et al., 2009). In addition, others and our current results are suggestive that hippocampal levels of BDNF in female offspring were more susceptible than males to be affected by prenatal morphine (Nasiraei-moghadam & Sherafat, 2012). Given that BDNF is implicated in synaptic plasticity in the adult hippocampus (Ying et al., 2002), more depression in LTP in the females morphine-exposed offspring which occurred in both PS and EPSP elements of LTP, could be partly attributed to more decreased BDNF levels in female offspring.

We also found that postnatal exercise and EE improved LTP in both prenatally saline and morphine-exposed male and female rats. The exact mechanisms underlying these beneficial effects are not known precisely. Previous studies show that exercise increases dendritic complexity and the hippocampal levels BDNF and PSD-95 (Shih, Yang, & Wang, 2013). Exercise also activates NMDA receptor and increases the phosphorylated form of NMDA receptor in the hippocampus (Dietrich et al., 2005).

On the other hand, EE has been shown to increase the functional response of the pre-synaptic NMDA receptors which modulate noradrenaline release in mouse hippocampus (Grilli et al., 2009) which can

counteract the decreased level of NMDA receptor subunits in prenatally morphine-exposed rats (Lin et al., 2009). Others and our current results also show that BDNF levels are increased in EE animals compared to those housed in standard conditions (Gobbo & Mara, 2004; Sun et al., 2010).

As a third observation, our results are suggestive that prenatal exposure to morphine decreases BDNF protein level in the hippocampus of female offspring. This finding is in agreement with a study which showed prenatal morphine exposure reduced the expression of BDNF precursor protein in adolescent and adult female offspring (Nasiraei-Moghadam et al., 2013). Based on our findings, female offspring were more susceptible than males to be affected by prenatal morphine in the case of changes in hippocampal levels of BDNF. It has been shown that sex steroid hormones have modulatory effects on BDNF expression during adolescent development (Hill, Wu, Kwek, & Van den Buuse, 2012); male mice showed significant changes in BDNF expression which corresponded significantly with a surge in serum testosterone and in female rats ovariectomy increased BDNF expression. Recently we have shown that estrogen and estrous cycle in female rats can modulate memory impairments due to interventions such as glucocorticoid injections (Mohammadkhani, Darbandi, Vafaei, Ahmadalipour, & Rashidy-Pour, 2015).

It has also been demonstrated that some of the morphine-induced deficits such as impaired performance in passive avoidance retention task, persisted in female offspring even after puberty. Sex-dependent changes in hippocampal BDNF levels due to different interventions is also reported in other studies (Bakos et al., 2009; Venezia, Guth, Sapp, Spangenburg, & Roth, 2016). For example, it is demonstrated that EE influences hormonal status and hippocampal BDNF levels in a sex-dependent manner (Bakos et al., 2009). It is also reported that impact of long-term voluntary wheel running on the transcriptional and post-translational regulation of BDNF may be sex-dependent (Venezia et al., 2016). In addition, it has been demonstrated that prenatal morphine exposure induces sex-dependent changes in seizure susceptibility such that prenatally morphine-exposed, adult male rats are more sensitive to excitatory amino acid receptor-mediated seizures than control males, control females, or morphine-exposed females (Vathy, 2001). We also showed that postnatal exercise and EE increased BDNF levels of the hippocampus in female morphine group (MO/EX and MO/EE). BDNF plays various roles in regulating neuronal structure, function, and long-term survival in the developing and adult brain and also in plasticity-related changes in long-term drug exposure or abuse (Akbarian et al., 2001; Bolaños & Nestler, 2004; Hatami et al., 2007). In addition, there are several studies demonstrating that the impact of exercise on the level of BDNF may be sex-dependent (Onakomaiya, Porter, Oberlander, & Henderson, 2014; Venezia et al., 2016).

EE and exercise promote synaptic plasticity and neurogenesis while drug addiction induces impairment of synaptic organization and neurogenesis. Males showed greater effect than females in the most reports (Zhou et al., 2016). So synaptic plasticity and neurogenesis seem to be the probable mechanism driving behavioral changes in male and BDNF is the probable mechanism driving behavioral changes in female rats. Current data suggest that BDNF is not the only mechanism driving behavioral changes and other mechanisms must be involved in the alteration of memory function due to prenatal and postnatal interventions.

Adolescence and young adulthood represent critical periods of development during which enriched living, increased cage complexity and increased physical activity have been reported to be associated with lots of improvements of neurochemical and neurobehavioral changes such as an increased basal function of HPA (Laviola et al., 2004). These changes can relate adolescence period to earlier ages and adulthood.

In summary, the results of our study showed that prenatal morphine exposure impaired the water maze performance and hippocampal LTP in both male and female offspring and postnatal exercise and EE improved the performance in the water maze and enhanced hippocampal LTP in prenatally morphine and saline exposed male and female rats. Prenatal morphine exposure also caused a reduction in the hippocampal BDNF levels in female rats, and postnatal exercise and EE had improving effects on this deficit induced by prenatal morphine contact. Our results demonstrate that postnatal exercise and EE as two non-drug therapeutic strategies can improve deficits in water maze performance and hippocampal BDNF levels caused by prenatal morphine exposure. However, more research needs to be undertaken before the association between prenatal and postnatal life influences is more clearly understood.

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Conflict of interest statement

We attest 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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