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Effect of Morphine Based CPP on the Hippocampal Astrocytes of Male Wistar Rats

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ABSTRACT

Opioid abuse is an increasing problem worldwide and the reinforcing effects of opioid analgesics make them susceptible to diversion and illicit use and abuse. As morphological changes in different parts of the brain including the hippocampus and dentate gyrus that have Plasticity is looking for a lot of drug injection in human and animal studies has been viewed. Also other evidences have shown that Astrocytes actively participate in synaptic plasticity. Therefore the aim of this study was obtain of Conditioning Place Preference (CPP) on cell density (Astrocytes number) of rat hippocampal formation. In present study, 48 male Wistar rat weighted average 220-250 g were used. For behavioral tests, rats divided into eight groups and experimental groups daily (three days) received morphine at different doses (2.5, 5, 7.5 mg kg⁻¹) by subcutaneous injection and sham groups, received saline dose (1 mL kg⁻¹) and then CPP test in them were investigated. 48 h after behavioral testing animals were decapitated under chloroform anesthesia and their brains fixed and after tissue processing, slices stained with GFAP immunohistochemistry techniques. Present results showed the most dose responses of morphine were observed in 7.5 mg kg⁻¹. It also revealed that don't exist any differences between the number of astrocytes in CA₁ and CA₃ in control group and control-saline, but this difference between control and the other groups was significant statistically (p<0.05). We concluded that the phenomenon of morphine based conditioned place preference can cause a significant increase in the number of astrocytes in experimental groups with compared to controls.

Key words: Conditioning place preference, immunohistochemistry, morphine, astrocytes, hippocampus rats

INTRODUCTION

Researches over the last few decades on the biological principles of chemical dependency suggested that some brain areas and neurotransmitter systems involved in drug reward phenomena, especially alcohol dependence, opioids and Cocaine have relied on a common path biochemical mechanisms (Koob *et al.*, 1992; Geller, 1996).

Opioids, including morphine, are critical for pain management but are highly rewarding and for some individuals their use can lead to a lifelong cycle of addiction, withdrawal and relapse. Opioid abuse is an increasing problem worldwide and the reinforcing effects of opioid analgesics make them susceptible to diversion and illicit use and abuse (Spanagel and Welss, 1999; Compton and Volkow, 2006).

Several evidence supports that hippocampus is also involved in opiates and addiction of other drugs (White, 1996; Hyman and Malenka, 2001; Nestler, 2001).

In the past astrocytes were thought to play only a secondary, nonregulatory and permissive role in nervous function. However, based on diverse research lines in recent decades have demonstrated that astrocytes are critical role in CNS.

Although, the traditional view of opioid actions is that they are neurally-mediated, recent research has suggested an important modulatory role for glia (Astrocytes and microglia) in opioid actions, particularly in the areas of analgesia, tolerance and dependence (Hutchinson *et al.*, 2007).

Astrocytes have privileged access to synapses and we are beginning to learn that as a consequence they are able to regulate both the pre and postsynaptic terminal of excitatory and inhibitory synapses. Because of the reciprocal signaling that can occur between astrocytes and synaptic terminals, these structures have been termed the 'Tripartite Synapse' (Araque *et al.*, 1999).

The plasticity of astrocytes in some activity such as learning and in some disease (Diabetes mellitus) has been studied previously with me (Jahanshahi *et al.*, 2007, 2008, 2009).

There are some reasons indicate that a direct relationship exists between behavioral searching for drug, motor activity, reward and learning and drug use. And also the frequent use of drugs will facilitate learning (Mitchell and Stewart, 1990).

Addictive drugs via the mesolimbic dopaminergic pathway induce the synaptic plasticity changes in the neural network (Martin-Soelch *et al.*, 2001).

The purpose of this study was demonstrated morphine effect and conditioned place preference effect on cellular structure (number of astrocytes) in rat hippocampus with using immunohistochemical techniques for detection of astrocytes.

MATERIALS AND METHODS

Adult male Wistar rats (Pasteur institute, Tehran, Iran) weighing 220-270 g were used. They were housed four in a cage and had free access to food and water and kept at $22\pm 2^{\circ}\text{C}$ under a 12/12 h light-dark cycle (light beginning at 7:00 am). All experiments were carried out during the light phase between 8:00 and 14:00.

After one week animal environment adaptation, rats distributed in eight groups (control, control-saline, three shams groups were: sham 1 = 2.5 mg kg⁻¹, sham 2 = 5 mg kg⁻¹ and sham 3 = 7.5 mg kg⁻¹ morphine and experimental groups were: CPP 1 = 2.5, CPP 2 = 5 and CPP 3 = 7.5 mg kg⁻¹ morphine+CPP test). The rats in sham groups only received morphine in different doses and in CPP groups rats tested with CPP apparatus. For behavioral tests in each group at least six rats were used.

The place conditioning apparatus is based on that used by Carr and White with a minor modification and consisted of three wooden compartments. Compartments A and B were identical in size (40×30×30 cm) but differed in shading. The compartment A was white with black horizontal

stripes 2 cm wide on the walls and also had a textured floor. The other compartment (B) was black with vertical white stripes 2 cm wide and also had a smooth floor.

Compartment C (40×15×30 cm) was painted red and was attached to the rear of compartments A and B; it had removable wooden partitions that separated it from the other compartments. When the partitions were removed, the animal could freely move between the two compartments (A and B) via compartment C (Zarrindast *et al.*, 2006).

The CPP paradigm took place on 5 consecutive days by using an unbiased procedure. The experiment consisted of the three following phases.

Pre-conditioning: On day 1, the animals were accustomed to the conditioned place preference apparatus for 15 min. The removable wall was raised, thereby allowing each rat to freely explore the three compartments. The amount of time spent in each compartment was measured to assess unconditioned preference (the position of the rat was defined by the position of its front paws). In the particular experimental setup used in the study, the animals did not show an unconditioned preference for either of the compartments. The animals were then randomly assigned to one of two groups for place conditioning and a total of eight animals were used for each subsequent experiments.

Conditioning: Place conditioning phase started 1 day after the pre-conditioning phase. This phase consisted of six 45 min sessions (Three saline and three drug pairings). These sessions were conducted twice each day (from day 2 to 4) with a 6 h interval. On each of these days, separate groups of animals received one conditioning session with morphine and one with saline. During these sessions, the animals were confined to one compartment by closing the removable wall. The animals of each group were injected with morphine and were immediately confined to one compartment of the apparatus for 45 min. Following administration of saline, the animals were confined to the other compartment for 45 min.

Treatment compartment and order of presentation of morphine and saline were counterbalanced for either group. Conditioning was conducted as previously described in detail, using an unbiased procedure (Martin-Soelch *et al.*, 2001).

Testing: The testing phase was carried out on day 5 (1 day after the last conditioning session), in a morphine-free state. Each animal was tested once only. For testing, the removable wall was raised and the animals had a free choice in the apparatus for 15 min. The time spent in drug-paired compartment was recorded for each animal and the change of preference was calculated as the difference (In seconds) between the time spent in the drug-paired compartment on the testing day and the time spent in this compartment on the pre-conditioning day (Zarrindast *et al.*, 2006).

The drug used in the present study was morphine sulfate (Temad Co., Tehran, Iran), The rats were anaesthetized with chloroform and intracardially perfused with 0.9% saline in 0.1 M phosphate buffer (PBS; pH 7.4) followed by 10% phosphate-buffered formalin. After perfusion, brains were cut coronally to obtain the hippocampus and embedded into paraffin blocks.

Using a rotary microtome 10 µm-thick sections were cut through the hippocampal formation. Sections were processed for GFAP immunocytochemistry.

Briefly, after deparaffinizing the sections, they were permeabilized in Tris Buffer Saline (TBS) containing 0.1% Triton X-100, followed by a 30 min pre-incubation in 1% human serum (Sigma, USA) dissolved in TBS. After this blocking step, a polyclonal primary antibody (rabbit anti-GFAP

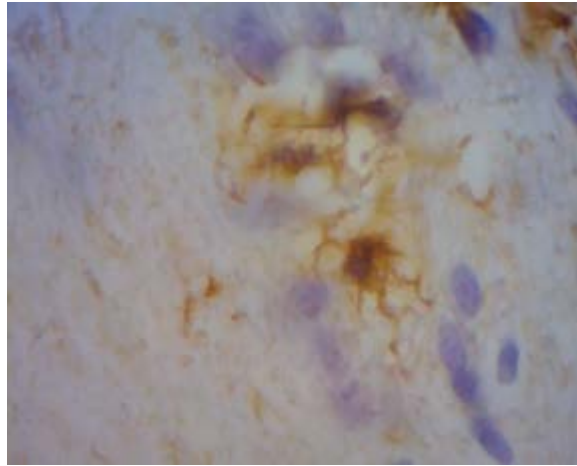


Fig. 1: Brown cells are astrocytes 100x

abcam-USA) was applied at 1:800 dilution and the sections were incubated for 24 h at 48°C. Sections were washed three times in TBS with Triton X-100, incubated in biotinylated secondary goat anti-rabbit IgG antibody (1:30 dilution; Pierce, USA) in 10% bovine serum for 30 min and dipped three times in TBS with Triton X-100. The sections were then incubated with an avidin-biotin horseradishperoxidase complex (Vectastain ABC-Ultrasecutive, Elite Kit; Pierce, USA) for 1 h at room temperature, washed twice in TBS with Triton X-100, rinsed in TBS and then visualized with DAB (Sigma, USA). Finally, the sections were dehydrated in ethanol, cleared in xylene and coverslipped with Entellan (Merck, USA).

Stained slides were visualized using a system composed of a binocular microscope (Olympus BX51, Japan) equipped with a digital video camera connected to a video monitor (Fig. 1).

Comparisons between groups were made with one-way analysis of variance (ANOVA). Following a significant F value, post hoc analysis (Tukey test) was performed for assessing specific group comparisons. A difference with $p < 0.05$ between the experimental groups was considered statistically significant. Calculations were performed using the SPSS statistical package.

RESULTS AND DISCUSSION

The behavioral test results showed the greatest response was in the 7.5 mg kg^{-1} morphine dose with an average 361.83 sec delay and the less delay time was in 2.5 mg kg^{-1} with 90.33 sec. Also the difference delay time between experimental CPP groups with the saline group was statistically significant (Fig. 2).

The mean number of astrocytes in $75,000 \mu\text{m}^2$ in all groups is depicted on Table 1 and 2. The findings showed that any differences don't exist between the number of astrocytes in CA₁ and CA₃ in control group and control-saline, but this difference between control and the other groups was significant statistically ($p < 0.05$). The density of astrocytes in sham groups was more than control and also was less than the CPP groups all area of hippocampus. On the other hand, conditioning place preference can increase the astrocytes in hippocampus ratio to morphine injection. Data showed that the increase of astrocytes was dose dependence and in high dose we showed the most number of astrocytes.

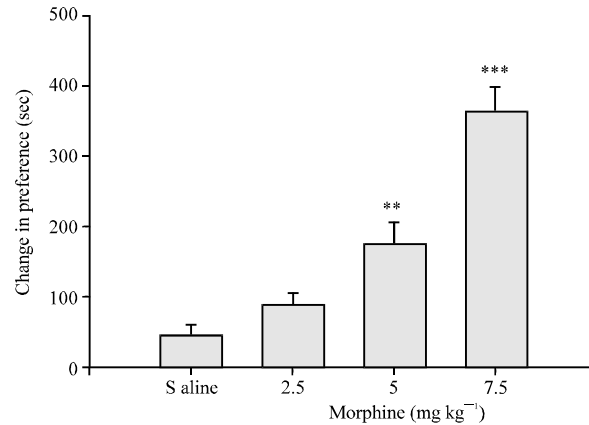


Fig. 2: The mean of delay time in all groups tested with CPP. ** $p \leq 0.01$, *** $p \leq 0.001$

Table 1: Mean and SD of astrocytes number in CA₁ area of hippocampus

Groups-CA ₁	Mean	SD	p-value
Control	10.44	3.02	
Control-saline	9	2.28	0.965
Sham-1	14.63	5.86	0.030
Sham-2	20.75	4.18	0.000
Sham-3	25.29	7.91	0.000
Cpp1	23.13	9.81	0.000
Cpp2	23.42	6.45	0.000
Cpp3	27.33	7.79	0.000

Table 2: Mean and SD of astrocytes number in CA₃ area of hippocampus

Groups- CA ₃	Mean	SD	p-value
Control	13.90	03.80	
Control-saline	11.75	02.82	0.956
Sham-1	28.20	16.32	0.000
Sham-2	30.53	09.43	0.000
Sham-3	36.55	05.86	0.000
Cpp1	33.22	11.80	0.000
Cpp2	36.96	07.93	0.000
Cpp3	43.04	07.37	0.000

The less number of astrocytes in all area of hippocampus showed in CA₁. In compare between control and control-saline in all area the density of astrocytes was decreased but this difference was not significant.

CPP is an animal model widely used to measure the rewarding properties of addictive drugs as reflected by the rewarding reinforced association of context and drugs of abuse. Our present findings demonstrate that the number of astrocytes was increased due to morphine injection and conditioning test. Otherwise, injection of saline can not cause changes in density of astrocytes.

It is not surprising then that many drugs can affect astrocytic physiology not only directly but also through alterations in the release of neurochemicals from surrounding neurons (Volterra and Meldolesi, 2005).

Moreover, glia may be involved in reward. A key finding in this regard was study of Narita *et al.* (2006) who demonstrated that microinjection of astrocyte conditioned media in the anterior cingulate cortex and the Nucleus Accumbens (NAc), but not the caudate putamen, enhanced morphine Conditioned Place Preference (CPP), a well characterized measure of the motivational effects of various drugs (Tzschentke, 2007).

The inactive effects of anterior tegmental area in expression of morphine-induced conditioning was investigated by Moaddab *et al.* (2009). In conditioning stage, during the three days, each rat received six injections, including three morphine doses (1, 2.5, 5, 7.5 and 10 mg kg⁻¹) and three injections of saline subcutaneously in the morning and afternoon with 6 h interval. In their study the most response to conditioning test was 5 mg kg⁻¹ dose of morphine (Moaddab *et al.*, 2009).

Miyatake *et al.* (2005) with purpose of evaluation of metamphetamines-induced conditioned place preference and role of protein kinase c in neuronal-glia communication described that metamphetamines -induced conditioned place preference cues phosphorylat of protein kinase c in astrocytes and neurons and finally activate the astrocytes in the cortex. In fact, after drug administration, the sensitivity of astrocytes increases to glutamate and dopamine and by activated astrocytes uptake and regulate synaptic glutamate through the synaptic space. These results indicate that astrocytes play an important role in drug dependency.

Also some changes have been observed in expression of astrocytes GFAP after morphine, amphetamines and their derivations exposure. One study in 2002 on the astrocytes of rats' dentate gyrus under the influence of cocaine showed that GFAP expression in the affected cocaine group has increased compared to control. Also in morphological and morphometric analysis, they were observed significant changes in size and number of astrocytes (Fattore *et al.*, 2002). In our study, followed by administration of morphine in different groups, the number of astrocytes was increased compared to control group.

Some previous studies on changes in cortical pyramidal neurons 15 days following administration of morphine, showed particularly morphine effect on the structure, size and terminal dendritic branches and dendritic spines in the pyramidal neurons of third layer in the motor cortex and perilimbic Region. Ballesteros-Yanez *et al.* (2006) showed that morphine administration leads to reducing the size of the terminal branches of pyramidal neurons in the motor cortex. Against the pyramidal neurons, perilimbic neurons' dendrits were taller after morphine administered. However, both areas had increased density of dendritic spines. These results suggest that morphine administration can lead to changes in the structure of pyramidal cells .

CONCLUSION

According to the above findings can be concluded that the morphine alone and morphine based conditioned place preference can cause a significant increase in the number of astrocytes in all area of hippocampus in experimental groups compared to controls and this increase of cell density can be dose dependence.

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