

## Original Article

# Female Rat Hippocampal Cell Density after Conditioned Place Preference

(female rats / conditioning place preference / neurons / astrocytes / hippocampus)

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**Abstract.** The hippocampus is important for learning tasks, such as conditioned place preference (CPP), which is widely used as a model for studying the reinforcing effects of drugs with dependence liability. Long-term opiate use may produce maladaptive plasticity in the brain structures involved in learning and memory, such as the hippocampus. We investigated the phenomenon of conditioning with morphine on the cell density of female rat hippocampus. Forty-eight female Wistar rats weighing on average 200–250 g were used. Rats were distributed into eight groups. Experimental groups received morphine daily (three days) at different doses (2.5, 5, 7.5 mg/kg) and the control-saline group received normal saline (1 ml/kg), and then the CPP test was performed. Three sham groups received only different doses (2.5, 5, 7.5 mg/kg) of morphine without CPP test. Forty-eight hours after behavioural testing animals were decapitated under chloroform anaesthesia and their brains were fixed, and after tissue processing, slices were stained with cresyl violet for neurons and phosphotungstic acid haematoxylin for astrocytes. The maximum response was obtained with 5 mg/kg of morphine. The density of neurons in CA1 and CA3 areas of hippocampus after injection of morphine and CPP was decreased. The number of astrocytes in different areas of hippocampus was increased after injection of morphine and CPP. It seems that the effective dose was 5 mg/kg, as it led to the CPP. We concluded that both injection of mor-

phine and CPP can decrease the density of neurons and also increase the number of astrocytes in the rat hippocampus.

## Introduction

The conditioned place preference (CPP) model has been commonly used as a classic model for studying the reinforcing effects of drugs with dependence liability (Zarrindast et al., 2003).

Many drugs such as cocaine, amphetamine, morphine, heroin and ethanol produce supporting effects and induce CPP for the drug-paired side after several conditioning sessions (Wei et al., 2005). Opioids can exert their effects through various classes of opioid receptors, including  $\mu$ ,  $\delta$  and  $\kappa$  receptors (MOR, DOR, and KOR, respectively) (Pennock and Hentges, 2011). Several evidences indicate that morphine ( $\mu$  opioid receptor agonist) (Narita et al., 2006) injected into laboratory animals can act as a stimulus and the reward is induction of CPP (Zataly et al., 2005). Opioid receptors belong to a large family of G protein-coupled receptors and play an important physiological role (Piestrzeniewicz et al., 2006). Recent reports support the view that the hippocampus is also involved in addiction to opiates and other drugs. Some studies suggest that the hippocampus is important for reward-related learning tasks, such as conditioned place preference (Wei et al., 2005; Zataly et al., 2005; Sharifzadeh et al., 2006; Liu et al., 2010).

The hippocampus is essential for the formation of new memories and episodic memory, which is used in animal studies to evaluate preferences for environmental stimuli that have been associated with a positive or negative reward (Liu et al., 2010). The hippocampus is also involved in psychological drug dependence and drug-seeking behaviour (Rezayof et al., 2007), and lesions of the dorsal hippocampus block psychostimulant-induced CPP (Dong et al., 2006; Rademacher et al., 2006). Previous studies have indicated that chronic opiate treatment can significantly modulate synaptic plasticity in the hippocampus, leading to an opiate dependence of the plasticity, and it has been suggested that up-regulation of the cAMP pathway is likely one of the

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Abbreviations: CPP – conditioned place preference, DOR –  $\delta$  opioid receptor, KOR –  $\kappa$  opioid receptor, MOR –  $\mu$  opioid receptor, PTAH – phosphotungstic acid haematoxylin.

underlying mechanisms of the observed phenomena (Sharifzadeh et al., 2006).

At the neuroanatomical level, drugs of abuse given for a long period of time induce structural changes in the dendritic branching and spine density not only in medium spiny neurons of the nucleus accumbens and in pyramidal cells of the medial prefrontal cortex (Robinson and Kolb, 2004), which are primarily involved in the reward circuitry, but also in the hippocampus, which has been discovered only recently (Crombag et al., 2005). We have investigated the phenomenon of conditioning with morphine on the cell density (number of neurons and astrocytes) in the female rat hippocampus.

## Material and Methods

### *Animals*

Forty-eight adult female Wistar rats (Pastor Institute, Tehran, Iran), weighing 200–250 g, were used in this study. They were given free access to normal laboratory chow and water. Temperature of the animal house was  $22 \pm 3$  °C. The rats were divided randomly into eight groups [control, control-saline (1 ml/kg), three sham groups were: sham 1 = 2.5 mg/kg, sham 2 = 5 mg/kg and sham 3 = 7.5 mg/kg morphine, and experimental groups were: CPP 1 = 2.5, CPP 2 = 5 and CPP 3 = 7.5 mg/kg morphine + CPP test]. The rats in the sham groups only received morphine in different doses, and in CPP groups the rats were tested with a CPP apparatus. For behavioural tests, in each group at least six rats were used.

### *Drugs*

Morphine sulphate (Temad, Tehran, Iran) was dissolved in normal saline (0.9%) and administered subcutaneously.

### *Behavioural apparatus*

The three-compartment conditioned place preference apparatus, based on the design of Carr and White (1983), was used and was made of wood. Two of the compartments (A and B) were identical in size (40×30×30 cm) but differed in shading and texture. Compartment A was white with black horizontal stripes on the walls. The second compartment (B) was black with white vertical stripes on the walls. The third compartment (C) was a red tunnel, i.e. its dimensions were different from the A and B compartments. Between the C and other compartments, a wooden gate was moving, completely separating the three compartments (Shaabani et al., 2011).

### *Behavioural procedures*

CPP includes three 5-day schedules with three distinct phases: precondition, condition and postcondition.

**Precondition.** At this stage (first day) rats were placed in compartment C for 15 min, and at that time the guillotine valve was opened. Simultaneously with this action, the length of the time spent in each compartment was measured by a stopwatch.

**Condition.** At this stage (from second to fourth day) rats received morphine and saline, twice daily (morning and evening session), according to the model as follows:

The first day at the morning session (9 h), rats received morphine subcutaneously and were placed in the white compartment (A) for 45 min. Six h later (15 h, evening session), rats received saline subcutaneously and were placed in the black compartment (B) for 45 min. On the second day, rats received the saline injections in the morning session and morphine in the evening session. The third day of conditioning had the same schedule as the first day.

**Postcondition.** At this stage (fifth day), rats were placed in compartment C for 15 min, and at that time the guillotine valve was opened. Simultaneously with this action, the length of time spent in each compartment was measured by a stopwatch and used to calculate the degree of place preference. Locomotor activity was simultaneously recorded throughout conditioning.

### *Histology*

Forty-eight hours after the last injection, animals were anaesthetized with chloroform and their chest was opened and a cannula placed in the left ventricle. Through the open right atrium wall and the left ventricle a combination of saline and 4% paraformaldehyde in 0.1 M phosphate buffer was administered and continued until complete fixation. After perfusion, the brains were removed carefully and described and then fixed for two weeks in 4% paraformaldehyde solution. Different degrees of alcohol were used for dehydration followed by clarification with xylol. After histological processing, tissue was impregnated and then embedded in paraffin wax.

Ten  $\mu$ m thick coronal sections were serially collected from the bregma  $-3.30$  mm to  $-6.04$  mm of the hippocampal formation (Paxinos and Watson, 1998). An interval of 20  $\mu$ m was placed between each two consecutive sections. The sections were stained with cresyl violet for neurons and phosphotungstic acid haematoxylin (PTAH) for astrocytes, according to routine laboratory procedures (Bancroft and Gamble, 2004; Jahanshahi et al., 2008, 2009, 2011).

### *Morphometry*

A photograph of each section was produced using an Olympus BX 51 microscope (Olympus, Tokyo, Japan) and a DP 12 digital camera (Olympus) under a magnification of 400. Marked images after calibrating the device, packed grid and central houses were counted in each field, and finally data from each slide and counting of neurons and astrocytes in each group were classified.

### *Data analysis*

All the data were entered into and analysed by SPSS 11.5 software. The data were expressed as mean  $\pm$  SD. The statistical analysis was performed using the one-way analysis of variance (ANOVA) test. After confirmation of normality, the means was compared by the ANOVA post-hoc Tukey test;  $P < 0.05$  was considered significant.

Table 1. Female pre-conditioning and conditioning time in black and white chambers

|           |                     | Pre-conditioning   |                    | Conditioning       |                    | Conditioning score  |                     |
|-----------|---------------------|--------------------|--------------------|--------------------|--------------------|---------------------|---------------------|
|           |                     | Black              | White              | Black              | White              | Black               | White               |
| Sham      | Time                | 2.11 <sup>cc</sup> | 3.17 <sup>cc</sup> | 2.12 <sup>cc</sup> | 3.06 <sup>cc</sup> | 0.01 <sup>cc</sup>  | -0.11               |
|           | Locomotion activity | 22.33              | 21.16              | 20.8               | 22.8               | 20                  | 22                  |
| 2.5 mg/kg | Time                | 2.08 <sup>cc</sup> | 5.00 <sup>cc</sup> | 2.03 <sup>cc</sup> | 2                  | -0.05 <sup>cc</sup> | -3.00 <sup>cc</sup> |
|           | Locomotion activity | 25.2               | 33.4               | 33.2               | 23.6               | 33                  | 23                  |
| 5 mg/kg   | Time                | 1.55 <sup>cc</sup> | 3.44 <sup>cc</sup> | 3.54 <sup>cc</sup> | 1.45               | 1.59 <sup>cc</sup>  | -2.01 <sup>cc</sup> |
|           | Locomotion activity | 28.2               | 36.2               | 36                 | 24.6               | 36                  | 24                  |
| 7.5 mg/kg | Time                | 1.35 <sup>cc</sup> | 4.56 <sup>cc</sup> | 4.07 <sup>cc</sup> | 1.45 <sup>cc</sup> | 2.32 <sup>cc</sup>  | -3.10 <sup>cc</sup> |
|           | Locomotion activity | 24                 | 37.8               | 37.6               | 16.5               | 37                  | 16                  |

## Results

### Behavioural results

Table 1 shows the dose-response place conditioning induced by morphine in the rats. Subcutaneous (s.c.) administration of morphine (2.5, 5 and 7.5 mg/kg) during conditioning induced CPP. Significant conditioning was observed at doses of 2.5, 5 and 7.5 mg/kg. The maximum response was obtained with 5 mg/kg of morphine. However, increases in the morphine dose decreased CPP. Table 1 also indicates that morphine administration (s.c.) had no significant effect on the locomotor activity compared to the control group.

### Histological results

As we showed in Table 2, the number of neurons in CA1 and CA3 areas of hippocampus after conditioning

significantly decreased. In sham groups that received morphine in different doses, the number of neurons was decreased, but the difference was not statistically significant.

In dentate gyrus, the decrease of neurons in all groups was not significant.

The highest decrease of the neuron number was shown in CA1 of 5 mg/kg conditioned group (Table 2).

There was no significant difference in the number of neurons between the sham groups (that only received morphine) and conditioned groups, but the numbers of neurons were more decreased in the CPP groups.

The number of astrocytes in different areas of hippocampus was increased and the highest increase was observed with 7.5 mg/kg doses of sham morphine (Table 3). There was no significant difference in the number of astrocytes between the sham groups (that only received morphine) and the conditioned groups.

Table 2. Mean and SD of neuron number in the female hippocampus (30,000  $\mu\text{m}^2$  for CA1 and CA3, 3,600  $\mu\text{m}^2$  for DG)

| Groups           | CA1 Mean $\pm$ SD | P value | CA3 Mean $\pm$ SD | P value | DG Mean $\pm$ SD  | P value |
|------------------|-------------------|---------|-------------------|---------|-------------------|---------|
| Control          | 26.33 $\pm$ 2.903 | -       | 14.35 $\pm$ 1.86  | -       | 16.62 $\pm$ 2.771 | -       |
| Control - saline | 25.28 $\pm$ 6.102 | 0.948   | 14.20 $\pm$ 2.334 | 0.998   | 17.53 $\pm$ 2.944 | 0.840   |
| Sham 2.5         | 23.90 $\pm$ 3.334 | 0.149   | 14.85 $\pm$ 2.713 | 0.985   | 16.75 $\pm$ 2.790 | 1.000   |
| Sham 5           | 24.95 $\pm$ 4.242 | 0.811   | 16.88 $\pm$ 2.623 | 0.000 * | 16.30 $\pm$ 3.291 | 1.000   |
| Sham 7.5         | 24.78 $\pm$ 4.435 | 0.699   | 14.65 $\pm$ 3.093 | 0.999   | 15.28 $\pm$ 3.486 | 0.383   |
| CPP 2.5          | 23.82 $\pm$ 3.713 | 0.123   | 14.00 $\pm$ 2.592 | 0.998   | 16.07 $\pm$ 2.379 | 0.988   |
| CPP 5            | 20.98 $\pm$ 4.048 | 0.000 * | 12.28 $\pm$ 2.230 | 0.004 * | 14.35 $\pm$ 2.455 | 0.008 * |
| CPP 7.5          | 21.33 $\pm$ 3.354 | 0.000 * | 13.05 $\pm$ 1.894 | 0.258   | 16.12 $\pm$ 2.015 | 0.993   |

All groups compared to the control (\* means significant)

Table 3. Mean and SD of astrocyte number in the female hippocampus (30,000  $\mu\text{m}^2$  for CA1 and CA3, 3,600  $\mu\text{m}^2$  for DG)

| Groups           | CA1 Mean $\pm$ SD | P value | CA3 Mean $\pm$ SD | P value | DG Mean $\pm$ SD   | P value |
|------------------|-------------------|---------|-------------------|---------|--------------------|---------|
| Control          | 8.97 $\pm$ 5.456  | -       | 6.13 $\pm$ 2.090  | -       | 13.45 $\pm$ 6.181  | -       |
| Control - saline | 8.42 $\pm$ 4.809  | 1.000   | 7.52 $\pm$ 5.364  | 0.680   | 15.77 $\pm$ 6.183  | 0.896   |
| Sham 2.5         | 6.90 $\pm$ 4.372  | 0.701   | 9.52 $\pm$ 4.279  | 0.001 * | 16.18 $\pm$ 7.56   | 0.790   |
| Sham 5           | 9.65 $\pm$ 6.889  | 0.999   | 8.05 $\pm$ 4.551  | 0.277   | 14.18 $\pm$ 10.780 | 1.000   |
| Sham 7.5         | 13.05 $\pm$ 7.772 | 0.024 * | 9.93 $\pm$ 3.190  | 0.000 * | 20.12 $\pm$ 7.904  | 0.014 * |
| CPP 2.5          | 9.00 $\pm$ 5.233  | 1.000   | 5.87 $\pm$ 2.151  | 1.000   | 12.82 $\pm$ 9.647  | 1.000   |
| CPP 5            | 8.35 $\pm$ 3.498  | 1.000   | 8.85 $\pm$ 4.080  | 0.023 * | 19.4 $\pm$ 4.627   | 0.021 * |
| CPP 7.5          | 9.45 $\pm$ 4.992  | 1.000   | 9.60 $\pm$ 2.228  | 0.001 * | 14.15 $\pm$ 9.162  | 1.000   |

All groups compared to the control (\* means significant)

## Discussion

The present study demonstrates that CPP is able to affect the hippocampal cell density. The number of neurons decreased and that of astrocytes increased after CPP. In our previous study we showed that the number of astrocytes increases after CPP in male Wistar rats (Shaabani et al., 2011) and in the present study we showed that this number also increased in female rats. It seems that there is no difference between male and female rats.

Many studies have investigated the phenomenon of conditioning with opioids such as morphine (Zhai et al., 2008) and cocaine (Hernandez-Rabaza et al., 2008). In agreement with previous studies, the administration of morphine produced conditioned place preference (CPP) in a dose-dependent manner. Our finding is similar to those of other studies to which we refer. For example, Zarrindast et al. (2006) showed that administration of different doses of morphine (0.5, 1, 3, 6 mg/kg) during conditioning induced CPP in male Wistar rats. The maximum response was observed with 3 mg/kg of the opioid (Zarrindast et al., 2006). Another study demonstrated that subcutaneous administration of various doses of morphine sulphate (1, 3, 6, 9 mg/kg) induced CPP in a dose-dependent manner (Sharifzadeh et al., 2006).

Our data are consistent with those previously reported showing that CPP can affect neurons in the hippocampus; for example, Eisch et al. (2000) studied the effect of long-term morphine exposure on the birth of new neurons in the adult male Sprague-Dawley rat hippocampus. They demonstrated that morphine decreases the number of BrdUrd-positive cells in the subgranular zone of the dentate gyrus.

Kahn et al. (2005) demonstrated repeated morphine (20 mg/kg) treatment effects on the cell proliferation and neuronal phenotypes in the dentate gyrus-CA<sub>3</sub> region of the adult male Sprague-Dawley rat hippocampus.

Additionally, Rademacher et al. (2006) used immunohistochemistry for a neuronal activity marker (c-Fos), a marker for synaptogenesis (synaptophysin) and a neurotrophic factor receptor that mediates synaptic plasticity (tyrosine kinase B receptor) to investigate the neural substrates of amphetamine-induced conditioned place preference in male Sprague-Dawley rats. Conditioned place preference was induced by both 1.0 and 0.3 mg/kg doses of amphetamine. Furthermore, amphetamine conditioning increased the density of c-Fos-immunoreactive cells and these cells were fully co-localized with the tyrosine kinase B receptor in the dentate gyrus and CA<sub>1</sub>.

## Conclusion

It may be concluded that subcutaneous injection of morphine and the phenomenon of morphine-based conditioning could decrease the density of neurons, but in many areas of the hippocampus these changes were not statistically significant. Only in the 5 mg/kg CPP group that achieved maximum response of conditioning, the

differences were significant in all areas. We also concluded that both the injection of morphine and CPP can increase the number of astrocytes in the hippocampus.

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## References

- Bancroft, J. D., Gamble, M. (2004) *Theory and Practice of Histological Techniques*. Churchill Livingstone, Edinburgh.
- Carr, G. D., White, N. M. (1983) Conditioned place preference from intra-accumbens but not intra-caudate amphetamine injections. *Life Sci.* **33**, 2551-2557.
- Crombag, H. S., Gorny, G., Li, Y., Kolb, B., Robinson, T. E. (2005) Opposite effects of amphetamine self-administration experience on dendritic spines in the medial and orbital prefrontal cortex. *Cerebral Cortex* **15**, 341-348.
- Dong, Z., Han, H., Wang, M., Xu, L., Hao, W., Cao, J. (2006) Morphine conditioned place preference depends on glucocorticoid receptors in both hippocampus and nucleus accumbens. *Hippocampus* **16**, 809-813.
- Eisch, A. J., Barrot, M., Schad, C. A., Self, D. W., Nestler, E. J. (2000) Opiates inhibit neurogenesis in the adult rat hippocampus. *Proc. Natl. Acad. Sci. USA* **97**, 7579-7584.
- Hernandez-Rabaza, V., Hontecillas-Prieto, L., Velazquez-Sanchez, C., Ferragud, A., Perez-Villaba, A., Arcusa, A., Barcia, J. A., Trejo, J. L., Canales, J. J. (2008) The hippocampal dentate gyrus is essential for generating contextual memories of fear and drug-induced reward. *Neurobiol. Learn. Mem.* **90**, 553-559.
- Jahanshahi, M., Sadeghi, Y., Hosseini, A., Naghdi, N., Marjani, A. (2008) The effect of spatial learning on the number of astrocytes in the CA3 subfield of the rat hippocampus. *Singapore Med. J.* **49**, 388-391.
- Jahanshahi, M., Ghalipour, M., Afshar, M. (2009) The effect of *Urtica dioica* extract on the number of astrocytes in the dentate gyrus of diabetic rats. *Folia Morphol. (Praha)* **68**, 93-97.
- Jahanshahi, M., Khoshnazar, A. K., Azami, N. S., Heidari, M. (2011) Radiation-induced lowered neurogenesis associated with shortened latency of inhibitory avoidance memory response. *Folia Neuropathol.* **49**, 103-108.
- Kahn, L., Alonso, G., Normand, E., Manzoni, O. J. (2005) Repeated morphine treatment alters polysialylated neural cell adhesion molecule, glutamate decarboxylase 67 expression and cell proliferation in the adult rat hippocampus. *Eur. J. Neurosci.* **21**, 493-500.
- Liu, F., Jiang, H., Zhong, W., Wu, X., Luo, J. (2010) Changes in ensemble activity of hippocampus CA1 neurons induced by chronic morphine administration in freely behaving mice. *Neuroscience* **171**, 747-759.
- Narita, M., Miyatake, M., Shibasaki, M., Shindo, K., Nakamura, A., Kuzumaki, N., Nagumo, Y., Suzuki, T. (2006) Direct evidence of astrocytic modulation in the development of rewarding effects induced by drugs of abuse. *Neuropsychopharmacology* **31**, 2476-2488.

- Paxinos, G., Watson, C. (1998) *The Rat Brain in Stereotaxic Coordinates*. Academic Press, San Diego.
- Pennock, R. L., Hentges, S. T. (2011) Differential expression and sensitivity of presynaptic and postsynaptic opioid receptors regulating hypothalamic proopiomelanocortin neurons. *J. Neurosci.* **31**, 281-288.
- Piestrzeniewicz, M., Fichna, J., Janecka, A. (2006) Opioid receptors and their selective ligands. *Postepy Biochem.* **52**, 313-319. (in Polish)
- Rademacher, D. J., Kovacs, B., Shen, F., Napier, T. C., Meredith, G. E. (2006) The neural substrates of amphetamine conditioned place preference: implications for the formation of conditioned stimulus-reward associations. *Eur. J. Neurosci.* **24**, 2089-2097.
- Rezayof, A., Razavi, S., Haeri-Rohani, A., Rassouli, Y., Zarrindast, M. R. (2007) GABAA receptors of hippocampal CA1 regions are involved in the acquisition and expression of morphine-induced place preference. *Eur. Neuro-psychopharmacol.* **17**, 24-31.
- Robinson, T. E., Kolb, B. (2004) Structural plasticity associated with exposure to drugs of abuse. *Neuropharmacology* **47**, 33-46.
- Shaabani, R., Jahanshahi, M., Nowrozian, M., Sadeghi, Y. (2011) Effect of morphine based CPP on the hippocampal astrocytes of male Wistar rats. *Asian Journal of Cell Biology* **6**, 89-96.
- Sharifzadeh, M., Haghghat, A., Tahsili-Fahadan, P., Khalaj, S., Zarrindast, M. R., Zamanian, A. R. (2006) Intra-hippocampal inhibition of protein kinase AII attenuates morphine-induced conditioned place preference. *Pharmacol. Biochem. Behav.* **85**, 705-712.
- Wei, X. L., Su, R. B., Lu, X. Q., Liu, Y., Yu, S. Z., Yuan, B. L., Li, J. (2005) Inhibition by agmatine on morphine-induced conditioned place preference in rats. *Eur. J. Pharmacol.* **515**, 99-106.Y
- Zarrindast, M. R., Faraji, N., Rostami, P., Sahraei, H., Ghoshouni, H. (2003) Cross-tolerance between morphine- and nicotine-induced conditioned place preference in mice. *Pharmacol. Biochem. Behav.* **74**, 363-369.
- Zarrindast, M. R., Massoudi, R., Sepehri, H., Rezayof, A. (2006) Involvement of GABAB receptors of the dorsal hippocampus on the acquisition and expression of morphine-induced place preference in rats. *Physiol. Behav.* **87**, 31-38.
- Zataly, Y., H., Zarrindast, M. R., Rezayof, A., Haeri-Rohani, A., Razavi, S. (2005) Involvement of muscarinic receptors of the dorsal hippocampus on the acquisition of CPP caused by morphine in rats. *New Cognition Science* **2**, 21-22. (in Persian)
- Zhai, H., Wu, P., Chen, S., Li, F., Liu, Y., Lu, L. (2008) Effects of scopolamine and ketamine on reconsolidation of morphine conditioned place preference in rats. *Behav. Pharmacol.* **19**, 211-216.