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**Original Article**

# The Effect of Valproic Acid Administration on Learning, Social Interaction, and Depression Induced by Withdrawal Syndrome in Morphine-dependent Mice

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**Abstract**

**Background:** Opioids can lead to mood disorders, anxiety, depression, and cognitive impairment. Valproic acid (VPA) has neuroprotective effects that can prevent neural degeneration. This study aims to examine the impact of VPA on learning, social interaction, and depression in mice dependent on morphine.

**Methods:** Subjects were divided into four groups and received injections of saline, VPA, morphine, or a combination of VPA and morphine for eight days. Behavioral tests were conducted on day 8, and then administration of VPA and morphine was stopped, leading to spontaneous withdrawal syndrome. Behavioral tests were repeated on day 11, and histological analysis was performed on the hippocampus.

**Findings:** The preference index (PI%) decreased in the novel object recognition test in the VPA and morphine sulfate (MOR) groups compared to the control (CTL) group in the chronic phase. The concomitant administration of VPA and morphine caused an increase in social interaction criteria in both the chronic and withdrawal phases. The decrease in immobility time in the VPA and MOR+VPA groups compared to the CTL group in the withdrawal phase was not statistically significant in the tail suspension test (TST). In Nissl staining, the combination of MOR+VPA led to a significant decrease in the DC/All cell ratio compared to the individual MOR and VPA groups ($P<0.05$).

**Conclusion:** VPA may improve social relationships and depression indices during morphine withdrawal. VPA may potentially mitigate the cellular changes in the CA1 of the hippocampus induced by morphine.

**Keywords:** cognitive dysfunction, Opioid, Valproic acid, Substance withdrawal syndrome

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## Introduction

Opioids are highly potent drugs with different receptors (μ, κ, δ, etc.) in the central nervous system. These receptors are expressed in different parts of the brain.[1] As a μ-receptor agonist, morphine has a very high affinity for binding to these receptors, causing changes in the brain's normal functioning by acting on these receptors.[2] Long-term use of morphine causes changes in the release of serotonin, dopamine, acetylcholine, and nor-adrenaline in the brain, which play critical roles in regulating neuronal activity.[3,4] Opioid addiction causes mood disorders and increases anxiety, depression, and cognitive impairment.[5] Opioids also impair learning, memory, attention, reasoning, and impulse control.[6] One of the major problems associated with long-term abuse of drugs such as morphine is addiction, which is characterized by withdrawal syndrome.[7] The exact mechanisms of tolerance, dependence, and withdrawal symptoms have not been clearly defined. However, neurobiological changes of dopaminergic neurons in the ventral tegmental area and nucleus accumbens and noradrenergic neurons in the locus coeruleus may induce withdrawal symptoms. GABAergic mechanisms in the basal ganglia mediate anxiety-like behaviors, and the reduction in GABA activity induces fear and anxiety-like behaviors.[8]

Preventing anxiety-like behaviors and depression is crucial in treating addiction during and after withdrawal. Sodium valproate is an antiepileptic drug that controls seizures in children and adults with epilepsy. It is also prescribed for other neurological disorders, including depression and neuralgia caused by diabetes. Sodium valproate controls seizures by reducing abnormal

electrical activity in the brain.[9] Valproic acid (VPA) is not routinely used to treat anxiety-like disorders. Despite strong evidence for its anxiolytic properties, animal studies suggest that it acts through a different pathway than benzodiazepine.[10]

The use of VPA can be assessed based on the degree of anxiety-like and depressive-like behaviors caused by the withdrawal syndrome.[11,12] It has been shown that chronic morphine use induces depression and anxiety-like behaviors, reduces motor activity in the open maze test, and leads to impairments in learning and memory performance.[13] While opioid-related compounds impair learning and memory,[14-16] VPA can affect brain cells through various mechanisms,[17-19] including GABAergic mechanisms.[20] It may interfere with the opioid system[21] and induce changes in various behaviors. This study aimed to investigate the chronic administration of VPA on novel object recognition behaviors, social interaction, and depression during morphine addiction and spontaneous withdrawal.

## Methods

The present study used male NMRI mice (2 months old) weighing 20–30 g. The subjects were under standard laboratory conditions and had access to adequate food and water. The animals were housed in standard cages (three mice in each cage) under the same care conditions with a 12-hour light-dark cycle at room temperature. The animals were randomly divided into four groups ($n=6$). The control (CTL) group received 1 cc saline as intraperitoneal and subcutaneous injections for 8 days. The VPA group (Sigma, St. Louis, MO) was injected intraperitoneally with 240 mg/kg VPA. VPA was injected for 8 days in 1 cc of physiological saline.[22] The animals in the morphine sulfate (MOR) group were injected subcutaneously with 10 mg/kg morphine sulfate (Tamad, Iran) for 8 days. The MOR + VPA group received two injections, valproate and morphine, similar to the VPA and MOR groups. Four rats from the morphine group were randomly selected and then injected with naloxone (5 mg/kg). The withdrawal symptoms, including teeth chattering, sneezing and salivation, wet dog shakes, grooming, and diarrhea, were monitored (Table S1). The animals were then assessed on day 8 and their abilities in the novel object recognition, social interaction, and tail-hanging tests were evaluated. The administration of VPA and morphine was discontinued from day 8, and the animals entered the spontaneous withdrawal syndrome phase. The behavioral tests were repeated on day 11 (Figure 1).

### *Novel object recognition task (NORT)*

The NORT evaluates episodic memory. The animals were placed in a bright and object-free (40 × 60 × 60 cm) plastic box for a 10-minute acclimatization session. twenty-four hours after the acclimatization session, each animal was allowed to search for two other similar objects for 5 minutes (training session). Forty-five minutes later, a test session was performed in which a new object was placed in the place of one of the old objects, and the animal was allowed to identify the new object for 3 minutes. A camera above the maze recorded the subject's behaviors. To avoid animals' innate preference for one place or another, we randomly changed the positions of the new subjects. Between each trial, the objects and box were cleaned with alcohol. Exploration time was defined as the time the subject smelled or touched an object.[23,24] Preference index (PI%) was considered the object identity index in the NORT. The preference index is calculated with this formula: Novel object exploration time / (Novel + Old object exploration times) × 100.

### *Social Interaction Test (SI)*

This test was performed to study social interactions

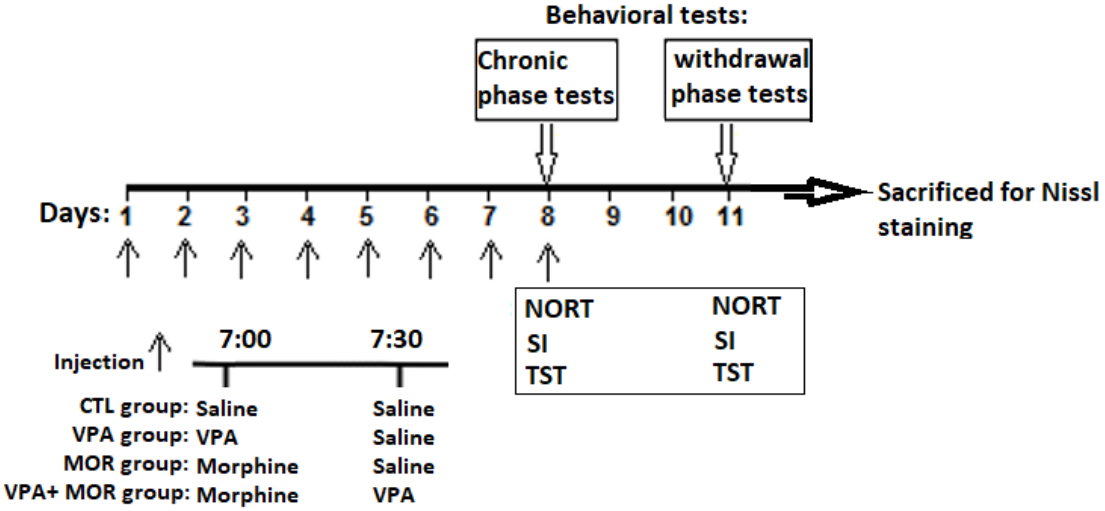


**Figure 1.** Experiment timeline. NORT: novel object recognition test; SI: social interaction test; TST: tail suspension test; CTL: control; VPA: valproic acid; MOR: morphine

in rats. The same NORT maze box was used, and the animals in the experimental groups were fully acclimated to the conditions of the maze. One animal from each experimental group was then separated and exposed to a new animal from another experimental group in the center of the box for 10 min. Then, the behavior of the test subject was evaluated. The total time each mouse spent on social behavior, such as following, sniffing, touching, and licking any part of a stimulus rat's body, was considered the social interaction time.[25]

### *Tail suspension test (TST)*

This test is performed after the object recognition and social interaction cognitive tests. First, each rat was hung from a crossbar 20 cm above the table by a rope connected to the animal's tail. The experimental duration was six minutes, and the time when the mouse was completely stationary during the last four minutes was recorded as the immobilization time.

### *Histological evaluation*

After the behavioral tests (day 11), three rats per group were deeply anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg). The coronal sections of the hippocampus were prepared at a thickness of 7 microns and then stained with 0.1% cresyl violet, dehydrated through graded alcohol (50%, 70%, 95%, 100%), placed in xylene, and mounted. Thirty-six fields on the slides in each group were randomly selected. The fields were matched among groups according to the atlas of the adult rat brain.[26] The samples were observed by an optical microscope with a magnification of × 400, and only pyramidal neurons in the CA1 area of the hippocampus on the right side and clear and visible nuclei were considered alive and intact cells.

### *Data analysis*

Data were analyzed using GraphPad Prism (version 9.3.1.471) and SPSS (version 16.0). *P* values less than 0.05 were considered statistically significant. After performing the normality test of the data (Shapiro-Wilk test), two-way ANOVA and post hoc LSD tests were used for data analysis.

## Results

### *Novel object recognition test*

In the VPA group, PI% significantly decreased compared to the CTL group ($P<0.01$). In the MOR group, PI% also decreased significantly compared to the CTL group ($P<0.01$). There was a statistically non-significant decrease in the MOR + VPA group compared to the CTL group ($P=0.06$). Data analysis showed no statistically significant differences among the MOR, VPA, and MOR + VPA groups.

In the withdrawal phase, PI% decreased in the VPA group compared to the CTL group ($P<0.001$). Furthermore, exposure to morphine at this stage also reduced PI% in the MOR ($P<0.001$) and MOR + VPA ($P<0.01$) groups compared with the CTL group. Further data analysis revealed no statistically significant differences among the VPA, MOR, and MOR + VPA groups (Figure 2).

### *Social interaction test*

Each subject's total time with the stranger mouse was considered social interaction (SI) time. In the chronic phase, a decrease in SI time was observed in the VPA group compared to the CTL group ($P<0.001$). SI time in the MOR group also significantly decreased compared to the CTL group ($P<0.05$). During the chronic phase, the VPA + MOR group showed a significant improvement in SI time compared to both the MOR ($P<0.01$) and VPA ($P<0.01$) groups.

In the withdrawal phase, there was also an apparent decrease in SI time in the MOR group compared to the CTL group ($P<0.001$). Interestingly, in the VPA + MOR group ($P<0.05$), the SI time significantly improved compared to the MOR group.

In both phases, there was no statistically significant difference between the MOR + VPA and CTL groups

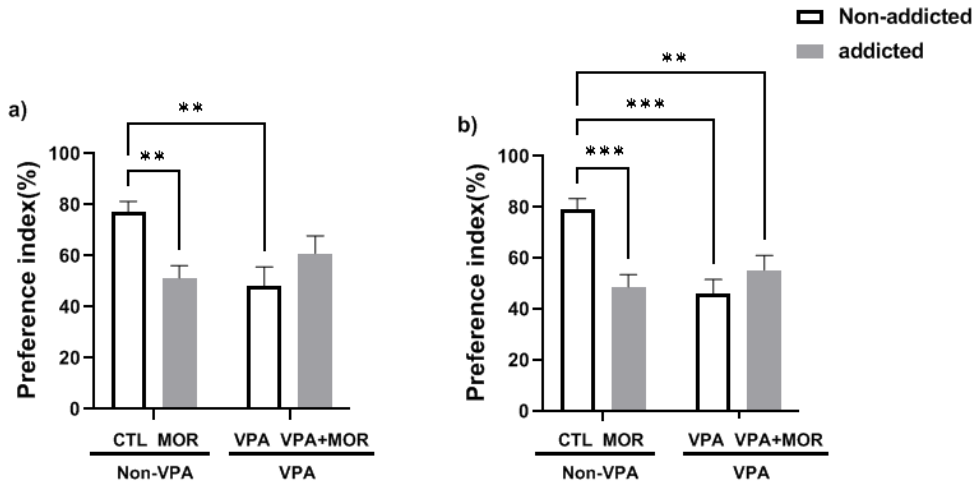


**Figure 2.** Preference index percentage (PI%) in the chronic (a) and withdrawal phases (b) in the NORT among the control (CTL), morphine (MOR), and valproic acid (VPA), and MOR + VPA groups. Data are shown as mean ± SEM ($n=6$). *$P<0.05$, **$P<0.01$, and ***$P<0.001$

(Figure 3).

***Tail suspension test***

The increase in immobility time in the TST was considered an indicator of depression. In the chronic phase, exposure to morphine caused a significant increase in the immobility time of the MOR group compared to the CTL group ($P<0.01$). In contrast, it was found that immobility time decreased in the VPA group ($P<0.01$) compared to the CTL group. The MOR + VPA group exhibited a significant decrease in immobility time compared to the CTL group ($P<0.05$) and the MOR group ($P<0.001$).

In the withdrawal phase, the immobility time in the MOR group did not show a statistically significant change compared with the CTL group. In addition, immobility time in the VPA group showed a significant decrease compared to the CTL group ($P<0.01$). However, the decrease in the immobility time of the MOR + VPA group compared to both CTL and MOR groups in the withdrawal phase was not statistically significant (Figure 4).

***Nissl staining***

The number of pyramidal CA1 cells did not change among the experimental groups (Figure 5). As depicted in Figure 5, the cells had dark and pyknotic nuclei, and dense and dark cytoplasm decreased in the MOR + VPA group compared to the MOR and VPA groups. A significant increase in the DC/All cell ratio of the MOR group was revealed compared to the CTL group ($P<0.001$). The DC/All cell ratio of the VPA group also increased compared to the CTL group ($P<0.001$). In the MOR + VPA group, the DC/All cell ratio showed a significant decrease compared to the CTL group; however, in this group, the DC/All cell ratio decreased significantly compared to the MOR ($P<0.001$) and VPA ($P<0.01$) groups (Figure 6).

## Discussion

In the present study, the effect of VPA drug administration on learning, social interaction, and depression indicators was studied in healthy and addicted rats. The present study showed that chronic treatment with VPA decreased novel object recognition in mice. Similarly, the effects of VPA remained during the drug withdrawal period, and the recognition of new objects decreased. In this regard, consistent with this study, it has been found that

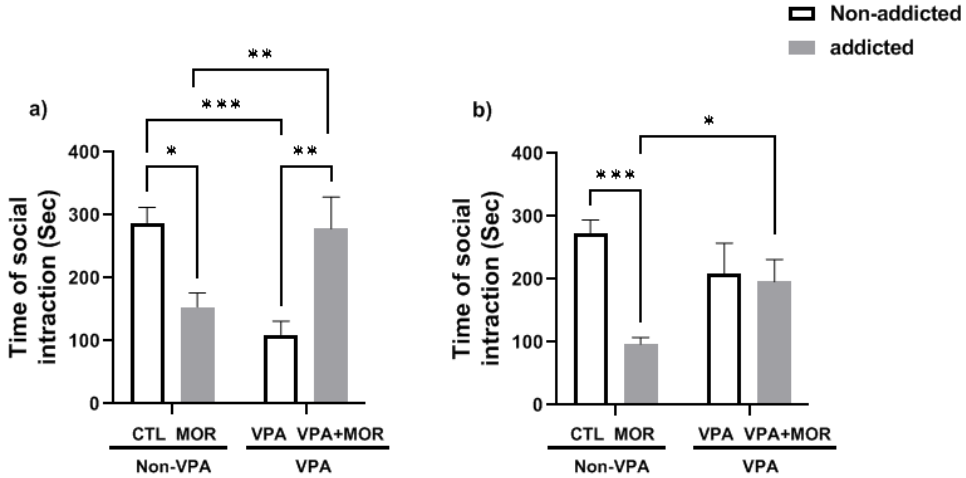


**Figure 3.** Social interaction (SI) time in the chronic (a) and drug withdrawal (b) phases in the social interaction test among the control (CTL), morphine (MOR), and valproic acid (VPA), and MOR + VPA groups. Data are shown as mean ± SEM ($n=6$). *$P<0.05$, and **$P<0.01$

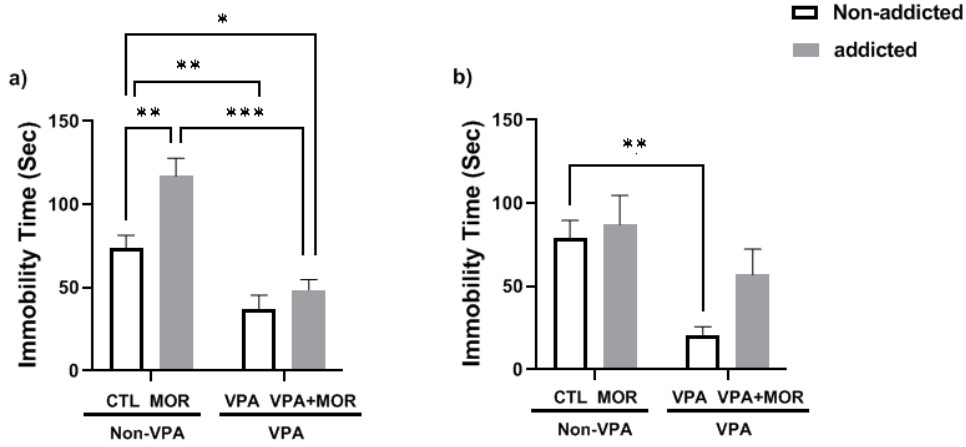


**Figure 4.** Immobility time in chronic phases (a) and drug withdrawal (b) in tail suspension test among the control (CTL), morphine (MOR), valproic acid (VPA), and MOR + VPA groups. Data are shown as mean ± SEM ($n=6$). *$P<0.05$, **$P<0.01$, and ***$P<0.001$

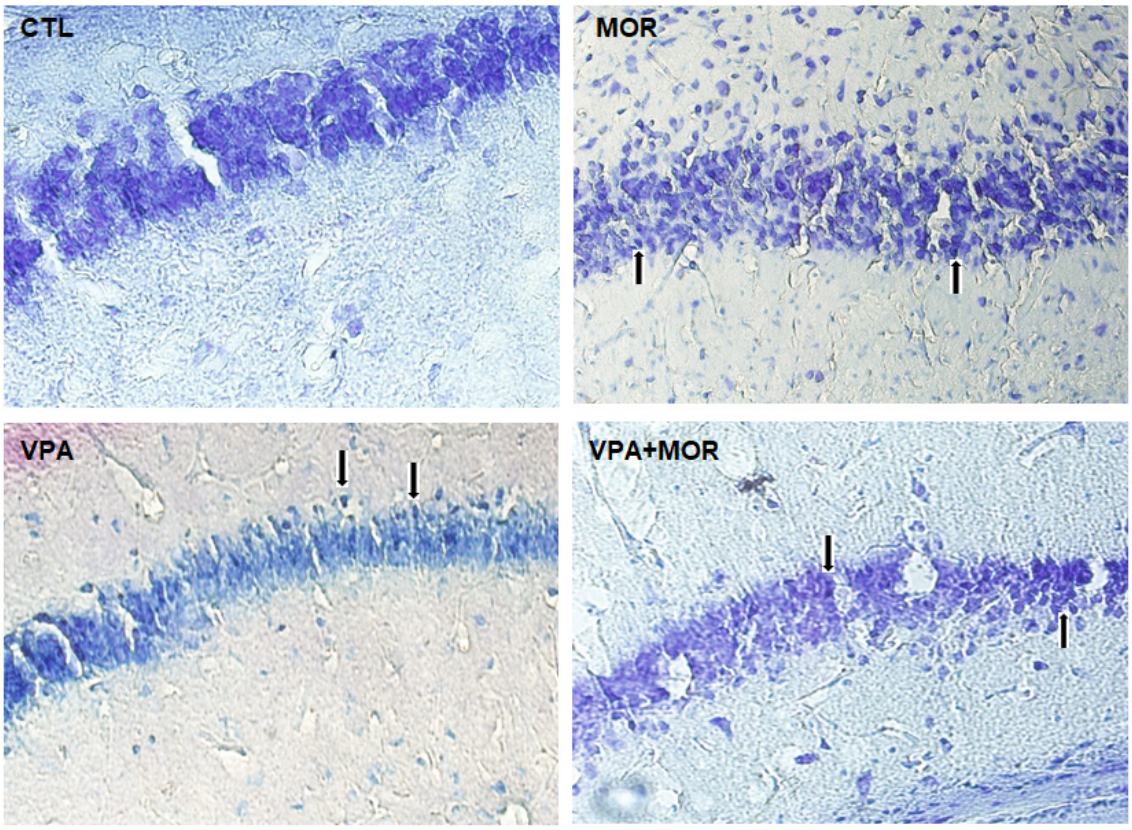


**Figure 5.** Micrograph of hippocampal CA1 region of a sample mouse among the control (CTL), morphine (MOR), and valproic acid (VPA), and MOR + VPA groups. The degenerated cells were observed in the VPA, MOR, and VPA + MOR groups (arrowhead). Magnification factor:10 × 40

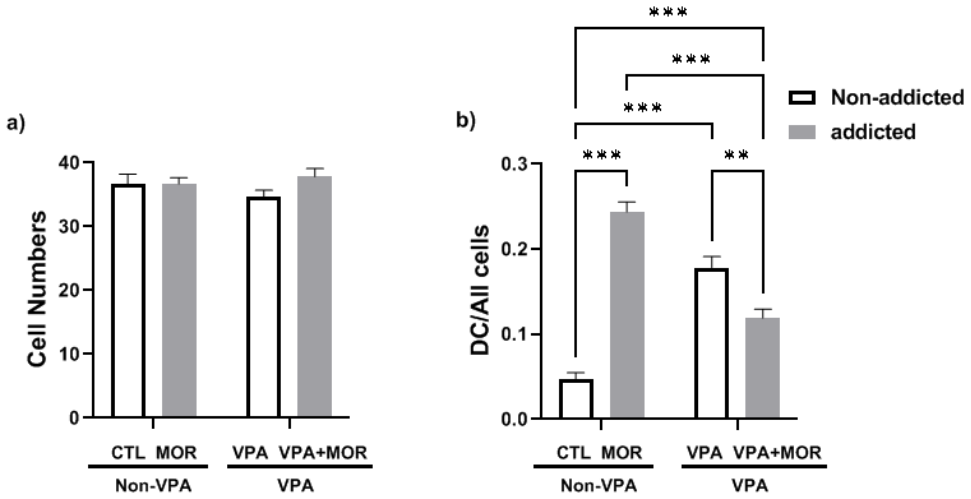


**Figure 6.** The number of degenerated cells (DC) to number of cells (all) of the CA1 region of the hippocampus among the control (CTL), morphine (MOR), and valproic acid (VPA), and MOR + VPA groups. Data are shown as mean ± SEM ($n = 4$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$

animals under VPA treatment took approximately the same amount of time to find new and familiar objects.[27] Another study indicated that VPA notably reduced PI% as a discrimination index in the novel object recognition test. It appears that VPA may exert its effects by reducing the levels of Ki-67, BrdU/NeuN, and DCX-positive cells in the hippocampus, leading to a decline in memory.[19] The histological results show that the VPA group has more damaged cells than the CTL group, supporting the NOR test results.

The present study also revealed that the percentage of PI decreased in addicted rats, and this decrease was also observed during the morphine withdrawal phase. The histology results show that the MOR group has more degenerated cells than the CTL group, confirming the NOR test results. Previous studies have also established that the use of morphine and other opioids may have a deleterious effect on learning and memory. These studies show that even parental use of opioids leads to impaired learning.[14,28] In this context, abrupt cessation of morphine

after chronic morphine exposure was found to impair memory and working memory.[29] One of the main findings of this study was that VPA combined with morphine increased the likelihood of recognizing novel objects by more than 50% (comparable to the CTL group; $P = 0.06$). This change was insignificant compared to the MOR or the VPA groups. During the morphine withdrawal phase, the level of new object recognition in the MOR + VPA group was also significantly lower than in the CTL group, so these two drugs showed no synergistic or additive effects on new object recognition. However, the present results also indicate that the number of degenerated cells in the VPA + MOR group was less than in the MOR and VPA groups, suggesting a protective effect of VPA against neurodegeneration. VPA is commonly used to treat epilepsy, seizures, and bipolar disorder.[9] In contrast, prenatal exposure to VPA can lead to brain malformations and behavioral abnormalities similar to those of autism spectrum disorder.[30] VPA's exact mechanism of therapeutic action is not yet known. VPA increases GABA levels[17] and inhibits histone deacetylase (HDAC) activity, which affects gene expression.[31] HDAC inhibitors have been found to prevent the development of tolerance to morphine in mice, which is significant in understanding drug effectiveness.[32] Thus, the positive impact of VPA on morphine tolerance may contribute to its protective effects on cognition, depressive behavior, and social interactions.

The present study showed that treatment with VPA decreased the social interaction time in mice treated with this drug in the chronic phase. Previous research has shown that VPA reduces sniffing behaviors.[33-36] In the drug withdrawal phase, VPA improved social interaction in the mice. These social interaction deficits in VPA-exposed rats have been determined in three-chamber and SI tests.[37] These data support our previous findings that prenatal exposure to VPA in rats induces a wide range of social defects during development, ranging from social interaction deficits to texture discrimination deficits.[38]

The present study showed that in rats addicted to morphine (the MOR group), social interaction time decreased in the chronic phase of morphine administration. Even when the administration of morphine was stopped, the social interaction time in the addicted rats of the MOR group was meaningfully reduced. In previous studies, two doses of morphine (1.25 and 2.5 mg/kg) have been shown to reduce sociability, motivation, and palatable food intake in male and female rats, independent of cognitive performance or motor activity.[39] One of the main findings of this study was that the concomitant administration of VPA and morphine caused an increase in the social interaction time in both the chronic and withdrawal phases.

The results of the TST in the chronic phase of the current study showed that VPA treatment reduced the immobilization time of mice in the VPA group. This effect was also observed during the withdrawal period. This is consistent with previous studies showing that VPA improves depressed mood.[40,41]

In the present study, morphine exposure in the MOR group was found to increase the duration of immobility of dependent rats during both the chronic and withdrawal phases. This finding was in contrast with previous studies which reported that opioids reduce immobility time in rats.[42-44]

Finally, VPA drug treatment was found to significantly reduce the immobilization time of the mice in the MOR + VPA group. Based on these results, it can be concluded that VPA mitigates the effects of morphine and exerts antidepressant effects in drug withdrawal syndrome. Importantly, antidepressants reduce immobility[40,41] and spontaneous locomotor activity.[45] In one study, the effects of combined methadone and VPA administration on behavioral morphine withdrawal of male rats were previously studied.[46] The results of the study showed a reduction of anxiety-like behavior in the VPA + methadone group compared with the morphine group. In the suspension trial of the study, immobility time was reduced in the VPA + methadone group.[46] As mentioned above, the effects of VPA can be assessed based on the degree of anxiety-like and depressive-like behaviors caused by the withdrawal syndrome. Farzad et al conducted an experimental study to study the effects of VPA on the anxiety-like levels of morphine-dependent rats, and their results showed that the use of VPA significantly reduces anxiety-like levels in rats.[47] Bach et al conducted a randomized clinical trial in Switzerland to use VPA for anxiety-like behaviors, and the results of their study showed that its use significantly reduced these behaviors.[48] In addition, Salehi et al conducted a randomized clinical trial aimed at controlling the effects of VPA in the management of withdrawal symptoms in patients with opiate addiction, and their results showed that the average score of the physical symptoms of withdrawal syndrome did not differ significantly between the two groups on different days. However, in controlling psychotic symptoms, VPA had a significant effect compared with placebo.[49] Motaghinejad et al have shown that chronic morphine use induces depression and anxiety-like behaviors, reduces motor activity in the open maze test, and leads to impairments in learning and memory performance. Of course, treatment with progressive intervals, irregular intervals, and a reduced dose regimen of morphine significantly reduced depression and anxiety-like behaviors and improved cognitive performance compared with the control group.[13] Therefore, reducing stress in rats during morphine withdrawal may be part of the beneficial effects of VPA in improving social interaction and depression indices; of course, these results need further investigation.

Additionally, considering the epigenetic mechanisms between VPA and morphine described above, VPA has

been shown to elevate the level of GABA in the brain through competitive inhibition of GABA transaminase, thus promoting the availability of synaptic GABA and facilitating GABA-mediated responses.[17] VPA has been shown to have the potential as a therapeutic medication[50] for drug abuse by inhibiting the establishment of morphine-induced conditioned place preference (CPP) in rats.[51] This effect is thought to be due to VPA's ability to inhibit GABA transaminase,[42] which results in decreased GABA degradation and increased GABA synthesis, leading to enhanced GABA system activity.[52] This, in turn, attenuates the morphine-induced enhancement of dopaminergic transmission, which is responsible for the rewarding effects of morphine. Additionally, VPA has been reported to alter the activity of protein kinase C and early inducible genes belonging to the AP-1 family of transcription factors.[18] These findings suggest that VPA may be a useful pharmacological adjunct in the treatment of drug abuse.

Studies have reported that VPA decreases the total number of days of cocaine use and reduces the frequency and amount of marijuana use in some patients.[53] In animal studies, consecutive ICV injections of VPA before morphine treatment significantly attenuated the establishment of chronic morphine-induced CPP. There are two hypotheses to explain the inhibitory effect of VPA on morphine's rewarding effects.[21] One is that VPA has antagonistic activity towards morphine, thus disturbing its action in the central nervous system.[54] The other is that VPA itself has adverse effects that counteract the rewarding effect of morphine.[55] However, according to the results of VPA group studies, the first hypothesis is more reliable,[56] as VPA has no averse or rewarding effects.[21]

## Conclusion

The present study's findings suggest that chronic VPA treatment reduced novel object recognition and social interaction time in mice while decreasing depressive behavior. Moreover, the study indicated that VPA might exert its effects by increasing degenerated hippocampal cells, leading to memory decline. Notably, combining VPA and morphine increased the likelihood of recognizing novel objects but did not exhibit synergistic or additive effects on new object recognition. The results demonstrate that VPA's impact on learning, social interaction, and depression indicators in healthy and addicted mice is intricate and multifaceted. Additionally, VPA was observed to have a protective effect against neurodegeneration in the context of morphine addiction. The study also underscored VPA's potential in enhancing social interaction and depression indices during morphine withdrawal. Further research is necessary to fully comprehend the complex effects of VPA in the context of addiction and its potential therapeutic implications.

### Authors' Contribution

**Conceptualization**: Mohammad Reza Afarinesh.
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**Writing–original draft**: Melika Farhadi and Mohammad Reza Afarinesh.
**Writing–review & editing**: Mohammad Reza Afarinesh, Mansoureh Sabzalizadeh, Melika Farhadi, and Ali Shamsara.

### Competing Interests

The authors declare no potential conflicts regarding this article's research, authorship, and/or publication.

### Ethical Approval

The study protocol was approved by the local Medical Ethics Committee for the Use and Care of Animals at Kerman University of Medical Science (ethics approval code IR.KMU.REC.1400.459).

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### Supplementary File

Supplementary file contains Table S1.

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