

Long-term morphine addiction reduces neurogenesis and memory performance and alters emotional reactivity and anxiety levels in male rats

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Introduction: Substance abuse is a behavioral disorder associated with a wide variety of devastating effects. Neurogenesis in dentate gyrus of hippocampus is essential for brain functions like memory formation. Therefore, this may play an important role in achieving successful withdrawal.

Methods and materials: Twenty Sprague-Dawley rats were randomly divided into two experimental groups: control and addiction. To induce morphine dependence, animals received morphine (0.75 mg/rat) for 21 days. The performance of animals in Morris water maze and elevated plus maze tests was evaluated after day 20. At the end of the study, the rats were decapitated, and their brains were sectioned to study neurogenesis by counting BrdU-positive cells.

Results: Hippocampal neurogenesis was significantly reduced in rats in the addicted group. Also, reference and working memory performance were impaired in animals in the addicted group. A decrease in emotional reactivity and anxiety was observed in animals in the addicted group when compared with that in the control group.

Conclusion: Addiction adversely affects brain functions and neurogenesis; thus treatment to increase neurogenesis is the better option for the persons with substance abuse.

Keywords: hippocampal neurogenesis, morphine addiction, striatum, morphine-induced, hippocampus, substance abuse

Introduction

Neurogenesis is a process that occurs in some parts of brain like dentate gyrus of hippocampus and subventricular zone of lateral ventricles.¹ Adequate neurogenesis is essential for brain functions, but it may be influenced by many pathological conditions like Alzheimer, depression, and addiction.²⁻⁴ Drug addiction, a compulsive urge to take drugs like cocaine, heroin, and alcohol, can adversely affect mesolimbic system.⁵ Mesolimbic system is the most known region of brain that is responsible for side effects of drugs.⁶ It is important to study adult hippocampal neurogenesis because cognitive functions are an important part of brain function and healthy life.⁷⁻¹⁰ Also, hippocampus, as part of the limbic system, may be necessary for tolerance of drugs and reduction of relapse. However, mechanisms involving addiction-induced side effects are not elucidated to date. Previous studies have shown that cocaine has adverse effect on neurogenesis; however, the effect of long-term use of other substances such as morphine has not been studied. The aim of this research is to study the effect of long-term morphine addiction on neurogenesis and brain functions like memory, emotional reactivity, and anxiety levels to delineate the behaviors that help addiction

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exerts and improves its various adverse effects. We further studied to find whether improved neurogenesis can prevent drug relapse.

Methods and materials

Animals

Twenty male Sprague-Dawley rats (weighing 200–250 g) were used in this study. Animals were divided into two groups ($n=10$ each): control and addiction. Housing took place under standard 12 hour day/night cycle at room temperature 22°C, and animals had free access to water and chow ad libitum. All the experiments were performed in accordance with the guidelines for the care and use of laboratory animals published by the US National Institutes of Health (NIH Publication No 85-23, revised 1996). The experimental protocol was approved by the institutional care and use committee of Tehran University of Medical Sciences (Tehran, Iran).

Experimental procedure

At the start of experiment, rats were injected with BrdU (50 mg/kg) and morphine (0.75 mg) intraperitoneally, and these injections lasted for 21 days. On day 20, the memory of rats was first tested by Morris water maze for two days, and then anxiety was tested by elevated plus maze. Then rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and then sacrificed. The brain was perfused with paraformaldehyde 4% for fixation of brain, and then it was removed for preparing sections.

Addiction

To induce drug dependence and addiction, animals received 0.75 mg morphine Sulfate intraperitoneally for 3 weeks.

Morris water maze

This apparatus was used to assess two types of memory: working and reference memory. During this task, animals had to find extra-maze cues to locate the platform. This setup consisted of a large circular black tank (diameter 183 cm) placed in the center of the room. After performing the first trial for visible test with clear water, the tank was filled with water (27°C) that was made opaque with nontoxic black tempera paint and divided into four quarters (North, East, West, and South). An escape platform (10 cm in diameter) was submersed 0.5 cm below the surface of water in the southeast quadrant. Furthermore, non motile visual cues were placed around the water tank. Since the animals were kept in opaque water and the platform was not visible, they had to rely on external maze cues to find their route of escape. During

the probe trial, the platform was retracted from the bottom of the maze to assess if animals can swim to the quadrant where the platform was located previously. Large red geometrical shapes with white backgrounds were placed around the maze to provide external cues. During the entire experiment, the animals' performance was videotaped using a video camera mounted above the maze and interfaced with a computerized tracking system. In this 3 days experiment, two trials were performed each day. However, on the third day, the probe trial was performed. The first trial was considered for the visible test.¹¹ First trial of each session was considered as the test for reference memory and the later one for working memory. In addition, swimming speed was also recorded to evaluate the emotional state of the animal.¹²

Visible test

This test was used to assess nonspatial learning and to rule out the possibility that the spatial learning deficit detected might actually be a product of deficient escape motivation or impairment of vision and/or motor skills. In this test, the location of platform was highlighted by attaching it to a high-contrast striped flag rising above the water. This gave spatial cues to animals for escaping from water. This simple associative nonspatial task is believed to be independent of hippocampal function.

Spatial acquisition test

In these trials, the animals had to locate the hidden platform using extra-maze cues and find the platform that is now located under water in the southeast of the quadrant of the tank. A transparent Lucite platform (10×10 cm) was submerged beneath the surface of the water (0.5 cm) in the southeast quadrant of the tank. Each rat participated in 16 spatial trials with invariable start positions. For each trial, a 60-second time period was provided to reach the platform. If the animal had failed to reach the platform within 60 seconds, the experimenter placed the animal on the platform. A 20-second rest period was observed between each trial. Swimming time (seconds), swim distance (cm), and swim speed (s/cm) were recorded. For swimming time and distance, lower values were indicative of better performance, whereas higher values indicated better swimming speed.

Probe Trial for Spatial Memory

This trial was performed to evaluate how well the animal had learnt the location of platform. The platform was removed and made unavailable for 60 seconds. For time spent in the fourth quadrant and swim distance in the fourth quadrant,

higher numbers were indicative of better performance in contrast to the swimming speed.

- Fourth quadrant is the quadrant in which the platform had been placed and was removed in the probe trial.

Elevated plus maze

During this task, the level of anxiety of animals was assessed. It consisted of a plus-shaped apparatus with two open and two closed arms, each with an open roof. The arms were elevated 70 cm from the floor. Each animal was placed in the center of the apparatus and then allowed to move freely in the four arms for 5 minutes. The number of entries to the open arms and time spent in the open arms were recorded. Reduced anxiety levels were indicated by more time spent and increased number of entries into open arms.¹³

Immunohistochemistry

For 21 days, all animals received 50 mg/kg BrdU intraperitoneally. On day 22, rats were anesthetized using ketamine (100 mg/kg) and xylazine (10 mg/kg) and then sacrificed. After thoracotomy, all animals were first perfused with normal saline and then with paraformaldehyde 4% via intracranial infusion. After fixation, the brains were removed from skull. For the first 2 days, the brains were kept in PBS + paraformaldehyde 4% and then on day 3, in sucrose 10% + paraformaldehyde 4% + PBS. Throughout day 4, the brains were kept in sucrose 20% + paraformaldehyde 4% + PBS, and for the remaining days, they were kept in sucrose 30% + paraformaldehyde 4% + PBS. The cryosections (30 μ m) were prepared from dentate gyrus of the hippocampal region. Immunohistochemistry was performed for ten sections from each brain, five of which were stained for BrdU-positive neurons with anti-BrdU antibody kit (5-Bromo-2'-dU Labeling and Detection Kit II; Roche, Germany; Cat No 11299964001-en-17). BrdU-positive cells in dentate gyrus were counted using light microscope (400 \times).¹⁴

Statistics

Data were analyzed using SPSS version 22 and Graph pad prism 5. Independent two-tailed sample *t*-test was performed for all experiments. Data were represented as mean \pm SEM, and $P < 0.05$ was considered significant.

Results

Morris water maze

Working memory

In animals in the addicted group, working memory performance was markedly reduced when compared with that of the control group (34.45 vs 28.54, $P < 0.0344$, Figure 1).

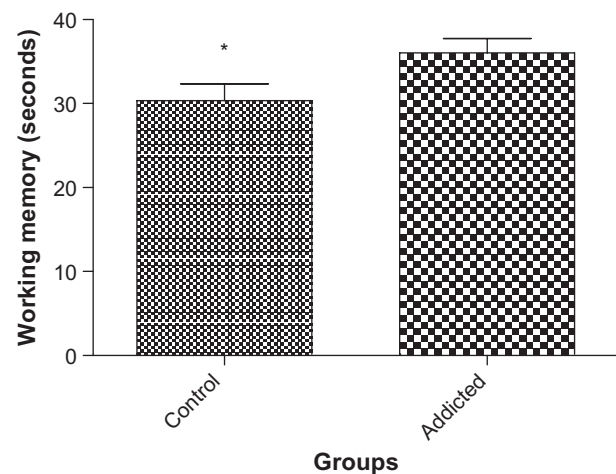


Figure 1 Working memory performance in control and addicted rats.

Notes: Data are represented as mean \pm SEM. * $P < 0.05$.

Abbreviation: SEM, standard error of the mean.

Spatial task acquisition

Swimming speed, as an indicator of emotional reactivity, was lower in animals in the addicted groups than the animals in the control group (30.28 cm/s vs 27.04 cm/s, $P < 0.0246$, Figure 2).

Probe trials

Animals in the control group spent more time in the fourth quadrant than the animals in the addicted group (25.93 seconds vs 20.30 seconds, $P = 0.0388$, Figure 3A). Speed of swimming was found to be significantly higher in rats in the addicted group than that of rats in the control group (33.77 cm/s vs 26.73 cm/s, $P = 0.0139$, Figure 3B). These results indicate better reference memory performance in the control group.¹⁵

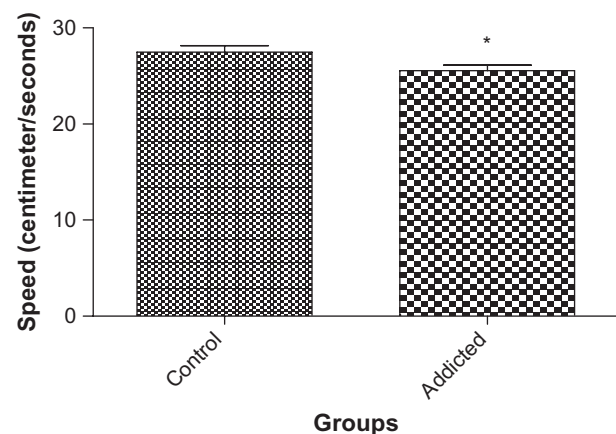


Figure 2 Speed of swimming in spatial task acquisition (n=10).

Notes: Data are represented as mean \pm SEM. * $P < 0.05$.

Abbreviation: SEM, standard error of the mean.

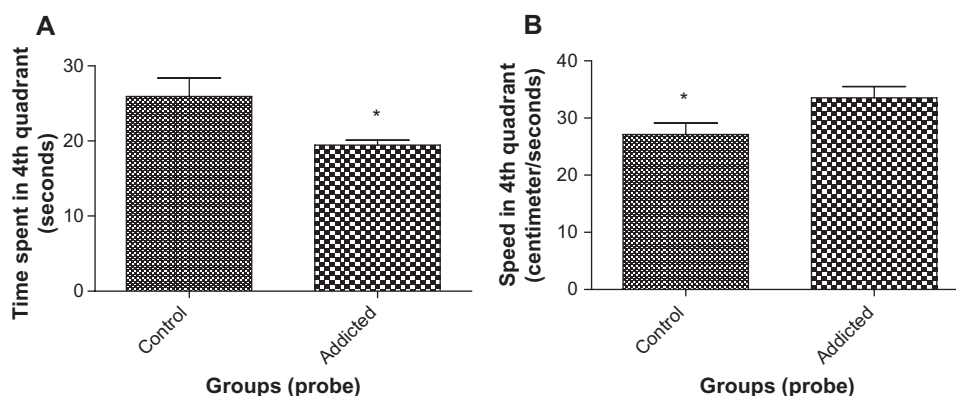


Figure 3 (A) Time spent in fourth quadrant in probe trial ($n=10$). (B) Speed of swimming in fourth quadrant in probe trial ($n=10$).

Notes: Data are represented as mean \pm SEM. * $P<0.05$.

Abbreviation: SEM, standard error of the mean.

Elevated plus maze

Anxiety levels

Rats in the addicted group showed higher number of entries (3.667 vs 1.4, $P=0.0268$, Figure 4A) and also spent more time in the open arms (42.78 seconds vs 11.7 seconds, $P=0.0049$, Figure 4B) in comparison with the animals in the control group. These measures are suggestive of lower anxiety levels in rats in the addicted groups.

Neurogenesis

The number of BrdU-positive cells in dentate gyrus of hippocampus was considerably higher in animals in the control group compared with that of the addicted group (251.4 vs 158.2, $P=0.0386$, Figures 5 and 6).

Discussion

In this study, we examined the effect of long-term morphine abuse on neurogenesis and brain functions. We found that morphine addiction reduces neurogenesis and memory performance in rats in the addicted group. Increased anxiety and emotional state was observed in rats in the control group.

Adequate neurogenesis is important for brain functioning. Therefore, morphine-induced reduction in neurogenesis can adversely affect memory performance, learning, emotional reactivity, and anxiety levels. Furthermore, intact learning abilities are required for successful drug abstinence. It is possible that along with reduction in learning abilities, adaptation in rewarding system occurs, and morphine abusers cannot overcome this dysfunction for avoiding morphine abuse.¹⁶ As we know, along with abuse of morphine, brain activates rewarding system to discard morphine abuse. Two types of rewarding systems are involved: 1) one for quitting drug abuse that activates behaviors that are necessary for life such as feeding, and 2) the other is the rewarding system that causes relapse due to adverse effect on mesolimbic dopaminergic system.¹⁷ Exercise results in enhancement of neurogenesis, angiogenesis, and neuroplasticity, which in turn reduces side effects caused by drug abuse.¹⁸ Another study shows overall enhancement in neurogenesis, angiogenesis, and neuroplasticity, and this shows that one possible reason such as angiogenic factor can result in enhancement of all of them.¹⁹ But in drug addiction, this pathway decreases

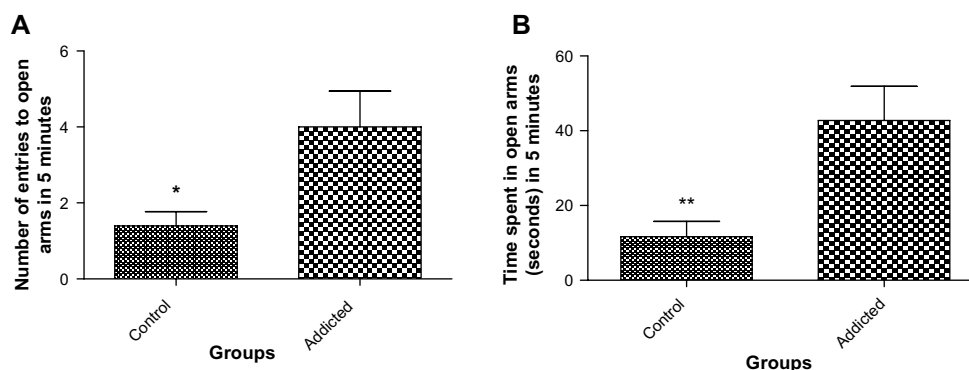


Figure 4 (A) Number of entries to open arms in elevated plus maze ($n=10$). (B) Time spent in open arms in elevated plus maze ($n=10$).

Notes: Data are represented as mean \pm SEM. * $P<0.05$, ** $P<0.01$.

Abbreviation: SEM, standard error of the mean.

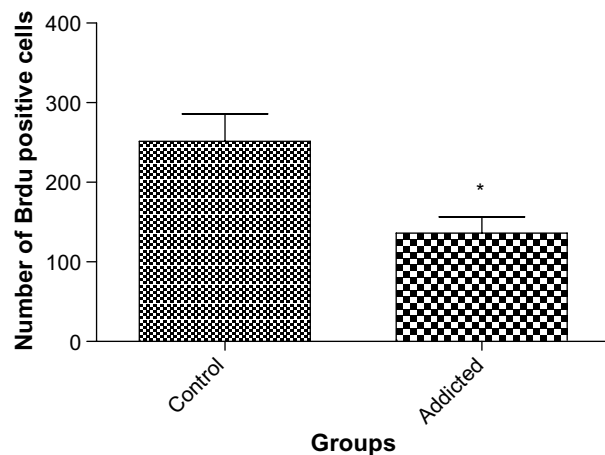


Figure 5 Neurogenesis in dentate gyrus of hippocampus (n=6).

Notes: Data are represented as mean \pm SEM. * $P < 0.05$.

Abbreviation: SEM, standard error of the mean.

and can result in loss of normal pleasurable activities. In drug addiction, pleasure center of the brain is hijacked by drugs, so it is the drug that gives brain the sense of joy.¹⁷ So

if improvement in neurogenesis helps this lost function of pleasure center, tolerance of drugs can be easier. This can be somehow because of loss of neuroplasticity in brain regions and improvement in neurogenesis directly or indirectly compensate this defect.²⁰ By destroying cells in dentate gyrus of hippocampus, drug abuse was increased. This suggests that rats' desire for drugs has increased.²¹ Noonan et al showed that self-administration of cocaine decreases neurogenesis, whereas cocaine withdrawal normalizes proliferation and survival of adult-generated neurons and enhances maturity of adult-generated neurons in subgranular zone of rats' brain.²¹ This indicates that cognitive functions are vital for drug abstinence, and restoration of neurogenesis is essential to reduce the risk of drug relapse.

In Morris water maze, different aspects of memory, such as reference memory and working memory, were assessed in a two-phase test: spatial task acquisition trial and probe trial. Our results showed that morphine abuse impairs reference memory and working memory; this is suggestive of morphine-induced disruption in brain signaling required for intact memory.¹⁵ Previously, it has been documented that successful drug withdrawal requires intact memory and proper prefrontal cortex function.^{22,23} This implies that adequate neurogenesis can improve brain functions, which may result in drug abstinence. During spatial task acquisition trial, we observed high emotional reactivity in rats in the control group.¹² This result suggests that decreased emotional state in rats in the addicted group may cause more depression and augment adverse effect of morphine. Striatum is a part of brain implicated in regulation of emotions and memory.^{24,25} In this study, along with the reduction in neurogenesis, memory and emotions were disturbed. So hippocampal neurogenesis is required for proper memory function and emotional regulations. Galanin is a neurotransmitter proposed to regulate cognitive functions and craving for drugs.^{26–28}

Broadly speaking, fear and anxiety are two different terms. Evidence of this difference is found in studies of humans and animals.²⁹ In animals, fear causes them to move away from danger, whereas anxiety causes the animal to move toward danger. This suggests that the defensive direction is an important factor.³⁰ Conversely, it has been implicated that the demarcation between fear and anxiety is independent of the conditioned or unconditioned nature of the stimuli.³⁰ We used elevated plus maze to access anxiety levels in rats. We observed that animals in the addicted group entered more frequently and spent more time in open arms, which indicates low anxiety levels and fear in these animals. These results imply that morphine abuse affects

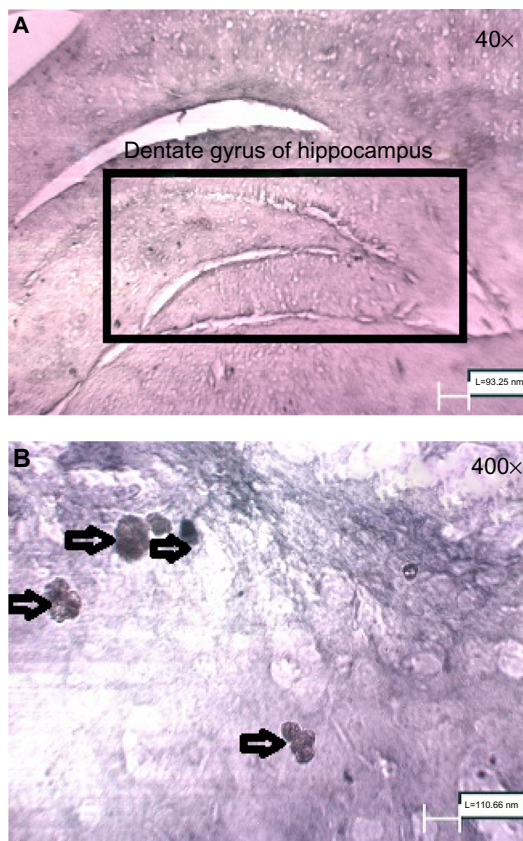


Figure 6 (A) Dentate gyrus of hippocampus of control rats (40 \times) (B) BrdU-positive cells in dentate gyrus of hippocampus of control rats (400 \times).

Notes: The above two pictures show dentate gyrus of hippocampus with different magnification under a light microscope. Note that arrows shows BrdU-positive cells in the same region. (A) shows 40X magnification. The enclosed box indicate the region that BrdU positive cells have been counted. (B) shows 400X magnification. BrdU positive cells as newly proliferated cells have been colored brown.

activity in amygdala, striatum, and cingulate cortex³¹ and further, addiction-induced reduction in neurogenesis may aggravate the outcome.³² Sufficient anxiety levels are required to combat the harmful effects of addiction. With reduction of anxiety level, the defending action of animals reduces because anxiety may recruit body compensatory mechanisms to prevent bad situations. Thus, our findings elaborate that reduced neurogenesis in animals in addicted group can affect memory, anxiety levels, and emotional reactivity.³³ Liu et al demonstrated the role of interleukin-17 in hippocampus in altering anxiety levels.³⁴

Comorbidity of mood and anxiety disorders is well documented in literature. Mood disturbances are attributed to alterations in circadian rhythms, and effectiveness of antidepressants is partially mediated by their ability to regularize abnormal circadian rhythms.³⁵ However, a number of experiments have been performed to study if hippocampal neurogenesis can independently cause anxiety. Some of these studies have observed that reduction of anxiety is associated with reduced neurogenesis.³⁶ In addition, Revest et al divided hippocampus into two parts: rostral for cognition and ventral for anxiety level regulation.³⁶ Taken together, neurogenesis can prevent devastating effects of drug abuse.

Treatments for improving neurogenesis

1. Deep brain stimulation: Electroconvulsive therapy (ECT) has been used in medicine for more than a century. The mechanism, which adjusts its effectiveness in treatment of neurological diseases, has been associated with neurogenesis.^{37,38} It has been shown that dose-dependent electroconvulsive brain stimulation can increase number and age of neurons.^{37,38} Evidence of effectiveness of increasing neurogenesis in treatment of depression comes from disruption of fear memory. ECT increases proliferation of quiescent progenitor cells, followed by that of amplifying progenitor cells.^{39,40} Also, applying chronic ECT causes synaptic rearrangements in dentate gyrus, which is associated with increase in long-term potentiation.⁴¹ Improved long-term potentiation further increases the number of new born neurons in the dentate gyrus,⁴² which in turn alters hippocampal circuits. The mechanism behind ECT-induced neurogenesis is not fully understood. However, studies have suggested the participation of some factors like brain-derived neurotrophic factor (BDNF), fibroblast growth factor-2, and vascular endothelial growth factor.^{43–45}

2. Long-term use of some antidepressants also induces neurogenesis. Among them, selective serotonin reuptake inhibitors are the most commonly used drugs. These drugs increase progenitor cells in dentate gyrus of hippocampus.³ A study using nestin-cyan fluorescent protein has shown that fluoxetine increases number of amplifying neuronal progenitor cells but does not exert any effect on stem cells.⁴⁶ Furthermore, Wang et al⁴⁷ have also reported fluoxetine-induced hippocampal neurogenesis.⁴⁸ Some factors such as BDNF and vascular endothelial growth factor are considered to regulate the microenvironment for adult neurogenesis.^{49–54} N-acetylcysteine has also shown useful properties for treatment of addiction.⁵⁵
3. Exercise therapy: Exercise induces neurogenesis,^{56–59} which improves memory performance and learning abilities in rats^{56,59} and humans.⁶⁰ Human brain continues to undergo neurogenesis; running helps to increase neurogenesis to exert its effect. A study on mice has shown that running increases BrdU-positive cells, learning, and long-term potentiation.⁵⁹ In addition, exercise can also activate quiescent cells in the hippocampus.⁵⁷ These neurons eventually become mature, and hence improve hippocampal function.

Conclusion

These findings suggest that drug abuse has devastating effects on the brain, and it impairs proper functioning of the brain. Thus, neurogenesis may be essential with successful drug abstinence.

Disclosure

The authors declare they have no conflicts of interest in this work.

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