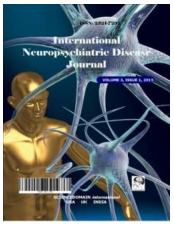

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Social Isolation Increases Risk of Morphine Addiction in Male Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author HF with the help of author MK designed the study and wrote the protocol. Also, author HF preformed the experiments, the statistical analysis, managed the literature search and wrote the first draft of the manuscript with assistance from author MK. In addition, author SF rewrote the final manuscript and edited the language. All authors read and approved the final manuscript.

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ABSTRACT

Background: Drug addiction, especially among adolescents, is one of the major concerns of human society. Identifying factors which predispose an individual to drug -seeking behavior, can be beneficial in reducing risk of addiction in society.
Materials and Methods: Forty two adult male Sprague-Dawley rats were divided into four groups: 1) pair 2) isolated 3) pair for biased-CPP (Conditioned Place Preference) test 4) isolated for biased-CPP test. At the end of experiment, rats were assessed for memory, mood, neurogenesis, BDNF (brain derived neurotrophic factor) and MDA (malondialdehyde) levels. In addition, rats in biased-CPP test groups were tested for drug abuse preference.
Results: Avoidance memory was markedly impaired in isolated rats. Furthermore, isolated rats demonstrated depressive - behavior and had reduced neurogenesis and BDNF levels. Lipid peroxidation (MDA) was significantly enhanced in isolated rats as compared to paired rats. Rats in isolation spent more time in non-preferred compartment than pair rats during biased-CPP test.

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Conclusion: Social isolation increases vulnerability to morphine addiction thus, creating socially interactive society can be beneficial in preventing drug abuse.

Keywords: Addiction; isolation; biased-CPP; neurogenesis; MDA and BDNF.

1. INTRODUCTION

Drug addiction continues to be a serious medical and social problem. Vulnerability to develop addiction to drugs is dependent on genetic, environmental, social and biological factors. Developing positive behaviors that improve brain functioning is vital for reducing risk of relapse to drug abuse. Morphine is an effective analgesic drug which has reward-seeking and addictive effects. Studies have reported dramatic associations between various behavioral personality traits and morphine addiction [1].

Neurogenesis occurs predominantly in two regions of brain, i.e., the subventricular zone of lateral ventricles and the subgranular zone of dentate gyrus of hippocampal formation [2,3]. Insufficient neurogenesis contributes to various disease processes like Parkinson's disease, Huntington's disease, Alzheimer's disease and chronic epilepsy [4]. Because of its connectivity with limbic system, reduced neurogenesis in hippocampus is also implicated in mood disorders like anxiety and depression. To date, all drugs of abuse which have been studied, suppress hippocampal neurogenesis. Furthermore, suppressed neurogenesis following compulsive drug - taking behavior, enhances resistance to extinction of drug-seeking behavior.

Enriched environment (EE) - an environment where animals are exposed to high levels of sensory, motor and cognitive stimulation, induces remarkable effects on neuronal functionality. EE has shown to improve brain processes and prognosis of disorders like depression, Alzheimer's disease, drug addiction and stroke. In addition, socially enriched environment in combination with physical enrichment enhances cognitive performances and mitigates ageing - induced dementia [5]. Conversely, social isolation dramatically affects health. It reduces cognitive capacities and life span while increasing the risk of obesity and type 2 diabetes. Perceived isolation depresses immune responses and may exacerbate infarct size and edema. Interestingly, social isolation reduces neurogenesis and alters neuroplasticity which is associated with deficits in learning and memory [6,7]. Combination paradigm of EE with physical exercise and proper diet improves spatial memory deficits and improves hippocampal neurogenesis.

The mesocorticolimbic dopaminergic system (reward system) which includes projections from ventral tegmental area (VTA) to nucleus accumbens (NAc), is principally associated with the rewarding effects of drug abuse [8]. Being an important regulator of motivational and emotional processes, the reward system has been implicated in a variety of psychiatric disorders like addiction, depression, anxiety and bipolar disorder [3,9]. Presumably because of neural connections between rewarding centre and hippocampus, hippocampal neurogenesis may influence functioning of rewarding centre [10,11]. Moreover, addiction causes aberrant learning and memory such that, the reward related learning becomes magnified and the learning associated with the negative consequences of drug abuse becomes impaired. Drug craving and relapse is associated with the persistence of maladaptive rewarding memories of the drug formed during drug intoxication. In addition, enhanced avoidance learning is beneficial for successful drug withdrawal [1].

On the basis of existing literature, we designed this study to evaluate if isolation increases risk of relapse to drug abuse by affecting neurogenesis, avoidance memory and mood balance. We also aimed to study the involvement of BDNF and MDA in this regards.

2. MATERIALS AND METHODS

2.1 Animal Care

The experimental protocols followed in this study were conformed to the Guidelines for the Care and Use of Laboratory Animals published by National Institutes of Health (NIH Publication No.85-23, revised 1996) and was approved by the institutional ethical committee of Tehran University of Medical Sciences (Tehran, Iran).

2.2 Animals

Forty two adult male Sprague-Dawley rats aged between 8 to 9 weeks (weighing 200-250 g) were used in this study. Rats were divided into 2 groups (each n= 8); isolated and socialized groups. Animals in the isolated group were

housed individually in cages covered with black plastic. In order to avoid disturbance in light and dark cycle, the roof of the cages was kept uncovered. In socialized group, rats were housed in pairs or triads and the walls of the cages were left transparent. Animals were caged for 1-week adaptation period followed by two weeks of experimental period [12,13].

2.3 Experimental Procedure

In each group, two experiments were performed. In first experiment, shuttle box and tail suspension tests were performed. Then, serum and CSF were obtained for MDA and BDNF, respectively. On day 17th, these animals were sacrificed and neurogenesis was studied using BrdU staining. In second experiment, biased - CPP test was performed on 15th day.

2.4 Assessment of Avoidance Memory

Shuttle box was used to assess short-term and long-term memory in isolated and socialized rats. This rectangular box (27×14.5×14 cm) consisted of 2 compartments separated by an automated guillotine door. One compartment was illuminated by an overhead electric bulb, while the other was kept dark. The box had a grid floor consisting of stainless steel bars at 1 cm intervals. Intermittent electric shocks (50 Hz, 1 sec, 1 mA intensity) could be delivered to the dark compartment floor by an insulated stimulator. On first day of training, each rat was placed in shuttle box to explore illuminated and dark compartments for 15 min. On second day of training, animal was gently placed in the light compartment and after 5 sec, the guillotine door was retracted, such that the animal could enter the dark compartment. Animals that waited more than 100 sec to cross were excluded from the study. Once the animal had completely moved into dark compartment (with all 4 paws), the door was closed and current shock was applied for 3 sec. After 20 sec the animal was returned to the home cage. For short term memory testing, each rat was placed in illuminated chamber 24 h after habituation, and 30 sec later, the door was raised. The time spent in the light compartment before entering the dark compartment was recorded [14].

2.5 Tail Suspension Test (TST)

In TST, a rat is suspended by its tail against a fixed metal rod such that the body faces downwards. Normally, the rat tries to escape from this stressful state by trying to climb up the metal rod. However, depressed rats struggle less and remain immobile. Therefore, we recorded the duration of immobility in a 5-minute period which was indicative of low mood or depression-like behavior [15].

2.6 Assessment of BDNF Levels

BDNF levels were assessed in CSF (0.4- 1 µl/rat) using commercially available ELISA kit (Promega, USA).

2.7 MDA Measurement in Serum

Serum sample (100 µl) was mixed with 1 mL 30% trichloroacetic acid (TCA, Sigma-Aldrich Co.) and 1 mL 0.375% thiobarbituric acid (TBA, Sigma-Aldrich Co.). The mixture was heated at 90℃ for 60 min and centrifuged at 12, 000 g for 5 minutes. The final product was measured at 532 nm using UV visible spectrophotometer. It was assessed by applying standard curve and expressed as µmol/L [7].

2.8 Immunohistochemistry

At the end of 14th day, animals were anesthetized with cocktail of ketamine (100 mg/kg) and xylazine (10 mg/kg). The brains were perfused with 100 ml normal saline and then, fixed with 100 ml paraformaldehyde 4% via intra - cardial infusion. After fixation, the brains were removed from skull. For the first 2 days, the brains were kept in PBS + paraformaldehyde 4% and then at day 3, in sucrose 10% + paraformaldehyde 4% + PBS. Throughout day 4, the brains were kept in sucrose 20% + paraformaldehyde 4% + PBS and for the rest of the days they were kept in sucrose 30% + paraformaldehyde 4% + PBS. The cryosections (30 µm) were prepared from hippocampal region and five sections per animal were selected and stained for BrdU positive neurons using a commercially available anti-BrdU antibody kit (5-Bromo-2-dU Labeling and Detection Kit II; Roche). BrdU-positive cells in dentate gyrus (Fig. 7) were counted directly under light microscope (Zeiss Co.) at 400X magnification. BrdU positive neurons appeared colored brown were observed as single cells or in clusters [16].

2.9 Biased-CPP (Conditioned Place Preference) Experiment

Apparatus: The set-up for performing biased-CPP paradigm consisted of an enclosed box

(60×30×30 cm) with open roof that was divided in two compartments (30×30×30 cm) by a removable wall. The floor of two compartments had different texture and color. For this experiment, the left side was covered by white paper and the right side by sandpaper. The experiment was divided into three phases: 1) preconditioning - day 1 2) conditioning - day 2 to 4 3) post conditioning - day 5.

2.9.1 Preconditioning

In this phase, rats were allowed to explore both compartments for 20 minutes. The compartment where rats spent more than 60% of total time was considered as the preferred side.

2.9.2 Conditioning

In this phase, rats received morphine (0.75 mg/i.p., Temad Co.) and saline (i.p.) and were placed in the non-preferred compartment for 30 minutes. The injection repeated after every 4 hours.

2.9.3 Post conditioning

In this phase of experiment, the dividing wall is retracted, and the rat was allowed to freely explore both compartments for 20 minutes. The time spent in each compartment is recorded. More time spent more in non-preferred side was indicative of addiction [17].

2.10 Statistics

Data were analyzed using SPSS version 22 and Graph Pad Prism version 5. Data were represented as mean ± SEM and two-tailed independent sample t-test was used to compare differences. A P-value < 0.05 was considered statistically significant.

3. RESULTS

3.1 Avoidance Memory

Time-lag for moving from light compartment to dark compartment was more in isolated rats as compared to paired rats. This is indicative of better avoidance memory in socialized group (Fig. 1).

3.2 Tail Suspension Test

Time of immobility was higher in isolated rats compared to paired rats. This indicates depressive state of isolated rats (Fig. 2).

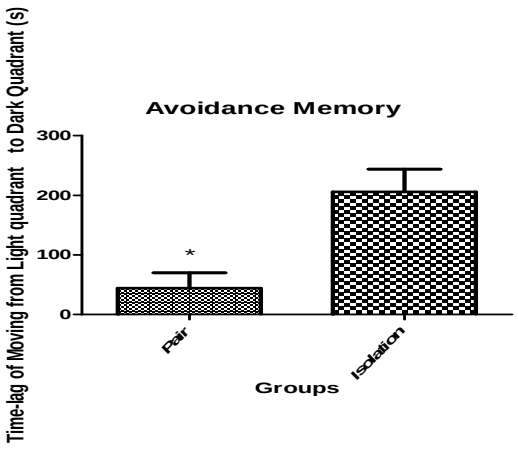

Fig. 1. Avoidance memory was assessed using shuttle box (n=8)

*Symbol * indicates significant difference between both groups at P<0.05. Data are represented with Mean ± SEM*

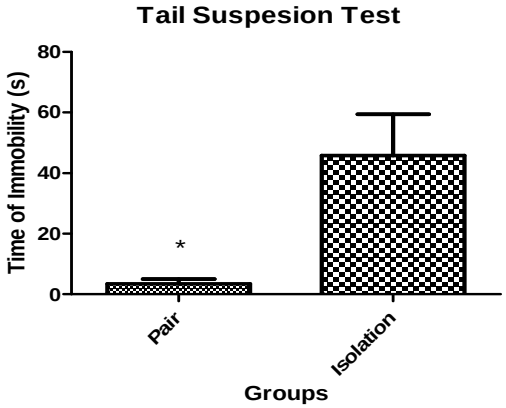

Fig. 2. Time of immobility in tail suspension test (n=8)

P<0.05 was considered significant

*Symbol * indicates significant difference between both groups at P<0.05. Data are represented with Mean ± SEM*

3.3 BDNF Levels

Isolated group had lower levels of BDNF in CSF as compared to pair group. Low levels of BDNF indicate poor brain functioning in isolated rats (Fig. 3).

3.4 Oxidative Stress

MDA levels were higher in isolated group as compared to pair group. Higher MDA level is indicative of more lipid peroxidation in isolated rats (Fig. 4).

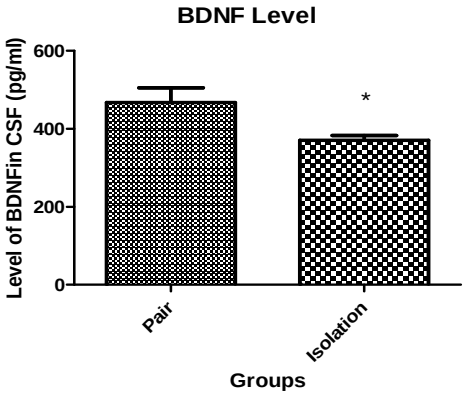

Fig. 3. Assessment of BDNF levels in CSF (n=8)

*Symbol * indicates significant difference between both groups at P<0.05. Data are represented with Mean ± SEM*

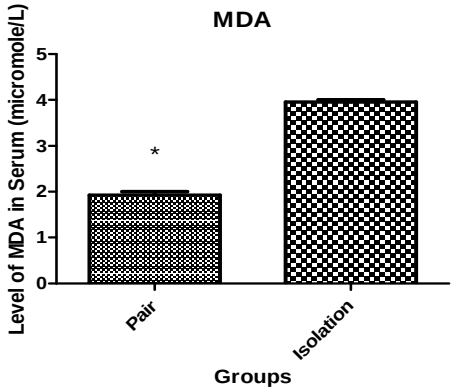

Fig. 4. Assessment of MDA level in serum (n=8)

*Symbol * indicates significant difference between both groups at P<0.05. Data are represented with Mean ± SEM*

3.5 Biased-CPP

Time spent in non-preferred compartment was higher in isolated group as compared to pair group. This indicates higher vulnerability to addiction in isolated rats (Fig. 5).

3.6 Neurogenesis

Number of BrdU positive cells was higher in pair group compared to isolated group. Reduced neurogenesis predisposes rats to develop addiction (Figs. 6 and 7).

4. DISCUSSION

The current study, for the first time shows that isolation increases risk of addiction by impairing avoidance memory and diminishing neurogenesis caused by reduction of BDNF and elevation of MDA levels.

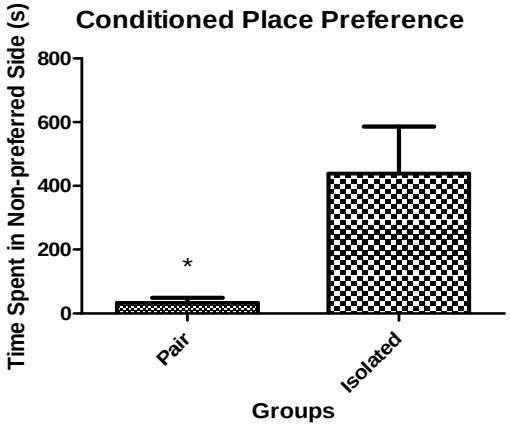

Fig. 5. Time spent in non-preferred compartment in biased-CPP test (n=4)

*Symbol * indicates significant difference between both groups at P<0.05. Data are represented with Mean ± SEM*

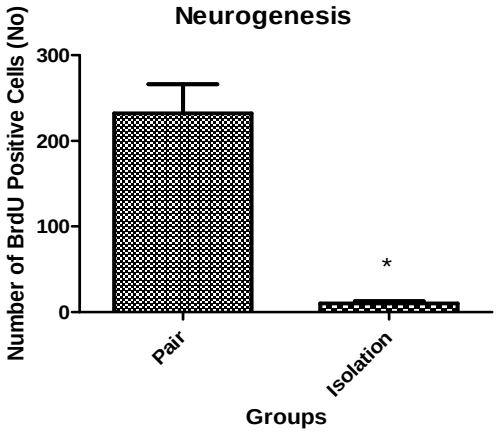

Fig. 6. Number of BrdU positive cells counted in dentate gyrus of hippocampus (n=6)

*Symbol * indicates significant difference between both groups at P<0.05. Data are represented with Mean ± SEM*

It is well - evident that social isolation impairs cognitive functions like learning [6] and memory [18] and disturbs mood balance [19]. We found that social isolation increases vulnerability to addiction by reducing neurogenesis. In addition, insufficient neurogenesis can be linked to isolation - induced reduction in BDNF and MDA levels. Suppression of hippocampal neurogenesis directly affects rewarding center and thus, cause euphoria and drug craving in isolated rats.

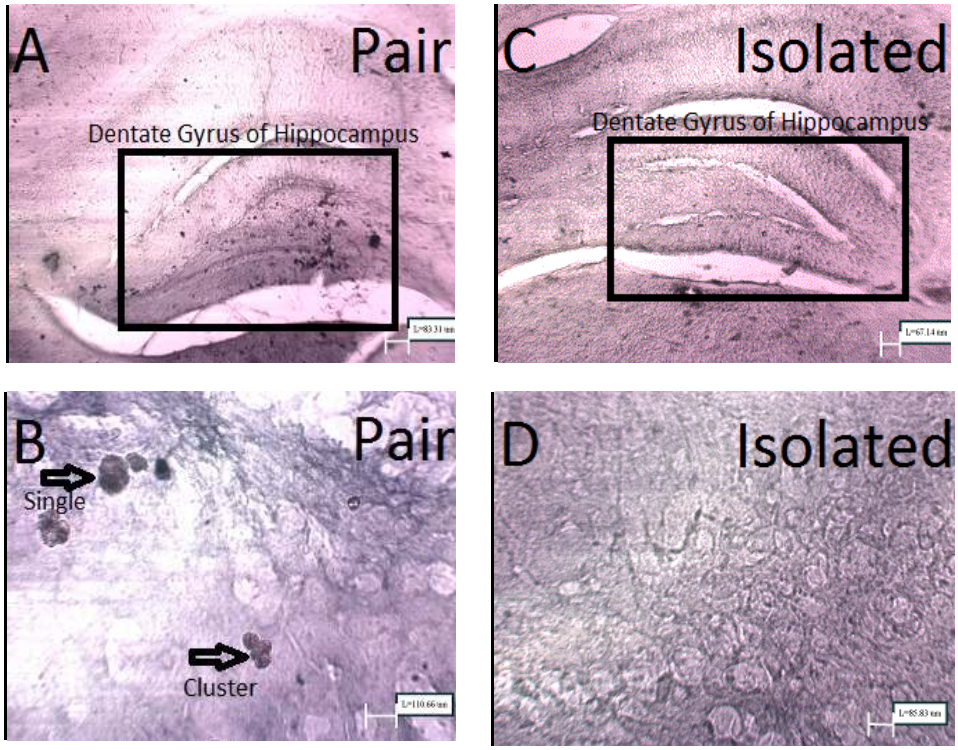

Fig. 7. A and C shows dentate gyrus of hippocampus at 40X B. Shows BrdU positive cells occurring singly and in cluster at 400X D. Indicates no BrdU positive cells in isolated rat at 400X

Social isolation adversely affects health. It escalates chronic illness [20], suppresses immune system [21] and inflammatory responses [22], and impairs myelin formation in prefrontal cortex [23]. Social isolation can be the outcome of depression, shame, or low self-worth. It can be presented by abandonment fears or social anxiety and avoiding social interactions. Chronic social isolation increases emotional reactivity to stress and produces hyperactivity of HPA axis in adult rats, particularly in males [24]. In addition, emotional isolation can occur as a result of social isolation, or when a person lacks any close confidant or intimate partner. Even though relationships are necessary for our well-being, they can trigger negative feelings and thoughts, and emotional isolation can act as defense mechanisms to protect a person from emotional distress. When people are emotionally isolated, they keep their feelings completely to themselves, are unable to receive emotional support from others, feel “shut down” or numb, and are reluctant or unwilling to communicate with others. Emotional isolation in humans can occur within an intimate relationship, particularly as a result of infidelity, abuse, or other trust issues [25]. To date, emotional isolation model has not been developed in rats, hence social and emotional isolation are often considered together.

Drug abuse is a chronic relapsing disorder characterized by several sequential stages. These include; binge/intoxication, withdrawal/ negative affect and preoccupation/anticipation (craving) [26]. It begins as an impulsive action which later turns into a compulsive behavior. . In addition, drug-taking involves both, positive and negative reinforcements. In impulse control disorder, there is a failure to resist temptation, and a sense of arousal precedes drug consumption. Therefore, this type of psychiatric disorder is associated with positive reinforcement mechanisms. In compulsive disorder, stress and tension precedes drug consumption and at the time of abuse, a sense of relief occurs. Thus, compulsive disorders take in account negative reinforcement. A wide variety of factors can predispose and individual to develop addiction. This study focuses on the role of social isolation in increasing the vulnerability to addiction. We found in biased-CPP test that socially isolated

rats spent more time in non - preferred compartment. This method is a widely used preclinical test to assess rewarding and aversive effects of drug. Furthermore, it can be used to study the aversive effect of drug withdrawal. Increase time spent in non-preferred compartment indicates that the drug - seeking behavior of animal. In a recent study, it has been demonstrated that CPP test impairment is associated with modulation of opioid system [27]. Therefore, it can be suggested that social isolation increases morphine sensitivity to opioid receptors in brain.

Addiction is considered as a disease of aberrant learning and memory [28]. Drug abuse hijacks neuronal circuits related to pursuit of reward. It enhances learning related with reinforcing effects of drugs and thus, relapse often follows detoxification. Studies have proposed the role of dopamine release and action in VTA - NAc circuit in producing reward related learning [29,30].

Neurogenesis is a phenomenon involving several stages. The new born neurons are integrated into pre-existing circuits. Neuronal precursors divide into neuronal stem cells niches and these niches are necessary for production of new neurons. Several markers for neuronal differentiation and maturation have been identified; such as nestin, sox2, Ki67, NeuN and calbindin. Lack of responses to trophic factors such as BDNF can arrest the proliferation and differentiation of new neurons [31]. In addition, MDA produced in response to oxidative stress, exerts aversive effects on neuronal growth and survival [32]. Besides proliferation, the inhibition of differentiation of new neuron to mature neurons can increase addictive behavior. This is important because reduce in dendritic spine density as occur during maturation of neurons can increase addictive behavior in nucleus accumbens [33]. In a recent study it has been postulated that neurons in any stages can be contributed to a special rewarding-experience [34]. In this study, inhibition of proliferation of new neurons decreases efficacy of hippocampus as part of rewarding circuit. In this study rewarding center dysfunction has been reflected in impaired CPP test. Also impaired decision-making (impaired avoidance memory and mood balance) can increase drug-taking behavior. Indeed impaired neurogenesis can impair frontal cortex function [35]. Alternation of subventricular zone neurogenesis has not been associated with addictive behaviors [36].

In this study, we propose the role of insufficient neurogenesis in increasing the risk of addiction or rewarding effects of drug. It is well-evident that hippocampal neurogenesis participates in formation of memory and learning processes. Similarly, we found impaired avoidance memory as a consequence of insufficient neurogenesis. It shall be noted that complete course of differentiation of neuronal precursors into mature neurons can take up to three weeks [30] thus, we our findings can be attributed to the effects of neuronal precursors in hippocampus. Furthermore, functional impairment of hippocampus is implicated in development of allostatic state manifested as compulsive drug - seeking and drug - taking behaviors. Because of neuronal connections between hippocampus and rewarding center, it has been suggested that diminished hippocampal neurogenesis can result in augmented rewarding effects of drug [8]. Caine et al demonstrated self - administration of cocaine in response to hippocampal damage [37]. Interestingly, increase blood flow to hippocampus is also associated with cue-induced craving for drugs [26].

The last mechanism acts through impulse control mechanism. In several studies impulse control along with other rewarding responses brain behaviors increases relapse to drug abuse. This is not supervising because reduction of hippocampal neurogenesis increases depression-like behaviors. It is well-known that loss of dopamine in rewarding center decreases rewarding pleasure of normal brain. So by decreasing normal pleasure loss of tolerance and decrease in coping styles develop and impulse control become impaired. So desire for replacing pleasure increases that desire to drug abuse increases. So housing condition can be a condition that can modulates brain areas that regulate pleasure.

We used tail suspension test to assess mood and degree of anhedonia in animals. Development of anhedonia has been ascribed to dysfunction of reward pathway, in which the nucleus accumbens plays a pivotal role [38]. Therefore, anhedonia frequently occurs in substance abuse disorders especially in withdrawal state. Furthermore, Ahmed and Khoob reported a transition from moderate to excessive drug intake due to change in hedonic set point. In this study, depressive - behaviors were increased in isolated animals, suggesting high vulnerability of these animals to drug abuse [39].

Brain-derived neurotrophic factor (BDNF) is one of the major neurotrophic factors which primarily supports growth and survival of cholinergic, dopaminergic, and motor neurons [40]. BDNF is synthesized by sensory neurons and glia cells, and has autocrine and paracrine functions in mediating activity-dependent plasticity. It is highly expressed in brain areas that are known to regulate cognition, emotions and rewards [41]. Hippocampal pyramidal neurons in CA3 region are profoundly responsive to changes in BDNF levels. In addition, BDNF plays a critical role in regulating reward associated functions of mesocorticolimbic dopaminergic system [42].

Oxidative stress status is one of the major determinants of health. Malondialdehyde (MDA) is a naturally occurring organic compound, often used as a marker of oxidative stress [43]. MDA is indicative of lipid peroxidation which in turn may alter neurogenesis by affecting neuronal membrane lipids [44]. Moreover, MDA levels have shown to positively correlate with memory impairment [45]. We found high MDA levels in isolated rats and these high levels can be attributed to lipid peroxidation of young neurons as mature ones have better tolerance. Increase in MDA production impairs neuronal signaling involved in proliferation and maturation and proliferation processes. In one study, oxidative stress and BDNF levels have been proposed as markers for cocaine use in early withdrawal period [46].

5. CONCLUSION

Taken together, the current study shows that social isolation reduces neurogenesis possibly by altering BDNF and MDA levels. In addition, socialization reduces risk of addiction by improving avoidance memory and mood balance.

CONSENT

It is not applicable.

COMPETING INTERESTS

The authors declare that they have no potential conflict of interests.

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