

Effects of single-dose ketamine infusion on behavioral parameters and neuronal activation in the medial prefrontal cortex of juvenile rats exposed to prenatal stress

Elif Polat Çorumlu¹, Osman Özcan Aydın¹, Emine Gülhan Aydın², Emel Ulupınar^{1,3}

¹Department of Interdisciplinary Neuroscience, Institute of Health Sciences, Eskişehir Osmangazi University, Eskişehir, Turkey

²Department of Pharmacology, Faculty of Medicine, Eskişehir Osmangazi University, Eskişehir, Turkey

³Department of Anatomy, Faculty of Medicine, Eskişehir Osmangazi University, Eskişehir, Turkey

Abstract

Objectives: Subanesthetic dose of ketamine administration produces antidepressant-like response especially in treatment-resistant depression patients. Ketamine's rapid and sustained actions have also been demonstrated in normal or chronically stressed animals, but no experimental studies have examined its effects in prenatally stressed rodents. Therefore, aim of the present study was to investigate the behavioral and structural consequences of single-dose ketamine application in juvenile rats exposed to prenatal stress.

Methods: Prenatal stress protocol was applied by immobilization of pregnant rats during the last week of their gestation for 3 hours a day. While treatment group received a single-dose (10 mg/kg) of intraperitoneal ketamine injection, control and stress groups received same amount of saline injections at P38. After completion of behavioral tests (sucrose preference, modified grip and forced swim), animals were sacrificed via intracardiac perfusion. Then, immediate gene expression in the medial prefrontal cortex was evaluated by c-Fos immunohistochemistry.

Results: Although the active coping and depressive-like behavior of juvenile animals exposed to prenatal stress did not significantly change, ketamine application caused alterations in the sucrose preference pattern of animals and immobility time in the forced swim test. Two-way ANOVA test results showed significant differences among groups and a group X gender interaction in the density of c-Fos expressing neurons present in the medial prefrontal cortex.

Conclusion: A single-dose ketamine treatment might differentially affect the depressive-like behaviors of juvenile animals exposed to prenatal stress and activate neurons in the medial prefrontal cortical region in a gender-dependent manner.

Keywords: c-Fos; depression; ketamine; prefrontal cortex; prenatal stress

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Introduction

Major depressive disorder (MDD) is the leading cause of disability worldwide.^[1] This disease is characterized by loss of interest in daily activities, sadness, and feelings of tiredness, guilt or low self-worth. Since these symptoms disturb sleep, appetite and concentration skills of individuals, depression can be defined as a debilitating disorder

by its growing direct and indirect financial burden on health care spending. The estimated lifetime prevalence varies across the cultures from 3% to 16.9%.^[2]

Currently, there are more than twenty different antidepressant medications targeting the monoamine systems. These drugs generally work by increasing the amount of serotonin or norepinephrine in the brain. While effective in most patients, sustained remission is

achieved only in one-third of patients after treatment which puts them at greater risk of alcohol and drug abuse, hospitalization, and even suicide attempts.^[3] The full clinical benefits of conventional antidepressants take weeks to months start working.^[4] This time lag to therapeutic efficacy is a serious problem especially in considering the high suicide risk associated with MDD. For patients with severe depression or those having thoughts of self-harm, immediate relief is needed and may even be lifesaving. Until now, electro-convulsive therapy (ECT) has been the only proven method of treating severe and life threatening depression with minimal delay. However, this is an invasive method, in which electrical shock induces seizures in patients, and failed to gain a general acceptance.^[5] Therefore, there is urgent need of fast-acting, well-tolerated and more effective treatment method especially for patients at the high risk of suicide.

For over a decade, scientists have been investigating the role of other neurotransmitters and mechanisms in understanding the pathophysiology of treatment resistant MDD. Now, a growing body of research indicates there may be a new hope: a well-known general anesthetic drug ketamine. Ketamine is a non-competitive glutamate N-methyl-D-aspartate (NMDA) receptor antagonist and subanesthetic dose of this drug is associated with antidepressant effect in patients with MDD. Stimulation of glutamate transmission by ketamine results in BDNF release and activation of signaling pathways critically involved in the formation, maturation and function of new synapses.^[6] Unlike other antidepressants, ketamine has a very rapid and dramatic onset of antidepressant response.^[7,8] This feature can be extremely helpful in emergency rooms for curbing suicidal thoughts and making it a potential lifesaver.^[9]

Effects of ketamine were tested in both patients with MDD and animal models of depression.^[10,11] In these basic and clinical studies, morphological and functional alterations have been shown in the brain regions that are critical for the regulation of mood and cognition, including the prefrontal cortex (PFC).^[12] Post mortem studies of patients with MDD revealed reduced number of synapses in the PFC.^[13] Stress exposure during prenatal or adult period, a well-known depression model in rodents, also causes deficits in medial PFC neurons.^[14,15] We have recently shown that in perinatally stressed animals, dendritic complexity and spine density of pyramidal neurons in the medial PFC display anxiety level- and sex-dependent reductions.^[16] Moreover, accumulating evidence suggests that a dysfunction in the glutamatergic system is evident in the prefrontal cortical neurons of MDD patients.^[17]

Imaging studies showed that subanesthetic doses of ketamine increased cerebral blood flow and glucose metabolic rate especially in the thalamus, frontal and parietal cortical regions.^[18] It has been shown that reduced activity in the PFC region of depressed patients is normalized following ketamine treatment.^[19] In a series of preclinical studies, ketamine has been shown to induce synaptic signaling proteins and neuroplastic changes in the PFC of rats.^[20-22]

Prolonged development of the PFC makes it susceptible to adverse conditions. Although recent well-designed studies are beginning to yield important insights into the cellular and molecular mechanisms of ketamine's antidepressant action, the role of ketamine in juvenile animal models of depression has not been investigated yet. Therefore, in this study, by using behavioral and morphological analyses, the effect of single-dose ketamine application was investigated in juvenile rats exposed to prenatal stress.

Materials and Methods

Stress protocol and animal treatment

Sprague-Dawley rats were obtained from the breeding colony at the Eskişehir Osmangazi University Animal Care Facility. The laboratory was maintained under constant temperature (21°C) and lighting (12:12 h light/dark cycle) conditions. After overnight mating with breeder male rats, pregnancy was confirmed by the sperm positivity in vaginal smear and this particular day was assigned as embryonic day (E) 0. Pregnant rats were housed individually in transparent cages by giving free access to food and tap water. Prenatal stress protocol was applied by keeping the dams (n=10) immobile in close-fitting wire mesh cylinders for 3 h, between E14 and E21. In the treatment group, prenatally stressed animals received a single-dose of ketamine injection (10 mg/kg) intraperitoneally, at P38. Control and prenatal stress groups (n=4 for each gender in each group) received same amount of saline solution. Experimental procedures were performed in accordance with protocols approved by the Institutional Animal Usage Committee of Eskişehir Osmangazi University Faculty of Medicine (Protocol #201/1).

Sucrose preference test

Animals were initially subjected to a palatable sucrose solution (1%; Sigma) for 48 hours and then exposed to a water deprivation period for 4 hours. At the end of this period, they were exposed to two identical bottles, containing either water or sucrose solution for one hour. Sucrose and water consumption were determined by measuring the change in the volume of fluid consumed.

The sucrose preference percentage (SPP) was calculated as follows: $SPP = [\text{sucrose solution intake (ml)} / (\text{sucrose solution intake (ml)} + \text{sterile water intake (ml)})] \times 100$. The cut off point for anhedonia, as an indicator of depressive symptom, is considered as under 0.65.^[23]

Modified grip suspension test

The endurance capacity and escape behavior of animals were determined by using an apparatus consisting of a string (50 cm length and 3 mm in diameter) pulled straight between two vertical supports, 80 cm above the blanket-covered tabletop.^[24] The front paws of the rats were placed on the horizontal string, at a point midway between the supports, and the latency before the animals fell from the string was recorded. In addition, escape behavior of an animal was rated by two blinded experimenters according to the following scale: 0 = Fall off; 1 = Hangs on string only by forepaws; 2 = As for 1, but attempts to climb on string; 3 = Hangs onto string by two forepaws plus one or both hind paws; 4 = Hangs onto string by all fore and hind paws plus tail wrapped around string; 5 = Staying on the string for more than 150 s or escape after 50 s; 6 = Escape in between 41 and 50 s; 7 = Escape in between 31 and 40 s; 8 = Escape in between 21 and 30 s; 9 = Escape in between 11 and 20 s; 10 = Escape in the first 10 s of the test. The highest score for each rat was obtained from two trials with an interval of approximately 15–20 minutes.

Forced swim test

Forced swim test (FST) is a two-day procedure performed as described previously.^[25] On the first day, rats were placed in large inescapable Plexiglas cylinders (30 × 45 cm) filled with 25°C water to a depth of 30 cm, and let to swim for 15 minutes. On the second day, animals were forced to swim again under the same conditions, for 5 minutes. The cylinders were emptied and cleaned at the end of each swimming test. Behavior of animals during swimming period was videotaped and the immobility time was recorded. The duration of immobility was defined as the period when the animals made no escape-oriented movement, as a sign of depressive behavior.

Tissue preparation and immunohistochemical analysis

In order to estimate the level of immediate gene expression, c-Fos immunohistochemistry was used. Since forced swimming was also used to stimulate neuronal activity, animals were sacrificed immediately after the FST by cardiac perfusion under the halothane anesthesia. All animals were perfused with phosphate-buffered saline (PBS) followed by

a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH=7.4). The brains were removed from the skulls and dissected into two hemispheres. Left hemispheres were left in the fixative solution for 24 h at 4°C, followed by dehydration in graded alcohols, then embedded in paraffin. All tissue blocks were fixed and processed under the same conditions. The tissue was sectioned at 5- μ m thickness and every 20th section was mounted on poly-L-lysine coated slides. After deparaffinization and rehydration, sections were treated with 0.3% H₂O₂ for 30 min, washed in PBS, and then boiled in antigen retrieval solution, containing sodium citrate (pH=6.00) for 5 min in a microwave oven. Unspecific binding was suppressed in a blocking solution for 30 min at room temperature. The rabbit polyclonal antibody to c-Fos (Merck-Millipore, Darmstadt, Germany) was used at 1:200 dilutions. After overnight incubation at 4°C, sections were rinsed with PBS and incubated in biotinylated anti-rabbit secondary antibody (1:100, Novostain Universal Detection Kit; Novocastra Laboratories, Leica Biosystems Newcastle Ltd., Newcastle, UK) for 30 min at room temperature. Bound antibody was detected by the ready-to-use streptavidin/peroxidase complex reagent (ABC-Elite Kit; Vector Laboratories, Burlingame, CA, USA), and diaminobenzidine (DAB) mixture as chromogen. Finally sections were dehydrated in alcohol series, cleared in xylene, and cover-slipped with permanent mounting medium.

Estimation of neuronal density

Quantitative analyses were done in sections obtained through the rostro-caudal extent, between Bregma 2.76 and 0.48, according to Paxinos and Watson's stereotaxic rat atlas.^[26] Systematic random sampling method was applied in every step and each slide was identified with a code not revealed until the end of quantifications. Photographs of the medial prefrontal cortical regions were used to analyze the mean number of c-Fos (+) nuclei within a two-dimensional counting frame (600 μ m × 600 μ m in size) by using a 40 \times objective. The size of the counting frame was set up to generate about 8–10 sampling sites on average, per tissue section. The counting frame locations were kept constant for each section, and the nuclear profiles were counted in every 20th section. Approximately 10 sections were used for quantitative analyses per animal.

Statistical analysis

The data presented in the text and tables represents mean \pm standard error of means (SEM). Normality distribution of data was assessed by the Shapiro-Wilk tests. Groups were compared using two-way ANOVA (group

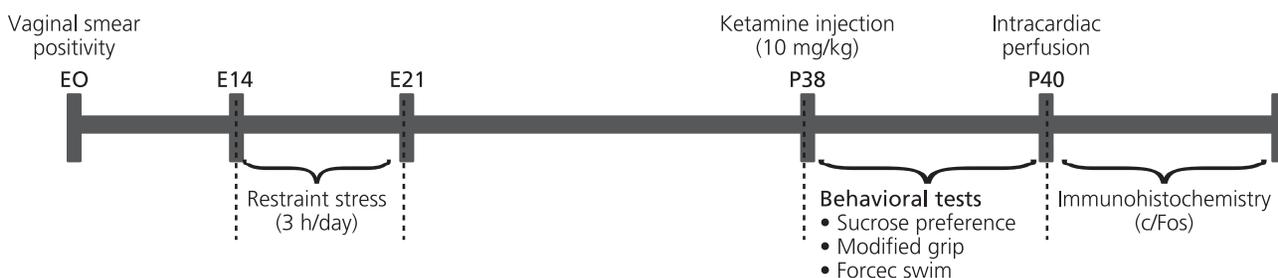


Figure 1. Time table of the experimental design.

× gender) followed by post-hoc pairwise Bonferroni multiple comparisons or Kruskal-Wallis test followed by Dunn's multiple comparisons test. Results were considered significant at a level of $p < 0.05$. All analyses were performed using SPSS Statistics Version 21 software.

Results

Prenatal stress exposure model has been successfully established and utilized as a depression model in our laboratory.^[24] In the present study, we kept pregnant dams immobile for 3 hours daily during the last week of their gestation period, and then evaluated the behavioral consequences of prenatal stress on pups following ketamine injection (Figure 1). In animals exposed to prenatal stress, behavioral parameters related to human depression symptoms, such as loss of active coping, social withdrawal and inability to feel pleasure (anhedonia) can be evaluated under appropriate experimental conditions.^[27] For instance, anhedonia can be assessed by comparing the preference for sucrose over water in home cages of animals. In our study, mean sucrose consumption of prenatally stressed rats was comparable to those of unstressed controls. However, when we considered the individual differences, sucrose consumption rate was below the threshold level (0.65%), indicating an anhedonic response, especially in males exposed to prenatal stress (Figure 2). Following ketamine injection, majority of both female and male animals preferred sucrose over water.

The performance of animals in the modified grip test remained consistent across different groups (Figure 3). Although grip test is more valuable for evaluation of the motor coordination ability, here, we have used this test to compare the active coping and endurance capacity of animals. In comparison to prenatally stressed males, average grip test score was increased after ketamine injection (8 versus 9), but their statistical comparisons did not reach to significance level.

Loss of coping is also seen as a sign of learned helplessness and it can be assessed by inescapable stress in the forced swim test.^[28] In this test, too, females displayed sim-

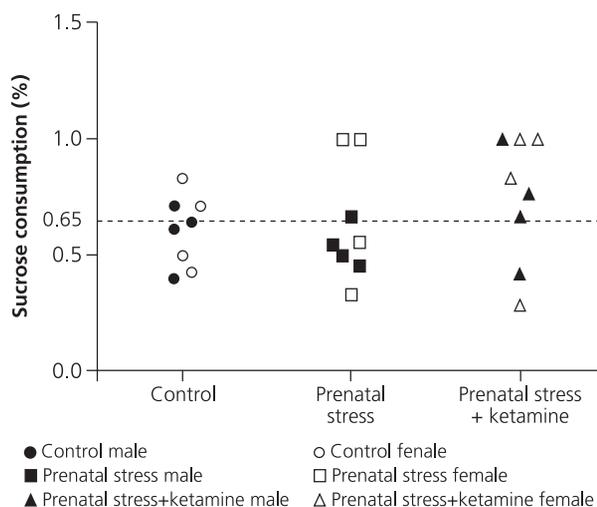


Figure 2. The sucrose preference test results of control, saline or ketamine administered prenatally stressed rats (n=8 animals for each group). Data shows the sucrose consumption ratio of each animal with white symbols indicating female and black symbols indicating male groups. Dashed line shows the threshold level (0.65%) of the anhedonic response.

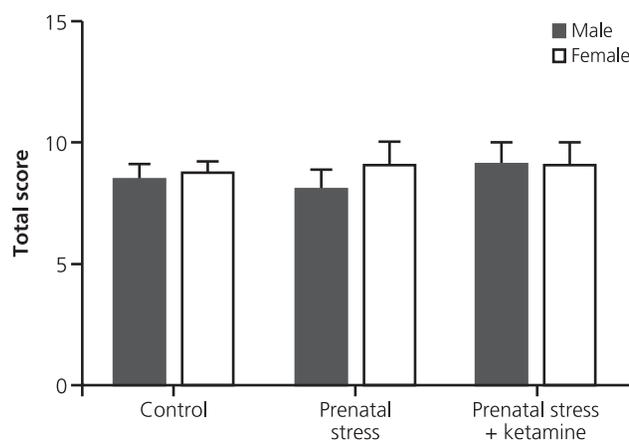


Figure 3. The modified grip suspension test results of control, saline or ketamine administered prenatally stressed rats (n=8 animals for each group) displayed no significant differences among groups. Data shows the average scores ± SEM of each group for male (black bars) and female (white bars) animals.

ilar immobilization time across groups no significant difference between control and stress groups (38.13 ± 17.1 in controls, 45.01 ± 20.9 in prenatal stress, and 46.25 ± 21.0 in prenatally stressed and ketamine treated group). However, in males, prenatal stress exposure increased the immobility duration from 27.88 ± 20.8 to 60.01 ± 24.2 seconds, in comparison to control animals (Figure 4). Immobility time of male animals displayed a reduction (21.00 ± 24.2 seconds) in the forced swim test following ketamine treatment.

After completion of behavioral tests, we examined stress-induced immediate gene expression by using c-Fos immunohistochemistry. The representative sections from the region of interest were shown in Figure 5 and the outline of the medial prefrontal cortical region is indicated in the middle section. Within this region, approximately 8–10 sampling sites were defined by systematic random sampling method, and c-Fos expressing nuclear profiles was counted to estimate the neuronal activity levels (Figure 5a). Immediate gene expression level was different in control and experimental groups as estimated by quantitative analyses (Figure 5b). Two-way ANOVA results displayed a significant group [$F(2,18)=8.47$, $p=0.0026$] and gender difference [$F(1,18)=25.71$, $p<0.0001$]. There was also a significant group-gender interaction [$F(2,18)=4.69$, $p=0.0229$]. These differences were due to the enhanced c-Fos expression in prenatally stressed females compared to control groups, and ketamine treatment decreased neuronal activation similar to control levels. In ketamine treated group, there is also a significant gender difference. On the other hand, there was no significant difference among male groups.

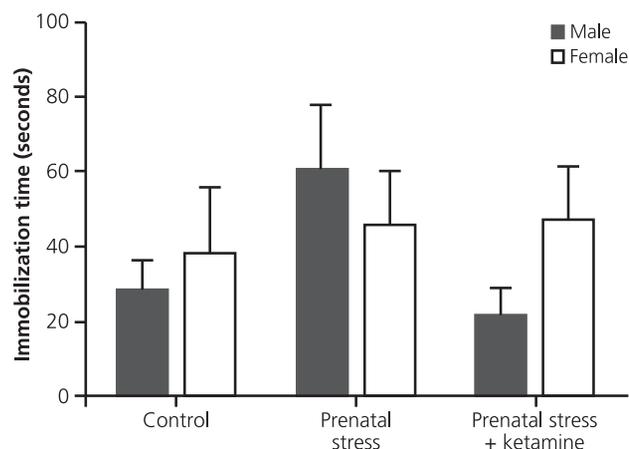


Figure 4. The forced swim test results of control, saline or ketamine administered prenatally stressed rats ($n=8$ animals for each group) displayed no significant differences among groups. Data shows the mean immobilization duration \pm SEM of each group for male (black bars) and female (white bars) animals.

Discussion

In this study, we have investigated the effect of single-dose ketamine application in prenatally stressed juvenile rats. Although stress protocol used in this study did not significantly alter the active coping and depressive-like behavior of pups, ketamine application caused alterations in some behavioral parameters and immediate gene expression in the prefrontal cortical region were observed in prenatally stressed animals. In previous studies, the antidepressant-like effect of single or repeated dose of ketamine treat-

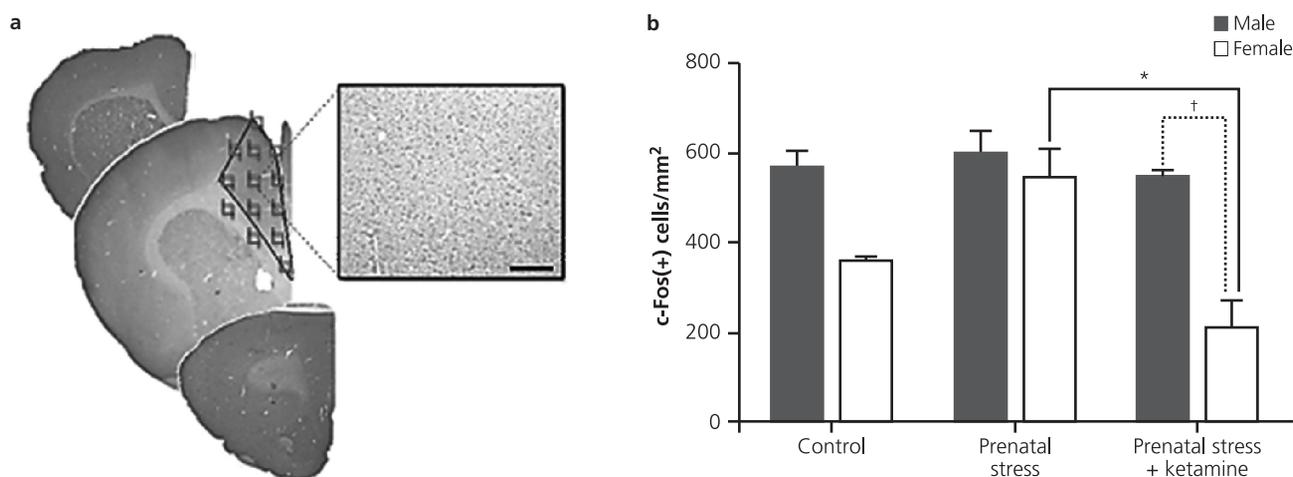


Figure 5. The estimation of neuronal density of c-Fos (+) neurons in the medial PFC outlined with black lines in (a) was done by using systematic random quantification method. Quantitative analyses showed significant ($*p<0.001$) differences between prenatally stressed and ketamine treated groups in females (b). There was also a significant gender difference ($†p<0.01$) in ketamine administered group. Data shows the mean \pm SEM of the number of c-Fos expressing neurons per unit area. Scale bar=250 μ m.

ment has been investigated in normal or chronically stressed animals, but none of the experimental studies examined its effects in prenatally stressed rodents. A general impairment of the hypothalamo-pituitary-adrenal (HPA) axis is characteristic for prenatally stressed animals and the prolonged corticosterone secretion in response to stress was positively correlated with alterations in behavioral tests, such as sucrose preference or FST.^[29,30] Therefore, prenatal stress exposure model has a face validity with some clinical features observed especially in juvenile depression patients.

In rodents, exposure to stressors in an unpredictable sequence over a prolonged time period satisfies most of the criteria of validity as an animal model of depression.^[31] By using this model, it has been shown that acute or chronic treatment with ketamine reverses the increase in adrenal gland weight, normalizes circulating corticosterone and ACTH levels and promotes regain of body weight.^[32] Although similar physiological alterations have also been shown by other investigators, in these studies, acute ketamine treatment failed to reverse anhedonic response in rats exposed to chronic mild stress.^[33] These results suggest that stress protocols might differentially influence the behavioral outcomes of ketamine treatment.

In experimental studies, anhedonia is widely measured by sucrose preference test and reduced sucrose consumption corresponds to a depressive-like symptom.^[34] Chronic unpredictable stress exposure for 3 weeks significantly decreases the preference for sweetened solution and increases the latency in novelty suppressed feeding test, and these effects are reversed by single-dose ketamine treatment.^[22] In our study, the number of prenatally stressed animals passing the threshold level of the sucrose consumption ratio showed an increase following ketamine treatment. Similarly, Garcia et al.^[32] showed that in rats exposed to chronic stress, repeated administration of ketamine (7 days) reversed the decrease in sucrose consumption. In another study, administration of a low dose of ketamine (0.5 mg/kg) for 10 days significantly increased sucrose consumption in Wistar-Kyoto rats.^[35] Furthermore, Li et al.^[22] reported that even a single-dose of ketamine produces a long lasting (up to 7 days) increase in sucrose preference relative to chronic unpredictable stress exposed animals. Protracted effects of acute ketamine treatment were also evident in mice exposed to chronic mild stress that is tested at 4, 6, and 8 days after a single ketamine treatment.^[36] These data suggest that sucrose preference of animals might be used as a sensitive screening test for rapid-acting antidepressant drugs like ketamine.

In addition to rewarding behaviors, the effects of ketamine have also been shown on aversive circumstances,

such as elevated plus-maze or FST.^[21,32,37-39] While elevated plus-maze is frequently used to measure anxiety behavior in rodents, FST is the most frequently used behavioral despair test for measuring depressive-like behavior, based on an immobility response induced by inescapable exposure to stress.^[40,41] FST has strong predictive validity because short-term administration of antidepressant compounds from a variety of pharmacological classes reduces immobility time in FST.^[42] The most commonly prescribed antidepressants such as tricyclic anti-depressants and selective serotonin reuptake inhibitors (SSRIs) have significant limitations with delayed onset of action and their effects do not last beyond a few hours following their acute administration.^[43] Whereas, effects of ketamine are distinct from those conventional drugs since it produces protracted behavioral effects persisting from one to several days after administration.^[29] The majority of studies indicate that the FST remains sensitive to the protracted effects of ketamine up to 1 week after a single injection.^[44-50] Different groups were reported diverse durations persisting for 8 days,^[36] 10 days,^[51] 12 days,^[52] and 2 weeks.^[53] Interestingly, repeated ketamine application (20 mg/kg) for 15 days has been shown to have anxiolytic- and antidepressant-like responses even 2 months after the cessation of treatment in adolescent rats.^[54] These results are in agreement with other studies utilizing a 10- or 12-day dosing regimen to establish longer-lasting effects of chronic ketamine on depressive-like activity in the FST.^[55,56] In the literature, there are significant dose, gender and strain-dependent differences in the sensitivity to ketamine treatment. For example, Wistar rats are insensitive to the antidepressant-like effects of low dose ketamine (2.5 and 5 mg/kg) in comparison to Wistar-Kyoto rats.^[55] Interestingly, in majority of studies, behavioral consequences of ketamine treatment have been investigated in adult male rats.^[35,46-49] In our study, the effects of ketamine were investigated in both females and males, but more prominent behavioral alterations were observed in males. In the literature, there is a clear consensus that efficacy of antidepressant drugs show gender-dependent variations. In one study comparing the behavioral and molecular effects of ketamine in both male and female rats, it has been demonstrated that low dose of ketamine does not have antidepressant-like effects in males.^[45] In contrast, females have a greater sensitivity to lower doses of ketamine, and these effects might be completely abolished when female rats were ovariectomized and then restored when physiological levels of estrogen and progesterone were supplemented. These results suggest that gonadal hormones play a critical role in determining the effectiveness of ketamine treatment in rats. Likewise, in mice subjected to chronic mild stress, a single-dose of ketamine

injection (10 mg/kg) can induce sex-dependent behavioral effects for five weeks. Although female mice were shown more reactive to the earlier effects of ketamine, the antidepressant potential of this drug was proved to last longer in male animals.^[56] A recent clinical study suggested that sex differences in glutamate receptor gene expression might also play a major role in depression and antidepressant efficacy of drugs.^[3] In a large cohort of postmortem subjects from MDD patients and controls, more generalized and severe disruption in the regulation of the glutamate receptors were found in females, but more specific alterations were reported in males.

Prenatal stress exposure not only leads to long-term behavioral consequences, but also causes neurobiological and morphological alterations in the offspring.^[23,57] Functional imaging studies revealed that stress experience dramatically decreased brain activity especially in the regions associated with the limbic system.^[58] Within this system, PFC has a particular importance, since it receives input from all other cortical areas and modulates motor, cognitive, affective and social behavior across time. Moreover, prolonged developmental pace makes this neocortical region more vulnerable to both developmental and environmental insults, which is often manifested in neuropsychiatric disorders.^[59] In a current study, we evaluated the morphological changes in the medial PFC of rats subjected to perinatal stress exposure. The dendritic complexity and spine density of pyramidal neurons located in this region displayed an anxiety level- and sex-dependent reduction, especially in the stressed males.^[16] Moreover, total number estimation of c-Fos (+) nuclei, indicating the neuronal activation upon stressful challenge, significantly increased in high anxious animals compared with low anxious and control groups, in both gender. These results suggest that medial PFC is a critical site of neural plasticity within the stressor controllability paradigm. Since morphological outcomes of early life stress show correlation with the behavior of animals, in the present study, to investigate the efficacy of ketamine treatment, we compared the immediate gene expression of neurons in the medial PFC between control and experimental groups. Our results showed that density of neuronal activation, estimated by using c-Fos immunohistochemistry, have significant gender- and group- dependent differences. However, in contrast to behavioral outcomes, alterations in the immediate gene expression were more prominent in females than those of males. We thought that these differences were high likely due to the diverse stimulation of reward circuitry in female and male animals. In a recent study, Chang et al.^[60] showed that activation of neurons in the ventrome-

dial PFC and projections from this region to the nucleus accumbens play a key role in regulation of reward-related processes and actions of diverse antidepressant drugs. Therefore, it is possible that in prenatally stressed female animals, enhanced activation of neurons in the medial PFC might cause an increase in the sucrose consumption of animals by stimulating the neurons located in the nucleus accumbens. In contrast, sustained activation of this pathway might contribute to the anhedonic response observed in prenatally stressed males.

Neuroimaging studies in humans supports these ideas by demonstrating that ketamine increases the activity of PFC.^[60,61] Likewise, rodent studies showed that ketamine treatment causes rapid increases of glutamate transmission in PFC.^[62] It is high likely that ketamine's actions on synapses might reverse the morphological and functional alterations in the medial PFC neurons caused by stress exposure. In a recent study, using optogenetic stimulation of infralimbic PFC, Fuckikami et al.^[63] showed that rapid and long-lasting antidepressant and anxiolytic effects of systemic ketamine administration is associated with increased number and function of spine synapses of layer V pyramidal neurons of PFC. Therefore, in the future studies, we are planning to assess the impact of ketamine application on neuronal plasticity by investigating the dendritic morphology of neurons located in different limbic regions.

Conclusion

Both clinical and animal studies report that subanesthetic dose of ketamine administration produces antidepressant-like response, especially in treatment-resistant depression cases. Ketamine's rapid and sustained actions have been investigated in normal or chronically stressed animals, but none of the experimental studies has examined its effects in prenatally stressed rats. Using a combination of behavioral tests and c-Fos immunohistochemistry, we demonstrated that a single-dose ketamine treatment produces antidepressant-like effect in juvenile male rats and enhances the neuronal activation in the medial prefrontal cortical region in female rats exposed to prenatal stress.

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Correspondence to: Emel Ulupınar, MD, PhD
Department of Anatomy, Faculty of Medicine,
Eskişehir Osmangazi University, 26040, Eskişehir, Turkey
Phone: +90 222 239 29 79 / 4436
e-mail: eulupi@ogu.edu.tr

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