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RESEARCH ARTICLE

The role of dopamine D2 receptors in the amygdala in metabolic and behavioral responses to stress in male Swiss-Webster mice

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OBJECTIVE: The D2 dopamine receptor is found in different parts of the amygdala. However, its contribution to stress is unknown. Thus, in the present study, we examined the effects of excitation and inhibition of D2 dopamine receptors in the amygdala on the metabolic and hormonal changes in response to stress.
METHODS: Bilateral amygdala cannulation was carried out in Swiss-Webster mice ($n = 7$). On recovery, different doses of the dopamine D2 receptor antagonist, sulpiride (1, 5 and 10 μg/mouse) or the dopamine D2 receptor agonist, bromocriptine (1, 5 and 10 μg/mouse) were injected into the amygdala. The animals were then placed in stress apparatus (communication box) where they received an electric shock (10 mV voltage, 10 Hz frequency and 60 s duration) after 30 min. The animal's activities were recorded for 10 min before and 10 min after the stress induction. Locomotion, rearing and freezing were investigated. Metabolic changes, such as food and water intake and anorexia, were studied.
RESULTS: The results show that stress increased the concentration of plasma corticosterone, which was followed by a decrease in locomotion and rearing and an increase in freezing behavior. Furthermore, both weight and water and food intake were reduced. Administration of bromocriptine led to a reduction of corticosterone at doses of 1 and 5 μg/mouse and an increase of corticosterone at 10 μg/mouse. Additionally, lower doses of bromocriptine (1 and 5 μg/mouse) caused an increase in locomotion and rearing and a decrease in freezing behavior. Similar results were observed with sulpiride injection.
CONCLUSION: D2 dopamine receptors can play a major role in the amygdala in stress. Both an agonist and an antagonist of the D2 receptor attenuate the metabolic and hormonal changes observed in response to stress

Keywords amygdala, anorexia, bromocriptine, corticosterone, D2 dopamine recepetor, sulpiride

Introduction

Stress is described as a general reaction of the mammalian central nervous system, which plays a vital role in the way an organism monitors internal conditions, as well as conditions of the world around it, in order to survive. Physical and psychological stresses are implicated at the onset of many diseases. The continued existence of stress can cause many diseases such as diabetes, metabolic disorders and diseases of the nervous system such as anxiety and depression (McEwen, 2007; Erfani et al., 2017). It has been shown that the mesolimbic dopamine pathway, of which the amygdala is a part, plays a role in stress responses (Asalgoo et al., 2015). The hippocampus is a brain region which is involved in memory function (Meftahi et al., 2014). Stressful stimuli such as tail-pinch or foot-shock, induce activation of the amygdala and the dopamine (DA) system is activated by maintaining stressful stimuli. Lesions of the amygdala tend to block stress-induced increases in dopamine levels in the prefrontal cortex (Belujon and Grace, 2015).

Different stressors affect synaptic plasticity within the amygdala or in the amygdala–nucleus accumbens pathway. Prolonged stress exposure leads to an increase in the size of the amygdala structure in rodents (Hölzel et al., 2009). Increased dendritic length and increased arborization were reported within the basolateral complex of the amygdala and in the extended amygdala as a result of exposure to chronic immobilization stress (Vyas et al., 2003). Stress alters dopamine-dependent behaviors (such as sniffing and rearing),

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and can also change brain functions such as locomotion, emotional response, emotion, appetite, cognition and ability to experience pleasure and pain, which are also controlled by dopamine (Brake et al., 2004; Meftahi et al., 2015).

The mesocorticolimbic dopamine system originates from the ventral tegmental area and innervates different parts of the limbic system and the prefrontal cortex. The release of dopamine in these areas increases during actions such as eating, drinking and sexual activity (Alcaro et al., 2007; Ghodrat et al., 2014; Motahari et al., 2016; Mohammadian et al., 2017). The amygdala, and in particular, the basolateral nucleus (BLA) receives a strong locus serolous projection, which is activated by stress (Rosen et al., 1998). Acute stress induces an increase in adrenergic-dependent long-term potentiation in the BLA, which suggests that dysfunctional stress integration, as observed in psychiatric disorders, may involve dysregulation of this circuitry (Sarabdjitsingh et al., 2012).

Based on the biochemical and pharmacological properties, at least five groups of dopamine receptors (D1-D5), with different physiologic functions, have been identified. D2 receptors are expressed at significant levels in the amygdala (Missale et al., 1998; Seeman, 2006; Sadeghi-Gharajehdaghi et al., 2017). Dopamine enhances the response of the amygdala by increasing excitatory sensory input through dopamine D2 receptor stimulation and reducing inhibitory prefrontal input to the amygdala through dopamine D1 receptor stimulation. Both D1 and D2 receptor stimulation directly increases the excitability of amygdala projection neurons through postsynaptic mechanisms (Rosenkranz & Grace, 2002; Yamamoto et al., 2007). Projection neurons in the amygdala express dopamine D1 and D2 receptors. D2 receptors also play a role in disinhibiting the amygdala response by decreasing inhibition onto projection neurons and increasing inhibition onto interneurons (Bissière et al., 2003).

As mentioned earlier, it seems that the amygdala plays an important role in the stress response, but the role of dopamine D2 receptors in the amygdala is not well-known. Thus, the aim of this study was to investigate the role of dopamine D2 receptors in the amygdala response to stress in male mice. To this end, we investigated metabolic, behavioral and hormonal responses to stress following administration of an agonist or an antagonist of the D2 receptor in the amygdala.

Materials and methods

Male Swiss-Webster mice, weighing 30±5 g, were kept in groups of four per cage in 12/12 light/dark conditions at 22-25°C. Food and water were provided *ad libitum.* Animals were deprived of food and water only during the experiment time when they were put in a communication box. The food and water intake was recorded for each animal at specific hours every day. Animal experiments were conducted in accordance with the Guidelines of the National Institute of Health (NIH) for the Care and Use of Laboratory Animals, and were approved by the local ethical committee (The Baqiyatallah University of Medical Sciences Committee on the Use and Care of Animals, 87/381, July 25, 2009).

Animal group

Animals were divided into eight groups at random (n = 7). The negative control group did not receive any treatment. The positive control group received 14 days of stress induction. In three groups of animals, bromocriptine (the D2-dopamine agonist receptor) was injected bilaterally into the amygdala at a concentration of 1, 5 or 10 μg/mouse, 5 min prior to the stress induction. In an additional three groups, sulpiride (the D2-dopamine antagonist receptor) was injected bilaterally into the amygdala at a concentration of 1, 5 or 10 μg/mouse, 5 min before the stress induction. The plasma corticosterone concentrations, food and water intake, weight changes and delay to eating (anorexia) were measured as metabolic criteria in all groups. Additionally, locomotion, rearing and freezing were measured as dopamine-related behaviors.

Surgical procedures

For the amygdala cannulation, the animals were anesthetized with ketamine (Sigma-Aldrich, CA, USA, 50-75 mg/kg) and diazepam (Sigma-Aldrich, CA, USA, 5-7 mg/kg), and the surgical area was shaved. The animals were placed in a stereotaxic apparatus. A small incision was made in the scalp to expose the skull. Using bregma and lambda as landmarks, the skull was leveled in the coronal and sagittal planes with one or two guide cannulas (gauge No. 23, World Precision Instruments) implanted into the skull 500 μm above the amygdala using the Paxinos and Watson atlas (2001); (the amygdala nucleus coordinates were 0.8 mm posterior to bregma, ±2.5 mm from the midline and 4.5 mm below the skull surface). Cannulas were fixed with dental acrylic cement. The animals were given 7 days to recover from the surgery. Dental needle heads No. 30 (Alibaba; INTR), polyethylene tubes and 10 μL Hamilton syringes were used for injections. Bilateral intra-amygdala administration of bromocriptine (Tolid Daru, Tehran, Iran; chemical formula $C_{32}H_{40}BrN_5O_5$) or sulpiride (Pars Mino, Tehran, Iran; chemical formula $C_{15}H_{23}N_3O_4S$) at a concentration of 1, 5 or 10 μg/mouse in 1 μl sterile saline at a rate of 0.2 μL/min, using a microinfusion pump, was injected daily for 5 min prior to the stress induction. This injection was gradual and lasted for 60 s. The animals were free to move during this time. The cannula remained in place for 2 min after completion of the injection. After completing the test, all the animals were anesthetized and transcardially perfused with 0.9% normal saline followed by 10% buffered formalin. The brains were removed, blocked, and cut coronally into 40 μM-thick sections via the cannula placements. The tissue

was stained with cresyl violet and examined by light microscopy by an unknown observer. Only the animals with correct cannula placements were included in the analysis.

Communication box

Stress induction was performed as previously described (Ghobadi et al., 2016; Dalooei et al., 2016). Briefly, after bilateral intra-amygdala bromocriptine or sulpiride injection, the animals were transferred to a communication box (made by Borje Sanat Co. in Tehran, Iran), which is comprised of 9 separate compartments with plexiglass walls and small holes, with a diameter of 2 mm, that enable communication between mice in separate compartments. The floor of the box had stainless steel bars connected to a generator, which was linked to a computer that controls the voltage and the duration of the shock (10 mV voltage, 10 Hz frequency and 60 s duration). The animals in the control group were also placed in a communication box that was switched off for 30 min. Stress induction was performed over 14 consecutive days. Blood samples were collected from the retro-orbital sinus of all animals on the 1st, 7th and 14th days of the test. The blood was centrifuged at 3000 rpm (revolutions per minute) for 5 min at 4°C. Serum was collected for the detection of corticosterone, as a stress hormone. The serum was collected and frozen at −20°C. Corticosterone concentrations were determined by ELISA kit (Rat Corticosterone ELISA kit; EIA-4164; DRG Instruments GmbH, Germany). Briefly, serum samples were added to 96-well plates containing biotinylated primary antibody and then incubated at 37°C for 45 min. The plates were then washed and horseradish peroxidase-conjugated streptavidin solution was added to the wells and incubated for an additional 30 min at 37°C. The 3,3′,5,5′-tetramethylbenzidine substrate was added and the plates were incubated for an additional 15 min at 37°C. Stop solution was added to the wells to terminate the reaction. The corticosterone concentration was determined using a standard curve.

Data analysis

Data were expressed as mean±standard error (Mean±SEM). Two-way analysis of variance (Two-Way-ANOVA) was applied using bromocriptine or sulpiride and stress as factors followed by Tukey post HOC. P values of < 0.05 were considered statistically significant.

Results

The effect of bilateral intra-amygdala administration of bromocriptine on weight changes in stress

Animals received different doses of bromocriptine (1, 5 or 10 μg/mouse) 5 min before stress induction for 14 consecutive days. The results demonstrated that weight changes were not altered significantly in the negative control group (without bromocriptine or stress induction). In the positive control group (with stress induction and saline injection), stress induction led to weight reduction until the 7th day, followed by weight increase. The mean weight on the 14th day was higher than that of the first day. In the group that received low doses of bromocriptine (1 or 5 μg/mouse), the weight increase was smaller than that observed in the positive control group. The group that received high doses of bromocriptine (10 μg/mouse) showed gradual weight increase, but this increase was smaller than the increase observed in response to low doses of bromocriptine. In other words, bromocriptine in low doses (1 and 5 μg/mouse) reduced the effect of stress induction on weight changes, while at high dosage (10 μg/mouse), the effect of stress induction were increased (Fig. 1 A). [Repeated Measurement ANOVA For Day F (24.439) = 13, $P < 0.0001$, for weight gain]

The effect of the bilateral intra-amygdala administration of bromocriptine on food and water intake in stress induction

Food and water intake were assessed daily. In the negative control group, food intake was fairly constant over the 14 test days. The positive control group showed an initial decrease in food intake followed by an increase, which indicates that the animals adapted to stress. Food intake in the group that received low doses of bromocriptine (1 or 5 μg/mouse) gradually increased. This increase was greater than that observed in the positive control group. An increase in food intake was also observed in the group that received high doses of bromocriptine (10 μg/mouse); however, this increase was smaller than that observed at low doses (Fig. 1 B). [Repeated Measurement ANOVA For Day F (34.185) = 13, $P < 0.0001$ for food intake].

The results showed that, in the negative control group, water intake during the 14 test days remained relatively stable. In the positive control group, water intake first decreased over the test period, and then increased. Similarly, administration of bromocriptine at different doses prior to stress induction showed that water intake first decreased over the test period, and then increased (Fig. 1 C). [Repeated Measurement ANOVA For Day F (21.647) = 13, $P < 0.0001$ for water intake]

The effect of stress induction and bilateral administration of bromocriptine into the amygdala on delay to eating (anorexia) in mice

Thirty minutes after stress induction, the experimental groups were returned to their holding cages and the duration of anorexia (delay to eating) was measured. The results showed

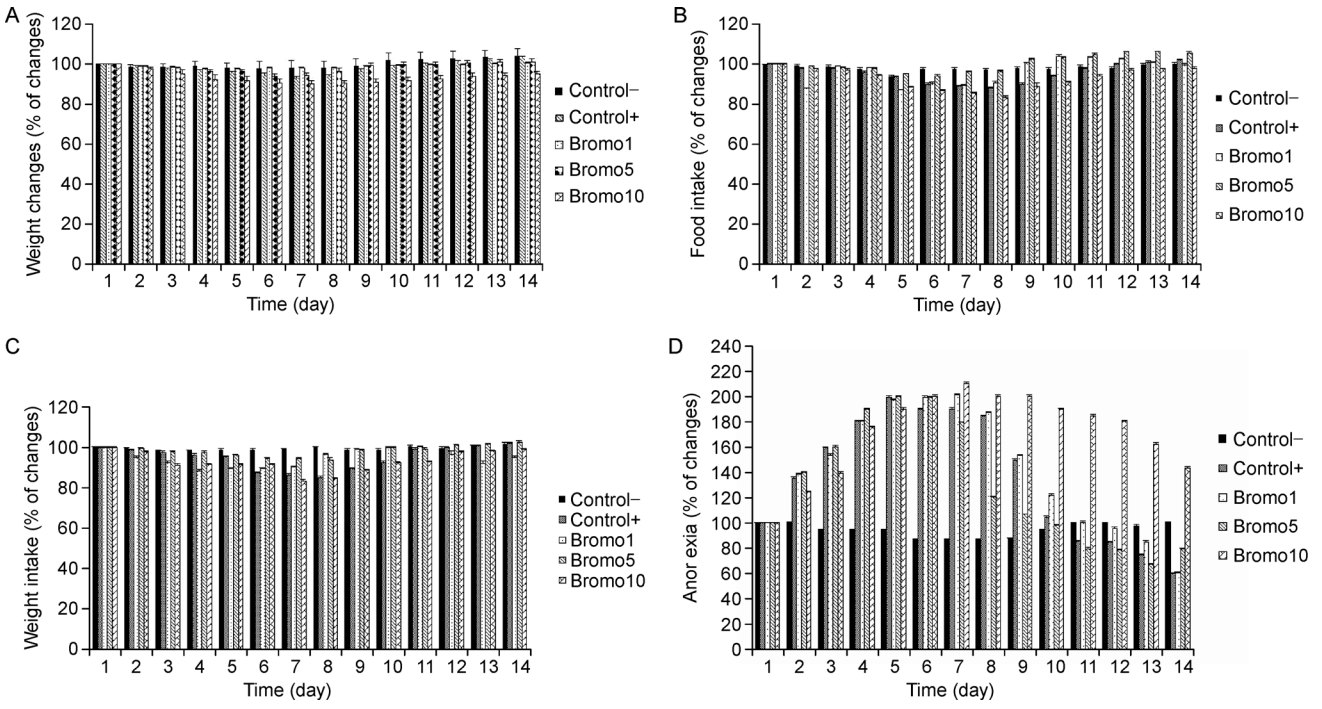

Figure 1 The effect of bilateral intra-amygdala administration of different doses of bromocriptine (1, 5 or 10 μg/mouse) and induced stress on weight changes (A), food intake (B), water intake (C) and anorexia (D). The mice were investigated at a specific time every day for 14 consecutive days. The results obtained on the first day were taken as 100 and were used as a point of reference for measurements made on subsequent days (percentage).

that, in the negative control group, the duration of anorexia within the 14 days remained relatively stable. In the positive control group, anorexia initially increased and then decreased. Anorexia was reduced on the 14th day as compared to the first day. Likewise, in the group that received low doses of bromocriptine (1 or 5 μg/mouse), anorexia was reduced. However, this reduction was smaller than that observed in the positive control group. In animals that received the high dose of bromocriptine (10 μg/mouse), the duration of anorexia initially increased. The duration then decreased, but this decrease was much smaller than that observed in other groups. Thus, it appears that low doses of bromocriptine reduce stress-induced anorexia, to some extent, whereas, high doses of bromocriptine enhance stress-induced anorexia (Fig. 1 D). [Repeated Measurement ANOVA For Day F (164.90) = 13, $P < 0.0001$ for anorexia]

The effect of stress induction and bilateral administration of bromocriptine into the amygdala on serum corticosterone levels

The results showed that serum corticosterone levels on the 7th and 14th days after the stress induction were increased in the positive control group as compared to the negative control group. However, in the positive control group, the corticosterone concentration was slightly lower on the 14th day than on the 7th day. Bilateral injection of bromocriptine at a dose of 1 μg/mouse in the amygdala reduced the corticosterone concentration following 7 and 14 days of stress induction ($P < 0.001$). Serum corticosterone levels in the groups that received 5 μg/mouse bromocriptine showed a significant increase on the 7th day as compared to the negative control group. However, there was no significant difference in the corticosterone concentration, as compared to the negative control group, on the 14th day after stress induction ($P < 0.001$). Bromocriptine at a 10 μg/mouse dose showed a significant increase in corticosterone levels, as compared to the negative control group, on the 7th day. This trend was also observed on the 14th day following stress induction. Thus, low doses of bromocriptine reduced corticosterone concentration after stress induction, while at high doses of bromocriptine, corticosterone concentration was increased (Fig. 2).

The effect of the bilateral intra-amygdala administration of bromocriptine on dopamine related behaviors in stress induction

On each test day, animal behaviors were filmed for 10 min before and after stress induction. Locomotion, rearing and freezing were measured. The results showed that locomotion was significantly decreased on the first day after stress induction in the positive control group as compared to the negative control group ($P < 0.001$). Reduced locomotion was

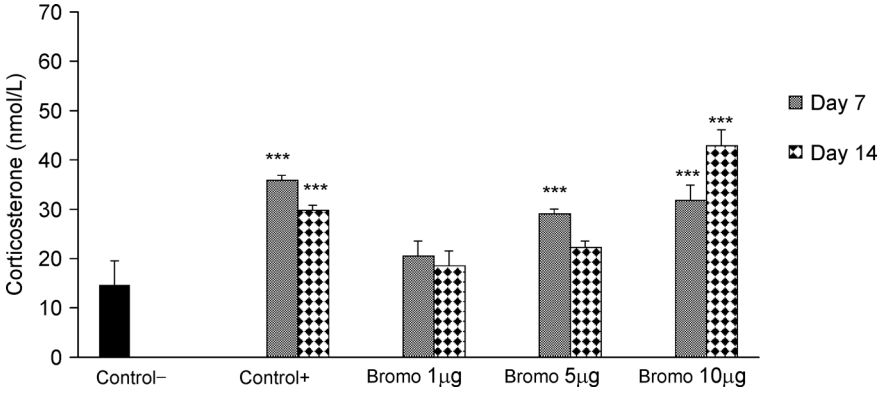

Figure 2 Stress and bilateral intra-amygdala administration of bromocriptine induced changes in serum corticosterone concentrations. Five minutes before the stress, different doses of bromocriptine (1, 5 or 10 μg/mouse) or saline were injected bilaterally into the amygdala. Five minutes before the stress, saline bilateral was injected into the amygdala. To measure the serum corticosterone concentration, blood samples were taken from all mice on the first, seventh and fourteenth days after stress. The sample was taken from the corner of the animal's eye after the experiment. The Mean±SEM are presented for 6 mice. ***$P < 0.001$ shows a significant difference as compared to the negative control group (n = 7 mouse/group).

also observed at a dose of 10 μg/mouse bromocriptine. Groups that received low doses of bromocriptine (1 and 5 μg/mouse) did not show a significant change in locomotion before or after stress induction as compared to the negative control group (Fig. 3 A).

The positive control group showed a significant decrease in rearing on the first day after stress induction, as compared to the negative control group ($P < 0.001$). The results showed that bilateral injection of bromocriptine into the amygdala decreased rearing after stress. It should be noted that the number of rearing after stress induction in the groups that received low-doses of bromocriptine (1 or 5 μg/mouse) initially decreased (as measured on the 7th day) and then increased (as measured on the 14th day). However, in the groups that received a high-dose of bromocriptine (10 μg/mouse), the number of rearing before and after stress induction was lower on the 14th day than on the first day (Fig. 3 B).

Additionally, the results showed that stress induction increased freezing before and after the administration of

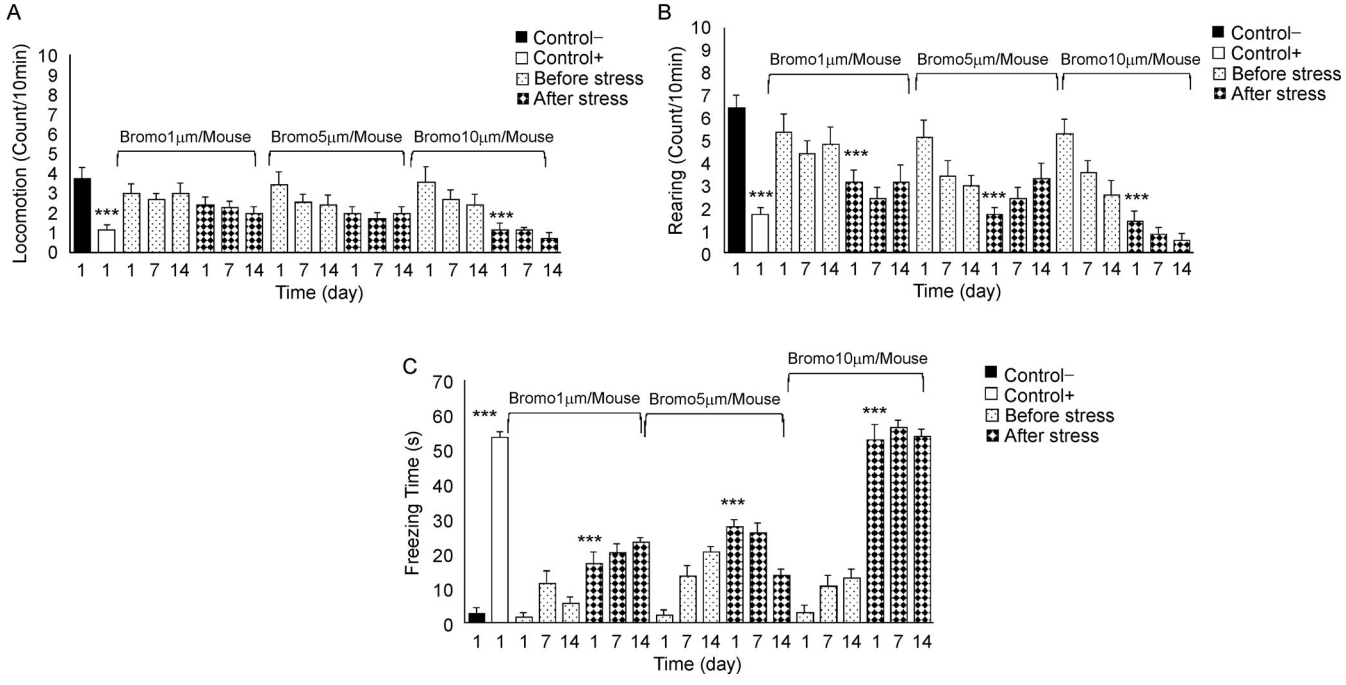

Figure 3 The effect of induced stress and bilateral intra-amygdala administration of different doses of bromocriptine (1, 5 or 10 μg/mouse) on dopamine related behavior. Changes in locomotion (A), rearing (B) and freezing time (C). ***$P < 0.001$ shows a significant difference as compared to the negative control group.

bromocriptine. Freezing increased significantly in the positive control group on the first day after stress induction, as compared to the negative control group ($P < 0.001$). This increase was observed at all doses of bromocriptine, but the difference was more significant at 10 μg/mouse than at 1 or 5 μg/mouse (Fig. 3 C).

The effect of bilateral intra-amygdala administration of sulpiride on weight changes in stress induction

In these experiments, as in the previous section, the animal's bodyweight was measured every day before the induction of stress. For these experimental groups, a dose of sulpiride (1, 5 or 10 μg / mouse) were bilaterally injected into the amygdala (0.25 ml/side), 5 min before stress induction. The results showed that changes in bodyweight remained relatively stable in the negative control group. In the positive control group (saline injection and stress induction) an initial decrease in bodyweight was followed by an increase. On the 14th day, the mean weight was higher than that of the first day. Thus, in this group, the bodyweight increased within 14 days of stress induction. However, in the group that received different doses of sulpiride (1, 5 or 10 μg/mouse), the weight was decreased on the fourteenth day compared to the first day. In the group that received different doses of sulpiride (1, 5 or 10 μg/mouse) before stress induction, the bodyweight decreased. This decrease in response to low doses of sulpiride (1 μg/mouse) was smaller than that in response to the high dose (5 or 10 μg/mouse). This suggests that sulpiride dose-dependently reduced bodyweight following stress induction (Fig. 4 A). [Repeated Measurement ANOVA For Day F (45.021) = 13, $P < 0.0001$, for weight gain]

The effect of bilateral intra-amygdala administration of sulpiride on food and water intake before stress induction

The food and water intake in the experimental groups were measured every day. The results showed that food intake was relatively stable during the 14 test days in the negative control group. However, in the positive control group, food intake initially decreased and then slightly increased after 5 days. After bilateral injection of sulpiride in the amygdala, food intake decreased. In the groups that received high doses of sulpiride (5 or 10 μg/mouse), the decrease in food intake was larger than in the group that received a low dose of sulpiride (1 μg/mouse) (Fig. 4 B). [Repeated Measurement ANOVA For Day F (122.611) = 13, $P < 0.0001$ for food intake]

The changes in water intake during the 14 test days show a similar trend to that of changes in food intake. Bilateral administration of sulpiride before stress induction also reduced water intake. This reduction was larger at the high dose than that observed at the low dose (Fig. 4 C). [Repeated Measurement ANOVA For Day F (101. 243) = 13, $P < 0.0001$ for water intake]

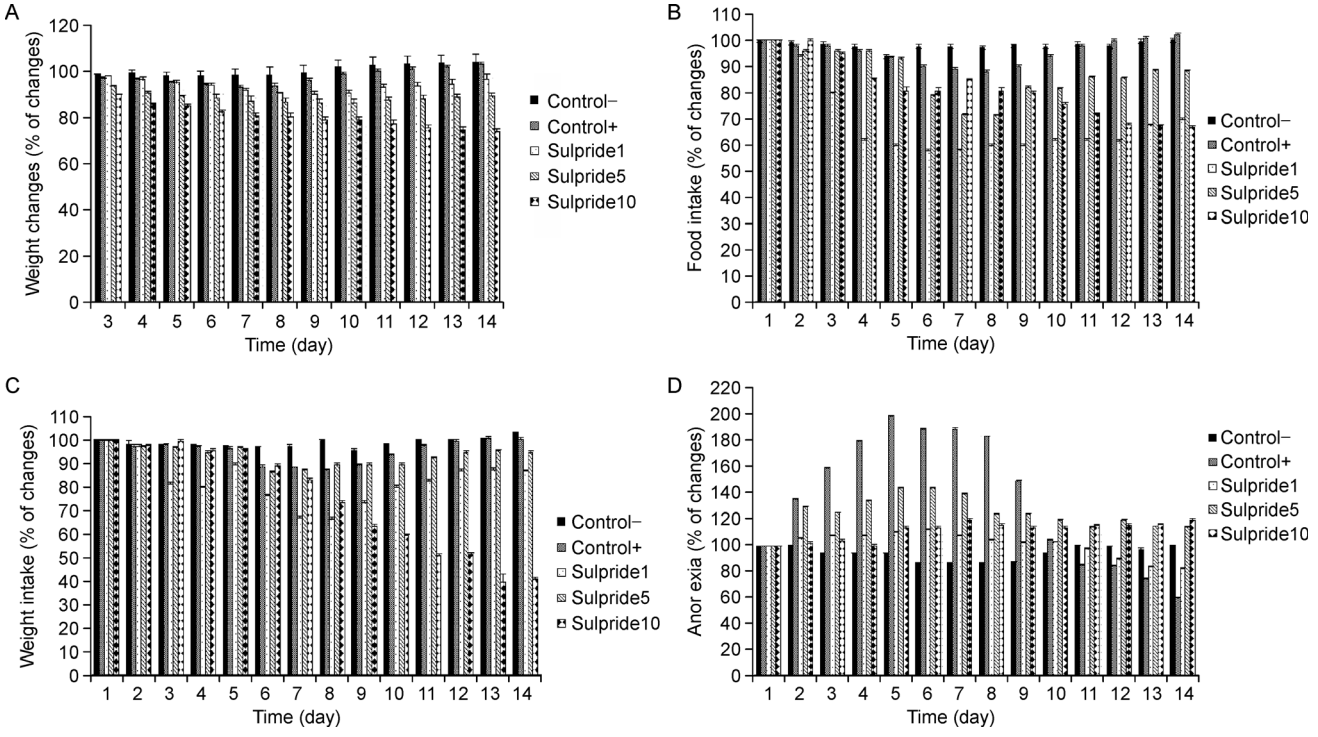

Figure 4 The effect of the bilateral intra-amygdala administration of different doses of sulpiride (1, 5 or 10 μg/mouse) and induced stress on weight changes (A), food intake (B), water intake (C) and anorexia (D). The results obtained on the first day were taken as 100 and were used as a point of reference for measurements made on subsequent days (percentage).

The effect of stress and bilateral administration of sulpiride in the amygdala on delay to eating (anorexia)

The results showed that the delay to eating was relatively stable during the 14 test days in the negative control group. The positive control group showed an initial increase followed by a decrease in delay to eating. In this group, the delay to eating was reduced on the 14th day, as compared to the first day. Additionally, the duration of anorexia in the group that received sulpiride (1, 5 or 10 μg/mouse) initially increased and then decreased; however this reduction was much smaller than that observed in the control group. Thus, it appears that inhibition of dopamine D2 receptor in amygdala before stress induction reduced the animal's adaptation to stress, as compared to the control group. The anorexia time was increased at high dose of sulpiride (10 μg/mouse) (Fig. 4 D). [Repeated Measurement ANOVA For Day F (229.774) = 13, $P < 0.0001$ for anorexia]

The effect of stress and bilateral intra-amygdala administration of sulpiride on serum corticosterone concentration

The results showed that serum corticosterone levels increased in the positive control group 7 days after stress induction. The serum corticosterone level on the 14th day was also high in this group. Corticosterone levels, measured on the 7th and 14th days following stress induction, in the positive control group showed a significant increase as compared to the negative control group ($P < 0.001$). Bilateral administration of different doses of sulpiride (1, 5 or 10 μg/mouse) did not significantly change serum corticosterone levels on days 7 and 14, as compared to the positive control group. Plasma corticosterone levels on the 14th day in these groups were similar to that of the positive control group; however, the levels were slightly lower than that of the 7th day. It should be noted that sulpiride, at a dose of 10 μg/mouse, significantly increased serum corticosterone level. It appears that this dose exacerbates the effect of stress on serum corticosterone level (Fig. 5).

The effect of bilateral intra-amygdala administration of sulpiride on dopamine related behaviors in stress

On the first day of stress induction, locomotion significantly decreased in the positive control group, as compared to the negative control group ($P < 0.001$). In the groups that received different doses of sulpiride (1, 5 or 10 μg/mouse), there was no significant difference in locomotion as compared to the negative control group, on the first day of stress induction. Administration of different doses of sulpiride (1, 5 or 10 μg/mouse) led to a locomotion decrease on the 1st, 7th and 14th days after stress induction, as compared to before stress induction. This reduction was greater at the 10 μg/mouse dose than at 1 μg/mouse dose. Locomotion after administration of 10 μg/mouse sulpiride was decreased at days 1, 7 and 14, as compared to the group that received 1 μg/mouse sulpiride (on days 1, 7 and 14 pre-stress; Fig. 6 A).

There was a significant decrease in rearing on the first day of stress in the positive control group, as compared to the negative control group ($P < 0.01$). Similar results were observed in the groups that received sulpiride (1, 5 or 10 μg/mouse). Administration of sulpiride at doses of 1 and 5 μg/mouse reduced rearing on the seventh day before and after stress induction. The rate of rearing was significantly reduced after administration of 10 μg/mouse sulpiride, which continued until the 14th day (Fig. 6 B).

The results showed that freezing time was significantly

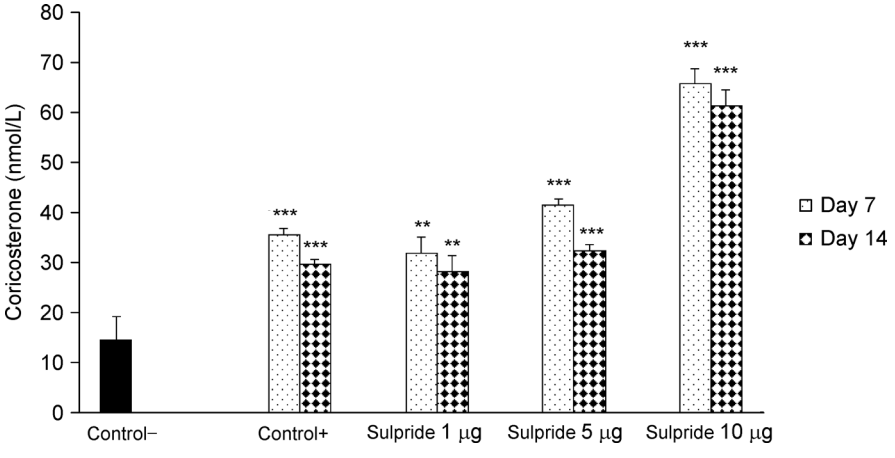

Figure 5 Stress and bilateral intra-amygdala administration of different doses of sulpiride (1, 5 or 10 μg/mouse) induced changes in serum corticosterone concentrations. Five minutes before the stress, different doses of sulpiride (1, 5 or 10 μg/mouse) or saline were injected bilaterally into the amygdala. Five minutes before the stress, saline bilateral was injected into the amygdala. To measure the serum corticosterone concentration, blood samples were taken from all mice on the first, seventh and fourteenth days after stress. The samples were taken from the corner of the animals eyes after the experiment. The Mean±SEM are presented for 6 mice. ** $P < 0.01$ and ***$P < 0.001$ show a significant difference as compared to the negative control group. n = 7 mouse/group.

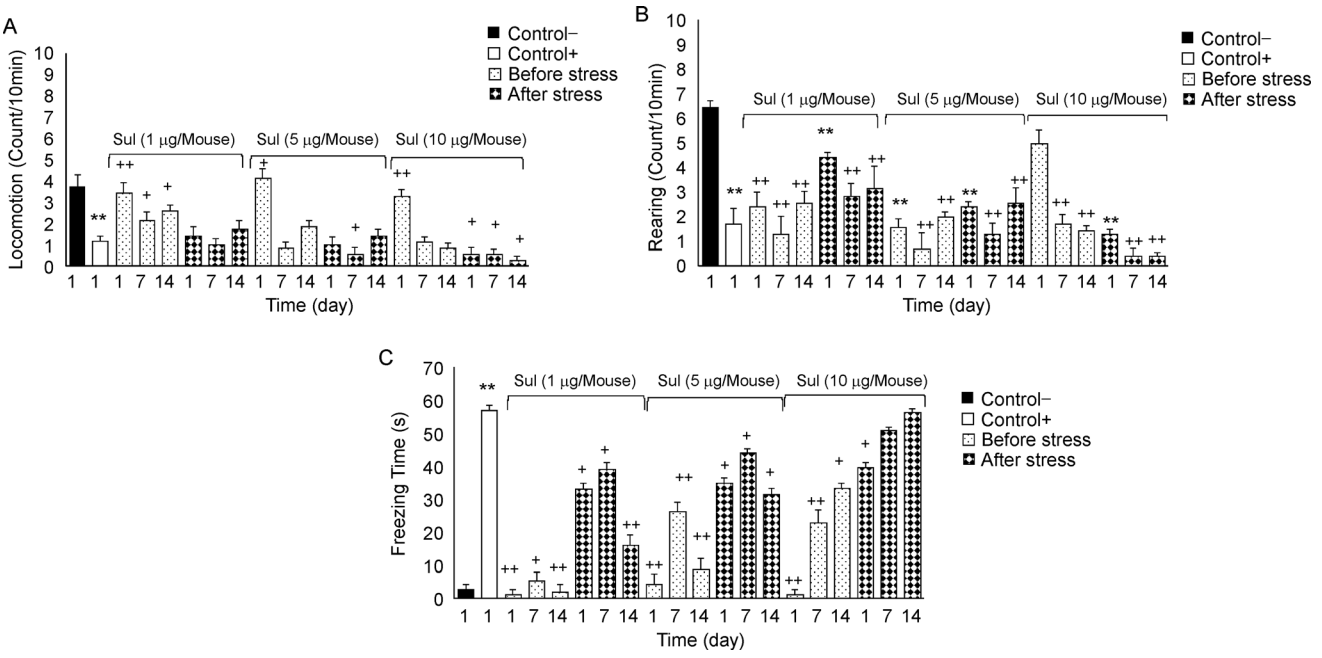

Figure 6 Effects of bilateral intra-amygdala administration of different doses of sulpiride (1, 5 or 10 μg/mouse) on dopamine related behavior before stress. Changes in locomotion (A), rearing (B), and freezing time (C). ***$P < 0.001$ shows a significant difference as compared to the negative control group. ++ $P < 0.01$ and + $P < 0.05$ shows a significant difference as compared to the positive control group. $n = 7$ mouse/group.

increased in animals on the first day of stress induction ($P < 0.01$). Administration of sulpiride significantly increased freezing time after stress induction. This result was more pronounced at 10 μg/mouse dose than at the 1 μg/mouse dose. In the groups that received 1 or 5 μg/mouse sulpiride, freezing time was slightly decreased on the 14th day. However, a reduction in freezing time was not observed at the 10 μg/mouse dose on the 14th day (Fig. 6 C).

Discussion

Any event that changes the internal environment of an organism that can be interpreted, by the brain, as extreme or threatening, is considered to be stress. Additionally, any factor that can change cellular and molecular activity is considered a stressor (Habib et al., 2001; Ehteram et al., 2017). Stress causes abnormal activity and disruption of tonic activity in the mesolimbic dopamine system. In the stressed condition, activity in the mesocorticolimbic pathway is increased. This leads to increases in dopamine-dependent behaviors, such as locomotion, sniffing and freezing (Trainor, 2011). In the present study, the role of dopamine D2 receptors in behavioral, hormonal and metabolic changes in response to inescapable stress was investigated. Stress has two components: mental and physical. In this research, stress with a strong physical component (inescapable foot-shocks) was performed.

It is well-known that stress increases the activity of the HPA axis and leads to enhanced secretion of corticosteroids. In rodents, corticosterone is considered the main glucocorticoid involved in regulation of stress responses and it is therefore used as a stress biomarker (Kim et al., 2013). Consistent with other studies, the present results showed that corticosterone levels increased following inescapable stress. We found that the corticosterone concentration was slightly decreased on the 14th day as compared to that of the 7th day.

The HPA axis is activated in response to a stressful stimulus. Neural inputs from the central and peripheral nervous system converge to the hypothalamus and signal for enhanced synthesis and release of CRH. CRH is released into the hypophyseal portal blood and carried to the anterior pituitary, with increased release of ACTH. ACTH is carried via the blood to its target organ, the adrenal cortex, where it stimulates secretion of corticosterone in rodents. Increasing concentrations of glucocorticoids mediate a variety of metabolic effects that help the body to respond to the stressor, such as reduced anorexia, decreased movement, weight loss, increased irritability and high blood sugar (Ginsberg et al., 2003; Kasckow et al., 2001).

In the present study, foot shock stress led to dopamine-related behaviors such as decreased rearing and locomotion and increased freezing time. The reason for this could be the function of the dopamine mesocorticolimbic system (reward system) that acts in parallel with the stress system. There is growing evidence that dopamine neurons have pivotal roles in behavioral responses to stress and addiction (Trainor, 2011; Chalabi-Yani et al., 2015; Hosseini et al., 2015; Husseini et

al., 2016). Stressors can activate opposing dopamine behavioral effects depending on the duration of stress experienced. In severe stress, there is increased stress system activity because of the predominance of the activity of neurons, which secrete CRF. Because of the decreased dopamine release, there is a decrease in dopamine-related behavior such as rearing and locomotion as well as an impaired response to rewarding and aversive stimuli. Exposure to repeated and variable stress promotes deficits in responding to rewarding stimuli, as indicated by a marked and persistent reduction in the intake of palatable liquids and food (Cabib and Puglisi-Allegra, 1996). Isovich et al. (2000) showed that chronic psychosocial stress reduces the expression of dopamine transporter binding sites in motor-related brain areas. The observed reduction in locomotor activity is related to the downregulation of dopamine transporter binding sites. In the current study, it is probable that stress induction decreased extracellular dopamine concentrations in animals, which led to increased freezing time and decreased rearing and locomotion. Moreover, following repeated variable stressful experiences, rats presented increased levels of dopamine and reduced specific binding to dopamine D2 receptors in the limbic forebrain, but not in the caudate nucleus or septal area (Bruijnzeel et al., 2001). Because the amygdala is an important brain area involved in stress, and given that the function of dopamine D2 receptors in this region is not well-studied. Here, we studied the function of dopamine D2 receptors in stress induction. For this purpose, prior to stress induction, an agonist (bromocriptine) or an antagonist (sulpiride) of dopamine receptors was injected into the amygdala.

Bilateral intra-amygdala administration of bromocriptine showed dose-dependent responses to stress induction. At 1 and 5 μg/mouse doses, bromocriptine reduced corticosterone concentration. Whereas, at 10 μg/mouse, bromocriptine increased corticosterone concentration. It should be noted that after administration of bromocriptine into the amygdala, in the stress group (positive control group), the corticosterone concentration initially increased (until the seventh day) and then decreased. This indicates adaptation of animals to stress, which is also seen in other studies. This suggests that, after a period of time, the HPA system and corticosterone secretion lost sensitivity. Thus, there was no increase after stress (Bruijnzeel et al., 2001; Bahari et al., 2015). The activity of the HPA axis plays a critical role in restoring homeostasis following acute stressor exposure. Long-term exposure to stress reduced HPA axis sensitivity (Chrousos, 2009).

The results showed that repeated exposure to foot shock stress resulted in an adaptation of the HPA axis, which was manifested as a habituation to stress-induced corticosterone secretion. This is consistent with previous reports (Girotti et al., 2006; Jaferi & Cabib & Bhatnagar, 2006; Bahari et al., 2014). Repeated exposure to the same stimulus has been found to result in a decline in both neuronal activation of the corticolimbic circuit regulating activation of the HPA axis and the synthesis and release of glucocorticoids hormones from the adrenals. Theories of stress adaptation propose that a system level attenuation of neuronal responses to stressful stimuli within sensory limbic regions of the brain produces the habituation of HPA axis activation and glucocorticoid secretion (Hill et al., 2010). However, while chronic treatment with glucocorticoids results in inhibition of basal and stress-induced CRH mRNA in the paraventricular nucleus in rats, intense chronic stress has been shown to increase CRH mRNA (Girotti et al., 2006).

One other explanation for habituation of stress induced CRH gene induction could be a change in the presynaptic input to the CRH neurons after repeated stress. Alterations in presynaptic inputs to the CRH neurons could involve either reduced excitatory afferent activity or an activation of inhibitory GABAergic projections (Herman et al., 2004). However, administration of a high dose of bromocriptine (10 μg/mouse) into the amygdala increases the corticosterone concentration, as compared to the control, and prevents their habituation. Therefore, it seems that bromocriptine has a different effect in HPA axis adaptation during stress through the activation of different dopamine D2 receptors.

Dopamine D2 receptors have mainly presynaptic and postsynaptic localization and functions. Both presynaptic and postsynaptic receptors were found in the amygdala. Activation of these differently localized receptors could lead to different functions. Stimulation of presynaptic D2 receptor can inhibit dopamine release, leading to a reduced effect of dopamine (De Mei et al., 2009). The dopamine D2 receptor has a high degree of functional diversity, which is very likely determined by intrinsic and extrinsic events that converge on dopamine neurons to modulate their functions. Indeed, dopamine, acting in an autocrine manner, stimulates D2 receptor expression in dopaminergic neurons (here referred to as autoreceptors). Autoreceptor activation suppresses DA synthesis and release. Dopamine also activates D2 receptors on neurons receiving dopamine afferents that control the release of heterologous neurotransmitters such as glutamate, GABA and acetylcholine, which in return could stimulate or inhibit dopamine release (Anzalone et al., 2012).

Inglis and Moghaddam (1999) showed that dopamine release in the amygdala is enhanced in response to stress. Dopamine D2 receptors play a role in disinhibiting the amygdala response by reducing inhibition onto projection neurons and increasing inhibition onto interneurons (Bissie`re et al., 2003). Goldstein et al. (1996) showed that bilateral amygdala lesions lead to a blockade of the mesocortical monoaminergic responses to stress induced by exposure to stimuli paired previously with an unconditioned stressor. They also showed that amygdala lesions attenuated associated adrenocortical activation, freezing and defecation. Dziedzicka-Wasylewska et al. (1997) showed that stress reduced the D2 receptor message in the substantia nigra and the lateral part of the ventral tegmental area (VTA). Also, imipramine (a D2 agonist) increased the D2 receptor message

in the VTA, but only in a non-stressed group.

Intra-amygdala administration of bromocriptine showed a dose-dependent effect. It appears that, at low doses, the effect is probably through pre-synaptic influences and at high doses, the effect is probably through postsynaptic influences. Thus, at a high dose, bromocriptine reinforces the effects of stress. In other words, low doses of bromocriptine (1, 5 μg/mouse) increased locomotion and rearing and reduced freezing time, as compared to the positive control group. However, at the high dose (10 μg/mouse), bromocriptine decreased locomotion and rearing, and did not alter freezing time, as compared to the positive control group. It should be noted that bromocriptine binds with high affinity at the dopamine D2 receptor but also has high affinity for the dopamine D3 receptor (Perachon et al., 1999). These effects may also be due to the varying degrees of D3 dopamine receptor activation at different bromocriptine doses. Thus, activation of dopamine D2 receptors in the amygdala can dose-dependently decrease (at low dose) or increase (at high doses) freezing time. This could be the reason that the dopamine D2 receptor in the amygdala is involved in stress.

After administrating a high-dose of bromocriptine before stress, rearing did not show significant changes as compared to the positive control group. When animals received low and medium doses of bromocriptine, they showed a significant increase in rearing as compared to the high-dose group and the positive control group. This suggests that stimulation of the dopamine D2 presynaptic receptor or the D3 receptor by bromocriptine (at low doses) in the amygdala reduces stress behaviors in mice. It is probable that both types of these receptors were involved in the behavioral effects of low doses of bromocriptine.

The metabolic effects of administration of different doses of bromocriptine in the amygdala also showed similarities to the behavioral and hormonal effects. In low doses, the effect of bromocriptine on anorexia in stress was adaptable. With increasing doses of bromocriptine, anorexia could not adapt to stress. Conversely, anorexia increased in the groups that received low doses of bromocriptine before the stress until the 7th day, however on the 14th day there was a decreasing trend. At high doses of bromocriptine, anorexia increased until the fourteenth day. Similar to the results of the current study, Seo and Kuzhikandathil (2015) showed that repeated restraint stress episodes over a 5-day period led to a reduction in locomotor activity during the initial period in a novel environment. They also showed that there was a decrease in D3 receptor expression in the amygdala of adult males subjected to preadolescent stress. The D3 receptor is an inhibitory dopamine receptor. Thus, the mice that experienced preadolescent stress and social isolation might have reduced inhibitory control of the excitatory glutamatergic cells in the amygdala resulting in increased excitability of the amygdala in male mice. Diaz et al. (2011) showed that, in rats, the dopamine D3 receptor in the basolateral amygdala regulates GABAergic neurotransmission and modulates anxiety-like behavior. Consistent with the results of the present study, Liu et al. (2013) showed that acute restraint stress induces anorexia, reduces food intake and activates the HPA axis.

Chronic glucocorticoid elevation and acute stress inhibit food intake by reducing both the amount of time spent consuming a meal and the amount of food consumed. Stress related effects on feeding are mediated by a dispersed network of neurons that express CRF. CRF is necessary and sufficient to induce a wide range of stress-associated behaviors such as food intake suppression and rapid elevations in plasma levels of corticosetrone. The reduced food intake that occurs after restraint stress is inhibited by intracerebroventricular infusion of a CRF antagonist. CRF action at forebrain sites, delivery of stress factors to the brainstem reduces short-term and 24-h food intake, maybe by modulating the strength of negative feedback signals (cholecystokinin) stimulated by visceral, oral, gut neural afferent signals, or gut hormone signals that act on the nucleus tractus solitaries (Schwartz & Zeltser, 2013). This mechanism could explain the changes in food intake observed in this study. In this study it seems possible that low doses of bromocriptine reduced CFR secretion, which decreased corticoestrone concentration and led to reduced anorexia. A possible reason that high doses of bromocriptine could not reduce anorexia in stress is that, at this concentration, bromocriptine inhibits all types of D2 and D3 dopamine receptors leading to a change in CRF function, and an increase in anorexia during stress. Similar findings were obtained for the changes in food and water intake and bodyweight. It is probable that bromocriptine has changed these factors through the mentioned mechanisms.

Chang and Grace (2013) showed that suppressing noradrenergic modulation of the basolateral amygdala nucleus (through the infusion of the beta-adrenergic antagonist propranolol) inhibits the decrease in ventral tegmental area dopamine neuron activity and the stress-induced attenuation of amphetamine locomotor response observed following restraint stress. Additionally, during withdrawal from sustained stress, hyperactivity of the basolateral amygdala nucleus would suppress dopamine neurons via the ventral pallidum, thereby, reducing the effect of subsequent behaviorally relevant stimuli (Belujon & Grace, 2015).

In another part of this study, the findings showed that administration of sulpiride can cause significantly increased corticosterone concentration. In this regard, Belda and Armario (2009) showed that dopamine acting through both D1 and D2 receptors exerts a stimulatory effect on the activation of the HPA axis in response to an intense stressor. Dopamine is involved in the maintenance of post-stress activation of the HPA axis, which is critically dependent on how long high levels of glucocorticoid are maintained after stress. Casolini et al. (1993) showed that damage of dopaminergic neurons of the ventral tegmental area lead to a decrease in the corticosterone level in both basal and

restraint stress in rats. Moreover, Puri et al. (1994) have reported increased corticosterone response to restraint stress after administration of the D2 antagonists, sulpiride and haloperidol.

Sulpiride, like bromocriptine, could affect both pre- and post-synaptic receptors in a dose-dependent manner. Although these drugs are dopamine D2 receptor antagonist and agonist, respectively, they showed similar effects on hormonal, metabolic and behavioral responses to stress when administrated intra-amygdala. Perhaps, the main reason for this is that the D2 receptors in the amygdala have a wide distribution in both pre- and post-synaptic neurons. Another reason could be that stress activates different parts of the brain and causes numerous responses that could somewhat explain the similar effects seen in response to these drugs with opposing actions. As mentioned earlier, the corticosterone concentration increased during stress and sulpiride strengthened the effects of stress. Especially in animals that received high doses of the sulpiride (10 μg/mouse), adaptation to stress was prevented.

Sulpiride administration before stress induction showed similar results on dopamine-related behaviors (locomotion, rearing and freezing). Animals that received high doses of sulpiride (10 μg/mouse) showed a significant reduction in locomotion and rearing, while freezing increased significantly. This increase in freezing continued until the 14th day of stress. Additionally, similar results were obtained for metabolic changes (food intake, water intake and anorexia) in animals that received sulpiride.

It has been shown that dopamine has opposing functioning mechanisms in the fear/anxiety processes. Depending on the type of conditioned or unconditioned situation, dopamine D2 receptor antagonists may decrease or enhance the aversiveness of the situation. For instance, intra-basolateral administration of D2 receptor antagonists decreases conditioned fear in rats that are subjected to animal models of anxiety. Therefore, dopamine seems to mediate conditioned fear by acting at rostral levels of the brain, and dopamine in midbrain areas seems to regulate unconditioned fear, likely by decreasing the sensorimotor gating of aversive events (Brandão et al., 2015).

In conclusion, the current study extends the growing literature implicating dopamine D2 receptors in the amygdala in the coordination of hormonal, metabolic and dopamine related behavioral responses to foot shock stress. Stress can result in an overactive HPA axis and trigger disorders in corticosterone concentration, eating and effective dopamine related behavior. Additionally, the present study provides evidence that dopamine D2 receptors signaling in the amygdala are involved in the regulation of emotional and feeding behaviors and the endocrine responses to stress. We propose that abnormal D2 receptor activity in the amygdala may contribute to the pathophysiology of foot-shock stress. Therefore, the D2 receptor in the amygdala is a strong candidate for mediating the stress response.

Although this study was carefully done, the limitations of this study were that we could not investigate using a highly specific D2 agonist or antagonist because bromocriptine and sulpiride have partial affinity for the D3 receptor.

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Compliance with ethice guidelines

Maryam Hassantash, Hedayat Sahraei, Zahra Bahari, Gholam Hossein Meftahi and Roshanak Vesali declare that they have no conclict of interest. All Experiments were done in accordance with standard ethical guidelines and approved by the local ethical committee (The Baqiyatallah University of Medical Committee on the Use and Care, 81/021, July 10, 2015).

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