Research report

Effects of immobilization stress on emotional behaviors in dopamine D3 receptor knockout mice

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A central problem in understanding the dopamine system in anxiety and depression is to specify functions of different members of the dopamine receptor family. Recent studies have reported that the dopamine D2/D3 receptor agonist pramipexole exerts an antidepressant-like effect in the chronic mild stress model and in the behavioral despair model, suggesting dopamine D3 receptor may be an important target for antidepressant actions. The aim of the present study was to examine the role of dopamine D3 receptor on the anxiety-like and depression-like behaviors induced by immobilization stress. We subjected D3 receptor knockout (D3KO) mice to a series of behavioral paradigms after acute (1 h) or chronic (1 h a day for 14 days) immobilization stress. The results showed that immobilization stress significantly altered the anxiety-like behaviors (open field test and elevated plus maze) and depression-like behaviors (tail suspension test) in both D3KO mice and their wild-type littermates. Moreover, further analysis of the data indicated that the D3KO mice, but not their littermates, failed to show a change in immobility time in the tail suspension test after the acute and chronic stress as compared to intact controls, suggesting an increased resistance to the immobilization stress given before behavioral tests. Although our study did not suggest a significant role of D3 receptor in regulating basal anxiety-like and depression-like behaviors, it demonstrated the mice lacking D3 receptor might be more resistant to stressful procedure than their WT littermates.

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1. Introduction

The pervasiveness of anxiety and depression has enormous costs for modern society. With a prevalence rate of approximately 5% worldwide, major depressive disorder (MDD) is the most common mood disorder [1]. MDD occurs in up to 60% of people with anxiety disorders [2]. Comorbid anxiety and depression is associated with more severe symptoms, greater functional impairment and persistent course of illness than either depression or anxiety alone [3]. Antidepressants such as selective serotonin reuptake inhibitors (SSRIs) are the most commonly prescribed drugs for the treatment of MDD and several anxiety disorders [4]. With regards to the poor prognosis of a large proportion of patients, further understanding of the neurobiology of depression and anxiety at the molecular and cellular level is necessary so that more effective treatments can be developed.

Stressful events appear to play a role in the onset, progression and treatment outcomes of several psychiatric disorders, including anxiety disorders and MDD [5–8]. Stress that induces
alterations in neuroendocrine systems and brain activity promotes the changes in physiological and behavioral responses [9, 10]. The hypothalamus–pituitary–adrenal (HPA) axis has been most frequently discussed as one of the major pathways through which the central nervous system exerts its influence on neural functions under stressful conditions [11, 12]. Increasing amounts of evidence implicated a putative role of dopamine system in the regulation of the HPA axis response to stress [13, 14]. The fact that exposure to stress results in enhanced dopamine release in limbic areas [15], and the observation that dopamine D1 and D2 receptor agonists activate the HPA axis [16] supports the assumption that dopamine plays a stimulatory role in the control of the neuroendocrine stress axis. A central issue in understanding the dopamine system in the stress-related process is to link various dopaminergic functions to different members of the two DA receptor classes, the D1 class (D1 and D5) and the D2 class (D2, D3, and D4) receptors [17].

The dopamine D3 receptor has recently been associated with anxiety-like behaviors in rats [18] and was postulated as a potential therapeutic target for depression [19]. Unlike the dopamine D2 receptor, which is widely distributed throughout the brain, the D3 receptor has a specific distribution to limbic areas, such as the nucleus accumbens (NAC), the olfactory tubercle, the islands of Calleja, and to a lesser extent, the dorsal striatum and hippocampus [17, 20]. Chronic antidepressant treatment has been reported to enhance D3 receptor binding in the rat brain [21]. Lammers et al. have shown a selective increase in dopamine D3 receptor gene expression after chronic treatment with fluoxetine and tranylcypromine, as well as chronic electroconvulsive therapy (ECT) [22]. Although these studies have focused on the potential role of D3 receptor in depression-like behaviors, the results from Chourbaji et al. do not indicate an evident involvement of the D3 receptor in the development of a depression-like phenotype in knockout studies [23].

Whereas the activation of the HPA axis is associated with dopamine release in limbic areas, the role of dopamine receptors, particular D3 receptor, in the control of the emotional behaviors under stress conditions is less clear. In this study, we used the immobilization stress model in different genotypes of mice, including D3 receptor knockout (D3KO) mice and their wild-type (WT) littermates, to examine the changes in anxiety-like and depression-like behaviors.

2. Materials and methods

2.1. Animal

The D3KO mice were previously generated by Xu et al. as described [20]. Homozygous mutant and WT littermates were produced from heterozygous breeding. The genetic background of the D3KO mice was initially 50% 129SvJ and 50% C57BL/6J, and was bred with C57BL/6J mice for three generations [24–26]. Mice 10–12 weeks of age were group housed in a temperature-controlled environment under a 12 h dark/light cycle and allowed to acclimate to new housing for at least 5 days before experimental manipulation. Only male mice were used in this study. All experimental procedures were approved by the Animal Care and Use Committee of Xi’an Jiaotong University.

2.2. Genotyping

To confirm the genotypes of D3KO and WT mice at the time of weaning, genomic DNA extracted from the tail was analyzed by PCR as described [20, 24]. The sequences of four oligonucleotide primers are: 5’-GCT CAC TAG GTA GTT G-3’; 5’-ACC CCT GAG CCA GAT AAG C-3’; 5’-GAT TCT GCA GGA TCA AAA ACC G-3’; and 5’-TTT TTC GCC AGC ACC AAG GTC C-3’.

2.3. Immobilization stress

To create a reproducible animal model of stress-induced anxiety-like and depression-like behaviors, we administrated a 1-day or 14-day immobilization stress to mice and subsequently performed behavioral assessments.

D3KO and WT mice were subjected to immobilization stress by restraint using a plastic cylinder with a diameter of 3 cm and height of 12 cm. For the acute immobilization stress (AIS) groups (2 groups/genotype for all behavioral testing), only one 1-h immobilization stress was applied, each behavioral test (mentioned in the 2.4 behavioral testing) was conducted 30 min after the immobilization. For the chronic immobilization stress (CIS) groups (2 groups/genotype for all behavioral testing), 1-h immobilization stress was applied once daily for 14 days, each behavioral test (mentioned in the behavioral testing) was conducted 23 h after the last immobilization. Control mice were handled as the stressed animal but without any stress.

2.4. Behavioral testing

Prior to each experiment, the animals were brought to the testing room for at least 30 min. Tests were conducted during the light cycle between 8:00 am and 3:00 pm. For all paradigms, 8–10 D3KO mice/experiment were used with a matched number of WT littermates. In the acute paradigm, behavioral tests were conducted as follows...
every two days with two days of rest without any treatment. In the chronic paradigm, behavioral tests were conducted every other day with a day of rest between each test while the CIS continued as previously mentioned. For each genotype, tests were applied in the following sequence, one group: novel cage test, elevated plus maze and tail suspension test; the second group: open field test, light–dark box and forced swimming test.

2.5. Open field test (OFT)

Mice were placed individually into a 45 × 45 × 45 cm open field chamber during a 1 h test session and data were recorded by a video tracking system (SMART, Panlab SL, Barcelona, Spain). The open field was divided into a central field (center, 20 × 20 cm) and an outer field (periphery). Total distance traveled in 1 h and numbers of entries to field’s central zone in first 10 min were measured.

2.6. Novel cage test (NCT)

Novel cage test is used to determine the exploratory behavior of an animal in a new environment by measuring vertical activity. Mice were placed into a new empty cage with a thin layer of sawdust in a brightly lit environment, and exploratory behavior was analyzed by counting the number of rearing for 6 min.

2.7. Light–dark box (L–D)

The light–dark box consisted of one brightly lit chamber (15 × 25 cm) connected by a small tunnel to a darkened second chamber (15 × 25 cm). Mice were placed into the light chamber and allowed to move freely to any of the chambers. Number of transitions between chambers was recorded for 6 min with a video tracking system (SMART, Panlab SL, Barcelona, Spain).

2.8. Elevated plus maze (EPM)

The elevated plus-maze consisted of two open arms (25 × 5 cm) and two closed arms (25 × 5 × 20 cm), and was elevated 50 cm from the ground. Mice were placed in the central platform (5 × 5 cm) which at the cross of the open and closed arms, and allowed to explore the maze for 6 min. The following variables were measured: numbers of entries to open arms and total time spent in the open arms.

2.9. Tail suspension test (TST)

Mice were suspended by the tails to the edge of a shelf 80 cm above the floor. The tail was secured to the shelf by adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time was recorded during a 6-minute session. The data of mice displayed tail climbing were removed from the test.

2.10. Forced swimming test (FST)

Mice were placed in 15 cm of water (22 ± 1 ℃) in Plexiglas cylinders (25 cm height × 15 cm diameter) for 6 min. The behavior of mice were recorded by a video tracking system (SMART, Panlab SL, Barcelona, Spain) and the immobility time was recorded during the 6-minutes session.

2.11. Statistical analysis

All data were expressed as mean ± S.E.M. Body weight data were analyzed by means of repeated measures (RM) two-way analysis of variance (ANOVA). The unpaired Student t-test was used to compare baseline data of NCT, L–D, OFT, EPM, TST and FST between D3KO and WT mice. Statistical differences among different stressed groups were estimated by Two-way ANOVA with a post hoc multiple comparison (Bonferroni t-test). A critical value for significance of p < 0.05 was used throughout the study.

3. Results

3.1. D3KO mice show normal development

As described initially by Xu et al. [20], we observed that D3KO mice show normal physiological development and inconspicuous behaviors under home cage conditions. There were no body weight differences between D3KO mice and their WT littermates on week 5, week 9 and week 13 from birth (supplemental Fig. 1).

![Fig. 2. Effect of AIS and CIS on anxiety-like behaviors of D3KO mice. (A) The number of rearing in the novel cage test (NCT) and (B) the number of transitions in the light–dark box test (L–D) were unchanged under basal, AIS and CIS conditions. For the elevated plus maze (EPM), the number of entries to the open arm (C) and percent time in the open arm (D) were significant increased under CIS but not AIS condition. Values represent mean ± SEM (n = 8–10 for each group). *p < 0.05, WT CIS versus WT baseline; #p < 0.05 D3KO CIS versus D3KO baseline.](image)
3.3. Effects of D3 receptor knockout on basal locomotor, anxiety-like and depression-like behavior

To evaluate the role of D3 receptors in modulating baseline locomotor activity (OFT), anxiety-related behaviors (NCT, L–D, OFT and EPM) and behavioral despair (TST and FST), we determined the baseline activity of both the D3KO and the WT mice in the behavioral assays. In the OFT, examination of the time course of activity indicated that the D3 mutants were significantly more active during the first 10 min of the test (Fig. 1B; $t = 3.970; df = 13; p = 0.0016$), whereas there was no significant difference between the two groups when the entire 1 h was analyzed (Fig. 1A; $t = 0.1223; df = 13; p = 0.9046$). Moreover, D3KO group showed more entries into the field’s central region (Fig. 1C; $t = 2.678; df = 13; p = 0.019$). There were no significant differences between D3 mutants and WT littermates in other anxiety-like behaviors such as NCT (Fig. 2A), L–D (Fig. 2B) and EPM (Fig. 2C and D). For baseline depressive-related behaviors, no significant differences were observed in the TST (Fig. 3A) and FST (Fig. 3B).

3.3. Effects of AIS and CIS on the anxiety-like behaviors in D3KO mice

The NCT, L–D, OFT and EPM were performed to determine the anxiety level of the D3KO and WT mice under acute and chronic immobilization stress. For the three parameters measured in the OFT, a significant interaction effect (but neither genotype nor treatment effects) was found in the distance traveled in the first 10 min [Fig. 1B; $F_{(2,39)} = 5.179, p < 0.05$], whereas there is no significant genotype, treatment or interaction effect in total distance traveled (Fig. 1A) or numbers of entries to the central zone (Fig. 1C). Post hoc Bonferroni tests revealed highly significant effects in comparison with no-stress treatment for AIS and CIS respectively ($p < 0.05$, Fig. 1B) for the distance traveled in the first 10 min. There were no significant interaction, genotype or treatment differences among groups in the NCT (Fig. 2A), L–D (Fig. 2B). In the EPM, the treatment was statistically significant in numbers of entries to the open arms [Fig. 2C; $F_{(2,38)} = 14.62, p < 0.001$] and in the percent time spent in the open arms [Fig. 2D; $F_{(2,38)} = 14.94, p < 0.001$]. As shown in Fig. 2C and D, the CIS mice exhibited a 2.7-fold increase in numbers of entries to the open arms and 2.2-fold increase in the percent time spent in the open arms. However, there was no genotype or interaction effect on these parameters in the EPM.

3.4. Effects of AIS and CIS on the depression-like behaviors in D3KO mice

The TST and FST were applied to measure the depression-like characteristics of the mice lacking D3 receptor under stress conditions. As shown in Fig. 3A, CIS animals showed a significant reduction in the immobility time during the 6 min in the TST [$F_{(2,39)} = 15.13, p > 0.001$], although no genotype effect was found [$F_{(1,39)} = 0.121, p > 0.05$]. Significant differences between stressed group and no-stress controls were found in WT mice (for both AIS and CIS, $p < 0.05$), but not in the D3KO mice (Fig. 3A). There was no significant difference among groups in the FST (Fig. 3B).

4. Discussion

Accumulating evidence implicates stress as an important factor in the onset of many psychiatric disorders, such as MDD and anxiety disorders. Dopamine receptor-mediated signal transmission, along with serotonin, adrenaline/noradrenaline, and histamine systems, has long been linked to stress responsiveness, but the role of specific dopamine receptors is unclear. Here we show that a battery of tests measuring basal anxiety- and depression-like behaviors uncovered no phenotypic abnormalities in D3KO mice. Compared with WT littermates, D3KO mice exhibit a resistance to behavioral changes induced by AIS and CIS in the TST. Thus, our data support the involvement of the D3 receptor in the stress-related responses in rodents and the D3 receptor as a potential target for pharmacotherapy of stress-related disorders need be further investigated.

There is evidence that dopamine D2 class receptors but not D1 class receptors are involved in the regulation of anxiety. Previous pharmacological studies have indicated that sulpiride, the D2/D3 receptor antagonist, produces an anxiolytic effect in the PPM, while quinpirole, the D2/D3 receptor agonist, induces an anxiety-like behavioral response at the higher dose (0.5 mg/kg)[27]. However, the D1 receptor agonists or antagonists appear to have no effect on the performance of the task[27]. Possible functions of D3 receptors mediating anxiety-like behaviors have also been suggested in knockout studies, where an increased central locomotion in OFT and open arm exploration in EPM was observed[28]. Our findings that D3KO mice show greater activity in the initial period of the OFT, while deletion of D3 receptor has no effect on the exploratory behavior when the entire time period was analyzed are in agreement with former reports[20,24]. Lack of significant changes in NCT, L–D and EPM, in D3KO mice, as demonstrated in the present and past studies[23], suggests that dopamine D3 receptor do not play a critical role in regulating anxiety-like behaviors. The greater activity of D3KO mice displayed in OFT could be attributed to enhanced locomotor responses to novel environments relative to controls. However, several studies on mice generated by Accili and...
co-workers revealed reduced thigmotaxis in the OFT and anxiety-related behavioral changes on the EPM [28–30]. The reasons for the dissimilar results of D3KO mice on locomotion and anxiety are not apparent, but it should be noted that these studies utilized mice with different genetic background, which may have contributed to the observed discrepancy. Moreover, the D3KO mice exhibited normal basal anxiety-like behaviors were generated by targeted disruption of the exon 1 of the dopamine D3 receptor gene in the present studies and others, whereas former studies on mice generated by Accili and co-workers disrupted the D3 gene at sequences coding for the second intracellular loop of the receptor. It cannot be excluded that the truncated forms of the D3 receptors may have an effect on anxiety-like behaviors, although the full-length functional D3 receptors was undetectable in the different mutant strains by different means. Also, it is possible but unlikely that other factors (such as environmental differences and variations in test protocols) accounts for the difference in results.

Pharmacological studies have suggested that D3 receptor stimulation exhibit an antidepressant-like effect. 7-OH-DPAT, a preferential dopamine D3 receptor agonist, showed antidepressant-like effect in the FST at higher doses in rats. Co-treatment with 7-OH-DPAT and imipramine resulted in a stronger effect in FST than administration of either drug alone [31]. BP 897, a partial D3 receptor agonist, showed no effect in the FST when given alone but it enhanced the antidepressant-like effect of imipramine [31]. Furthermore, pramipexole, a dopamine D2/D3 receptor agonist, alleviates the depressive symptoms in patients suffering from Parkinson’s disease [32,33]. Previous studies have also indicated that pramipexole produce an antidepressant-like effect in animal models of depression such as mouse FST and the olfactory bulbectomy rats [21,34]. Siciak and Fujiwara have argued that the antidepressant-like effects of pramipexole were mediated by D2 rather than D3 receptors because these effects could be blocked by pretreatment with D2 receptor selective antagonist, whereas not by pretreatment with D3 receptor selective antagonist nor in D3KO mice [35]. Consistent with previous studies [23,35,36], we found that D3KO mice showed normal behavioral responses to forced swim stress. In addition, just as in FST, mice lacking the D3 receptor exhibited similar immobility duration under the less stressful situation in TST. Based on these results, we propose that D3 receptors are unlikely to be involved in depression-like behaviors and we believe that the putative ligands of the D3 receptors that have been used to draw conclusions about the functional roles lack the necessary in vivo selectivity to support such views. Although the constitutive knockout mice may provide valuable insights into the possible functions of the missing D3 receptors, a major problem which could hinder the interpretation of any phenotype is the fact that developmental complications may have taken place during mouse development. Future studies using a D3 receptor knockdown strategy (e.g., viral expression of D3 receptor shRNA or D3 receptor conditional/inducible deletion mutant mice) might be helpful to resolve this issue.

Although lack of D3 receptors did not evoke alterations in basal emotional behaviors in mice, the performances of D3KO mice under the stressful situation were probably different. For example, the duration of immobility time in the FST in mice injected with vehicle (distilled water) was significantly increased compared with that in the untreated animals. However, vehicle-injected D3KO mice failed to show such an increased in FST as compared to WT controls [36], suggesting an increased resistance to injection-induced stress before. In rodent models, immobilization stress is known to be a severe stressor which can activate the HPA axis, accelerate dopamine synthesis in mesolimbic dopamine neurons and enhance dopamine release in limbic brain regions [37–40]. Recent work has shown that dopamine, acting through both D1 and D2 receptors, exert stimulatory effect on the activation of the HPA axis in response to immobilization stress [41]. The D2-like receptors take part in the central mechanisms of dopamine inactivation of the HPA axis in response to stress as the effect of pergolide, a blood-brain-barrier (BBB)-permeable D2-like receptor agonist, was not blocked by the D2-like receptor antagonist domperidone, which does not cross the BBB [42]. In the present study, D3KO mice displayed a resistance to the acute and chronic immobilization stress compared with their littermates in the TST, suggesting the involvement of D3 receptor in modulating stress-related responses. Given a large number of D3 receptor-containing neurons co-express D1 receptors in limbic brain regions (such as NAc), one possible explanation for the observation is that the D1 and D3 receptors exhibit synergistic effects on stress-induced behavioral changes and the dopamine mediated-activation of the HPA axis could be inhibited by the dysfunction of D3 receptors. Otherwise, a higher basal dopamine level in the synapse due to the lacking D3 autoreceptors might dampen the stress responses in mice since high tonic dopamine in NAc affects the coping strategies against stressors [43].

In summary, to determine whether the D3 receptor mutation altered the emotional behavioral response induced by stress, we compared the behavioral effects of A5 or C5 on the D3KO and WT mice using a well-established behavioral test battery. Our results provide no evidence that dopamine D3 receptors play an important role in mediating the basal anxiety-like and depression-like behaviors. However, the D3KO mice exert a certain resistance to the immobilization stress in the former, indicating a modulatory role of D3 receptor on the stress-related responses. The regulating mechanisms on the molecular level between D3 receptor signaling and critical hormones in NAc neurons should be focused in the future studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2013.01.019.

References
