Research Report

Pitx3 deficient mice as a genetic animal model of co-morbid depressive disorder and parkinsonism

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Article info

Article history:
Accepted 15 January 2014

Keywords:
Parkinson’s disease
Depression
Stress
c-Fos

Abstract

Approximately 40–50% of all patients with Parkinson’s disease (PD) show symptoms and signs of depressive disorders, for which neither pathogenic understanding nor rational treatment are available. Using Pitx3-deficient mice, a model for selective nigrostriatal dopaminergic neurodegeneration, we tested depression-related behaviors and acute stress responses to better understand how a nigrostriatal dopaminergic deficit increases the prevalence of depressive disorders in PD patients. Pitx3-deficient mice showed decreased sucrose consumption and preference in the two-bottle free-choice test of anhedonia. Acute restraint stress increased c-Fos (known as a neuronal activity marker) expression levels in various brain regions, including the prefrontal cortex, striatum, nucleus accumbens, and paraventricular nucleus of the hypothalamus (PVN), in both Pitx3+/+ and −/− mice. However, the stress-induced increases in c-Fos levels in the cortex, dorsal striatum, and PVN were significantly greater in Pitx3−/− than +/+ mice, suggesting that signs of depressive disorders in parkinsonism are related to altered stress vulnerability. Based on
1. Introduction

Depressive disorders occur in greater than 40% of patients with Parkinson’s disease (PD; Cummings, 1992; Lemke, 2008). For reasons independent of the motor deficits, the quality of life in depressed PD patients is markedly reduced (Kuopio et al., 2000). Measurement and diagnosis of depressive disorders in PD is difficult due to the considerable overlap between the symptoms and signs of the two illnesses (Schrag, 2006; McDonald et al., 2003). As we mentioned in a previous study (Ardayfi et al., 2008), the increased prevalence rate of non-motor symptoms, such as depressive disorders, in PD patients may be caused by biochemical and behavioral abnormalities in coping with stress (Charlett et al., 1998; King et al., 1997).

Stress may play a critical role in the development of PD related and other depressive disorders (Hennerle et al., 2012). Animals subjected to maternal separation stress during the neonatal period can have depression-related signs and increased motor deficits as assessed by behavior. Furthermore, adult rats treated with 6-hydroxydopamine to induce dopaminergic cell death (Matthews and Robbins, 2003; Pienaar et al., 2008) and individuals with PD show stress-induced reduction in hedonic responses (Macht et al., 2007). Restraint stress and elevated corticosterone levels can enhance nigral neuronal loss and motor symptoms in a rat PD model (Smith et al., 2008) and induce depression-related signs (Kim and Han, 2006; Wolkowitz et al., 2009). Hypersecretion of cortisol and dysfunction of the hypothalamic–pituitary–adrenal (HPA) axis are seen in both PD and patients with depressive disorder when compared to healthy age-matched controls (Charlett et al., 1998; Pariante and Miller, 2001). However, animal model systems to investigate the comorbidity of depressive disorders and parkinsonism are not well established.

Pitx3 is a transcription factor important for the appropriate development and survival of midbrain dopaminergic neurons in mammals (Smidt et al., 2000; Chung et al., 2005; Vasudevan et al., 2012). Pitx3 gene expression is restricted to the developing eye and DA progenitor cells formed from embryonic day 11 throughout adult life in mice (Gage et al., 1999; Smidt et al., 1997). In the brain, cytoplasmic TH and nuclear Pitx3 are co-expressed only in the SN and VTA neurons (van den Munchhof et al., 2003; Maxwell et al., 2005). Thus, Pitx3’s expression is specific in midbrain dopaminergic neurons. Pitx3-deficient mice (aphakia mice; ak) contain a double-deletion mutation in the Pitx3 gene, a minor 652 bp deletion located 2.5 kb upstream of the transcription start site and a major 1423 base-pair deletion in the upstream promoter region. Pitx3 mice have arrested lens development (Rieger et al., 2001) and selective loss of dopamine neurons in the substantia nigra (SN). Although DA cells in VTA and A8 of newborn mice are intact (Hwang et al., 2003; Nunes et al., 2003; van den Munchhof et al., 2003), these are progressively affected postnatally (Kas et al., 2008; van den Munchhof et al., 2003).

Biochemical, behavioral, and pharmacological data indicating parkinsonism phenotypes in ak mice have been reported (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004; Hwang et al., 2005; van den Munchhof et al., 2006; Kas et al., 2008; Ding et al., 2007). Furthermore, impairments of striatal-dependent cognition and unique behavioral responses to psychotropic drugs have also been reported in Pitx3-deficient mice (Hwang et al., 2005; Ardayfi et al., 2008, 2010). Whether there are any signs of depression or alterations in the response to stress in Pitx3-deficient mice has not yet been investigated. Approximately 40–50% of all patients with Parkinson’s disease (PD) show symptoms and signs of depressive disorders. The underlying mechanisms of depression-like behavior in PD patients are not known and it is probably caused by complex mechanisms including the affected nigrostriatal pathway as well as other neuronal systems. In aphakia mice, our data suggest that the impaired nigrostriatal system directly contributes to this behavior because of its selective degeneration. It is interesting to note that not all animal models of PD (e.g., as toxin-based and genetic models) exhibit depressive phenotypes. Here, we examined anhedonia (the loss of capacity for pleasure) to analyze depression-like signs in Pitx3+/– mice. Stress-induced c-Fos expression patterns (a marker of neuronal activity) were examined in various brain regions, including the cortex, striatum, nucleus accumbens, and paraventricular nucleus of hypothalamus (PVN). In addition, blood levels of stress hormone were determined. We found that Pitx3+/– mice exhibit a markedly increased susceptibility to stress as well as depression-related signs.

2. Results

2.1. Pitx3+/– mice (ak mice) display depression-like behavior in the sucrose preference test

To determine whether Pitx3+/– mice show depressive-related phenotypes, we performed the sucrose preference test in Pitx3+/+ and +/- mice. Two-way ANOVA analysis on consumption of sucrose demonstrated the main effects of genotype ($F_{(1,56)}=8.55$, $P=0.0111$) and sucrose concentration ($F_{(4,56)}=64.14$, $P<0.0001$), but there was no genotype x sucrose concentration interaction ($F_{(4,56)}=2.40$, $P=0.0611$) (Fig. 1A). The effect of sucrose concentration ($F_{(4,56)}=25.56$, $P<0.0001$) and genotype x sucrose concentration interaction ($F_{(4,56)}=3.03$, $P=0.0248$) on water consumption were significant; however, there was no genotype effect ($F_{(1,56)}=4.02$, $P=0.0648$) (Fig. 1B). We also observed a significant effect of genotype ($F_{(1,56)}=4.90$, $P=0.044$) and sucrose concentration ($F_{(4,56)}=64.58$, $P<0.0001$)}
but there was no genotype × sucrose concentration interaction ($F_{(4,56)}=1.92$, $P=0.12$) in the sucrose preference ratio (Fig. 1C).

Chronic antidepressant treatment can reverse some depression-related behaviors in mouse models of depressive disorders (Krishnan and Nestler, 2008, 2011; Wallace et al., 2009). We therefore examined whether this depression-related phenotype caused by nigrostriatal dopamine deficits in Pitx3−/− mice could be improved with antidepressant treatment. Two-way ANOVA analysis on sucrose preference demonstrated the main effects of imipramine ($F_{(4,40)}=10.15$, $P=0.0097$) and sucrose concentration ($F_{(4,40)}=11.58$, $P<0.001$), but there was no imipramine × sucrose concentration interaction ($F_{(4,40)}=1.68$, $P=0.172$) in the vehicle and imipramine-treated Pitx3+/− mice (Fig. 1F). The effect of sucrose concentration ($F_{(4,40)}=35.76$, $P<0.0001$) and imipramine effect ($F_{(4,40)}=5.06$, $P=0.0481$) on sucrose consumption was significant in the vehicle and imipramine-treated Pitx3+/− mice (Fig. 1D).

Chronic imipramine treatment of Pitx3+/+ mice showed no significant effects on sucrose consumption ($F_{(4,48)}=0.00$, $P=0.974$) and on sucrose preference ($F_{(4,48)}=0.24$, $P=0.632$, Fig. 1D and F).

The effect of imipramine (Pitx3+/+ mice, $F_{(4,48)}=0.12$, $P=0.7308$; Pitx3−/− mice, $F_{(4,40)}=4.01$, $P=0.0732$) on water consumption was not significant in either genotype, but sucrose concentration effect (Pitx3+/+ mice, $F_{(4,48)}=22.64$, $P<0.0001$; Pitx3−/− mice, $F_{(4,40)}=12.99$, $P<0.0001$) was significant in Pitx3+/+ mice and Pitx3−/− mice and indicated groups at $P<0.05$ and $P<0.01$ and * denotes significant differences between vehicle and imipramine in Pitx3−/− mice at $P<0.05$, respectively (Bonferroni’s post hoc analysis after two-way ANOVA).

2.2. Stress-induced c-Fos expression in the dorsal striatum and nucleus accumbens

To analyze stress-dependent neural activity, alterations in c-Fos protein expression levels were examined following the exposure of mice to restraint stress. Pitx3+/+ and −/− mice were restrained for 2 h, and then c-Fos induction in both genotypes was detected in the dorsal and ventral (nucleus accumbens) striatum (Fig. 2). A two-way ANOVA test revealed that an interaction occurred between the two genotypes (genotype, $F_{(1,39)}=18.39$, $P=0.001$; stress effect, $F_{(1,39)}=85.16$, $P<0.001$; interaction, $F_{(1,39)}=18.55$, $P<0.001$) in the dorsolateral striatum of the brain (Fig. 2A). In the dorsomedial striatum of the brain, a two-way ANOVA revealed that an interaction also occurred between the two genotypes (genotype, $F_{(1,40)}=40.93$, $P<0.001$; stress effect, $F_{(1,40)}=129.01$, $P<0.001$; interaction, $F_{(1,40)}=46.16$, $P<0.001$) (Fig. 2B). In the NAC, a two-way-ANOVA revealed overall effects of both stress and genotype; however, there was no interaction seen between genotype and stress (genotype, $F_{(1,40)}=11.58$, $P=0.0015$; stress effect, $F_{(1,40)}=113.03$, $P<0.001$; interaction, $F_{(1,40)}=2.41$, $P=0.128$) (Fig. 2C). A significant difference was
found between genotypes when c-Fos expression levels in the striatum and NAc were compared from mice that had been exposed to stress ($P<0.01$). In these brain regions, control levels of c-Fos expression did not differ between genotypes ($P>0.05$).

### 2.3. Stress-induced c-Fos expression in the prefrontal cortex and PVN

In the ventral aspect of medial prefrontal cortex, a two-way ANOVA revealed that an interaction occurred between the two genotypes (genotype, $F_{(1,27)}=4.26$, $P=0.0487$; stress effect, $F_{(1,27)}=58.94$, $P<0.001$; interaction, $F_{(1,27)}=4.81$, $P=0.0371$) (Fig. 3A, D, and E).

In the prefrontal cortex, a two-way ANOVA revealed that an interaction occurred between the two genotypes (genotype, $F_{(1,23)}=16.45$, $P<0.001$; stress effect, $F_{(1,23)}=148.96$, $P<0.001$; interaction, $F_{(1,23)}=15.38$, $P<0.001$) (Fig. 3B, D, and E). c-Fos induction levels after stress exposure in the prefrontal cortex of Pitx3$^{-/-}$ mice were significantly higher than from wild-type mice ($P<0.01$).

c-Fos expression levels in the PVN of the hypothalamus demonstrated the main effects of genotype ($F_{(1,18)}=5.34$, $P<0.05$) and stress ($F_{(1,18)}=243$, $P<0.001$), and a significant genotype $\times$ stress interaction ($F_{(1,18)}=8.87$, $P<0.001$) (Fig. 3C, D, and E). Stress-induced levels of c-Fos expression were significantly increased in the PVN of Pitx3$^{-/-}$ mice relative to wild-type mice ($P<0.01$).

### 2.4. Stress-induced corticosterone levels in plasma of Pitx3$^{-/-}$ mice

To determine the susceptibility of Pitx3$^{-/-}$ mice to stress, mice were subjected to restraint for 2 h and plasma corticosterone levels were measured. Plasma corticosterone levels in unstrained Pitx3$^{-/-}$ mice were higher than in unstrained Pitx3$^{+/+}$ mice, but it was not significant (Fig. 4; $P>0.05$; one-way ANOVA, Tukey’s multiple comparison test). Two hours of restraint stress significantly increased plasma corticosterone levels in both genotypes ($P<0.001$, one-way ANOVA, Tukey’s multiple comparison test). Although the basal level of corticosterone was higher in Pitx3$^{-/-}$ mice than Pitx3$^{+/+}$ mice, it was statistically insignificant. Interestingly, stress-induced
corticosterone level was significantly higher in Pitx3−/− mice compared to stress-induced Pitx3+/+ mice (Pitx3+/+ mice, 241.6 ng/mL ± 24.5; Pitx3−/− mice, 342.0 ng/mL ± 22.0, *P < 0.001, one-way ANOVA, Tukey’s multiple comparison test) (Fig. 4). Two-way ANOVA analysis on corticosterone level demonstrated the main effects of genotype (F(1,18) = 16.28, P < 0.001) and stress (F(1,18) = 164.18, P < 0.0001), but there was no genotype × stress interaction (F(1,18) = 1.67, P = 0.2126).

3. Discussion

Pitx3−/− (ak) mice have previously been reported as a useful genetic animal model of parkinsonism, as they have selective nigrostriatal dopaminergic cell death and motor dysfunction (Hwang et al., 2003, 2005; Nunes et al., 2003; Smidt et al., 2004; van den Munckhof et al., 2006; Kas et al., 2008; Ding et al., 2007). Here, we demonstrate that Pitx3 deficiency in mice results in depression-related signs. Anhedonia seen in Pitx3−/− mice using the sucrose preference test can be reversed by chronic treatment with an antidepressant. Interestingly, while both Pitx3+/+ and −/− mice showed significant increases in c-fos expression levels in various brain regions in response to restraint stress, these increases were significantly greater in the cortex, dorsal striatum, and PVN of Pitx3−/− mice. These data suggest a role for the nigrostriatal pathway in responding to stressful situations and in maintaining mood.

c-Fos is an immediate early gene (IEG) that is activated in the brain by various external stressors (Senba and Ueyama, 1997). c-Fos induction upon emotional or physical challenges has been reported to be a functional marker for activated neurons (Kovács, 1998). Anatomical mapping of IEG, such as c-Fos, expression levels in the brain is useful for identifying...
the susceptibility to stressors and the hereditary predisposition to stress-related diseases for an individual (Senba and Ueyama, 1997). The prefrontal cortex is known to be a region that is responsive to stressful stimuli (Cullinan et al., 1995; Girotti et al., 2006). In response to stress, Pitx3+/− mice showed a greater increase in c-fos expression levels in the cingulate and motor cortex subregions of the prefrontal cortex when compared to Pitx3+/+ mice (Fig. 3). The medial part of the prefrontal cortex (medial prefrontal cortex; mPFC) plays an important role in regulating the HPA axis response to stress (Diorio et al., 1993). c-Fos activation levels in the ventral mPFC regions in response to restraint stress were also increased in Pitx3+/− mice when compared with wild-type mice (Fig. 3A). However, lesion studies suggest that the ventral mPFC inhibits stress-induced HPA responses (Diorio et al., 2006; Radley et al., 2006). Furthermore, exposure to restraint stress resulted in increased c-fos expression levels in the dorsal striatum (Fig. 2). The stress-induced c-Fos expression levels of dorsomedial striatum is higher than that of dorsolateral striatum in both genotypes. In addition, stress-induced activation of neurons in these areas increased to a greater extent (Fig. 2A and B) in Pitx3+/− mice compared to wild-type control mice, whereas stress-induced activation of neurons in NAc was increased to a lesser extent in Pitx3 deficient mice (Fig. 2C). Degeneration of dopaminergic nigral neurons causes a cascade of functional modifications and increased activity of the excitatory neurotransmitter, glutamate (Blandini et al., 1996). Pitx3+/− mice have an intact behavioral response to the non-competitive NMDA receptor antagonist MK-801 and slightly increased locomotor activity during the day (Ardyfio et al., 2010). Therefore, while glutamate is likely to be involved in the stress-induced increase in neural activity in the dorsal striatum (Moghaddam et al., 1993), further work is required to determine which neurotransmitter systems are modulated in response to stress in the NAc and other areas of the striatum in Pitx3+/− mice.

Forced swim and tail suspension tests are widely used to find depression-related phenotypes in rodents (Cryan and Mombereau, 2004). Our experiments using Pitx3+/− mice revealed an abnormal vertical swimming behavior during the forced swim test and a hind-limb claspng behavior during tail suspension test, associated with motor impairment (Supplemental Fig. 1). The hind limb clamping may be indicative of some sort of rigidity or spasticity. Hind limb clamping is revealed in mice with neurodegenerative disorders (Carter et al., 1999; Takahashi et al., 2010), indicating neurological dysfunction of the central motor control system. Pitx3+/− mice show severe motor deficits in behavior tests that are sensitive for the nigrostriatal pathway (Hwang et al., 2005). In this study, we assessed anhedonia, the inability to feel pleasure, as a sign of depression (Fig. 1). We found decreases in sucrose intake and preference in Pitx3+/− mice, which were relieved by chronic administration of an antidepressant (Fig. 1D, E and F). These studies suggest a role for A9 dopaminergic neurons in mediating mood and behavioral response to a tricyclic antidepressant (TCA). Treatment with the dopaminomimetic compound, pramipexole, slightly decreased anhedonic responses in Pitx3+/− mice (Supplemental Fig. 2). These results indicate that the antidepressive effects observed in Pitx3+/− mice with chronic imipramine treatment (Fig. 1) may be related to neurotransmitter systems, involving dopamine signaling. Studies have reported that depressive behavior can result from genetic predispositions and environmental stress (Nestler et al., 2002). Resilience, or stress-resistance, is mediated by neurobiological adaptive changes involving numerous neurotransmitter and molecular systems (Feder et al., 2009). PD patients with dopamine depletion in the striatum also have various neurological abnormalities including decreased availability of acetylcholine and monoamine neurotransmitters, abnormal protein aggregations, mitochondrial dysfunction, and heightened responses to inflammatory and oxidative stimuli (Caviness et al., 2011). These changes in the brain may cause altered neurological or behavioral reactions to environmental stress in PD patients. Interestingly, it was also demonstrated using ak mice that the nigrostriatal DA circuit plays a role in maintaining normal responsiveness to psychostimulants and antipsychotic drugs (Ardyfio et al., 2010). Here, we demonstrated that ak mice, which have phenotypes characteristic of parkinsonism, show an abnormal ability to cope with restraint stress. Stress-induced neural activity is increased in the prefrontal cortex, dorsal striatum, and PVN regions of Pitx3+/− mice, but decreased in the NAc (Figs. 2 and 3) relative to wild-type mice. The increased PVN activity, a region important for the stress response, may underlie the overactive HPA axis in Pitx3+/− mice (Fig. 4). Taken together, Pitx3+/− (ak) mice may serve as a useful genetic animal model to further investigate the relation between the nigrostriatal degeneration and stress responses and depressive behavior.

Please cite this article as: Kim, K.-S., et al., Pitx3 deficient mice as a genetic animal model of co-morbid depressive disorder and parkinsonism. Brain Research (2014), http://dx.doi.org/10.1016/j.brainres.2014.01.023
Gender differences in susceptibility to stress and effects of anti-depressant have been reported (Xing et al., 2013). Ovarian hormones can affect baseline locomotor activity levels, and anxiety-/depression-like behavior (Barros and Ferigolo, 1998; Elliott and Grunberg, 2005; Mora et al., 1996). Monoamine neurotransmitter systems, dopamine, serotonin, and noradrenaline, are regulated by the ovarian cycle and sex differences in monoamine concentration in various brain regions, including prefrontal cortex, striatum, and nucleus accumbens, were observed (Simpson and Kelly, 2012). The evidences for gender differences in various commonly employed behavioral, physiological, and neurochemical parameters suggest that males and females perceive and respond differentially to same stimuli. While the present study does not address the gender differences in the stress and anti-depressant treatment, these studies warrant further investigations.

Despite a high prevalence of depressive disorders in PD patients, a combined depression/parkinsonism animal model is not available. In this study, Pitx3−/− mice that have depression-related signs were responsive to treatment with an antidepressant, imipramine, as is observed in animal models of depression and patients. Moreover, abnormal responses to acute stress were revealed in Pitx3−/− mice. An altered response to stress caused by nigrostriatal dopamine loss may underlie non-motor PD symptoms and signs. Accordingly, we suggest that the Pitx3-deficient mouse model is useful for the study of potential pathogenic mechanisms and therapeutic targets for depression in PD patients and for exploring the functional neuroanatomy underlying the response to stressful situations in PD patients.

4. Experimental procedure

4.1. Animals

Pitx3-deficient aphakia mice used in this study were acquired from Jackson Labs (Bar Harbor, ME, USE). Mice were transferred to and then expanded in the Animal Care Facility at McLean Hospital (described in Hwang et al., 2005). Male aphakia mice were crossed to female C57BL/6j mice to generate heterozygous offspring. Heterozygotes were then intercrossed to generate Pitx3+/−, +/+ and −/− mice. From these crosses, Pitx3+/+ and −/− mice were used in this study. All mice were housed in regular polycarbonate plastic cages in a temperature (21–22°C) and humidity (50–60%) controlled environment with a 12 h light/dark cycle (lights on from 0700 to 1900 h) and were maintained on an ad libitum diet of standard lab chow (Hanlan Laboratory, Inc., Seoul, Korea) and water. All materials used to house animals were autoclaved, and gamma-irradiated lab chow was supplied. Animal rooms were maintained in specific-pathogen-free (SPF) conditions. Experimental mice were weaned at 4 weeks of age and socially housed (2–4 per cage). Males ranging in age from 10 to 16 weeks-old and weighing between 23 and 30 g were used for all main experiments (except those of Supplemental Fig. 3 in which female mice were used). All animal experiments were approved and conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology (KRIIB, Daejeon, Korea).

4.2. Drugs

Imipramine hydrochloride and sucrose were purchased from Sigma Aldrich (St. Louis, MO, USA). Imipramine and sucrose were dissolved in saline and drinking water, respectively. Mice were subjected to once daily injections of imipramine (20 mg/kg, intraperitoneal injection) or vehicle control for 14 days. Treatment with 20 mg/kg/day imipramine for 14 days was sufficient to reveal the anti-depressive effect in C57BL/6j mice showing depressive phenotype (Kim and Han, 2006).

4.3. Sucrose preference test

The sucrose preference test was performed as previously reported (Wallace et al., 2009). Animals were given the choice between water and a sucrose solution after an initial habituation to two bottles of water for 5 days in the home cage. At the start of the experiment, animals were allowed unlimited access to water in one bottle and ascending concentrations of sucrose (0%, 0.25%, 0.5%, 1% and 2% solutions; wt/vol; 24 h at each concentration) in the other bottle. To prevent the possible effects of a side-preference in drinking behavior, the position of the bottles in the cage was switched after 24 h during the test. The total volume of liquid consumed was measured each day by weighing the bottles before and after testing. In the experiment with antidepressant treatment, the sucrose preference test was performed after chronic treatment with imipramine for 14 days. Imipramine was also administered during the testing period. Sucrose preference was indicated as a ratio of volume of sucrose solution consumed relative to the total volume of fluids consumed.

4.4. Restraint stress

The restraint protocol used in this study was performed as previously described (Kim and Han, 2006, 2009). To avoid any nonspecific response all mice were initially handled for a week. To minimize external noise, vibration, and cross contamination between control and stress groups, animal cages were separated by physical barriers during treatment. Male mice weighing 22–25 g were individually placed head first into well-ventilated 50 ml polypropylene conical tubes. Non-stressed control mice were placed in their home cages before sacrifice. Mice were then completely restrained in this device for 2 h. Blood collection and transcardiac perfusion were performed immediately after restraint stress (Kim and Han, 2006).

4.5. Corticosterone assay

The corticosterone assay used in this study has been previously described (Wallace et al., 2009). Animals were given the choice between water and a sucrose solution after an initial habituation to two bottles of water for 5 days in the home cage. At the start of the experiment, animals were allowed unlimited access to water in one bottle and ascending concentrations of sucrose (0%, 0.25%, 0.5%, 1% and 2% solutions; wt/vol; 24 h at each concentration) in the other bottle. To prevent the possible effects of a side-preference in drinking behavior, the position of the bottles in the cage was switched after 24 h during the test. The total volume of liquid consumed was measured each day by weighing the bottles before and after testing. In the experiment with antidepressant treatment, the sucrose preference test was performed after chronic treatment with imipramine for 14 days. Imipramine was also administered during the testing period. Sucrose preference was indicated as a ratio of volume of sucrose solution consumed relative to the total volume of fluids consumed.
This assay has low cross-reactivity with other major steroid hormones and a detection limit of approximately 30.0 pg/ml.

4.6. Immunohistochemistry

Mice were transcardially perfused with PBS followed by 4% formaldehyde in PBS. Perfused brains were dissected; post-fixed overnight, and then cut into 40 μm coronal sections on a vibratome (Leica, Vibratome VT1000A). Sections were incubated in 5% normal fetal bovine serum in PBS for 1 h at RT and then incubated with rabbit anti-c-Fos antibody (1:1000, Santa Cruz) for 16 h at 4°C. Immunohistochemistry was then performed using biotinylated secondary anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA), avidin-biotinylated peroxidase complex (ABC kit, Vector Laboratories), and 3,3’-diaminobenzidine (Sigma, Saint Louis, MO, USA). Sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior Sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior Sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior Sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior Sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior Sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior Sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior Sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior Sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior Sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior Sections containing the paraventricular nucleus of the hypothalamus (PVN) at sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior Sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior Sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior Sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior Sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior Sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior Sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior Sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior Sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior Sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior

1.0 mm), and nucleus accumbens (NAc; 0.37 mm ventral aspect of medial prefrontal cortex (mPFCv, 0.37 mm anterior to bregma were also used. c-Fos positive nuclei from both hemispheres were counted. Sections containing the paraventricular nucleus of the hypothalamus (PVN) at anteroposterior –0.8 mm from the bregma were also used. c-Fos positive cells were evaluated under the microscope. Qualitatively, valuations of c-Fos induction levels were performed manually in a blind manner in terms of genotypes and restraint applied, by following the procedure introduced by Kim and Han (2009). Males, groups of 5–12 animals, and 2–3 sections for each region per each animal, were used for counting.

4.7. Statistical analysis

GraphPad PRISM software (GraphPad Software Inc., CA, USA) was used to perform statistical analyses. One-way (corticosterone assay) or two-way ANOVA tests (sucrose preference test, genotype × sucrose concentration; c-Fos induction assay, genotype × stress effect; and corticosterone assay, genotype × stress effect), followed by Tukey’s and Bonferroni post hoc tests, were performed. All data were presented as the mean ± S.E.M. and statistical differences at the 5% level were considered significant.

Acknowledgments

This work was supported by a Grant from KRIIB Research Initiative Program, the Basic Science Research Program through the National Research Foundation of Korea (NRF) Grant funded by the Korea Government (MEST) (2012R1A2A2A02014520), the Next-Generation BioGreen 21 Program (No. PJ0080220202012), Rural Development Administration, Republic of Korea, and National Institute of Health Grants (MH48866, NS070577 and MH87903). The authors thank Dong-Hee Choi for technical assistance.

Appendix

Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.brainres.2014.01.023.

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Please cite this article as: Kim, K.-S., et al., Pitx3 deficient mice as a genetic animal model of co-morbid depressive disorder and parkinsonism. Brain Research (2014), http://dx.doi.org/10.1016/j.brainres.2014.01.023.


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