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Peroxiredoxin 6 Overexpression Induces Anxiolytic and Depression-Like Behaviors by Regulating the Serotonergic Pathway in Mice

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Abstract

Peroxiredoxin 6 (PRDX6) is a bifunctional protein with both glutathione peroxidase and calcium-independent phospholipase activity. Recently, we reported that PRDX6 plays an important role in dopaminergic neurodegeneration in Parkinson's disease. However, the relationship between PRDX6 function and emotional behavior remains elusive. In the present study, we examined depression- and anxiety-like behaviors in PRDX6-overexpressing transgenic (PRDX6-Tg) mice using the forced swim test, tail suspension test, open field paradigm, and elevated plus-maze. PRDX6-Tg mice exhibited depression-like behaviors and low anxiety. In particular, female PRDX6-Tg mice exhibited anxiolytic behavior in the open field test. Furthermore, the serotonin content in the cortex and 5-hydroxytryptophan-induced head twitch response were both reduced in PRDX6-Tg mice. Interestingly, levels of dopa decarboxylase expression in the cortex were decreased in male PRDX6-Tg mice but not in female mice. Our findings provide novel insights into the role of PRDX6 in 5-HT synthesis and suggest that PRDX6 overexpression can induce depression-like behaviors via downregulation of the serotonergic neuronal system.

Key Words: Peroxiredoxin 6, Depression, Anxiety, L-amino acid decarboxylase, Serotonin

INTRODUCTION

Peroxiredoxin 6 (PRDX6), the only 1-Cys member of the peroxiredoxin (PRDX) family (Chae *et al*., 1994), is a bifunctional enzyme with glutathione peroxidase (GPx) and phospholipase A_2 (PLA_2) activities, called aiPLA$_2$ (Fisher *et al*., 1999; Manevich *et al*., 2004). Recently, we reported that PRDX6 plays an important role in dopaminergic neurodegeneration in a mouse model of Parkinson's disease (PD) (Yun *et al*., 2015). Moreover, an imbalance in the redox system can be associated with psychiatric symptoms (Vaccarino *et al*., 2008). However, the potential role of PRDX6 in psychological behavior remains poorly clarified. Insufficiency of the serotonergic central nervous system (CNS) may play a key role in the pathophysiology of depression (Stockmeier, 2003). Patients with depression exhibit reduced cerebrospinal fluid levels of 5-hydroxyindoleacetic acid (5-HIAA), as well as tryptophan depletion-induced transient relapse during successful treatment with selective serotonin (5-hydroxytryptamine, 5-HT) reuptake inhibitors (SSRIs) (Owens and Nemeroff, 1994). The pharmacological management of depression currently involves drugs that frequently target monoamine transporters, including SSRIs, noradrenaline (NA) inhibitors (NRIs), or a combination of both (SNRIs) (Hillhouse and Porter, 2015).

The association between 5-HT receptors, depression, and anxiety has been well-established. Abnormalities in the synthesis, degradation, and transport of neurotransmitters can lead to diverse neurological manifestations, including developmental delay, motor disorders, epilepsy, autonomic dysfunction, and neuropsychiatric features, which might be associated with neurotransmitter synthetic enzymes or transporters such as tyrosine hydroxylase, aromatic L-amino acid decarboxylase

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(AADC), and dopamine transporter (DAT) (Ng *et al*., 2014; Siu, 2015). AADC is a homodimeric pyridoxal phosphate-dependent enzyme responsible for synthesizing dopamine and 5-HT. AADC deficiency is reportedly associated with PD (Zou *et al*., 2016). In the present study, we examined depression- and anxiety-like behaviors in PRDX6-overexpressing transgenic (PRDX6-Tg) mice. Furthermore, we investigated the association between AADC and neurotransmitter imbalances in PRDX6-Tg mice.

MATERIALS AND METHODS

Animals

PRDX6-Tg (C57BL/6J) mice were purchased from Jackson Laboratory (ME, USA) and C57BL/6 (non-Tg) mice were purchased from DBL (Eumseong, Korea). Animals were maintained in conventional housing at 23 ± 2°C under a controlled 12 h light/dark cycle, with drinking water and rodent chow provided throughout the experiment. Behavioral analyses were conducted on mice aged 2-5-months. Animals were used for each behavioral test only once in the following order: one group of mice was subjected to the open field test, elevated plus-maze, inclined screen test, forced swim test, tail suspension test, grip strength test, and rotarod test; the second group was used to examine the 5-hydroxy-L-tryptophan (5-HTP, Sigma-Aldrich, St. Louis, MO, USA)-induced head twitch response. This study was conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experiments were approved by the Guidelines for the Care and Use of Animals (Animal Care Committee of Chungbuk National University, Cheongju, Korea [CBNUA-436-12-02]). All mice were sacrificed using CO_2.

Open field test

The open field test was performed according to a previously described method with minor modifications (Lopatina *et al*., 2014). The open field consisted of a square box with a total diameter of 60 cm×60 cm×45 cm, divided into 16 squares, each 15 cm×15 cm. In the middle of the open field, a central zone was set up in 30×30 cm squares. The light (50 W) was positioned 100 cm above the floor center. Each mouse was placed in a section of the central zone and allowed to explore the environment freely for 10 min. The time spent in each zone and the total distance traveled were measured using images captured on video (SMART-LD program; Panlab, Barcelona, Spain).

Elevated plus-maze test

The elevated plus-maze test was performed according to a previously described method, with minor modifications (Lister, 1987; Miyamoto *et al*., 2002). The elevated plus-maze consisted of two open (30×5 cm) and two closed (30×5×20 cm) arms, extending from a common central platform (5×5 cm) to form a plus shape. The entire apparatus was elevated to a height of 50 cm above the floor. The test was initiated by placing a mouse on the central platform of the maze facing an open arm. The frequency of entry into the open and closed arms and the total distance traveled were measured for 5 min using images captured on video (SMART-LD program; Panlab).

Forced swim test

The forced swim test was performed according to a previously described method, with minor modifications (Porsolt *et al*., 1977; Murai *et al*., 2007; Can *et al*., 2012). Each mouse was placed in a transparent cylindrical tank (30 cm high, 20 cm in diameter) containing water at 25°C to a depth of 15 cm and was forced to swim for 6 min. Video files were transmitted directly from the camera to a connected computer to analyze the swimming duration. We used on-screen stopwatch software (Xnote Stopwatch, dnSoft Research Group, Cheboksary, Russia) for the time measurements. Immobility time was calculated as follows:

Immobility time (s)=Total time–swimming time

Tail suspension test

The tail suspension test was performed according to a previously described method, with minor modifications (Steru *et al*., 1985; Cryan *et al*., 2005; Tomida *et al*., 2009). Each mouse was placed in a transparent rectangle (20 (width)×12 (length)×50 (deep) cm). Mice were suspended by their tails using a string attached to the tails with adhesive tape (~1 cm from the tip of the tail), and the string was looped around a hook. The distance between the tip of the nose of each mouse and the floor was approximately 10 cm. Mice were suspended for 6 min. Video files were transmitted directly from the camera to a connected computer to analyze the mobility duration. We used on-screen stopwatch software (Xnote Stopwatch, dnSoft Research Group) for time measurements. Immobility time was calculated as follows:

Immobility time (s)=Total time–mobility time

5-HT and dopamine in the cortex

The 5-HT metabolism was measured as previously described (Miyamoto *et al*., 2002; Kasahara *et al*., 2006). The mice were sacrificed after behavioral analysis, and levels of 5-HT and dopamine were determined using high-pressure liquid chromatography (HPLC). Each frozen brain sample was weighed and homogenized with an ultrasonic processor in 0.2 M perchloric acid containing isoproterenol as an internal standard. The homogenates were placed on ice and centrifuged at 20,000 g for 15 min. The supernatants were mixed with 1 M sodium acetate to adjust the pH to 3.0 and injected into an HPLC system (Shiseido, Tokyo, Japan), equipped with a reversed-phase ODS column (SC-5ODS, 150×2.1 mm, EICOM, Kyoto, Japan) and an electrochemical detector (EICOM).

5-HTP-induced head twitch response

The head twitch response was measured according to a previously described method with minor modifications (Nabeshima *et al*., 1992). Mice were treated with 5-HTP (150 mg/kg, i.p.) or vehicle (1% dimethyl sulfoxide). Head twitch responses were recorded for 2 min at 10, 20, and 30 min after injection.

Quantitative real-time PCR (qPCR)

For mRNA quantification, total RNA was extracted using an easy-Spin™ total RNA extraction kit (iNtRON Biotech, Daejeon, Korea). Complementary DNA was synthesized from the total isolated RNA using a SuperScript III first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA).

qPCR was performed using the SYBR® GreenER™ qPCR SuperMix Universal (Invitrogen) specific for glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*, 5'-TGTCAAGCTCATTTCCTGGT-3' and 5'-CTTACTCCTTGGAGGCCATG-3'), tryptophan hydroxylase 2 (*Tph2*, 5'-ATGCCTAATCACCACCTTTC-3' and 5'-TTTCAATGCTCTGCGTGT-3'), dopa decarboxylase (*Ddc*, 5'-CGTGATGATGGACTGGCTGG-3' and 5'-ACTGGCACTTCCCTGGATCA-3'), solute carrier family 6 member 4 (*Slc6a4*, 5'-GGTTCTCCTGGCGTTTGCTA-3' and 5'-GCTCGTCATGCAGTTCACCA-3'), 5-hydroxytryptamine receptor 1A (*Htr1a*, 5'-TGAGACAGGGTGAGGACGAC-3' and 5'-GATTCGCTGGGCAGAGGAAG-3'), 5-hydroxytryptamine receptor 2A (*Htr2a*, 5'-CCGCTTCAACTCCAGAACCA-3' and 5'-AAGTTGTCATCGGCGAGCAG-3') and 5-hydroxytryptamine receptor 2C (Htr2c, 5'-TGCCATCGTTTGGGCAATATCA-3' and 5'-CGAAGGACCGCATGAGAACG-3'). All reverse transcription reactions were run in an iCycler IQ5 (Bio-Rad, Hercules, CA, USA) using universal cycling parameters (10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C). cDNA was included in a 25 μL volume PCR reaction with the following components: 0.125 μL each of forward and reverse primer, 12.5 μL SYBR green, and 0.5 μg cDNA with sterilized water. The results were normalized to *GAPDH* and quantified relative to expression in control samples. For relative quantification calculation, the $2^{-\Delta\Delta Ct}$ formula was used.

$\Delta\Delta Ct=(Ct_{target}–Ct_{GAPDH})$ experimental sample–$(Ct_{target}–Ct_{GAPDH})$ control sample

Statistics

Data represent mean ± standard error (SE). Data were analyzed using Student's t-test or two-way repeated-measures ANOVA followed by Bonferroni *post-hoc* t-test using SigmaPlot 14.5 software (Systat Software, San Jose, CA, USA).

RESULTS

Anxiety-like behaviors were decreased in PRDX6-Tg mice

In the open field test, the time spent in the center of the open field, an indicator of decreased anxiety-like behavior (Crawley, 1999), was increased in PRDX6-Tg mice when compared with the non-Tg mice (Fig. 1A). To further investigate anxiety-like behavior in PRDX6-Tg mice, the mice were subjected to the elevated plus-maze test, which also measures anxiety-like behavior based on the natural aversion of rodents to open and elevated areas. PRDX6-Tg mice spent more time in the open arm than non-Tg mice (Fig. 1B), whereas the time spent in closed arms did not differ between non-Tg and PRDX6-Tg mice (Fig. 1C). These results suggested that PRDX6-Tg mice show decreased anxiety-like behaviors in these tests.

Depression-like behaviors were increased in PRDX6-Tg mice

Oxidative stress is also associated with depression-like be-

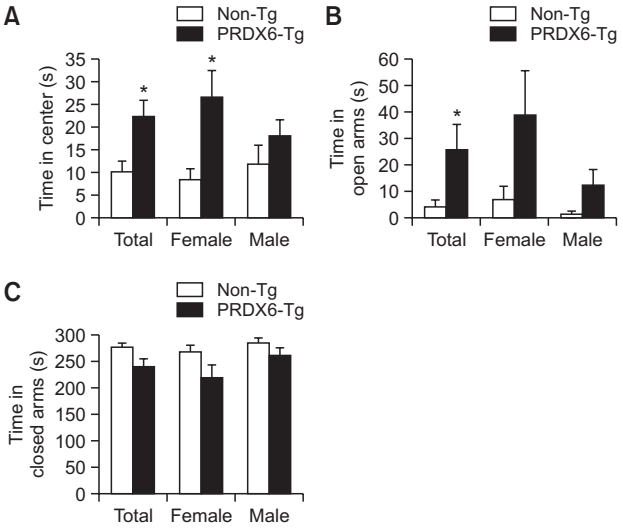

Fig. 1. Anxiety-like behaviors in PRDX6-Tg mice. (A) Time in the center was measured using images captured on video (SMART-LD program; Panlab) in the open field test for 10 min. Data are expressed as mean ± standard error (SE) (n=4-8) and were analyzed using Student's t-test (*p<0.05 *vs*. non-Tg group). (B) Time in open arms or (C) closed arms were measured using images captured on video (SMART-LD program; Panlab) in the elevated plus-maze test for 5 min. Data are expressed as mean ± SE (female n=4; male n=4; total n=8) and were analyzed using Student's t-test (*p<0.05 *vs*. non-Tg group). PRDX6, Peroxiredoxin 6; PRDX6-Tg, PRDX6-overexpressing transgenic.

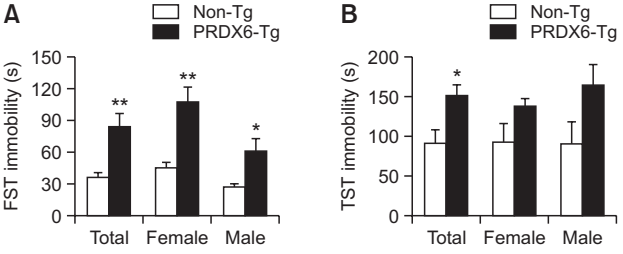

Fig. 2. Depression-like behaviors in PRDX6-Tg mice. The immobility in (A) FST and (B) TST was measured using on-screen stopwatch software (Xnote Stopwatch; dnSoft Research Group) for 6 min. Data are expressed as mean ± standard error (SE). (female n=4; male n=4; total n=8) and were analyzed using Student's t-test (*p<0.05 and **p<0.01 *vs*. non-Tg group). FST, forced swim test; TST, tail suspension test; PRDX6, Peroxiredoxin 6; PRDX6-Tg, PRDX6-overexpressing transgenic.

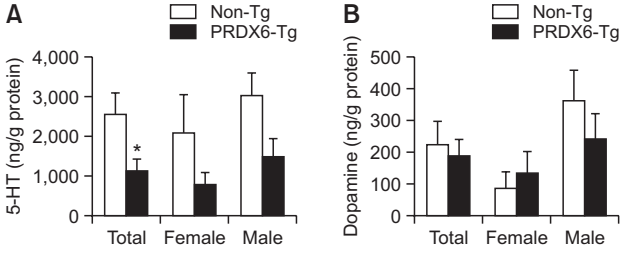

Fig. 3. 5-HT and dopamine content in the cortex of PRDX6-Tg mice. The contents of (A) 5-HT and (B) dopamine in mice cortex were measured using HPLC. Data are expressed as mean ± standard error (SE). (female n=4; male n=4; total n=8) and were analyzed using Student's t-test (*p<0.05 *vs*. Non-Tg group). 5-HT, 5-hydroxytryptamine; HPLC, high-pressure liquid chromatography; PRDX6, Peroxiredoxin 6; PRDX6-Tg, PRDX6-overexpressing transgenic.

havior; however, the behavioral results from open field tests in animal models of depression remain controversial (McHedlidze *et al*., 2011). Therefore, to examine the depression-like responses of non-Tg and PRDX6-Tg mice, we performed forced swim and tail suspension tests. PRDX6-Tg mice showed an elevated duration of immobility when compared with non-Tg mice in the forced swim and tail suspension tests (Fig. 2A, 2 B). These results suggested that PRDX6-Tg mice show increased depression-like behavior in these tests.

5-HT content was reduced in the cortex of PRDX6-Tg mice

We speculated that the increased depression-like behavior of PRDX6-Tg mice might be related to alterations in the 5-HT content. We assessed 5-HT levels in the cortex, as depression-like behavior might be associated with dysfunctions in 5-HT neurotransmission (Mouri *et al*., 2012). The 5-HT content in the cortex of PRDX6-Tg mice was significantly lower than that in the cortex of non-Tg mice (Fig. 3A). In addition, the dopamine content was found to be slightly lower in the cortex of PRDX6-Tg mice than that in non-Tg mice, but the difference was not significant (Fig. 3B).

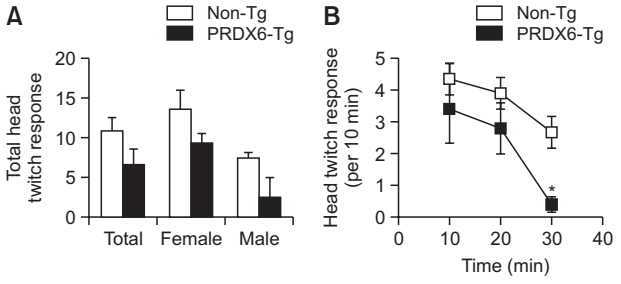

Fig. 4. 5-HTP-induced head twitch response in PRDX6-Tg mice. (A) The cumulative head twitch response for each group was measured after 5-HTP (150 mg/kg, intraperitoneal [i.p.]) injection in mice. Data are expressed as mean ± standard error (SE) (non-Tg female n=5, male n=4, total n=9; PRDX6-Tg female n=3, male n=2, total n=5) and were analyzed using Student's t-test (*vs*. non-Tg group). (B) The head twitch response of each time zone was scored for 2 min at 10 min after 5-HTP (150 mg/kg, i.p.) injection in mice. Data are expressed as mean ± SE (non-Tg female n=5, male n=4, total n=9; PRDX6-Tg female n=3, male n=2, total n=5) and were analyzed using two-way RM ANOVA followed by Bonferroni post-hoc t-test (*p<0.05 *vs*. non-Tg group in each time zone). 5-HT, 5-hydroxytryptamine; PRDX6, Peroxiredoxin 6; PRDX6-Tg, PRDX6-overexpressing transgenic.

5-HTP-induced head twitch response was inhibited in PRDX6-Tg mice

Serotonergic dysfunction is well-known to be associated with depression (Mouri *et al*., 2012). The head twitch response in rodents induced by 5-HTP, a precursor of 5-HT, is considered a specific behavioral model for the activation of serotonergic neurons (Corne *et al*., 1963; Schreiber *et al*., 1995). Therefore, we measured the 5-HTP-induced head twitch response in non-Tg and PRDX6-Tg mice. PRDX6 overexpression did not alter the total head twitch response (Fig. 4A); however, PRDX6-Tg mice showed a lower head twitch response than non-Tg mice 30 min after 5-HTP injection (Fig. 4B; Gene condition: $F(1,24)=2.57$, $p=0.135$; time condition: $F(2,24)=12.832$, $p<0.001$; interaction: $F(2,24)=1.37$, $p=0.337$).

AADC expression level was decreased in PRDX6-Tg mice

Serotonergic neuronal function is associated with several factors, including tryptophan hydroxylase (TPH), serotonin transporter, and serotonin receptors. 5-HT is synthesized from 5-HTP via the AADC. Therefore, we compared mRNA levels of 5-HT in the cortex of non-Tg and PRDX6-Tg mice; however, we detected no significant changes in total mRNA levels in female and male mice (Fig. 5A). When separated by sex, mRNA levels of *Htr2c* and *Ddc* were significantly regulated by PRDX6 overexpression (Fig. 5B, 5C). In particular, PRDX6-Tg mice exhibited reduced *Ddc* mRNA expression. Compared with male non-Tg mice, male PRDX6-Tg mice, but not female mice, displayed significantly reduced *Ddc* mRNA levels in the cortex.

DISCUSSION

Redox balance is closely associated with several psychological disorders. Among antioxidant enzymes, PRDX6 exhibits unique bifunctional activity. We have previously reported that PRDX6 plays a role in dopaminergic neurodegeneration by modulating 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity in a PD model (Yun *et al*., 2015). However, we demonstrated that PRDX6-Tg mice exhibit emotional behavioral phenotypes. In the elevated plus-maze test,

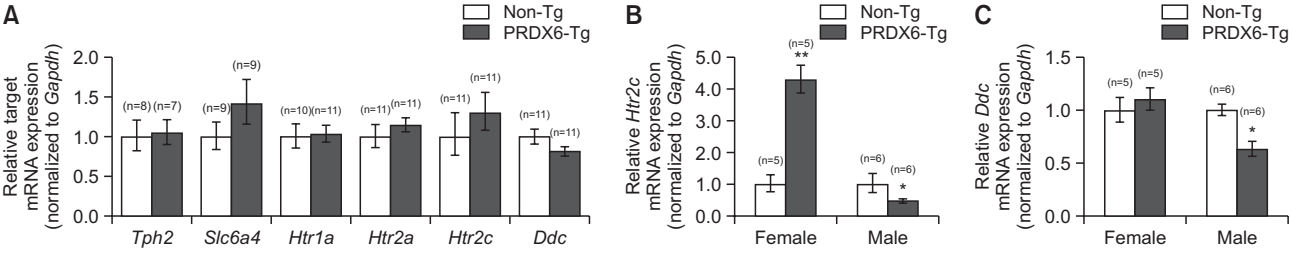

Fig. 5. mRNA expression of 5-HT-related genes in PRDX6-Tg mice. (A) mRNA levels of 5-HT-related genes were examined in male and female mice using qPCR and normalized to *Gapdh*. Data are expressed as mean ± standard error (SE) (n=7-11) and were analyzed using Student's t-test. The female or male mRNA levels of (B) *Htr2c receptor* and (C) *Ddc* were detected by qPCR and normalized to *Gapdh*. Data are expressed as mean ± SE (n=5-6) and were analyzed using Student's t-test (*p<0.05 and **p<0.01 *vs*. non-Tg group). *Tph2*, Tryptophan hydroxylase 2; Slc6a4, Solute carrier family 6 member 4 (serotonin transporter). Htr1a, 5-hydroxytryptamine receptor 1A; Htr2a, 5-hydroxytryptamine receptor 2A; Htr2c, 5-hydroxytryptamine receptor 2C; Ddc, Dopa decarboxylase; PRDX6, Peroxiredoxin 6; PRDX6-Tg, PRDX6-overexpressing transgenic.

the time spent in the open arm was higher in PRDX6-Tg mice than in non-Tg mice. Interestingly, female mice exhibited more anxiolytic behavior than male mice. To elucidate the mechanism underlying this behavior, we measured expression levels of serotonin receptors in the cortex. *Htr2c* mRNA levels were increased in PRDX6-Tg female mice but not in male mice. According to a previous study, *Htr2c* knockout mice display anxiety-like behaviors (Rosenzweig-Lipson, 2011). In addition, 5-HT2C receptor agonists induce anxiolytic-like activity in the elevated plus-maze test (Nic Dhonnchadha *et al*., 2003). Therefore, we postulated that the anxiolytic behaviors of PRDX6-Tg mice could be associated with upregulated *Htr2c* levels. In particular, the profound increase in *Htr2c* in female PRDX6-Tg mice might explain their marked anti-anxiety behaviors when compared with those of male PRDX6-Tg mice.

We also observed that PRDX6-Tg mice exhibited increased depression-like behavior in the forced swim and tail suspension tests. Furthermore, 5-HT levels were reduced in the cortex of PRDX6-Tg mice. Deficits in 5-HT neuronal transmission are well-known to be associated with depression (Jacobsen *et al*., 2012). In the cortex, the 5-HT content was decreased in PRDX6-Tg mice, while dopamine levels were unaltered. Furthermore, the head twitch response, a typical behavior induced by 5-HTergic neuronal activation, was reduced in PRDX6-Tg mice (Canal *et al*., 2010; Canal and Morgan, 2012). These results suggest that depression-like behaviors in PRDX6-Tg mice are related to downregulated 5-HT neurotransmission.

We further measured AADC expression levels in PRDX6-Tg mice, as the head twitch response is evoked by 5-HT metabolized from 5-HTP (a precursor) via Ddc in our model (Colpaert and Janssen, 1983; Darmani, 1996). *Ddc* mRNA levels were lower in male PRDX6-Tg mice than in non-Tg mice but not in female mice. Low *Ddc* expression in male PRDX6-Tg mice may be associated with greater depression-like behavior observed in the tail suspension test. The precise molecular mechanism remains unknown; however, PRDX6 transcription of Ddc may be regulated by the POU protein, which is associated with PRDXs (Millevoi *et al*., 2001; Oliviero *et al*., 2015). In the present study, the levels of PRDX6 expression in the cortex of male and female mice did not differ significantly. Furthermore, the natural expression pattern of PRDX6 has been reported in both males and females (Birzniece *et al*., 2002; Balasinor *et al*., 2010; Buonora *et al*., 2015). One human study has reported that males exhibit approximately 20% higher plasma levels of *Prdx6* than females (Buonora *et al*., 2015). The physiological and pathophysiological roles of the sex-specific expression patterns of PRDX6 need to be elucidated in future studies. Collectively, our results suggest that PRDX6 regulates the expression of anxiolytic and depression-like behaviors by modulating 5-HTergic neurotransmission in the cortex.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

ACKNOWLEDGMENTS

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