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Impaired motor coordination in Pitx3 overexpression mice

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ABSTRACT

Midbrain dopaminergic (DA) neurons are involved in the regulation of voluntary movement and in emotion-related behaviors and are affected in Parkinson's disease (PD). The homeodomain transcription factor Pitx3, which is uniquely expressed in midbrain DA neurons, plays a critical role in the development, function and maintenance of midbrain DA neurons. Pitx3 deficiency results in selective deficits of midbrain DA neurons in the substantia nigra pars compacta (SNc), reminiscent of the specific DA neuronal loss observed in PD. In this study, we found that selective overexpression of Pitx3 in intact midbrain DA neurons significantly affects the function of midbrain DA neurons. We observed changes in DA levels and gene expressions in mice overexpressing Pitx3. Furthermore, motor coordination and locomotion activities are significantly affected in mice overexpressing Pitx3, suggesting that the expression level of Pitx3 plays an important role in the function of midbrain DA neuron *in vivo*.

1. Introduction

Parkinson's disease (PD) is a severe neurological disease primarily characterized by dysfunction of the dopaminergic (DA) nigrostriatal system as a result of the progressive degeneration of the DA neurons in the substantia nigra of midbrain [2,5,8,20]. Midbrain DA neurons play an essential role in the regulation of voluntary movement and other behaviors including learning and reward [25]. The development and function of midbrain DA neurons are complex processes that require the participation of numerous genes and several specific transcription factors [20]. Understanding the molecular mechanisms of DA neuronal development and function has led to significant improvements in cell therapies for PD.

The transcription factor Pitx3 plays critical roles in the development, function and maintenance of midbrain DA neurons [1,25]. Pitx3 is strictly expressed in the midbrain DA neurons in the mouse brain and Pitx3-deficient ak mice recapitulate selective nigrostriatal DA neuron loss, leading to locomotor deficits resembling those seen in PD [6,13,16,18,21–24]. Pitx3 is a homeodomain containing transcription factor with binding activity to a conserved bicoid response element (GGCTTT) just a few bases upstream of the TATA box of the TH gene in the rat, mouse and human [7,11]. Moreover, Pitx3 exerts its impact by directly regulating the expression of a cascade of many downstream genes in DA neurons, including *Vmat2*, En1, *Bdnf* and *Gdnf*, indicating that Pitx3 expression is important for the function of adult midbrain DA neurons [14,15].

Furthermore, the development and function of midbrain DA neurons requires specific and coordinated control mechanisms, and Pitx3 is one of the key factors that regulate the precise spatial and temporal expression patterns of specific genes in midbrain DA neuron development and function. Previously, we and others have reported that there is a feedback mechanism between Pitx3 and miR-133b in the development and function of midbrain DA neurons to control expression the of Pitx3 [9,12,17]. Thus, if Pitx3 mRNA is a functional target of miR-133b, the question remains why Pitx3 expression is negatively regulated in the midbrain DA neurons. In this context, we asked whether elevated Pitx3 expression *in vivo* might be implicated in DA neuronal dysfunctions such as behavioral disorders.

To address this question, we generated a mouse model selectively expressing Pitx3 in midbrain DA neurons. We observed that the ectopic expression of Pitx3 in midbrain DA neurons significantly altered gene expression levels in DA neuronal genes, and monoamine levels were changed in the mice overexpressing Pitx3. Furthermore, we found that locomotor behaviors in mice overexpressing Pitx3 are significantly different from those of wild-type controls. Taken together, these data suggest that the expression level of Pitx3 is critical for midbrain DA neuron functions *in vivo*.

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2. Methods and materials

2.1. Generation of Pitx3–AAV2

The full length murine Pitx3 gene was amplified by PCR and the Pitx3 PCR product was cloned into pAAV2-IRES-hrGFP cloning vector (Agilent Technologies). Constructs consisting of eGFP was used as controls. Packaging of the Pitx3–AAV2 virus was carried out according to the standard protocol (Stratagene AAV Helper-Free System, La Jolla, CA). Briefly, to produce virus, AAV-293 cells grown in a 15 cm tissue culture dish were transfected with pHelper, AAV expression plasmid (Pitx3-flag, GFP) via calcium phosphate precipitation. The cells were harvested 72 h later, and the virus was concentrated by using Virakit AAV purification kit (Virapur, LLC). The titer of viral particles was about 108 particles/ml, determined after transduction of HEK cells.

2.2. AAV injection

All animal procedures were approved by the Dongguk University Animal Care and Use Committee. Adult (12 weeks) male C57BL/6 mice were obtained from Charles River Laboratories. Mice were anesthetized with ketamine/xylazine and placed in a stereotaxic frame (Leica) with a mouse adapter. Artificial eye ointment was applied to cover and protect the eye. The animals were operated upon with a midline scalp incision and a drilling hole on the skulls above somatosensory cortex. The tip of a pulled glass pipette (diameter) was inserted to stereotaxic coordinates AP: −3.1 mm; ML: ±1.1 mm; DV: −4.4 mm, relative to bregma. Viral vector suspension in a volume of 2 μl was microinjected into the both hemisphere. After injection, the needle was kept in place for at least 5 additional minutes and then slowly withdrawn. 12 weeks following AAV injection, AAV virus transduction of DA neurons of the midbrain was confirmed by double immunolabeling for FLAG and TH and real time PCR for Pitx3 mRNA.

2.3. Behavioral analyses

The open field test was performed as described previously [9]. Briefly, 12 week after Pitx3 AAV injection, mice were placed in automated 16 × 16 in. chambers (AccuScan Instruments) for 60 min. Total distance traveled (centimeters) and time spent in the center were detected in 5 min bins. Two weeks later, the same mice were placed in 8 × 8 in. chambers, and distance traveled was similarly detected for 60 min. 2 mg/kg per kilogram of amphetamine was immediately administered by i.p. injection, the mice were returned to their chambers, and locomotor activity was recorded for a further 120 min. For the rotarod test, mice were trained for four trials per day over 3 days on a rotarod device (Med Associates) at a steady speed of 24 rpm. The latency to fall was recorded as the time at which the mouse fell completely off the rod or failed to stay atop the rod. The intertrial interval was 5 min. On the fourth day the mice underwent an accelerating rotarod task, where rotation velocity increased from 4 to 40 rpm over 5 min. Performance on the task was assessed by measuring the average latency to fall off the rod over three trials.

2.4. Immunostaining

8 weeks after AAV injections, mice were sacrificed via anesthetic overdose (ketamine/xylazine) and perfused with ice-cold 0.9% saline followed by 4% paraformaldehyde (PFA; Boston Bioproducts). Whole brain was fixed overnight, equilibrated in 15% sucrose, and embedded in OCT solution. 50 μm frozen coronal serial sections were collected using a cryostat (Leica CM1850). Sections were permeabilized in TBS containing 0.25% Triton-X-100 for 30 min. The tissue sections were blocked in TBS + 3% bovine serum albumin (BSA) for 1.5 h at room temperature then incubated overnight at 4 °C with the primary antibodies sheep anti-TH (AbCam) and rat anti-flag (Sigma) each diluted 1:1000 in TBS + 1% BSA. The secondary antibodies Alexa Fluor donkey anti-sheep 594 and Alexa Fluor goat anti-rat 488 (Molecular Probes, Invitrogen) were diluted 1:1000 in the same diluent and incubated with the tissue for 1.5 h at room temperature. Sections were mounted with Vectashield mounting medium with DAPI (Vector Laboratories).

2.5. Stereological neuronal counts

Unbiased stereological estimation of the total number of TH immunoreactive cell bodies in the SNpc and VTA was performed using the optical fractionator method. Every second section was collected through the midbrain and every fourth section was analyzed for cell counting, totaling 10 sections per animal.

2.6. Measurement of DA and DA metabolites

Mice were sacrificed by anesthetic overdose seven days after the final MPTP or saline injection. Animals used for these biochemical measurements had not undergone amphetamine-induced rotational behavior testing. Nucleus accumbens, and dorsal striatum were rapidly dissected on a cold plate, flash-frozen in liquid nitrogen, and stored at −80 °C for high performance liquid chromatography with electrochemical detection (HPLC) analysis performed by the Neurochemistry Core, School of Medicine, Vanderbilt University as described previously [9]. The levels of DA and DA metabolites were normalized to mg of protein input.

2.7. Expression analysis

RNA was extracted from the striata or SN using the RNeasy mini kit (Qiagen). After DNase treatment the RNA was purified using the RNeasy MinElute Clean-up kit (Qiagen) and used as a template for cDNA synthesis with cDNA Reverse Transcription Kit (Applied Biosystems). The final cDNA reaction was diluted sevenfold and 5 μl of the reaction was used in duplicate 15 μl SYBR-Green (Applied Biosystems) PCR reactions with primers specific to the target gene. Cycle numbers were normalized to the numbers obtained for a GAPDH expression, and fold-induction of the gene of interest was calculated in relation to the uninjected striatal or SN hemisphere of the same animal by the ΔΔCt method. Each sample was measured in triplicate.

3. Results

3.1. Generation of Pitx3 overexpression mice

To induce specific overexpression of Pitx3 in the midbrain DA neurons, we have generated Pitx3 adeno-associated virus serotype 2 (Pitx3–AAV2) that allow efficient transduction into the midbrain DA neurons. The AAV2 system has a particular affinity for the neurons of the SN pars compacta, which makes it possible to express proteins stably, and at high levels in the nigrostriatal DA neurons in adult mice and rats [10]. AAV2 expressing Pitx3 or GFP were bilaterally transduced into the midbrain with a stereotaxic frame (AP: −3.1 mm; ML: ±1.1 mm; DV: −4.4 mm) (Fig. 1A). At 12 weeks after AAV2 injection, transduction of AAVs harboring Pitx3-flag or GFP vectors by stereotaxic injection into adult mouse brain leads to robust expression of Pitx3 that is restricted to midbrain DA neurons (Fig. 1B). Immunochemical analysis showed that exogenous

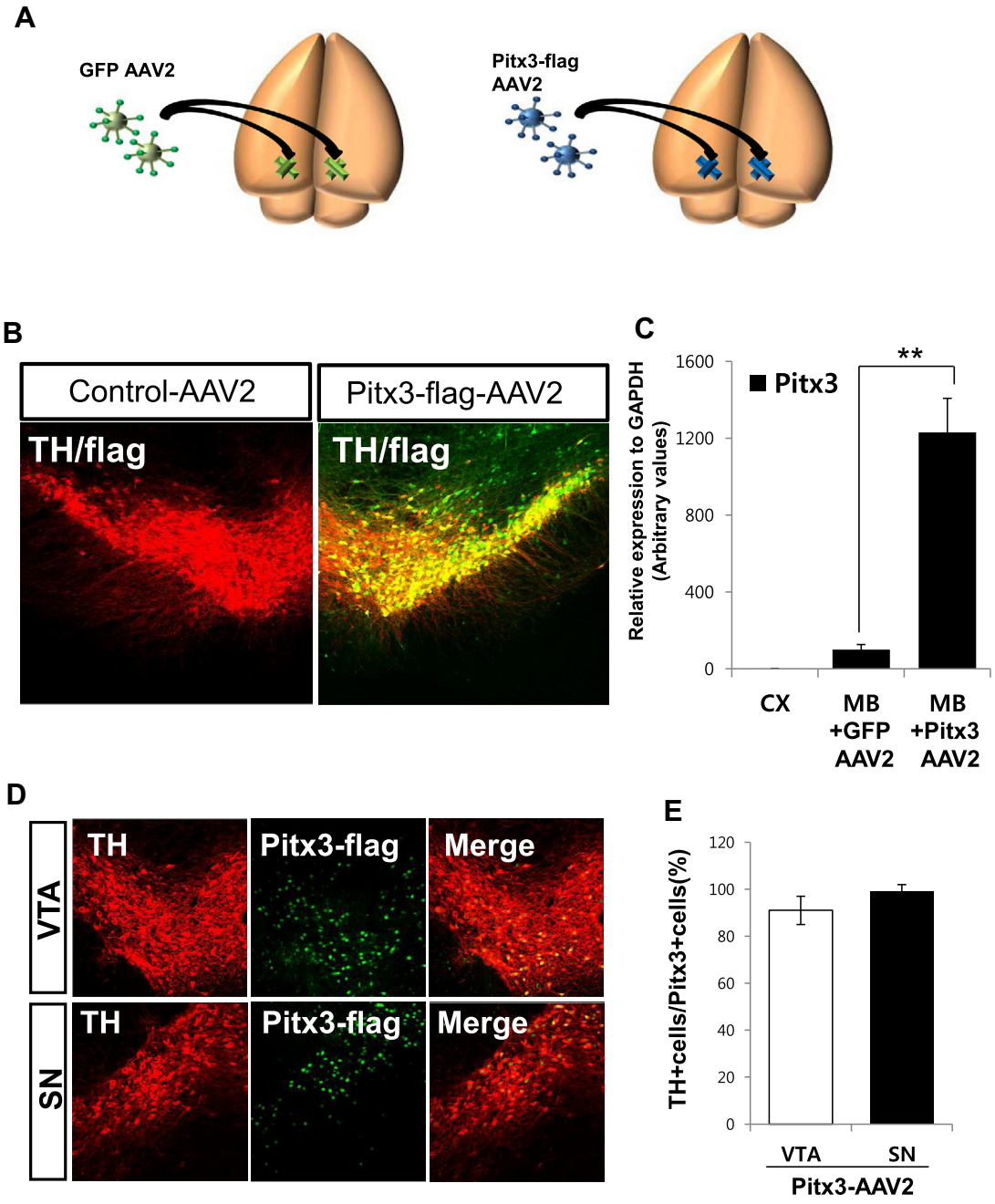

Fig. 1. Generation of Pitx3 overexpression mouse. (A) Schematic of Pitx3 overexpression strategy. Pitx3-flag and GFP expressing Adeno-associated virus 2 (AAV2) were bilaterally transduced into the midbrain with a stereotaxic frame. (AP: −3.1 mm; ML: ±1.1 mm; DV: −4.4 mm) (B) TH and flag immunohistochemistry of midbrain at 12 weeks after GFP and Pitx3-flag AAV2 injected mouse.**$p < 0.05$, Student's *t*-test. (C) Quantitative Pitx3 RNA expression in control brain and Pitx3 overexpression brain. Expression was normalized to *Gapdh*, $n = 6$ in control and Pitx3 overexpression mice. Error bars indicate SEM. **$p < 0.05$, Student's *t*-test. CX : cerebral cortex, MB :midbrain (D) Pitx3 AAV2 viral transduction in Substantia Nigra (SN) and Ventral Tegmental Area (VTA) DA neurons were confirmed by double immunostaining for Pitx3-flag (green) and TH (Red). (E) The quantification of TH+/Pitx3 + cells in the midbrain DA neurons at 12 weeks after transduction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

expression of Pitx3-flag was specifically present in the nuclei of midbrain DA neurons, and Pitx3-flag was not detected in midbrain DA neurons treated with the control vector (Sup. Fig. 1A and B). We also observed a significant increase in Pitx3 mRNA in the midbrain of mice injected with Pitx3–AAV2 (Fig. 1C). Transgene expression with AAV2 transduction was observed in >95% of cells in the substantia nigra (SN) and >90% of cells in the ventral tegmental area (VTA) (Fig. 1D and E), suggesting that AAV2-based transduction in midbrain DA neurons is a highly efficient method for the overexpression of Pitx3.

3.2. Overexpression of Pitx3 in mice changes gene expression in midbrain DA neurons

Pitx3 has been shown to regulate differentiation, function and maintenance in mouse midbrain DA neurons [13]. Therefore, we asked whether Pitx3 overexpression in mouse midbrain might have changed the numbers of mDA neurons. The general morphology of the midbrain and striatum of mice overexpressing Pitx3 was indistinguishable from those of control mice (data not shown), and unbiased stereological counts of TH+ cells and Pitx3+/TH+ in

midbrain were also unchanged at 2, 4 and 6 months post-injections (Fig. 2A). Moreover, we found no difference in the number of cells positive for activated caspase-3 between control and Pitx3 overexpression mice (Fig. 2B), suggesting that overexpression of Pitx3 does not cause degeneration of mDA neurons. Furthermore, neuronal survival and apoptosis, as quantified by staining for Annexin V, is unchanged in the transduced cells (Sup. Fig. 2A).

Although Pitx3 overexpression does not appear to affect the number of DA neurons *in vivo*, it may act by regulating the expression of genes that affect neuronal function. Therefore, we measured the expression levels of several genes important for mDA neuronal function. Surprisingly, we observed significant upregulation of *TH*, *DAT*, AADC, VMAP2, Girk2 and a-Synuclein in midbrain DA neurons overexpressing Pitx3 (Fig. 2C). To more specifically determine whether protein expression was altered in midbrain dopaminergic, GABAergic or cholinergic neurons, protein levels of GAD1, ChAT, and 5HT were quantified in control and Pitx3-overexpressing mice. Consistent with previous results, the levels of *GAD1*, *ChAT*, and 5HT expressed in GABAergic, cholinergic, serotonergic neurons,

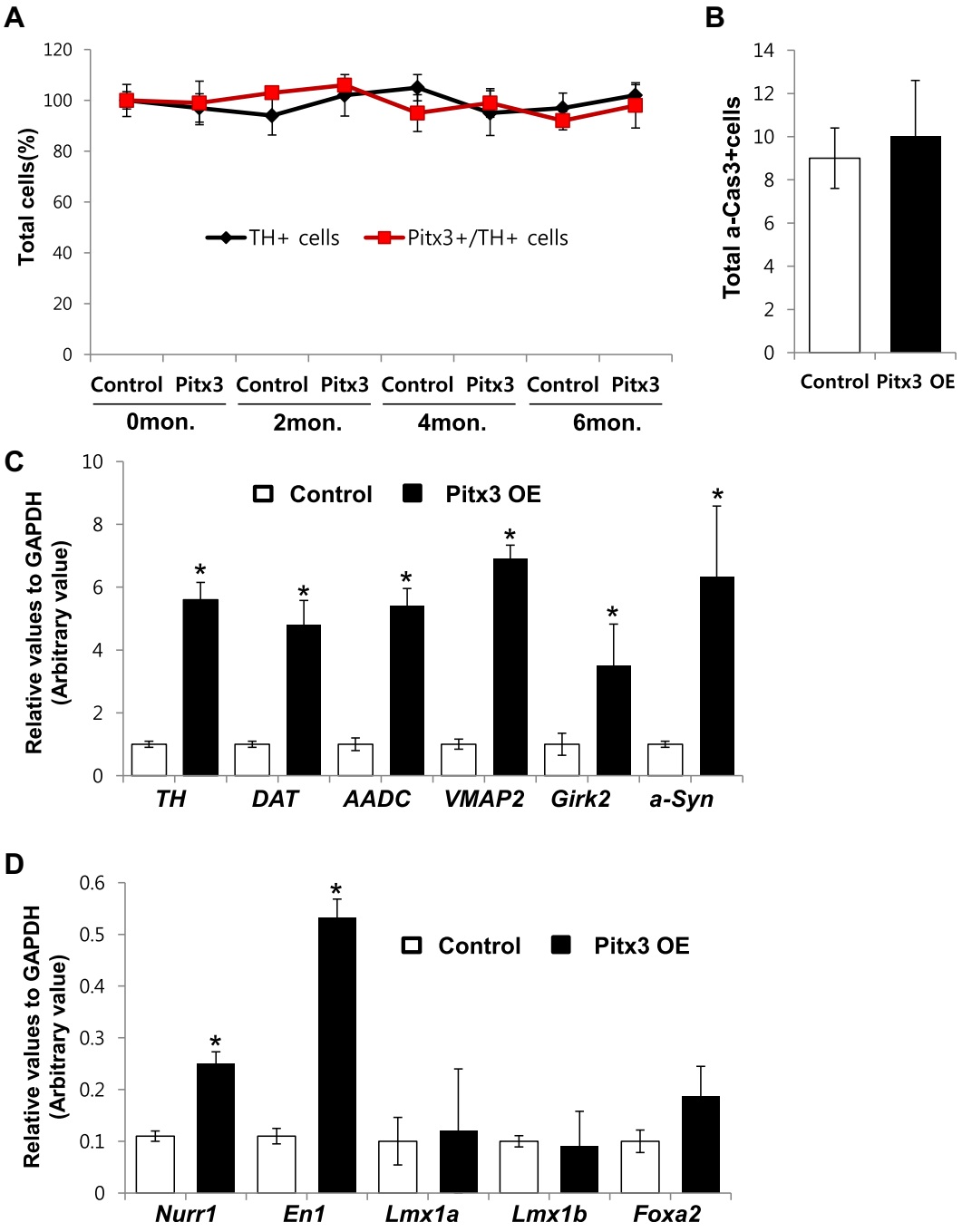

Fig. 2. Altered DA gene expression in Pitx3 overexpression (Pitx3 OE) mice (A) Number of TH+ cells/Pitx3+ and TH+ cells in control and Pitx3 overexpression midbrain at 2, 4, and 6 month after AAV2 injection ($n = 5$) Error bars indicate SEM. (B) The quantification of activated caspase 3+(a-Cas3) cells in the midbrain DA neurons at 6 months after AAV2 injection. (C) Quantitative RNA expression for TH, DAT, AADC, VMAP2, Girk2 and alpha-synuclein in control brain and Pitx3 overexpression brain. Expression was normalized to *Gapdh*, $n = 6$ in control and Pitx3 overexpression mice. Error bars indicate SEM. $^*p < 0.05$, Student's *t*-test. (D) Quantitative RNA expression for Nurr1, En1, Lmx1a, Lmx1b, and Fox2a in control brain and Pitx3 overexpression brain. Expression was normalized to Gapdh, $n = 6$ in control and Pitx3 overexpression mice. Error bars indicate SEM. $^*p < 0.05$, Student's *t*-test.

respectively, were also unchanged (Sup. Fig. 2B). Furthermore, the expression of the glial markers GFAP and OSP was unaltered in the midbrain in Pitx3-overexpressing mice (Sup. Fig. 2B). Because Pitx3 interacts with several transcription factors in the development and function of DA neurons, Pitx3 overexpression may affect the expression of other transcription factors. We observed that Lmx1a and Lmx1b, two transcription factors that are normally expressed early at the midbrain–hindbrain junction and which serve as markers for the identity of this region, are not expressed in the majority of adult midbrain DA neurons and appear unaltered by Pitx3 transduction (Fig. 2D). However, induction of Nurr1 and EN1 was observed in the Pitx3-overexpressing animals (Fig. 2D), suggesting that the transcriptional patterns in midbrain DA neurons are affected by overexpression of Pitx3.

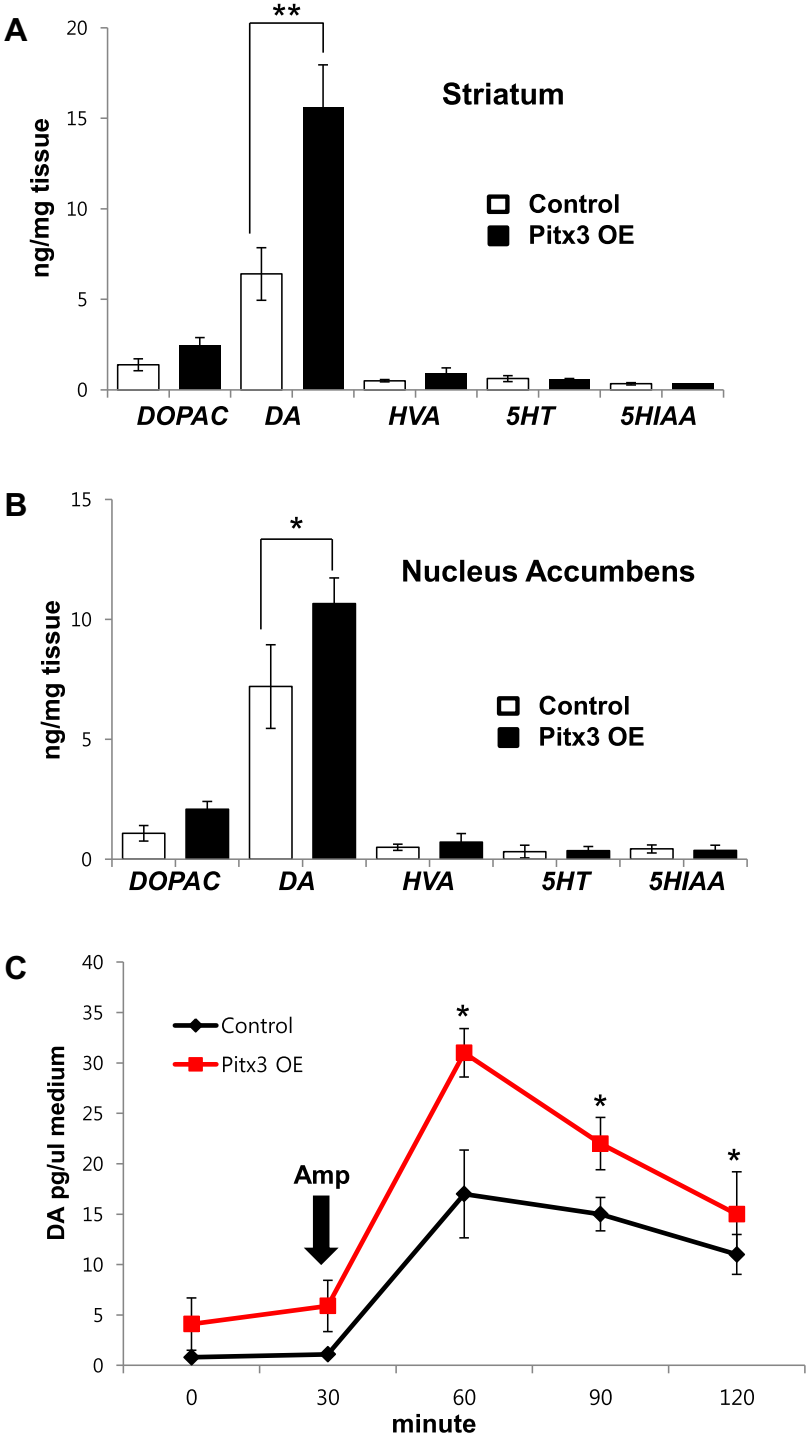

Fig. 3. Altered monoamine neurotransmitter levels in Pitx3 overexpression (Pitx3 OE) mice. (A and B) Neurochemical analysis of whole-tissue monoamine contents in striatum (A) nucleus accumbens (B) from 8 weeks post injection of control and Pitx3 overexpression mice; $^{**}p < 0.05$, Student's *t*-test. $n = 6$ control and Pitx3 overexpression mice. Error bars indicate SEM. (C) Neurochemical analysis of baseline and amphetamine-stimulated extracellular DA levels in dorsal striatum of 3-month-old male WT and Pitx3 OE mice. Amphetamine at 100 μM was continually infused via reverse microdialysis after 40 min (arrow); $n = 6$ for control and overexpression mice. Error bars indicate SEM.

3.3. Monoamine neurotransmitters are altered in mice overexpressing Pitx3

To evaluate whether the activity of midbrain DA neurons was altered in mice overexpressing Pitx3, whole-tissue monoamine neurotransmitter levels were quantified by HPLC at 12 weeks after AAV2 transduction. Consistent with the previous data, significant induction of DA and DOPAC levels was observed, whereas the levels of 5-HT, was unchanged in the striatum (Fig. 3A) and nucleus accumbens (Fig. 3B) of mice overexpressing Pitx3. To determine whether extracellular DA release was altered following Pitx3 overexpression, baseline and amphetamine-stimulated dorsal striatal microdialysis perforates were collected from control and Pitx3-overexpressing mice, and the levels of DA release were quantified by HPLC. We observed that amphetamine-induced DA release was significantly induced in Pitx3-overexpressing samples,

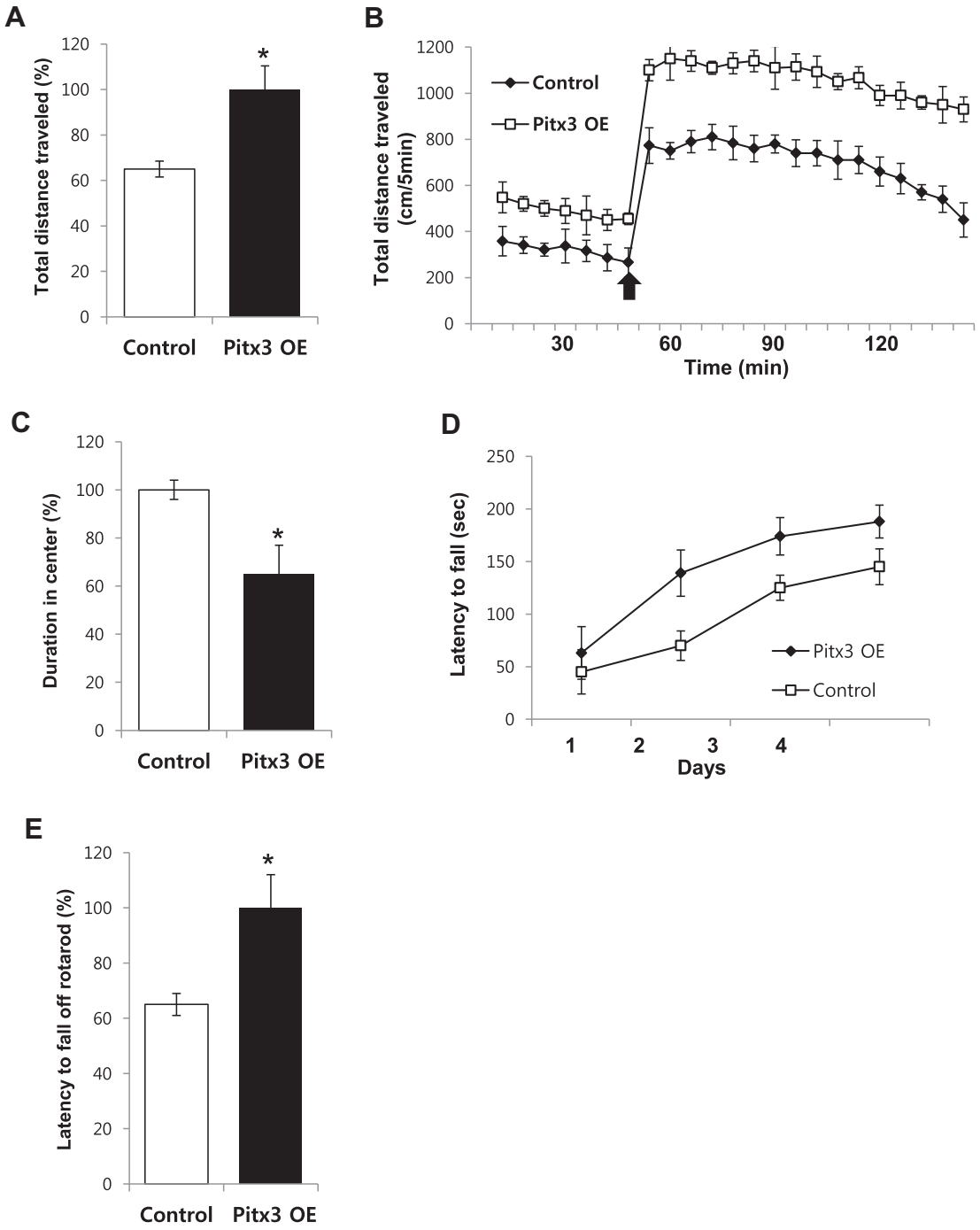

Fig. 4. Locomotor behaviors in Pitx3 overexpression mice (Pitx3 OE). (A) Spontaneous locomotor activity of control and Pitx3 overexpression mice at 12 weeks after AAV2 transduction in the open field test; $^{*}p < 0.05$, Student's *t*-test. $n = 10$ control and 10 overexpression mice. (B) Amphetamine-induced locomotor activity of control and Pitx3 overexpression mice in the open field test. 2 mg/kg of amphetamine was administered by i.p. injection at 45 min (arrow); $n = 8$ control and 8 overexpression mice. Error bars indicate SEM. (C) Pitx3 overexpression mice spend more time in the center of the open field chamber during the first 30 min of the open field test; $^{*}p < 0.05$, Student's *t*-test. $n = 10$ control and 10 overexpression mice. (D) Average latency to fall of control and Pitx3 overexpression mice trained on a rotarod at a fixed speed of 24 rpm for 4 trials per day over 4 days. (E). Latency to fall from an accelerating rotarod (4–40 rpm) on the fifth day, averaged over 3 trials; $^{*}p < 0.05$, Student's *t*-test. $n = 10$ WT and 10 Pitx3 overexpression mice. Error bars indicate SEM.

with a similar increase in extracellular DA upon administration of amphetamine (~20-fold above baseline), followed by a gradual decline (Fig. 3C). These data suggest that the Pitx3 overexpression increased the total DA production in mDA neurons and DA release in the dorsal striatum of mice overexpressing Pitx3.

3.4. Locomotor activity in mice overexpressing Pitx3 is differs from that of wild-type mice

Movement and motivation-related behaviors are directly linked to midbrain DA neuronal function [21]. To determine whether Pitx3-overexpressing mice have an underlying detrimental in the midbrain DA system, Pitx3-overexpressing mice were submitted to a battery of behavioral tests. Surprisingly, we observed that Pitx3 overexpression mice exhibited increased spontaneous locomotor behavior in the open field test (Fig. 4A). The response mice overexpressing Pitx3 to injection of amphetamine, which elevates extracellular DA levels, significantly increased from that of control mice (Fig. 4B). Moreover, Pitx3 overexpressing mice spent more time in the center of the open field during the first 30 min of the test as compared to control mice (Fig. 4C), suggesting an increase in anxiety-like behavior.

In addition, motor coordination was tested on the rotarod which is used to measure balance, coordination, motor function and learning [26]. As shown in Fig. 4D, performance on the rotarod was similar in the two groups on day 1. However, Pitx3-overexpressing mice improved their performance over the next 3 days, as assessed by the duration of their attempt to stay on the rod (Fig. 4D). To further assess the magnitude of rotarod learning over several days, we compared the difference between rotarod latency on day 5 between the control and Pitx3 overexpression groups. Pitx3-overexpressing mice demonstrated a marked improvement over the 4 days compared to control mice, consistent with impaired motor learning in Pitx3 overexpression mice (Fig. 4E). Taken together, these data suggest that misregulation of Pitx3 expression affects motor functions, consistent with inappropriate midbrain DA neuron function.

4. Discussion

Here, we show that mice overexpressing Pitx3 show significant differences in midbrain DA neurons function, suggesting that the expression level of Pitx3 is critical for the functions of adult midbrain DA neurons. We observed significant abnormalities in gene expression, monoamine levels, and motor and anxiety-like behaviors associated with DA neurons in the Pitx3-overexpressing mice. Several groups have demonstrated that Pitx3 deficiency in ak mice causes selective loss of A9 DA neurons in the midbrain and have shown impaired performance on motor coordination tasks [6,13,16,18]. In this study, for the first time, we report that mice overexpressing Pitx3 also show these behavior abnormalities, as demonstrated by an enhanced baseline locomotor activity compared to control mice. These data directly suggest that although Pitx3 expression persists throughout the life, a precise dosage of Pitx3 expression is essential for function of midbrain DA neurons *in vivo*. The development and function of midbrain DA neurons requires complex changes in transcriptional circuits of regulating gene expression [23]. Pitx3 may play important roles in these transcriptional regulatory circuits, providing a mechanism for rapid changes in its transcription level during the development and functions of midbrain DA neurons.

Previously, we have shown that Dicer midbrain knockout mice have increased Pitx3 levels in the midbrain DA neurons [9]. This result directly suggested expression of Pitx3 is down-regulated by miRNAs. Moreover, we proposed that one role of miR-133b is to control the expression of Pitx3 via a negative feedback loop. One interesting effect of this negative feedback loop may be to create a safe mechanism for avoiding high levels of Pitx3 activity. This interpretation is supported by the observations that overexpression of Pitx3 in adult mice led to behavioral changes. We observed that high Pitx3 levels are potentially dangerous for the function of DA neurons. Numerous studies support a role for the precise timing and dosage of transcription factors for their proper functions. For example, the report of the successful generation of dopamine neurons from mouse ESC using a Nestin-enhancer-Lmx1a construct suggests that precise dosage and timing of Lmx1a are essential for the differentiation of DA neurons from immature cells [1]. Thus, our results contribute to the understanding of the regulatory mechanisms for down-regulating Pitx3 expression and are relevant to the development and function of DA neurons.

There is growing evidence that Pitx3 is involved in neurodegenerative disorders [4], possibly presenting new directions for therapeutic intervention [19]. Pitx3 genetic variants have been identified in the midbrain of PD patients and Pitx3 depletion promotes PD progression. However, we did not detect midbrain DA neuron degeneration in aged animals overexpressing Pitx3. Moreover, no association has been found between Pitx3 overexpressing genetic variants and PD [3]. These results suggest that Pitx3 overexpression may not be causal in PD neurodegeneration. However, we cannot exclude the possibility that the overexpression of Pitx3 may enhance the susceptibility of midbrain DA neurons to neurodegeneration resulting from PD or toxic insults. This could be tested in the future by comparing PD-like disease progression in control and Pitx3-overexpressing mice following MPTP or 6-OHDA treatment. Although Pitx3 overexpression does not appear to play a causal role in midbrain neurodegeneration, high level of Pitx3 in midbrain DA neurons *in vivo* does cause profound changes in neuronal functions. Therefore, understanding the Pitx3-based transcriptional network in midbrain DA neurons may contribute to neuronal development and survival. *In vivo* analyses of this transcriptional regulation may pave the way for novel therapeutic strategies in PD and other disorders of the midbrain DA system.

Acknowledgment

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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.bbrc.2014.03.085.

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