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Restoration of Noradrenergic Function in Parkinson's Disease Model Mice

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Abstract

Dysfunction of the central noradrenergic and dopaminergic systems is the primary neurobiological characteristic of Parkinson's disease (PD). Importantly, neuronal loss in the locus coeruleus (LC) that occurs in early stages of PD may accelerate progressive loss of dopaminergic neurons. Therefore, restoring the activity and function of the deficient noradrenergic system may be an important therapeutic strategy for early PD. In the present study, the lentiviral constructions of transcription factors Phox2a/2b, Hand2 and Gata3, either alone or in combination, were microinjected into the LC region of the PD model VMAT2 Lo mice at 12 and 18 month age. Biochemical analysis showed that microinjection of lentiviral expression cassettes into the LC significantly increased mRNA levels of Phox2a, and Phox2b, which were accompanied by parallel increases of mRNA and proteins of dopamine β-hydroxylase (DBH) and tyrosine hydroxylase (TH) in the LC. Furthermore, there was considerable enhancement of DBH protein levels in the frontal cortex and hippocampus, as well as enhanced TH protein levels in the striatum and substantia nigra. Moreover, these manipulations profoundly increased norepinephrine and dopamine concentrations in the striatum, which was followed by a remarkable improvement of the spatial memory and locomotor behavior. These results reveal that over-expression of these transcription factors in the LC improves noradrenergic and dopaminergic activities and functions in this rodent model of PD. It provides the necessary groundwork for the development of gene therapies of PD, and expands our understanding of the link between the LC-norepinephrine and dopamine systems during the progression of PD.

Keywords

locus coeruleus, norepinephrine, tyrosine hydroxylase, dopamine, dopamine β-hydroxylase, Morris water maze

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Introduction

The locus coeruleus (LC, A6) is the primary source of norepinephrine (NE) in the brain, and sends divergent projections throughout the central nervous system (Aston-Jones et al., 2000). The hippocampus (HP) and frontal cortex (FC) are the brain regions to receive sole noradrenergic innervation from the LC (Haring and Davis, 1985; Samuels and Szabadi, 2008). Correspondingly, the substantia nigra pars compacta (SN, A9) and ventral tegmental area (VTA, A10) (Anden et al., 1966; Domesick, 1988) are the primary source of dopamine (DA) in the brain, each of which sends variant axons throughout the striatum and other regions of the cerebral cortex. NE and DA play

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important roles in spatial memory, attention, motor functions, as well as emotional behaviors (Robbins and Everitt, 1995; Bromberg-Martin et al., 2010). Although the noradrenergic and dopaminergic systems account for different functions and activities in the brain, however, they are closely correlated in normal and pathogenic conditions, likely due to the fact that NE and DA are on the same biosynthesis pathway.

Parkinson's disease (PD) is a neurodegenerative disease in which both the noradrenergic and dopaminergic systems share a progressive neuronal loss. Nevertheless, a number of studies have showed that a constant and extensive neuronal loss in the LC of PD patients is even greater than loss of dopaminergic neurons, and this pathology appears earlier to the dopaminergic neuronal loss in the SN (Cash et al., 1987; Bertrand et al., 1997; Zarow et al., 2003). Recent observation showed that a transgenic mouse, expressing human wild-type α-synuclein controlled by dopamine β-hydroxylase (DBH) promoter, developed α-synuclein in LC neurons with degeneration of LC fibers, decreased striatal DA metabolism, and non-motor symptoms of PD (Butkovich et al., 2020). Therefore, NE deficiency is an early indication of PD progression in the brain (Espay et al., 2014). Moreover, LC-NE pathologies are directly associated with the severity of parkinsonism, including cognitive inflexibility (McGaughy et al., 2008). More importantly, an intact LC-NE neuronal system has neuroprotective effects on dopaminergic neurons and an increased NE concentration in the brain can facilitate the recovery of the damaged dopaminergic system (Delaville et al., 2011; Isaias et al., 2011; Paredes-Rodriguez et al., 2020). Therefore, restoration of the damaged central LC-NE system may positively influence the recovery process of degeneration to the dopaminergic system, ultimately facilitating the treatment of PD.

Over the past several years, substantial progress has been made in uncovering critical effects of transcription factors such as Phox2a, Phox2b, Hand2 and Gata3 on the brain's noradrenergic system. It is well known that during the embryonic period, these transcription factors coordinately control the differentiation of noradrenergic neurons (Brunet and Pattyn, 2002; Schmidt et al., 2009; Tsarovina et al., 2010). Recent studies also revealed that these transcription factors have potential regulatory roles for noradrenergic properties in the adult brain. Supportive evidence includes that all four transcription factors are present in fully differentiated noradrenergic neurons *in vitro* and in noradrenergic neurons in the brain throughout life (Zhu et al., 2005; Doxakis et al., 2008; Zhao et al., 2008; Schmidt et al., 2009; Pellegrino et al., 2011). Furthermore, they are required for maintenance of mature noradrenergic neurons *in vivo* and for the continued expression of DBH and tyrosine hydroxylase (TH) (Lucas et al., 2006; Schmidt et al., 2009; Card et al., 2010; Tsarovina et al., 2010). Our previous studies *in vitro* and *in vivo* showed that these transcription factors could increase the transcription of DBH and TH (Fan et al., 2009; Fan and Zhu, 2010; Fan et al., 2011). Therefore, it is feasible to enhance the function of the noradrenergic system in PD animal models by overexpressing these transcription factors in the LC.

The neural specific vesicular monoamine transporter 2 (VMAT2) is a transmembrane protein and is a key regulator of monoamine homeostasis. VMAT2 plays an important role in monoamine storage, protects neurotransmitters from oxidation and controls quantal secretion of these neurotransmitters (Erickson et al., 1992). As such, loss of VMAT2 function has been considered to be conductive to the pathology of PD (Lotharius and Brundin, 2002). A transgenic mouse (called as VMAT2 Lo) has been created with ∼95% shutdown in endogenous VMAT2 expression (Mooslehner et al., 2001; Colebrooke et al., 2006). These mice display substantial reductions of DA and NE levels in the striatum and cortex, and progressive degeneration in dopaminergic and noradrenergic neurons with formation of α–synuclein containing inclusions in the SN (Caudle et al., 2007; Taylor et al., 2009, 2011). This model also shows motor- and non-motor symptoms with the VTA unaffected, thereby replicating important pathogenic features of PD. More importantly, unlike other chemical and genetic models of PD, neuronal loss in the LC of VMAT2 Lo mice starts earlier (at 12 months, and with a larger reduction at 18 months of age) than occurs in the SN (beginning at 18 months and reaching significant degeneration at 24 months and maximal severity at 30 months) (Caudle et al., 2007; Taylor et al., 2014). Furthermore, the LC undergoes a much more severe degeneration than the SN in the VMAT2 Lo mice (Taylor et al., 2011, 2014). Therefore, these mice are an excellent model for the analysis of restoring noradrenergic activity and further affecting the dopaminergic system.

In the present study, effects of forced overexpression of transcription factors on activities and function of central noradrenergic and dopaminergic systems were examined. The lentiviral construction cassettes of Phox2, Hand2 and Gata3, alone or combined, were microinjected into the LC region in VMAT2 Lo mice. The expression levels of noradrenergic and dopaminergic phenotypes in brain areas were measured. The results demonstrated that overexpressed transcription factors upregulated DBH and TH expression in the LC and its main projection areas, and increased NE/DA concentrations in the striatum. Furthermore, these treatments also enhanced TH expression in the SN and striatum and improved cognitive and locomotor performance. These data indicate that microinjection of these transcription

factors can be an alternative approach for gene therapy in PD.

Method and Materials

Lentiviral Vector Production

Lentiviral vectors were prepared and used by the similar methods as published previously (Fan et al., 2011), which was approved by the Institutional Biosafety Committee of the East Tennessee State University. Briefly, a lentiviral vector pLenti6/V5, and the packaging vector pCMV△R8.9 and pVSVG were used in this preparation. Dual promoters to drive the genes of interest (*Phox2a*, *Phox2b*, *Hand2* or *Gata3* from mice), and the reporter gene (enhanced green fluorescent protein, eGFP), an index for verification of viral delivery to the LC site in mouse brains, were constructed as lentiviral-mediated expression cassettes, based on the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). The cassette for the control in the experiments contained eGFP only. After modification, transferring, co-infection, and untracentrifugation, the lentiviral stock was obtained and their viral titers were measured. A stock with viral titer ($1x10^8$ TU) was stored at −80°C until use.

Animals and in Vivo Stereotaxic Microinjection

Both male and female VMAT2 Lo mice (RRID: MGI_3758030), bred in the animal facility of the XXX State University, were used in the present study. As described above, neuronal loss in the LC of VMAT2 Lo mice starts at 12 months, and with a larger reduction at 18 months of age (Taylor et al., 2014). Considering these characteristics, two ages of mice were selected for microinjection treatments: 12 months of age (the LC begins degeneration), and 18 months of age (significant LC neuronal loss and dopaminergic neurons in the SN beginning to degenerate). Although VMAT2 Lo mice at 24 month of age showed more severely neuronal loss in the SN with major motor deficits, they were determined to be of too advanced age to bear the microinjection surgery based on preliminary observations. Tissues were used without regard to the sex of the animal so we are unable to assess sex-dependent effects.

These mice were housed on a 12 h light/dark cycle and had free access to food and water. All animal handling procedures were approved by the Animal Care and Use Committee of East Tennessee State University, which are consistent with the NIH Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). VMAT2 Lo and age-matched wild-type littermates were randomly assigned to the experimental and control/sham-operation groups. The animal numbers used in groups were estimated by a power analysis. This is based on our preliminary studies, by assuming the weaker of the two effects (i.e., Post-BUP). Microinjection was performed based on previous works (Carlezon and Neve, 2003) with some modifications. Briefly, under gas anesthesia through isoflurane (1.5–2.0%), animals were placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Two burr holes were drilled into the skull above the LC region with an electric hand-drill to the level of the dura mater. A sterile 26-guage Hamilton syringe, loaded with recombinant lentivirus preparations (1 µl at 1×10^8 TU of titer per milliliter, or 2 µl in the combination of two genes), was lowered to the LC ([AP] = −5.4 mm, [LAT] = ±0.9 mm, and [V] = −3.9 mm) (Paxinos and Franklin, 2001). Microinjection was carried out at a rate of 0.1 µl/30 seconds over a period of 10 minutes (20 min in the combination of two genes: either Phox2a+Hand2, Phox2b+Gaba3, or Phox2b+Hand2, Phox2b+Gaba3). After injection, dental cement was used to fill the holes and the incision closed with sutures. In the control mice, the lentiviral cassette carrying eGFP only was bilaterally microinjected, whereas in sham-operated mice an empty Hamilton syringe was lowered into the LC region without injection. Mice given surgery were allowed to recover, and 21 days later were sacrificed by decapitation (for biochemical measurements) or perfusion (for immunofluorescence staining). After decapitation mouse brains were removed, rapidly frozen in 2-methyl-butane on dry-ice, and stored at −80°C until dissection of different brain regions (see below for the details of the LC dissection). Perfused mouse brains were also stored at −80°C until section on a cryostat. To protect newly synthesized NE and DA, the no-selective monoamine oxidase inhibitor phenelzine (10 mg/kg, i.p.) (Griebel et al., 1998; Dwivedi et al., 2006; Sakata et al., 2013) was administered daily for 21 days for all VMAT2 Lo mice (except for those without injected mice), as well as corresponding control and sham-operation groups. Microinjection into the LC region was verified by examining eGFP fluorescence under a fluorescence microscope to confirm the delivery of the viruses to the LC region. Mice with missed placements were eliminated from the study.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qPCR) Analysis for mRNAs of Phox2a, Phox2b, Hand2, Gata3, TH and DBH in the LC

The brains of some mice were microdissected at −20°C. First, along the line between the posterior border of the cerebral cortex and cerebellum was vertically cut with a razor blade to separate the brain stem from the brain cortex to show the midbrain aqueduct. Then a slight trimming to sure the appearance of the 4th ventricle, which was used for orientation of the LC. From there a

posterior section from appearance of 4th ventricle to the place where the 4th ventricle becomes a triangle shape a section of about 350 μM thickness was cut again (approximately −5.34 to −5.70 mm from the Bregma). On this coronal section a 2 mm trephine tool was used to punch the LC region near two corners of the 4th ventricle. The LC tissues were collected and stored at −80°C for RNA isolation. The RNA isolation and qPCR analysis were similar to those described previously (Zha et al., 2011). Briefly, RNAzol reagent (Molecular Research Center, Inc., Carlsbad, CA) was used to extract total RNA from dissected brain LC regions of mice. The superscript III First-Strand Synthesis Kit (Applied Biosystems/Life technologies, Forster City, CA, USA) was used to convert cDNAs, based on the manufacturer's protocol. Then qPCR analysis was performed using primers listed as follows. Mouse DBH: 5′- CTCAGGAGACTGCCTTT GTGTTG-3′ and 5′- GAAGCTGAGAGGCAAAGAT GTGG-3′. Mouse TH: 5′-AGCCGTGCAGCCCTACC A-AGATCAA-3′ and 5′- AATGGGCGCTGGATACG AGAGGCAT-3′; Mouse Phox2a: 5′-CGAGGAACTGG CGCTCAAGA-3′ and 5′- CGCTCCTGTTTGCGGAA CTTG-3′; Mouse Phox2b: 5′-GGGCTAAGTTTCGC AAGCAG-3′ and 5′- CAGTGCTGTCGGGATCAG TG-3′; mouse *β-actin*: 5′-CAACGAGCGGTTCCGA TG-3′ and 5′- GCCACAGGATTCCATACCCA-3′. For analyzation of the relative changes in gene expression from qPCR, the value of each interest gene was normalized to *β*-actin (ΔCt). The comparative threshold cycle method ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001) was used to compare mRNA levels of each respective gene expression calculated as the inverse log of ΔΔCT to give the relative fold change. These measurements were performed in triplicate of each cDNA aliquot.

Immunofluorescence Staining for DBH and TH

This method was similar to previous work from our laboratory (Fan et al., 2011). Briefly, the slides containing the sections of LC regions were pre-incubated in 5% bovine serum albumin in phosphate-buffer saline (PBS) supplemented with 0.2% Triton-X 100. These slides were further exposed to primary monoclonal antibody solution (for DBH: 1:2000, ab19353, RRID: AB_73185, Abcam, Cambridge, MA, USA; for TH: 1:1000, MA1-24654, RRID: AB_79566, ThermoFisher Scientific, Waltham, MA, USA) overnight at 4°C. After washing, slides were then probed with 2nd antibodies (for DBH: Alexa Fluor 488-conjugated goat anti-rabbit IgG; for TH: Alexa Fluor 488-conjugated Goat anti-mouse IgG; both from Abcam, Cambridge, MA, USA), followed by 4 time rinses with 0.1 M PBS. A Leica TCS SP2 confocal microscope system (Leica Microsystems Inc., Bannockburn, IL, USA) was used to observe and acquire immunofluorescence labeling. These images were quantitatively quantified using ImageJ software (Rasband, US National Institutes of Health, Bethesda, http://rsbweb.nih.gov/ij, 2010). Non-immunoreactive portions of brain sections adjacent to the LC region were taken as the reference background levels.

Measurement of NE/DA by High-Performance Liquid Chromatography (HPLC)

Measurement of NE/DA in mouse striatum was similar to those described before (Fan et al., 2020). Briefly, animals were sacrificed and brains were dissected. The striatum was homogenized in an ice-cold solution containing 0.2 M perchloric acid, 1×10^{-7} M ascorbic acid, and dihydroxybenzylamine (2 μg/ml) which was used as the internal standard. Following centrifugation (10,000 xg at 4°C for 5 minutes) and filtration in 0.2 μm nylon disposable syringe filters, the supernatant (5 μL) was injected into the HPLC system. This chromatography system includes an Ultrasphere ODS reverse-phase column (Beckman), electrochemical detection and a Hitachi D-2500 Chromato-Integrator. The mobile phase consisted of 4% acetonitrile, 0.1 M sodium nitrate, 0.08 M sodium dihydrogen phosphate, 0.2 mM sodium octyl sulfate, and 0.1 mM EDTA. A standard curve was constructed with known amounts of NE/DA to calculate the NE/DA concentration. Before centrifugation and filtration, the protein concentration in tissue homogenate of the striatum was measured, which was used to define the NE/DA presentation in homologies as pg/mg proteins.

Behavior Tests: Morris Water Maze (MWM) & Locomotor Behavior Tests

The MWM is a behavior test for animal spatial learning and reference memory and the method used for this task was similar to previous work (Brown et al., 2001). Briefly, animals were administered a total of 4 days of testing. Each day two trial blocks of four trials were given to each animal, for a total of 24 training trials during acquisition. On each trial, mice were released with their snout pointing towards the wall of the pool in the east, north, south and west (these directions do not indicate compass points) quadrants of the pool and allowed a swim time of 60 s to reach the platform. If the mouse did not find the platform, it was placed there by the experimenter. Regardless, if the animal found the platform, it spent the last 10 s of each trial on the platform. Both swim distance and escape latency (time to find the platform) were recorded on each trial. Following the last trial, the platform was removed from the pool and a probe trial was administered. For the probe trial, the mean search difference (MSD) and mean search difference (MZD) scores (Brown et al., 2000; Gonzalez et al., 2000) were used to analyze

search behavior. The total time spent searching and swim pattern was recorded via a digital camera (Rockhouse Products, NJ, USA). The AnyMaze behavioral scanning system (Stoelting, Co, Wood Dale, IL, USA) was used to analyze all behavioral measurements.

The locomotor activity test is a mean of assessing spontaneous locomotor activity and arousal in mice, and is necessary to establish baseline levels of motor activity. The method used was similar to a previous publication (Brown et al., 2011). Briefly, animals were placed individually in a square arena (30 cm/side) with Plexiglas floor and walls (Kinder Inc., Poway, CA, USA) and allowed to freely move for 60 min. All animal locomotor activities were recorded by the AnyMaze behavioral software and distance traveled during the 60 min period was recorded and analyzed.

Western Blot Analysis

Western blotting was performed similar to previous work from our laboratory (Fan et al., 2011). About 30 µg proteins from each sample were loaded into a 10% SDS–polyacrylamide gel and electrophoretically separated, and electro-blotted onto a nitrocellulose membrane (Amersham Life Sciences, Buckinghamshire, UK). These blots were respectively incubated with antibodies of monoclonal anti-TH antibody (H-16, 1:1,000 dilution, AMAb91112, RRID: AB_2665805, Sigma, USA), or monoclonal antibody against DBH (sc-365710, 1:100 dilution, RRID: AB_1084404, Sanda Cruz Biotechnology, Dallas, TX, USA), which was followed by being exposed to the second antibody (1:5,000 dilution; Amersham Life Sciences, Buckinghamshire, UK). Immunoreactive bands were visualized and detected by G:Box Imaging (Fyederick, MD, USA) after membranes were probed by enhanced chemiluminescence (ECL, Amersham Life Sciences, Buckinghamshire, UK) or super ECL (Sigma Chemical Co., St Louis, MO, USA). By the similar steps, ß-actin reactivates were determined as an index of equal loading and transfer effectivities after membranes were re-probed using antibody against mouse ß-actin.

Statistics: All experimental data are presented in the text and graphs as the mean ± SEM, with an enumerated replicate number (N = x/group) in the figure legends. Statistical analysis was carried out using one-way analysis of variance (ANOVA) when comparing multiple treatment groups for most experiments (SigmaStat, Systat Software Inc., Richmond, VA). Then *post-hoc* Duncan's Multiple Range tests were performed for planned comparisons. A two-way ANOVA was used for MWM acquisition statistics.

Results

Microinjection of Lentiviral Expression Cassettes Enhanced mRNA Levels of Phox2a/2b, DBH and TH in the LC Region

The cassettes of recombinant lentiviral vector-cDNAs (*Phox2a* or *Phox2b*, plus *Hand2* or *Gata3*) were bilaterally microinjected into the LC region of VMAT2 Lo mice. Age-matched wild-type littermates were used as the control (microinjected with lentiviral constructs containing eGFP only) and sham operation groups (an empty Hamilton syringe lowered into the LC region without injection). All mice were sacrificed on the 22nd day after microinjection, a period that long enough for overexpression of transcription factors based on past works (Fan et al., 2011). Figure 1A showed that microinjection was accurately delivered to the LC region as indicated by eGFP fluorescence in the target area. The first experiment was to examine mRNA levels of Phox2a/2b, Hand2 and Gata3 in these mice by quantitative PCR (qPCR). As shown in Figure 1B and C, microinjection of these lentiviral expression cassettes significantly influenced mRNA levels of Phox2a and Phox2b in the LC regions (Figure 1A for Phox2a, 12 month: $F_{6,64,} = 6.85$, $p < 0.01$; 18 month: $F_{6,63} = 4.18$, $p < 0.05$. Figure 1B for Phox2b, 12 month: $F_{6,56,} = 9.25$, $p < 0.001$; 18 month: $F_{6,58} = 7.37$, $p < 0.01$). Comparison of the control and sham-operation groups showed no significant difference, indicating microinjection operation did not cause marked effects on these gene expressions. The comparison results for the control and sham-operation groups were similar for all following experiments. *Post hoc* analysis revealed that whereas both control and sham-operated groups demonstrated a significantly higher Phox2a mRNA expression than those in VMAT2 Lo mice without microinjection (Lo) ($p < 0.05$ or 0.01 respectively), microinjection of *Phox2a, Phox2a+Hand2* and *Phox2a+Gata3* resulted in a markedly increased Phox2a mRNA level ($p < 0.01$), as compared to those of Lo mice. However, there was no significant difference between these microinjection groups and control groups (Figure 1A). A similar expressive pattern was found for Phox2b mRNA measurements (Figure 1B). Similarly, microinjection of these cassettes also markedly influenced mRNA levels of Hand2 and Gata3 in the LC (Figure 1D for Hand2, 12 months: $F_{6,64,} = 5.33$, $p < 0.01$; 18 month: $F_{6,63} = 5.24$, $p < 0.01$. Figure 1E for Gata3, 12 month: $F_{6,56,} = 6.78$, $p < 0.01$; 18 month: $F_{6,58}=6.27$, $p < 0.01$). It is noteworthy that comparing to those in the control, there are a relatively lower expression level of Phox2a or Phox2b in the LC in Lo mice of both 12 and 18 month-old, and relatively lower levels of Hand2 and Gata3 in the LC of 18 month-month. It indicates that in VMAT2 Lo mice the expression of these transcription factors was also reduced accompanied with the loss of noradrenergic neurons.

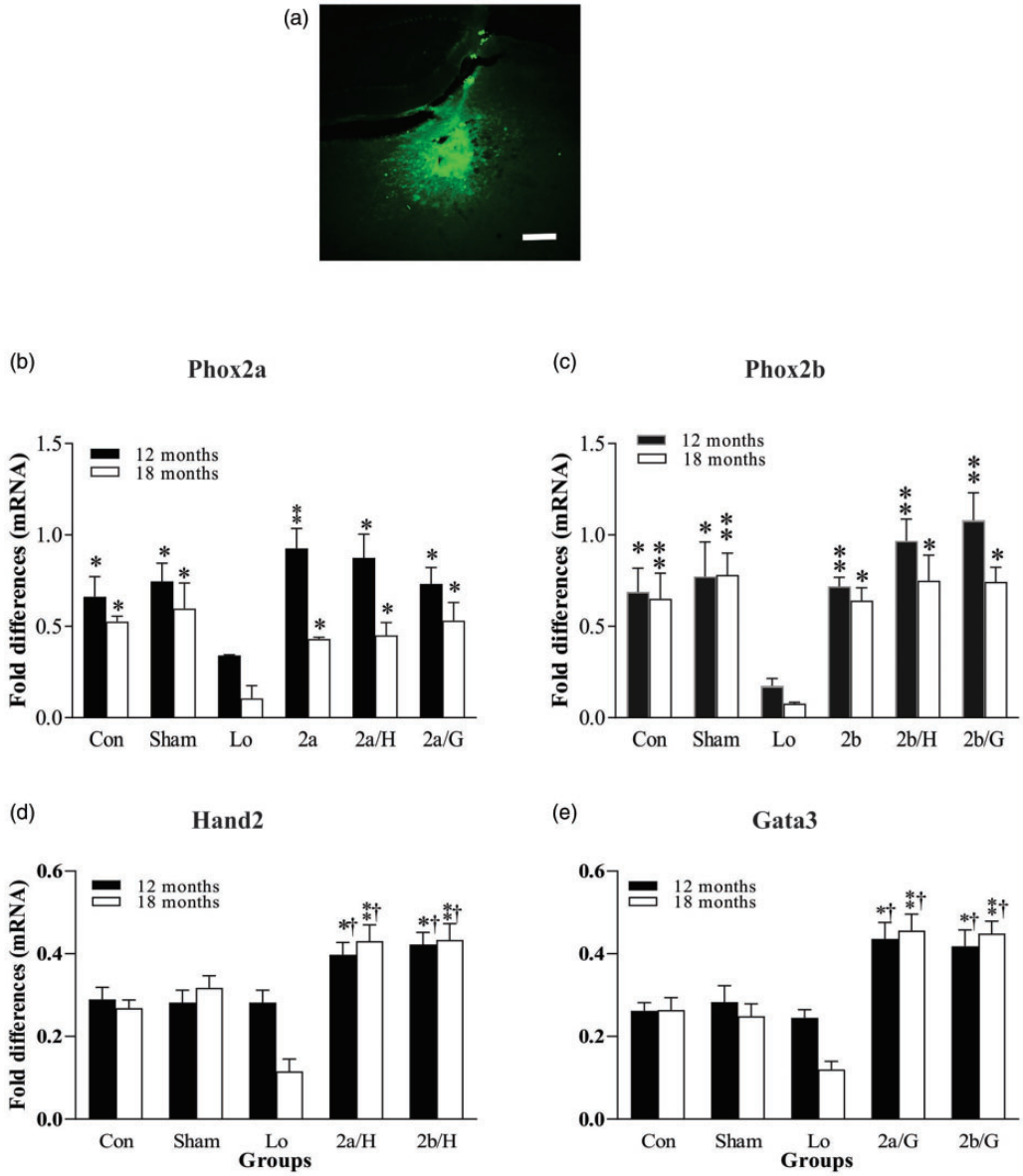

Figure 1. Effects of microinjection on mRNAs of Phox2a/2b, Hand2 and Gaba3 in the LC region as indicated by eGFP fluorescence in the target area. mRNA expression levels of Phox2a (A), Phox2b (B), Hand2 (C) and Gata3 (D) in the LC of 12 or 18 month-old mice after microinjection of lentiviral expression cassettes, measured by qPCR ($N = 6$). * $p < 0.05$, ** $p < 0.01$, compared to the Lo. † $p < 0.05$, compared to the control. Scale bar = 100 μm in (A). Abbreviations: Con: control; Sham: sham-operation; Lo: VMAT2 Lo mice without microinjection; 2a: microinjection with *Phox2a*; 2 b: microinjection with *Phox2b*; 2a/H or 2 b/H: microinjection with *Phox2a* or *Phox2b*+*Hand2*; 2a/G or 2 b/G: microinjection with *Phox2a* or *Phox2b*+*Gata3*.

Next, mRNA levels of DBH and TH in the LC were measured aiming to elucidate effects of over-expression of these transcription factors on the noradrenergic phenotype. qPCR analysis showed that microinjection of lentiviral expression cassettes markedly changed mRNA levels of DBH and TH (Figure 2A for 12 month-old, DBH: $F_{8,54} = 7.15$, $p < 0.01$; TH: $F_{8,54} = 3.21$, $p < 0.05$. Figure 2B for 18 month-old, DBH: $F_{8,52} = 7.25$, $p < 0.001$; TH: $F_{8,52} = 6.53$, $p < 0.01$). *Post hoc* analysis demonstrated that in 12 month-old Lo mice, mRNA levels of DBH and TH were not significantly different from the control (Figure 2A), indicating a relatively intact distribution of noradrenergic neurons at that developmental period. However, in 18 month-old Lo mice (Figure 2B), mRNA levels of DBH and TH were markedly reduced as compared to those in the control, a consistent finding with previous work (Taylor et al., 2014). In the LC of 12 month-old VMAT2 Lo mice, a significantly increased DBH mRNA level was found in the groups microinjected with *Phox2b*,

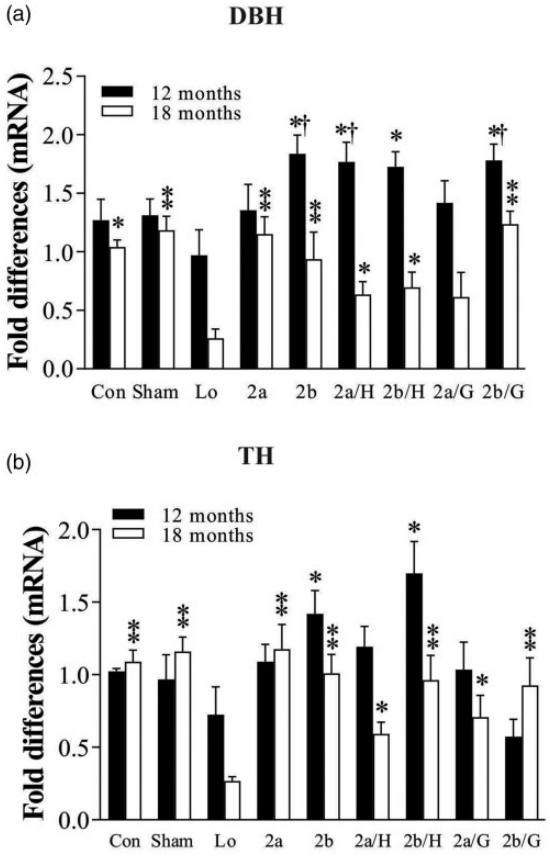

Figure 2. Effects of microinjection on mRNAs of DBH and TH in the LC of 12 or 18 month-old mice after microinjection of lentiviral expression cassettes, measured by qPCR ($N = 6$). *$p < 0.05$, **$p < 0.01$, compared to the Lo. †$p < 0.05$, compared to the control. See Figure 1 legend for abbreviations. 12-m or 18-m: 12 or 18 month-old mice.

Phox2a+Hand2, *Phox2b+Hand2* and *Phox2b+Gata3*, respectively, as compared to Lo animals. Interestingly, DBH mRNA levels in the group of microinjection of *Phox2b*, *Phox2a+Hand2*, *Phox2b+Hand2* and *Phox2b+Gata3* were even higher than those of the control (Figure 2A). Whereas microinjection of *Phox2b+Hand2* increased TH mRNA levels, other groups did not show a statistical difference at 12 months of age, as compared to the Lo group (Figure 2A). Nevertheless, in 18 month-old mice, all microinjection groups showed an enhanced mRNA level of DBH or TH in the LC, as compared to those Lo mice (Figure 2B, $p < 0.05$ or $p < 0.01$).

Microinjection of Lentiviral Expression Cassettes Increased Protein Levels of DBH and TH in the LC Region

Immunofluorescence analysis was used to measure protein levels of DBH and TH in the LC after microinjection of lentiviral expression cassettes in the brain of 12 and 18 month-old mice. Our previous study demonstrated that protein levels of DBH and TH in the LC as measured by immunofluorescence staining and western blotting were very similar (Fan et al., 2020). Therefore, only immunofluorescence staining was used for DBH and TH proteins in the LC in the present study. As shown in Figure 3, overexpression of transcription factors in the LC significantly affected DBH protein levels in the LC (12 month-old: $F_{8,54} = 4.15$, $p < 0.05$; 18 month-old: $F_{8,63} = 6.78$, $p < 0.01$). DBH immunoreactivities in the LC of 12 month-old Lo mice were not markedly different from those of the control. However, 18 month-old Lo mice exhibited a markedly reduced DBH immunoreactivities as compared to those of control ($p < 0.01$, Figure 3B), indicating a severe loss of noradrenergic neuron in the LC of 18 month-old VMAT2 Lo mice. *Post hoc* analysis demonstrated that in the LC of 12 month-old mice, the microinjection of all injected groups produced a significant increase of DBH immunoreactivities, as compared to those of the control (Figure 3A). In the LC of 18-month-old mice, whereas all VMAT2 Lo mice microinjected with lentiviral expression cassettes showed a marked enhanced DBH immunoreactivities than those in the Lo mice, DBH immunoreactivities in the groups microinjected with *Phox2a+Hand2*, and *Phox2a+Gata3* were higher than those in the control group ($p < 0.05$) (Figure 3B).

The TH immunofluorescence staining in the LC also showed similar results as those of DBH. Compared to the control, TH immunoreactivity in the LC of Lo mice at the age of 12 month-old were not significantly reduced, but those of Lo mice at 18 month-old did show a reduction compared to the control (Figure 4). Microinjection of lentiviral expression cassettes highly influenced the TH immunoreactivity in the VMAT2 Lo mice (12 month-old: $F_{8,64} = 5.65$, $p < 0.01$; 18 month-old: $F_{8,62} = 7.88$, $p < 0.01$). *Post hoc* tests revealed that where TH immunoreactivity in all microinjection groups were markedly higher than those of Lo mice ($p < 0.05$ or $p < 0.01$), they were also significantly higher than those of the control in 12 month-old VMAP2 Lo mice ($p < 0.05$ or 0.01). In mice of 18 month-old, LC TH immunoreactivities after microinjection of *Phox2a/2b+ Hand2* or *Gata3* were higher than those of the control ($p < 0.01$, Figure 4B).

Microinjection of Lentiviral Expression Cassettes Increased Protein Levels of DBH and TH in Other Brain Regions

In the brain the LC is the primary source of noradrenergic projections to the FC and HP. Therefore, DBH protein levels in these regions were measured by western blotting. As shown in Figure 5, DBH protein levels in the FC and HP of 12 month-old Lo mice were not

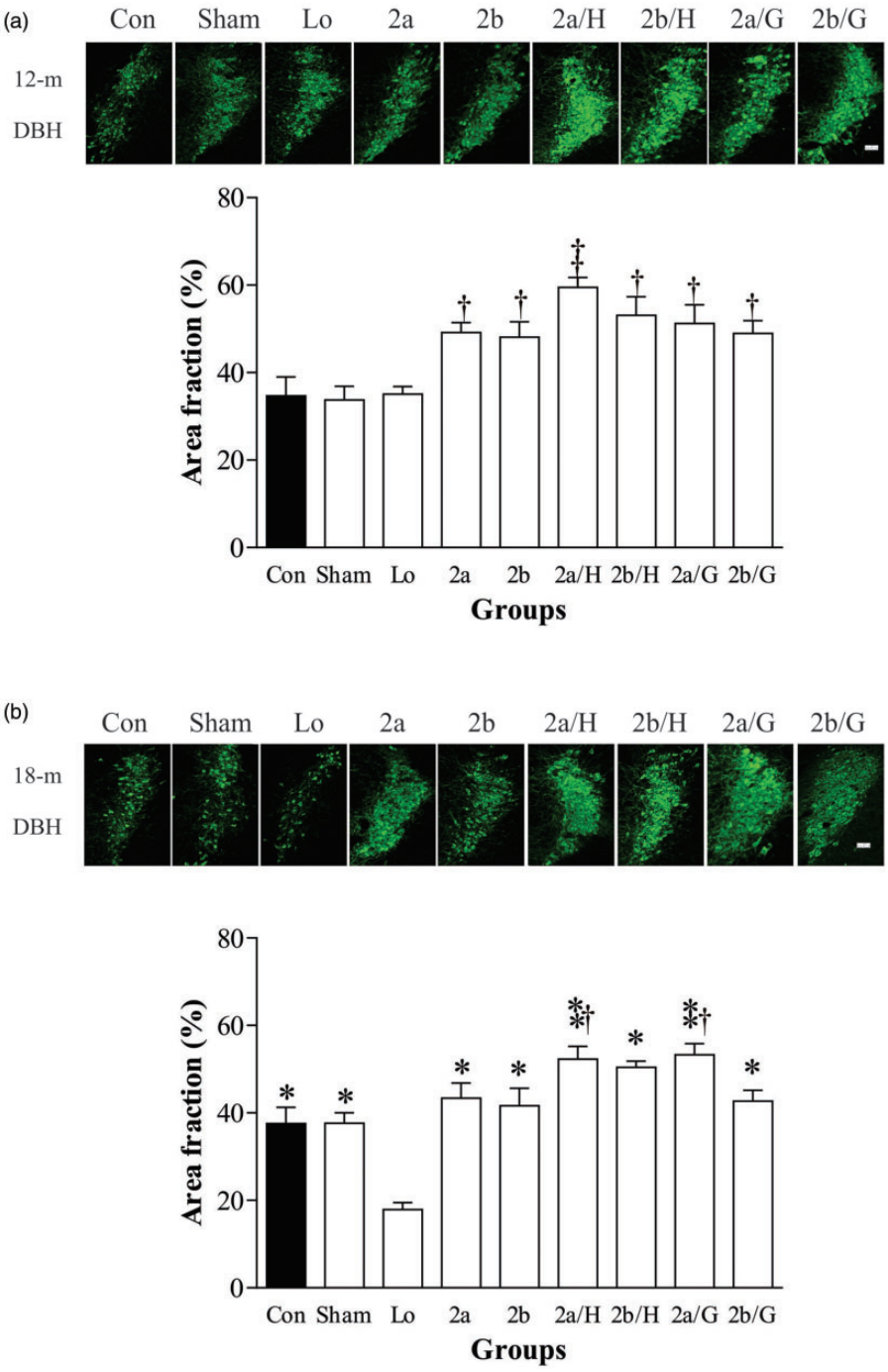

Figure 3. Effects of microinjection on DBH proteins in the LC after microinjection of lentiviral expression cassettes measured by immunoflurescence in mice at age of 12 month-old (N = 6) (A) and 18 month-old (N = 5) (B). Upper panels in A and B are representative micrographs of DBH immunofluorescence. Lower panels in A and B: quantitative analysis of DBH immunofluorescence. *$p < 0.05$, **$p < 0.01$, compared to the Lo mice; †$p < 0.05$, compared to the control. See Figure 1 legend for abbreviations. 12-m or 18-m: 12 or 18 month-old mice. Scale bar: 25 μm for all images.

significantly changed compared to the control. Further, microinjection of lentiviral expression cassettes markedly influenced DBH protein levels in these two regions in VMAT2 Lo mice at 12 and 18 month-old ages (Figure 5A: 12 month-old FC: $F_{8,64} = 3.55$, $p < 0.05$; 18 month-old FC: $F_{8,62} = 5.78$, $p < 0.01$. Figure 5B: 12 month-old HP: $F_{8,56} = 3.75$, $p < 0.05$; 18 month-old HP: $F_{8,60} = 5.97$, $p < 0.01$). *Post hoc* tests demonstrated that in the FC (Figure 5A) of 12 month-old VMAT2 Lo mice microinjection of *Phox2b* and *Phox2b+Hand2*

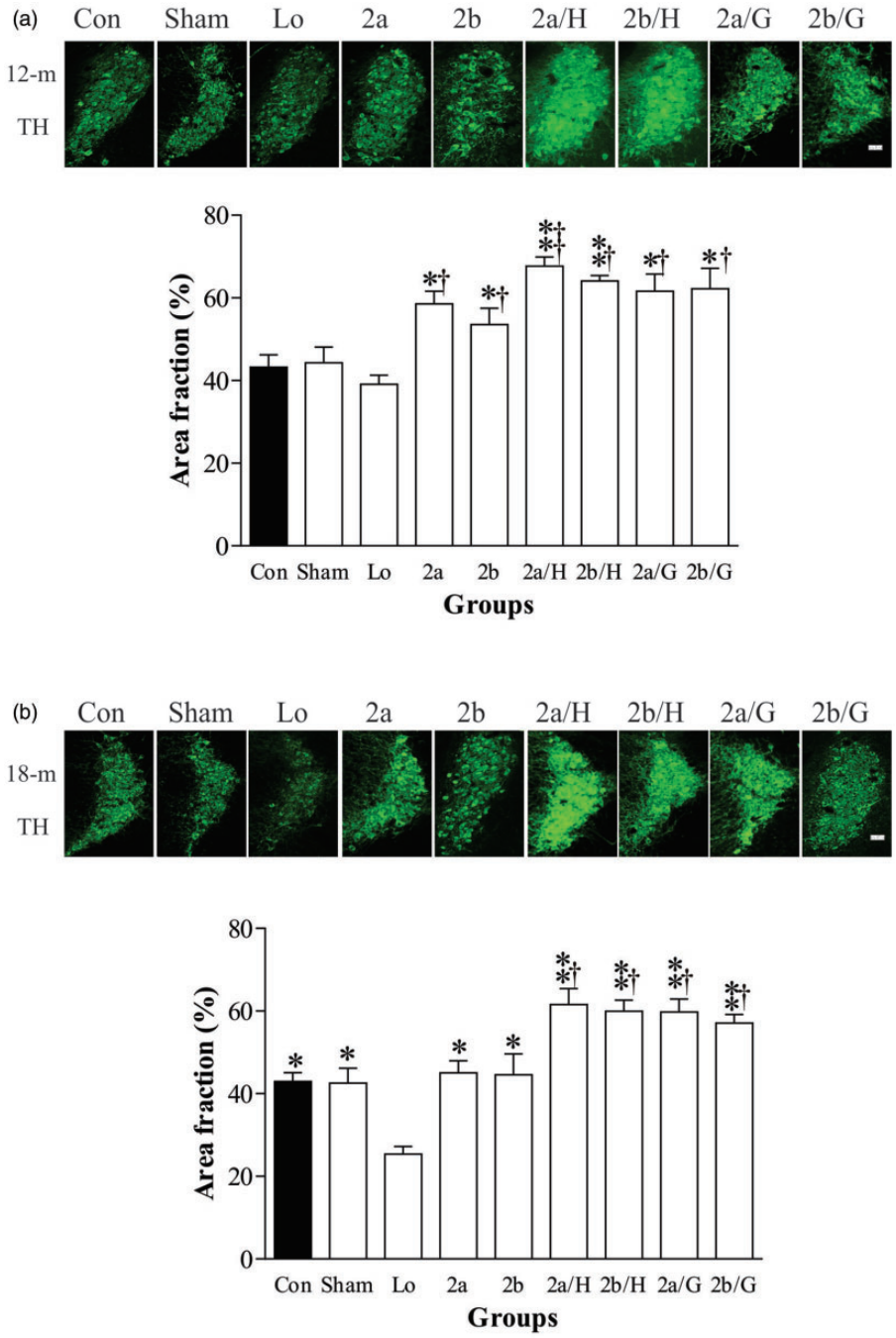

Figure 4. Effects of microinjection on TH proteins in the LC. after microinjection of lentiviral expression cassettes (N = 6) measured by immunoflurescence in mice at age of 12 month-old (A) and 18 month-old (N = 5) (B). Upper panels in A and B are representative micrographs of TH immunofluorescence. Lower panels in A and B: quantitative analysis of TH immunofluorescence. *$p < 0.05$, **$p < 0.01$, compared to the Lo mice † $p < 0.05$, compared to the control. See legends of Figures 1 and 2 for abbreviations. Scale bar: 25 μm for all images.

significantly increased DBH protein levels ($p < 0.05$). However, although DBH protein levels in all microinjection groups in 18 month-old mice were higher than those in Lo mice, only those microinjected with *Phox2a*, *Phox2a+Gata3*, and *Phox2b+Gata3* reached statistical significances ($p < 0.05$). In the HP (Figure 5B), 12 month-olds that received microinjection of *Phox2a* and *Phox2a+Gata3* markedly enhanced DBH protein levels, as compared to those in Lo mice. In the 18 month-old groups, microinjection of *Phox2a* and

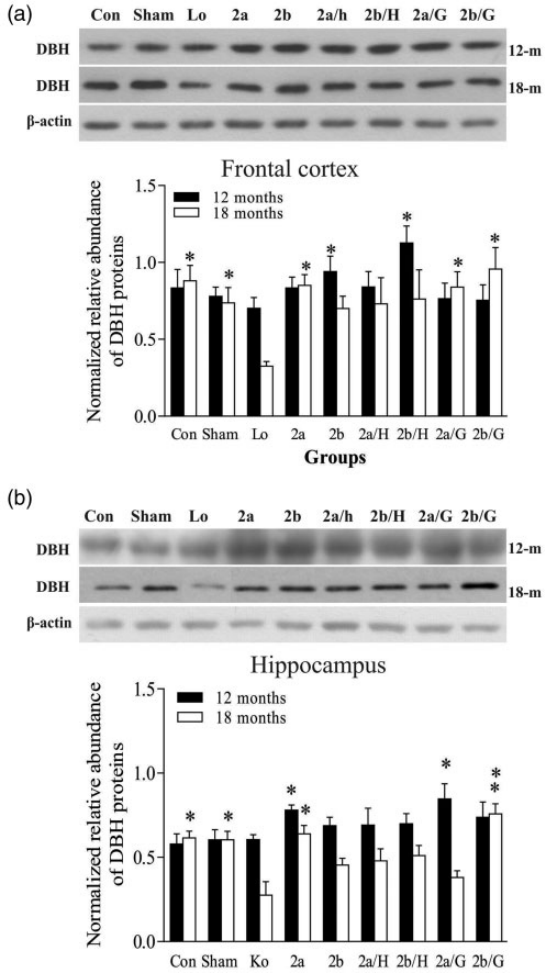

Figure 5. Effects of microinjection on DBH proteins in the FC and HP (A) and hippocampus (B) of mice measured by western blotting (all N = 5). The upper panels in A and B show autoradiographs obtained by western blotting. The lower panels in A and B show quantitative analysis of band densities. *$p < 0.05$, **$p < 0.01$, compared to the Lo mice. See legends of Figures 1 and 2 for abbreviations.

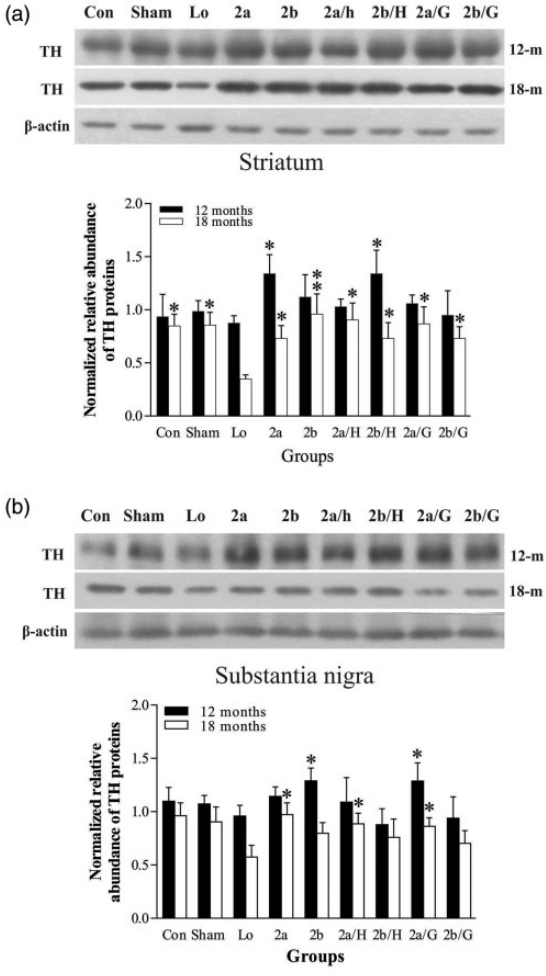

Figure 6. Effects of microinjection on DBH proteins in the striatum and SN (A) and substantia nigra (SN, B) of mice measured by western blotting (all N = 5). The upper panels in A and B show autoradiographs obtained by western blotting. The lower panels in A and B show quantitative analysis of band densities. *$p < 0.05$, **$p < 0.01$, compared to the Lo mice. See legends of Figures 1 and 2 for abbreviations.

Phox2b+Gata3 significantly increased DBH protein levels ($p < 0.05$ or $p < 0.01$), although DBH levels in all injection groups were higher than those of Lo mice (Figure 5B).

The striatum and SN are the major regions where dopaminergic neuron are distributed in the brain. To assess effects of overexpression of transcription factors in the LC on dopaminergic neurons, TH protein levels, as a dopaminergic phenotype, in these regions were examined. Compared to the control, TH protein levels of the striatum (Figure 6A) and SN (Figure 6B) in Lo mice of 12 month-old mice were not significantly changed ($p > 0.05$). However, 18 month-old Lo mice exhibited a significantly reduced TH protein level in these two brain areas ($p < 0.05$). Microinjection of lentiviral expression cassettes markedly influenced TH levels in these two regions (Figure 6A: 12 month-old striatum: $F_{8,59} = 3.78$, $p < 0.05$; 18 month-old striatum: $F_{8,64} = 6.48$, $p < 0.01$. Figure 6B: 12 month-old SN: $F_{8,60} = 4.28$, $p < 0.05$; 18 month-old SN: $F_{8,64} = 6.17$, $p < 0.01$). *Post hoc* tests revealed that in 12 month-old VMAT2 Lo mice, microinjection of *Phox2a* and *Phox2b+Hand2* markedly enhanced TH protein levels in the striatum ($p < 0.05$). In 18 month-old VMAT2 Lo mice, all microinjection groups showed a significant increase of TH levels in the striatum, as compared to those in Lo mice ($p < 0.05$ or $p < 0.01$, Figure 6A). In the SN, there were an enhanced TH protein level in microinjection of *Phox2b* and *Phox2a+Gata3* in 12 month-old mice, and microinjection

of *Phox2a*, *Phox2a+Hand2* and *Phox2a+Gata3* exhibited an increased TH protein level in 18 month-old mice ($P < 0.05$, Figure 6B).

Microinjection of Lentiviral Expression Cassettes Increased NE/DA Levels in the Striatum

To examine effects of overexpression of transcription factors in the LC on noradrenergic and dopaminergic transmitters in the brain, NE and DA concentrations in the striatum of VMAT2 Lo mice and littermates were measured by HPLC using similar methods as previous work (Fan et al., 2020). In both 12 and 18 month-old Lo mice, NE and DA concentrations in the striatum were significantly reduced, as compared to those in the control ($p < 0.01$), a consistent finding to previous report (Taylor et al., 2014). As shown in Figure 7, microinjection of lentiviral expression cassettes significantly affected NE/DA concentrations in the mouse striatum (Figure 7A: 7 to 12 month-old: NE: $F_{8,54} = 7.89$, $p < 0.01$; DA: $F_{8,54} = 7.23$, $p < 0.01$. Figure 7B: 18 month-old: NE: $F_{8,52} = 5.23$, $p < 0.01$; DA: $F_{8,52} = 7.68$, $p < 0.01$). *Post hoc* tests demonstrated that although NE concentrations in the striatum in all groups of 12 month-old and 18 month-old VMAT2 Lo mice were much lower than those in the control or sham groups, they were markedly higher than those in the Lo mice ($p < 0.05$). Similar results were revealed for DA concentrations in the striatum in all groups of 12 month-old (Figure 7A) and in three groups of 18 month-old VMAT2 Lo mice (microinjection of *Phox2a*, *Phox2b* and *Phox2a+Hand2* ($P < 0.05$ or 0.01, Figure 7B). The increased NE/DA concentrations in the striatum indicate that overexpression of transcription factors in the LC improved upon a significantly degenerated noradrenergic and dopaminergic system, although the levels of NE and DA were significantly lower than those of controls.

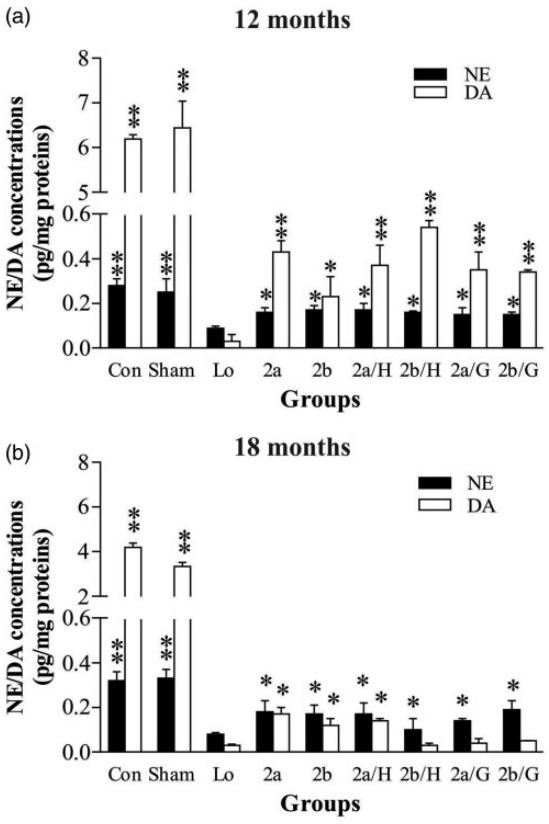

Figure 7. Effects of microinjection of *lentiviral* expression cassettes on NE/DA levels in the striatum measured by HPLC (all $N = 6$) in 12 (A) and 18 month-old (B) mice. *$p < 0.05$, **$p < 0.01$, compared to the Lo mice. See Figure 1 legend for abbreviations.

Microinjection of Lentiviral Expression Cassettes Altered Cognitive and Locomotor Behavior

The MWM behavioral test was performed in these PD model mice. The acquisition latency and mean search difference (MSD) were used to judge effects of overexpression of transcription factors in the LC on cognitive behavior. A two-way ANOVA was used for the statistics of accusation in which the treatment and trial block were two effectors. The analysis revealed that a significant main effect of treatment ($F_{1,70} = 9.11$, $p < 0.001$), trial block ($F_{1,70} = 47.43$, $p < 0.001$), and a significant interaction of treatment x trial block ($F_{1,70} = 2.92$, $p < 0.04$. Both controls and sham groups demonstrated significant lower latencies than all other groups from trial blocks through 5, especially compared to Lo group (ko mice). As shown in Figure 8A and B, Lo mice in both 12 and 18 month-old exhibited a longer acquisition latency and lower MSD scores, as compared to those in the control, indicating a cognitive deficit, and it should be noted that there were no significant differences in swim speed (m/s) across groups. In 12 month-old VMAT2 Lo mice, microinjection with *Phox2a+Gata3* markedly improved acquisition latency performance ($p < 0.05$) from trial blocks 3 to 5, and microinjection with *Phox2b+Gata3* also improved acquisition latency in trial block 5, as compared to Lo mice (Figure 8A).

In addition, microinjection with *Phox2b*, *Phox2a + Gata3* and *Phox2b+Gata3* markedly increased MSD scores on the probe trial ($F_{8,44}$=3.87, $p < 0.05$), as compared to those in Lo mice (Figure 8B), indicating improvement of search behavior for the platform. In 18 month-old mice, microinjection with *Phox2a*, *Phox2a+Hand2* and *Phox2b+Gata3* significantly reduced acquisition latency ($p < 0.05$). Also, except for the microinjection with *Phox2b,* all other groups showed a significantly increased MSD score ($F_{8,44} = 4.52$, $p < 0.05$), compared to the Lo mice (Figure 8D). These results demonstrated that overexpression of specific transcription factors in the LC ameliorated deficits in spatial memory performance without disrupting swim

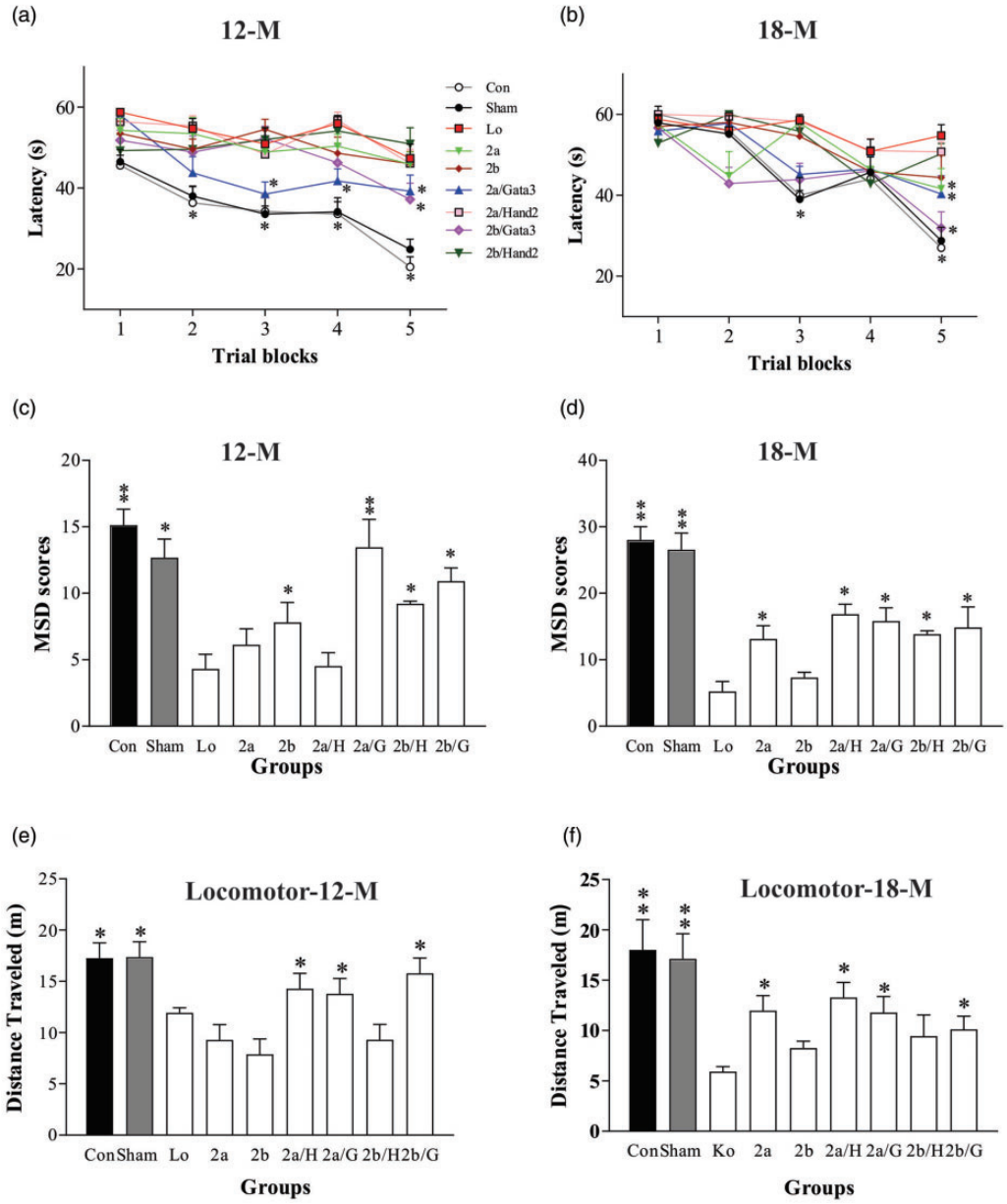

Figure 8. Behavior tests, Effects of Microinjection of Lentiviral Expression Cassettes on Mouse Behavior Performance Measured by MWM or Locomotor Tests (N = 5). Acquisition latency (A, B) is represented as a function of trial blocks. Trial blocks consisted of four training trials each. Mean search difference scores (C, D) are represented as a function of group. Distance traveled is represented as the locomotor test result in 12 month-old (E) and 18 month-old (F). *$p < 0.05$, **$p < 0.01$, compared to the Lo mice. See legends of Figures 1 and 2 for abbreviations.

speed, indicating that cognitive function appears to have been improved in microinjected VMAT2 Lo mice.

For locomotor activities, as shown in Figure 8E and F, Lo mice in both 12 and 18 month-old showed a significant reduction in distance traveled ($p < 0.05$ or $p < 0.01$). Microinjection of *Phox2a* + *Hand2*, *Phox2a* + *Gata3* and *Phox2b* + *Gata3* in 12 month-old mice, as well as all microinjected groups except for *Phox2b* groups in 18 month-old mice demonstrated no change in locomotor activity. Therefore, it appears that several manipulations of these transcription factors alleviated spontaneous motor deficits due to knock-down of VMAT2.

Discussion

This study is an extension of our previous investigations in adult and aged rats (Fan et al., 2011, 2020). In the present study, VMAT2 Lo mice, a mouse PD model at ages of 12 or 18 month-old, were microinjected with

transcription factors *Phox2a/2b* or their combination with *Hand2* or *Gata3* in the LC for 3 weeks to determine whether these manipulations influence expression levels of noradrenergic and dopaminergic phenotypes in the brain. The results showed that after microinjection, mRNA levels of Phox2a/2b, as well as DBH and TH in the LC were significantly increased, as compared to those of Lo mice. Furthermore, protein levels of DBH and TH in the LC, FC and HP, as well as TH protein levels in the striatum and SN were markedly elevated. These upregulated gene expressions were accompanied by an increased level of NE and DA in the striatum and an improved cognitive and locomotor behavior. The current observations not only support our previous findings that overexpression of these transcription factors in the LC can upregulate the expression of noradrenergic phenotypes, but also provide the evidence that restoring a damaged noradrenergic function can facilitate the recovery of injured dopaminergic system.

VMAT2 Lo mice display substantial reductions of DA levels in the striatum and cortex, and progressive neurodegeneration in the SN with formation of α–synuclein containing inclusions (Taylor, 2014). This model also shows progressive degeneration in the LC with deficits in motor and non-motor phenotypes, thereby replicating important pathogenic features of PD (Caudle et al., 2007; Taylor et al., 2009, 2011). More interestingly, neuronal loss in the LC of these mice begins at 12 months of age, and with a larger reduction at 18 months of age, whereas neuronal loss in the SN begins at 18 months and reaches significant degeneration at 24 months and later (Caudle et al., 2007; Taylor et al., 2014). Considering one of goals of this study is to examine whether a restored noradrenergic system can bring improvement to damaged dopaminergic neurons, two age groups such as 12 and 18 month-old were selected for over-expression manipulation. The reason for the selection of these ages is based on the consideration that at ages of 12 or 18 months, the neuronal loss of noradrenergic or dopaminergic neurons in the LC or SN should be just beginning to emerge and at this stage PD model mice should have ability to repair damaged neurons, if an appropriate strategy is provided. The data showed that in VMAT2 Lo mice at age of 12 months the microinjection increased mRNA levels of Phox2a/2b and DBH/TH, as well as increased protein levels of DBH/TH in the LC and other brain regions were generally higher than those in 18 month-old groups. Furthermore, consistent with these effects, the microinjections of these transcription factors raised NE/DA concentrations in the striatum of 12 month-old VMAT2 Lo mice which were also relatively higher than those in 18 month-old mice. Moreover, mRNA levels of DBH and TH in the LC induced by microinjection in 12 month-old mice, rather than 18 month-old mice, were even higher than those in the control (Figure 2A). This comparison reveals that the interference strategy for PD would get better benefit at the early stage.

In the present study the lentiviral expression constructs of *Phox2a* or *Phox2b* were bilaterally microinjected into the LC region in combination with constructs of transcription factors *Hand2* or *Gata3* for the goal to assess the synergistic effects of these transcription factors. The test is based on the fact that they work as a complex co-transcriptional regulatory network to strengthen transactivation of various genes (Howard, 2005) and interact in a combinatorial fashion as a transcription factor scaffold at the DBH promoter (Xu et al., 2003; Howard, 2005). The consequence of synergistic actions in combination of *Phox2* and *Hand2* (Howard et al., 2000), or *Phox2* and *Gata3* (Lim et al., 2000; Tsarovina et al., 2004) is the significant enhancement of transcriptional activities at the promoters of noradrenergic marker genes (Xu et al., 2003; Pellegrino et al., 2011). It resulted for instance in a 5-fold increase in DBH expression than that with *Phox2a* alone (Rychlik et al., 2003). However, our data showed although there was a mild increase for DBH and TH in the LC, as well as DBH and TH in other brain regions in some groups microinjected with combination cassettes when compared to those microinjections of *Phox2a* or *Phox2b* alone, this increase did not reach statistical significance. It indicates that an expected synergistic action did not occur. Currently we do not have a satisfactory explanation for this discrepancy between those findings in the literature and the present study. One possible contribution is the difference between *in vitro* and *in vivo* experiments. Among those reports regarding the synergistic action from *Phox2* and *Hand2* or *Gata3*, except for two reports which were related to development in embryos (Lim et al., 2000; Tsarovina et al., 2004), all other reports were *in vitro* studies. To the best of our knowledge, the present research is the first study *in vivo* to examine the synergistic effects of these transcription factors. Generally, there has been a growing body of evidence defining the value of using cell culture as an appropriate *in vitro* model in order to elucidate mechanisms associated with the *in vivo*. However, *in vitro* experiments may be under non-physiological conditions, as *in vivo* there are stronger capacities to buffer or interfere with the transactivity. Therefore, the interpretation for *in vitro* results should be cautious. Nevertheless, a tendency towards the synergism was observed in the present study and it deserves further investigation.

Coincident with our previous study in rats, the present observation demonstrated that microinjection of lentiviral expression cassettes in the LC improved MWM acquisition and probe trial performance, indicating an advancement in cognitive behavior in the microinjected VMPT2 Lo mice. In addition, the current study showed an ameliorative locomotor activity. Accompanying DA

reduction, significant depletion of NE (>80%) in different brain regions was reported in the postmortem PD brain (Taquet et al., 1982) due to the neuronal loss in the LC (Freed, 1990; Zarow et al., 2003). In a consequence, progressive cognitive disturbances such as memory loss, impaired ability in learning, attention and decision making are typical problems in PD (Baggio et al., 2014). Animal studies showed that the reduction of these neurotransmitters leads to the functional deficits in frontal cortex functions, including cognitive inflexibility (Tait et al., 2007; McGaughy et al., 2008). A locomotor deficit is an important hallmark of PD and most PD models have reduced locomotor activity (Taylor et al., 2010). In PD mouse models treated with MPTP, the degeneration of dopaminergic neurons was followed by a marked reduction of locomotor (Colotla et al., 1990; McNaught et al., 1996; Zeng et al., 2014; Gratuze et al., 2019). As showing in the present study, Lo mice exhibited the similar cognitive and locomotor deficits (Figure 8), possibly due to substantial reductions in NE/DA in the brain as observed in the striatum in the present study and reported in the literature (Taylor, 2014). Our study also showed that overexpression of transcription factors in the LC enhanced NE/DA concentrations in the striatum, compared to those in Lo mice (Figure 7), as a result parallel to upregulated noradrenergic and dopaminergic phenotypes. An improved MWM and locomotor activity results (Figure 8) fits relatively well with these alterations in the brain, especially in 18 month-old groups. It has been reported that stimulation of the noradrenergic system or NE infusion enhance MWM acquisition (Hatfield and McGaugh, 1999). In recently developed transgenic mice expressing α-synuclein in the LC, adrenergic receptor antagonists rescued non-motor symptoms of PD (Butkovich et al., 2020). A selective monoamine oxidase B inhibitor L-deprenyl alleviated acquisition and probe trial performance deficits caused by scopolamine (Yavich et al., 1993; Gelowitz et al., 1994). Similarly, DA antagonists impair MWM learning (McNamara and Skelton, 1993) and transgenic mice lacking DA D1 receptors showed severely impaired MWM acquisition (Smith et al., 1998); and administration of D1 receptor agonists enhanced acquisition performance (Hersi et al., 1995). Likely, administration of smilagenin (He et al., 2019) or overexpression pre-enkephalin in the striatum (Bissonnette et al., 2014) to increase TH positive neurons in SN significantly improved the locomotor ability. Therefore, enhanced noradrenergic and dopaminergic activities resulting from overexpression of these transcription factors in the LC significantly improves the function of the NE and DA systems. It consequently improves behavioral performance, because both NE and DA modulate the behavioral-affective components of the whole-animal response (Antelman and Caggiula, 1977; Morilak et al., 2005).

It is worthwhile to note that the Morris water maze is well documented to be a hippocampal-dependent task (D'Hooge and De Deyn, 2001). Compared to the TH in the striatum and SN, in mice of 18- month-old the effects of microinjection of expressional cassettes on DBH in the hippocampus were not especially robust although were higher than Lo group, and even were not significant different from those of the controls, expect for one group of "2a" (Figure 5B). However, those groups that showed a significantly increased DBH protein levels such as *Phox2a* in 18-month-old and *Phox2a+Gata3* and *Phox2b+Gata3* in the hippocampus at age of 12 months, demonstrated an improvement on the latency (Figure 8B) and Mean Search Difference score (MSD) of the probe trial (Figure 8C/8D). The probe trial, without the platform in the pool, is an excellent measurement of spatial memory, and has been argued to be more accurate relative to hippocampal integrity in past work (Whishaw et al., 1995). The importance of the hippocampus in Morris water maze performance, and especially on the probe trial of this task demonstrates that these groups and enhanced DBH positively correlate with spatial memory on this task.

However, there is a discrepancy between behavior and biochemical measures in some groups (Figure 8). While this inconsistency between biochemical and behavioral events has been reported in the literature, especially when monoamine oxidase inhibitors were applied (Pani et al., 1990; Blaha et al., 1996), this discrepancy may be attributed by following reasons. First, the LC-NE system is a critical component of the neural architecture. As such, it appears reasonable to propose that dysfunction of this system might contribute to the alteration in the cognitive processes, and vice versa. However, the data in the literature show some difference. For example, rats with 6-OHDA-induced LC ablation did not show any signs of impairment in learning tasks (Mason and Fibiger, 1979). Furthermore, the noradrenaline uptake inhibitor desipramine failed to improve learning and memory impediment caused by the neurotoxin DSP4 (Ichihara et al., 1993). Similarity also happened to the DA system: intra-cerebral injection of DA did not efficiently elicit behavioral changes (Costall and Naylor, 1975). Therefore, there is a notion that the LC-NE system may be viewed as a general and global modulator of neuronal circuits that guide behavioral action (Itoi, 2008; Itoi and Sugimoto, 2010). Second, behavioral tests like the MWM is influenced by numerous factors, including other transmitter systems (D'Hooge and De Deyn, 2001). It may at least partly account for that behavioral performances in PD animal models are more complex than the change in gene expression and neurotransmitter levels. Finally, age may be another possible effector. Mice at the age of 18 month are relatively old. It

is reported that young and aged animal have different effect in water maze performance (Sirvio et al., 1991). It is documented that MWM performance declines with increasing age of the animals (Brandeis et al., 1989). Whereas more studies are needed to clarify correlation between the behavioral alterations and gene overexpression, nevertheless, the present study provides some evidence for functional output of genetic manipulations in this PD model.

It is well known that significant neuronal loss (about 70%) occurs in the LC in PD, and continues throughout PD (Mavridis et al., 1991; Gesi et al., 2000). Disturbance of the LC-NE system influences both the onset and progression of neuronal damage in the dopaminergic tract. A functional LC-NE system benefits the recovery of damaged dopaminergic neurons (Espay et al., 2014). Therefore, restoring the damaged central LC-NE system in PD by augmenting noradrenergic neurotransmission positively impacts the recovery process of degenerated dopaminergic neurons (Paredes-Rodriguez et al., 2020). However, the strategies targeting the LC-NE system in PD are currently limited. Although gene therapies for PD have been used in clinical trials (Nakata et al., 2012; O'Connor and Boulis, 2015), and the lentiviral vector-based gene therapy has been used in patients with advanced PD at ages 48-65 with promising results (Palfi et al., 2014). However, whereas none of clinical applied gene therapy has resulted in significant clinical benefits, these gene therapies target the genes of the dopaminergic circuitry that are downregulated in PD. There is no similar gene therapy aiming at the noradrenergic phenotype. The identification and testing of new therapeutic genes in this field would be necessary (Nakata et al., 2012; Bartus et al., 2014; O'Connor and Boulis, 2015). Therefore, the present study is the first to use genetic approaches to augment LC-NE function and further positively impact the recovery process of dopaminergic neurons in PD. It expands our understanding of molecular mechanisms involved in the facilitating effects of NE on the DA phenotype expression. It may also provide opportunities for the development of novel therapies for the treatment of PD.

In summary, the present study demonstrated that overexpression of transcription factors in the LC of PD model VMAT2 Lo mice upregulated expression of DBH/TH in the LC and in the HP and FC, as well as TH protein levels in the striatum and SN. The enhancement of noradrenergic and dopaminergic phenotype expression may further increase NE and DA concentrations in the brain, as measured in the striatum, which was accompanied by the functional improvement as indicated by the behavioral performance in a cognitive task and spontaneous activity. This study carefully investigated the possibility that symptomatic relief in PD patients as a result of therapeutic intervention might be related, at least in part, to the NE neurotransmitter system. These results reveal that over-expression of these transcription factors in the LC can improve noradrenergic and dopaminergic activities and functions in this PD model. It provides necessary groundwork for the development of gene therapies of PD, and expands our understanding of the link between LC-norepinephrine and dopamine systems during the progression of PD.

Summary Statement

The present study employs forced overexpression of transcription factors in brains to reverse the deficiencies of noradrenergic neurons in Parkinson's disease model mice. The results showed a biochemical and functional amelioration in degenerating dopamine neurons possibly through an improved noradrenergic system.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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