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Transcription factors Phox2a/2b upregulate expression of noradrenergic and dopaminergic phenotypes in aged rat brains.

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Abstract

The present study investigated the effects of forced overexpression of Phox2a/2b, two transcription factors, in the locus coeruleus (LC) of aged rats on noradrenergic and dopaminergic phenotypes in brains. Results showed that a significant increase in Phox2a/2b mRNA levels in the LC region was paralleled by marked enhancement in expression of DBH and TH per se. Furthermore, similar increases in TH protein levels were observed in the substantia nigra and striatum, as well as in the hippocampus and frontal cortex. Over-expression of Phox2 genes also significantly increased BrdU-positive cells in the hippocampal dentate gyrus and NE levels in the striatum. Moreover, this manipulation significantly improved the cognition behavior. The *in vitro* experiments revealed that norepinephrine treatments may increase the transcription of TH gene through epigenetic action on the TH promoter. The results indicate that Phox2 genes may play an important role in improving the function of the noradrenergic and dopaminergic neurons in aged animals, and regulation of Phox2 gene expression may have therapeutic utility in aging or disorders involving degeneration of noradrenergic neurons.

Keywords

tyrosine hydroxylase; dopamine- β -hydroxylase; locus coeruleus; dopamine; neurogenesis; Morris water maze

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Introduction

The locus coeruleus (LC) is the primary source of norepinephrine (NE) in brains. The LC not only sends ascending projections to the cortex and hippocampus (HP), NE arising from the LC also influences activity of the substantia nigra (SN) and ventral tegmental area (VTA) by enhancing the release of dopamine (DA) in projections to the striatum (Grenhoff et al., 1993). As the important enzymes in the NE synthesis, dopamine β -hydroxylase (DBH) (Kaufman and Friedman, 1965) and tyrosine hydroxylase (TH) (Udenfriend, 1966) are considered as the noradrenergic hallmarks. Since widely expressing in dopaminergic neurons, TH is also recognized as the dopaminergic hallmark (Blanchard et al., 1993; Raisman-Vozari et al., 1991). As such, the expression of both DBH and TH is bound to the activities and functions of both noradrenergic and dopaminergic neurons in brains. While a progressively neuronal decline in the LC and SN has been observed in aging (Manaye et al., 1995; Rudow et al., 2008) and degenerative diseases (Rudow et al., 2008), a reduced expression of DBH and TH has been taken as the index for aging (Chan-Palay and Asan, 1989; Rollo, 2009) and these diseases (Benarroch, 2018).

It is now known that the disturbance, and/or a functional enhancement of the LC-NE system influences both the onset and progression of neuronal damage to the DA nigrostriatal tract (Delaville et al., 2011; Hassani et al., 2020; Isaías et al., 2011). For example, animal studies have shown that neurotoxin-induced reductions in LC activity and functions are accompanied by a dopaminergic neuronal loss or activity reduction in the SN and VTA (Af Bjerken et al., 2019; Bing et al., 1994; Guiard et al., 2008). As a consequence, there was a significant reduction in striatal concentrations of DA and its metabolites (Fornai et al., 1996; Mavridis et al., 1991; Srinivasan and Schmidt, 2003), and an alteration of DA-related behavior (Antelman and Caggiula, 1977; Wang et al., 2010). Whereas administration of NE protected dopaminergic neurons from neurotoxin-induced cell death *in vitro* (Troade et al., 2001) and *in vivo* (Kilbourn et al., 1998; Rommelfanger et al., 2004), treatment of rats with NE facilitates burst firing of the SN (Grenhoff and Svensson, 1993), and significantly improves parkinsonian behavior (Marien et al., 1994). Accordingly, to restore the function of the degenerating central LC-NE system by augmenting noradrenergic neurotransmission may positively affect the recovery process of degenerated dopaminergic neurons.

Phox2a and Phox2b, two transcription factors in the brain, are the master regulator of the noradrenergic phenotypes during the embryonic period and coordinately control the specification and differentiation of noradrenergic neurons (Pattyn et al., 2000). Recent studies revealed that these transcription factors have potential regulatory roles for noradrenergic properties in adult brains. For example, they were reported to be required for maintenance of matured noradrenergic neurons *in vivo* and for the continued expression of DBH and TH (Card et al., 2010; Lucas et al., 2006; Schmidt et al., 2009; Tsarovina et al., 2010). Our previous studies found that these transcription factors can not only transactivate the DBH gene *in vitro* (Fan et al., 2009), but also markedly increased DBH expression in the LC after they were delivered into the LC of adult rats (Fan et al., 2011). Considering there may be much difference in the activity and function between adult and aged subjects, further pursue whether these transcription factors can similarly act on aged animals should be interesting. As aging is considered as a major risk for the degenerative diseases such as

Parkinson's disease (PD) and Alzheimer's disease (AD) (Rodriguez et al., 2015;Swerdlow, 2011), to elucidate effects of these transcription factors in aged animals may also provide interesting information for the treatment strategy of these diseases.

The goal of the present study is to investigate whether forced overexpression of *phox2a/2b* on the LC of aged rats can similarly increase the expression of noradrenergic and dopaminergic phenotypes in the brain. The lentiviral construction of cDNAs of *Phox2a* and *Phox2b* (*vPhox2*) were microinjected into the LC of rats at age of 23 months. The results showed that an increased mRNA level of *Phox2a/2b* in the LC was accompanied by enhanced expression of DBH and TH per se. Furthermore, there was a parallel increase of TH protein level in the SN and striatum. Animals treated with *vPhox2a* and *vPhox2b* were also tested on the Morris water maze (MWM), which is a spatial memory test primarily dependent on hippocampal integrity. We utilized this behavioral test to analyze whether manipulating this system may improve cognitive performance. Manipulating *Phox2* genes or activity have been shown to affect long-term potentiation, which is closely related to cognitive performance (Ma et al., 2011). The *in vitro* experiments showed that a treatment with NE can upregulate the transcription binding of transcription factor SP1 with the TH promoter. The present study suggests that overexpression of *Phox2* transcription factors in the LC may influence the activities of central noradrenergic and dopaminergic systems in aged animal brains.

Material and methods

Preparation of lentiviral vectors

The preparation and use of lentiviral vectors are similar to those described previously (Fan et al., 2011). Briefly, cDNAs of *Phox2a* or *Phox2b* and enhanced green fluorescent protein (eGFP) were constructed into the pENTR/D-TOPO vector, which was then transferred into a lentiviral vector pLenti6/V5, and packaged into vectors pCMVΔR8.9 and pVSVG based on the instruction of the manufacture (Invitrogen, Carlsbad, CA, USA). In the recombinant expressional cassettes there are two promoters respectively to drive *Phox2a/2b*, or eGFP. The eGFP is a reporter gene as an index to verify viral delivery to the LC site and was also separately constructed into an individual cassette used as a control in the experiments. A lentiviral stock of these cassette preparations was obtained after verification, ultracentrifugation and viral titer determination. High-titer viral stocks (1×10^8 TU) were stored at -80°C until use.

Animals and in vivo stereotaxic microinjection

Male and female Fischer 344 rats from Harlan Laboratories Inc. (Indianapolis, IN, USA) were 23 months of age and maintained on a 12 h light/dark cycle and with *ad libitum* access to food and water. The reason not to use animals that were older than 23 months of age was based on our preliminary study that rats over 23 months of age did not survive anesthesia for surgery at a very high rate. All animal procedures were approved by the East Tennessee State University Animal Care and Use Committee and complied with the NIH Guide for the Care and Use of Laboratory Animals. All rats were randomly assigned to different experimental groups after an acclimation period of 7 days. Microinjection protocol is similar

to that reported previously (Fan et al., 2011). Briefly, rats were anaesthetized with ketamine/xylazine (100mg/10mg/kg. i.p.). On a stereotaxic apparatus, burr holes were drilled into the skull above the LC region with an electric hand-drill. A sterile Hamilton syringe with 26-gauge needle, which was loaded with expression cassettes with a titering of 1×10^8 TU of titer per milliliter, was lowered into the LC regions ([AP]= -10.0 mm, [LAT]= ± 1.3 mm, and [V]= -6.0 mm) (Paxinos and Watson, 2005). Bilateral microinjection of cassettes (2 μ l/each side) were delivered into the LC region through the syringe. The burr holes on the skull were then filled with dental cement and sutures were used to close the incision. Rats in the control group were microinjected with expression cassettes inserting eGFP only. For those in the sham group the syringe needle was lowered into rat LC region without injection. All rats, including those for the behavior test (see 2.9 below), were sacrificed on 22nd day after microinjection by decapitation or perfusion. Fluorescence of eGFP in the LC region was examined to verify the microinjection site. Rats with missed placements were eliminated from further experiments.

In situ hybridization

The *in situ* hybridization was carried out based on our previous publication (Fan et al., 2011). Briefly, brain sections (16 μ m), which contained the stem LC regions, were fixed with 4% (w/v) paraformaldehyde. After acetylation with acetic anhydride, an increasing concentrations of alcohol (50, 70, 95 and 100% [vols]) were used to extract lipids. [³⁵S]-labeled cRNA probes were transcribed *in vitro* from cDNAs for rat Phox2a (0.85 kb), Phox2b (0.95 kb), and DBH (1.4 kb) in pGEM-3Zf vectors with T3 or T7 RNA polymerase. After incubation with hybridization solution containing the radiolabeled probes at 55°C for 3–5 h, sections were exposed to Biomax autoradiographic films (Kodak; Rochester, NY). Sections were further dipped in Kodak NTB2 emulsion (Fisher, Pittsburgh, PA) to get the higher resolution studies. The quantitative analysis was performed using the MCID CORE 7.0 program (Imaging Research Inc.; Linton, England).

RNA isolation and quantitative real-time polymerase chain reaction (qPCR) analysis for mRNA of DBH.

The method is similar to that reported previously (Deng et al., 2016; Huang et al., 2015). Briefly, total RNA was extracted using RNeasy reagent (Molecular Research Center, Inc., Carlsbad, CA) from dissected brain LC regions and cDNAs were converted using the superscript III First-Strand Synthesis Kit (Applied Biosystems/Life technologies, Foster City, CA, USA) following the manufacturer's protocol. qPCR was conducted using the SYBR green Platinum Quantitative PCR supermix (Invitrogen, Carlsbad, CA, USA) in Stratagene Mx3000P (Agilent Technologies, Santa Clara, CA, USA). The primers for q-PCR were as follows: rat DBH: 5'-CCAGGATCCCATACTAGTA-3' and 5'-CTGGATACCCATCAGGACTA-3'; β -actin: 5'-GTTGCCAATAGTGATGACCT-3' and 5'-GGACCTGACAGACTACCTCA-3'. Measurements were normalized to β -actin (Ct) and comparisons calculated as the inverse log of 2^{-CT} to give the relative fold change in respective DBH gene expression levels using the 2^{-CT} method (Livak and Schmittgen, 2001). All reactions were run in triplicate, each using separate sets of samples.

Western blotting analysis

Western blotting was conducted to measure protein levels of DBH and TH in brain LC, SN, striatum, HP and FC from old rats. The brain tissues were lysed in sample buffer containing sodium lauryl sulfate (SDS) and β -mercaptoethanol, supplemented with protease inhibitors. After centrifugation at 1000 *g* and protein assay, equal quantities of sample proteins were loaded 10% SDS-polyacrylamide gels and separated by electrophoresis, and then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking, the membranes were then probed with primary antibodies [either anti-DBH from rabbit (1:400 dilution; sc-15318, Santa Cruz Biotechnology Inc., CA, USA), or anti-TH from mouse (1:1000 dilution; T-1299, Sigma-Aldrich, Saint Louis, MO, USA)]. After incubation with secondary antibodies against rabbit or mouse, bands were acquired with enhanced chemiluminescence (ECL, Amersham; Piscataway, NJ, USA). Densitometric values of DBH and TH signals, analyzed by imaging software (Molecular Dynamics IQ solutions, Molecular Dynamics, Inc., Sunnyvale, CA, USA), were normalized versus β -actin signals, which were determined on the same blot after stripped and re-probed. Normalized values were then averaged for all replicated gels and used to calculate the relative changes on the same gel.

Immunofluorescence staining for DBH or TH

Immunofluorescence staining was conducted as described previously (Fan et al., 2011). Briefly, after pre-incubation (5% bovine serum albumin plus 0.2% Triton-X 100), slides containing brain sections were probed with polyclonal antibody from rabbit against DBH (1:500 dilution, CA-301, Protos Biotech Corp, New York, NY, USA) or monoclonal antibody from mouse against TH (1:500 dilution, MAB7566, Novus Biologicals, Centennial, CO, USA) overnight at 4°C. After being washed, sections were then incubated with secondary antibodies (for DBH: Alexa Fluor 488-conjugated goat anti-rabbit IgG, Invitrogen, Carlsbad, CA, USA; for TH: Alexa Fluor 488-conjugated Goat anti-mouse IgG, from Abcam, Cambridge, MA, USA) for 2 h at room temperature. Slides were washed, and covered by coverslips using Citifluor mounting medium. Immunofluorescence labeling was acquired through a Leica TCS SP2 confocal microscope system (Leica Microsystems Inc., Bannockburn, IL, USA). ImageJ software (Rasband, US National Institutes of Health, Bethesda, <http://rsbweb.nih.gov/ij>, 2010) was used to quantify immunofluorescence images.

Measurement of NE by high-performance liquid chromatography (HPLC)

The striatum was dissected from animals microinjected with *vPhox2* and were sonicated in 1.5 ml Eppendorf tubes, which contained 400 μ l of tissue homogenizing solution (0.2 M perchloric acid, 1×10^{-7} M ascorbic acid, chilled on ice) containing dihydroxybenzylamine (2 μ g/ml) as the internal standard for catecholamines. Following tissue homogenization, 30 μ l of homogenate was saved for the protein assay and samples were centrifuged at 10,000 *g* at 4°C for 5 minutes. The supernatant of samples was filtered using 0.2 μ m nylon disposable syringe filters and stored at -80°C until HPLC assay.

The supernatant samples were subsequently analyzed by HPLC/electrochemical detector apparatus as described previously (Church, 2005; Zhu et al., 2008). NE in the samples was separated on an Ultrasphere ODS reverse-phase column (Beckman) by isocratic elution,

using a mobile phase consisting 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, adjusted to pH 2.7 with phosphoric acid. The chromatograms were recorded and analyzed with a Hitachi D-2500 Chromato-Integrator. The concentration of NE was calculated using standard curves constructed with known amounts of NE. The recoveries were determined using isoproterenol as an internal standard and found to be about 86%. The NE concentration in the tissue preparation was expressed as pg/mg tissue proteins.

Immunohistochemical labeling for 5-Bromo-2-deoxyuridine (BrdU)

BrdU injection and immunohistochemical staining were performed as descriptions reported previously (Fan et al., 2011). The microinjection of *vPhox2* for animals was the same as description in *Animals and in vivo stereotaxic microinjection* above. Beginning on day 17 after microinjection with *vPhox2*, BrdU (100 mg/kg/day, Sigma-Aldrich, St. Louis, MO, USA) was injected (i.p., daily) for 5 consecutive days. After the last injection rats were transcardially perfused 24 h with saline and followed by 4% paraformaldehyde. Rat brains were cut through the rostral/caudal extent of the HP (30 μ m) (bregma -1.88 mm to -5.20 mm) (Paxinos and Watson, 2005). Brain sections were stored in the long-term storage solution (sucrose/ethylene glycol/sodium azide in PBS) at -20°C until analysis.

BrdU labeling was performed with free-floating sections, which were treated with 50% formamide-2x standard saline citrate for 2 h at 65°C and denaturated in 2 N HCl at 37°C for 30 min. After washing, the sections were processed preincubation, and then probed with a BrdU-specific mouse monoclonal antibody (1:600; MAB3262F, Sigma-Aldrich, Saint Louis, MO, USA) overnight at 4°C . On the next day, sections were probed with a biotinylated mouse secondary antibody (1:200; Vector Laboratories, Burlingame, CA, U.S.A.) for 1 h, and continued to be incubated with VECTASTAIN (Elite ABC Kit, 1:100; Vector Laboratories, Burlingame, CA, USA) for another 1 h. Three, 3'-diaminobenzidine containing nickel chloride (nickel-DAB; 40 mg/mL) was used to visualize BrdU-positive cells.

For BrdU-labeled cell image analysis, a light microscope (Zeiss Axio Observer Z1, Carl Zeiss Microimaging, LLC, Jena, Germany) was used. Cells were imaged at 40x or 60x objective lens using a modified unbiased stereology protocol (Eisch et al., 2000; Malberg et al., 2000). All BrdU-labelled cells were counted in the subgranular zone of the DG regions. The values of BrdU-positive cells were averaged and expressed as mean number of BrdU-positive cells in subgranular zone of the DG.

Behavior test-MWM

The MWM is primarily a test for checking spatial learning and reference memory and was performed based on the methods of a previous publication (Brown et al., 2001). Briefly, animals began training on the MWM at 18th day after the microinjection for three consecutive days. All animals were tested at approximately the same time of the day for about 8 trials a day, yielding a total of 24 training trials for each rat. These trials let rats learn to find the hidden platform from a randomly designed location such as north, south, east or west within a water-filled tank. When the trial begins, rats were gently placed once into the

tank from one of four start locations by facing the sidewall of the tank. They were given 60 s to navigate to the platform. If the rat failed to find the platform at the end of 60 s, the experimenter guided it to the platform and let it stay there for 10 s. On each trial different starting location was selected. On each training trial the acquisition latency was recorded, which is the time when the rat swam from the start location to the platform in the tank. On the final day after the last trial, a probe trial was performance in which the platform was removed from the tank. The performance of rats was recorded via a video camera (Rockhouse Products, NJ, USA) which mounted above the tank, and the video tape was analyzed using a Videomex scanning system (Columbus Instruments, Columbus, OH, USA) as described previously (Brown et al., 2001).

Cell cultures, reporter gene construction and luciferase activity assay.

MN9D, a dopaminergic cell line from mouse (Choi et al., 1991), was used in this experiment. The growth medium for this cell line was Dulbecco's modified Eagle's medium, which was added with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) (Gibco-Invitrogen, Carlsbad, CA, USA) at 37°C in humidified air containing 5% CO₂ as described previously (Zhu et al., 2019). The cell viability was measured using trypan blue exclusion for all experimental groups following cell harvesting, and was >95 % for all experimental cells.

The methods for the reporter gene and deletion constructs were the similar as previous reported (Zha et al., 2011). Briefly, a parental promoterless and enhancerless luciferase reporter vector pGL3-basic was used for TH promoter-luciferase reporter construction. The upstream primers containing KpnI site and the downstream primer bearing the HindIII site were designed: pGLrTH-798: F: 5'-TTT CTC TAT CGA TAG TCG AGA CCC ATG ATA CAG G-3'; R: 5'-CCG GAA TGC CAA GCT TGG TCC CGA GTT CTG TCT C-3'; pGLrTH-471: F: 5'-TTT CTC TAT CGA TAG TTT GTT ACA TGG GCT GGG GG-3'; R: 5'-CCG GAA TGC CAA GCT TGG TCC CGA GTT CTG TCT CC-3'; pGLrTH-349: F: 5'-TTT CTC TAT CGA TAG CCT TAG GAA GTC CAG CAT GGT TCT C-3'; R: 5'-CCG GAA TGC CAA GCT TGG TCC CGA GTT CTG TCT CC-3'; pGLrTH-231: F: 5'-TTT CTC TAT CGA TAG GTG ATT CAG AGG CAG GTG C-3'; R: 5'-CCG GAA TGC CAA GCT TGG TCC CGA GTT CTG TCTCC-3'; pGLrTH-104: F: 5'-TTT CTC TAT CGA TAG CGC AGG AGG TAG GAG GTG G-3'; R: 5'-CCG GAA TGC CAA GCT TGG TCC CGA GTT CTG TCTCC-3'. These primers were then synthesized by Integrated DNA Technology (Coralville, IA, USA). The locations of the primers in the putative TH promoter are illustrated in Fig. 6A. The genomic DNA was prepared from 2×10⁷ MN9D cells in a 150-mm culture dish. Aliquots of the DNA solution were used for cloning according to standard molecular cloning methods. Restriction digestion and direct sequencing were used to screen and confirm clones, respectively. Using these designed primer sets, six different deletion constructs with a DNA insert of 798-, 471-, 349-, 231- and 104-bp 5'-upstream from the translation start codon ATG were obtained, respectively, and constructed with the reporter gene (Fig. 6A).

For transfection, MN9D cells were seeded in 24-well plates at a density of 1×10⁵ per well 24 h before transfection. Transient transfections were performed using the Lipofectamine

2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's manual. Each transfection was carried out with 2 ng Renilla reniformis luciferase plasmid pRL-TK (Promega, Madison, WI, USA) to normalize transfection efficiencies. Twenty-four hours after transfection, NE (100 nM) was added for another 24 hrs. Then, cells were harvested, and luciferase activity was determined by a Modulus Luminometer (Turner BioSystems, Sunnyvale CA, USA).

Chromatin immunoprecipitation assay (ChIP).

An EZ-Magna ChIP™ A kit was used for ChIP (Millipore Biotechnology, Billerica, MA, USA) based on the method as described previously (Zhu et al., 2019). Briefly, lysed cells were sonicated at 4°C to shear chromatin to 200–500 bp. After centrifugation, supernatants were recovered. Sheared chromatin was immunoprecipitated respectively using Rabbit polyclonal antibodies anti-Specificity protein 1 (Sp1), or anti-methylation-Histone H3 (both from MilliporeSigma, Burlington, MA, USA), or normal rabbit IgG as recommended by the manufacturer. One-tenth of the lysate was kept to quantitate the amount of DNA present in different samples before immunoprecipitation. DNA purified from both the immunoprecipitated and pre-immune (*pre*) samples was subjected to PCR amplification using the following primers listed below for the promoter regions of TH to detect the enriched genomic DNA fragments. ChIP-PCR-derived DNA was also electrophoresed through 2% agarose gels and stained with ethidium bromide. Primers for quantitative real-time PCR were F: 5'-CCAGTGAGAGGGCTTCTA-3' and R: 5'-CACCTGCCTCTGAATCAC-3', which recognize the -340 to +1 bp region of the TH promoter.

Statistics

All experimental values were shown as the means $\pm$ SEM. The number of replicates is enumerated in the figure legends (N=x/group). Statistical significance was determined using oneway analysis of variance (ANOVA, SigmaStat, Systat Software Inc., Richmond, USA), followed by Student-Newman-Keuls' multiple comparisons *post-hoc* test.

Results

Microinjection of vPhox2a/2b increased mRNA and protein levels of DBH in the LC region.

To test effects of over-expression of Phox2 genes in the LC region on the expression of the noradrenergic phenotype in the same area, we stereotactically microinjected the vPhox2a or vPhox2b (about 1×10^8 TU/ml) into the bilateral LC region of rats. All rats were sacrificed on days 22 after microinjection. *Ex vivo in situ* hybridization was performed to measure mRNA levels of Phox2a and Phox2b in the LC region. As shown in Table 1, microinjection of vPhox2a or vPhox2b significantly affected mRNA levels of Phox2a and Phox2b in the rat LC ($F_{3,56}=8.57$, $p<0.001$ for vPhox2a; $F_{3,47}=3.32$, $p<0.05$ for vPhox2b). There were no significant differences between the control and sham groups in both Phox2a and Phox2b mRNA. It indicates microinjection surgery did not influence the expression of these genes in the LC region (as the similar analysis results were found between the control and sham groups in protein assays, this description would not be repeated in the following sections of this study). *Post hoc* analysis indicated that Phox2a and Phox2b mRNA levels were

significantly increased by 245% ($p<0.001$) and 50% ($p<0.05$), respectively, compared to the control group. These experiments demonstrated that microinjection of *vPhox2* resulted in over-expression of these genes in the LC region.

Next, we examine mRNA levels of DBH in the LC region after microinjection. As shown in Fig. 1, microinjection of *vPhox2a* significantly influenced mRNA levels of DBH in the LC region, as measured by qPCR or *in situ* hybridization [$F_{3,47}=6.83$, $p<0.05$, for qPCR (Fig. 1A); $F_{4,49}=5.98$, $p<0.05$, for *in situ* hybridization (Fig. 1B)]. However, microinjection of *vPhox2b* did not significantly affect DBH mRNA in the LC region. These data are in agreement with our previous study that showed there was no synergistic effect on noradrenergic phenotypes after simultaneous microinjection of *vPhox2a* and *vPhox2b* in adult rats (Fan et al., 2011). Because of these previous data, we did not perform the experiments for microinjection of both *vPhox2a* and *vPhox2b* in the same in the present study. Immunofluorescence and western blotting were performed to examine DBH protein levels in the LC region after microinjection of *vPhox2*. Analysis results showed that microinjection of *vPhox2a* markedly increased DBH protein levels in the LC region [$F_{4,54}=8.27$, $p<0.01$, for *western blotting* (Fig. 1C); $F_{3,65}=9.15$, $p<0.01$, for immunofluorescence (Fig. 1D)]. Similarly, although there is a tendency towards an increased DBH immunoreactivity in the LC region after microinjection of *vPhox2b*, this increase did not reach the statistically significant levels ($p>0.05$) (Figs. 1C and 1D).

Microinjection of *vPhox2* increased protein levels of TH in the LC region.

TH protein levels were examined after microinjection of old rats by immunofluorescence staining and western blotting. As shown in Fig. 2, microinjection of *vPhox2* had a significant effect on protein levels of TH in the rat LC region ($F_{3,49}=8.51$, $p<0.01$ for immunofluorescence staining; $F_{3,46}=3.35$, $p<0.05$ for western blotting). *Post hoc* analysis revealed that both microinjection with *vPhox2a* or *vPhox2b* significantly increased immunoreactivities of TH region (32% by *vPhox2a*, $p<0.05$; 82% by *vPhox2b*, $p<0.01$) as measured by immunofluorescence staining. However, western blotting showed that TH protein levels were significantly increased only by microinjection of *vPhox2b* ($p<0.05$, Fig. 2B).

Microinjection of *vPhox2* increased NE levels in the striatum and increased neurogenesis in the DG of the HP.

In separate experiments, all control and microinjected rats were sacrificed on 22nd day after injection and the brain striatum was dissected. After homogenization and centrifugation, the supernatants were further processed to measure NE concentration by HPLC. As shown in Fig. 3A, the NE levels in the striatum were significantly affected after microinjection of *vPhox2a* or *vPhox2b* ($F_{3,98}=7.03$, $p<0.01$). *Post hoc* analysis revealed that microinjection of *vPhox2a* or *vPhox2b* significantly increased NE levels by 135% or 91%, respectively, as compared to those of the controls.

Whether over-expression of *Phox2* in the rat LC and subsequent elevation of DBH and TH could have downstream postsynaptic effects in the hippocampal DG, a LC neuronal terminal area in the brain, was evaluated by measuring neurogenesis following *vPhox2*

microinjection. BrdU injection was given to aged rats that were microinjected with *vPhox2* for 21 days. Microinjection of *vPhox2a* had a substantial effect on the number of BrdU-positive cells in the DG of the HP, as compared to control rats ($F_{3,19}=5.15$, $p<0.05$; Fig. 3B). However, microinjection of *vPhox2b* did not affect the neurogenesis in the DG of the HP.

Microinjection of *vPhox2* increased protein levels of TH in the SN, striatum, HP and FC.

In order to assess effects of over-expression of Phox2 genes in the LC on some other brain areas, TH protein levels in the SN, striatum, HP and FC were measured by western blotting following microinjection with *vPhox2*. As shown in Fig. 4A, microinjection of *vPhox2a* and *vPhox2b* markedly affected protein levels of TH ($F_{3,20}=6.57$, $p<0.01$) in the SN. *Post hoc* comparisons revealed that TH protein levels were significantly increased (by 102%, $p<0.01$) after microinjection of *vPhox2a*, compared with those of the control group. Similarly, microinjection of *vPhox2b* also markedly increased TH protein levels by 83%. Western blotting analysis in the striatum showed that microinjection of *vPhox2* significantly affected TH protein levels in the striatum ($F_{3,20}=4.68$, $p<0.05$). *Post hoc* comparisons revealed that TH protein levels were significantly increased by 51% or 54% (both $p<0.05$) after microinjection of *vPhox2a* or *vPhox2b*, respectively, as compared with those of the control group (Fig. 4B).

Similarly, TH protein levels in the HP and FC, two regions to receive sole innervation from the LC (Haring and Davis, 1985; Morrison et al., 1979), were measured by western blotting in aged rats. As shown in Figs. 4C and 4D, microinjection of *vPhox2a* or *vPhox2b* significantly affected TH protein levels in the HP and FC ($F_{4,20}=3.92$, $p<0.05$ for HP, $F_{3,20}=4.16$, $p<0.05$ for FC). *Post hoc* comparisons showed that microinjection of *vPhox2a* significantly increased TH protein levels in the hippocampus by 63% ($p<0.05$), while microinjection of *vPhox2b* failed to do so. In the FC region, both microinjection of *vPhox2a* or *vPhox2b* markedly increased TH protein levels by 53% or 65%, respectively, as compared to those in the control.

Microinjection of *vPhox2* altered spatial memory as test on the MWM.

The MWM behavioral test showed that for acquisition latency the performance of the control and sham group was similar from trial blocks 2 to 8. However, microinjection with Phox2a and Phox2b markedly reduced acquisition latency (Fig. 5A). As shown in Fig. 5B, microinjection of *vPhox2* significantly increased mean search difference scores ($F_{3,20}=4.16$, $p<0.05$), compared to the control group (23 month-old). These results revealed that overexpression of Phox2a and Phox2b in the LC improved MWM acquisition and probe trial performance, indicating an improvement in cognitive behavior in the microinjected rats.

NE-induced increases in TH transcriptional activity are mediated through the epigenetic mechanisms.

At the TH gene promoter regions there are several binding sites (cis-elements) for transcription factors. whether these *cis*-acting elements are involved in the NE-induced transactivation remains to be elucidated. Also, chromatin remodeling has been recognized as a key control point for modulating gene expression in neurons (Verdone et al., 2005). For example, modification of histone proteins such as acetylation and methylation can change

interactions between DNA and histone, allowing the transcriptional machinery access to the gene promoters (Gibbons, 2005; Narlikar et al., 2002; Roeder, 2005) and resulting in an increase of transcription (Grunstein, 1997). To explore possible mechanisms underlying NE-induced upregulation of TH expression in the SN and striatum after forced overexpression of Phox2 genes in the LC, experiments to examine the effect of transcription factor Sp1 and H3 methylation were performed. First, to determine the regions of the TH promoter necessary for the actions of NE, a number of TH promoter deletion constructs were generated and tested in MN9D cells. The luciferase activity assay showed that treatment of 100 nM NE exhibited 2- to 40-fold increases in promoter activities (Fig. 6A), compared to pGL3-basic, especially for the segment of pGLrTH-349. This mapping indicated that NE responsiveness falls in these regions within the TH promoter. Second, since many *cis*-acting elements are concentrated in these regions, ChIP assay was performed to confirm the binding of the transcription factors to these *cis*-acting elements in the TH promoter. As shown in Fig. 6B, the transcription factor Sp1 was captured by specific antibody against Sp1, with an increased signal, compared to those of the IgG control. Furthermore, our previous study showed that NE treatment increased H4 acetylation in the TH promoter (Zhu et al., 2019). The further experiment currently showed that 100 nM NE treatment also markedly increased the methylation of H3 in the TH promoter (Fig. 6C).

Discussion

In the present study, lentiviral vectors expressing eGFP and rat Phox2a or Phox2b (*vPhox2a* or *vPhox2b*) with dual-promoters were microinjected into the LC region of aged rats to examine effects of Phox2 over-expression on noradrenergic and dopaminergic phenotypes in the LC and other regions of rats. Microinjection of the *vPhox2* resulted in elevated Phox2a and Phox2b mRNA levels in the LC. Elevated Phox2a expressions were accompanied by parallel and significant increases in mRNA and proteins of DBH in the LC, as well as protein levels of TH in the LC and other brain areas such as the SN, striatum, HP and FC. Furthermore, microinjection of *vPhox2* increased NE levels in the striatum and neurogenesis in the DG area of the HP, which was accompanied by improvement of cognitive behavior as measured by MWM. *In vitro* experiments revealed that NE could act on the TH promoter through enhancing of binding of transcription factor Sp1 and epigenetic mechanism to transactivation of TH gene. The findings, coupled with our previous study (Fan et al., 2011), support the hypothesis that Phox2a and Phox2b, the determinant for development of noradrenergic phenotypes during embryogenesis, continue to maintain the phenotype of noradrenergic neurons in adult and aged rat brains. Furthermore, they may also play a role to influence on the dopaminergic system.

The present study is the extension of our previous *in vivo* investigation in which over-expression with rat Phox2 genes in the LC of adult rats caused a parallel increase in the expression of DBH and NET (Fan et al., 2011). Comparing two studies, there are some differences. First, the previous study used adult rats that were 3 months of age and the present study used aged rats that were 23 months of age. Human studies showed that aging causes structural (neuronal loss) and functional decline in the LC, leading to NE deficiency in the brain (Chan-Palay and Asan, 1989; DeKosky and Palmer, 1994; German et al., 1988; Manaye et al., 1995). Animal studies also showed that an instant age-associated LC

neuronal loss was observed (Leslie et al., 1985; Sturrock and Rao, 1985; Tatton et al., 1991). Therefore, a relatively lower action of microinjection of *vPhox2* in the aged rats is expected. However, the current study showed that in aged rats overexpression of Phox2 genes, especially Phox2a, in the LC still can significantly increase the expression of DBH or TH in the LC. It indicates that forced expression of transcription factors such as Phox2 can also be used to restore the noradrenergic activity in aged subjects.

Second, HPLC analysis revealed an increased NE level in the striatum. The present study did not measure NE in other brain regions, however, as a result of microinjection-induced increase in DBH and TH there, an increased amount of NE in the brain is reasonable. NE plays an important role in learning, memory (Lee et al., 1993), arousal and attention (Aston-Jones et al., 2000). There is an extensive body of evidence that an increased NE levels in the brain improve cognitive performance. For example, desipramine and reboxetine, both block NET and increase synaptic NE availability, reduced intermittent swim stress-induced deficits in the learning trial, acquisition and retrieval in the MWM, however, clonidine enhanced such deficits (Warner and Drugan, 2012). Also, treatment mice with L-DOPS, a NE precursor, improved learning in MWM (Kalinin et al., 2012) and cognitive dysfunction in DBH knockout mice (Hammerschmidt et al., 2013). Using selective neurotoxin DSP4 depletion of central NE impaired spatial memory in MWM (Lapiz et al., 2001). Rats with lesions of the LC exhibited impaired learning directly associated with decreased levels of cortical NE (Anlezark et al., 1973) and produced cognitive deficits in animals (Cole and Robbins, 1992; Devauges and Sara, 1990). These deficits can be reversed by enhance of brain NE. Coincident with these reports, our study demonstrated that microinjection with *vPhox2a* resulted in an improvement in both MWM acquisition and probe trial performance, whereas microinjection of *vPhox2b* induced an improvement in probe trial performance, compared to non-treated controls. Further, BrdU labelling also demonstrated an increased neurogenesis in the DG of the HP. One of characterizations of aging is a progressive decline of memory and cognitive performance (Keller, 2006; Rapp and Heindel, 1994; van Groen et al., 2002). This cognitive decline is linked to damaged LC functional/neuronal integrity. For example, older individuals with mild cognitive impairment exhibited lower functional connectivity between the LC and parahippocampal gyrus (Jacobs et al., 2015). Postmortem study revealed a reduced neuronal density in the LC of older adults (Buchman et al., 2012), which was associated with cognitive decline and controlling for LC neuronal density could diminish cognitive decline (Wilson et al., 2013). Thus via its regulation of healthy cognition and central neuronal function, the LC-NE system may mediate the protective effects of reserve on cognitive aging processes (Robertson, 2013). It was reported that consequent NE increases caused by stimulation of the LC improved cognitive, perceptual, and memory performance in monkeys and rodents (Benarroch, 2018; Berridge and Waterhouse, 2003; Sara, 2009). On other hand, age-related reduction in neurogenesis may underlie hippocampal volume reduction, which could be related to behavioral deficits including cognitive decline (Driscoll et al., 2006; Verret et al., 2007). Therefore, in the present study increased NE levels resulted from restored noradrenergic system, together with an enhanced neurogenesis in the DG of the HP after overexpression of Phox2a/2b in the LC may account for the improvement in cognitive performance, which is consistent with past work (Puumala et al., 1998).

Third, in the present study, TH expression as a noradrenergic and dopaminergic marker was measured in the LC, SN, Striatum and FC. The results showed that microinjection of *vPhox2* in the LC increased the expression of DBH and TH in the LC, and TH in the HP and FC which Sp1 are innervated by the LC (Haring and Davis, 1985; Morrison et al., 1979). Furthermore, microinjection of *vPhox2* in the LC also increased TH protein levels in the SN and striatum, indicating that the restoration of the noradrenergic system also benefits the recovery of declined dopaminergic system in the brain of aged rats. These observed increases of NE may play an important role in this restoration. Currently, the mechanisms by which how NE influences the expression of the dopaminergic phenotype remain to be determined. Speculatively, TH promoter activity in several segments of the promoter in MN9D cells can be activated by 100 nM NE (Fig. 6), indicating some elements within these segments are responsible for NE-induced transactivation of the TH gene. Furthermore, ChIP assays revealed that both transcription factor Sp1 and methylation of histone H3 may play a role for NE-induced activation. Sp1 is the first transcription factor identified to be a sequence-specific DNA-binding protein that activated a broad and diverse spectrum of genes (Safe and Abdelrahim, 2005), including TH (Kim et al., 2003). Sp1, via its interaction with the Sp1-like motif residing in the TH promoter area, critically regulated promoter activity of TH gene (Yang et al., 1998). On other hand, epigenetic modifications to histone proteins such as acetylation and methylation can alter the structure of chromatin, allowing the transcriptional machinery to access gene promoters (Gibbons, 2005; Narlikar et al., 2002; Roeder, 2005) and result in transcriptional activation (Grunstein, 1997). Our previous study demonstrated that exposure of MN9D cells to NE significantly increased H4 acetylation (Zhu et al., 2019). These data suggest that this NE-induced enhancement of H4 acetylation may contribute to increased TH expression. The present investigation showed NE-induced methylation of histone H3, further providing the evidence for the involvement of epigenetic mechanisms in NE-induced TH upregulation. However, given the role of histone acetylation and methylation in TH gene regulation is not fully established (Lenartowski and Goc, 2011), more studies are needed to clarify this point.

One point worth noting is that in the present study overexpression of Phox2a and Phox2b did not equivalently affect mRNA and protein levels of DBH in the LC (Fig. 1) as in the previous study (Fan et al., 2011). Rather, only microinjection of *vPhox2a* markedly increased mRNA and protein levels of DBH. Similar phenomenon was also seen in the TH levels in the HP and BrdU assay. We do not have a satisfactory explanation for these results. One possibility may be that after microinjection of *vPhox2b* viral vector into the LC of aged rats, the increased Phox2b mRNA levels were not as high as those of Phox2a in rats microinjected with *vPhox2a* (Table 1). Therefore, more Phox2a may be available to transcript DBH genes in the brain of these aged rats. Furthermore, although Phox2a and Phox2b have identical transcription activity on noradrenergic phenotypes (Stanke et al., 1999), Phox2a was proposed to be a master regulator of these genes, especially for DBH (Swanson et al., 1997; 1998; Valarche et al., 1993). Evidence strengthening this hypothesis is that DBH expression was dependent on Phox2a (Morin et al., 1997), and compared to Phox2b, Phox2a is expressed in all neurons that contain DBH (Tiveron et al., 1996; Zellmer et al., 1995). During development of LC, phox2a is expressed before phox2b and it is responsible of TH and DBH expression and phox2a knock-out mice show a selective

agenesis of LC (Morin et al., 1997). Thus, it may be possible that *phox2b* is not so required for DBH expression maintenance conversely to TH. Also, based on their distribution in adult rat brain (Card et al., 2010; Kang et al., 2007), *Phox2a* may play a dominant role for the maintenance of noradrenergic phenotype in the adult and aged brain. Finally, another possible explanation is that endogenous *Phox2b* levels may be saturating with respect to the transcription of this gene, as some studies showed forced *Phox2b* does not affect TH, DBH and *trkA* (Parodi et al., 2012; Reiff et al., 2010). Nevertheless, to clarify the potentially difference between *Phox2a* and *Phox2b* in the aged brain, more studies are warranted.

The present study demonstrated that forced expression of *Phox2* genes in the LC region of aged rats resulted in an increased expression of DBH and TH in the LC, which was accompanied by an increase of NE levels in the striatum and improvement of cognitive performance on a test of spatial navigation. Furthermore, this restored noradrenergic activity and function were accompanied by enhanced expression of TH in the SN and striatum. These findings imply that *Phox2* genes continue to regulate the phenotype of noradrenergic neurons in older animals and improve dopaminergic activity, which may be possibly mediated through epigenetic mechanisms. Future studies should focus on elucidating the sequence of events leading to the *Phox2*-induced transactivation of the noradrenergic phenotype, and ultimately on the potential therapeutic utility of such a strategy for the treatment of aging-related disorders that demonstrate noradrenergic deficits, such as PD and AD.

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Abbreviation:

AD	Alzheimer's disease
BrdU	5-Bromo-2-deoxyuridine
ChIP	Chromatin immunoprecipitation assay
DA	dopamine
DBH	dopamine β -hydroxylase
DG	dentate gyrus
ECL	enhanced chemiluminescence
FBS	fetal bovine serum
FC	frontal cortex
GCL	granule cell layer
HPLC	high-performance liquid chromatography

HP	hippocampus
LC	locus coeruleus
MWM	Morris water maze
NE	norepinephrine
PBS	phosphate-buffer saline
PD	Parkinson's disease
qPCR	quantitative real-time polymerase chain reaction
SDS	sodium lauryl sulfate
SN	substantia nigra
Sp1	Specificity protein 1
TH	tyrosine hydroxylase
VTa	ventral tegmental area

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Figure 1:

DBH mRNA and protein expression in rat LC after microinjection of *vPhox2*, measured by (A) qPCR (N=4/group), (B) *in situ* hybridization (N=5/group), (C) western blotting (N=5/group) and (D) immunofluorescence (N=5/group). Upper panel in B, C and D are representative micrographs of DBH *in situ* hybridization, western blotting or immunofluorescence, respectively. Lower panel in B: quantitative analysis of DBH mRNA levels in the LC of old rats obtained with emulsion-dipped slides, DBH protein levels. Lower panel in c and D quantitative analysis of DBH immunofluorescence or band densitometry of western blotting in the LC of old rats. * $p < 0.05$, compared to the control. Abbreviations: 2a: microinjection with *vPhox2a*; 2b: microinjection with *vPhox2b*. Scale bar: 25 μ m for all images.

Figure 2.

TH protein expression in the rat LC after microinjection of *vPhox2* measured by immunofluorescence (A) (N=4/group) and western blotting (N=5/group) (B). Upper panel in A and B are representative micrographs of TH immunofluorescence or autoradiograph obtained by western blotting. Lower panel in A and B: quantitative analysis of TH immunofluorescence or band densitometry of western blotting in the LC of old rats. * $p < 0.05$, ** $p < 0.05$, compared to the control. See Fig. 1 for abbreviations. Scale bar: 25 μm for all images.

Figure 3.

A: Microinjection of *vPhox2* increased NE levels in the striatum measured by HPLC (n=5/group). B: Microinjection of *vPhox2* increased BrdU-positive cells in the DG areas of rat hippocampus (N=5) measured by immunohistochemical staining. Shown are representative examples of BrdU-positive cells in the DG (upper panels, Scale bar: 25 μ m) and counted BrdU-positive cell numbers (low panel). * $p < 0.05$, ** $p < 0.05$, compared to the control. See Fig. 1 for abbreviations.

Figure 4. Microinjection of *vPhox2* increased TH protein in rat substantia nigra (A), striatum (B), hippocampus (C) and frontal cortex (D) (all N=5/group) measured by western blotting. The upper panels in A, B, C and D show autoradiographs obtained by western blotting. The lower panels in A, B, C and D show quantitative analysis of band densities. * $p < 0.05$, ** $p < 0.05$, compared to the control. See Fig. 1 for abbreviations.

Figure 5.

Effects of microinjection of *vPhox2* on rat behavior performance measured by MWM test (N=5/group). Acquisition latency (A) is represented as a function of trial blocks. Trial blocks consisted of four training trials each. Mean search difference scores (B) are represented as a function of group. * $p < 0.05$, ** $p < 0.01$, compared to the control. See Fig. 1 for abbreviations.

Figure 6.

A: Mapping of NE-responsive regions on the rat TH gene promoter by serial deletion analysis. Relative luciferase activity of each expression construct was measured as firefly luciferase/renilla luciferase and expressed as fold change from pGL3-basic vector transfected cells (0). B: ChIP assay to identify the binding of the transcription factor to the *cis*-acting elements in the TH promoter using anti-Sp1. C: ChIP assay showed methylation of histone (H3) in MN9D cells in response to NE. "Input" serves as a loading control and rabbit IgG immunoprecipitation serves as a negative control. Band indicates the TH promoter in response to NE treatment. Low panel in B and C: Quantitative real-time PCR of the TH promoter regions from immunoprecipitation with antibody against Sp1 or methylation of H3. The fold enrichment value is shown as the normalized ChIP signals divided by the normalized input signal. Each bar from both pictures represent data obtained from 4 separate experiments (N=4). NE: treatment with 100 nM NE.

Table 1.

Effects of overexpression of lentiviral constructs of Phox2 gene on their niRNA levels in the LC of aged rats as measured by *in situ* hybridization.

Groups	Control	Sham	2a	2b
<i>vPhox2a</i>	231 ± 16	224 ± 17	798 ± 16 **	N/A
<i>vPhox2b</i>	195 ± 11	190 ± 22	N/A	293 ± 28*

Unit: Relative abundance silver grains. 2a: microinjected with *vPhox2a*; 2b: microinjected with *rPhox2b*.

*
p<0.05.

*
p<0.01: Compared to control groups.