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Transcription factor Phox2 upregulates expression of norepinephrine transporter and dopamine β-hydroxylase in adult rat brains

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Abstract

Degeneration of the noradrenergic locus coeruleus (LC) in aging and neurodegenerative diseases is well documented. Slowing or reversing this effect may have therapeutic implications. Phox2a and Phox2b are homeodomain transcriptional factors that function as determinants of the noradrenergic phenotype during embryogenesis. In the present study, recombinant lentiviral eGFP-Phox2a and -Phox2b (*vPhox2a* and *vPhox2b*) were constructed to study the effects of Phox2a/2b over-expression on dopamine β-hydroxylase (DBH) and norepinephrine transporter (NET) levels in central noradrenergic neurons. Microinjection of *vPhox2* into the LC of adult rats significantly increased Phox2 mRNA levels in the LC region. Over-expression of either Phox2a or Phox2b in the LC was paralleled by significant increases in mRNA and protein levels of DBH and NET in the LC. Similar increases in DBH and NET protein levels were observed in the hippocampus following *vPhox2* microinjection. In the frontal cortex, only NET protein levels were significantly increased by *vPhox2* microinjection. Over-expression of Phox2 genes resulted in a significant increase in BrdU-positive cells in the hippocampal dentate gyrus. The present study demonstrates an upregulatory effect of Phox2a and Phox2b on the expression of DBH and NET in noradrenergic neurons of rat brains, an effect not previously shown in adult animals. Phox2 genes may play an important role in maintaining the function of the noradrenergic neurons after birth, and regulation of Phox2 gene expression may have therapeutic utility in aging or disorders involving degeneration of noradrenergic neurons.

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Keywords

Phox2; norepinephrine transporter; dopamine-β-hydroxylase; locus coeruleus; noradrenergic neurons; neurogenesis

The major source of norepinephrine in brains is the locus coeruleus (LC), from which noradrenergic neurons project to numerous areas including the hippocampus and frontal cortex. Noradrenergic neurons exclusively express dopamine β-hydroxylase (DBH, EC 1.14.2.1), which converts dopamine to norepinephrine (Kaufman and Friedman, 1965), and the norepinephrine (NE) transporter (NET), a membrane protein which transports NE into noradrenergic neurons (Barker and Blakely, 1995). Their specific expression makes DBH and NET as the markers of noradrenergic neurons (Valarche et al., 1993, Lorang et al., 1994, Comer et al., 1998, Schroeter et al., 2000). The expression of DBH and NET is also tightly bound to the propagation and function of noradrenergic neurons. For example, substantial loss of LC neurons has been identified in aging, Parkinson' disease (PD), and Alzheimer's disease (AD) (Zarow et al., 2003). As a direct consequence of the LC neuron loss, the expression of DBH and NET is proportionately reduced in advancing age (Chan-Palay and Asan, 1989, Tejani-Butt and Ordway, 1992), PD (Nagatsu and Sawada, 2007) and AD (Iversen et al., 1983).

Phox2a and Phox2b are two closely related homeodomain transcription factors with similar expression patterns. They play a critical role as determinants for the development of noradrenergic neurons during embryogenesis. For example, inactivation of the Phox2 genes in mice leads to the agenesis of the LC (Morin et al., 1997), disruption of noradrenergic differentiation and failed phenotype expression (Pattyn et al., 1999, Pattyn et al., 2000). It has been reported that while Phox2a expression is maintained through life, that of Phox2b is substantially reduced. Recent study demonstrated that loss of one Phox2 protein can be fully compensated by expression of the other (Coppola et al., 2010). However, whether these genes still have modulatory effects on noradrenergic neurons and phenotypes in adult brains has not been elucidated. Our previous *in vivo* study showed that a significant reduction in mRNAs of NET and DBH in the LC and adrenal glands of aging rats was paralleled with a decline in mRNA levels of Phox2 (Zhu et al., 2005), suggesting a possible relationship between these marker genes and Phox2 genes in noradrenergic neurons after birth. Furthermore, *in vitro* investigations (Yang et al., 1998, Stanke et al., 1999, Kim et al., 2002, Fan et al., 2009) demonstrated direct transcriptional activation of DBH and NET genes by Phox2 genes. Collectively, these findings implicate the possible modulatory effect of Phox2 genes on the expression of NET and DBH after birth. Given that the cellular mechanisms and potential therapeutic implications of LC neuronal loss in aging and neurodegeneration diseases remain unknown, exploring this modulation may provide important insight for better management of these diseases.

In the present study, we sought to test the hypothesis that Phox2a and Phox2b genes are able to upregulate the expression of NET and DBH after birth. Recombinant lentiviral eGFP-Phox2 and -Phox2b (*vPhox2a* and *vPhox2b*) were microinjected into the LC of adult rats. We observed that forced over-expression of Phox2a and Phox2b in the rat LC markedly influence the expression of NET and DBH in noradrenergic neurons. The present study suggests that Phox2a and Phox2b possess modulatory roles in the control of the expression of NET and DBH in adult animal brains.

EXPERIMENTAL PROCEDURES

cDNA cloning of rat Phox2a/2b

Total RNA was prepared from the LC area and adrenal glands of Fischer 344 rats using TRIzol reagent (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. Full-length cDNAs encoding Phox2a and Phox2b were prepared by RT-PCR using these total RNA samples. The following primers were used for *Phox2a* (NM_053869; forward primer, ACG CGT CGA CC ATG GAC TAC TCC TAC CTC AAT TCG; reverse primer, TGT CTA GAG CGG CTA GAA GAG GTT TGTC) and for *Phox2b* (XM_344239; forward primer, ACG CGT CGA CC ATG GAC TAC TCC TAC CTC AAT TCG; reverse primer; TGT CTA GAT CAG AAC ATA CTG CTC TTC ACT AAG). Annealing temperatures of 62°C and 51°C for Phox2a and Phox2b, respectively, were required for optimal amplification. Amplified fragments were gel purified using QIAEX II beads according to the manufacturer's instructions (Qiagen, Valencia, CA) and cloned into the SalI/Xba of the pCMV Sport 6 expression vector (Invitrogen, NY). Construct identity was confirmed by DNA sequencing.

Cell cultures

The cell types used in the present study were human embryonic kidney (HEK) 293T (ATCC #CRL-11268), 293FT cells (Invitrogen R700-07), human fibrosarcoma cells HT1080 (ATCC #CCL-121) and rat PC12 (ATCC #CRL-1721.1). HEK293T, 293FT and HT1080 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 200 mM L-glutamine, 10 mM MEM non-essential amino acids, 100 mM MEM sodium pyruvate and G418 (500 μg/mL). PC12 cells were maintained in RPMI 1640 medium. All these media were also supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL), at 37°C in humidified air containing 5% CO_2.

Lentiviral vector production

The preparation and use of all viral vectors were carried out in a bio-safety level 2 laboratory with the approval of the Institutional Biosafety Committee of the East Tennessee State University. All reagents and kits were purchased from Invitrogen (Carlsbad, CA). Lentiviral-mediated expression cassettes, with dual promoters to drive the gene of interest (Phox2a or Phox2b), and the reporter gene (enhanced green fluorescent protein, eGFP) which allows direct observation of viral delivery to the LC site, were constructed according to the manufacturer's instruction. One cassette containing eGFP alone was used as a control in the experiments. Briefly, cDNAs of eGFP and rat Phox2a or Phox2b were modified using PCR to include the sequence CACC at the 5′ end, in order to be complimentary to the overhang sequence GTGG in the pENTR/D-TOPO vector. These cDNAs were inserted into the pENTR/D-TOPO vector and the produced cDNA-vectors were further transferred into a lentiviral plasmid pLenti6/V5-DEST by LR-clonase. The identity of the recombinant expression vector pLenti6/V5-eGFP-Phox2a (or 2b) was confirmed by restriction analysis, PCR reaction, and sequencing. Recombinant expression cassettes were finally produced by co-infection of HEK293T cells with the recombinant expression vector pLenti6/V5-eGFP-Phox2a (or 2b) and packaging vectors, pCMVΔR8.9 and pVSVG, using the TransIT®-293 Transfection Reagent. A lentiviral stock was produced and satisfactory eGFP expression was verified by fluorescence microscopic analysis by fluorescence-activated cell sorter (FACS) analysis. Lentiviral supernatant was harvested 72 h post-infection after microscopic verification of fluorescence and filtered through a 0.45 μM membrane. The non-concentrated virus titer was greater than 5×10^6 TU/ml. High-titer viral stocks were obtained by untracentrifugation at 25,000 rpm for 2 h and the viral pellet was reconstituted overnight (4°C) in artificial cerebrospinal fluid (aCSF). Viral titers were determined by infecting

HT1080 cells. The cells were plated at a density of 1×10^5 cells per well in six-well tissue culture dishes. Serial dilutions of the vector stock were added, and blue-stained colonies were counted to determine the titer after blasticidin was added for colony screening. Viral stocks were stored at −80°C until use.

Animal and *in vivo* stereotaxic microinjection

Male Fischer 344 rats (Harlan Laboratories Inc., Indianapolis, IN) weighing 220-250 g at the time of initiation of experiments were housed on a 12 h light/dark cycle and with food and water provided *ad libitum*. 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. After an acclimation period of 5 days, rats were randomly assigned to the experimental and placebo groups. Microinjection was performed following previously published procedures (Carlezon Jr. and Neve, 2003) with modifications. Briefly, using a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA), two burr holes for bilateral injection (1 mm in diameter) were drilled to the level of the dura in the skull according to the coordinates of the LC. Hamilton syringes (30 gauge beveled needle) loaded with recombinant lentiviruses were lowered to the LC ([AP]= −10.0 mm, [LAT]= ±1.3 mm, and [V]= −6.0 mm) (Paxinos and Watson, 2005). Once at the desired depth, microinjections of vector constructs (2 μl at 1×10^8 TU of titer per milliliter) were delivered into the LC region at a rate of 0.1 μl/30 seconds over a period of 10 minutes. The microinjection volume of 2 μl for viral vectors is a widely used parameter from previous reports (Carlezon et al., 1998, Azzouz et al., 2002, Carlezon Jr. and Neve, 2003). To allow maximum diffusion from the microinjector tip and absorption in the tissue, the syringe needle was kept in the injection place for additional 5 min, then withdrawn very slowly. The skull holes were sealed with dental cement to prevent cerebrospinal fluid loss. The incision was closed with sutures and covered with antibiotic ointment. Some rats were bilaterally microinjected with lentiviral cassette carrying eGFP only as the control, while some were sham-operated (the injection syringe is lowered into the LC region without injection) to rule out possible effects of the surgery. Rats were allowed to recover, following which they were sacrificed on the designated day as described in Results. 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.

In situ hybridization

After euthanized, rat brains were removed, rapidly frozen in 2-methyl-butane on dry-ice, and then stored at −80° C until sectioning. Sections (16 μm) through the pontine LC region were cut on a cryostat, mounted on SuperFrost Plus slides (Fisher Scientific; Pittsburg, PA), and stored at −80° C. On the day of hybridization experiment, slides were fixed with 4% (w/v) paraformaldehyde followed by acetylation with acetic anhydride. Lipids were extracted by washing with increasing concentrations of alcohol (50, 70, 95 and 100% [vols]). [^{35}S]-labeled cRNA probes (Perkinelmer, MA) were transcribed *in vitro* from cDNAs for rat Phox2a (0.85 kb), Phox2b (0.95 kb), NET (0.5 kb), and DBH (1.4 kb) in pGEM-3Zf vectors with T3 or T7 RNA polymerase. Pre-hybridized sections were incubated with a hybridization solution containing the radiolabeled probes, at 55° C for 3-5 h. The brain tissue sections were then washed extensively and apposed to Biomax autoradiographic films (Kodak; Rochester, NY). For higher resolution studies, sections were also dipped in Kodak NTB2 emulsion (Fisher, Pittsburgh, PA) and quantitatively analyzed with the MCID CORE 7.0 program (Imaging Research Inc.; Linton, England).

Detection of protein levels

Protein levels of Phox2a/b, NET, and DBH in cultured cells (for *in vitro* experiments), or rat hippocampus and frontal cortex from rats were determined by Western blotting. These

samples were homogenized in sample buffer containing sodium lauryl sulfate (SDS) and β-mercaptoethanol. After centrifugation at 1000 xg, protein concentrations of supernatants were measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Equal amounts of samples (10 μg of protein per lane) were loaded on 10% SDS-polyacrylamide gels for electrophoresis and transferred by electro-blotting to a polyvinylidene diflouride membrane. The membranes were incubated overnight at 4°C with primary antibodies [either anti-Phox2a or Phox2b from rabbit (1:200 dilution, Sigma-Aldrich, St. Louis, MO), anti-NET from rabbit (1:330 dilution; Alpha Diagnostic Intl. Inc, San Antonio, TX), or anti-DBH from rabbit (1:400 dilution; Santa Cruz Biotechnology Inc., CA). Membranes were then washed and incubated with secondary antibodies (horseradish peroxidase-conjugated anti-rabbit IgG, 1:3000; Amersham Biosciences, Little Chalfont, UK). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Amersham; Piscataway, NJ). Bands were detected by the Kodak Image station (New Haven, CT), or exposed on films and scanned by Quantity One imaging devices (Bio-Rad, Hercules, CA). Band densities were quantified using imaging software (Molecular Dynamics IQ solutions, Molecular Dynamics, Inc., Sunnyvale, CA). A linear standard curve was created from optical densities (ODs) of bands with a dilution series of total proteins prepared from 293-FT, PC12 cells, or brain tissues. OD values of Phox2, NET, and DBH signals were compared and normalized with β-actin immunoreactivities, which were determined on the same blot. Normalized values were then averaged for all replicated gels and used to calculate the relative changes on the same gel.

Immunofluorescence staining with NET and DBH

For the immunofluorescence staining experiments, rats were transcardially perfused under anesthesia with pentobarbital (40mg/kg, i.p.) using 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4. Brains were stored in 10% sucrose followed by 30% sucrose and then sectioned at 30 μm using a sliding microtome. Immunofluorescence staining was performed using commercial monoclonal antibodies from mice to NET (MAb Technologies, Inc., TN), and polyclonal antibody from rabbit against DBH (Protos Biotech Corp, New York, NY). Brain sections were washed three times in phosphate-buffered saline (PBS) and preincubated in 5% bovine serum albumin in PBS supplemented with 0.2% Triton-X 100 for 1 h at room temperature, followed by incubation in primary antibody (1:500 dilution, in PBS containing 0.2% Triton-X 100) overnight at 4° C. After washing, sections were then incubated with secondary antibodies (for NET: Alexa Fluor 488-conjugated goat anti-mouse IgG; for DBH: Alexa Fluor 488-conjugated goat anti-rabbit IgG; all from Invitrogen, Carlsbad, CA) for 2 h at room temperature, followed by 4 rinses with 0.1M PBS. Slides were then covered by coverslips using Citifluor mounting medium. In order to reduce inter-animal staining variability, each immunofluorescence staining experiment was performed on all tissue sections at the same time by an investigator blinded to the animal group. Immunofluorescence labeling was observed and acquired under a Leica TCS SP2 confocal microscope system (Leica Microsystems Inc., Bannockburn, IL, USA). Images were semiquantitatively analyzed by using ImageJ software (Rasband, US National Institutes of Health, Bethesda, http://rsbweb.nih.gov/ij, 2010). Reference background levels were obtained from non-immunoreactive portions of brain sections adjacent to the LC region by determining the optical density on a 0 to 255 grayscale (0 being white and 255 black). The area fraction of immunofluorescence was quantified at three levels of each section and two sections from each rat.

5-Bromo-2-deoxyuridine (BrdU) injection, tissue collection and processing

BrdU immunohistochemical staining was used for the detection of newly generated cells in the dentate gyrus (DG) of the hippocampus. This experiment was performed in rats that were *in vivo* stereotaxically microinjected with *vPhox2* for 21 days. BrdU (100 mg/kg/day, Sigma-Aldrich, St. Louis, MO) was intraperitoneally injected once daily for 5 successive

days starting on day 15 after administration of *vPhox2*. Rats were perfused 24 h after the last injection. Serial sections (30 μm) were cut through the rostral/caudal extent of the hippocampus (bregma −1.88 mm to −5.20 mm) (Paxinos and Watson, 2005) using a sliding microtome. Every sixth section was coded and stored in the long-term storage solution (sucrose/ethylene glycol/sodium azide in PBS) at −20°C.

For BrdU labeling, these free-floating sections were washed 3 times in PBS and 2xSSC, and then incubated in 50% formamide-2x standard saline citrate at 65°C for 2 h. Following denaturation in 2 N HCl at 37°C for 30 min, the sections were washed in 2xSSC and PBS, preincubated in 1% Triton-X/4% goat serum/PBS, and then incubated overnight at 4°C with a BrdU-specific mouse monoclonal antibody (1:600; Sigma-Aldrich). The next day, sections were incubated for 1 h with a biotinylated mouse secondary antibody (1:200; Vector Laboratories, Burlingame, CA. U.S.A.), followed by another 1 h incubation with VECTASTAIN (Elite ABC Kit, 1:100; Vector Laboratories). Cells were visualized with 3,3′-diaminobenzidine containing nickel chloride (nickel-DAB; 40 mg/mL).

Quantitation of BrdU-positive cells was performed at 400x and 1000x magnifications under a light microscope (Zeiss Axio Observer Z1, Carl Zeiss Microimaging, LLC, Jena, Germany) using a modified unbiased stereology protocol (Eisch et al., 2000, Malberg et al., 2000). BrdU-positive cells were counted in the granule cell layer (GCL) and subgranular zone, which was defined as a two-cell body-wide zone along the border of the GCL. The total number of BrdU-positive cells was calculated by multiplying the cell number counted in each 6^{th} section by 6 and reported as cells per DG.

Statistics

All experimental data are presented in the text and graphs as the mean ± SEM. Data were analyzed by one way analysis of variance (ANOVA, SigmaStat, Systat Software Inc., Richmond) when multiple treatment groups were compared. A *post-hoc* Student-Newman-Keuls test was performed for further examination of group differences.

RESULTS

cDNA cloning of rat Phox2a/2b

Sequence analysis of the cloned cDNAs of rat Phox2 revealed an open reading frame of 846 base pairs for rat Phox2a, implicating a protein consisting of 281 amino acids; and 945 base pairs for rat Phox2b, implicating a protein consisting of 314 amino acids. At the nucleotide level, rat Phox2a and mouse Phox2a share 97% identity, while rat Phox2a and human Phox2a show 90% identity (data not shown). In the deduced amino acid sequences, rat Phox2a possesses 98.9% and 79.6% identity to its mouse and human counterparts respectively (Fig. 1). Similarly, rat Phox2b and mouse Phox2b share 95% identity of the nucleotide, while rat Phox2b and human Phox2b show 91% identity (data not shown). For the deduced amino acid sequences, rat Phox2b possesses 100% identity to its mouse and human counterparts respectively. It is noteworthy that the nucleotide sequence of our cloned rat Phox2b cDNA is exactly the same as that of the ORF of predicted rat Phox2b in the Genebank (XM_344239.2, GI:62660435). The cloned rat Phox2 cDNAs provide the opportunity to investigate the effects of microinjection of Phox2 cDNAs on the expression of noradrenergic phenotypes in adult rats.

Lentivirus-mediated expression of Phox2 in 293FT and PC12 cells

To test the infection efficiency of the *vPhox2 in vitro*, 293FT cells were infected with a lentiviral stock for 72 hours. As shown in Fig. 2A, GFP was found to be expressed in 91% of cells as measured by a fluorescence-activated cell sorter (FACS, see description in the

legend of Fig. 2A). Next, the ability of the expression cassette to induce expression of transgenes *in vitro* was confirmed following transduction of PC12 cells with *vPhox2a* or *vPhox2b* at 1:2, 1:4, 1:8, 1:16 and 1:32 dilutions of non-concentrated lentiviral stocks with the viral titer (TU/ml) for *vPhox2a*: 4×10^5 and for *vPhox2b*: 5×10^6. The reason to use non-concentrated lentiviral stock *in vitro* tests is based on the consideration that concentrated lentivirus preparations are mainly used for *in vivo* experiments for the sake of using a relatively small volume (2 μl). Three days later, cells were harvested. Western blotting for Phox2a or Phox2b showed that the *vPhox2a* and *vPhox2b* significantly increased protein levels of Phox2a and Phox2b in a concentration-dependent manner (Figs. 2B, 2C; $F_{5,30}=2.87$, $p < 0.05$ for *vPhox2a*; $F_{5,30}=5.51$, $p < 0.01$ for *vPhox2b*). These i*n vitro* expression tests demonstrated that transduction with the *vPhox2* significantly increased levels of Phox2 proteins, indicating that a relatively high titer lentiviral packaging platform was preliminary established. We then examined overexpressed Phox2 on protein levels of DBH in the same cell preparations. Transduction of the *vPhox2a* or *vPhox2b* markedly enhanced protein levels of DBH ($F_{5,30}=4.83$, $p < 0.01$ for *vPhox2a*; $F_{5,24}=4.45$, $p < 0.01$ for *vPhox2b*). *Post hoc* tests showed that the *vPhox2a* preparation at the dilution of 1:8, 1:4 and 1:2 increased DBH proteins by 89%, 129% and 105%, respectively (all $p<0.05$). Similarly, the *vPhox2b* preparation at the dilution of 1:4 and 1:2 increased DBH protein levels by 85% and 69%, respectively (all $p<0.05$, Figs. 2D and 2E). These results are consistent with our previous report in which transfection with cDNAs of human Phox2a or Phox2b significantly increased expression of DBH *in vitro* (Fan et al., 2009).

Microinjection of vPhox2 increased mRNA levels of NET and 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 stereotaxically microinjected the *vPhox2* (about 1×10^8 TU/ml) into the bilateral LC region of rats. All rats were euthanized on days 7, 14 and 21 after injection. Fig. 3A shows that the *vPhox2* was accurately delivered to the LC region, as there is clear GFP fluorescence in the target area. Rats with missed placements were eliminated from further experiments. We then performed *ex vivo in situ* hybridization to measure mRNA levels of Phox2a and Phox2b in the LC region. As shown in Figs. 3B and 4, microinjection of *vPhox2a* or *vPhox2b* significantly affected mRNA levels of Phox2 in the rat LC ($F_{4,75}=11.95$, $p < 0.001$ for *vPhox2a*; $F_{4,60}=4.95$, $p < 0.05$ for *vPhox2b*). *Post hoc* contrasts revealed that there were no significant differences between the control and sham groups in the expression of Phox2a and Phox2b genes, demonstrating no effect of surgery. Likewise, there was a consistent lack of differences in expression levels of noradrenergic phenotypes comparing control and sham groups for all following experiments, and this is not mentioned again. Compared to the control group at the same time points, Phox2a mRNA levels were robustly increased 119%, 146 and 141%, on days 7, 14 and 21 (all $p<0.01$), respectively, after microinjection of *vPhox2a*. Levels of Phox2b mRNA in the LC region were significantly increased by 41% ($P<0.05$) 21 days after microinjection with *vPhox2b*, as compared to those of the control group. Although there was a trend towards an increase for Phox2b mRNA on days 7 and 14 (by 23% and 20%, respectively) after microinjection of *vPhox2b*, these increases did not reach significance (Fig. 4). Nevertheless, these experiments demonstrated that microinjection of *vPhox2* resulted in over-expression of these genes in the LC region.

To examine biological effects of over-expressed Phox2a and Phox2b in the LC region on noradrenergic phenotypes, we measured mRNA levels of DBH and NET in the LC region in the same rats used for the measurement of Phox2 mRNAs. As shown in Fig. 5, microinjection of *vPhox2a* or *vPhox2b* significantly influenced mRNA levels of DBH in the LC region ($F_{4,65}=11.74$, $p < 0.001$, for microinjection of *vPhox2a*; $F_{4,70}=10.48$, $p<0.001$, for microinjection of *vPhox2b*). Further comparison revealed that DBH mRNA levels were

increased by 57% ($p<0.01$), 42% ($p<0.05$) and 66% ($p<0.01$), respectively, on days 7, 14 and 21 following microinjection of *vPhox2a*. Similarly, 21 days after microinjection of *vPhox2b*, DBH mRNA levels in the LC region were significantly increased by 57% ($p<0.01$). Interestingly, although Phox2b mRNA levels were only modestly increased 7 and 14 days after microinjection of *vPhox2b* (Fig. 4), DBH mRNA levels were significantly increased by 39% ($p<0.05$) and 66% ($p<0.01$) during the same period.

Microinjection of *vPhox2a* or *vPhox2b* also markedly affected mRNA levels of NET ($F_{4,60}=4.44$, $p < 0.01$, for microinjection of *vPhox2a*; $F_{4,70}=8.92$, $p<0.01$, for microinjection of *vPhox2b*; Fig. 6). NET mRNA levels in the LC were increased by 78% ($p<0.05$) 14 days after microinjection of *vPhox2a*. Small increase in NET mRNA levels was observed 7 (by 39%) and 21 (by 35%) days after microinjection of *vPhox2a*, but these increases did not reach statistical significance when compared with the control (both $p>0.05$). Comparatively, microinjection with *vPhox2b* has stronger effects than *vPhox2a* on NET mRNA in the LC. As shown in Fig. 6, NET mRNA levels in the LC were significantly increased 7, 14 and 21 days after microinjection by 111%, 139% and 68% (all $p<0.01$), respectively, compared to that of the control group.

Microinjection of vPhox2 increased protein levels of DBH and NET in the LC region

DBH and NET protein levels were examined 21 days after microinjection by immunofluorescence staining. As shown in Figs. 7 and 8, microinjection of *vPhox2* had a significant effect on protein levels of DBH and NET in the rat LC region ($F_{3,16}=7.03$, $p < 0.001$ for DBH; $F_{3,36}=3.35$, $p < 0.05$ for NET). Microinjection with *vPhox2a* or *vPhox2b* significantly increased immunoreactivities of DBH in the LC region (52% by *vPhox2a*, $p<0.01$; 27% by *vPhox2b*, $p<0.05$). Co-microinjection of *vPhox2a* and *vPhox2b* did not produce synergistic effects on DBH protein levels (increased by 50%, $p<0.05$; Fig. 7). Similar to these findings, microinjection of *vPhox2a* or *vPhox2b* markedly increased NET immunoreactivity in the LC by 34% and 41% (both $p<0.05$), respectively. Likewise, no synergistic effect was observed for NET immunoreactivity after simultaneous microinjection of *vPhox2a* and *vPhox2b*, compared to those of single microinjections with *vPhox2a* or *vPhox2b* (Fig. 8).

Microinjection of vPhox2 increased protein levels of DBH and NET in the terminal areas of the LC neurons

In order to assess effects of over-expression of Phox2 genes in the LC on the noradrenergic phenotype in its terminal areas, protein levels of DBH and NET in the hippocampus and frontal cortex were measured by Western blotting following microinjection with *vPhox2*. Tissue samples were dissected from the same rats that were used for measurement of mRNA levels described above. Similar to the mRNA measurement mentioned above, there was no difference for these protein levels between the control and sham groups. As shown in Figs. 9 and 10, microinjection of *vPhox2a* or *vPhox2b* markedly affected protein levels of DBH ($F_{4,28}=3.36$, $p < 0.05$, for microinjection of *vPhox2a*; $F_{4,29}=5.40$, $p < 0.01$, for microinjection of *vPhox2b*) and NET ($F_{4,34}=7.43$, $p < 0.001$, for injection of *vPhox2a*; $F_{4,42}=6.89$, $p < 0.001$, for injection of *vPhox2b*) in the hippocampus. *Post hoc* comparisons revealed that DBH protein levels were significantly increased in the hippocampus 21 days (by 71%, $p<0.05$) after microinjection of *vPhox2a*, compared with those of the control group. However, no effect was observed 7 and 14 days after *vPhox2a* microinjections (Fig. 9A). Microinjection of *vPhox2b* seemed to have somewhat greater effects on DBH protein levels in the hippocampus. On days 7, 14 and 21 after microinjection, DBH protein levels were increased by 57% ($p<0.05$), 82% ($p<0.01$) and 59% ($p<0.05$) respectively (Fig. 9B). Similarly, 7, 14 and 21 days after microinjection of *vPhox2a*, NET protein levels in the hippocampus were increased by 167% ($p<0.01$), 77% ($p<0.05$), and 74% ($p<0.05$),

respectively (Fig. 10A). On days 7, 14 and 21 after microinjection of *vPhox2b*, NET protein levels in the hippocampus were also increased by 40% ($p>0.05$), 70% ($p<0.01$) and 63% ($p<0.01$; Fig. 10B), compared to those in the control group.

As shown in Fig. 11, microinjection of *vPhox2a* or *vPhox2b* significantly affected NET protein levels in the frontal cortex ($F_{4,33}=3.86$, $p<0.05$ for *vPhox2a*; $F_{4,27}=4.22$, $p<0.01$ for *vPhox2b*). On days 14 and 21, but not on day 7 after microinjection of *vPhox2a*, NET protein levels in the frontal cortex were increased by 33% and 42%, respectively, (both $p<0.05$, Fig. 11A). On days 7 and 14, but not on day 21 after microinjection of *vPhox2b*, NET protein levels were increased by 57% and 52%, respectively (both $p<0.05$, Fig. 11B). Nevertheless, although on days 7, 14 and 21 after microinjection with *vPhox2a* or *vPhox2b*, DBH protein levels in that area were increased by 6.7%, 28.2% and 35.1%, or by 9.5%, 18.6% and 17.1%, respectively, as compared to the control group, all these increases did not reach the statistical significance ($F_{4,33}=1.62$, $p>0.05$ for *vPhox2a*; $F_{4,29}=1.36$, $p>0.05$ for *vPhox2b*; data not shown).

We also assessed whether there were synergistic effects of over-expression of Phox2a and Phox2b on DBH and NET protein levels in the hippocampus and frontal cortex. These rats were simultaneously microinjected with mixed preparation of *vPhox2a* and *vPhox2b* in both sides of the LC. Co-microinjection with *vPhox2a* and *vPhox2b* significantly affected both DBH and NET in the hippocampus, and NET in the frontal cortex ($F_{4,31}=3.08$, $p<0.05$ for DBH in the hippocampus, Fig. 9C; $F_{4,42}=5.76$, $p<0.01$ for NET in the hippocampus, Fig. 10C; $F_{4,31}=5.07$, $p<0.01$ for NET in the frontal cortex, Fig. 11C). However, co-microinjection with *vPhox2a* and *vPhox2b* had no additive or synergistic effects on the protein levels of DBH in the hippocampus, or NET in the hippocampus or frontal cortex, when compared to microinjection with either *vPhox2a* or *vPhox2b* alone. These results were consistent with measurements of immunofluorescence observed in the LC region after microinjection with Phox2 (Figs. 7 and 8).

Microinjection of vPhox2 increased neurogenesis in the DG

Whether over-expression of Phox2 in the rat LC and subsequent elevation of DBH 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 rats that were microinjected with *vPhox2* for 21 days. Microinjection of *vPhox2* had a substantial effect on the number of BrdU-positive cells in the DG of the hippocampus, as compared to control rats ($F_{3,273}=9.01$, $p<0.01$; Fig. 12). The increase in BrdU-positive cell numbers was significant comparing control and *vPhox2b*-microinjected rats ($p<0.05$), as well as comparing control and co-microinjected (*vPhox2a* and *vPhox2b*) rats ($p<0.01$). No significant difference was observed comparing control and *vPhox2a*-microinjected rats. Co-microinjection with *vPhox2a* and *vPhox2b* resulted in a greater increase in BrdU-positive cells than either *vPhox2a* or *vPhox2b* microinjection alone ($p<0.05$).

DISCUSSION

In the present study, dual-promoter lentiviral vectors expressing eGFP and rat Phox2a or Phox2b, i.e. *vPhox2a or vPhox2b,* were constructed. After *in vitro* expression testing, these expressional cassettes were microinjected into the rat LC region to examine effects of continuous Phox2 over-expression on noradrenergic phenotypes in intact LC neurons of rats. Intra-LC delivery of the *vPhox2* resulted in sustained elevation of Phox2a and Phox2b mRNA levels. Elevated gene expressions were accompanied by parallel significant increases in DBH and NET protein levels in the LC and its terminal areas such as the hippocampus and frontal cortex. To our knowledge, this is the first study of forced over-expression of

Phox2a and Phox2b in the LC of adult rats. The present study is the extension of our previous *in vitro* investigation, in which over-expression with cDNAs of human Phox2 or shutdown of endogenous Phox2 genes with shRNAs caused a parallel increase or reduction, respectively, in the expression and function of DBH and NET in SK-N-BE(2)C cells (Fan et al., 2009). The findings, coupled with a previous report (Card et al., 2010), support the hypothesis that Phox2a and Phox2b, determinants for development of noradrenergic phenotypes during embryogenesis, continue to maintain the phenotype of noradrenergic neurons in adult rat brains and may play a role in homeostasis.

Phox2b expression in the mouse LC has been reported to be transient and shut down at E11.5 (Pattyn et al., 1997, Pattyn et al., 2000). At birth, only Phox2a expression is detectable in the LC and subcoeruleus (Tiveron et al., 1996, Pattyn et al., 1997), and expression continues into adulthood in mice (Tiveron et al., 1996) and rats (Card et al., 2010). Recent studies show that A5, A6 (LC) and A7 cell groups do not express Phox2b in adult Sprague-Dawley rats (Kang et al., 2007). However, the present results using *in situ* hybridization clearly demonstrate that Phox2b mRNA is detectable in the LC of adult Fischer 344 rats. Possible explanations for the discrepancy between our results and previous observations (Pattyn et al., 2000, Kang et al., 2007) include different rat strains and different methods for measuring Phox2 (*in situ* hybridization versus immunostaining). It is worthwhile to note that mRNA levels of Phox2b in the LC of Fischer 344 are much lower (Fig. 4) than those of Phox2a (Fig. 3B). Nevertheless, the presence of Phox2b gene expression in the LC of adult Fischer 344 rats and the expression enhancement of DBH and NET after forced expression of Phox2b in the LC suggest that Phox2b plays a role in maintaining and modulating noradrenergic phenotypes in the adult brain.

Viral gene delivery systems are powerful tools for the expression of exogenous cDNAs especially in neuronal cells. Among them, lentiviral vectors remain most promising for the long-term, stable expression of transgenes *in vivo* owing to their ability to integrate into the genome of the dividing and non-dividing host cells (Naldini et al., 1996) after retrotranscription (Lois et al., 2002, Pfeifer et al., 2002). After lentiviral injection in the rat brain, the majority of transduced cells are neurons (Naldini et al., 1996). In the present study we chose lentiviral vectors because of their pantropic nature in the nervous system after *in vivo* delivery. Seven days after microinjection of lentiviral cassettes, Phox2a mRNA levels were increased over two folds and this higher expression level persisted over the next two weeks, demonstrating efficient transduction and stable expression of Phox2 genes. This time point and increased range are consistent with previous reports in which lentiviral vector-induced transduction and stable expression of marker genes were first observed in adult rat brains (Naldini et al., 1996). Compared to that of Phox2a, however, forced expression of Phox2b appeared relatively slower. Although there was a modest increase in mRNA levels of Phox2b on days 7 (about 23%) and 14 (about 20%) after *vPhox2b* microinjection, a significant increase of Phox2b mRNA in the LC was observed only 21 days after microinjection (Fig. 4). Whether the relatively low expression of endogenous Phox2b genes in the LC accounts for this different transgene pattern, as compared to Phox2a, remains to be elucidated. Nevertheless, even these modest increments of Phox2b mRNAs still markedly increased the expression of DBH and NET in the LC and in LC terminal areas. Indeed, Phox2b has been reported to be the main regulator of the noradrenergic phenotype (Coppola et al., 2005), as Phox2b not only binds to and transactivates the promoters of these phenotypes, but also even regulates the expression of Phox2a by directly binding to its 5′ regulatory region (Yang et al., 1998, Adachi et al., 2000, Flora et al., 2001).

Neuronal loss in the LC in brains of the aged subjects and patients suffering from progressive neurodegenerative diseases such as PD and AD has been well documented (Mann et al., 1983, Bondareff et al., 1987, Cash et al., 1987, Chan-Palay and Asan, 1989,

Forstl et al., 1994, Zarow et al., 2003). Dysfunction of the central noradrenergic system resulting from LC neuronal loss and LC neuron degeneration-induced NE deficiency may contribute to changes in attention, learning, information processing, and motor function appearing in aging populations, and also to symptoms such as dementia, memory loss, depression, anxiety, postural dysfunction observed in PD and AD. However, the causes and cellular mechanisms of LC neuronal loss in aging and in neurodegenerative diseases are poorly understood. A number of studies have drawn attention to the involvement of Phox2 genes in these mechanisms, specifically: (1) Phox2a/2b genes are exclusively expressed in the noradrenergic neurons. The co-localization of Phox2 genes with noradrenergic markers have been demonstrated in mouse embryos (Pattyn et al., 1999) and neonates (Tiveron et al., 1996), as well as in the brain of adult rats (Card et al., 2010). Their co-expressional pattern is especially found in the LC region, as only neurons in this region are noradrenergic and there are no interneurons there. (2) They are essential and determinant for the generation and development of noradrenergic neurons during embryogenesis; (3) Phox2a genes are commonly considered to be persistently present in adult brains; and (4) Phox2a/2b can directly transactivate the promoters of DBH and NET, both characteristic factors of noradrenergic neurons. Our previous study demonstrated that reduced Phox2 mRNA levels in the LC of aged rats was accompanied by parallel reduction of DBH and NET mRNA levels (Zhu et al., 2005), indicating the possibility of a causal relationship between Phox2 genes and expression of the noradrenergic phenotypes during aging. If so, then delivery of related transcription factors into the LC of brains to up-regulate the phenotype may be a strategy to arrest or counteract the neurodegenerative process in aging or degenerative diseases. The demonstration here that forced over-expression of Phox2 genes in the LC markedly enhances expression of DBH and NET in the LC and its terminal areas in adult rats raises the possibility that such an approach could have therapeutic implications for aging, AD and PD (Costantini et al., 2000) and other disorders where noradrenergic deficits have been demonstrated. While the essential effects of these transcription factors on the noradrenergic phenotype during embryogenesis have been well documented (Goridis and Rohrer, 2002, Howard, 2005, Huber, 2006), their roles in the adult brain need to be elucidated. Likewise, whether increased noradrenergic gene expression resulted from an increased noradrenergic neuronal number or an increase NE synthesis in existing neurons is also an important issue to be addressed.

The mechanisms by which forced over-expression of Phox2 genes in the LC of rats increases expression of the noradrenergic phenotype remain to be determined. Enhanced expression may result from a direct transactivation of target genes by these transcription factors. Multiple *cis*-elements and homeodomain (HD)-binding sites in the promoter of DBH and NET genes have been identified (Kim et al., 1998, Yang et al., 1998, Kim et al., 1999, Kim et al., 2002), and binding of Phox2 to these *cis*-regulatory elements and HD-binding sites on promoters to transactivate these target genes have been observed (Tissier-Seta et al., 1993, Zellmer et al., 1995, Yang et al., 1998, Adachi et al., 2000, Kim et al., 2002). It is also possible that signal transduction pathways such as cAMP/PKA signaling may be involved. The activation of the DBH promoter by Phox2a is potentiated by cAMP/PKA and this effect is mediated by a CRE/AP-1 site, essential for Phox2a-mediated reporter activity, as well as its potentiating by Hand2 (Swanson et al., 1997, Swanson et al., 2000, Adachi and Lewis, 2002, Xu et al., 2003). Other transcription factors such as bone morphogenetic protein (BMPs) (Lo et al., 1999), Mash1 (Hirsch et al., 1998), dHand (Xu et al., 2003) may also take part in this Phox2-mediated enhancement of noradrenergic phenotypes.

An important finding in the present study is that over-expression of Phox2 genes in the LC region increased total number of BrdU-positive cells in the DG area of the hippocampus. This result demonstrates that transgenic upregulation of Phox2 genes in the LC has

functional consequences in LC projection areas in adult rats. Generally, in animals killed 24 h after BrdU injection, the BrdU-positive cells in the DG represents the newly generated cells. As one of the active neurogenesis areas in the adult mammalian brain, the hippocampal DG receives a dense noradrenergic input from LC neurons. The increased BrdU-positive cells observed here could be a result of increased NE released from LC terminals, secondary to a Phox2 over-expression-induced upregulation of noradrenergic neurons. Supporting this interpretation is evidence of the role of NE in modulating neurogenesis. For example, NE can enhance proliferation (Popovik and Haynes, 2000) and plays a crucial role after injury (Kolb and Sutherland, 1992). Administration of desipramine and reboxetine, the NE reuptake inhibitors which increase synaptic NE availability, increases neurogenesis in the hippocampus (Malberg et al., 2000, Chen et al., 2006), presumably as a result of increase NE availability in the synapse. In contrast, depletion of NE with DSP4, a selective noradrenergic neurotoxin, resulted in a 63% reduction in the proliferation of DG progenitor cells (Kulkarni et al., 2002). Interestingly, the age-dependent reduction of noradrenergic innervations of the DG in rats and human is associated with declining neurongenesis in this area (Kuhn et al., 1996, Gould et al., 1999, Ishida et al., 2000). In the present study, brain NE levels were not measured and we do not have direct evidence of increased NE in the synapse. Generally, an increased DBH expression may increase NE levels as DBH possesses the regulatory effect on NE biosynthesis based on the literature (Wise et al., 1977, Thomas et al., 1995, Thomas et al., 1998, Schroeder et al., 2010, Steele et al., 2011). Certainly, an increased NET could simultaneously enhance the reuptake of NE and thereby reduce NE concentrations in the synapse. However, NET is regulated by many different mechanisms (Mandela and Ordway, 2006) and an increase in NET proteins does not necessarily mean that the new protein is expressed on the cell surface, the only place where it is functional and can affect synaptic NE. Even on the surface, its activity can also be regulated. Second, our latest findings demonstrate a significant increase of tyrosine hydroxylase (TH) protein levels, the rate limiting enzyme in NE biosynthesis, in the brain LC after overexpression of Phox2 genes in these transgenic rats (Fan and Zhu, 2010), supporting the possibility of increased NE levels in the brain of these transgenic rats. We plan to measure NE concentrations in rats that are microinjected with *vPhox2a* and *vPhox2b* in future experiments to verify this functional consequence.

In the present study, microinjection with *vPhox2* significantly increased NET protein levels in the frontal cortex (Fig. 11). However, no significant changes were found in DBH protein levels in the frontal cortex after microinjection at any time period. Currently we do not have a satisfactory explanation for this discrepancy between protein levels of NET and DBH after microinjection of *vPhox2*. One possible cause is that their distribution pattern in the frontal cortex may be a factor. NET and DBH are exclusively co-expressed in cell bodies of noradrenergic neuron such as the LC, but not always in the terminal areas (Lorang et al., 1994, Schroeter et al., 2000). This distributional difference is particularly in cortical regions where DBH expression levels are lower (Schroeter et al., 2000). Therefore, the effect from microinjection with *vPhox2* on DBH in the frontal cortex may be smaller than on NET, although DBH expression there still shows a potential enhancement.

The present study demonstrated successful construction of two recombinant lentivirus vectors containing cDNAs of rat Phox2a or Phox2b. With forced expression of Phox2 genes after microinjection of these recombinants into the rat LC region, an upregulatory effect of Phox2a and Phox2b on the expression of NET and DBH in the adult rat brains was observed. These findings imply that Phox2 genes continue to regulate the phenotype of the noradrenergic neurons after birth. 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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Highlights

Phox2 genes may regulate the expression of noradrenergic phenotypes after birth. > Recombinant lentiviral eGFP-Phox2 was microinjected into rat locus coeruleus. > Overexpression of Phox2 upregulated NET and DBH in adult rat brain. >These findings may have possibly therapeutic implications for aging, AD and PD.

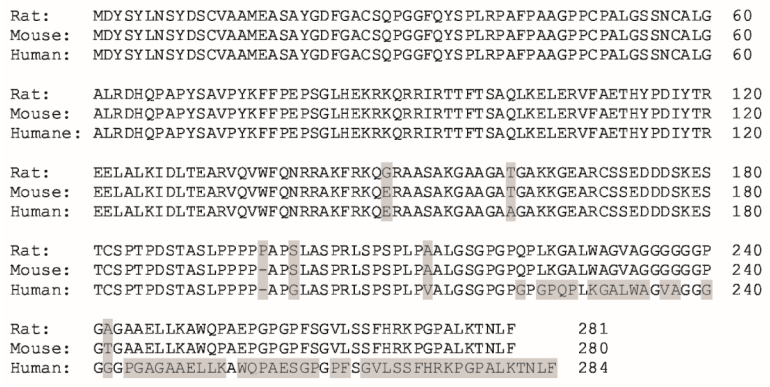

Figure 1.

Comparison of the amino acid sequences of Phox2a in rat, mouse, and human using CLUSTAL W. Shaded areas show different amino acids. Numbers on the right refer to the last amino acid on each line.

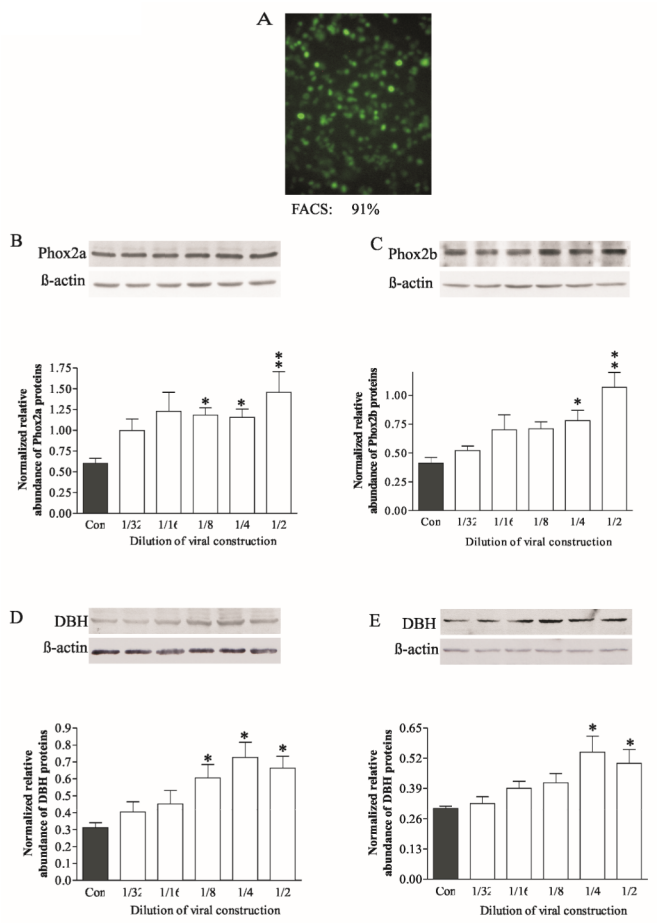

Figure 2.

Expression of GFP, Phox2 and DBH in HEK 293FT and PC12 cells infected with recombinant lentiviral eGFP-Phox2 cDNAs (*vPhox2*). The expression of the GFP marker in 293FT cells (panel A) showed a gene transduction efficiency of 91% by fluorescence microscopy and fluorescence-activated cell sorter (FACS) analysis (green cells/total cells × 100%). Protein levels of Phox2a (panel B), Phox2b (panel C) and DBH (panels D and E) were measured by Western blotting after PC12 cells were infected with *vPhox2a* or *vPhox2b* (all n=5/group). Con: the control group; 1/32, 1/16, 1/8, 1/4, and 1/2 represents dilutions of the original viral stock of *vPhox2a* or *vPhox2b*. **P*<0.05 and ** *P*<0.01, compared to the control group.

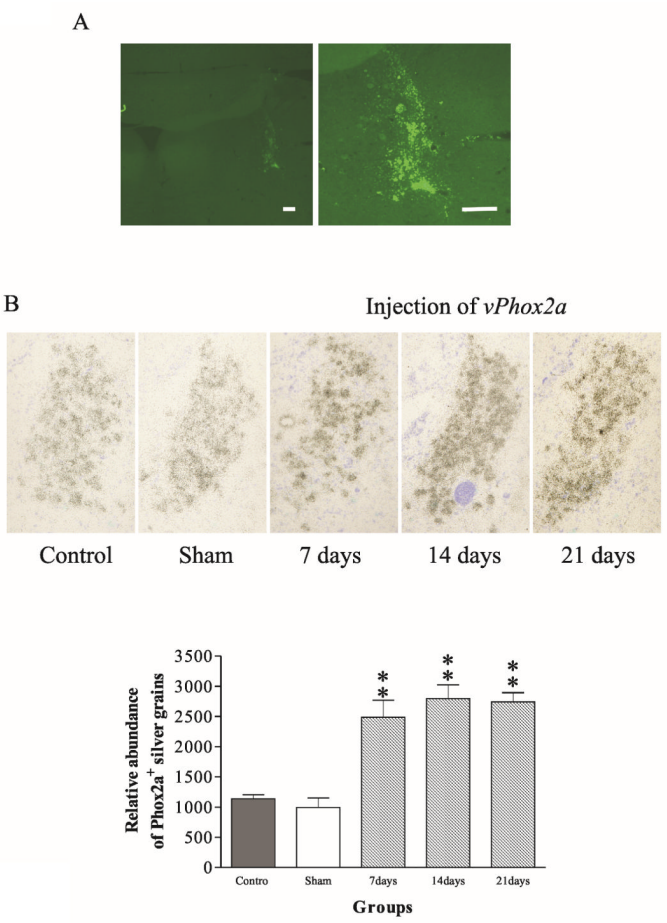

Figure 3.

A. Transduction efficiency of *vPhox2* in the brain as showed by fluorescence in the LC region of rats observed under fluorescence microscopy magnified as indicated. Scale bar = 100 μm for all images. B. Expression of Phox2a mRNA levels in rat LC region at different times after microinjection with *vPhox2a* in the LC region. Upper panel: Phox2a mRNA detected by *in situ* hybridization in the LC of rats (n=6/group). Lower panel: quantitative analysis of mRNA obtained with emulsion-dipped slides, ** $P<0.01$, compared to the control group.

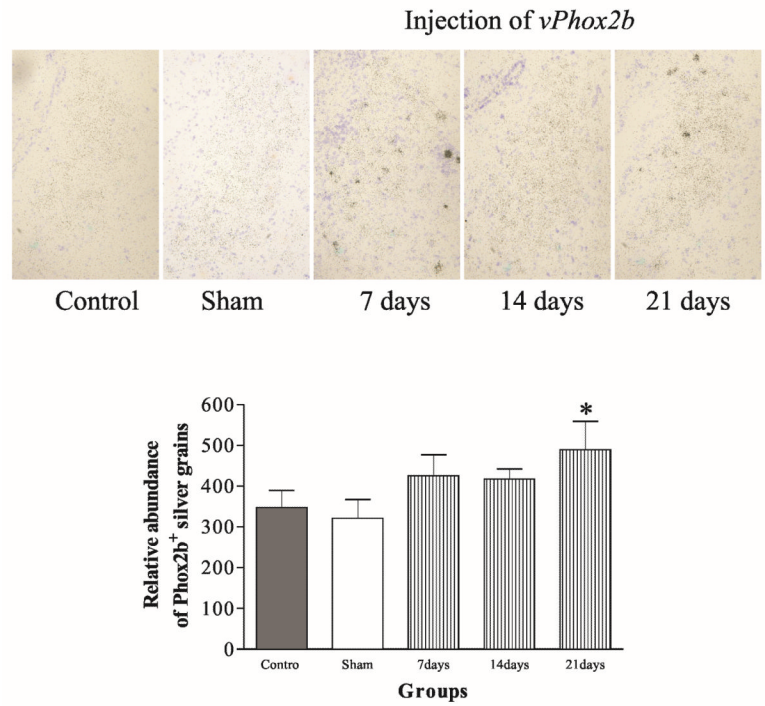

Figure 4.

Expression of Phox2b mRNA levels in rat LC region at different times after microinjection with *vPhox2b* in the LC region. Upper panel: Phox2b mRNA detected by *in situ* hybridization in the LC of rats (n=6/group). Lower panel: quantitative analysis of mRNA obtained with emulsion-dipped slides, * $P<0.05$, compared to the control group.

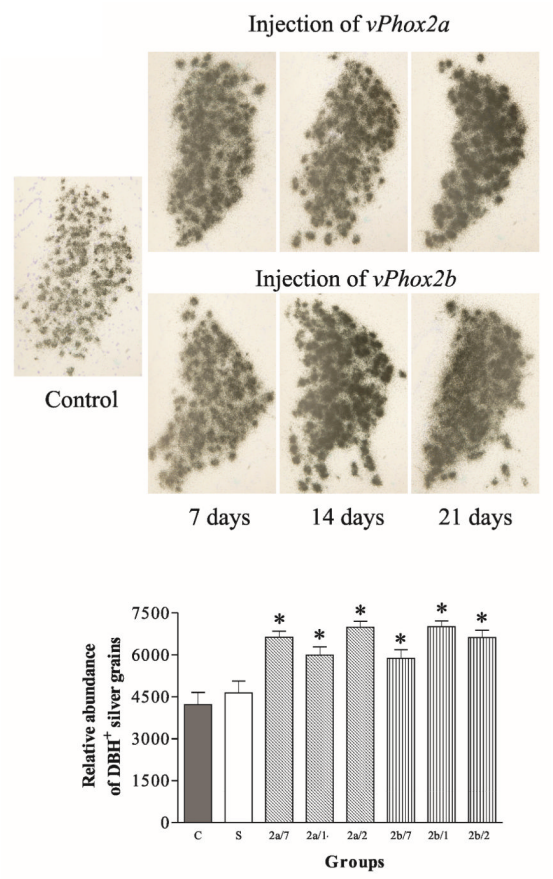

Figure 5.

Effects of over-expression of Phox2 on DBH mRNA levels in rat LC region, determined at different times after microinjection with *vPhox2a* or *vPhox2b*. Upper panel: DBH mRNA detected by *in situ* hybridization in the LC of rats (n=6/group). Lower panel: quantitative analysis of DBH mRNA obtained with emulsion-dipped slides, * $P < 0.05$, compared to the control. C, control; S, sham; 2a/7-21, 3 time points after *vPhox2a* injections; 2b/7-21, 3 time points after *vPhox2b* injections.

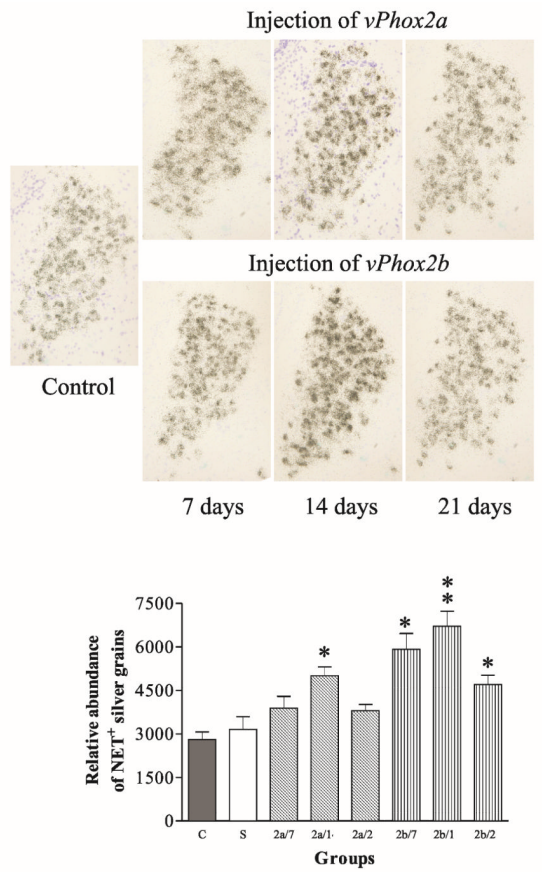

Figure 6.

Effects of over-expression of Phox2 on NET mRNA levels in rat LC region, determined at different times after microinjection with *vPhox2* or *vPhox2b*. Upper panel: NET mRNA detected by *in situ* hybridization in the LC of rats (n=6/group). Lower panel: quantitative analysis of NET mRNA obtained with emulsion-dipped slides, * $P<0.05$, ** $P<0.01$, compared to the control. See Fig. 5 for abbreviations.

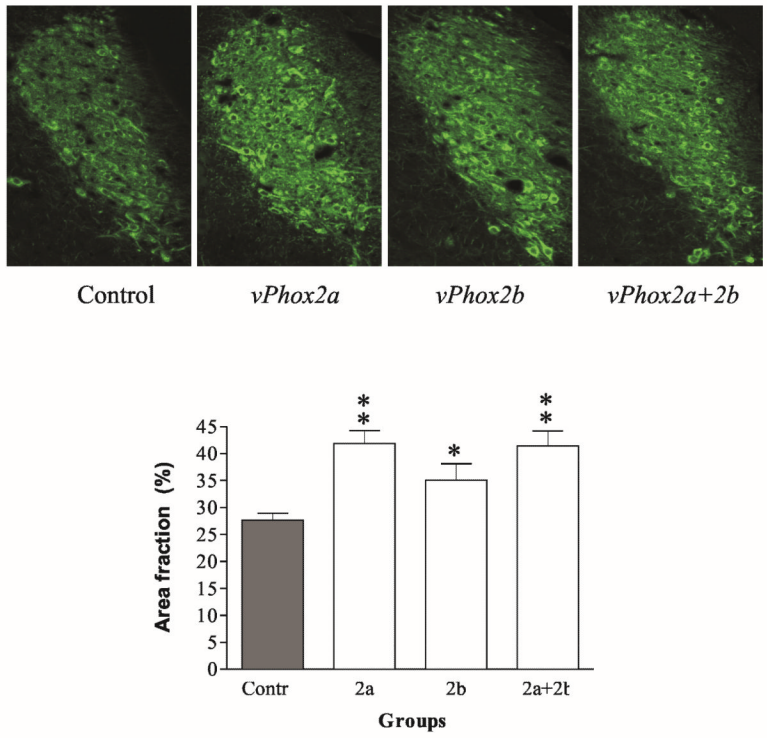

Figure 7.

Expression of DBH-immunoreactivity in the LC after microinjection of *vPhox2* into the rat LC region. The top panels are representative micrographs of DBH immunofluorescence in the LC region of control, *vPhox2a*-injected, *vPhox2b*-injected and *vPhox2a+vPhox2b* microinjected rats. The bottom panels show measurements of the area fraction of DBH immunofluorescence in the LC region (n=6/group). * $P<0.05$, ** $P<0.01$, compared to the control.

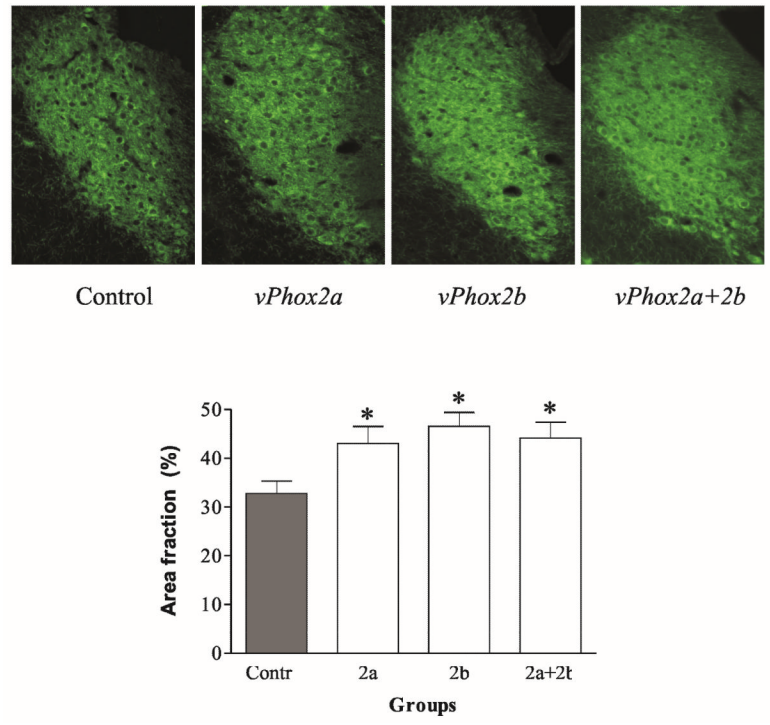

Figure 8.

Expression of NET-immunoreactivity in the LC region after microinjection of *vPhox2* into the rat LC regions. The top panels are representative micrographs of NET immunofluorescence in the LC region of control, *vPhox2a*-injected, *vPhox2b*-injected and *vPhox2a*+*vPhox2b* injected rats. The bottom panels show measurements of the area fraction of NET immunofluorescence in the LC regions (n=6/group). * $P<0.05$, compared to the control.

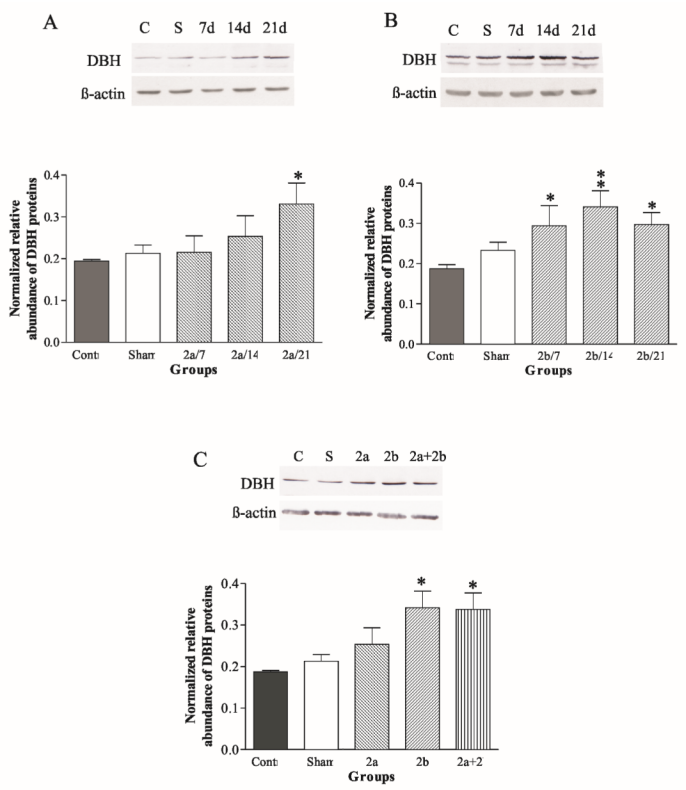

Figure 9.

Effects of over-expression of Phox2 on DBH protein levels in rat hippocampus, determined at different times after microinjection with *vPhox2* into the LC region. The upper figure of each panel show autoradiographs obtained by Western blotting of DBH after microinjection with *vPhox2a* (panel A), *vPhox2b* (panel B), and co-injection (panel C) (all n=6/group). The lower graph in each panel shows quantitative analysis of band densities. Values of DBH bands were normalized to those of β-actin probed on the same blot. **P*<0.05, ** *P*<0.01, compared to the control group. C, control; S, sham; 2a/7-21, 3 time points after *vPhox2a* microinjections; 2b/7-21, 3 time points after *vPhox2b* microinjections. 2a, microinjection with *vPhox2a*; 2b, microinjection with *vPhox2b*; 2a+2b, co-microinjection with *vPhox2a* and *vPhox2b*.

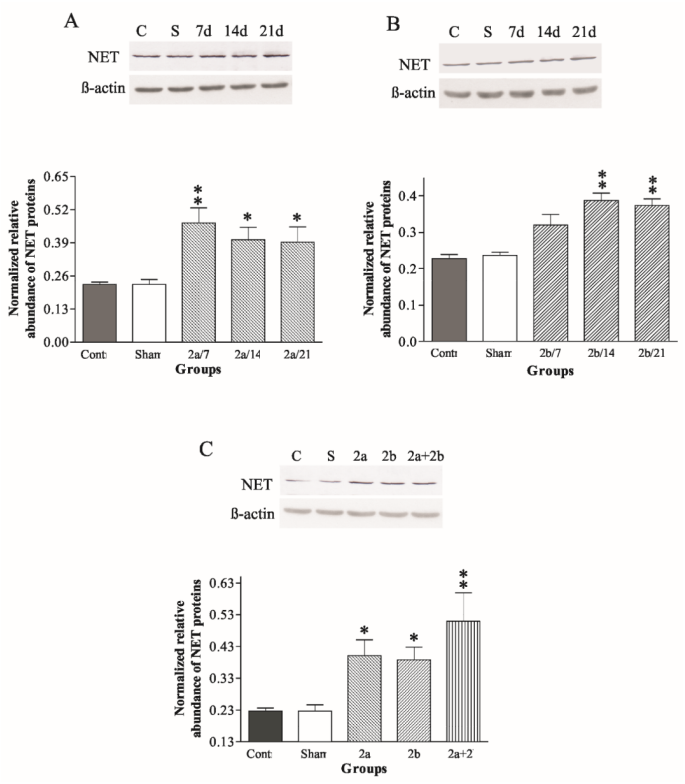

Figure 10.

Effects of over-expression of Phox2 on NET protein levels in rat hippocampus, determined at different times after microinjection with *vPhox2* into the LC region. The upper figure of each panel show autoradiographs obtained by Western blotting of NET after microinjection with *vPhox2a* (panel A), *vPhox2b* (panel B), and co-microinjection (panel C) (all n=6/group). The lower graph in each panel shows quantitative analysis of band densities. Values of NET were normalized to those of β-actin probed on the same blot. **P*<0.05, ** *P*<0.01, compared to the control group. See Fig. 9 for abbreviations.

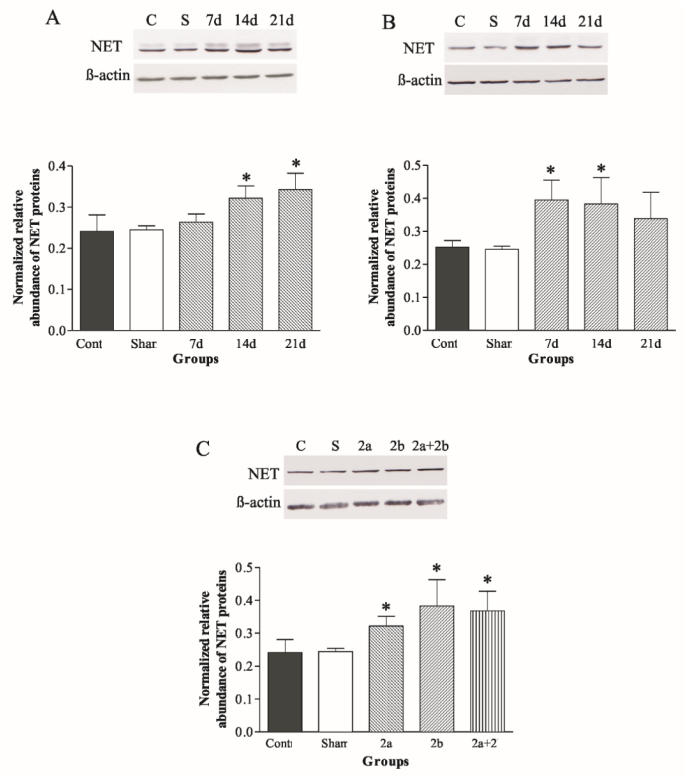

Figure 11.

Effects of over-expression of Phox2 on NET protein levels in rat frontal cortex, determined at different times after microinjection with *vPhox2a* or *vPhox2b* into the LC region. The upper figure of each panel show autoradiographs obtained by Western blotting of NET after microinjection with *vPhox2a* (panel A), *vPhox2b* (panel B), and co-microinjection (panel C) (all n=6/group). The lower graph in each panel shows quantitative analysis of band densities. Values of NET were normalized to those of β-actin probed on the same blot. **P*<0.05, compared to the control group. C, control; S, sham; 2a, microinjection with *vPhox2a*; 2b, microinjection with *vPhox2b*; 2a+2b, co-microinjection with *vPhox2a* and *vPhox2b*.

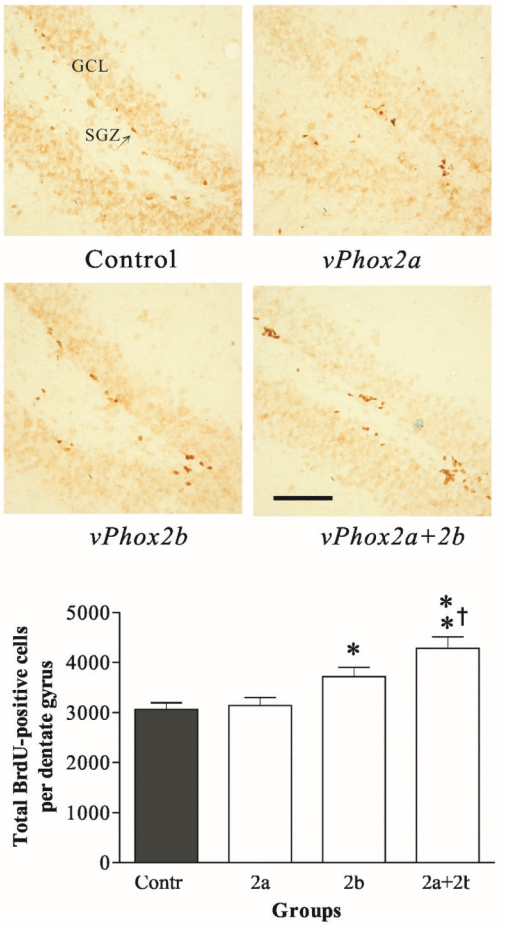

Figure 12.

Microinjection of *vPhox2* increased the number of BrdU-positive cells in the DG of the hippocampus. Shown are representative examples of BrdU-positive cells in the DG (upper panels, Scale bar: 100 μm) and counted BrdU-positive cell numbers (low panel, all n=6/group). **P*<0.05, ** p<0.01, compared to the control group; † p<0.05, compared to the group microinjected with *vPhox2a* or *vPhox2b*. 2a, microinjection with *vPhox2a*; 2b, microinjection with *vPhox2b*; 2a+2b, co-microinjection with *vPhox2a* and *vPhox2b*. SGZ: the subgranular zone (indicated by *arrow*); GCL: the granule cell layer.