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Effects of transcription factors Phox2 on expression of norepinephrine transporter and dopamine β-hydroxylase in SK-N-BE(2)C cells

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Abstract

Phox2a and Phox2b are two homeodomain proteins that control the differentiation of noradrenergic neurons during embryogenesis. In the present study, we examined the possible effect of Phox2a/2b on the *in vitro* expression of the norepinephrine transporter (NET) and dopamine β-hydroxylase (DBH), two important markers of the noradrenergic system. SK-N-BE(2)C cells were transfected with cDNAs or short hairpin RNAs specific to the human *Phox2a* and *Phox2b* genes. Transfection of 0.1 to 5 μg of cDNAs of Phox2a or Phox2b significantly increased mRNA and protein levels of NET and DBH in a concentration-dependent manner. As a consequence of the enhanced expression of NET after transfection, there was a parallel increase in the uptake of [^{3}H]norepinephrine. Co-transfection of Phox2a and Phox2b did not further increase the expression of noradrenergic markers when compared with transfection of either Phox2a or Phox2b alone. Transfection of shRNAs specific to *Phox2a* or *Phox2b* genes significantly reduced mRNA and protein levels of NET and DBH after shutdown of endogenous *Phox2*, which was accompanied by a decreased [^{3}H]norepinephrine uptake. Furthermore, there was an additive effect after cotransfection with both shRNAs specific to *Phox2a* or *Phox2b* genes on NET mRNA levels. Finally, the reduced DBH expression caused by the shRNA specific to *Phox2a* could be reversed by transfection with Phox2b cDNA and vice versa. The present findings verify the determinant role of Phox2a and Phox2b on the expression and function of NET and DBH *in vitro*. Further clarifying the regulatory role of these two transcription factors on key proteins of the noradrenergic system may open a new avenue for therapeutics of aging-caused dysfunction of the noradrenergic system.

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Keywords

dopamine β-hydroxylase; norepinephrine transporter; over-expression; Phox2 genes; short hairpin RNAs; SK-N-BE(2)C cells

The norepinephrine (NE) transporter (NET) is located presynaptically on noradrenergic nerve terminals. Reuptake of synaptically released NE by NETs is the major physiological mechanism by which released NE is inactivated (Barker and Blakely 1995). In the rat brain NET expression is restricted to noradrenergic neurons (Lorang et al. 1994; Comer et al. 1998; Schroeter et al. 2000) and has not been found in the serotonergic, dopaminergic, or adrenergic neurons. This unique distribution indicates that NET is essential for phenotypic specification and is a hallmark protein of noradrenergic neurons. Dopamine β-hydroxylase (DBH, EC 1.14.2.1) is another hallmark protein of noradrenergic neurons (Valarche et al. 1993) because of its specific activity to catalyze the conversion of dopamine to form NE (Kaufman and Friedman 1965) and its highly restricted expression pattern in noradrenergic neurons. In most cases, NET and DBH are co-expressed in noradrenergic neurons of CNS and PNS (Lorang et al. 1994; Schroeter et al. 2000). Therefore, as the hallmarks of noradrenergic circuit, the expression of NET and DBH has been tightly bound to the generation and function of noradrenergic neurons *in vitro* and *in vivo*.

Since the last decade, substantial progress has been made in uncovering critical extracellular signals and transcriptional regulators that control the specification and differentiation of catecholaminergic neurons. Among them, Phox2a and Phox2b, two closely related homeodomain (HD) transcription factors with similar expression patterns, are pivotal for the development of noradrenergic neurons throughout the nervous system during embryogenesis (Morin et al. 1997; Hirsch et al. 1998; Pattyn et al. 2000). For instance, inactivation of the *Phox2a* gene in mice leads to the agenesis of the locus coeruleus (LC) (Morin et al. 1997). The silencing of the *Phox2b* gene disrupts noradrenergic differentiation throughout the CNS and PNS (Pattyn et al. 1999, 2000). Also, *Phox2* genes are involved in the transcriptional control of the noradrenergic neurotransmitter phenotypes as they regulate the gene expression of tyrosine hydroxylase (TH) and DBH. For example, in the Phox2a−/− or Phox2b−/− embryos the expression of DBH is abolished (Morin et al. 1997; Pattyn et al. 1999). Phox2b−/− embryo died at mid-gestation (Pattyn et al. 2000) with signs similar to those that appeared in DBH knock-out animals (Thomas et al. 1995). While these data established *Phox2* genes as a determinant for development of noradrenergic neurons and neurotransmitter phenotypes during embryogenesis, whether these genes are a master regulator of the noradrenergic neurons and neurotransmitter phenotypes beyond their role in embryogenesis has not been wholly explored.

Previously, the *in vitro* study demonstrated that forced expression of *Phox2* genes can induce DBH in cultured neural crest cells from quail (Stanke et al. 1999) and activate DBH promoter activity (Yang et al. 1998). A recent study also demonstrated that Phox2a interacts with HD-binding sites of the NET promoter (Kim et al. 2002). These studies indicate that Phox2a/2b may participate in direct transcriptional activation of *DBH* and *NET* genes *in vitro*. Our study showed that a significant reduction in mRNAs of Phox2 in the LC and

adrenal glands of aging rats was accompanied by a decline in mRNA levels of NET and DBH (Zhu et al. 2005), suggesting a possible relationship between *Phox2* genes and these marker genes in noradrenergic neurons after birth. Age-related reductions in [^{3}H]nisoxetine binding to NET (Tejani-Butt and Ordway 1992) and DBH immunoreactivities (Chan-Palay and Asan 1989) in the human LC have been reported. Therefore, a thorough examination of regulation of the NET and DBH by *Phox2* genes is of paramount importance for better management of aging and age-related degeneration diseases.

As the first step to test our hypothesis that *Phox2* genes may have regulatory role on the noradrenergic circuit after birth, the present study aimed to examine whether *Phox2a* and *Phox2b* genes regulated the expression and function of NET and DBH in SK-N-BE(2)C cells, a human neuroblastoma cell line that expresses essential noradrenergic phenotypes (Ciccarone et al. 1989; Ishiguro et al. 1993). Using transfection of cDNAs and short hairpin RNAs (shRNAs) specific to *Phox2* genes to over-express or shutdown the expression of Phox2a and Phox2b, respectively, we observed that these manipulations forcefully influenced the expression and function of NET and DBH in cells. The present study suggests that Phox2a and Phox2b are sufficient to control the expression and function of NET and DBH in this cell line.

Materials and methods

Cell culture and transient transfection

The human neuroblastoma cell line SK-N-BE(2)C was maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL), at 37°C in humidified air containing 5% CO_2 as described previously (Zhu et al. 2002). The density of viable cells/mL was measured for all experimental groups following cell harvesting. Viability was determined by trypan blue exclusion and was >95% for control and transfection cells.

Transient transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for cDNAs and Arrest-In (OPEN Biosystems, Huntsville, AL, USA) for shRNAs according to the manufacturer's instruction. For the experiment to over-express Phox2 proteins, SK-N-BE(2)C cells were transfected with pCMV6-XL5 vectors (for the control) or pCMV6-XL5 vector carrying cDNAs of human Phox2a or Phox2b. Briefly, cells were grown to about 90% confluence on 6-well plates and transfected with gradient amounts of plasmid DNAs. Cells were harvested 3–4 days after transfection for different measurements. For RNA interference experiment, shutdown of endogenous *Phox2a* and *Phox2b* genes was performed by transfecting SK-N-BE(2)C cells with shRNAs specific to *Phox2a* or *Phox2b* genes. These shRNAs were commercially purchased from The RNAi Consortium Human shRNA Library (OPEN Biosystems). Each shRNA clone was constructed within the lentivirus plasmid vector pLKO.1 (Moffat et al. 2006) and purified using a Qiagen column (Santa Clarita, CA, USA). SK-N-BE(2)C cells were grown to about 50–60% confluence on 6-well plates and transfected with 2 μg of shRNA plasmid DNA for 5 or 7 days after transfection for different measurements. Cells in the control group were transfected with pLKO.1 empty vectors based on the instruction from the manufacturer by the same procedure.

RNA isolation and RT-PCR

The transfected cells were collected from 6-well plates and total RNAs were isolated using Trizol reagent (Invitrogen) following manufacturer's instructions. Quality and quantity of total RNAs were detected using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies LLC, Wilmington, Delaware, CA, USA) at 260/280 nm. Equal amounts of total RNA (1 μg) from each sample were primed with random primers and reverse transcribed to cDNAs using the superscript First-Strand Synthesis Kit (Invitrogen) following the manufacturer's recommendation. Aliquots of first-strand cDNA (5 μL for Phox2a and Phox2b, 1 μL for NET or DBH, and 0.5 μL for β-actin) were amplified by PCR in a 25 μL reaction mix containing platinum PCR supermix (Invitrogen) and primers at appropriate concentrations. PCR amplification of the cDNAs (2.5 μL each) was carried out using specific primers for human *β-actin, Phox*2a, *Phox*2b, *NET*, and *DBH* genes in an Eppendorf Thermal Cycler (Eppendorf, Hamburg, Germany). Primers were, respectively, synthesized at the Invitrogen facility and Integrated DNA Technologies (Coralville, IA, USA). Their sequences used were β-actin: Forward: 5′-TGTGCCCATCTACGAGGGGTATGC-3′; Reverse: 5′-GGTACATGGTGGTGCCGCCAGACA-3′; Phox2a: Forward: 5′-TTTCGCTGAGACCCACTACC-3′; Reverse: 5′-ACTCCTTGGAATCGTCGTCC-3′; Phox2b: Forward: 5′-AGTGGCTTCCAGTATAACCCG-3′; Reverse: 5′-GGTCCGTGAAGAGTTTGTAAGG-3′; NET: Forward: 5′-ACTGTTCCTTATCATCGCGG-3′; Reverse: 5′-CGATCAGGATGACAGCATAGC-3′; DBH: Forward: 5′-TCAACAACGAGGATGTCTGC-3′; Reverse: 5′-CAGTGTGGAGATGACCTTGG-3′. Optimization was performed for all primer sets to determine the optimal cycle number within the logarithmic phase of amplification. Generally, amplification was carried out for a total of 30 cycles consisting of an initial 5 min at 94°C followed by denaturing at 94°C for 30 s, annealing at different T_m according to the structure of primers for 1 min and extension at 72°C for 1 min. Following this, there was a final extension at 72°C for 1 min. PCR conditions for the rest of the transcripts were the same except in the number of cycles and final extensions. Both Phox2a and Phox2b were amplified for a total of 30 cycles with a final extension of 4 min at 72°C. NET and DBH transcripts were amplified for a total of 35 cycles and extended at 72°C for 5 min. For visualization of products, 5–7 μL was run on an ethidium bromide-stained 1% agarose gel. Bands were visualized using Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA, USA), where appropriate semi-quantitative analysis of PCR signals was carried out by densitometry using a Kodak1D Image Analysis software (Eastman Kodak Company, Rochester, NY, USA). The intensities of targets were normalized to those of β-actin.

Detection of protein levels

Protein levels of Phox2, NET, DBH, and TH (EC 1.14.16.2) were determined by western blotting. After washing twice with ice-cold phosphate-buffered saline, SK-N-BE(2)C cells were solubilized in the sample buffer containing sodium lauryl sulfate and β-mercaptoethanol. Protein concentrations of solubilized samples were measured using the Bio-Rad protein assay (Bio-Rad Laboratories). Equal amounts of samples (10 μg of protein per lane) were loaded on 10% sodium lauryl sulfate–polyacrylamide gels and electrophoretically fractionated. Protein bands in gels were transferred to polyvinylidene diflouride membranes by electroblotting. The membranes were incubated with primary

antibodies [two polyclonal antibodies from rabbit, respectively, against Phox2a or Phox2b (both 1 : 200 dilution, Sigma-Aldrich, St Louis, MO, USA), one polyclonal antibody against NET from rabbit (1 : 330 dilution; Alpha Diagnostic International Inc, San Antonio, TX, USA), one monoclonal antibody against DBH from rabbit (1 : 400 dilution; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA)], or monoclonal antibody against TH (Sigma, Milwaukee, WI, USA; 1 : 5000) overnight at 4°C. Membranes were further incubated with secondary antibodies (horseradish peroxidase-conjugated anti-rabbit IgG, 1 : 3000; Amersham Biosciences, Little Chalfont, UK). Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham; Piscataway, NJ, USA). Bands were detected by the Kodak Image station (New Haven, CT, USA), or exposed on film and scanned by Quantity One imaging devices (Bio-Rad Laboratories). Band densities were then quantified by imaging software (Molecular Dynamics IQ Solutions, Molecular Dynamics, Inc., Sunnyvale, CA, USA). A linear standard curve was created from optical densities of bands with a dilution series of total proteins prepared from cells without transfection on each blot. Optical densities values of Phox2, NET, DBH, and TH signals were compared and normalized with β-actin immunoreactivities which were determined on the same blot to assess equal protein loading. Normalized values were then averaged for all replicated gels and used to calculate the relative changes of the same gel.

Uptake of [^{3}H]NE

[^{3}H]NE uptake in SK-N-BE(2)C cells was performed using the procedure described previously (Zhu and Ordway 1997). Briefly, SK-N-BE(2)C cells transfected with Phox2 cDNAs or shRNAs were washed twice with 4 mL Krebs–Ringer–HEPES buffer (KRH; in mM: NaCl 130, KCl 1.3, $CaCl_2$ 2.2, $MgSO_4$ 1.2, KH_2PO_4 1.2, HEPES 10, D-glucose 10, ascorbic acid 0.1, and pargyline 0.1; pH adjusted to 7.4 with HCl). Cells were then pre-incubated in 1.5 mL warm KRH buffer at 37°C for 5 min. Uptake assays were initiated by adding 90 nM [^{3}H]NE (Perkin-Elmer Life Science, Boston, MA, USA) at 37°C for 5 min. Uptake was terminated by aspiration of incubation solutions and followed by two rapid washes with ice-cold KRH buffer. Cells were lysed with 1 mL 0.1% v/v Triton X-100 (in 5 mM Tris–HCl, pH 7.4) and 0.5 mL of the lysed cell preparation was used to quantify radioactivity using a liquid scintillation counter (Beckman LS3801; Beckman Coulter Inc., Irvine, CA, USA). The specific uptake of [^{3}H]NE by the NET was computed as the difference between uptake in the absence of and in the presence of 100 μM desipramine (added during both the pre-incubation and incubation periods of the uptake assay). Protein concentrations of cell lysates were measured by using the Bio-Rad protein assay. Results were expressed as a percentage of control uptake values. Control cells were incubated in parallel and were treated with reserpine (10 μM) 24 h before uptake assay. For comparison, another group of control cells were grown in normal medium and were not exposed to reserpine before uptake determination.

Statistics

All experimental data were presented in the text and graphs as the mean ± SEM. Data were analyzed by one-way ANOVA when multiple treatment groups were compared such as those in the experiment of over-expression of Phox2 cDNAs, TH protein levels after shutdown of Phox2 genes, [^{3}H]NE uptake, and the shutdown-over-expression expression experiments.

Then *post hoc* Newman–Keuls tests were performed for planned comparisons. The unpaired Student's *t*-test was used to analyze data when there were only two groups in a comparison such as those experiments to determine the effects of shRNAs specific to *Phox2* genes on the expression of Phox2, DBH, and NET.

Results

Over-expression of Phox2 increases mRNA and protein levels of NET and DBH in SK-N-BE(2)C cells

To examine whether *Phox2* genes affected the expression of NET and DBH, cDNAs of Phox2a and Phox2b were transfected into SK-N-BE(2)C cells for 3 (for mRNA assay) or 4 days (for protein assay). mRNA levels of Phox2a, Phox2b, NET, and DBH were measured by RT-PCR. Transfection of Phox2a cDNAs produced a significant effect on mRNA levels of NET ($F_{3,15}$, = 4.57, $p < 0.05$) and DBH ($F_{3,15} = 8.52$, $p < 0.01$) in a concentration-dependent manner. The Student–Newman–Keuls test revealed that transfection of 1 and 5 μg Phox2a cDNA significantly increased Phox2a mRNA by 68.29% ($p < 0.05$) and 167.99% ($p < 0.01$), respectively. However, only one concentration of Phox2a cDNA (5 μg) increased mRNA levels of NET and DBH by 38.61% ($p < 0.05$) and 82.26% ($p < 0.01$), respectively (Fig. 1a). Likewise, there was a significant effect of over-expression of Phox2b on mRNA levels of NET ($F_{3,15} = 10.12$, $p < 0.01$) and DBH ($F_{3,15} = 4.31$, $p < 0.05$), respectively. *Post hoc* contrasts showed that transfection of cells with 1 and 5 μg Phox2b cDNAs increased mRNA levels of Phox2b by 113.06% ($p < 0.01$) and 260.81% ($p < 0.01$), respectively. These two concentrations of Phox2b cDNAs increased NET mRNA levels by 31.46% ($p < 0.05$) and 39.95% ($p < 0.01$) as well as increased DBH mRNA levels by 53.15% ($p < 0.05$) and 71.17% ($p < 0.01$), respectively (Fig. 1b).

The effect of Phox2a and Phox2b on protein levels of NET and DBH was measured by western blotting. As shown in Fig. 2, transfection with cDNAs of Phox2a and Phox2b resulted in a significant influence on protein levels of NET ($F_{3,11} = 6.72$, $p < 0.05$ from Phox2a; $F_{3,15} = 4.41$, $p < 0.05$ from Phox2b) and DBH ($F_{3,15} = 4.43$, $p < 0.05$ from Phox2a; $F_{3,15} = 15.13$, $p < 0.01$ from Phox2b). The Student–Newman–Keuls test revealed that transfection with 0.1, 1, and 5 μg Phox2a cDNAs increased protein levels of Phox2a by 7.69% ($p > 0.05$), 43.08% ($p < 0.05$), and 107.69% ($p < 0.01$), respectively. Protein levels of NET and DBH were uniformly increased. In line with the results of mRNAs (Fig. 1), only transfection with 5 μg cDNA of Phox2a yielded significant increases in protein levels of NET by 67.36% ($p < 0.05$) and DBH by 46.95% ($p < 0.05$, Fig. 2a). Also, transfection of 1 and 5 μg cDNAs of Phox2b increased Phox2b protein levels by 45.0% ($p < 0.05$) and 91.67% ($p < 0.01$), respectively, which was accompanied by increased protein levels of NET (by 33.77% and 51.56%, both $p < 0.05$) and DBH (by 28.73% and 39.31%, both $p < 0.05$). Protein levels of Phox2b, NET, or DBH were not significantly changed after transfection with 0.1 μg cDNAs of Phox2 (Fig. 2b).

We further examined whether there was a synergistic effect of Phox2a and Phox2b on the expression of NET and DBH. cDNAs of Phox2a and Phox2b (5 μg for each) were transfected into SK-N-BE(2)C cells alone or together for 3 or 4 days. Similar to the data reported above, cDNAs of either Phox2a or Phox2b significantly increased mRNA

and protein levels of NET or DBH. However, simultaneous transfection with both cDNAs of Phox2a and Phox2b did not further increase mRNA (Fig. 3a) and protein (Fig. 3b) levels of NET or DBH, compared with those in which cDNAs of Phox2a or Phox2b was transfected alone. This indicated that there was no additive effect of Phox2a and Phox2b over-expression on the expression of NET or DBH. However, because endogenously expressed Phox2a and Phox2b were present, we could not rule out an interaction between the two isoforms.

Shutdown of Phox2 genes reduced mRNA and protein levels of NET and DBH in SK-N-BE(2)C cells

Next we aimed to test whether shutdown of *Phox2* genes would interfere with the expression of NET and DBH in cells. To ablate endogenous *Phox2* genes, we used the commercial shRNAs targeted to human *Phox2a* and *Phox2b* genes. To assess the effectiveness of shRNAs on the expression of *Phox2* genes in cells, we quantified the extent to which shRNAs could induce silencing of *Phox2* genes. First, we screened the efficiency of shRNA constructions for Phox2a or Phox2b (five for each) provided by the manufacturer. SK-N-BE(2)C cells were initially transfected with shRNAs using the amount (2 μg) suggested by the manufacturer for 3–7 days. RT-PCR and western blotting were performed. From these preliminary experiments, one shRNA construction for each Phox2a and Phox2b was identified, which showed the best efficiency to silence Phox2 genes (data not shown), and with the transfection time of 5 (for RT-PCR) or 7 days (for western blotting).

As shown in Fig. 4, transfection with the shRNA specific for the *Phox2a* gene significantly reduced mRNA levels of Phox2a by 47.3% ($p < 0.01$), which was accompanied by a marked reduction of mRNA levels of NET by 18.0% ($p < 0.05$) and DBH by 23.8% ($p < 0.05$). Similarly, transfection with the shRNA specific to the *Phox2b* gene significantly reduced the mRNA levels of Phox2b by 55.6% ($p < 0.01$), which was paralleled by marked reduction in mRNA levels of NET (by 45.6%, $p < 0.05$) and DBH (by 35.2%, $p < 0.05$). Western blotting was performed to verify protein levels after shRNA transfection. Transfection with shRNAs specific to *Phox2a and Phox2b* genes significantly diminished Phox2 protein expression in these cells, compared with the controls that were transfected with pLKO.1 empty vector. As shown in Fig. 5a, transfection with the shRNA specific to *Phox2a* gene reduced protein levels of Phox2a, NET, and DBH by 50.0% ($p < 0.01$), 47.0% ($p < 0.01$), and 29.4% ($p < 0.05$), respectively. Similarly, transfection with shRNA specific to the *Phox2b* gene decreased protein levels of Phox2b, NET, and DBH by 49.8% ($p < 0.01$), 36.2% ($p < 0.05$), and 24.0% ($p < 0.05$,), respectively (Fig. 5b). Similar to the over-expression experiments above, we further examined whether there was a synergistic effect for both shRNAs specific to *Phox2a* and *Phox2b* genes. Cells were transfected with the shRNA specific to the *Phox2a* or *Phox2b* alone or together. RT-PCR and western blotting results showed a significant effect on the expression of NET and DBH after transfection with shRNAs specific either to the *Phox2a* or *Phox2b* gene ($F_{3,23} = 12.5$, $p < 0.001$ for NET mRNA and $F_{3,23} = 16.79$, $p < 0.001$ for DBH mRNA; $F_{3,32} = 13.09$, $p < 0.001$ for NET proteins; $F_{3,30} = 6.21$, $p < 0.01$ for DBH proteins, respectively). Student–Newman tests showed that cotransfection with both shRNAs to *Phox2a* and *Phox2b* resulted in a significant lower NET mRNA level than that of transfection with single shRNA specific to *Phox2a* ($p < 0.05$, Fig. 6a). However, these

cotransfections did not cause further reduction in DBH mRNA levels (Fig. 6a) and protein levels of both NET and DBH (Fig. 6b) when compared with transfection with shRNAs specific to either *Phox2a* or *Phox2b* gene alone.

Effects of over-expression or shutdown of Phox2 genes on TH protein levels in SK-N-BE(2)C cells

As expression of Phox2a or Phox2b has previously been found to have no effect on TH promoter activity in SK-N-BE(2)C cells (Yang et al. 1998), we examined whether manipulation of *Phox2* genes affected TH protein levels under our experimental conditions. Using the same procedures as described in the experiments related to Figs. 3 and 6 above, SK-N-BE(2)C cells were transfected with cDNAs (5 μg) of Phox2a and Phox2b, or shRNAs (2 μg) targeted to human *Phox2a* or *Phox2b* genes. The TH protein levels after transfection with cDNAs of Phox2a, Phox2b, or the two in combination were 2.96 ± 0.38, 3.10 ± 0.24, and 2.90 ± 0.43 (arbitrary unit), respectively, which did not reach significance when compared with the control (3.21 ± 0.01; $F_{3,12} = 1.55$, $p > 0.5$). Similarly, after transfection of cells with shRNAs specific to *Phox2a*, *Phox2b*, or the two in combination, the TH protein levels were 1.26 ± 0.06, 1.27 ± 0.05, and 1.15 ± 0.06 (arbitrary unit), respectively, which again did not reach significance when compared with the control (1.23 ± 0.06; $F_{3,15}$, = 0.98, $p > 0.05$).

Effects of over-expression or shutdown of Phox2 genes on uptake of [^{3}H]NE in SK-N-BE(2)C cells

The uptake of [^{3}H]NE was examined in an effort to assess whether *Phox2* gene-induced changes in the expression of NET in SK-N-BE(2)C cells were associated with alterations in the transport of NE. First, SK-N-BE(2)C cells were transfected with cDNAs of Phox2a or Phox2b for 4 days using the same concentrations as those in the over-expression experiments reported above. Uptake assay results showed that transfection with Phox2 cDNAs significantly affected the uptake of [^{3}H]NE ($F_{3,21} = 4.20$, $p < 0.5$ for Phox2a and $F_{3,23} = 4.08$, $p < 0.05$ for Phox2b, respectively, Fig. 7a and b). The Student–Newman–Keuls test showed that transfection with cDNAs of Phox2a or Phox2b markedly increased [^{3}H]NE uptake in concentration-dependent manner, which was similar to those of the RT-PCR and western blotting assays above. Likewise, SK-N-BE(2)C cells were transfected with 5 μg cDNAs of Phox2a and Phox2b, alone or together for 4 days. Consistent to those results of RT-PCR and western blotting, there was no synergistic effect on [^{3}H]NE uptake when Phox2a and Phox2b were co-transfected (Fig. 7c). Second, SK-N-BE(2)C cells were transfected with shRNAs specific to *Phox2a* and *Phox2b* genes alone or together for 7 days. One-way ANOVA analysis for [^{3}H]NE uptake assay showed that transfection with shRNAs specific to *Phox2* genes significantly influenced [^{3}H]NE uptake ($F_{3,22} = 8.16$, $p < 0.01$). *Post hoc* contrasts revealed that transfection with shRNAs specific to either *Phox2a* or *Phox2b* pronouncedly reduced [^{3}H]NE uptake by 35.3% and 44.3%, respectively (both $p < 0.01$). However, there was no further reduction of [^{3}H]NE uptake when co-transfection with shRNAs specific to *Phox2a* or *Phox2b* genes, compared with the group which was transfected with single shRNA specific to *Phox2a* or *Phox2b* gene (Fig. 8).

Interchangeable effects of Phox2a and Phox2b on protein levels of DBH in SK-N-BE(2)C cells

In order to examine whether either Phox2a or Phox2b independently regulated the expression of the noradrenergic phenotype, two similar experiments were carried out. In the first experiment, SK-N-BE(2)C cells were transfected with either shRNA (2 μg) specific to *Phox2a* or this shRNA plus Phox2b cDNA (5 μg) for 5 (for RT-PCR) or 7 days (for western blotting). Another experiment was carried out with Phox2b shRNA and Phox2a cDNA using the same parameters as the first one. The mRNA and protein levels of DBH were measured. As shown in Fig. 9, these manipulations significantly affected DBH expression ($F_{2,8} = 4.35$, $p < 0.05$ for RT-PCR; $F_{2,11} = 9.64$, $p < 0.01$ for western blotting). *Post hoc* contrasts revealed that the reduced mRNA ($p < 0.05$; Fig. 9a) and protein levels ($p < 0.05$; Fig. 9b) of DBH caused by the shRNA specific to *Phox2a* were reversed by cotransfection with Phox2 shRNA and Phox2b cDNA, although these effects of cotransfection did not reach significance when compared with that of transfection with the shRNA alone. The experiment with shRNA specific to *Phox2b* and Phox2a cDNA produced similar results (data not shown).

Discussion

Phox2 genes are considered to be the key determinant for the noradrenergic phenotype in the embryonic development. However, the potential role of these genes on the expression and function of these phenotypes beyond the development is largely uncertain, based on the relatively few amount of research papers in the literature. In the present study, the over-expression or shutdown of these genes in SK-N-BE(2)C cells by transfection with cDNAs or shRNAs specific to human *Phox2* genes resulted in coincident changes in mRNA and protein levels of DBH, which was similar to the observation in embryogenesis (Morin et al. 1997; Pattyn et al. 1997). Also, these manipulations of *Phox2* genes caused a significant alteration in mRNA and protein levels of NET, which was paralleled by changes in [^{3}H]NE uptake. These results demonstrated that *Phox2* genes could control the expression and/or function of DBH and NET in SK-N-BE(2)C cells. Caution must be exercised, however, when we tried to interpret that the molecular interaction between *Phox2* and NET (or DBH) as a common mechanism may apply to the mature noradrenergic neurons. The reason might lie in the fact that SK-N-BE(2)C cells were established *in vitro* from bone marrow biopsy specimens from a 2-year-old male with disseminated neuroblastoma (Biedler et al. 1978). Therefore, this cell line represents an early stage in neuronal development. Nevertheless, some findings about the karyotypic features of these cells may indicate their closeness to the adult properties. For example, while generally embryonic cells lack normal diploid, SK-N-BE(2)C cells have chromosome numbers in the diploid range. Thus, these cells may more closely reflect properties of normal neuronal cell (Biedler and Spengler 1976). Furthermore, the morphological and structural analysis showed that embryonic cells were stellate, and had abundant free ribosomes, moderate Golgi complexes, and scant granular endoplasmic reticulum. On the contrary, many cells from this cell line contains relatively large perikaryon, more prominent Golgi complex, and more extensive granular endoplasmic reticulum (Barnes et al. 1981). Also, the cytoplasm of these cells contains extensive rough-surfaced endoplasmic reticulum as well as other organelles such as mitochondria

and neurofilaments, among which the rough-surfaced endoplasmic reticulum is a particular criterion for indication of the cytological differentiation (Barnes et al. 1981). Moreover, the adult-like characteristics of this cell line are indicated by their abilities to elaborate neurite-like processes and the expression of NET, DBH, and TH, the phenotype of catecholamine neurons (Biedler et al. 1978; Kim et al. 1999). Finally, this cell line was phenotypically stable and had the constancy of certain cellular properties. Karyotypic analysis performed between approximately the week 6 and 35 of serial passage showed consistent marker chromosomes (Biedler et al. 1973, 1978). As the phenotype expression was the main target in our study, this characteristic would be important for the interpretation of our data from this cell line.

In line with the previous report (Yang et al. 1998), the present study demonstrated that over-expression or shutdown of Phox2a or Phox2b did not significantly affect TH protein levels in SK-N-BE(2)C. Previously, forced expression of either *Phox2a* or *Phox2b* has been found to induce TH *in vitro* in cultured mouse or chicken neural crest cells (Lo et al. 1999; Stanke et al. 1999) and *in vivo* in the hindbrain of zebrafish (Guo et al. 1999) and chicken (Vogel-Hopker and Rohrer 2002). Whether the discrepancy between those findings and the present study may lie in the used different cell lines or tissue origins is not known at present. Further work is necessary to address this issue.

Both DBH and NET are hallmark proteins of noradrenergic neurons as they are accountable for biosynthesis and reuptake of NE, respectively, in the nervous system, although DBH is not the rate-limiting enzyme in the synthesis of NE and the consequence of a change in this enzyme on NE levels is less completely studied. In consonance with this functional correlation, they are co-expressed in the majority of NE-containing cell bodies. While the regulatory role of *Phox2* genes on the expression of DBH is well known, whether these genes control the expression and function of NET is still a matter of debate. It was reported that the promoter activity of the NET seemed not to be activated by Phox2a (Kim et al. 1999) and Phox2b (Kim et al. 2002). In contrast to these reports, the present study clearly demonstrated that Phox2a and Phox2b mastered the expression and function of NET in SK-N-BE(2)C cells. This was evident when both over-expression with Phox2 cDNAs and shutdown by shRNAs specific to endogenous *Phox2* genes were performed. The following several points might explain the discrepancy between our results and previous reports (Kim et al. 1999, 2002). First, the reporter gene construction with the relatively short upstream sequence of the NET promoter was transfected in previous studies. Those constructions might not contain enough copies of Phox2-binding sites for transcriptional activation of NET, which influenced the transcription of NET. As indicated in their reports, a minimal DBH promoter, instead of the DBH promoter including multiple Phox2-binding sites which resulted in multiplefold activation, also failed to activate DBH reporter gene expression (Kim et al. 2002). Transactivation of promoter activity by Phox2a/2b may be detectable in transient transfection assays only when the promoter contained multiple binding sites. In the present study, whole cDNAs of Phox2a and Phox2b, which were controlled under strong CMV promoter, were used. As such, over-expressed Phox2 proteins have enough activity to activate the expression of NET. Second, most observations from previous reports resulted from the transfection in non-neural cell lines (NET/DBH/Phox2a-negative cell lines) (Kim et al. 1999, 2002). The transcriptional regulation of NET by *Phox2* genes may need some

other factors which are normally present in the neural environment. SK-N-BE(2)C cells used in the present study are of neural crest origin (Biedler et al. 1978) and exhibit high activity of noradrenergic biosynthetic proteins including NET (Biedler et al. 1978; Ross et al. 1981). This typical noradrenergic environment may be helpful for exerting the regulatory role of *Phox2* genes on NET. Nevertheless, it might be pivotal that shRNAs used in the present study to shutdown endogenous *Phox2* genes significantly reduced NET expression and function, which provided another circumstantial evidence for the direct regulatory role of *Phox2* genes on NET.

So far the molecular mechanisms underlying the regulation of the expression of DBH and NET by *Phox2* genes remain to be elucidated. One possibility is the direct transactivation of the target genes by binding *Phox2* to the *cis*-regulatory elements and HD-binding sites on the promoter in these genes. The promoter of DBH has multiple *cis*-elements and HD-binding sites (Kim et al. 1998; Yang et al. 1998). Similarly, these structures have also been identified in the promoter region of NET (Kim et al. 1999, 2002). Phox2a or Phox2b can transactivate the *DBH* gene by interacting at the *cis* binding sites for *Phox2a/2b* genes in DBH promoters (Tissier-Seta et al. 1993; Zellmer et al. 1995; Swanson et al. 1997; Yang et al. 1998; Lo et al. 1999; Adachi et al. 2000; Seo et al. 2002). The similar direct activation may also contribute to *Phox2*-induced NET transcription, as Phox2a was found to interact with the middle subdomain of the proximal promoter regions of the *NET* gene (Kim et al. 2002). This suggests, but does not prove, that such molecular interaction is a common mechanism for *Phox2*-induced regulation. The similar example is that Phox2a transactivates expression of $p27^{Kip1}$, a protein for inactivating cyclin-dependent kinase to coordinate cell cycles, by interacting with the HD *cis*-acting elements in $p27^{Kip1}$ promoter (Paris et al. 2006). Phox2a was also reported to activate the panneural *NCAM* gene by binding to the promoter of this gene (Valarche et al. 1993). However, more clarifying investigations are needed to verify this notion.

Although *Phox2* genes are essential for the development of noradrenergic neurons in the LC, several additional factors such as Mash1 (Hirsch et al. 1998), Gata2/3 (Lim et al. 2000), dHand (Xu et al. 2003), bone morphogenetic protein (Lo et al. 1999), AP2β (Hong et al. 2008b), and Trim11 (Hong et al. 2008a) are involved in the expression of the noradrenergic phenotype in the central and peripheral noradrenergic systems. Some of them synergize with or are required for actions of *Phox2* genes (Lim et al. 2000; Xu et al. 2003). Also, in the forced expression experiments, several second messenger pathways involving cAMP, protein kinase A, protein kinase C, calcium/cAMP-response element or AP1 collaborate with *Phox2* genes to fulfill their transcriptional action (Swanson et al. 1997, 2000; Lo et al. 1999; Yokoyama et al. 1999; Benfante et al. 2007). Therefore, *Phox2* genes activate expression of DBH and NET not only by Phox2 binding to the promoter regions of these markers, but also by direct protein–protein interaction between Phox2 and other transcription factors, as well as involving extracellular signal transduction pathways. The present study took a straight forward approach to examine the regulatory effects of *Phox2* genes on the expression of DBH and NET in this cell line. However, the potential effect of these additional transcription factors as well as the related signal transduction pathway has not been examined. Further work is necessary to address these issues.

The present study demonstrated that co-transfection with cDNAs of Phox2a and Phox2b did not result in an obvious synergistic effect on the expression of DBH and NET. However, there was a synergistic effect of co-transfection with two shRNAs specific to *Phox2a* and *Phox2b* on NET mRNAs (Fig. 6a). It indicates that Phox2a and Phox2b may somewhat cooperatively regulate NET expression. The present study also showed that Phox2a and Phox2b, respectively, regulated the expression and function of DBH and NET. It was reported that Phox2a and Phox2b functioned similarly and independently in activating DBH transcription *in vivo*, suggesting they had interchangeable roles (Adachi et al. 2000). To assess whether this phenomenon also occurred in this cell line, we performed the shutdown-over-expression experiment with these two transcription factors. Our data demonstrated that the reduced expression of DBH caused by shRNA of *Phox2a* could be reversed by Phox2b cDNA (Fig. 9) and vice versa. In this regard, Phox2a and Phox2b seem to be interchangeable for the expression of DBH in the present study. However, given the incomplete elimination of the expression of either Phox2a or Phox2b by shRNA manipulations (Figs. 5, 6, and 9), it is possible that some level of Phox2a or Phox2b is still reciprocally required for their effects. Therefore, more studies are needed to verify this notion in this cell line.

The transactivation of *Phox2a/2b* genes on the expression of DBH or NET has been studied. However, these previous experiments focused primarily on promoters of DBH or NET *in vitro*. Our results demonstrated that a single transcription factor, Phox2a or Phox2b, might control expression of a phenotype of noradrenergic neurons in cultured cells. Our previous experiments also demonstrated that reduced Phox2 mRNAs in the LC and adrenal medulla of aging rats were accompanied by a decline in mRNA levels of DBH and NET (Zhu et al. 2005). Taken together, these observations prompt us to hypothesize that *Phox2* genes may also play a key role in maintaining the expression of the phenotype of the noradrenergic neurons in matured animals, including aged ones. So far loss-of-function or gain-of-function for such transactivative regulation has not been performed. As the hallmarks of noradrenergic circuit, the regulation of transcription factors on the expression of DBH and NET is tightly bound to the generation and dissociation of LC neurons. It is conceivable that structural or functional changes in the LC contribute to dysfunction associated with aging. Interestingly, many studies report that there is a reduction of about 25% to 50% of LC neurons between the fourth and ninth decades of life (Mann et al. 1983; Lohr and Jeste 1988; Chan-Palay and Asan 1989). In fact, as a direct consequence of LC neuron loss in advancing age, the expression of DBH and NET was proportionately reduced (Chan-Palay and Asan 1989; Shores et al. 1999). Therefore, the implications of the strategy to enforcement of *Phox2* genes into aged animals, if confirmed, were significant. Clearly, further studies are necessary to elucidate the possibility for this regulation and mechanisms underlying regulatory effects of *Phox2* genes on the expression of DBH and NET. On the other hand, only one NET protein has been identified in tissues, suggesting that any change in its expression may significantly alter NE neurotransmission. Given altered NET expression may contribute to the pathophysiology of major depression (Klimek et al., 1997), it may be possible to use transcription factors such as Phox2 to control the phenotype of the noradrenergic system including NET for therapeutic purposes.

In summary, in the present study forced expression with cDNAs of Phox2 or shutdown of endogenous *Phox2* genes with shRNAs caused a parallel increase or reduction in

the expression of DBH and NET in SK-N-BE(2) cells. These results, demonstrating the transcriptional regulatory effects of *Phox2* genes on DBH and NET in SK-N-BE(2)C cells, agree with previous observations in the experiments with embryo and reporter gene constructions. The present study will serve as the initial exploration for further examining the gene regulation of DBH and NET by some transcription factors.

Acknowledgements

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Abbreviations used:

DBH	dopamine β-hydroxylase
HD	homeodomain
KRH	Krebs–Ringer–HEPES
LC	locus coeruleus
NE	norepinephrine
NET	NE transporter
shRNA	short hairpin RNAs
TH	tyrosine hydroxylase

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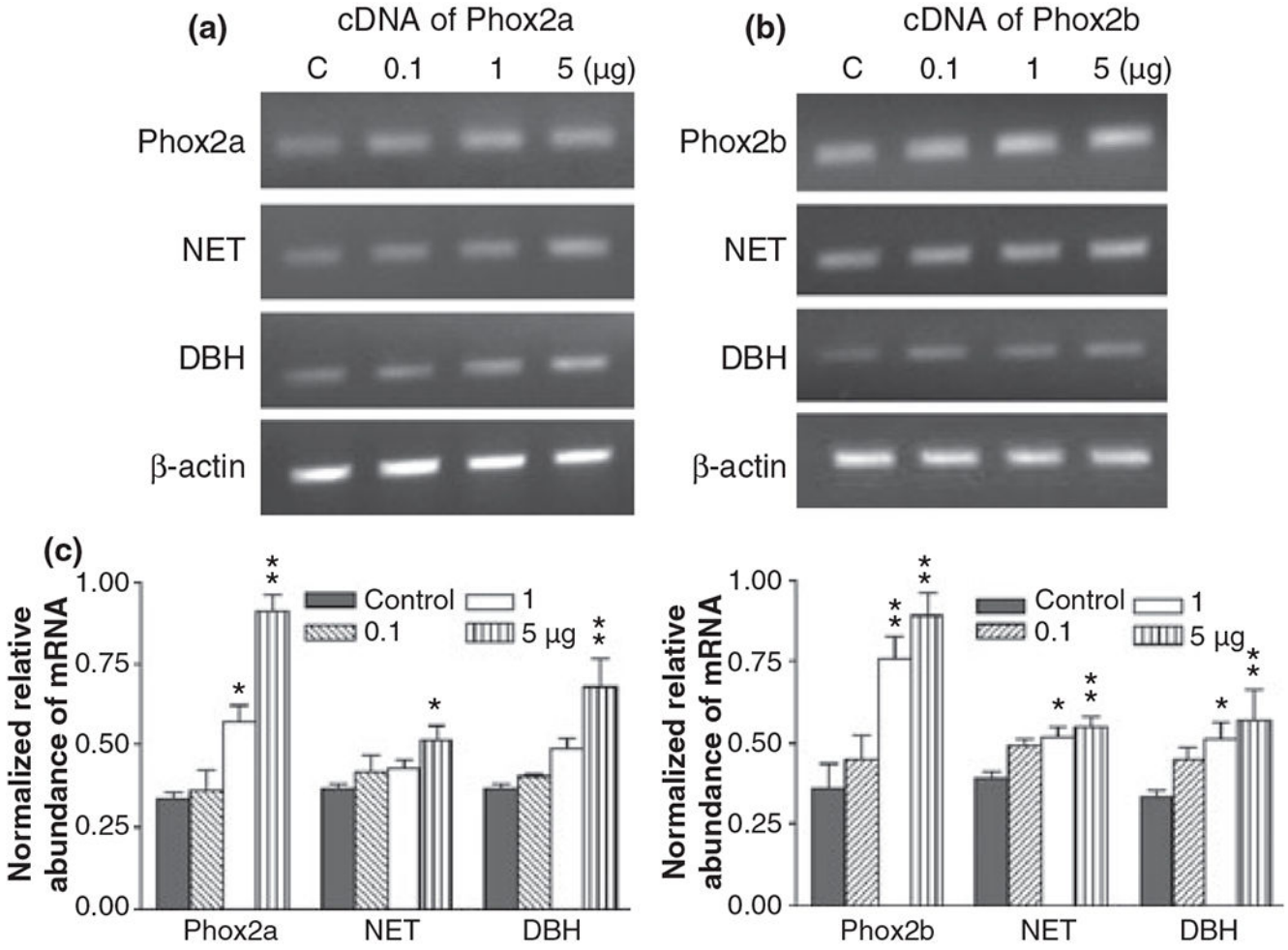

Fig. 1.

Effects of over-expression of Phox2 on mRNA levels of NET and DBH. Upper panel: autoradiogram obtained by RT-PCR products of Phox2, NET, DBH, and β-actin after transfection of cDNAs of Phox2a (a) and Phox2b (b) in SK-N-BE(2)C cells (both $n = 4$). Lower panel (c): graphical representation of semi-quantitative analysis by RT-PCR. Values of targets were normalized to those of β-actin. *$p < 0.05$ and **$p < 0.01$ compared with the control.

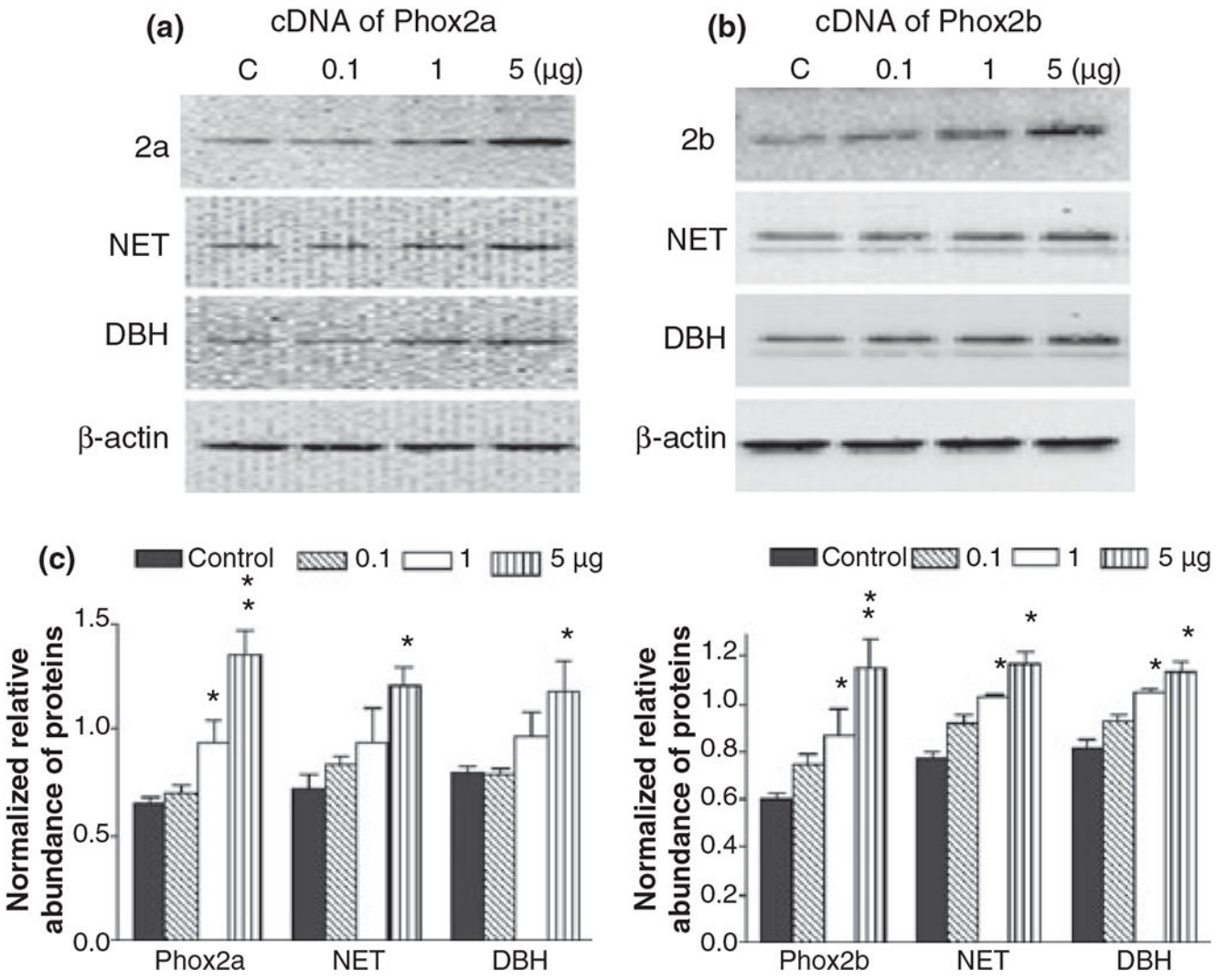

Fig. 2.

Effects of over-expression of Phox2 on protein levels of NET and DBH. Upper panel: autoradiogram obtained by western blotting of Phox2, NET, DBH, and β-actin after transfection of cDNAs of Phox2a (a) and Phox2b (b) in SK-N-BE(2)C cells (both $n = 4$). Lower panel (c): graphical representation of semi-quantitative analysis by densitometry of bands after western blotting. Values of targets were normalized to those of β-actin. *$p < 0.05$ and **$p < 0.01$ compared with the control.

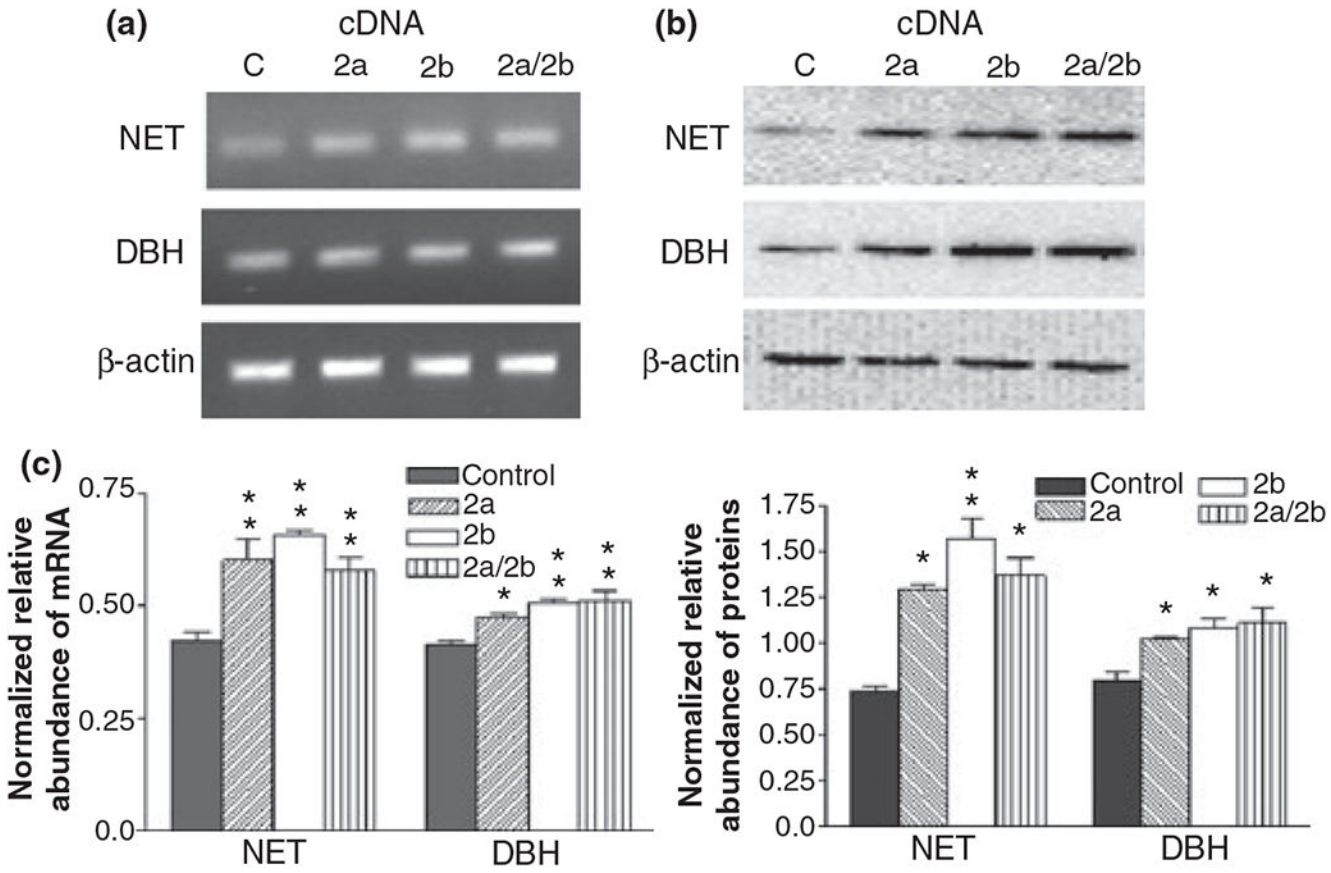

Fig. 3.

Effects of cotransfection of cDNAs of Phox2a and Phox2b on expression of NET and DBH. Upper panel: autoradiogram obtained by RT-PCR (a) and western blotting (b) of Phox2, NET, DBH, and β-actin after transfection of cDNAs of Phox2a and Phox2b in SK-N-BE(2)C cells (both $n = 4$). Lower panel (c): graphical representation of semi-quantitative analysis by RT-PCR or densitometry of bands after western blotting. Values of targets were normalized to those of β-actin. *$p < 0.05$ and **$p < 0.01$ compared with the control.

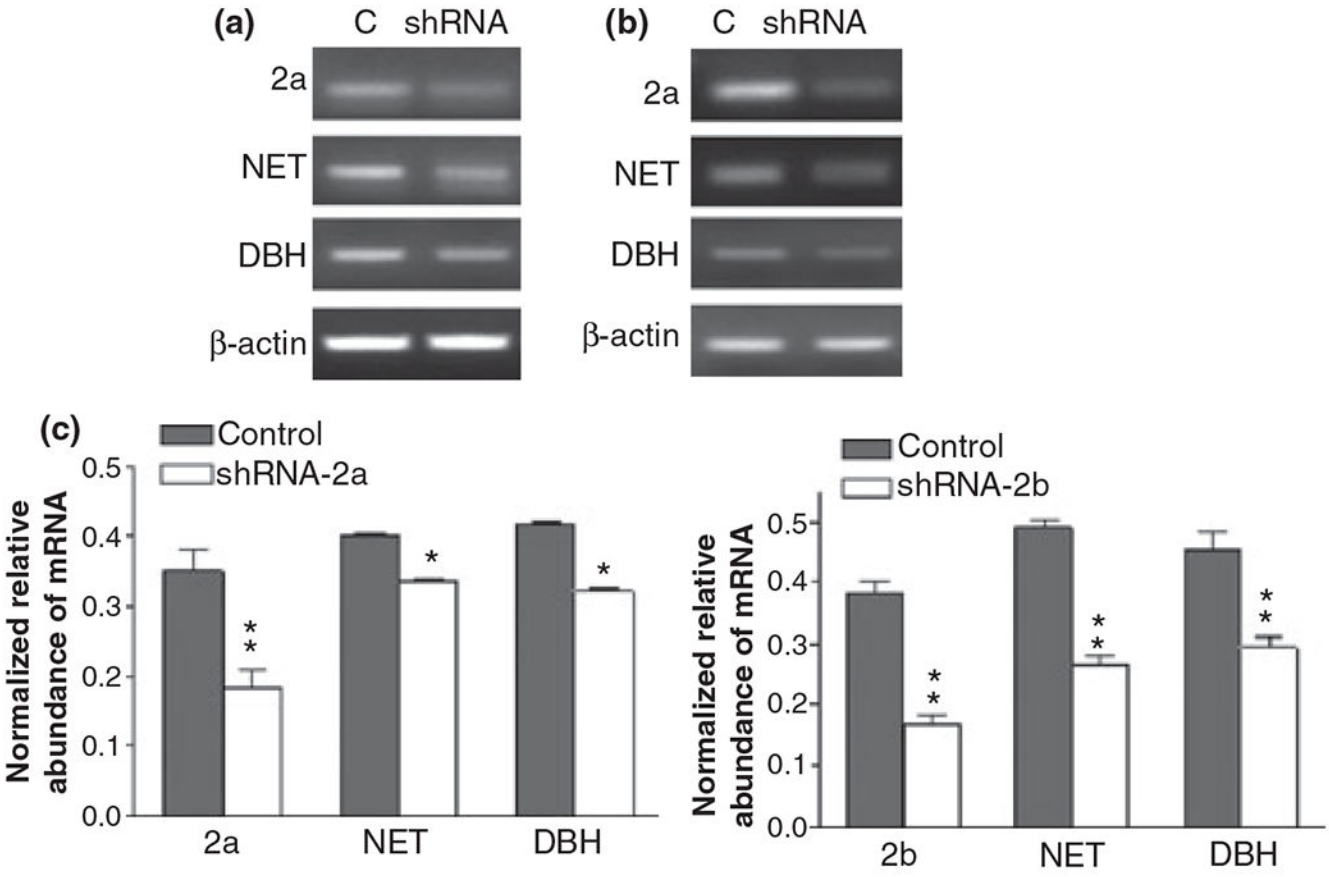

Fig. 4.

Effects of shutdown of endogenous *Phox2* genes on mRNA levels of NET and DBH. Upper panel: autoradiogram obtained by RT-PCR products of Phox2, NET, DBH, and β-actin after transfection of shRNAs specific to *Phox2a* (a) and *Phox2b* (b) in SK-N-BE(2)C cells (both $n = 5$). Lower panel (c): graphical representation of semi-quantitative analysis by RT-PCR. Values of targets were normalized to those of β-actin. *$p < 0.05$ and **$p < 0.01$ compared with the control.

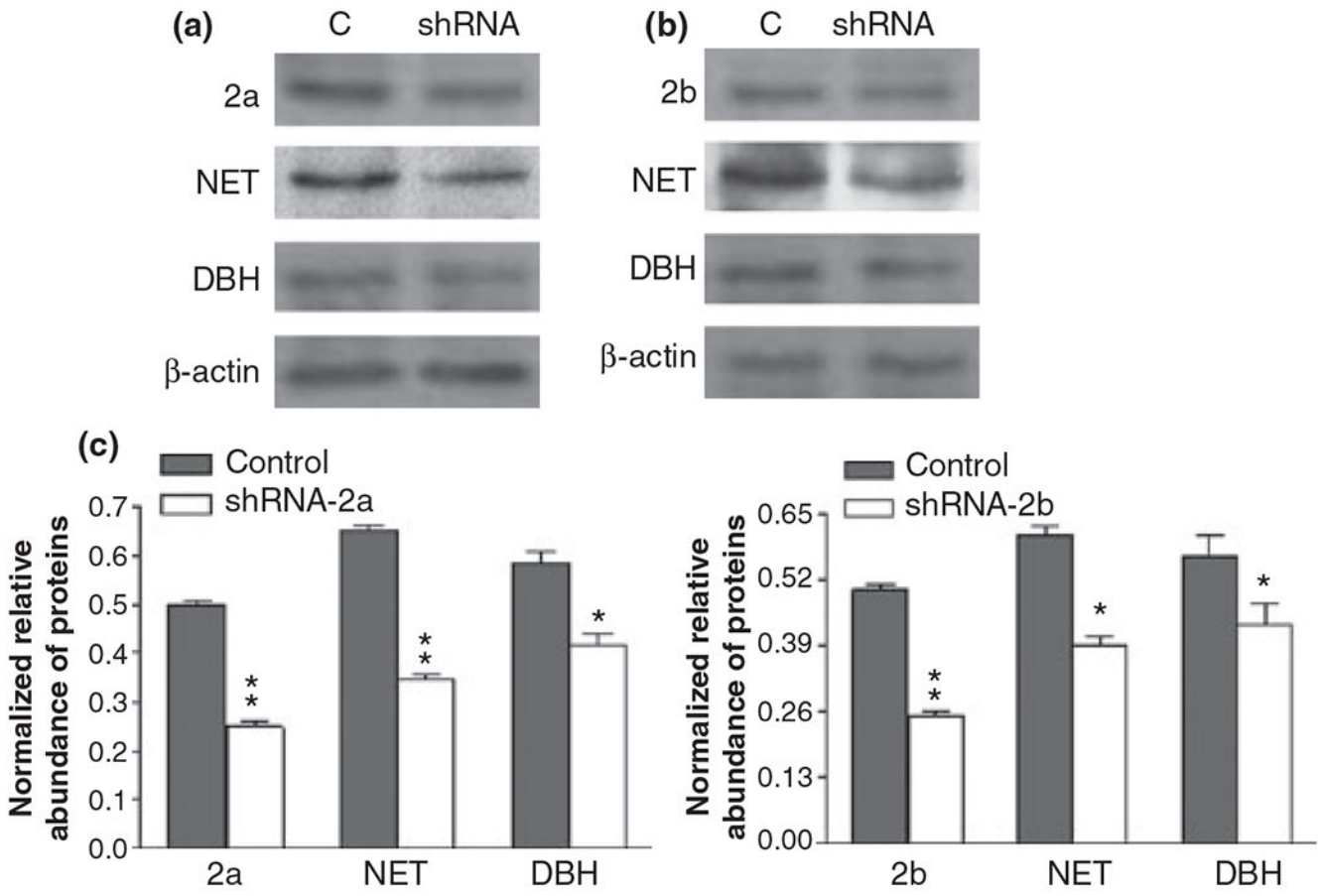

Fig. 5.

Effects of shutdown of endogenous *Phox2* genes on protein levels of NET and DBH. Upper panel: autoradiogram obtained by western blotting of Phox2, NET, DBH, and β-actin after transfection of shRNAs specific to *Phox2a* (a) and *Phox2b* (b) in SK-N-BE(2)C cells (both $n = 5$). Lower panel (c): graphical representation of semi-quantitative analysis by densitometry of bands after western blotting. Values of targets were normalized to those of β-actin. *$p < 0.05$ and **$p < 0.01$ compared with the control.

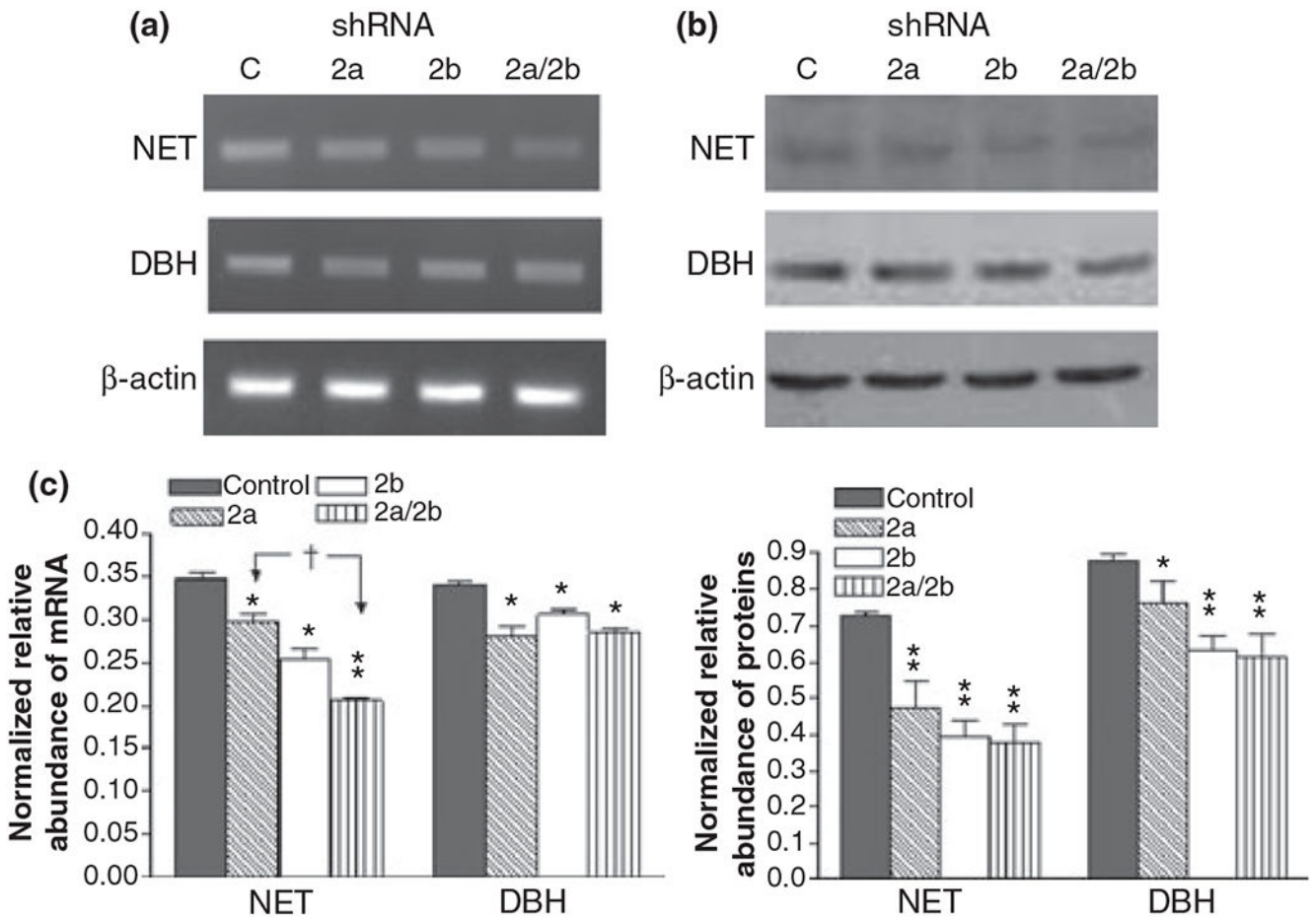

Fig. 6.

Effects of cotransfection of shRNAs specific to *Phox2a* and *Phox2b* on expression of NET and DBH. Upper panel: autoradiogram obtained by RT-PCR (a) and western blotting (b) of NET, DBH, and β-actin after transfection of shRNAs specific to *Phox2a* and *Phox2b* in SK-N-BE(2)C cells alone or together (both $n = 5$ or 6 for each group). Lower panel (c): graphical representation of semi-quantitative analysis by RT-PCR or densitometry of bands after western blotting. Values of targets were normalized to those of β-actin. *$p < 0.05$ and **$p < 0.01$ compared with the control. †$p < 0.05$, compared with the group transfected with shRNA specific to *Phox2a*.

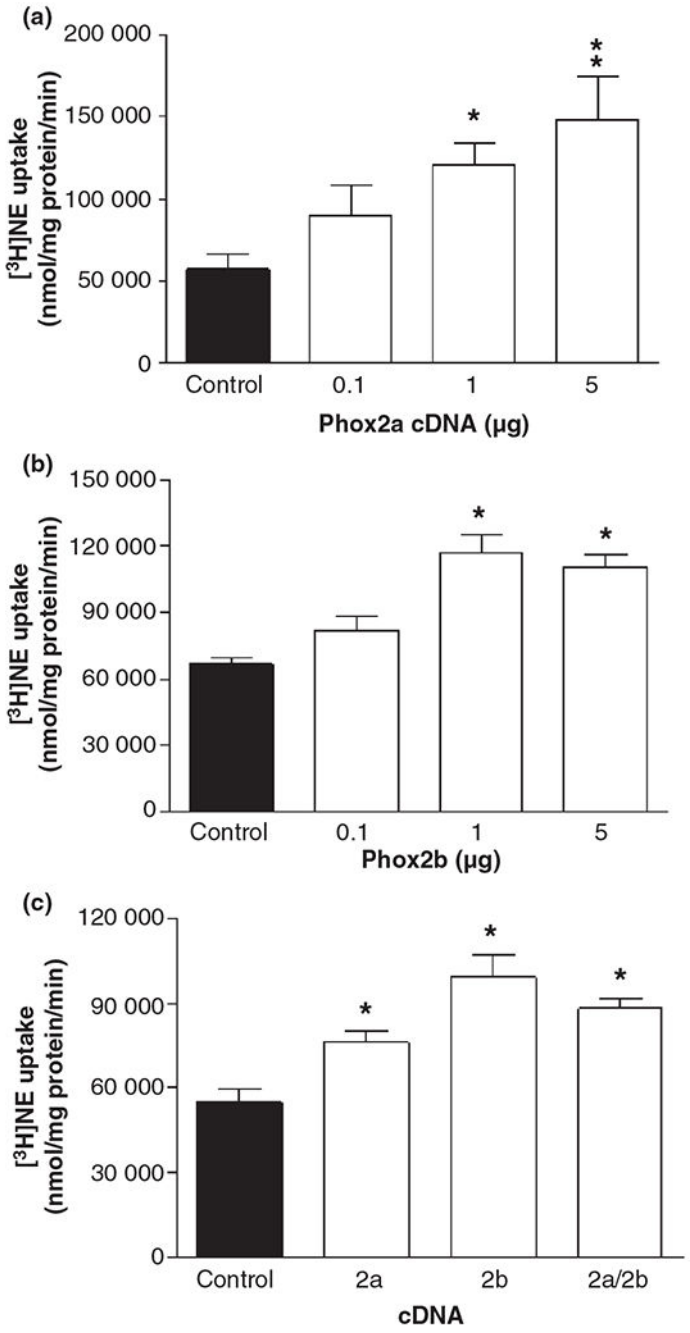

Fig. 7.

Effect of transfection of cDNAs of Phox2a (a), Phox2b (b), and cotransfection of Phox2a/2b (c) on the ability of SK-N-BE(2)C cells to transport [^{3}H]NE. Each bar represents data obtained from 5 (for Figure 7a and 7b) and 4 (for Figure 7c) separate experiments. *$p <$ 0.05, **$p < 0.01$ compared with the control.

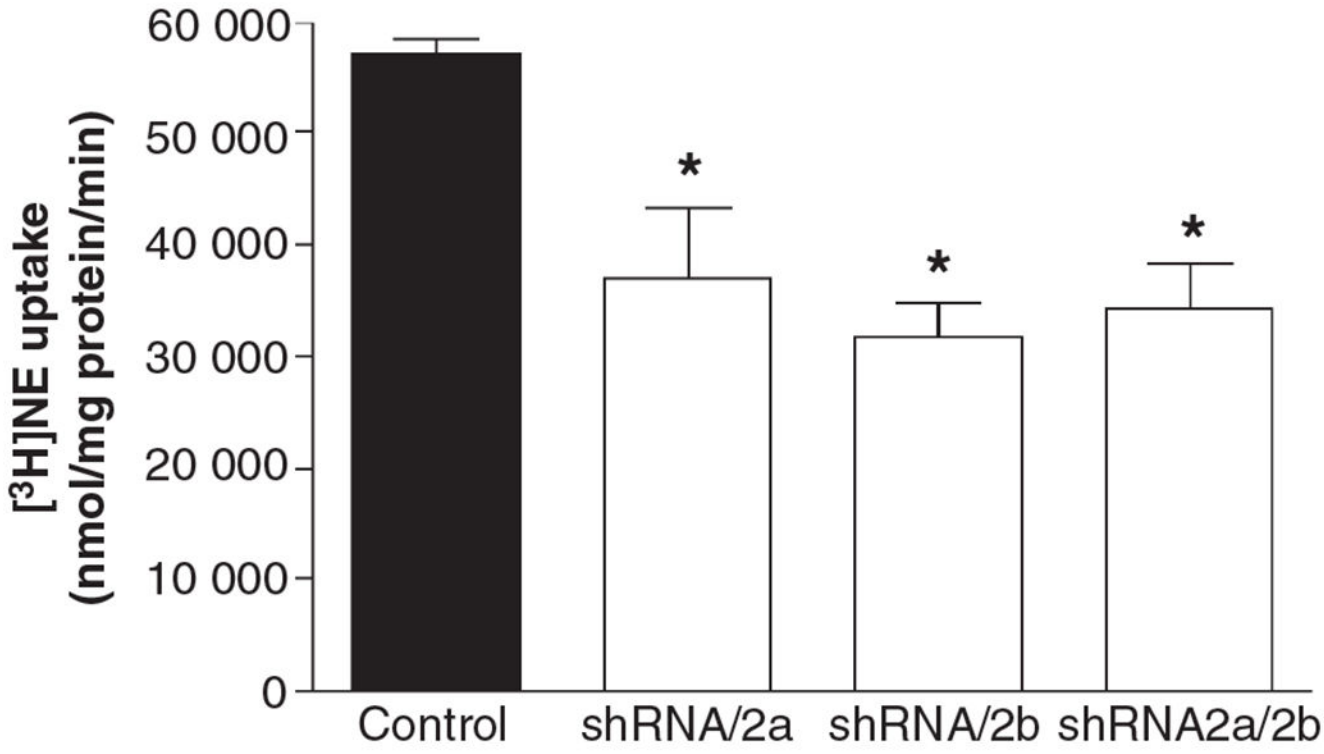

Fig. 8.

Effect of transfection of shRNAs specific to *Phox2a* and *Phox2b*, alone or together, on the ability of SK-N-BE(2)C cells to transport [^{3}H]NE. Each bar represents data obtained from five separate experiments. *$p < 0.01$ compared with the control.

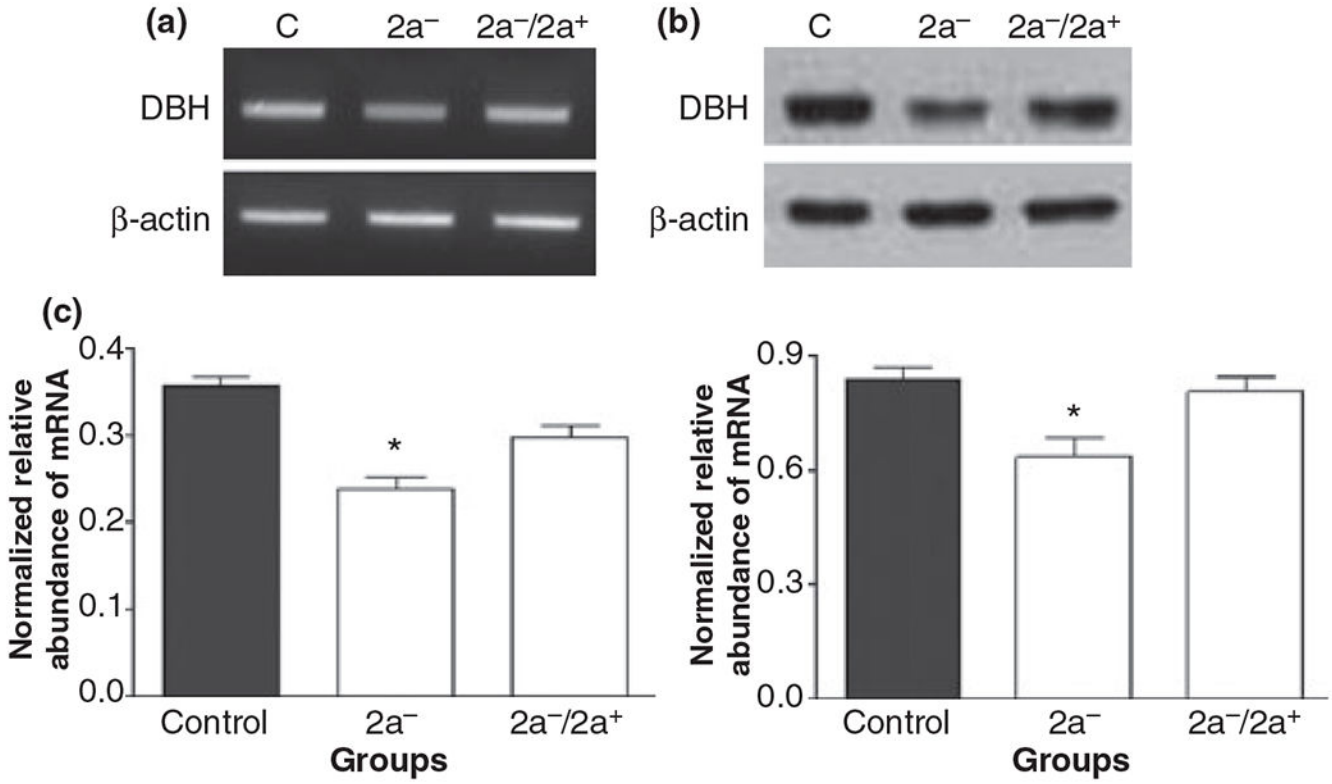

Fig. 9.

Effects of transfection of either shRNA specific to *Phox2a* (2a−) or Phox2a shRNA plus Phox2b cDNA (2a−/2b+) on DBH expression. Upper panel: autoradiogram obtained by RT-PCR (a, $n = 3$) and western blotting (b, $n = 4$) of DBH and β-actin after transfection in SK-N-BE(2)C cells. Lower panel (c): graphical representation of semi-quantitative analysis by RT-PCR or densitometry of bands after western blotting. *$p < 0.05$ compared with the control.