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Age-associated changes in mRNA levels of Phox2, norepinephrine transporter and dopamine β-hydroxylase in the locus coeruleus and adrenal glands of rats

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Abstract

Age-related changes in the gene expression of the transcription factors, Phox2a and 2b, and two marker proteins, nor-epinephrine transporter (NET) and dopamine β-hydroxylase (DBH), of noradrenergic neurons were characterized in the locus coeruleus (LC) and adrenal glands using *in situ* hybridization. Analysis of changes was performed in rats that were 1–23 months of age. Compared to 1-month-old rats, there was a 62% increase of Phox2a messenger RNA (mRNA) in the LC of 3-month-old rats, and a decline of 37% in 23-month-old rats. In contrast, levels of Phox2b mRNA in the LC remained unchanged in 3-month-old rats, but declined to a 30% reduction in 23-month-old rats. Interestingly, mRNA levels of NET in the LC decreased with increasing age to a reduction of 29%, 30% and 43% in 3-, 8- and 23-month-old rats, respectively. Similarly, DBH mRNA in the LC declined with increasing age to a 56% reduction in 23-month-old rats. mRNA levels of Phox2a, Phox2b, NET and DBH in the adrenal medulla of 23-month-old rats were significantly lower than those of 1-month-old rats. Semi-quantitative reverse transcription assays of the same genes yielded data similar to *in situ* hybridization experiments, with β-actin mRNA levels being unchanged across the ages. Taken together, these data reveal that reduced Phox2 mRNAs in the LC and adrenal medulla of aging rats are accompanied by a coincidental decline in mRNA levels of NET and DBH and suggest a possible relationship between Phox2 genes and the marker genes in noradrenergic neurons after birth.

Keywords

dopamine β-hydroxylase; gene regulation; locus coeruleus; norepinephrine transporter; Phox2; rat brain

Phox2a and Phox2b are two transcriptional factors that are closely related members of the Phox/aristaless family of paired-class homeobox genes. These paralogous homeodomain proteins are mostly co-expressed with dopamine β-hydroxylase (DBH, EC 1.14.2.1; Valarche *et al*. 1993), the enzyme that catalyzes the final β-hydroxylation step in the conversion of 3,4-dihydrophenylethylamine to form norepinephrine (NE; Kaufman and Friedman 1965), in all central and peripheral noradrenergic neurons of the embryo (Morin *et al*. 1997; Pattyn *et al*. 1999). It is believed that Phox2 genes play an important role in the transcriptional control of neurogenesis of noradrenergic neuronal systems. Many

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experiments have demonstrated that both *Phox2a* and *Phox2b* are determinants of the noradrenergic phenotype. For example, the loss of function of *Phox2a* leads to agenesis of the main noradrenergic nucleus of the brain, the locus coeruleus (LC) (Morin *et al*. 1997). Inactivation of *Phox2b* disrupts noradrenergic differentiation throughout the nervous system (Pattyn *et al*. 1999, 2000). Besides their role in embryogenesis, some studies have shown that endogenous Phox2 genes regulate the expression of *DBH* by transactivating the DBH promoter in cultured cells (Swanson *et al*. 1997; Kim *et al*. 1998; Yang *et al*. 1998). Phox2b$^{-/-}$ embryos die at mid-gestation with signs of cardiovascular congestion (Pattyn *et al*. 2000), similar to symptoms appearing in DBH and TH knock-out animals (Thomas *et al*. 1995). These Phox2b$^{-/-}$ embryos can be rescued up to birth by the administration of noradrenergic agonists to the mothers (Pattyn *et al*. 2000). Furthermore, several studies have demonstrated that forced expression of Phox2a or Phox2b can induce TH and DBH in cultured neuronal crest cells from mice and chicken *in vitro*, or zebrafish and chicken *in vivo* (Guo *et al*. 1999; Lo *et al*. 1999; Stanke *et al*. 1999; Vogel-Hopker and Rohrer 2002).

The expression of Phox2a has been identified in DBH-positive neurons such as A1, A2, A5 and the LC in postnatal mice (P12) (Tiveron *et al*. 1996). However, the expression of Phox2b in the LC appears to be transient and disappears around embryonic day 11.5 (E11.5), rather than continuing up to birth as observed for Phox2a (Pattyn *et al*. 2000). To date, the possible roles of Phox2a after birth in the physiology or the plasticity of the noradrenergic neuronal circuits in which they are expressed remain largely unknown. Given the requirement of both Phox2a/2b genes in the formation of the LC and other noradrenergic neurons during ontogeny, it would be interesting to study their developmental expression after birth.

As a main nucleus responsible for 70% of NE in the brain, the LC plays an important role in the global response to external stimuli and changes in the state of autonomic function (Moore and Bloom 1979). The NE transporter (NET) is an important hallmark of noradrenergic neurons. A decrease of NET mRNA levels in the LC has been reported in middle-aged rats with no significant change in the LC of aged rats (Shores *et al*. 1999). Although there is evidence that the numbers of NET in synaptosomes and [^{3}H]nisoxetine binding to NET decline with age (Tejani-Butt and Ordway 1992; Snyder *et al*. 1998), the age-associated changes in NET expression has not been clearly defined. Furthermore, the dynamic expression of DBH, another hallmark protein of the noradrenergic circuit, over the entire life span has not been extensively studied.

In the present study, we used a combination of *in situ* hybridization and semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) to characterize the expression profiles of mRNA levels of Phox2a/2b, together with those of NET and DBH, in the LC and adrenal glands of rats aged from 1 to 23 months. Our results indicate that the age-associated reduction of *Phox2a/2b* expression in the LC and adrenal glands coincides with a decrease in the expression of *NET* and *DBH*, leading to a presumption that decreased *Phox2a/2b* expression may, at least in part, underlie the expressional changes of *NET* and *DBH* during aging.

Materials and methods

Animal preparation

Male Sprague-Dawley rats ages 1, 3, 8 and 23 months were ordered from the National Institute of Aging (Bethesda, MD, USA). Rats were housed under standard 12 h light/12 h dark conditions with food and water available *ad libitum*. One week after arrival, rats were killed by decapitation. Brains and adrenal glands were removed and rapidly frozen in 2-methylbutane on dry-ice. For *in situ* hybridization, 16-μm coronal sections were cut around

the pons-brainstem LC region. From the same rats, 16-μm sections were cut through adrenal glands embedded with Optimal Cutting Temperature (OCT) solution in a cryostat. Tissue sections were mounted on SuperFrost-Plus slides (Fisher Scientific, Pittsburg, PA, USA), and stored at −80°C until assayed. For RNA isolation, the LC and surrounding tissues were punched (2-mm diameter) bilaterally from frozen coronal sections, which were cut through several 40-μm blocks of pontine-brainstem tissue containing the LC.

^{35}S-Labeled complementary RNA probe preparation

Radioactive antisense and sense cRNA probes were generated by *in vitro* transcription using [^{35}S]UTP (1000 Ci/mmol; Perkin Elmer, Inc., Boston, MA) and a 10 mM stock solution of CTP, GTP and ATP (Ambion, Austin, TX, USA) from cDNAs for mouse Phox2a (1.4 kb), mouse Phox2b (1.6 kb), rat NET (0.5 kb) and rat DBH (1.4 kb) in pGEM-3Zf vectors with SP6 or T7 RNA polymerases (Zhu *et al*. 2002). Labeled probes were extracted with phenol-chloroform and separated from non-incorporated nucleotides using a NucTrap probe purification column (Stratagene, Cedar Creek, TX, USA). A limited alkaline hydrolysis was further performed for cRNA probes of Phox2a/2b and DBH to get the optimal sizes (about 0.5 kb) immediately after probe synthesis and purification. For hybridization, ^{35}S-labeled cRNA probes were diluted with hybridization buffer to a final concentration of 10^5 dpm/μL when assays were performed.

In situ hybridization

Slides containing LC regions or adrenal glands 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%). Tissue sections were incubated with a hybridization solution containing ^{35}S-labeled cRNA probes at 55°C for 3 h. Tissue sections were then washed extensively and exposed to Biomax autoradiographic film (Kodak, Rochester, NY, USA). For higher-resolution studies, sections were dipped in Kodak NTB2 emulsion (Fisher Scientific). The specificity of cRNA probes was tested using three criteria. First, sense probes synthesized from each cDNA were used to perform *in situ* hybridization in parallel with antisense probes. There were no specific signals on these slides. Second, antisense probes were used on control slides from the cerebellum and cortex and no hybridization signals were detected. Third, antisense probes were hybridized to slides that were treated with RNase A (20 μg/mL) and no hybridization signal was detected.

Quantitative analysis

Both densitometry and silver grain analyses were applied to quantitation of *in situ* hybridizations. Films were scanned with a digital scanning densitometer (Molecular Dynamics ImageQuant program, Sunnyvale, CA, USA) and quantified with the same program. Relative optical densities (volumes) from six consecutive sections (LC) or five adrenals were normalized by areas of LC or adrenal medulla. These OD values were then converted to nCi/mg tissue by interpolation from standard curve generated from standard plastic strips containing known amounts of ^{14}C (ARC Inc., St. Louis, MO, USA), which were exposed and developed in parallel with the hybridized tissue sections (Miller 1991; Ito *et al*. 1995; Woessner *et al*. 1998).

Silver grain density was semiquantitatively analyzed with the Bioquant Nova program (R.M. Biometrics, Inc., Nashville, TN, USA). After setting a threshold grain density, a fixed circle was randomly placed in the LC area or adrenal medulla covered by silver grains. Similarly, six consecutive sections of LC or five adrenals were analyzed. The silver grains from these sections were normalized by the area of circle. The data were expressed as grain number per mm^2.

Data from each analysis (densitometry and silver grain counting) were converted to percentage control and the means of pooled data from two analyses are presented.

RNA isolation and reverse transcription–polymerase chain reaction

Total RNA was extracted from micropunched LC (tissues from each group were pooled from four rats) using Trizol reagent (Gibco BRL, Gaithersburg, MD, USA). Briefly, tissues were homogenized in Trizol reagent using a Teflon homogenizer three times for approximately 30 s each. Quality and quantity of total RNA were detected spectrophotometrically using a DU 650 Spectrophotometer (Bio-Rad, Hercules, CA, USA) at 230/260/320 nm. Equal amounts of total RNA (1 μg) from each sample were primed with random primers and reverse transcribed with the ImProm-II Reverse Transcription System (Promega) following the manufacturer's recommendations. PCR amplification of the cDNAs (2.5 μL each) was carried out using specific primers for β-actin, *Phox2a*, *Phox2b*, *DBH* and *NET* genes in a minicycler (MJ Research Inc., Reno, NV, USA). Primers were synthesized at the Invitrogen (Carlsbad, CA, USA) facility and their sequences are as follows: β-actin F 5′-GCTCGTCGTCGACAACGGCTC and β-actin R 5′-CAAACATGATCTGGGTCATCTTCTC; Phox2a F 5′-CCTGGAGGCTTCCAATACAG and Phox2a R 5′-TGAGCTG-AGCACTCGTGAAC; Phox2b F 5′-TGAGACACACTACCCCG-ACA and Phox2b R 5′-CTGCAGCTGCCATTCTGTAA; NET F 5′-GGAGTGGGCCTATGCTGTGAT and NET R 5′-GTCATG-GATCCCACTGCTCT; DBH F 5′-GGATCGAGGTGAGATG-GAGA and DBH R 5′-CTCCTCCAGGATCCCATACA. Optimization was performed for all primer sets to determine the optimal cycle number within the logarithmic phase of amplification. For the β-actin transcript, amplification was carried out for a total of 22 cycles consisting of an initial 3 min at 94°C followed by 30 s at 94°C, 1 min at 55°C, and 1 min at 72°C. 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 2B 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), where appropriate semiquantitative analysis of RT–PCR signals was carried out by densitometry using a Kodak ID Image Analysis software (Eastman Kodak Company, Rochester, NY, USA). Values of targets were normalized to those of β-actin.

Statistics

Data for *in situ* hybridization are presented as means ± SEM. Data from *ex vivo* experiments involving *in situ* hybridization ($n = 6$) were analyzed by analysis of variance (ANOVA) and *post hoc* Newman–Keuls tests for planned comparisons.

Results

Expression of Phox2a and Phox2b in the locus coeruleus of rats with different ages

To investigate the influence of age on the expression of Phox2 genes in the LC of rats, we performed *ex vivo in situ* hybridization to measure mRNA levels of Phox2a and Phox2b in young (1-month-old, $n = 6$), adult (3- or 8-month-old, $n = 6$, respectively) and aged (23-month-old, $n = 6$) rats. As shown in Fig. 1, mRNA levels of Phox2a in the LC of 3-month-old rats increased significantly to about 62% ($F_{3,210} = 59.5$, $p < 0.001$), compared to that of 1-month-old rats. At 8 months the levels were about the same as those of 1-month-old rats, although they were significantly lower when compared to 3-month-old rats ($F_{3,210} = 63.7$, $p < 0.001$). However, Phox2a mRNA levels in the LC of 23-month-old rats were markedly lower than that of 1-month-old rats (by 37%, $F_{3,210} = 37.8$, $p < 0.05$), and that of 3-month-

old rats (by 64%, $F_{3,210} = 57.3$, $p < 0.001$). In all the rats examined, there was an intense, homogeneous labeling of Phox2b mRNA in the LC (Fig. 2), demonstrating that this gene is expressed in the LC during the entire lifespan of rats. Analyses of the data revealed that Phox2b mRNA levels in the LC of 3-month-old rats were not different from that of 1-month-old rats. In contrast, Phox2b mRNA levels in the LC of 8- and 23-month-old rats appeared to decline, compared to that of 1-month-old rats. But only changes in Phox2b mRNA in the 23-month-old rats were statistically significant, with an average decrease of 30% ($F_{3,8} = 17.48$, $p < 0.05$) (Fig. 2).

Expression of norepinephrine transporter and dopamine β-hydroxylase in the locus coeruleus of rats with different ages

The expression of *Phox2* is closely associated with that of *DBH* (Valarche *et al*. 1993). NET is also considered an important marker of the noradrenergic system (Lorang *et al*. 1994). Interested in comparing the expression of Phox2 genes with these two markers of the noradrenergic system, we measured mRNA levels of NET and DBH in the LC in the same rats used for the measurement of Phox2 mRNA. As shown in Figs 3 and 4, mRNA levels of NET and DBH in the LC of rats progressively decreased with advancing age. The mean value (relative abundance of silver grains representing NET) of NET mRNA in the LC of 1-month-old rats was 70.7 ± 3.8 grains/mm^2; whereas those in the 3-, 8- and 23-month-old rats were 55.6 ± 2.6, 48.5 ± 2.1 and 34.5 ± 2.3 grains/mm^2, respectively. The mRNA levels of NET in the LC in the 3- and 8-month-old rats significantly decreased about 21% and 31%, respectively, whereas NET mRNA levels in the LC of 23-month-old rats showed less than half of that found in the 1-month-old rats ($F_{3,176} = 44.96$, $p < 0.001$). A similar trend was found for the *DBH*, as mRNA levels in the LC of 3-, 8-and 23-month-old rats decreased by 44%, 43% and 68%, compared to the levels in the one-month-old rats ($F_{3,144} = 24.5$, $p < 0.001$) (Fig. 4).

Expression of Phox2a, Phox2b, norepinephrine transporter and dopamine β-hydroxylase in the adrenal glands of rats of different ages

The relative abundance of mRNAs for Phox2a, Phox2b, NET and DBH in the adrenal medulla of rats of different ages is shown in Figs 5 and 6. mRNA levels of Phox2a in 3-month-old rats was not much different from that of 1-month-old rats, whereas a significant increase (29%) was evident in 8-month-old rats. In contrast, the mRNA levels of Phox2a in 23-month-old rats significantly decreased by 35% ($F_{3,56} = 6.91$, $p < 0.05$), as compared to those of 1-month-old rats (Fig. 5a). These results indicate that peak levels of Phox2a mRNA in the adrenal glands appeared in later adulthood (8 months), which is in contrast to its expression in the LC. The expression pattern of Phox2b mRNA in the adrenal glands of rats of different ages appeared to be very similar to that of DBH mRNA in the adrenal glands, with progressive reductions in the 3-, 8- and 23-month-old rats. Compared to 1-month-old rats, the reductions of Phox2b mRNA in 3-, 8-and 23-month-old rats were about 43, 42 and 67%, respectively ($F_{3,60} = 27.08$, $p < 0.001$) (Fig. 5b). NET mRNA levels in the adrenal medullas of 3-month-old rats showed a significant increase of 46% above that of 1-month-old rats. This elevation of NET mRNA did not remain constant through adulthood, as the levels of the 8-month-old rats decreased 23% below that of 3-month-old rats. By the 23rd month, NET mRNA levels had declined 66% ($F_{3,60} = 31.97$, $p < 0.001$) (Fig. 6a). Figure 6(b) shows age-associated reductions of DBH mRNA in the adrenal medullas of 3-, 8- and 23-month-old rats, compared to that of 1-month-old rats, with percentage reductions of 37%, 29% and 53%, respectively ($F_{3,8} = 17.48$, $p < 0.001$).

Reverse transcription–polymerase chain reaction measurement of Phox2, norepinephrine transporter and dopamine β-hydroxylase mRNA in the locus coeruleus of 1-, 3-, 8- and 23-month-old rats

Semi-quantitative RT–PCR was employed to further validate the findings of *in situ* hybridization experiments. To control for variation between samples, we used the constitutively expressed housekeeping gene, β-actin. Figure 7 shows the RT–PCR products obtained for the different transcripts including β-actin. Semi-quantitative analysis revealed that the bands of *Phox2a, Phox2b, NET* and *DBH* in 8- and 23-month-old groups showed lower densities than those in 1- and 3-month-old groups. Although the alteration pattern of mRNAs among the different age groups is not exactly the same as that of *in situ* hybridization assay, the RT–PCR results verified our earlier observations that the expression of *Phox2, NET* and *DBH* were reduced in the LC of aged rats. There were no significant changes in band densities for β-actin mRNAs in the different age groups, indicating that changes in expression of these genes were not attributable to age-related decreases in overall RNA expression (Fig. 7).

Discussion

Phox2 genes are considered to be key determining transcription factors of the noradrenergic phenotype during embryogenesis. The dynamic expression of Phox2 genes after birth has been rather unexplored, although the expression of Phox2a through adulthood was reported (Pattyn *et al*. 2000). To gain an understanding of the developmental expression profile of Phox2 genes and their possible relationship with noradrenergic marker proteins, we measured mRNA levels of Phox2a, Phox2b, NET and DBH in the LC and adrenal glands in rats of different ages. Our results show that Phox2a and Phox2b mRNA levels in both the LC and adrenal glands declined significantly with increasing age, especially after adulthood. It is interesting to note that the changes in mRNA levels of NET and DBH in the LC and adrenal glands of aged rats appeared to coincide with alterations in those of Phox2 genes. These data suggest that even after birth, Phox2 genes may have continued regulatory effects on the expression of marker proteins in the noradrenergic systems, leading to a decreased expression of *NET* and *DBH* during aging.

Previously, Phox2b expression in the LC has been reported to be transient and shut down along E11.5 (Pattyn *et al*. 1997, 2000). At birth, only Phox2a is detectable in the LC and subcoeruleus (Tiveron *et al*. 1996; Pattyn *et al*. 1997) and its expression continues into adulthood (Tiveron *et al*. 1996). However, our present results using *in situ* hybridization supported by semiquantitative RT–PCR analysis clearly demonstrate that Phox2b mRNA is detectable in the LC areas as well as adrenal glands of rats with ages from age 1–23 months, indicating that *Phox2b* is persistently expressed in the LC of normal rats. Whether this decline in mRNA translates to a corresponding decrease in protein level remains to be determined. The discrepancy between our results and previous observations (Pattyn *et al*. 1997, 2000) may be at least partly accounted for by the following possibilities. One is that the expression of Phox2b gene during the embryonic and postnatal period may not be linear. That is, its expression may be below the detection limit (or even turned off) after E11.5, but rises just above the threshold after birth (or turned on). An example of developmentally regulated changes in mRNA detection is the expression of subtypes of $GABA_A$ receptors, which differ substantially in the embryogenic and neonatal development phases of rats (Laurie *et al*. 1999). Specifically, β2 subtype mRNA is detected at E17 but declines to undetectable levels at E19 and at birth, but is again strongly identifiable at postnatal day 12 and in adults (Laurie *et al*. 1999). Another example is the expression of *Mash1*, which is expressed in embryos between days 10.5 and 16.5 of gestation, as well as in adult mouse brain. However, Mash1 mRNA can not be detected at birth (Franco del Amo *et al*. 1993). Therefore, Phox2b gene expression may be turned on and off during the different

developmental stages as needed. On the other hand, this discrepancy may be related to developmental expression patterns that are species-specific, i.e. Pattyn *et al*. (1999 (2000) studied mice, whereas our study utilized rats. Although these possibilities need to be addressed in greater detail in the future, our current results not only demonstrate the continued presence of the Phox2b gene in rat LC after birth, but also suggest that it may play a role in the maintenance of the noradrenergic phenotype throughout life.

NET and DBH are expressed in the brain, but also in various cells and neuronal fibers of the rat adrenal medulla (Cubells *et al*. 1995; Kippenberger *et al*. 1999; Schroeter *et al*. 2000; Phillips *et al*. 2001). Another important observation from this study was that the mRNA levels of NET and DBH in the LC and adrenal glands declined with advancing age. Although no such systematic comparison has been previously reported, a few sporadic studies do exist. NET mRNA levels in the LC have been observed to decrease significantly in rats of middle age (12 months) but this reduction did not persist into old age (24 months) (Shores *et al*. 1999). In fact, NET mRNA levels in the 24-month-old group did decline compared to young rats (3 months) (Shores *et al*. 1999), but this decline did not reach statistical significance. Age-related reductions in [^{3}H]nisoxetine binding to NET in the human LC (Tejani-Butt and Ordway 1992), V_{max} of uptake of NE (reflecting NET number) in the rat cardiac synaptosomes (Snyder *et al*. 1998), and NET density in rat myocardial membrane preparations (Kiyono *et al*. 2002) have been reported. DBH activity in the LC of aged rats and mice did not show any significant changes when compared with 4-month-old animals (Reis *et al*. 1977). However, the reduction of DBH expression with increasing age was demonstrated in a human study in which DBH was used as an immunological marker of noradrenergic neurons in the LC to identify LC cell loss in aged humans (Chan-Palay and Asan 1989). Taken together, it is conceivable that the age-associated decline in mRNA levels of NET and DBH observed in the present study may reflect a true biological phenomenon occurring in humans and experimental animals, although in animals these age-related changes may be a species- or even strain-specific event.

The decreased mRNA levels of NET and DBH in the LC may reflect either biological changes in response to LC cell loss with aging or a decrease in the amount of their expression per cells. Previous studies have reported an age-associated LC cell loss in mice (Sturrock and Rao 1985) and human subjects (Vijayashankar and Brody 1979; Bondareff *et al*. 1982; Mann *et al*. 1983; Tomonaga 1983; Yoshinaga 1986; German *et al*. 1988; Lohr and Jeste 1988; Chan-Palay and Asan 1989). However, more recent cell counting studies show no age-related neuronal loss in the human LC up to 80 years of age (Mouton *et al*. 1994; Kubis *et al*. 2000). Furthermore, no age-related loss of LC neurons has been reported in Fischer 344 rats up to 32 months of age (Goldman and Coleman 1981), in Wistar rats up to 30 months of age (Riihioja *et al*. 1999), and in AA (Alko, Alcohol) rats up to 24 months of age (Lu *et al*. 1997; Rintala *et al*. 1998). Hence, current data regarding age-related loss of LC neurons is somewhat controversial. It seems most likely that decreased DBH or NET mRNA levels in the LC of aged rats reported in the present study result from a decrease in the amount of their expression per cell rather than from age-related LC cell loss. This conclusion is based on consistent reports of no loss of LC neurons in aged animals of the same species used in the present study (rats; Goldman and Coleman 1981; Lu *et al*. 1997; Rintala *et al*. 1998; Riihioja *et al*. 1999).

Other causes for age-dependent declines of NET and DBH mRNA in the LC and adrenal glands should be considered. These include pre-translational regulatory events, such as mRNA synthesis or stability. Reductions in mRNA could be due to a decrease in transcription of the gene and/or a change in mRNA stability. It is known that DNA binding activity of activating protein-1 transcription factor decreases with increasing age (Asanuma *et al*. 1995). This could contribute to a lower transcription rate of the A1-AR gene. Whether

this is relevant to the expression of NET and DBH mRNA is not clear and will need further investigation.

Regarding the age-associated reduction of mRNA levels of DBH in the adrenal glands, our results do not support previous reports in which DBH activity in adrenal medulla showed a progressive age-related increase in rats, Chinese hamsters and Mongolian gerbils (Kvetnansky *et al*. 1978; Banerji *et al*. 1984). The increased DBH activity has been interpreted to be associated with increased levels of adrenaline and NE in the adrenal medulla of aged rats (Banerji *et al*. 1984). However, in another study (Seals and Esler 2000), adrenaline secretion from the adrenal medulla was markedly reduced with increasing age in humans. Furthermore, no differences were found in the activity of adrenal DBH between 4- and 26-month-old rats (Reis *et al*. 1977). Although species differences or relative age of rats may account for the different results obtained across these studies, including ours, another explanation could be the lack of correlation between enzyme activity and the amount of DBH mRNA levels.

One of the interesting findings in the present study is the coincidental decline of mRNA levels of Phox2a/2b with those of NET and DBH in both the LC and adrenal glands of aged rats, possibly indicating a relationship between these genes. Further analysis of distribution patterns of these genes in the LC and adrenal glands in rats of different ages revealed a much closer relationship between *Phox2b* and NE markers, especially *DBH* (Figs 2, 3, 4, 5b and 6b). This may be explained by the established functional role of Phox2b. During embryogenesis, several genes seem to act in a linear cascade: *Mash1–Phox2a–Phox2b* for the generation of the LC noradrenergic center (Brunet and Pattyn 2002;Goridis and Rohrer 2002). In this cascade, Phox2b may be a master determinant, as Phox2b seems to regulate the expression of *Phox2a* by specifically interacting with the Phox2a promoter (Flora *et al*. 2001) and is required for the maintenance of Mash1 expression (Pattyn *et al*. 1999). However, at this stage we cannot say conclusively that Phox2 genes, especially Phox2b, have a regulatory or controlling effect on the expression of *NET* and *DBH* in these two regions. A regulatory role seems plausible for several reasons. Firstly, Phox2 genes are expressed in all noradrenergic neurons in the central and peripheral nervous systems (Morin *et al*. 1997). Secondly, Phox2 genes have been referred to as master regulators of the noradrenergic phenotype during embryogenesis, because the LC and NE cell types in the peripheral nervous system fail to differentiate in *Phox2a* and *Phox2b* knock-out mice, and genes for *TH* and *DBH* fail to switch on (Morin *et al*. 1997;Pattyn *et al*. 1999,2000). Their persistent expression throughout life, as demonstrated for the Phox2b mRNA in the present study, suggests a potential role in the maintenance of noradrenergic phenotypes. Thirdly, Phox2 genes transactivate the transcriptional activity of the DBH gene, a hallmark enzyme of noradrenergic neurons (Zellmer *et al*. 1995;Yang *et al*. 1998). This transcriptional regulatory mechanism may explain their possible influence on the expression of noradrenergic phenotypes in the nervous system. A very similar example is Nurr1, an orphan member of ligand-activated nuclear receptor family. Nurr1 is co-expressed with, and transcriptionally activates, endogenous tyrosine hydroxylase (Backman *et al*. 1999;Schimmel *et al*. 1999) and the dopamine transporter (Sacchetti *et al*. 1999). Nurr1 appears to be essential for the development and survival of midbrain dopaminergic neurons during embryogenesis (Law *et al*. 1992;Zetterstrom *et al*. 1996). Recently, much compelling evidence demonstrated that Nurr1, which is persistently expressed in normal adult tissue, is a potential regulator of gene expression of dopaminergic phenotypic markers in the brain (Sacchetti *et al*. 2001;Kim *et al*. 2003;Bannon *et al*. 2004;Bassett *et al*. 2004). The possible regulatory or control role of Phox2 genes in maintaining the expression of noradrenergic key proteins such as NET and DBH after birth or during normal aging, if confirmed, will provide new information relevant to understanding the physiology of aging and possibly the pathophysiology of some mental illnesses.

In this study, we used both *in situ* hybridization and semiquantitative RT–PCR to measure mRNA levels of Phox2, NET and DBH in the LC of rats of different ages. Both measurements showed a consistent reduction in the mRNA levels of Phox2, NET and DBH in 23-month-old rats. However, the expression patterns for mRNA levels of these genes in the 3- and 8-month-old rats from both assays were not exactly the same. For instance, Phox2a mRNA levels in the 3-month-old rats increased significantly as measured by *in situ* hybridization and there was almost no change in the 8-month-old rats. The RT–PCR assay did not show such changes, and showed reduced mRNA levels in the 8-month-old rats. Similar reductions were also found in DBH mRNA levels in 8-month-old rats. This discrepancy may in part be attributable to different features of the methods used. Although *in situ* hybridization can be used to measure cellular localization of RNA without amplification, probe penetration may be a problem. On the other hand RT–PCR performed *in vitro* does not have that problem and may thus be more quantitative than *in situ* hybridization. Nevertheless, the overall trend observed in mRNA expression for the different genes is similar based on the two methods.

The present study provides for the first time a comprehensive description of the age-associated changes in expression of *Phox2a/2b* and marker proteins of the noradrenergic system in rats. In summary, we conclude that (i) the Phox2b gene is persistently expressed in the LC after birth, (ii) Phox2a and Phox2b mRNAs decline in the LC and adrenal glands in aged rats and (iii) the mRNAs of NET and DBH in the same areas exhibit a decline very similar to those of the Phox2 genes with aging. These findings leave open the possibility that there is a relationship between the gene expressions of *Phox2* and *NET* and *DBH*. Clearly, further studies are necessary to elucidate the precise molecular mechanisms underlying the regulation and maintenance of noradrenergic marker gene expression by Phox2 genes.

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Abbreviations used

ANOVA	analysis of variance
cRNA	complementary RNA
DBH	dopamine β-hydroxylase
LC	locus coeruleus
mRNA	messenger ribonucleic acid
NE	norepinephrine
NET	norepinephrine transporter
OCT	optimal cutting temperature
RT–PCR	reverse transcription–polymerase chain reaction

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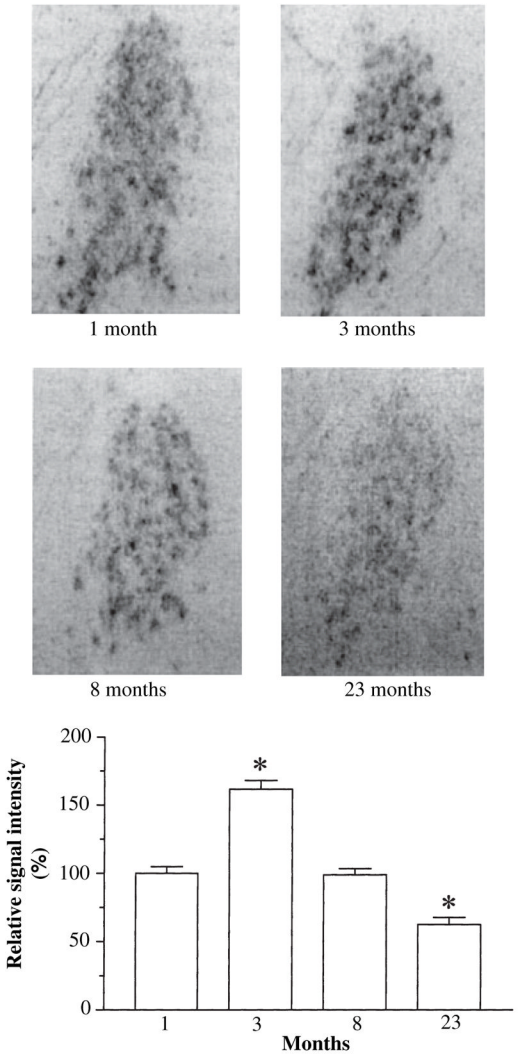

Fig. 1.

Upper panel: Phox2a mRNA detected by *in situ* hybridization in the locus coeruleus of rats of different ages ($n = 6$). Coronal brain sections were taken at 9.7 mm from bregma (corresponding to Plate 58 in the brain atlas of Paxinos and Watson 1998). Lower panel: quantitative analysis of mRNA in slides. *$p < 0.01$, compared to the group of 1-month-old rats.

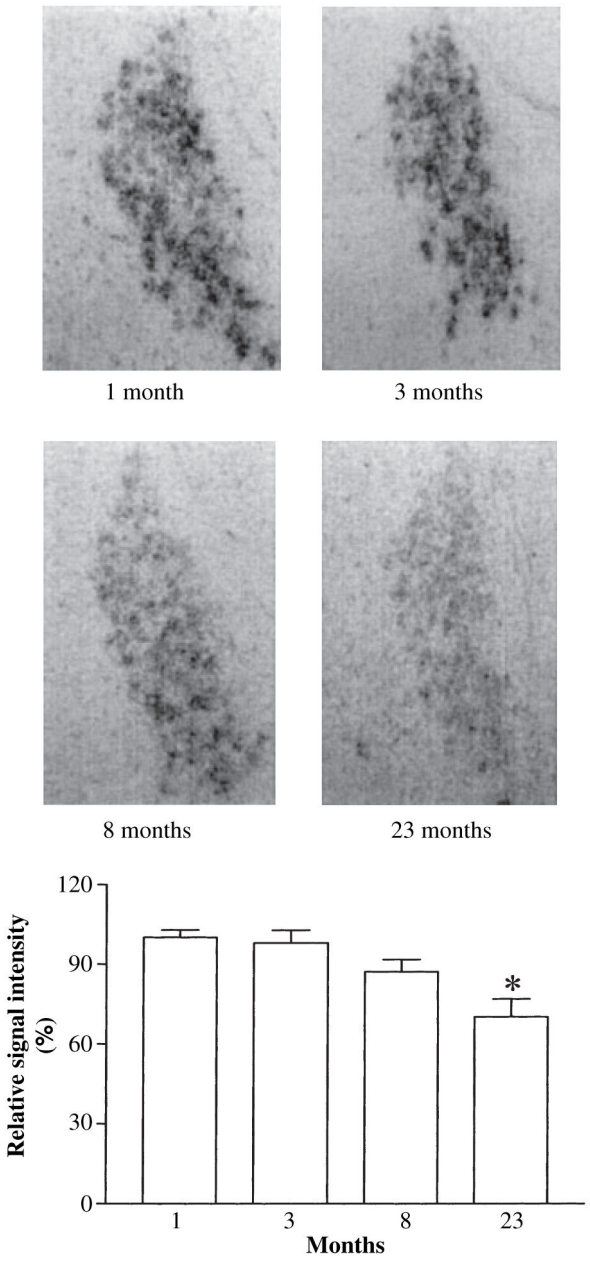

Fig. 2.

Upper panel: Phox2b mRNA detected by *in situ* hybridization in the locus coeruleus of rats of different ages ($n = 6$). Coronal brain sections were taken at 9.7 mm from bregma (corresponding to Plate 58 in the brain atlas of Paxinos and Watson 1998). Lower panel: quantitative analysis of mRNA in slides. *$p < 0.05$, compared to the group of 1-month-old rats.

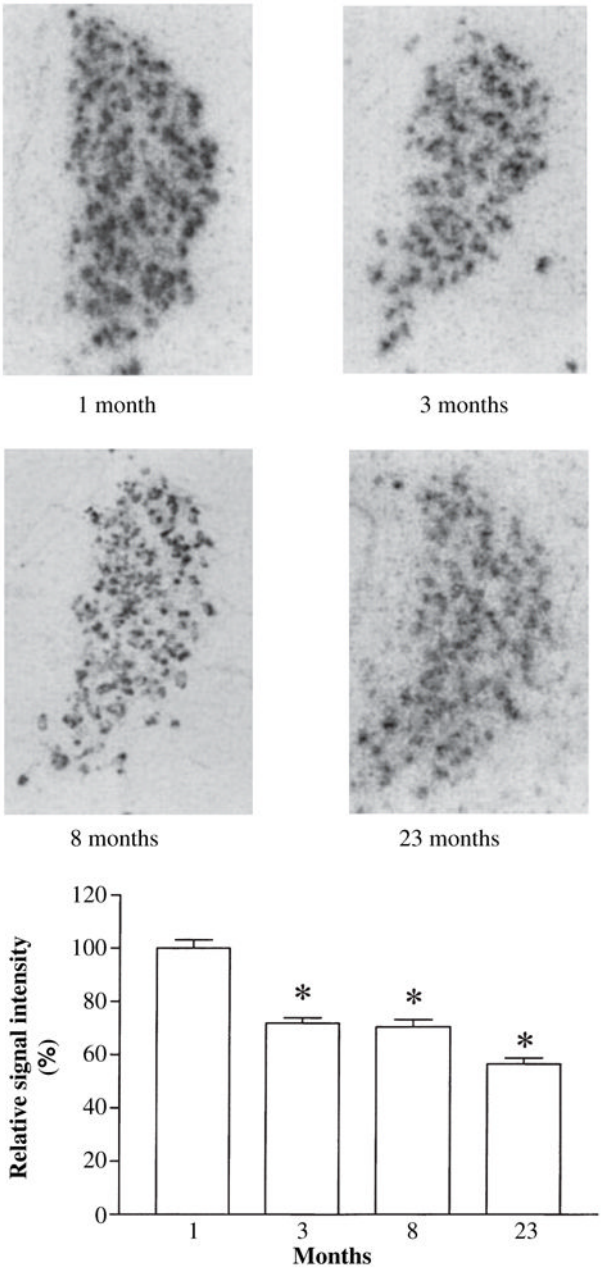

Fig. 3.

Upper panel: norepinephrine transporter mRNA levels detected by *in situ* hybridization in the locus coeruleus of rats of different ages ($n = 6$). Coronal brain sections were taken at 9.7 mm from bregma (corresponding to Plate 58 in the brain atlas of Paxinos and Watson 1998). Lower panel: quantitative analysis of mRNA in slides. *$p < 0.01$, compared to the group of 1-month-old rats.

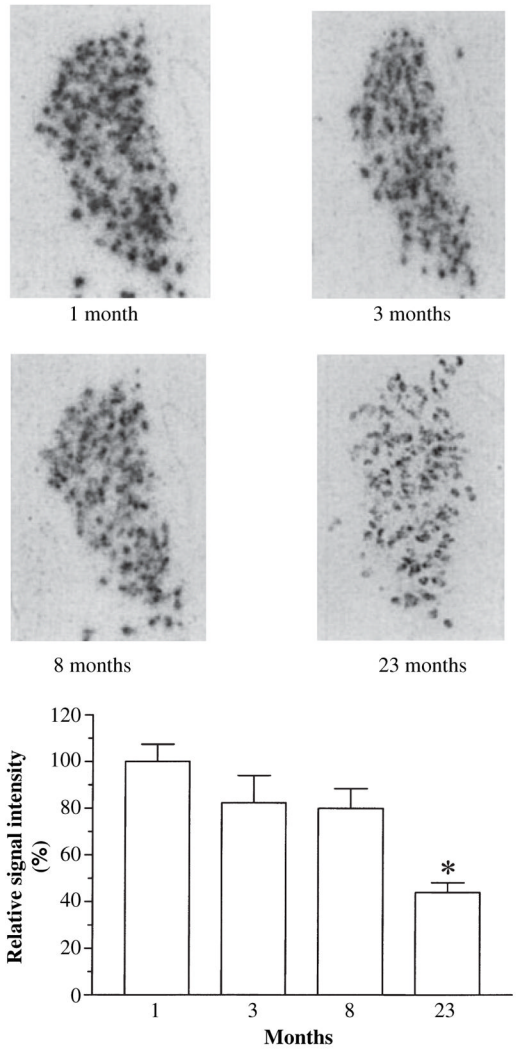

Fig. 4.

Upper panel: dopamine β-hydroxylase mRNA levels detected by *in situ* hybridization in the locus coeruleus of rats of different ages ($n = 6$). Coronal brain sections were taken at 9.7 mm from bregma (corresponding to Plate 58 in the brain atlas of Paxinos and Watson 1998). Lower panel: quantitative analysis of mRNA in slides. *$p < 0.01$, compared to the group of 1-month-old rats.

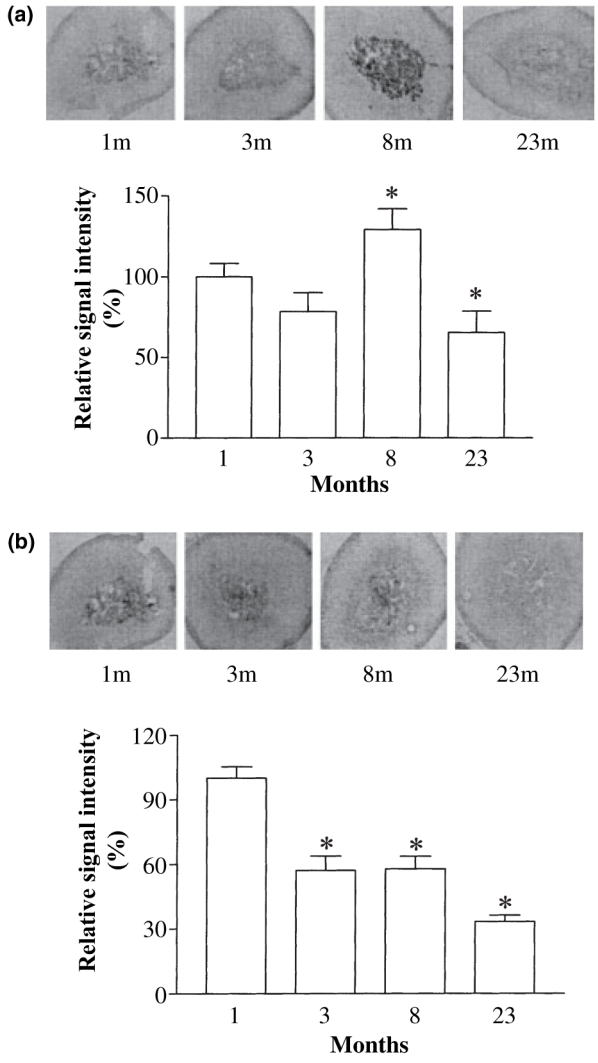

Fig. 5.

Upper panel: mRNA of Phox2a (a) and Phox2b (b) detected by *in situ* hybridization in the adrenal glands of rats of different ages ($n = 6$). Lower panel: quantitative analysis results of mRNA in slides. *$p < 0.01$, compared to the group of 1-month-old rats.

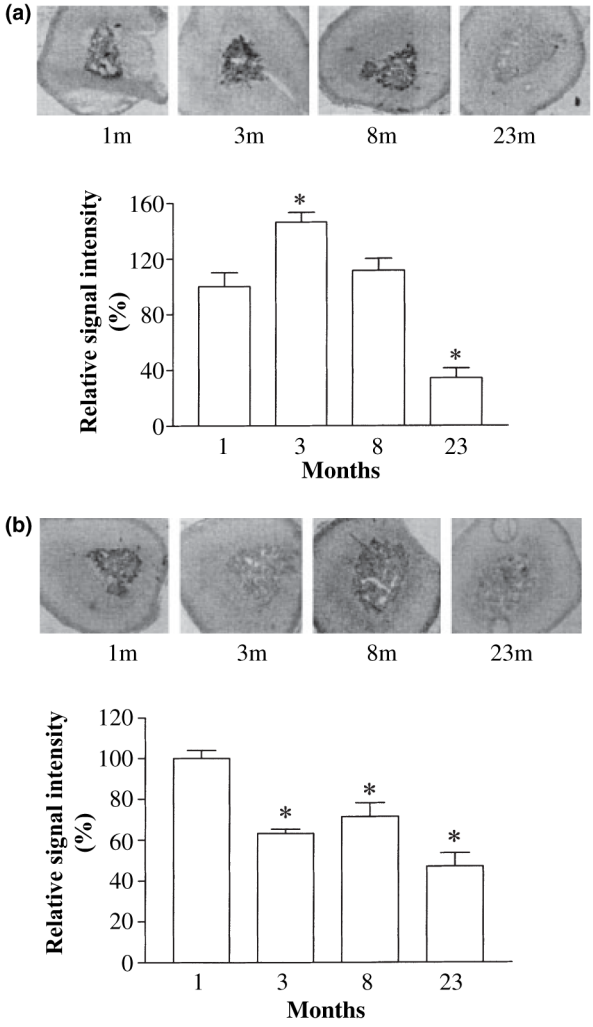

Fig. 6.

Upper panel: mRNA of norepinephrine transporter (a) and dopamine β-hydroxylase (b) detected by *in situ* hybridization in the adrenal glands of rats of different ages ($n = 6$). Lower panel: quantitative analysis results of mRNA in slides. *$p < 0.01$, compared to the group of 1-month-old rats.

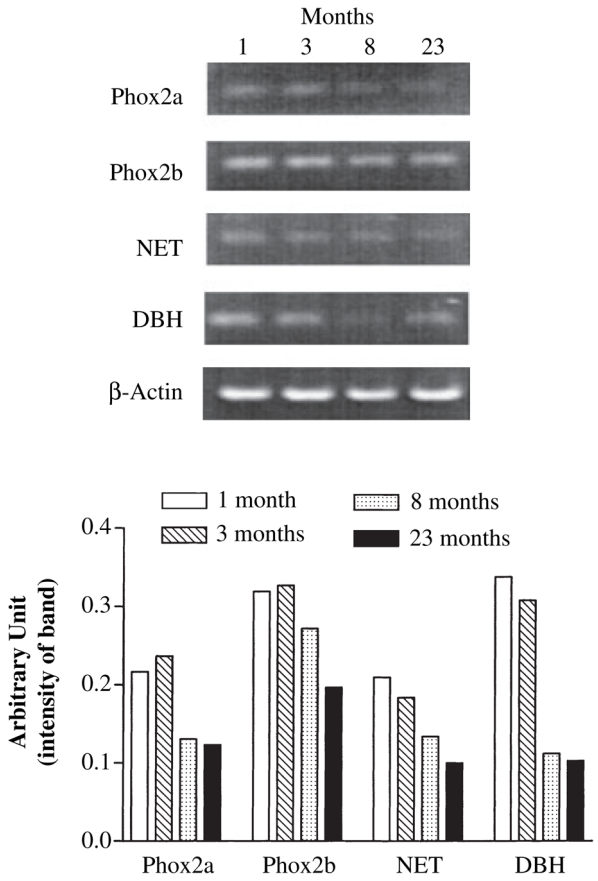

Fig. 7.

Upper panel: RT–PCR products of Phox2a, Phox2b, norepinephrine transporter (NET), dopamine β-hydroxylase (DBH) and β-actin obtained from RNAs isolated from the locus coeruleus of rats with different ages (n = 4). Lower panel: graphical representation of semiquantitative analysis by RT–PCR. Values of targets were normalized to those of β-actin.