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Cholinergic Differentiation Occurs Early in Mouse Sympathetic Neurons and Requires Phox2b

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The generation of neurotransmitter identity in the autonomic nervous system is a classical model system to study the development of neuronal diversity. Analysis of the expression of genes coding for enzymes of noradrenaline biosynthesis in the sympathoadrenal system allowed the characterization of factors involved in the differentiation of the noradrenergic transmitter phenotype. The development of cholinergic properties in the autonomic system is less well understood. Here we show that expression of mRNAs for choline acetyltransferase (ChAT) and the vesicular acetylcholine transporter (VAChT), both encoded by the cholinergic gene locus, is induced in mouse sympathetic ganglia at embryonic day 11 (E11). Positive cells amount to more than 50% of Phox2b-positive sympathetic cells at cervical levels. The proportion declines caudally, decreasing to ~20% of Phox2b-positive cells at lower thoracic levels. In the adrenal anlage, ChAT and VAChT mRNA are largely undetectable at E11 and E13. In mice homozygous for a mutational inactivation of the transcription factor Phox2b, ChAT and VAChT mRNA expression is absent from sympathetic ganglia. The data show that expression from the cholinergic gene locus is regulated differently in sympathetic neurons and adrenal chromaffin cells. Phox2b is required for development of cholinergic neurons but does not suffice to support cholinergic properties in chromaffin cells.

Key words: Sympathetic neuron; Adrenal chromaffin cell; Choline acetyltransferase; Vesicular acetylcholine transporter; Phox2b mutant; Development

INTRODUCTION

The sympathetic nervous system in mammals and birds has been exploited as a model system to study neuronal differentiation and diversification [(9,13,14) for review]. The development of two neuron populations differing in their neurotransmitter, noradrenaline, and acetylcholine, respectively, raised the question about the identity of the signals that regulate the developmental pathways leading to different transmitter phenotypes.

A major breakthrough was the characterization of bone morphogenetic proteins (BMPs) as aorta-derived growth factors necessary for the induction of tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH), enzymes required for noradrenaline biosynthesis and the acquisition of the noradrenergic transmitter phenoptype [(4,8) for review]. This finding was complemented by the characterization of Phox2 transcription factors being important for noradrenergic differentiation of sympathetic neurons (9,13,14,19) as well as adrenal chromaffin cells (16).

The situation for the cholinergic transmitter phenotype is less clear. It has been shown for chick (5) and mouse (1) sympathetic neurons that different signals are involved in the early induction of cholinergic differentiation and the subsequent, target-dependent maturation of cholinergic sympathetic neurons (6). Analysis of mice mutant for the tyrosine kinase receptor subunit *c-ret* demonstrates that signaling by

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ligands from the GDNF-related growth factor family is required for cholinergic development during the third embryonic week (1), when target innervation by sympathetic neurons starts. Mutational inactivation of *gp130* shows that cytokine signaling is necessary for the postnatal differentiation of cholinergic sympathetic neurons (23). However, the onset of the early induction of cholinergic properties and the molecular mechanisms involved still have to be characterized. Interestingly, Phox2 transcription factors, even though being appreciated for their involvement in the induction of noradrenergic differentiation, are expressed also in cholinergic sympathetic neurons of chick embryos (7). Moreover, Phox2b mutant mice lack autonomic neurons in general, including parasympathetic cholinergic neurons (2,19). In addition, the mutation affects cholinergic hindbrain motoneurons (2,20).

Here we determine the onset of choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT) mRNA expression in mouse embryos. We find that the respective mRNAs become detectable during embryonic day 11 (E11) in sympathetic neurons of wild-type but not Phox2b mutant mice. In adrenal chromaffin cells of wild-type animals, ChAT and VAChT expression is not detectable. Our observations indicate that Phox2b is required but not sufficient for cholinergic differentiation in cells of the sympathoadrenal lineage.

MATERIALS AND METHODS

Experimental Animals

Phox2b$^{+/lacZ}$ mice (19) were bred to obtain wild-type and Phox2b$^{lacZ/lacZ}$ animals. To support embryo survival beyond midgestation, pregnant females received 100 μg/ml L-phenylephrine, 100 μg/ml isoproterenol, and 2 mg/ml ascorbic acid with drinking water from E8.5 onwards. Pregnant females were killed by CO_2 asphyxation. Embryos were sacrificed on ice by hypothermia and tails were removed for genotyping by PCR. Specimen were fixed by immersion in 4% paraformaldehyde (8°C, overnight), dehydrated in 15% sucrose (8°C, overnight), and stored in OCT compound (Tissue Tek) at −70°C. Cryosections were taken at 12 μm on Superfrost slides from the head into the lumbar region.

In Situ Hybridization (ISH)

ISH was performed as described previously (1,5). Cryosections were incubated with digoxygenin-labeled cRNAs at 65°C overnight. Bound riboprobe was detected with anti-digoxygenin coupled to alkaline phosphatase (Fab fragments) (Roche, Mannheim) applied overnight at room temperature. Color reaction was performed with NBT/BCIP substrate (Roche, Mannheim) overnight at room temperature.

Riboprobes were synthesized with digoxygenin-labeling mixture (Roche, Mannheim) and appropriate RNA polymerases from the following cDNAs: ChAT (17), VAChT (1), DBH (19), Phox2b (19), SF1 (16), and lacZ (kindly provided by Dr. J. Strelau, IZN, Heidelberg). DBH and lacZ expression were used to localize sympathoadrenal cells and to control the PCR genotyping results. Steroidogenic factor 1 (SF1) was used as a marker to localize adrenal tissue (16).

Cell Counts

Numbers of Phox2b and VAChT mRNA-expressing cells were determined after ISH from sympathetic ganglion and adrenal anlagen in three wild-type animals (E11.5). For each animal cervical ganglion anlagen, thoracic primary sympathetic ganglia, and sympathoadrenal cells at adrenal levels were analyzed separately. Phox2b and VAChT-positive cells were determined on adjacent sections and every 10th section was counted. Cells with blue ISH reaction product sparing the nuclear region were judged positive. Identification of nuclear position was confirmed using DAPI staining in a selected number of sections. For every animal the percentage of VAChT-positive cells relative to Phox2b-positive cells was determined. Mean values (±SD) for each region were calculated from the three animals.

RESULTS

Expression of ChAT and VAChT mRNAs in the Mouse Sympathoadrenal System Becomes Detectable During Embryonic Day 11

In situ hybridization was performed to detect mRNAs for ChAT and VAChT in serial sections of mouse embryos at E10.5 to E11.5. Phox2b and DBH mRNA expression was analyzed to localize the anlagen of the cervical ganglia, the primary sympathetic ganglia of the thoracic region, and the more caudally positioned neural crest-derived sympathoadrenal precursors contributing to adrenal tissue as well as paravertebral and prevertebral sympathetic ganglia. As shown in Figure 1, at embryonic day 11.5 (E11.5), ChAT and VAChT mRNA overlaps with DBH expression. In all four wild-type embryos analyzed, expression of the cholinergic markers was observed. Comparison with expression of Phox2b mRNA, a marker for autonomic precursors and their neuronal

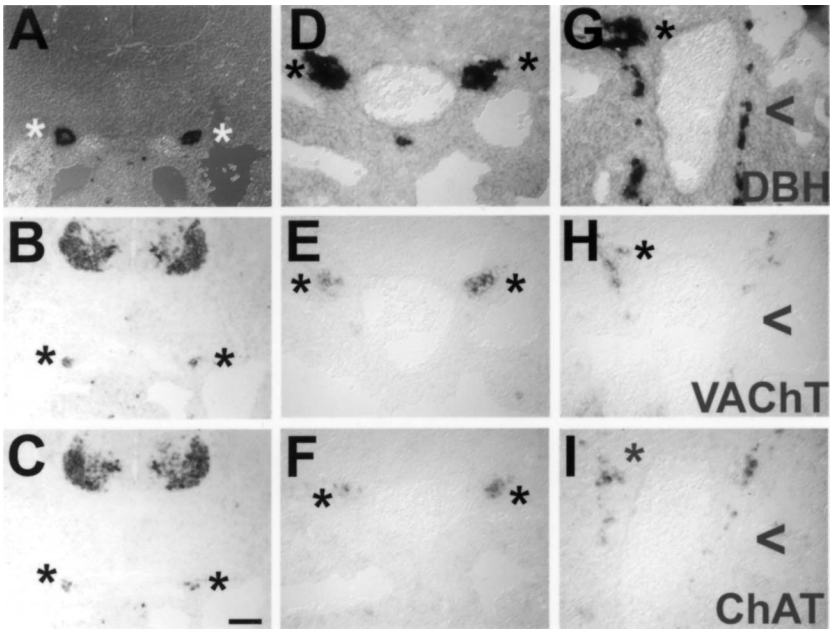

Figure 1. ChAT and VAChT mRNAs are expressed in sympathetic ganglion anlagen of 11.5-day-old mouse embryos. Serial sections from a 11.5-day-old wild-type mouse embryo, showing cervical sympathetic ganglion anlagen (A–C), thoracic primary sympathetic ganglia (D–F), and lower thoracic to lumbal adrenal and sympathetic ganglion anlagen (G–I), were hybridized to DBH (A, D, G), VAChT (B, E, H), and ChAT (C, F, I) riboprobes. Sympathetic ganglion anlagen (*) at all levels express noradrenergic and cholinergic properties. Cells lateral to the aorta in presumptive adrenal regions (<) express DBH but less frequently ChAT or VAChT mRNAs (G–I). VAChT and ChAT are expressed in developing motoneurons of the ventral neural tube (B, C). Scale bar: 80 μm (A–C), 40 μm (D–I).

and endocrine progeny, shows cholinergic properties in a substantial fraction of sympathetic ganglion cells (Fig. 2). A quantitative estimate was performed to determine the percentage of VAChT-positive cells in the sympathoadrenal Phox2b-positive cell population. In cervical regions along the carotid arteries, VAChT-positive cells amounted to more than half of Phox2b-positive cells (Table 1). The percentage declined caudally. At presumptive adrenal levels, VAChT-positive cells adjacent to the aorta amounted to approximately 20% of Phox2b-positive cells.

The decrease in percentage of VAChT-positive cells may suggest a developmental gradient. In addition, different regulation of the cholinergic gene locus in neuronal and chromaffin precursors may occur. Analysis in E11.5 animals showed different expression patterns of ChAT and VAChT mRNAs in presumptive adrenal regions and sympathetic ganglia (Fig. 2). In positions dorsolateral to the aorta (i.e., in presumptive ganglionic regions) cholinergic markers are expressed in a large fraction of cells, while in presumptive adrenal regions lateral to the aorta (10, 16), expression was rarely detectable. In E13.5 animals, VAChT and ChAT mRNA expression could be detected in only a few cells of adrenal tissue (Fig. 3). This expression pattern corresponds to the distribution of neuronal markers in adrenal tissue (10,16) and suggests that occasional neuronal cells but not developing chromaffin progenitors in adrenal tissue express detectable amounts of cholinergic markers. Correspondingly, abundant expression was detectable in catecholaminergic regions outside adrenal tissue, where neuronal cells in paravertebral and prevertebral sympathetic ganglia develop. The data indicate that expression of cholinergic properties differs in sympathetic neuronal and adrenal chromaffin lineages from the outset.

At E10.5 expression of VAChT and ChAT mRNA was analyzed by ISH in three wild-type animals (data not shown). Sympathetic ganglion anlagen were localized by Phox2b and DBH mRNA expression. One animal did not show detectable expression of cholinergic markers in the developing sympathetic system. The other two animals showed weak VAChT and ChAT signals in a small number of cells. These data indicate that expression from the cholinergic gene locus in sympathetic cells of mice is induced during embryonic day 11.

ChAT and VAChT mRNAs Are Undetectable in Sympathoadrenal Tissue of Homozygous Phox2b Mutant Mice

Phox2b is required for noradrenergic differentiation of sympathetic precursors and development of autonomic neurons in general (19). As expression

from the cholinergic locus in sympathetic neurons of wild-type mice starts at a time (embryonic day 11, this study) when sympathetic ganglia are still present in Phox2b mutant mice [(19), and this study], we analyzed whether ChAT and VAChT expression commences in these animals.

ChAT and VAChT mRNA expression patterns in heterozygous Phox2b mutant mice are similar to those in wild-type mice (data not shown). In homozygous animals ($n = 3$), no ChAT and VAChT mRNAs could be detected in sympathoadrenal sites at E11.5 (Fig. 4). Sympathetic ganglion anlagen at cervical (not shown) and thoracic (Fig. 4A) levels were detected by lacZ in situ hybridization. They appeared smaller than in wild-type or heterozygous animals corroborating earlier results (19). In adrenal regions of homozygous mutants, lacZ-positive cells were abundantly present (Fig. 4D) and distributed similar to DBH-positive cells in wild-type and heterozygous animals, as previously reported (16). Neither DBH (not shown), ChAT (Fig. 4B, E), nor VAChT (Fig. 4C, F) signals were detectable at sympathoadrenal sites, indicating that Phox2b is required for the development of noradrenergic as well as cholinergic properties.

TABLE 1
VAChT mRNA EXPRESSION IN THE SYMPATHOADRENAL ANLAGEN OF 11.5-DAY-OLD MOUSE EMBRYOS

VAChT-Positive Cells in % of Phox2b-Positive Cells		
Cervical Ganglion Anlagen	Thoracic Primary Ganglia	Adrenal and Adjacent Ganglion Anlagen
55.2 ± 8.3%	44.5 ± 6.6%	21.8 ± 7.4%

Three wild-type animals were analyzed and values are given as mean ± SD. Cervical ganglion anlagen adjacent to the carotid arteries and thoracic primary ganglia at the dorsal aorta were well demarcated. At adrenal levels, the adrenal anlage and adjacent ganglion anlagen and neuron clusters were not well delineated at this stage. Therefore, counts include all Phox2b and VAChT-positive cells surrounding the aorta.

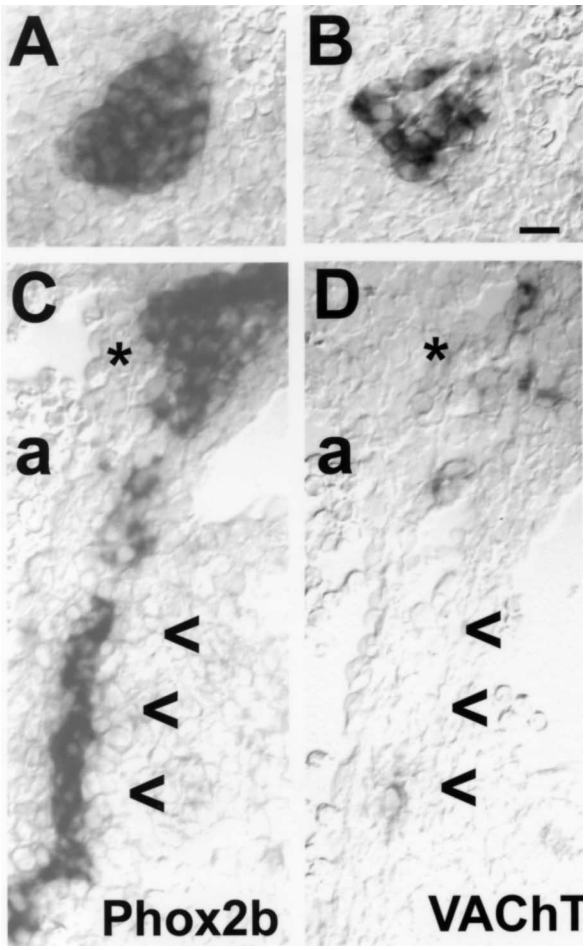

Figure 2. The proportion of VAChT mRNA expressing cells decreases from cervical sympathetic ganglia to adrenal anlagen in 11.5-day-old mouse embryos. Sections from a 11.5-day-old wild-type mouse embryo taken at cervical (A, B) and adrenal (C, D) levels were hybridized to Phox2b (A, C) and VAChT (B, D) riboprobes. In cervical ganglia, many cells express VAChT, often at high levels. At adrenal levels, positive cells are less abundant and the majority of VAChT-expressing cells are found dorsolateral to the aorta (a) in presumptive ganglionic sites (*). Lateral to the aorta (<), where adrenal tissue will differentiate, VAChT signals are observed infrequently. Scale bar: 20 μm.

DISCUSSION

Here we show that expression of cholinergic properties in sympathetic neurons occurs in a large number of cells in cervical and thoracic sympathetic ganglia of 11-day-old mouse embryos. ChAT and VAChT mRNA expression precedes the time when peripheral targets become innervated as analyzed for superior cervical ganglion neurons (11). Thus, similar to chick sympathetic neurons (5,6), expression of cholinergic markers in mouse sympathetic neurons starts at an early developmental stage, before innervation of peripheral targets has occurred, yet after induction of noradrenergic properties.

This early expression of cholinergic markers occurs in a large number, possibly the majority of sympathetic ganglion cells, suggesting coexpression with noradrenergic characters at least in part of the neurons. Coexpression of cholinergic and noradrenergic markers has been shown by double labeling in chick sympathetic neurons at embryonic day 7 (5). The incomplete overlap of cholinergic and noradrenergic features observed in these studies may be explained in two ways. First, distinct subpopulations of developing sympathetic neurons may be restricted to the

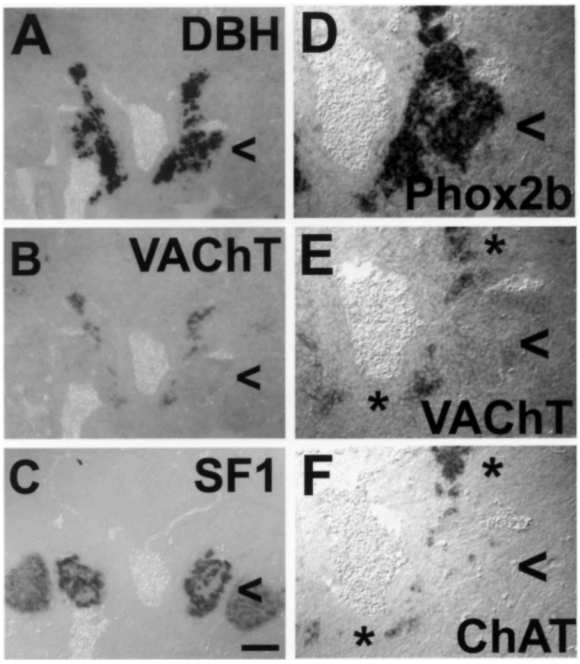

Figure 3. Cholinergic properties are largely absent from adrenal tissue of 13.5-day-old mouse embryos. Serial sections from the adrenal region of a 13.5-day-old mouse embryo were hybridized with DBH (A), VAChT (B, E), SF1 (C), Phox2b (D), and ChAT (F) riboprobes. SF1 expression shows the adrenal anlagen lateral to the aorta (<) expressing DBH and Phox2b. The majority of cells in adrenal tissue display no detectable VAChT or ChAT signals as shown in larger magnification (D–F). However, cholinergic marker expression is detectable in ganglion cell clusters (*) dorsal and ventral to the adrenal anlagen. Scale bar: 80 μm (A–C), 40μm (D–F).

expression of only one transmitter phenotype. A possible explanation could be the choice of different developmental paths by neurons generated at different times. Alternatively, all sympathetic neurons could at least transiently coexpress cholinergic and noradrenergic features. The incomplete overlap of cholinergic and noradrenergic features observed in tissue sections could be the result of the temporal profile of expression from the cholinergic gene locus including onset of expression spread across several days in a nonsynchronized precursor population as shown for noradrenergic features (10,16) and subsequent downregulation of expression in at least a subpopulation of differentiating neurons as suggested by the decrease in percentage of cholinergic sympathetic cells [compare this study and (1)]. In addition, low expression of cholinergic markers may compromise detection, in particular at early embryonic stages (1). Expression levels close to the detection threshold of in situ hybridization may go undetected such that our numbers may underestimate the percentage of sympathetic cells expressing the cholinergic gene locus.

At later stages segregation of the expression of cholinergic and noradrenergic markers into distinct neuron populations becomes evident. This time period coincides with peripheral target innervation. Indeed, for rat sympathetic neurons innervating sweat glands, induction of cholinergic properties by target tissue has been demonstrated (18).

Signals involved in the regulation of ChAT and VAChT expression differ between early embryonic and late embryonic to postnatal stages of cholinergic development. Whereas c-ret signal transduction is required for development of cholinergic sympathetic neurons during the third week of mouse embryonic development, mutational inactivation of c-ret does not compromise early induction of cholinergic marker gene expression (1). In addition, cytokine signaling is involved in later stages of cholinergic sympathetic development (3,12,23). gp130, the signal transducing subunit in the cytokine receptor complex, is required for the postnatal increase in the number of cholinergic neurons in mouse stellate ganglia (23).

Factors involved in the early phase of cholinergic sympathetic development still have to be characterized. Here we have shown that Phox2b is required not only for development of noradrenergic but also of cholinergic sympathetic neurons. No expression of cholinergic markers is detectable in homozygous Phox2b mutant mice at stages when ChAT and VAChT are expressed in wild-type sympathetic ganglia. At these stages there is still a significant although reduced number of cells present in mutant sympathetic ganglion anlagen [(19) and this study]. Because expression of noradrenergic traits as well as generic neuronal properties is blocked in sympathetic neurons of homozygous mutant mice (19), a general block of differentiation could prevent the acquisition of competence to express cholinergic properties. Alternatively, reduced ganglion cell number at E10.5–E11.5 and ongoing cell loss (19) could reflect the death of cells before the stage required for cholinergic differentiation. Irrespective of these possibilities, the question arises whether Phox2b may regulate expression from the cholinergic gene locus. Even though Phox2 transcription factors were initially appreciated for their importance in noradrenergic differentiation [(25); for review see (8)], they may regulate other neuronal genes such as NCAM (24). Overexpression experiments in the chick embryo show that Phox2b is able to induce ectopic neurons with noradrenergic and cholinergic properties (21). Because overexpression of Phox2b induces expression of other transcription factors involved in sympathetic neuron differentiation (15,22), this observation demonstrates that Phox2b may initiate sympathetic developmental programs leading towards both transmitter phenotypes but does not favor any of the above mechanisms. The

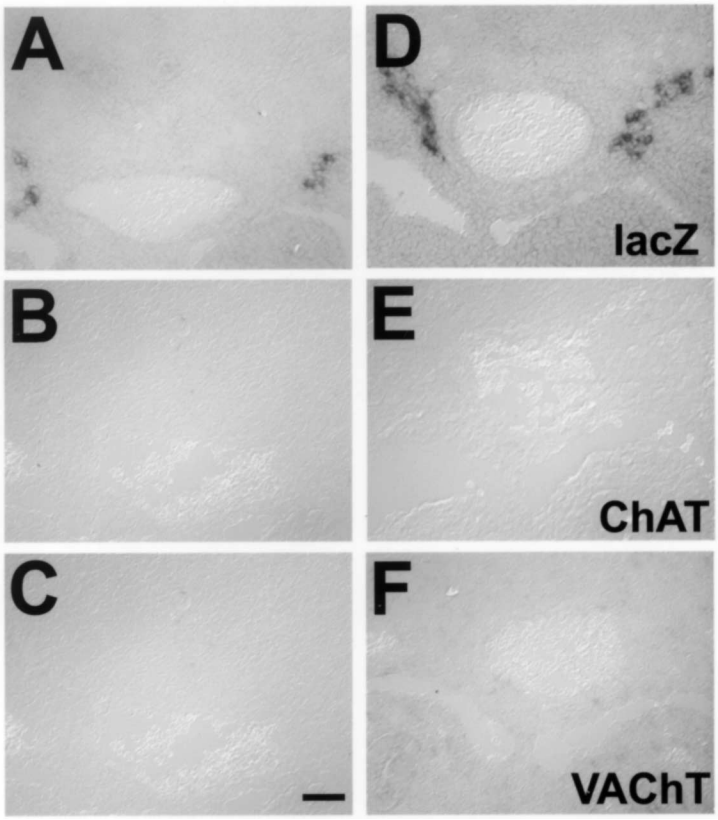

Figure 4. ChAT and VAChT mRNAs are undetectable in sympathetic ganglion anlagen of Phox2b mutant mice. Serial sections from a 11.5-day-old *Phox2b*$^{lacZ/lacZ}$ mouse embryo, showing thoracic primary sympathetic ganglion anlagen (A–C) and lower thoracic to lumbal adrenal and sympathetic ganglion anlagen (D–F) were hybridized to lacZ (A, D), ChAT (B, D), and VAChT (C, F) riboprobes. lacZ mRNA expression shows sympathetic ganglion and adrenal anlagen, which both lack ChAT and VAChT mRNA expression. Scale bar: 40 μm.

apparent lack of detectable ChAT and VAChT mRNA expression in adrenal tissue of mouse embryos (this study) as well as the delay between Phox2b expression and cholinergic induction in chick (5,7) and mouse [(19), and this study] suggest, however, that the Phox2b effect on cholinergic sympathetic development occurs indirectly via early developmental check points and/or cell death.

Thus, data from Phox2b mutant mice show that this transcription factor is required for differentiation not only of noradrenergic but also cholinergic sympathetic neurons. Lack of cholinergic properties in adrenal chromaffin precursors demonstrates that Phox2b is, however, not sufficient for cholinergic induction.

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