

# Orphan Nuclear Receptor Nurr1 Is Essential for Ret Expression in Midbrain Dopamine Neurons and in the Brain Stem

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The orphan nuclear receptor Nurr1 is essential for development of midbrain dopamine (DA) cells. In Nurr1-deficient mice, DA precursor cells fail to migrate normally, are unable to innervate target areas, and only transiently express DA cell marker genes. In the search for Nurr1-regulated genes that might explain this developmental phenotype, we found that expression of the receptor tyrosine kinase Ret is deregulated in these cells of Nurr1-deficient embryos. In addition, our analyses establish Nurr1 as an early marker for the dorsal motor nucleus (DMN) of the vagus nerve. Interestingly, Ret expression is absent also in these cells in Nurr1-targeted mice. Neural innervation of vagus nerve target areas appeared normal apart from a subtle disorganization of the DMN-derived nerve fibers. In conclusion, regulation of Ret by Nurr1 in midbrain DA neurons and in the DMN has implications for both embryonal development and adult physiology in which signaling by neurotrophic factors plays important roles.

## INTRODUCTION

Nuclear receptors are ligand-activated transcription factors that regulate gene expression by binding to specific hormone-response elements in the vicinity of regulated target genes. These evolutionarily related proteins include receptors for lipophilic ligands such as steroid hormones, retinoids, vitamin D, and thyroid hormone, signaling molecules whose activities are critical in cell fate determination, morphogenesis, and adult physiology (Mangelsdorf *et al.*, 1995). Moreover, the family also includes a large group of orphan receptors which lack identified ligands and thus indicate the

existence of novel and as yet uncharacterized signaling pathways (Giguère, 1999).

Nurr1 (nur-related factor 1; NR4A2) is an orphan nuclear receptor which is widely expressed in both the developing and the adult central nervous system (Law *et al.*, 1992; Zetterström *et al.*, 1996a,b). Expression of Nurr1 in the mouse is detected already at embryonic day (E) 10.5 in the ventral midbrain (VMB), which is the region where mesencephalic dopamine (DA) neurons develop (Zetterström *et al.*, 1997). These cells play fundamental roles in motor control, mechanisms of reward, and a number of other neural processes. Importantly, dopaminergic neurons degenerate in patients with Parkinson's disease and disturbances in DA neurotransmission are tightly linked to disorders such as schizophrenia and drug addiction. Previous studies in mice, in which the gene encoding Nurr1 has been targeted, revealed that Nurr1 is essential for DA cell development (Castillo *et al.*, 1998; Saucedo-Cardenas *et al.*, 1998; Zetterström *et al.*, 1997). Analyses demonstrated that Nurr1, which is first detected immediately peripheral to the ventricular zone of proliferating progenitor cells, is required for DA cell migration and axonal target area innervation (Wallén *et al.*, 1999). At birth, increased cell death is observed in the ventral midbrain of Nurr1-deficient mice and dopaminergic markers fail to be detected (Saucedo-Cardenas *et al.*, 1998; Wallén *et al.*, 1999; Zetterström *et al.*, 1997). Nurr1 is expressed also in adult DA neurons, suggesting that Nurr1 continues to influence the function of these cells during postnatal development and adulthood (Zetterström *et al.*, 1996a,b). Indeed, Nurr1 has been shown to regulate genes of importance for DA neurotransmission, includ-

ing the DA transporter and tyrosine hydroxylase (TH; Sacchetti *et al.*, 2001; Sakurada *et al.*, 1999; Schimmel *et al.*, 1999; Zetterström *et al.*, 1997). In contrast, Nurr1-regulated target genes that can explain the developmental DA cell phenotype remain to be identified.

Ret is a protein tyrosine kinase and a critical signal transducing subunit of receptors for glial cell line-derived neurotrophic factor (GDNF) and related neurotrophic factors (Baloh *et al.*, 2000). Ret gene targeting in mice results in early postnatal death due to developmental defects in, e.g., the kidneys and peripheral nervous system (Durbec *et al.*, 1996; Schuchardt *et al.*, 1994). Ret is also expressed in the dopaminergic neurons and GDNF and related factors promote their survival both *in vitro* and *in vivo* (Beck *et al.*, 1995; Hoffer *et al.*, 1994; Lin *et al.*, 1993; Sauer *et al.*, 1995; Strömborg *et al.*, 1993; Tomac *et al.*, 1995; Trupp *et al.*, 1996). Although DA cells are generated in Ret-targeted mice (Marcos and Pachnis, 1996) signaling by Ret might be essential for the correct maturation of these cells as well as for functions in mature dopaminergic neurons. Indeed, several studies have indicated that Ret ligands are expressed already in the developing striatum and play important roles both in developing and in mature DA cells (Golden *et al.*, 1999; Granholm *et al.*, 2000; Lin *et al.*, 1993; Messer *et al.*, 2000; Strömborg *et al.*, 1993).

Previous studies demonstrated that Nurr1 is required for sustained gene expression of dopaminergic markers, but that many genes are initially expressed even in the absence of Nurr1. Importantly, these include Ptx3, Engrailed, and Lmx1b, transcription factors previously implicated in dopaminergic development (Saucedo-Cardenas *et al.*, 1998; Simon *et al.*, 2000; Smidt *et al.*, 1997, 2000; Wallén *et al.*, 1999). Thus, an important goal is to identify genes that are regulated by Nurr1 and may explain defective DA neuron development in Nurr1-null mice. In this report we demonstrate that Ret, which is expressed soon after Nurr1 in the developing midbrain, fails to be induced in the Nurr1 mutant DA precursor cells and in the brain stem, where Nurr1 and Ret are colocalized in the dorsal motor nucleus (DMN) of the vagus nerve.

## RESULTS

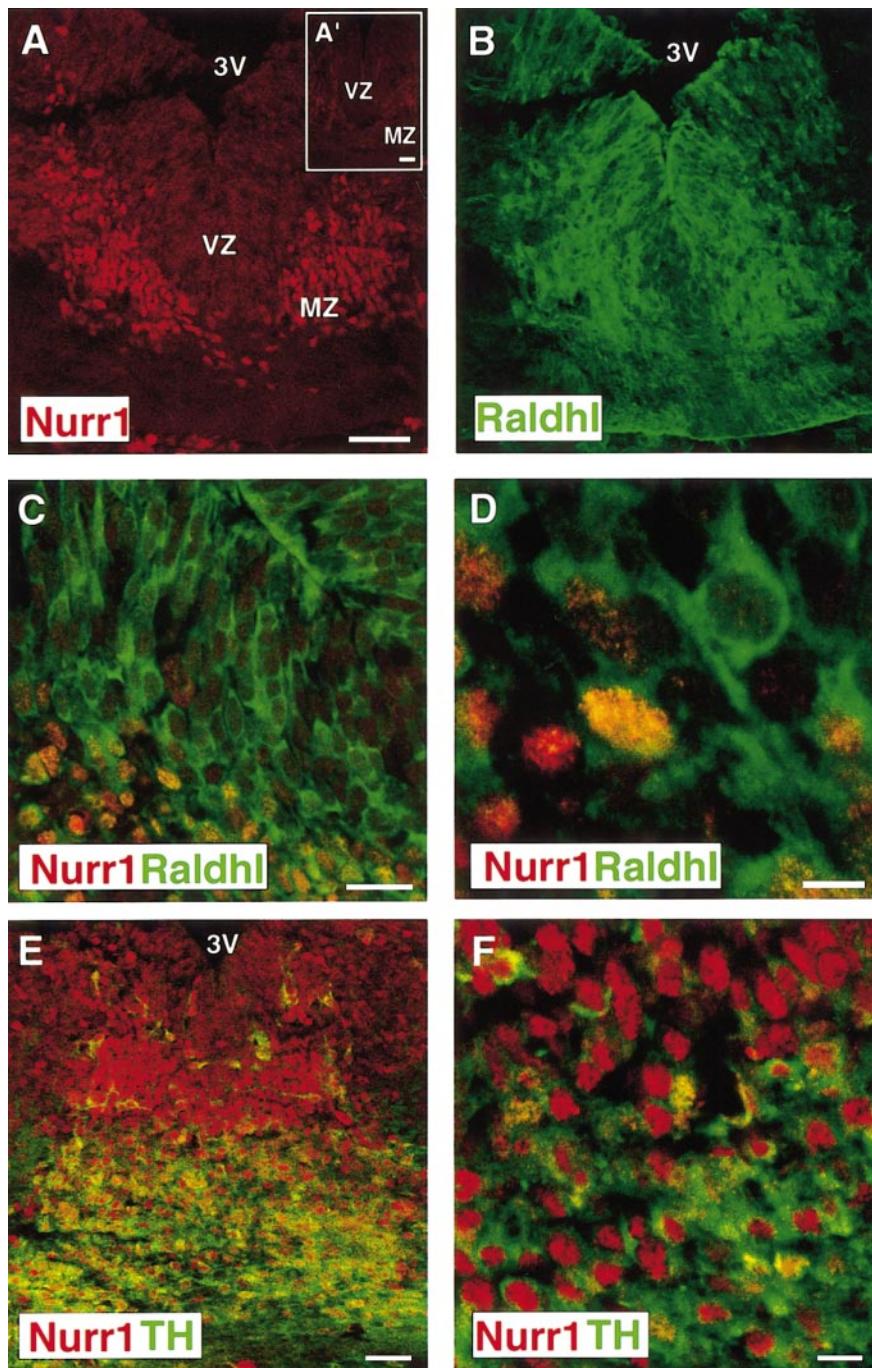
### *Nurr1 Protein Expression in the Ventral Midbrain*

Nurr1 mRNA expression in the mouse has previously been analyzed by *in situ* hybridization (ISH). Using a rabbit polyclonal antibody raised against the Nurr1 carboxy-terminal domain, Nurr1 immunoreac-

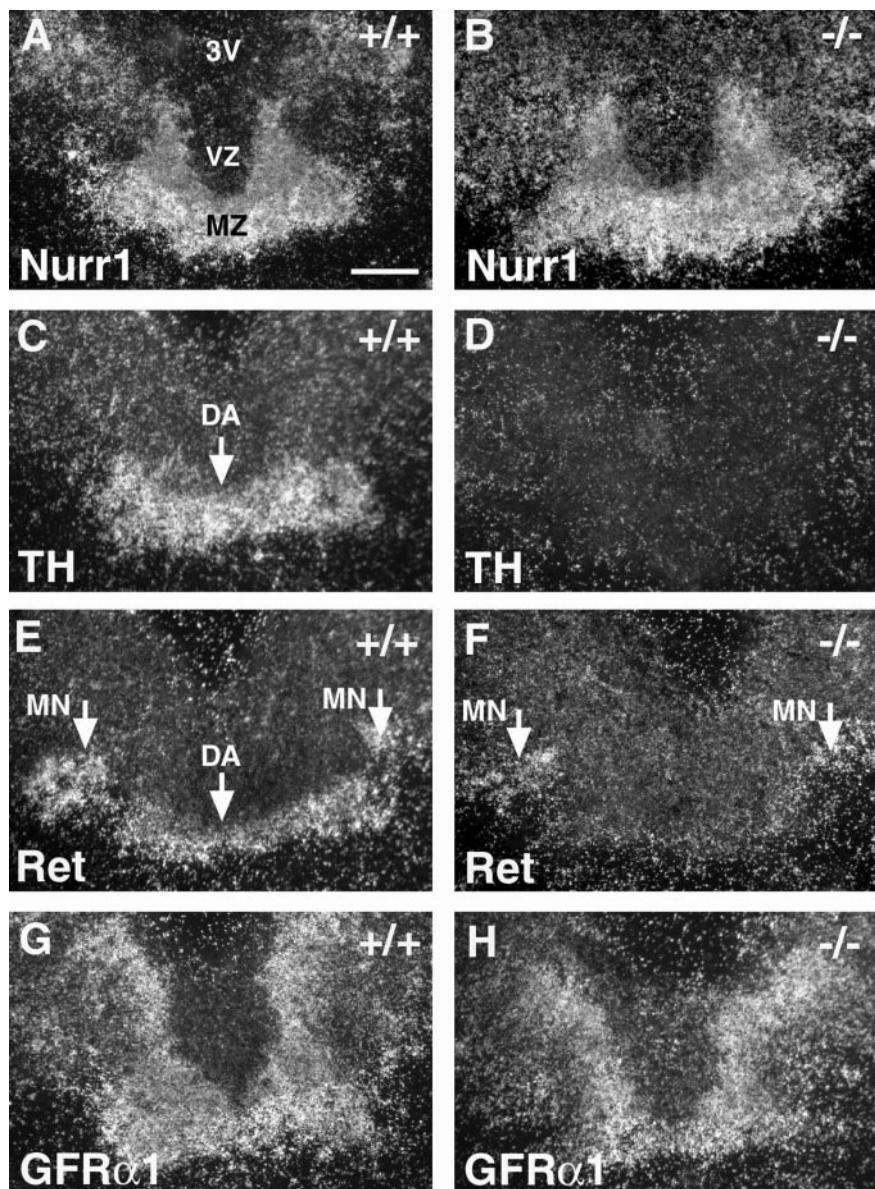
tivity (IR) was detected in the E12.5 mouse embryonal VMB. Distinct nuclear labeling outside the ventricular zone correlates well with the distribution of Nurr1 mRNA (Fig. 1A). The corresponding region in the Nurr1 mutant brain shows no Nurr1-specific IR (Fig. 1A'), demonstrating the specificity of the antibody. As expected, no tissue in the Nurr1 mutant embryo is positive for Nurr1-specific IR. Aldehyde dehydrogenase I (RaldhI, also known as AHD2; Lindahl and Evces, 1984) has been suggested as a marker for proliferating dopaminergic progenitors (Wallén *et al.*, 1999) and is expressed in both the proliferating cells in the ventricular zone and the mantle layer (Fig. 1B). Codection of Nurr1 and RaldhI shows strong cytoplasmic RaldhI IR in the proliferative ventricular zone as well as in the mantle layer of differentiating Nurr1-expressing cells. Nurr1 and RaldhI IR are perfectly colocalized in cells outside of the ventricular zone, firmly establishing RaldhI as a marker for dopaminergic progenitors (Figs. 1C and 1D). Colocalization of nuclear Nurr1 and cytoplasmic TH IR is identified in cells that have migrated farther from the ventricular zone (Figs. 1E and 1F).

### *Ret and GFR $\alpha$ 1 Expression in VMB of Nurr1 Mutant Mice*

In the search for Nurr1-regulated genes that might contribute to the Nurr1-deficient DA cell phenotype, we analyzed Ret and GFR $\alpha$ 1 expression. Ret mRNA expression can be detected in VMB by ISH in a pattern that is similar to that of TH mRNA at E11.5 (Fig. 2). At this stage, Nurr1 mRNA is expressed in a similar domain in the medial VMB and, additionally, in less mature cells closer to the ventricular cell layer (Fig. 2A). In the Nurr1 mutant, medial VMB cells can be detected with a probe that recognizes a transcript originating from the disrupted Nurr1 locus (Fig. 2B; Wallén *et al.*, 1999). TH and Ret are both induced at around E11.5, 1 day after the appearance of Nurr1 (Figs. 2C and 2E). Already at this early stage of development, Nurr1 deficiency results in the loss of Ret mRNA expression (Fig. 2F). Notably, Ret can still be detected in developing midbrain motor neurons (Fig. 2F), which normally are located lateral to the DA cells and are intact in the Nurr1 mutant midbrain (Wallén *et al.*, 1999). Analyses of VMB by ISH at several different developmental stages (E13.5, E16.5, and newborn) reveal that Ret fails to be expressed in developing DA cells in Nurr1 mutant mice. This finding was also confirmed by reverse transcriptase-coupled polymerase chain reaction of dissected VMB from wild-type and Nurr1-deficient embryos, respectively (data not shown). Thus, in addition



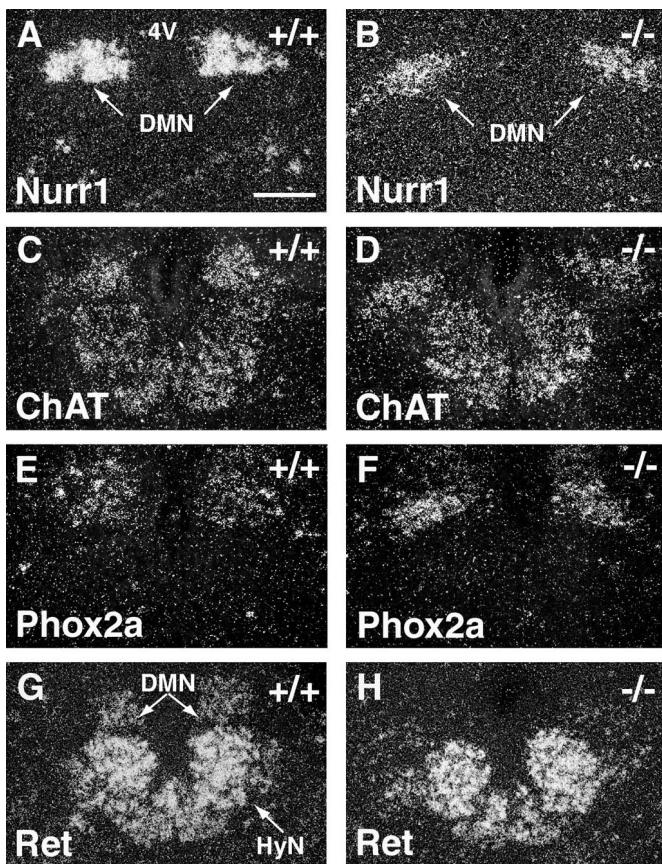
**FIG. 1.** Coronal sections showing Nurr1 protein by IR in the ventral midbrain at E12.5. Using an antibody raised against the carboxy-terminal region of Nurr1, specific nuclear IR (red) can be detected in the medial VMB outside the ventricular zone of the wild-type embryo (A), whereas no labeling is detected in the corresponding region of the Nurr1 mutant (A'). A Raldhl-specific antibody shows cytoplasmic IR (green) in both the ventricular and the periventricular zones in the same region (B). Double labeling for these two markers (Nurr1 in red and Raldhl in green) shows complete colocalization in the cells of the mantle layer (C and D). Costaining for Nurr1 (red) with TH (green) shows Nurr1 nuclear expression of differentiating DA cells with cytoplasmic TH IR (E and F). Scale bars: A (also applies for A' and B), 50  $\mu$ m; C, 20  $\mu$ m; D, 5  $\mu$ m; E, 50  $\mu$ m; F, 10  $\mu$ m. 3V, third ventricle; VZ, ventricular zone; MZ, mantle zone.



**FIG. 2.** *In situ* hybridization analysis of Ret and GFR $\alpha$ 1 mRNA in the ventral midbrain at E11.5. Adjacent coronal sections of Nurr1<sup>+/+</sup> (left) and Nurr1<sup>-/-</sup> (right) embryos showing expression patterns of Nurr1 (A and B; detection with the probe that recognizes also the disrupted Nurr1 transcript), TH (C and D), Ret (E and F), and GFR $\alpha$ 1 (G and H). As previously described, TH is not detected in the Nurr1 mutant midbrain (D). In the Nurr1<sup>+/+</sup>, Ret is detected in medial ventral midbrain DA cells as well as in the laterally located motor neurons (E). The Nurr1<sup>-/-</sup> VMB fails to display Ret expression in the medial cells, while it can be detected in the laterally located motor neurons (F). In contrast, at this stage GFR $\alpha$ 1 appears in the VMB cells in both the Nurr1<sup>+/+</sup> (G) and the Nurr1<sup>-/-</sup> (H) embryos. Scale bar: 200  $\mu$ m. 3V, third ventricle; VZ, ventricular zone; MZ, mantle zone; DA, DA cells; MN, motor neurons.

to TH (Fig. 2D), Ret is a second gene that is deregulated already from the earliest stages in the Nurr1 mutant medial VMB, where DA cells normally develop. In contrast, GFR $\alpha$ 1 is induced even in the absence of Nurr1 (Figs. 2G and 2H) although this expression, similar to

other dopaminergic markers, is abolished at later stages (E16.5) of development in mutant animals. In conclusion, impaired Ret mRNA expression in VMB DA neurons represents an early deficiency of Nurr1 gene-targeted embryos.



**FIG. 3.** *In situ* hybridization analyses of the brain stem in the newborn mouse. Serial coronal sections of Nurr1<sup>+/+</sup> (left) and Nurr1<sup>-/-</sup> (right) pups showing the mRNA expression of Nurr1 (A and B), ChAT (C and D), Phox2a (E and F), and Ret (G and H). In the newborn pup, Nurr1 can easily be detected in the DMN of the vagus nerve, whereas no labeling is detected in the ventrally located hypoglossal nucleus (A). In the mutant pup, the probe that recognizes the disrupted transcript labels the DMN, demonstrating the presence of these cells (B). Also, labeling with the markers ChAT (E and F) and Phox2a (E and F) appears normal in the Nurr1<sup>-/-</sup> compared to the Nurr1<sup>+/+</sup>. In contrast to the wild-type DMN, Ret cannot be detected in the Nurr1<sup>-/-</sup> nucleus at this stage (G and H). Scale bar: 400  $\mu$ m. 4V, fourth ventricle; DMN, dorsal motor nucleus; HyN, hypoglossal nucleus.

#### Nurr1 and Ret Expression in the Brain Stem

The results suggest that Nurr1 may be regulating Ret mRNA expression also at other sites where Nurr1 and Ret are coexpressed. Ret is expressed in cranial and spinal motor neurons. In the newborn mouse, Nurr1 was detected in the cranial DMN of the vagus nerve (Fig. 3A). In addition to Ret (Fig. 3G), this cholinergic nucleus also expresses choline acetyl transferase (ChAT; Fig. 3C) and the homeobox transcription factor Phox2a (Fig. 3E) (Tiveron et al., 1996). Both ChAT and

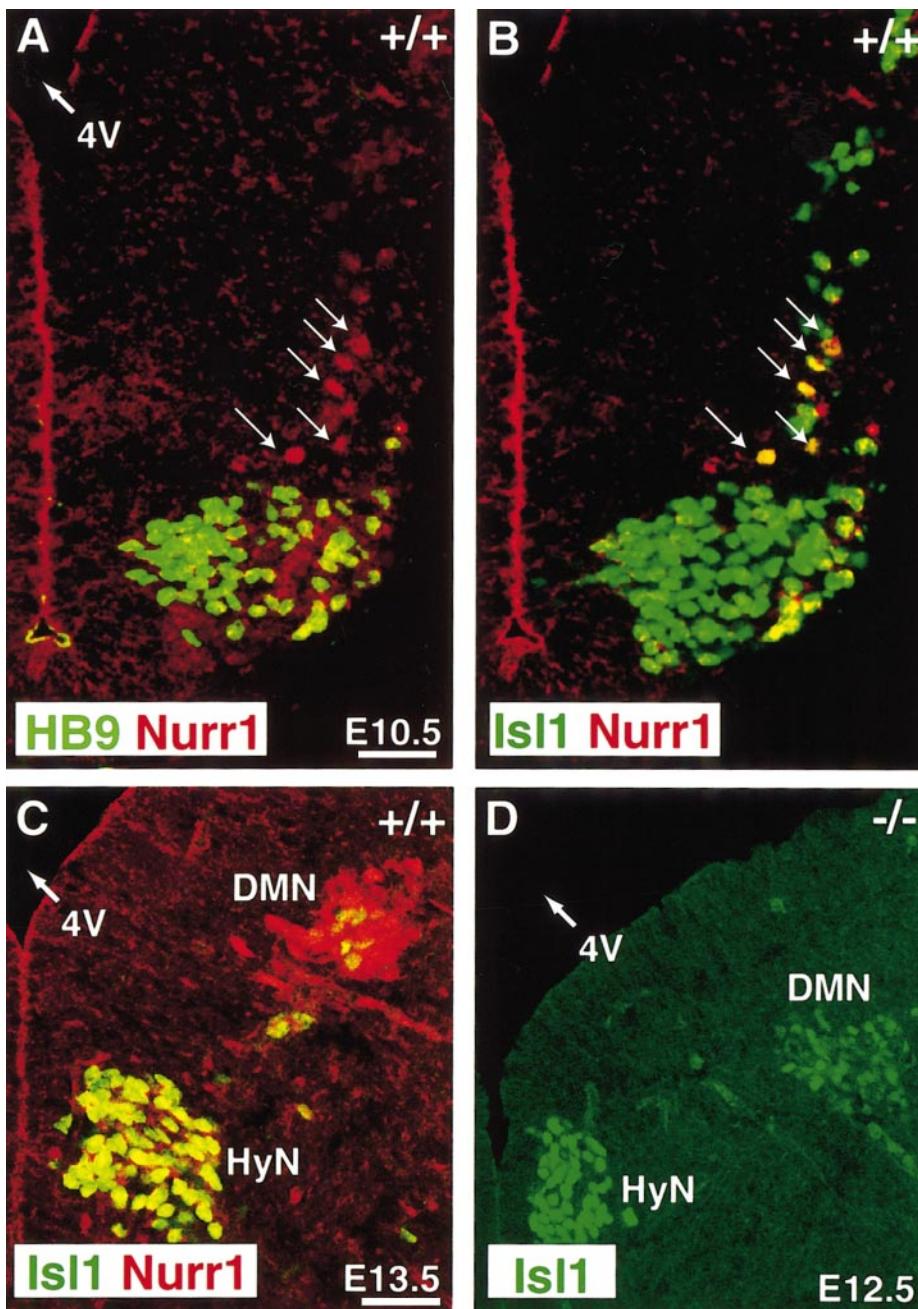
Ret are also expressed in the more ventrally localized hypoglossal nucleus. In Nurr1<sup>-/-</sup> brain stem, the DMN can be detected with the probe that specifically recognizes the disrupted Nurr1 transcript (Fig. 3B). Also, ChAT and Phox2a are expressed in these cells even in Nurr1<sup>-/-</sup> brains at birth, demonstrating that the generation of these cells does not require Nurr1 (Figs. 3D and 3F). However, neither Ret mRNA nor protein can be detected in Nurr1<sup>-/-</sup> DMN (Fig. 3H, data not shown) showing the requirement of Nurr1 for Ret expression at this stage.

Nurr1 expression in the brain stem was analyzed during development. Colocalization studies at E10.5 of Nurr1 with markers for somatic hypoglossal neurons (HB9 and Islet 1) and visceral motor neurons (Islet 1) (Briscoe et al., 2000; Ericson et al., 1997; Tsuchida et al., 1994) demonstrated that while Nurr1 is not expressed in hypoglossal cells (Fig. 4A), it is expressed already at this stage in dorsally migrating Islet 1-expressing visceral motor neurons (Fig. 4B). Nurr1 expression continues at E13.5 as the visceral motor neurons are detected at a more lateral site close to the fourth ventricle (Fig. 4C). At this stage, Islet 1 is almost completely downregulated in the DMN (Fig. 4C). Some weakly Nurr1-positive cells can also be detected in the area of the hypoglossal nucleus. In the Nurr1 mutant hindbrain, DMN Islet 1-positive cells are present (E12.5) and are localized lateral to the fourth ventricle at a normal location (Fig. 4D). Nurr1 is thus an early molecular marker for DMN cells, but Nurr1 deficiency does not lead to an apparent abnormal cellularity of this nucleus.

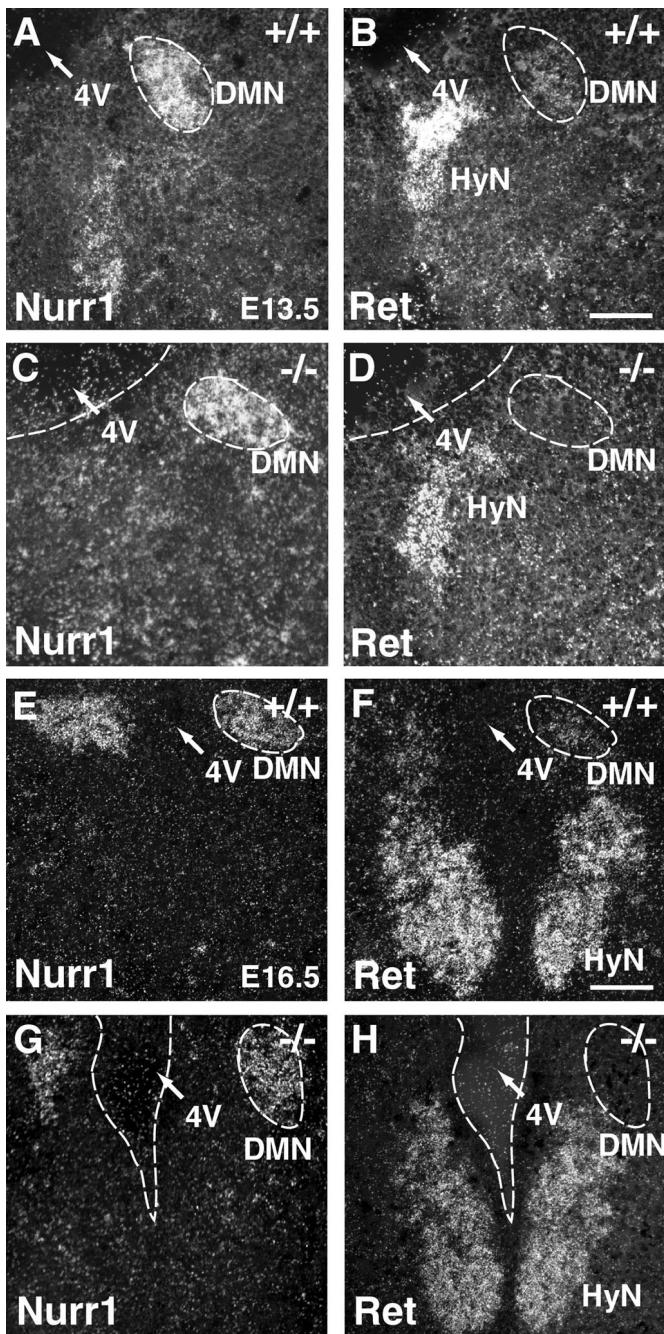
In the wild-type embryo, Nurr1 and Ret mRNA expression can be detected at E13.5 in the DMN (Figs. 5A and 5B). Although the disrupted Nurr1 transcript generated in the Nurr1 mutants can be detected (Fig. 5C), demonstrating the presence of the DMN cells, Ret expression is below detection level in the Nurr1 mutant DMN (Fig. 5D). Similarly, at E16.5 Ret expression is detected in the wild-type DMN, but is absent in Nurr1 mutant embryos (Figs. 5E–5H). Ret expression is normal in the ventral horns of the spinal cord and ureteric buds of the kidney, sites where Ret and Nurr1 are not coexpressed (data not shown). In conclusion, Ret expression is dependent on Nurr1 already from early developmental stages in both regions where these two genes are colocalized.

#### Normal Peripheral Cholinergic Innervation in Nurr1 Mutant Mice

Although DMN cells are generated in Nurr1-null mice, we speculated that the loss of Nurr1 and the



**FIG. 4.** Nurr1 protein expression in coronal sections of the developing brain stem. Localization at E10.5 (A and B), E13.5 (C), and E12.5 (D) of Nurr1 protein (A–C, red); HB9 (A, green), a marker for somatic motor neurons; and Islet 1 (B–D, green), a marker for both somatic and visceral motor neurons. At rhombomere 6–7 level, somatic motor neurons detected with an antibody to HB9 (green) do not express Nurr1 as no colocalization is detected (A). Nurr1-immunoreactive cells (marked by arrows) are mainly detected dorsal to the HB9-reactive somatic motor neurons that will form the hypoglossal nucleus. Nurr1 does colocalize with Islet 1 (B; yellow cells marked with arrows) in cells negative for HB9 (compare A and B), i.e., visceral motor neurons. At E13.5, the visceral DMN has formed lateral to the fourth ventricle and is positive for Nurr1, whereas Islet 1 is almost completely down-regulated (C). In the Nurr1 mutant brain stem, the DMN is present as shown by Islet 1 IR at E12.5 (D). Section is at an angle slightly different from that in (C). Scale bars: A, 65  $\mu$ m (also applies for B); C, 90  $\mu$ m (also applies for D). 4V, fourth ventricle; DMN, dorsal motor nucleus; HyN, hypoglossal nucleus; Isl1, Islet 1.



**FIG. 5.** Nurr1 and Ret mRNA expression in the brain stem. Serial coronal sections of E13.5 (A–D) and E16.5 (E–H) brain stems with ISH for Nurr1 (A, C, E, G) and Ret (B, D, F, H). DMN on right side (A–H) and 4V (C, D, G, H) are encircled with dotted lines for ease of identification. Nurr1 mRNA is detected in the DMN at E13.5 (A) and weak but distinct expression of Ret mRNA is detected in the DMN and in the hypoglossal nucleus (B). In the Nurr1 mutant, the detection of the DMN is by the probe that recognizes the disrupted Nurr1 transcript (C). No Ret signal is detected in the Nurr1 mutant DMN, but can be seen in the hypoglossal nucleus (D). Similarly, at E16.5, the DMN is recognized by ISH for Nurr1 in the wild-type and mutant

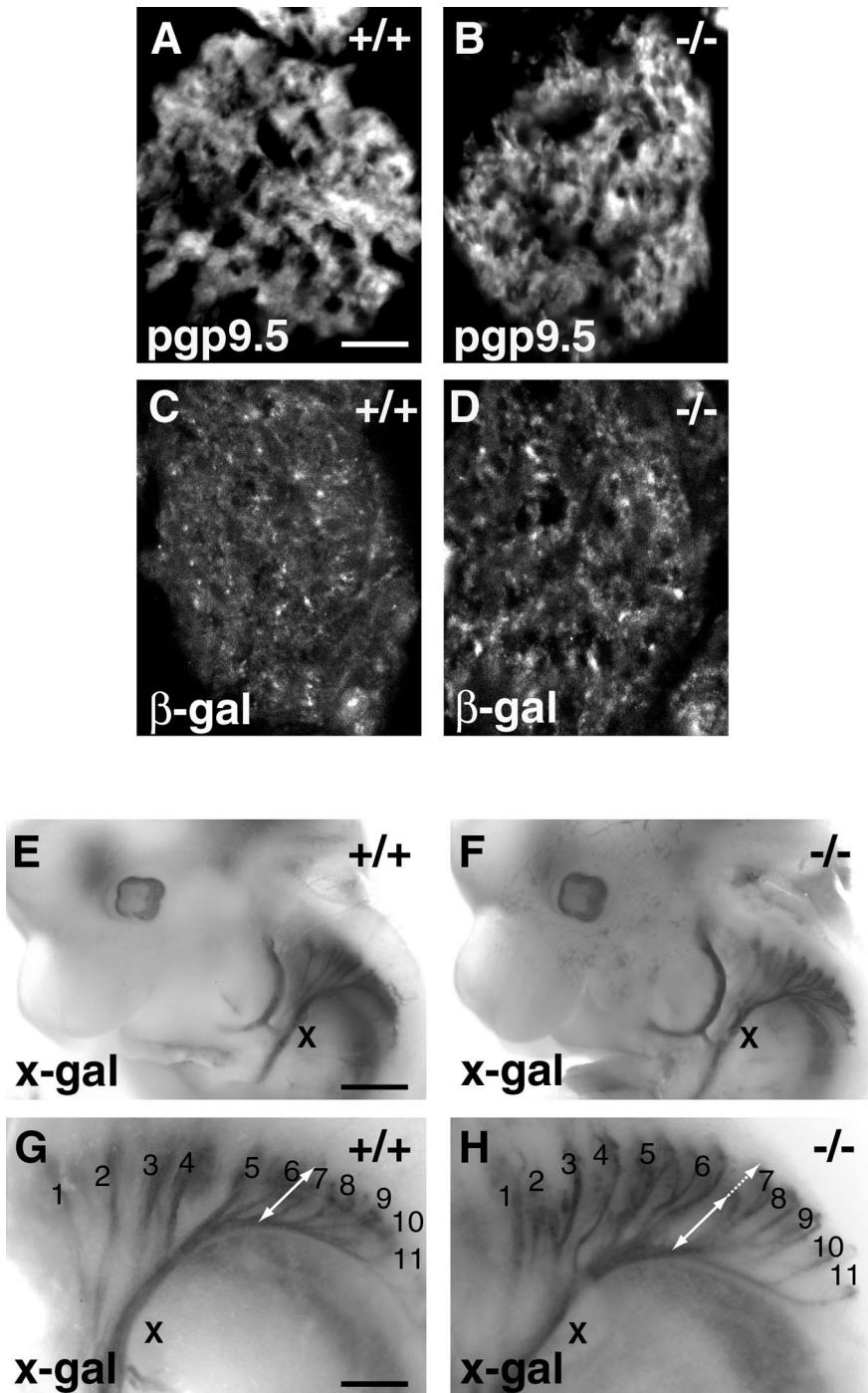
deregulation of Ret and possibly other genes might result in a functional deficiency of the vagus nerve or in its ability to correctly innervate peripheral targets. Immunohistochemical (IHC) analysis localized a strong Nurr1 expression in the DMN at E13.5 (Fig. 4), whereas a weaker expression could be detected in the nucleus ambiguus, an additional cranial nucleus which contributes efferents to the vagus nerve. In newborn mice, Nurr1 expression was detected only in the DMN (Fig. 3, data not shown).

Although the vagus nerve could be visualized by immunolabeling for the general neuronal marker pgp9.5 in both wild-type and Nurr1<sup>−/−</sup> embryos at E15.5 (Figs. 6A and 6B), it was important to specifically analyze DMN efferent fibers, which correspond to about 20% of total vagus nerve fibers. For this reason, mice heterozygous for an insertion of a tau-lacZ reporter gene in the locus of the Nkx6.2 gene were interbred with Nurr1 heterozygous mice. Nkx6.2 encodes a homeodomain transcription factor expressed in the nucleus ambiguus and DMN, but not in other neurons of the vagus nerve (J.E., unpublished results; Qiu *et al.*, 1998). As expected, a subset of pgp9.5-positive fibers (Fig. 6A) in horizontal cross sections at E15.5 show IR for lacZ-encoded β-galactosidase protein in Nkx6.2<sup>+/−</sup>:Nurr1<sup>+/+</sup> nerve bundles (Fig. 6C). The pattern is virtually identical in Nkx6.2<sup>+/−</sup>:Nurr1<sup>−/−</sup> nerves, indicating no gross outgrowth deficiency of vagus nerve efferents (Fig. 6D). However, whole-mount X-gal staining at E12.5 revealed a subtle abnormality characterized by disorganized fiber tracts at the cranial nuclei exit points (Figs. 6E–6H) in Nkx6.2<sup>+/−</sup>:Nurr1<sup>−/−</sup> embryos. Although the same number of fibers are present in both genotypes, they appear consistently extended in length in the Nkx6.2<sup>+/−</sup>:Nurr1<sup>−/−</sup> ( $n = 3/3$ ), whereas no Nkx6.2<sup>+/−</sup>:Nurr1<sup>+/+</sup> embryos show this phenotype ( $n = 0/4$ ). In these comparative analyses, the differences between the wild-type and the mutant embryos were confirmed by at least two observers blind to the genotype at the time of evaluation. In conclusion, although slightly distorted, no severe abnormality in the formation of vagus nerve efferent fibers is apparent in Nurr1-null mutant mice.

Ganglia of the lungs, heart, and gastrointestinal tract are primary target areas of preganglionic vagus nerve

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brain stem (E and G), whereas Ret mRNA can be detected in this nucleus in the wildtype (F) but not in the mutant (H). Scale bars: B, 80 μm (applies for A–D); F, 130 μm (applies for E–H). 4V, fourth ventricle; DMN, dorsal motor nucleus; HyN, hypoglossal nucleus.



**FIG. 6.** Analysis of the vagus nerve in the E15.5 and E12.5 embryo. (A–D) Horizontal sections at the thoracolumbar level of the mouse E15.5 trunk and (E–I) whole-mount X-gal-stained E12.5 *Nkx6.2<sup>+/-</sup>:Nurr1<sup>+/+</sup>* and *Nkx6.2<sup>+/-</sup>:Nurr1<sup>-/-</sup>* embryos. Labeling for the general neuronal marker pgp9.5 visualizes the vagus nerve in the *Nurr1<sup>+/+</sup>* embryo (A) as well as in the *Nurr1<sup>-/-</sup>* animal (B). To analyze efferent fibers only, sections from *Nkx6.2<sup>+/-</sup>:Nurr1<sup>+/+</sup>* and *Nkx6.2<sup>+/-</sup>:Nurr1<sup>-/-</sup>* embryos were analyzed for  $\beta$ -gal IR, which gives a punctuate pattern representing a subset of the pgp9.5-positive fibers (C and D). Side views (E and F) of E12.5 X-gal-stained embryos show the vagus nerve extending from the cranium into the trunk. Close-up of vagus nerve fibers (G and H) exiting in 11 fiber tracts from the cranial nuclei within the tissue. The fibers appear longer in the *Nkx6.2<sup>+/-</sup>:Nurr1<sup>-/-</sup>* brain (H) than in the *Nkx6.2<sup>+/-</sup>:Nurr1<sup>+/+</sup>* brain (G) as visualized by a full white arrow in the wildtype that is extended with a dotted arrow in the mutant to represent the total length of the fibers. Scale bars: A–D, 15  $\mu$ m; E and F, 100  $\mu$ m; G and H, 30  $\mu$ m. 4V, fourth ventricle; X, 10th cranial nerve, the vagus nerve.

fibers. To analyze innervation from the vagus nerve, pgp9.5 was used as a general neuronal marker, whereas cholinergic innervation was detected by using an assay that detects acetylcholine esterase (AChE) activity as well as IHC to vesicular acetylcholine transferase (VACHT) located in nerve terminals. Screening of target areas in lungs, esophagus, intestines (Fig. 7), and heart (not shown) for these markers revealed no abnormalities in E18.5 or newborn Nurr1 mutant animals. In the walls of lung bronchi, neuronal innervation of smooth muscle was detected by pgp9.5 and acetylcholine analyses (Figs. 7A, 7G, and 7M) and appeared normal in the mutant mice (Figs. 7D, 7J, and 7P). Also in the esophagus and intestines, neuronal innervation was normal as detected by pgp9.5 IR (Figs. 7B, 7C, 7E, and 7F), AChE assay (Figs. 7H, 7I, 7K, and 7L), and VACHT IR (Figs. 7N, 7O, 7Q, and 7R) in myenteric (Auerbach's) plexuses of external muscle wall and the Meissner's plexuses of the submucosa. In the wild-type esophagus and intestines, VACHT IR was also detected in the mucosal epithelium (Figs. 7S and 7T). Notably, in the esophagus, this VACHT IR was not detected to the same extent in the Nurr1 mutant pups (Fig. 7U), demonstrating an abnormality in this cell layer.

## DISCUSSION

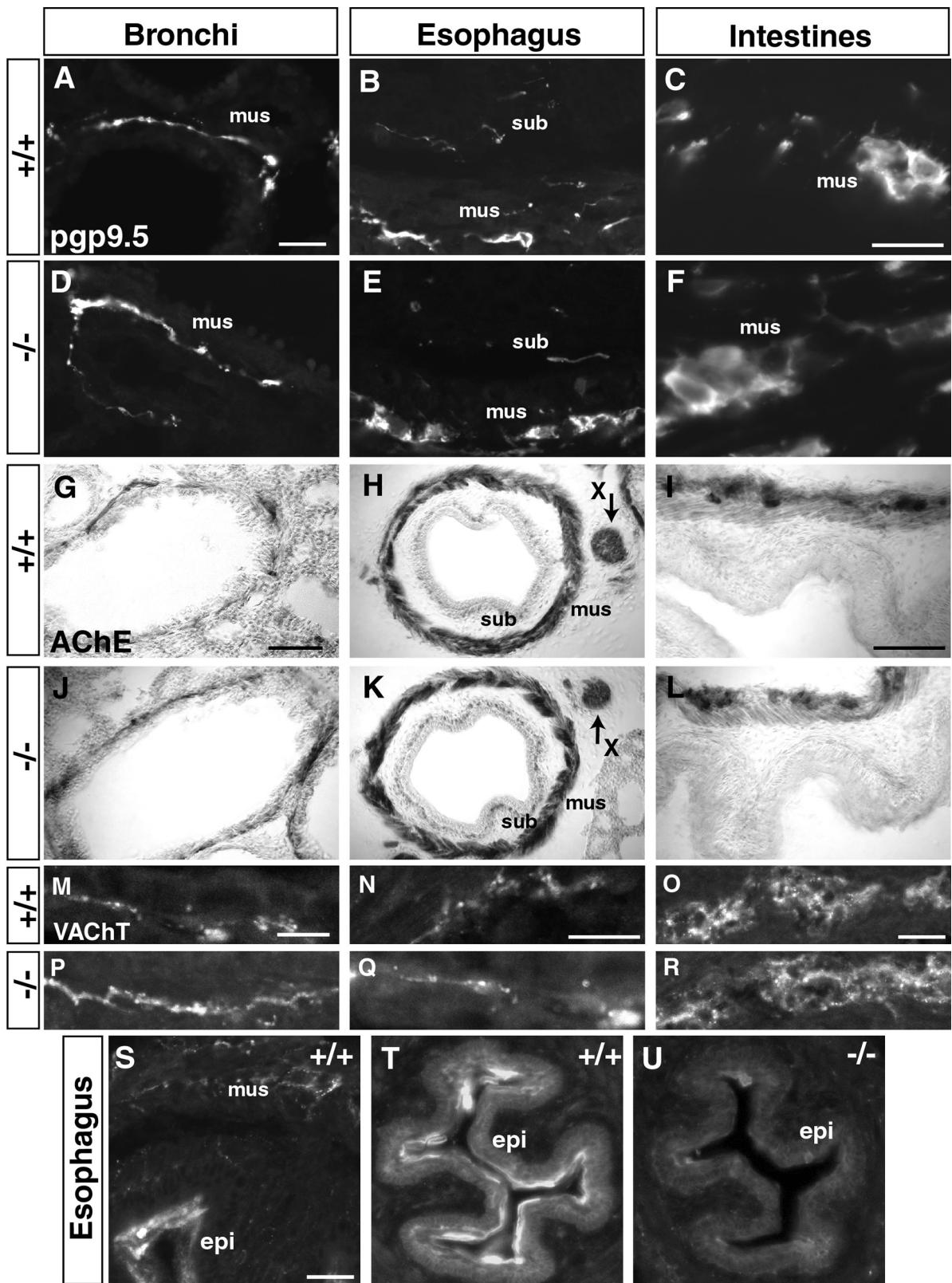
Recently, proteins important for DA cell neurotransmission, including TH and dopamine transporter, were shown to be regulated by Nurr1 in cells cultured *in vitro* (Sacchetti *et al.*, 2001; Sakurada *et al.*, 1999; Schimmel *et al.*, 1999). Regulation of TH is also manifested by the complete absence of expression in the Nurr1 mutant VMB. However, the severe developmental phenotype characterized by loss of dopaminergic markers, defective DA cell migration, and target area innervation, as well as early postnatal death, emphasizes the importance of identifying additional deregulated effector genes (Castillo *et al.*, 1998; Le *et al.*, 1999a; Saucedo-Cardenas *et al.*, 1998; Wallén *et al.*, 1999; Zetterström *et al.*, 1997). The results shown here represent the identification of the first Nurr1-regulated gene that is not related to the DA neurotransmitter phenotype but may be more directly linked to Nurr1 functions in the postnatal development of DA neurons and other functions in mature DA cells.

The regulation may be either direct or mediated via other transcription factors whose expression is regulated by Nurr1. Our preliminary characterization has not revealed a direct regulation of the Ret promoter in several cell lines transiently transfected with Nurr1.

Also, the endogenous Ret gene failed to be induced in these cell lines. However, the possibility that Nurr1 is binding to the Ret promoter and affecting its activity cannot be excluded from these experiments since additional regulatory regions not included in our reporter gene constructs may be required. Moreover, additional cell-type-specific factors not present in the tested cell lines may be essential for Nurr1-induced Ret expression. It is also possible that an as yet unidentified ligand not present in our *in vitro* culture experiments is required for Nurr1-dependent induction of the Ret gene.

Can deregulated Ret gene expression explain aspects of the Nurr1 mutant phenotype? As described, development of DA cells is severely affected in Nurr1 mutant mice and DA cell markers are not detected in VMB at birth in these animals. In contrast, DA neurons are generated in Ret-deficient animals (Marcos and Pachnis, 1996). However, detailed analyses of DA cell number, cell maturation, and the extent of target area innervation have not been reported. Thus, while deregulated Ret cannot alone explain the severe developmental deficiencies apparent in Nurr1<sup>-/-</sup> VMB, it remains possible that the absence of Ret may result in more subtle effects, e.g., on innervation density and/or axonal sprouting. Indeed, the Ret ligand GDNF is important for axonal sprouting upon striatal injury, and exogenously administered GDNF induces sprouting of DA neurons (Batchelor *et al.*, 2000; Tomac *et al.*, 1995). Moreover, transplantation of cells into adult striata have demonstrated that DA neurons from GDNF-deficient mice are severely impaired in their ability to survive and innervate host striatal tissue (Granholm *et al.*, 2000). These latter results indicate that Ret is influencing both postnatal survival and innervation. Such postnatal functions have not yet been possible to elucidate in knockout models since Ret<sup>-/-</sup> as well as Nurr1<sup>-/-</sup> mice die at an early postnatal age. In conclusion, Nurr1 is required for the embryonic expression of Ret and it seems likely that sustained Ret expression is dependent on the continuing postnatal expression of Nurr1. Nurr1 might thereby influence Ret-dependent development as well as postnatal Ret-dependent functions in DA cells.

It is well established that signaling via Ret promotes a robust survival pathway in many cell types, including VMB DA cells (Beck *et al.*, 1995; Hoffer *et al.*, 1994; Lin *et al.*, 1993; Oppenheim *et al.*, 1995; Sauer *et al.*, 1995; Tomac *et al.*, 1995; Williams *et al.*, 1996; Yan *et al.*, 1995). In models of Parkinson's disease in both rodents and primates, Ret ligands such as GDNF are efficiently influencing survival of DA cells and may likely become important in future therapies for Parkinson's disease (Björklund *et al.*, 2000; Kordower *et al.*, 2000; Olson,



2000). We propose that Nurr1 may influence responsiveness to these factors by affecting Ret gene expression. Since Nurr1 is an orphan receptor that may be regulated by as yet unidentified ligands it can be speculated that such molecules may promote survival of DA cells and stimulate DA nerve fiber growth by sensitizing DA cells to endogenous Ret ligands. Indeed, relatively small increases in the expression of the Ret coreceptor GFR $\alpha$ 1 have been shown to markedly enhance sensitivity to GDNF in superior cervical ganglion cells (Heng *et al.*, 2000). Conversely, GFR $\alpha$ 1 $^{+/-}$  mice are significantly less able to respond to GDNF as shown by the inability of GDNF preparations to counteract the effects of temporary middle cerebral artery occlusions in GFR $\alpha$ 1 heterozygotes (Tomac *et al.*, 2000). These observations support the possibility that modulation of Ret expression by Nurr1 could significantly affect cell survival via Ret signaling *in vivo*. Interestingly, this possibility is strengthened by the observation that Nurr1 heterozygous animals are more sensitive to the effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a neurotoxin that selectively induces DA cell death (Le *et al.*, 1999b).

In addition to early onset of Nurr1 expression in midbrain DA cells, we show here that Nurr1 can be detected already at E10.5 in visceral motor neurons of DMN of the vagus nerve. Also in this brain nucleus, Ret expression is colocalized with Nurr1 and dependent on its expression even from early developmental stages. This finding reveals the first Nurr1 abnormality that is independent of functions in the mesencephalic DA system. The analyses of the vagus nerve fibers derived from the DMN showed a subtle disorganization while the cholinergic target area innervation appeared normal. Interestingly, a clear decrease in VACChT IR was apparent in the epithelium of the esophagus mucosa. This region is not directly innervated and further analysis is required to establish the origin and identity of the VACChT IR. However, this defect was specific to Nurr1 mutant perinatal animals and therefore it must be directly linked to the Nurr1 deficiency. As Nurr1 is

not expressed in the esophagus or the enteric ganglia, it seems likely that defective VACChT IR results from the lack of Nurr1 in the DMN. In fact, the pattern of decreased VACChT IR supports this assumption. Cholinergic innervation derived from DMN is nonuniform with more extensive innervation occurring in posterior abdominal levels of the esophagus (Sang and Young, 1998). Strikingly, decreased VACChT expression in Nurr1-null mice was mainly observed at abdominal and not at cervical levels, in good agreement with the pattern of vagus nerve innervation.

Agenesis of DA neurons in Nurr1 knockout mice is unlikely to be the explanation for the early postnatal death of mutant animals. Nurr1 knockout mice can easily be identified due to lack of milk in their stomachs. Furthermore, milk has not been detected in the upper gastrointestinal tract in newborn Nurr1 $^{-/-}$  animals (our unpublished observation), demonstrating that Nurr1-null mice are unable to feed or are not fed by their mothers. It seems unlikely, however, that the subtle defect of esophageal mucosa is the reason for the inability to feed. Notably, the DMN is also essential for normal breathing in a circuitry also involving the nucleus ambiguus (Feldman *et al.*, 1986), an additional brain-stem region where Nurr1 is transiently expressed during development. Accordingly, Nurr1 might have important functions associated with respiration, which could also explain the early lethality of newborn mutant pups. It is also interesting to note that Ret deficiency results in abnormal respiration (Burton *et al.*, 1997). It will thus be important to specifically analyze breathing and oxygenation in Nurr1-null mice.

Deficient Ret mRNA expression in Nurr1 mutant mice in two defined brain nuclei identifies the first deregulated gene that is not directly related to DA neurotransmitter functions. Although this target gene cannot explain the severe DA cell developmental deficiencies observed in Nurr1 knockout mice, the results link Nurr1 to novel functions associated with cell survival and other activities exerted by ligands utilizing Ret as their signaling receptor subunit in cells where

**FIG. 7.** Horizontal trunk sections of vagus nerve target areas. Left (A, D, G, J, M, P), bronchi in the lungs; middle (B, E, H, K, N, Q), esophagus; right (C, F, I, L, O, R), intestines; and at bottom, additional sections of esophagus (S-U). (A-F) pgp9.5 IR in different target areas in the newborn wild-type (A, B, C) and Nurr1 mutant mouse (D, E, F), (G-L) AChE assay visualization of esterase activity in the same areas in the E18.5 wild-type (G, H, I) and Nurr1 mutant (J, K, L) embryo, and (M-R) VACChT IR in the same areas of newborn wild-type (M, N, O) and Nurr1 $^{-/-}$  (P, Q, R) pups show normal neuronal innervation of muscle in all areas and also of submucosa in esophagus and intestines. In addition to myenteric and submucosal innervation of the esophagus, staining was also detected in the mucosa epithelium in the wild-type pup (S and close up of epithelium in T); however, this was not detected to the same extent in the mutant epithelium (U). Scale bars: A (also applies for B, D, E), C (also F), M (also P), N (also Q), O (also R), S (also T and U), 20  $\mu$ m; G (also H, J, K) and I (also L), 40  $\mu$ m. mus, muscle; sub, submucosa; epi, epithelium; X, vagus nerve.

Nurr1 and Ret are coexpressed. Understanding the significance of these observations for cell survival of adult DA cells will be an important aim in further studies.

## EXPERIMENTAL METHODS

### Animals

The generation of Nurr1 mutant mice was described previously (Zetterström et al., 1997). Nurr1 heterozygous females were bred with Nkx6.2-tau-lacZ (J.E., unpublished data) males for generation of mice heterozygous for the two mutations and these were in turn crossed with Nurr1 heterozygous mice for generation of Nkx6.2-tau-lacZ<sup>+/−</sup>:Nurr1<sup>+/+</sup> and Nkx6.2-tau-lacZ<sup>+/−</sup>:Nurr1<sup>−/−</sup> embryos. Mice were mated during the night and females checked for vaginal plugs in the morning. Pregnant females were sacrificed by cervical dislocation and embryos collected from desired stages. For *in situ* hybridization, embryos were rapidly fresh frozen. For IHC, embryos were fixed 1–24 h in 4% phosphate-buffered paraformaldehyde (PFA) and stored in 30% sucrose. Genotyping was on tail- and amnion-derived DNA by PCR. Littermates were used in all comparative experiments. Cryosections on ProbeOn (Fisher Scientific) and SuperFrost Plus (Menzel-Gläzer) slides were prepared at 14–25  $\mu\text{m}$  thickness.

### Production of Nurr1-Specific Antibody

A GST-fusion protein of the Nurr1 ligand-binding domain was prepared according to the manufacturer's recommendations (Pharmacia). Rabbits were given a subcutaneous injection of 150  $\mu\text{g}$  fusion protein in Freund's complete adjuvant (Gibco Life Technologies) followed by booster injections every 2 weeks. For isolation of Nurr1-specific IgG, the Nurr1-GST fusion protein was coupled to CNBr-activated Sepharose and used for affinity purification of Ig from the immune sera. Nurr1-like IR was not detected in Nurr1 mutant embryos.

### In Situ Hybridization Histochemistry

Slides were incubated with end-labeled oligonucleotide probes at 42°C for 16–18 h, rinsed, and dipped in photo-emulsion (NTB2; Kodak) as previously described (Dagerlind et al., 1992; Zetterström et al., 1996a,b). Exposure was for 6 weeks followed by staining with cresyl violet and mounting. Oligoprobes to the following sequences were used: Nurr1 (both wildtype and mutant)

1430–1477 (Law et al., 1992; Wallén et al., 1999), Phox2a 29–78 (Valarce et al., 1993), ChAT 1818–1853 (Brice et al., 1989), Ret 2527–2576 (Iwamoto et al., 1993), GFR $\alpha$ 1 805–851 (Jing et al., 1996), TH 1441–1478 (Grima et al., 1985).

### Immunohistochemistry

Slides were air-dried, washed in phosphate-buffered saline (PBS), and incubated with blocking solution (3% bovine serum albumin or 0.1% fetal calf serum, 0.3% Triton X-100 in PBS or 10 mM Hepes buffer) before overnight incubation with primary antibody (AHD 2/RaldH1 1:400 (R. Lindahl),  $\beta$ -galactosidase 1:000, HB9 1:100, Islet 1 1:1000 (T. Jessell), Nurr1 1:2000, pgp9.5 1:400 (Biogenesis), Ret 1:50 (Immuno-Biological Laboratories Co. Ltd.), TH 1:100, and VACHT 1:1000 (Chemicon International, Inc.) diluted in blocking solution at 4°C. Following rinsing of slides, they were incubated with secondary antibody at 1:200 dilution (Alexa Fluor 488 and 594 IgG (Molecular Probes) or CY3-conjugated IgG (Jackson ImmunoResearch Laboratories)) for 1 h. After rinses in PBS, slides were mounted with Vectashield mounting medium (Vector).

### X-gal Staining of Embryos

Following dissection, embryos were fixed in 0.2% glutaraldehyde in PBS and incubated at 37°C overnight in staining solution (2 mM MgCl<sub>2</sub>, 0.02% NP-40, 0.01% Na-deoxycholate, 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>) with 1 mg/ml X-gal (5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactopyranoside). Embryos were postfixed in 4% PFA and placed in sucrose. Skin was removed to expose cranial nerve staining.

### Acetylcholine Esterase Activity

Sections were immersed in ice-cold 4% PFA for 15 min, washed in PBS, and incubated in staining solution (38 mM sodium acetate, 0.012% acetic acid, 4.8 mM sodium citrate, 3 mM copper sulfate, 0.08 mM tetraisopropyl pyrophosphoramido (Sigma), 0.5 mM potassium ferricyanide, 0.87 mM acetylthiocholine iodide) for 3 h after which they were rinsed in water, dehydrated, and mounted.

### Microscopical Evaluation and Image Collection

The analyses were performed on multiple embryos and pups and observations were confirmed by at least two persons. Structures were identified using published

atlases (Altman and Bayer, 1995; Kaufman, 1992; Paxinos *et al.*, 1994; Paxinos and Watson, 1986). For section analyses, data were evaluated and images were collected using a confocal microscope (Axiovert 100M; Zeiss) or regular microscopy (Eclipse E1000M; Nikon) coupled to a digital camera (Spot2; Diagnostic Instruments, Inc.). For whole-mount embryos, a dissection microscope (Nikon SMZ-2B) was used with a digital camera.

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