Phox2b controls the development of peripheral chemoreceptors and afferent visceral pathways

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Summary

We report that the afferent relays of visceral (cardiovascular, digestive and respiratory) reflexes, differentiate under the control of the paired-like homeobox gene *Phox2b*: the neural crest-derived carotid body, a chemosensor organ, degenerates in homozygous mutants, as do the three epibranchial placode-derived visceral sensory ganglia (geniculate, petrosal and nodose), while their central target, the nucleus of the solitary tract, which integrates all visceral information, never forms. These data establish *Phox2b* as an unusual 'circuit-specific' transcription factor devoted to the formation of autonomic reflex pathways. We also show that *Phox2b* heterozygous mutants have an altered response to hypoxia and hypercapnia at birth and a decreased tyrosine hydroxylase expression in the petrosal chemosensory neurons, thus providing mechanistic insight into congenital central hypoventilation syndrome, which is associated with heterozygous mutations in *PHOX2B*.

Key words: Transcription factor, Autonomic nervous system, Sensory neurons

Introduction

A key neuroanatomical substratum for the maintenance of bodily homeostasis is the set of reflex circuits of the autonomic [better termed 'visceral' (Blessing, 1997)] nervous system. These circuits modulate the activity of internal organs via a two-neuron efferent pathway that includes visceral motoneurons of the hindbrain and spinal cord, and their targets (the sympathetic, parasympathetic and enteric ganglionic neurons). The afferent pathway of visceral reflexes (Fig. 1A) conveys to the hindbrain baroreception, chemoreception (including taste) and osmoreception from blood vessels, airways and the digestive tract, and consequently modulates the discharge of visceral motoneurons as well as that of central respiratory rhythm generators. Primary visceral afferents make up the three epibranchial-derived cranial ganglia: the geniculate, petrosal and nodose. Among them, the chemoafferent neurons of the petrosal ganglion are in fact postsynaptic to bona fide chemosensors, in particular the glomus cells of the carotid body (Katz et al., 1987), responsible for sensing hypoxia, hypercapnia, low pH (Gonzalez et al., 1994) and, as recently established, hypoglycemia (Pardal and Lopez-Barneo, 2002). Primary visceral sensory neurons project onto second order visceral sensory neurons in the hindbrain, which form the nucleus of the solitary tract (nTS), the first central relay for visceral information (Blessing, 1997). Dorsal to the nTS and also projecting on it, the area postrema (AP), a circumventricular organ whose neurons are in direct contact with the bloodstream and cerebrospinal fluid, serves as a chemoreceptive center responsive to a variety of toxins and responsible for chemically induced vomiting and conditional taste aversion (Borison, 1989).

All the neuronal classes listed above (with the exception of spinal visceral motoneurons, i.e. sympathetic pre-ganglionic neurons) are, from the earliest phase of their differentiation (and irrespective of their developmental origin or eventual phenotype), marked by the expression of the same transcription factor: the paired-like homeobox gene *Phox2b* (and of its paralogue *Phox2a*) (Brosenitsch and Katz, 2002; Pattyn et al., 1997; Tiveron et al., 1996). Conversely, expression of Phox2 genes is largely restricted to these neurons, and is therefore a simple, non combinatorial predictor of their eventual integration in autonomic reflex loops. We previously showed that *Phox2b* is actually required for the differentiation of the two neuron efferent visceral reflex pathway (with the exception of sympathetic pre-ganglionic neurons, see above): in *Phox2b*-null mutants, parasympathetic ganglia never form, enteric neuron precursors never migrate past the gastroesophageal junction, and sympathetic ganglionic cells fail to undergo their pan-neural as well as type-specific differentiation and eventually degenerate (Pattyn et al., 1999). Moreover, hindbrain visceral motoneurons (i.e. pre-ganglionic enteric and parasympathetic neurons), normally born in the neuroepithelial pMNv domain of rhombomeres 2 to 7, are never generated (Pattyn et al., 2000b).

We show that, quite intriguingly, this *Phox2b*-dependency extends to the three-relay visceral sensory pathway comprising the carotid body, cranial ganglia and the nTS. In the context of our previous work, these new data reveal the developmental requirement for *Phox2b* throughout the nervous system to be unusually coherent and to correlate neither with neural phenotype, nor spatial coordinates, but connectivity.

Recently, heterozygous mutations in *PHOX2B* have been found to correlate with congenital central hypoventilation syndrome (CCHS) or Ondine's curse, a complex dysautonomic syndrome (Amiel et al., 2003). The dependency of visceral afferent pathways on *Phox2b* that we report here sheds light on the etiopathology of this disease. In addition, we show that heterozygous mutants display respiratory anomalies which partially model the impaired autonomic control of breathing, pathognomonic for CCHS.

Materials and methods

Mouse breeding, genotyping, and rescue

The generation and genotyping of *Phox2b* mutant mice have been reported previously (Pattyn et al., 1999). Homozygous *Phox2b* mutants, which normally die at midgestation, were rescued beyond E10.5 with noradrenergic agonists as described (Pattyn et al., 2000a).

Histology, immunodetection and quantitative analysis

In situ hybridization using *lacZ*, peripherin, *Rnx* (*Tlx3* – Mouse Genome Informatics), *Tbx20* and *Th* antisense riboprobes, immunohistochemistry using Phox2a, Phox2b or Lmx1b antisera, and combined in situ hybridization with immunohistochemistry were performed as previously described (Tiveron et al., 1996). Double-immunofluorescence experiments using Phox2b and Lmx1b antibodies were analyzed on a Leica microscope. Pictures were superimposed in Photoshop.

Quantitative analysis of cranial sensory ganglia in *Phox2b* mutants was carried out by calculating the surface of the ganglia on serial transverse sections stained with peripherin using the QFluoro program (Leica).

Whole-body plethysmography

Breathing variables were measured non-invasively in unanaesthetized, unrestrained pups using whole-body flow barometric plethysmography (Dauger et al., 2001). Frequency and amplitude of respiratory movements, and their product, ventilation, were calculated from the plethysmographic signal in air (baseline ventilation), and during hypoxia and hypercapnia. The respiratory tests were run and analyzed before genotyping 2 days after birth (P2) on 38 *Phox2b*$^{+/-}$ (weight, 1.52±0.15 g; mouth temperature, 33.7±0.75°C) and 44 *Phox2b*$^{+/+}$ pups (1.57±0.18 g and 33.5±0.58°C); at P6 on 18 *Phox2b*$^{+/-}$ (2.83±0.23 g and 32.1±0.35°C) and 13 *Phox2b*$^{+/+}$ pups (2.89±0.15 g, and 32.5±0.38°C); and at P10 on 18 *Phox2b*$^{+/-}$ (4.96±0.28 g and 32.69±0.25°C) and 21 *Phox2b*$^{+/+}$ pups (5.95±0.27 g and 33.5±0.12°C).

Results

Requirement of *Phox2b* for the formation of the nucleus of the solitary tract

We first examined the involvement of *Phox2b* in the development of the nTS. The nTS is an elongated nucleus which spans the dorsomedial medulla throughout most of its rostrocaudal extent (Fig. 1A). At the obex of the IVth ventricle, it is capped by the AP, a wedge of neural tissue dorsal to the lumen of the tube. We previously showed that, at birth, most if not all neurons in the nTS and AP express *Phox2b* (Pattyn et al., 1997). At E10.5, the dorsal stripe of *Phox2b* expression that extends from r4 to the caudal hindbrain (Pattyn et al., 1997) is, by its dorsoventral position and rostrocaudal extent, a likely candidate as the source of nTS precursors (Qian et al., 2001). To document the development of the nTS, we focused on levels caudal to r6. At E10.5, *Phox2b*$^{+}$ cells could be divided in a ventral and a dorsal population. The ventral population, where *Phox2b* expression started in the ventricular zone (i.e. in proliferative progenitors) corresponded to the visceral and branchial motor (vm/bm) neuronal precursors as evidenced by their post-mitotic coexpression of Phox2b and Islet1 (Fig. 1B,C) (Pattyn et al., 1997). The dorsal population (where *Phox2b* expression started in the mantle zone, i.e. postmitotically) co-expressed *Rnx* and *Lmx1b* (Fig. 1B,D,E) (Qian et al., 2002). At E11.5, the ventral bm/vm neurons had migrated dorsally to form the anlage of the dorsal motor nucleus of the vagus nerve (dmnX) and nucleus ambiguus (nA), while the dorsal population of Lmx1b^{+}/Phox2b^{+} cells had considerably expanded, suggesting an ongoing dorsal production of ventral-bound migratory cells (Fig. 1F,I). By E12.5, many of these cells had accumulated close to the incipient dmnX (Fig. 1G,I), while others were still dorsal, presumably still being born, in agreement with birth dating experiments (Taber Pierce, 1973). By E13.5, a dramatic spatial reconfiguration, which might involve active cell migration, passive displacement or both, had given to the Phox2b^{+}/Lmx1b^{+} population the recognizable, almost mature shape of the nTS (Fig. 1H,I). We compared this developmental sequence in heterozygous and homozygous mutants using *lacZ* as a marker. In *Phox2b*-null mutants the dorsal population of *lacZ*$^{+}$/Lmx1b^{+}/*Rnx*$^{+}$ cells was preserved until E11.5, showing that the nTS precursors are born in *Phox2b* mutants, express *Rnx* and Lmx1b (not shown) and start migrating ventrally (Fig. 1J-M). At E12.5, however, the pattern of mutant nTS precursors was massively altered: dorsally, *lacZ*$^{+}$ cells were present, but no *lacZ* expression was detectable more ventrally, where the majority of nTS precursors had accumulated by this stage in the heterozygotes (Fig. 1N,O). No cell death was detected by TUNEL analysis (not shown) suggesting that nTS precursors undergo a fate switch. At E13.5, *lacZ* expression in the mutants was restricted to scattered cells in the lateral medulla and no nTS was detectable (Fig. 1P,Q). At E18.5, on a dorsal wholemount view and on cross-sections of the medulla, a conspicuous loss of tissue affected the region where the nTS and AP were found in the wild type (Fig. 1R-U). Altogether, these data show that the nTS and AP never form in *Phox2b*-null mutants.

Degeneration of epibranchial placode-derived ganglia and of the carotid body in the absence of *Phox2b*

Projections to the nTS come from the geniculate, petrosal and nodose ganglia – the distal ganglia of, respectively, cranial nerves VII, IX and X. These ganglia develop from epibranchial placodes. Neuronal precursors first express *Phox2a* (Tiveron et al., 1996; Valarché et al., 1993) and, in mouse, *Ngn2* (Fode et al., 1998) [*Ngn1* in chick (Begbie et al., 2002)], then delaminate and start expressing *Phox2b* as they accumulate close to their site of aggregation (Begbie et al., 2002; Fode et al., 1998; Pattyn et al., 1997). We have previously shown that these ganglia form but become atrophic by midgestation in both, *Phox2a*$^{-/-}$ and *Phox2b*$^{lacZ/lacZ}$ embryos (Morin et al., 1997; Pattyn et al., 1999). We now show that, in *Phox2b*$^{lacZ/lacZ}$

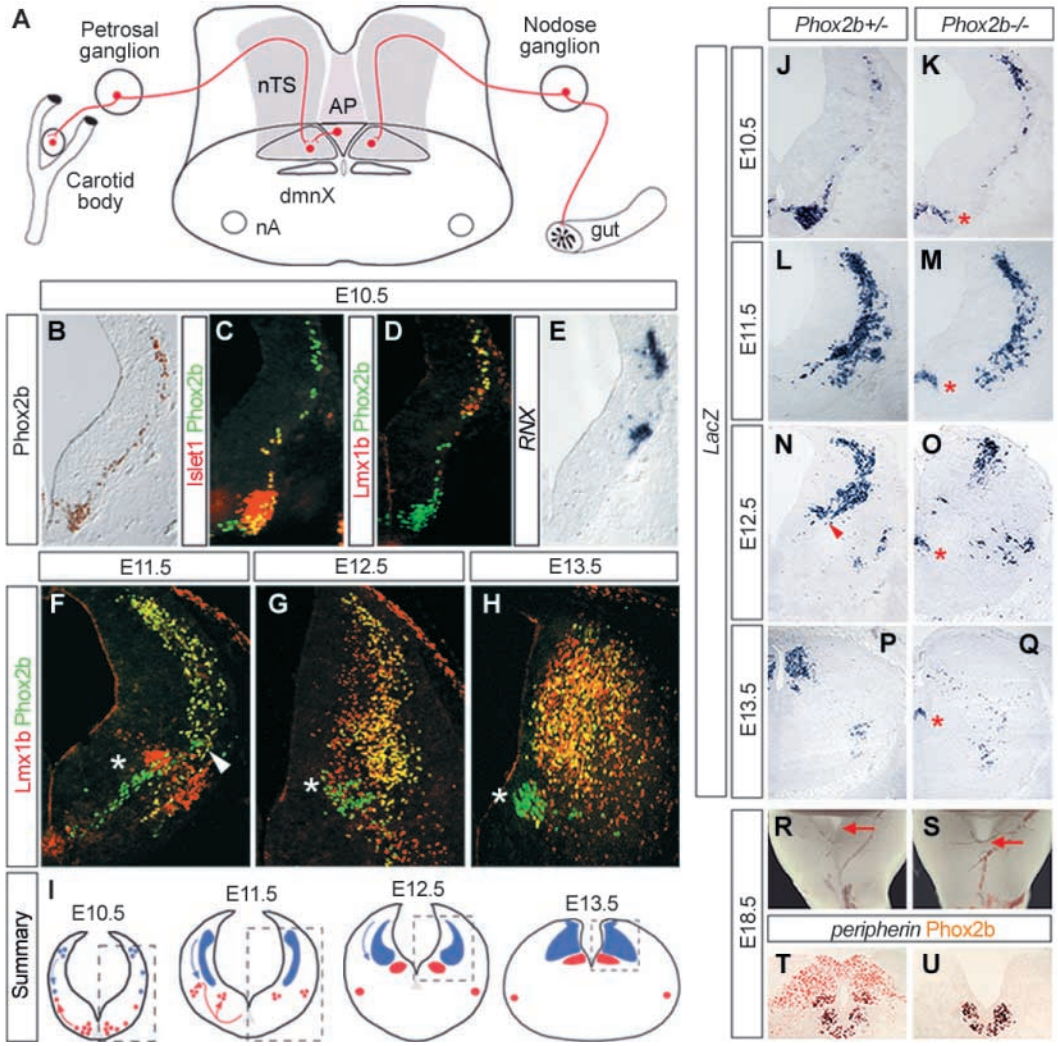

Fig. 1. The nTS and AP do not form in *Phox2b*$^{lacZ/lacZ}$ mutants. (A) Position of the mature nTS in the dorsal medulla and connections with the area postrema (AP) and cranial sensory ganglia. A fraction of sensory cells in the petrosal ganglion are postsynaptic to chemosensors of the carotid body. Most sensory cells in the nodose ganglion innervate sub-diaphragmatic organs, such as the gut. (B-I) Development of the nTS from Phox2b$^+$ dorsal precursors in the medulla of wild-type embryos. (B-E) Immunohistochemistry for Phox2b, Islet1 and Lmx1b (B-D) and in situ hybridization for *Rnx* (E) show that Phox2b$^+$ precursors at the level of r7 can be subdivided into motoneuron and nTS precursors according to their co-expression of Islet1 (C), or Lmx1b (D) and Rnx (E), respectively. Red cells in C correspond to Islet1$^+$/Phox2b$^-$ somatic motoneurons. (F-H) Immunohistochemistry for Phox2b and Lmx1b between E11.5 and E13.5 reveals the migration of both populations towards each other resulting in the formation of the dmnX (asterisk) and nA (arrowhead in F, not visible in G,H) (green), and of the nTS, whose cells co-express Phox2b and Lmx1b (yellow). Red cells correspond to several classes of Lmx1b$^+$/Phox2b$^-$ interneurons. (I) Schematic representation of the origin and migratory behavior of the dmnX, nA (red) and nTS (blue) precursors. The boxed areas are those photographed in B-H. (J-U) The nTS and AP do not form in *Phox2b*$^{lacZ/lacZ}$ mutants. (J-M) A normal complement of dorsal cells detected by *lacZ* in situ hybridization are born and migrate ventrally in a homozygous (K,M) compared with a heterozygous (J,L) mutant between E10.5 and E11.5. Note that ventrally, *lacZ* expression is preserved in the neuroepithelium of *Phox2b*$^{lacZ/lacZ}$ embryos (asterisk), where it persists until at least E13.5 (see Q), but not in the mantle layer. This reflects the fact that no bm/vm motor neurons are born (Pattyn et al., 2000b). (N,O) The dorsal emergence of *lacZ*$^+$ cells continues normally in the homozygous mutants at E12.5 but no ventral accumulation occurs, and the dmnX (arrowhead, N) does not form. (P,Q) The entire dorsal vagal complex is absent at E13.5, as assessed by *lacZ* expression. (R,S) Dorsal view of a hindbrain at E18.5 showing the obex of the fourth ventricle (red arrow) caudally displaced and wider in a homozygous (S) compared with a heterozygous (R) mutant. (T,U) Transverse sections caudal to the obex stained by immunohistochemistry for Phox2b and in situ hybridization for peripherin, showing a loss of tissue in the homozygote (U) affecting the region where the dmnX, nTS and area postrema (i.e. the dorsal vagal complex) are found in the heterozygote (T). Note that the peripherin$^+$ somatic motoneurons of the hypoglossal nucleus are preserved in the mutants. AP, area postrema; dmnX, dorsal motor nucleus of the vagus nerve; nA, nucleus ambiguus; nTS, nucleus of the solitary tract.

embryos, they lose expression of *Phox2a* and *lacZ* (i.e. of the *Phox2b* locus) as early as E13.5 (Fig. 2A-F) and continue to degenerate until virtual disappearance by E16.5 (Fig. 2G-I). The atrophy of the petrosal-nodose complex, visualized by peripherin expression, was estimated at 93% by surface area measurements (Fig. 2I) and, on most sections, the ganglion appeared fragmented (not shown). The atrophy was thus more complete than in Phox2a mutants where it was estimated at 77% with the ganglion retaining histological cohesion at birth (Morin et al., 1997). This denegeration is unlikely to be secondary to the absence of the nTS, as ganglionic neurons of *Rnx* mutants survive up to birth even in the absence of their normal central target (Qian et al., 2001). Therefore, *Phox2b* expression is absolutely required for the differentiation and survival of most, and possibly all, epibranchial placode-derived ganglionic cells.

Finally, we examined the development of the carotid body. Its chemosensors, the glomus cells, which are derived from the neural crest (Le Douarin et al., 1972), express a variety of neuropeptide and neurotransmitter synthetic enzymes,

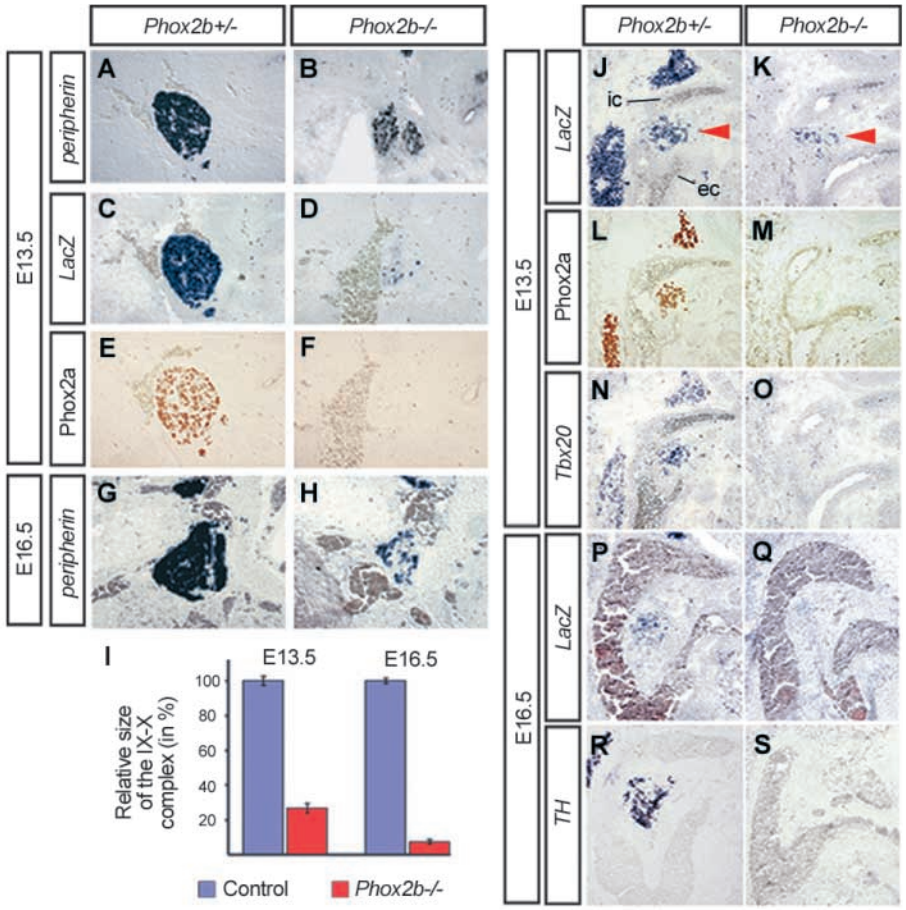

Fig. 2. Involution of the petrosal-nodose ganglionic complex and carotid body between E13.5 and E16.5 in *Phox2b*$^{lacZ/lacZ}$ mutants. (A-F) At E13.5 the petrosal-nodose complex is already markedly atrophic, as assessed by peripherin in situ hybridization (A,B, quantified in I) and has lost expression of *lacZ* (C,D) and Phox2a (E,F), which was initially expressed in the mutant placodal precursors at E10.5 (Pattyn et al., 1999). (G,H) In situ hybridization with peripherin showing that, at E16.5, the involution is almost complete. (I) Quantification of the ganglionic atrophy at E13.5 and E16.5. Measurements of control ganglia were considered as 100±5.5% at E13.5 (*n*=6) and 100±3.7% at E16.5 (*n*=4). The relative measurements of mutant ganglia are 25.8±5.3% at E13.5 (*n*=4) and 7±2% at E16.5 (*n*=4). (J-O) The anlage of the carotid body is first detected at E13.5 in a heterozygous mutant (arrowhead in J) by immunohistochemistry against Phox2a (L) or in situ hybridization for *lacZ* (J) or *Tbx20* (N). In a homozygous mutant, *lacZ* expression is already affected at this stage (arrowhead in K) and neither Phox2a (M) nor *Tbx20* (O) expression occurs. (P-S) At E16.5 the carotid body, which still expresses *lacZ* (P) and has switched on *Th* (R) in the heterozygote, is no longer detectable in the homozygote (Q,S). ec, external carotid artery; ic, internal carotid artery.

including tyrosine hydroxylase (TH) (Gonzalez et al., 1994) as well as the transcription factors Phox2a and Phox2b (Brosenitsch and Katz, 2002). In heterozygous mutants, at E13.5, when the carotid body was first detectable, a loose aggregate of cells expressing *lacZ,* Phox2a and *Tbx20* (Kraus et al., 2001) could be seen at the prospective site of carotid body formation, i.e. at the divergence of the internal and external carotids (Fig. 2J,L,N). In homozygous mutants, a smaller aggregate was also detected and expressed *lacZ*, but neither Phox2a nor *Tbx20* (Fig. 2K,M,O). At E16.5 no *Th* was detectable and *lacZ* expression itself was lost from the carotid sinus of null mutants (Fig. 2P-S). The small size of the carotid body and the abundance of naturally occurring cell death in the surrounding mesenchyme prevented a TUNEL analysis. Therefore, in *Phox2b*$^{lacZ/lacZ}$ embryos, the carotid body forms, but never expresses *Phox2a*, *Tbx20* or *Th* and then, presumably, degenerates.

A haploinsufficient respiratory phenotype in *Phox2b* mutants

From a physiopathological standpoint, our finding that *Phox2b* controls the ontogeny of afferent visceral pathways is directly relevant to the recent report that heterozygous mutations in human *PHOX2B* are frequently associated with CCHS syndrome (Amiel et al., 2003). The defining dysfunction of CCHS is in the autonomic control of breathing resulting in hypoventilation, most severe during the non-rapid eye movements phase of sleep (Gozal, 1998). The proposed mechanism of CCHS is an impairment of central integration of chemoreceptor input (Spengler et al., 2001). Furthermore, post-mortem examinations have revealed abnormal carotid bodies in two individuals (Cutz et al., 1997). Thus, the carotid body, the petrosal ganglion that innervates it and the nTS which integrates chemoreceptive inputs are all candidates for the main site(s) of dysfunction. The dependency of all three structures on *Phox2b* in mice provides the staightforward basis for a cell-autonomous mechanism of *PHOX2B* involvement in CCHS. It is unclear, however, to what extent the *PHOX2B* mutations implicated in CCHS [polyalanine expansions and C-terminal frame shifts (Amiel et al., 2003)] are functionally equivalent to the mouse mutation, which is presumably a null (Pattyn et al., 1999). To investigate whether some aspects of CCHS are modeled by heterozygous mutant mice, we studied the respiratory phenotype of freely moving *Phox2b*$^{lacZ/+}$ and *Phox2b*$^{+/+}$ pups. We first tested pups at 48 hours after birth to minimize the possible confounding effects of postnatal development and recovery processes. [Such recovery of respiratory impairments have been reported, for example, in heterozygous *Mash1*$^{+/–}$ (Dauger et al., 1999).] We examined the ventilatory changes caused by hypoxia (5% O_2), which are mainly mediated by afferences from the carotid body glomus cells to the nTS via the petrosal ganglion. In newborns, the initial increase in ventilation caused by hypoxia is followed by a strong ventilatory depression (Bissonnette, 2000). We found that hypoxia resulted in a similar increase in ventilation in *Phox2b*$^{lacZ/+}$ and *Phox2b*$^{+/+}$ pups, but the total duration of post-

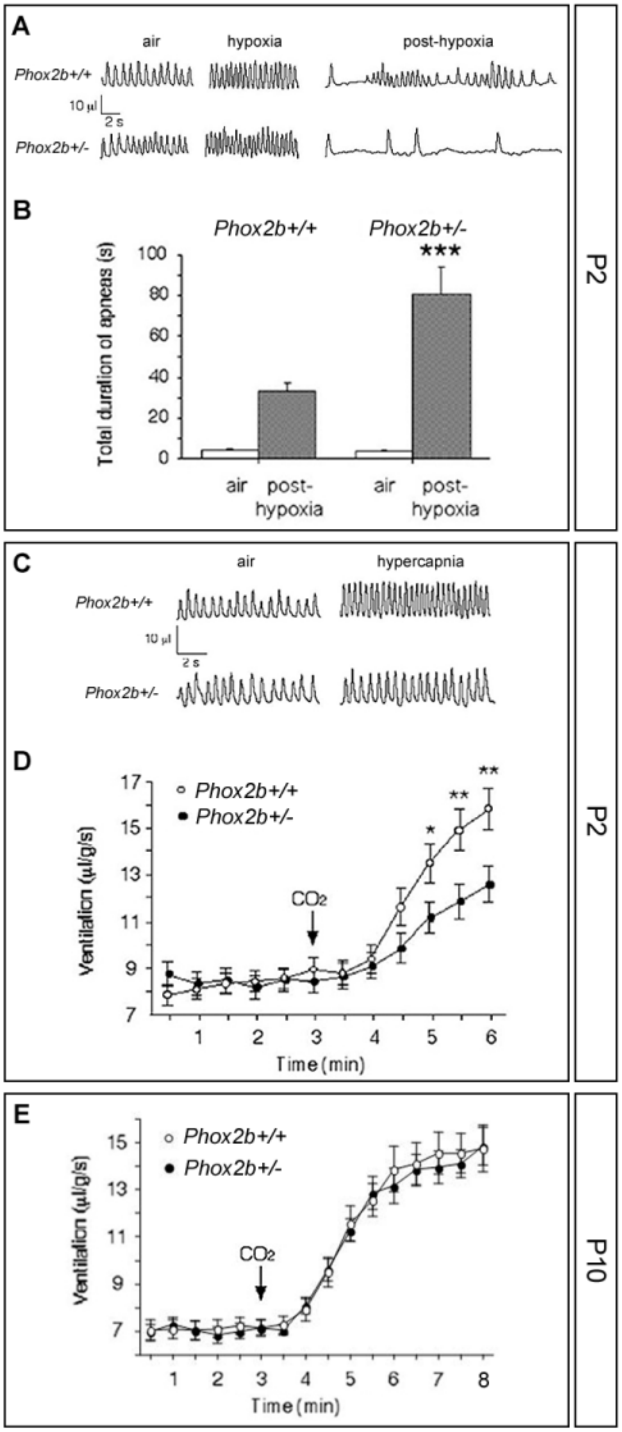

Fig. 3. Abnormal ventilatory response of heterozygous mutants to hypoxia and hypercapnia. (A) Ventilatory tracings in one *Phox2b*$^{+/+}$ pup (top) and one *Phox2b*$^{lacZ/+}$pup (bottom). Both pups had similar baseline ventilation (air) and initial increase during hypoxia, but the *Phox2b*$^{lacZ/+}$ pup showed long post-hypoxic apneas (flat respiratory tracing). (B) Total duration of apneas (defined as respiratory pauses longer than twice the duration of the preceding breathing cycle) in air condition (3 minutes) and post-hypoxic condition (6 minutes). Apneas (0.7 and 2.8 apneas/minute in *Phox2b*$^{lacZ/+}$ mice; 0.8 and 2.8 apneas/minute in *Phox2b*$^{+/+}$ mice) were strikingly longer in *Phox2b*$^{lacZ/+}$ mice (***P<0.001). Values are group means±s.e.m. (C) Ventilatory tracings in one *Phox2b*$^{+/+}$ pup (top) and one *Phox2b*$^{lacZ/+}$ pup (bottom). Both pups had similar baseline ventilation but the *Phox2b*$^{lacZ/+}$ pup showed a lower ventilatory response to hypercapnia. (D) The ventilatory increase during hypercapnia (three minutes) in *Phox2b*$^{lacZ/+}$ pups was about half that in *Phox2b*$^{+/+}$ pups, because of their lower increase in breathing frequency (not shown). *P<0.05; **P<0.01. Values are group means±s.e.m. (E) Ventilatory increase during hypercapnia at P10. The pups were exposed to 8% CO_2 for 5 minutes (instead of 3 minutes as in D) to examine possible changes in the time course of the hypercapnic response. No difference were observed between *Phox2b*$^{+/+}$ and *Phox2b*$^{lacZ/+}$ pups. Values are group means±s.e.m.

hypoxic apneas was strikingly longer in *Phox2b*$^{lacZ/+}$ than in *Phox2b*$^{+/+}$ pups (Fig. 3A,B). We also examined the ventilatory increase caused by hypercapnia (8% CO_2), which is mediated by CO_2/H^+-sensitive cells widely distributed within the brainstem and also by carotid body glomus cells (Nattie, 2001), which significantly contribute to this increase [40% in dogs (Rodman et al., 2001)]. We found that the ventilatory response to hypercapnia was markedly lower in *Phox2b*$^{lacZ/+}$ than in *Phox2b*$^{+/+}$ pups (Fig. 3C,D). It is notable that, in CCHS, a blunted response to hypercapnia is also the most prominent ventilatory defect (Spengler et al., 2001). We then examined the respiratory phenotype of *Phox2b*$^{lacZ/+}$ and *Phox2b*$^{+/+}$ pups at P6 and P10. We focused on the ventilatory response to hypercapnia, which presented the closest similarity between the CCHS and the *Phox2b*$^{lacZ/+}$ respiratory phenotypes. These responses were not distinguishable between *Phox2b*$^{+/+}$ and *Phox2b*$^{lacZ/+}$ pups at P10 (Fig. 3E), whereas 6-day-old pups presented an intermediate phenotype between 2-day- and 10-day-old pups (P<0.024, not shown).

We then looked for neuronal differentiation defects in heterozygous *Phox2b* mutants that could underlie this transient respiratory phenotype. We focused on *Th*, which is regulated by hypoxia and expressed in both glomus cells (Gonzalez et al., 1994; Wang et al., 1998) and the petrosal neurons that innervate them (Brosenitsch and Katz, 2002; Katz et al., 1987). Whereas *Th* expression was intact in glomus cells of late gestational (E16.5) heterozygous mutant embryos (Fig. 4A,B), it was decreased by 45% in the petrosal ganglion (P<0.01) (Fig. 4C,D,E). Whether this decrease is due to the disappearance of *Th*$^{+}$ neurons or their loss of *Th* expression is not known, but it shows a dose-related effect of *Phox2b* on the differentiation of at least one class of neurons known to be involved in respiratory control. At P10, when the respiratory phenotype was no longer detectable, a small but not statistically significant (P>0.1) difference in TH^{+} cell counts was found in the petrosal ganglion between wild type and heterozygous mutants (Fig. 4E).

Discussion

Phox2b as a neural circuit-specific transcription factor

We have previously shown that the two-neuron efferent pathway of parasympathetic and enteric reflexes is *Phox2b* dependent (Pattyn et al., 2000b; Pattyn et al., 1999). We now show that *Phox2b* is also required for the differentiation of peripheral afferent visceral pathways (carotid body and

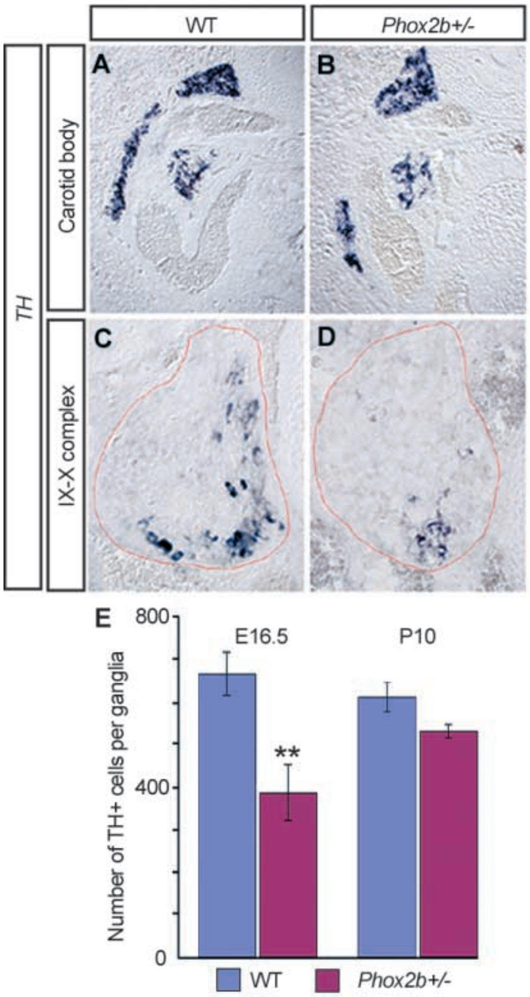

Fig. 4. Decrease in *Th* expression in the petrosal ganglion of *Phox2b*$^{lacZ/+}$ E16.5 embryos and normalization at P10. (A,B) In situ hybridization for *Th* expression shows that the heterozygous mutant glomus expresses normal levels of *Th*. (C,D) The subpopulation of petrosal ganglionic neurons, positioned ventrally, which expresses *Th* seen in the wild type in C is sparser in heterozygous mutants (D). The contour of the ganglion is outlined in red. (E) Quantification of *Th*-positive cells in the petrosal ganglion of E16.5 wild-type (n=6 ganglia) and heterozygous mutant (n=6 ganglia) embryos and P10 wild-type (n=4 ganglia) and heterozygous mutant (n=4 ganglia) pups. Values are means±s.e.m. (**P<0.01).

visceral sensory ganglia) and their central projection site, the nTS and associated AP. The afferent and efferent pathways thus defined constitute, via synapses of nTS interneurons onto visceral motoneurons, four- or five-neuron circuits that account for a variety of autonomic reflexes [examples can be found elsewhere (Blessing, 1997; Marshall, 1994)]. The dependence of the sympathetic reflex circuits is less complete, as it excludes spinal visceral motoneurons. However it does include noradrenergic sympathetic premotor neurons (Blessing, 1997; Pattyn et al., 2000a).

Conversely, the role of *Phox2b* seems largely restricted to the ontogeny of these pathways. The major class of *Phox2b*-dependent neurons, which seem not to fit in, are bm neurons (Pattyn et al., 2000b). However, from a phylogenetic perspective, this exception is only apparent. Although in amniotes, bm neurons have evolved voluntary functions in the control of head and jaw muscles, their original major function retained in fish and amphibia is the control of breathing mostly through the innervation of gill muscles. *Phox2b* thus appears to be dedicated to the differentiation of a set of neuronal classes whose sole, yet salient, point in common is to interconnect to form the reflex circuits of the visceral nervous system, most notably in its parasympathetic and enteric divisions. Indeed, the *Phox2b*-dependence of all those neurons is not paralleled by any common feature (other than their belonging to visceral reflex circuits), be it neurotransmitter phenotype, morphology, position or developmental origin (which ranges from dorsal and ventral neural tube to neural crest and neurogenic placodes).

After our suggestion of a hodological correlate for *Phox2* gene expression (Tiveron et al., 1996), *Rnx* (Qian et al., 2001) and *Math1* (Bermingham et al., 2001) have been proposed to play roles in specifying visceral and proprioceptive circuits, respectively. However, among the components of visceral reflex circuits, only the nTS and medullary noradrenergic centres have been shown to depend on *Rnx*, which is also required for proper formation of somatic sensory neurons and their connections (Qian et al., 2002). *Math1* is necessary for the development of several of the synaptic relays that partake in sensory proprioceptive pathways, but its requirement is limited to the afferent arms of these circuits, the outputs of which are under different genetic control. Hence, Phox2b stands out, so far, as a transcriptional regulator, dependence on which defines entire reflex pathways, including their afferent and efferent components. This unusually exhaustive correlation suggests that the expression of *Phox2b* is causal to the only common property of *Phox2b*-dependent neurons: their eventual synaptic connection to other *Phox2b*-dependent neurons. Remarkably, the closest structural relative of *Phox2b*, *Drg11*, is expressed in both, primary and secondary somatic sensory neurons (Saito et al., 1995) and required for projection of the former to the latter (Chen et al., 2001). However, in the case of *Phox2b*, such a role cannot be tested in simple knockouts, as abrogation of *Phox2b* function entails an early differentiation block (Pattyn et al., 2000a; Pattyn et al., 2000b; Pattyn et al., 1999).

How could expression of a same transcription factor by two (or more) neuronal classes determine their interconnection? As proposed by Lin et al. (Lin et al., 1998), the same transcription factor could, in two synaptic partners, regulate the expression of homophilic molecules, such as cadherins, which are thought to be required for synaptogenesis. This scenario could underlie some cases of matching expression of the Ets-class transcription factors PEA3 and ER81 by connected proprioceptive sensory and motor neurons of the spinal cord observed in chick (Lin et al., 1998) [but not in mouse (Arber et al., 2000)]. However, unlike PEA3 and ER81, Phox2b is expressed very early, before any neurite outgrowth. Therefore, no aspect of the axonal navigation of visceral neurons can be instructive for Phox2b expression – unlike PEA3 expression by spinal motor and sensory neurons (Haase et al., 2002; Patel et al., 2003) – and, conversely, axonal navigation itself should be under the control of Phox2b if Phox2b is to specify connectivity. One hypothesis is that *Phox2b* controls the expression in both presynaptic and postsynaptic partners of the same receptor for a chemotactic signal that steers the coordinated migration and appropriate positioning of their processes or cell bodies. In this respect, it is remarkable that the establishment of connectivity in visceral reflex circuits is often accompanied by such coordinated movements. For example, the nTS neurons and the dmnX neurons [the dendrites of which will eventually invade the nTS (Shapiro and Miselis, 1985)] are born at opposite ventral and dorsal poles of the rhombencephalon and migrate towards each other to form the compact and extensively connected 'dorsal vagal complex' (this study). Another example is provided by the di-synaptic extrinsic motor innervation of the enteric nervous system, which is preceded by the roughly simultaneous rostrocaudal invasion of the gut mesenchyme by enteric neuronal precursors (Taraviras and Pachnis, 1999) and vagal axons (Baetge and Gershon, 1989). Interestingly, in this case, *Phox2b* is required for the navigation of at least one partner, because, in enteric neurons, it controls the expression of Ret (Pattyn et al., 1999), a co-receptor for GDNF that is required for their migration (Natarajan et al., 2002). As *Ret* is also regulated by *Phox2b* in at least two other visceral neuronal types [sympathetic ganglia and cranial sensory ganglia (Pattyn et al., 1999)] and as GDNF-family ligands (GFLs) are also involved in the migration and/or axonal guidance of other visceral neurons, namely sympathetic and parasympathetic ganglionic neurons (Enomoto et al., 2001; Enomoto et al., 2000; Hashino et al., 2001; Honma et al., 2002), the Ret/GFL signalling system is an appealing candidate for a mechanistic underpinning of our model.

Phox2b heterozygous neonates as models of congenital central hypoventilation syndrome

Recently, the pleiotropic role of *Phox2b* in the ontogeny of the visceral nervous system was given a physiopathological dimension as human *PHOX2B* was found to be mutated in a majority of cases of a complex genetic dysautonomia: congenital central hypoventilation syndrome or Ondine's Curse (Amiel et al., 2003). It has already been noted by these authors that most of the incompletely penetrant symptoms of CCHS [such as multiple neuroblastomas, Hirschprung disease, paralysis of the pupils, cardiac rhythm disturbances or dysphagia (Croaker et al., 1998; Gozal, 1998)] involve *Phox2b*-dependent neuronal classes (Pattyn et al., 1999), in these cases, autonomic ganglionic neurons. The present study provides mechanistic insight into the main, defining symptom of CCHS (impaired autonomic control of breathing) by showing that three neuronal types involved in sensing hypoxia and hypercapnia are strictly dependent on *Phox2b* for their differentiation: the carotid body, the petrosal chemoreceptors that innervate it and the nTS on which they project (Finley and Katz, 1992). Moreover, our study demonstrates that partial loss of function of *Phox2b* (by heterozygoty) leads to dysfunction of the respiratory system, which partly model the respiratory phenotype of CCHS and to dysgenesis of petrosal chemoreceptors, which may underlie this neonatal respiratory phenotype. Our data do not preclude additional haploinsufficient defects in CO_2/H^+-sensitive cells located in the carotid body, the nTS or the locus coeruleus (Nattie, 2001), which all depend on *Phox2b* (this study) (Pattyn et al., 2000a).

The recovery of the ventilatory response to hypercapnia in elder mutant pups confirms the considerable postnatal plasticity of respiratory control (Feldman et al., 2003). It was paralleled by a normalization of the number of Th^+ neurons in the petrosal ganglion – most probably owing to the de novo expression of *Th* in $Phox2^+$ neurons (Brosenitsch and Katz, 2002) – consistent with a causative role of these cells in the transient respiratory anomalies of heterozygous mutants. However, it is possible that *Phox2b*-dependent respiratory inputs are superceded by CO_2/H^+-sensitive sites that do not express *Phox2b* and are therefore spared by its mutation [e.g. the midline raphé, the hypothalamus and the fastigial nucleus of the cerebellum (Feldman et al., 2003)]. Determining whether this recovery, not observed in individuals with CCHS, reflects a difference in the control of breathing between humans and mice, or in the severity of the *Phox2b* mutation will await future studies. The polyalanine extension and C-terminal frame shift mutation found in CCHS (Amiel et al., 2003) could lead to hypomorphic or dominant negative alleles or even cellular toxicity in the case of polyalanine extensions, which may not be modeled by the mouse mutation. However, as frame-shift mutations cause a clinically indistinguishable syndrome, a dose-related effect, resulting from haploinsufficiency or dominant-negative action, seems most likely.

It should be noted that *Phox2b* expression persists at postnatal stages in several neural structures, such as the carotid body (Brosenitsch and Katz, 2002) or the nTS (Pattyn et al., 1997). Therefore, it is conceivable that, apart from developmental defects such as the one we report in petrosal chemoreceptors, the respiratory disorder of CCHS could also reflect the disruption of post-developmental roles of *Phox2b*.

Finally, this study raises the possibility that *Phox2b* could be involved in cases of sudden infant death syndrome, in which anomalies in the catecholamine content of carotid bodies have been found (Perrin et al., 1984). More generally, given the richness and variability of the clinical picture of CCHS, it is tempting to speculate that mutations in this gene could underlie yet other congenital autonomic dysfunctions or dysplasia.

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References

Amiel, J., Laudier, B., Attié-Bitach, T., Trang, H., de Pontual, L., Gener, B., Torchet, D., Simonneau, M., Vekemans, M., Munnich, A. et al. (2003). Polyalanine expansion and frame shift mutations of the paired-like homeobox gene PHOX2B in congenital central hypoventilation syndrome (Ondine's curse). *Nat. Genet.* **33**, 459-461.

Arber, S., Ladle, D. R., Lin, J. H., Frank, E. and Jessell, T. M. (2000). ETS gene Er81 controls the formation of functional connections between group Ia sensory afferents and motor neurons. *Cell* **101**, 485-498.

Baetge, G. and Gershon, M. D. (1989). Transient catecholaminergic (TC) cells in the vagus nerves and bowel of fetal mice: relationship to the development of enteric neurons. *Dev. Biol.* **132**, 189-211.

Begbie, J., Ballivet, M. and Graham, A. (2002). Early steps in the production of sensory neurons by the neurogenic placodes. *Mol. Cell. Neurosci.* **21**, 502-511.

Bermingham, N. A., Hassan, B. A., Wang, V. Y., Fernandez, M., Banfi, S., Bellen, H. J., Fritzsch, B. and Zoghbi, H. Y. (2001). Proprioceptor pathway development is dependent on Math1. *Neuron* **30**, 411-422.

Bissonnette, J. M. (2000). Mechanisms regulating hypoxic respiratory depression during fetal and postnatal life. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **278**, R1391-R1400.

Blessing, W. W. (1997). *The Lower Brainstem and Bodily Homeostasis*. New York London: Oxford University Press.

Borison, H. L. (1989). Area postrema: chemoreceptor circumventricular organ of the medulla oblongata. *Prog. Neurobiol.* **32**, 351-390.

Brosenitsch, T. A. and Katz, D. M. (2002). Expression of Phox2 transcription factors and induction of the dopaminergic phenotype in primary sensory neurons. *Mol. Cell. Neurosci.* **20**, 447-457.

Chen, Z. F., Rebelo, S., White, F., Malmberg, A. B., Baba, H., Lima, D., Woolf, C. J., Basbaum, A. I. and Anderson, D. J. (2001). The paired homeodomain protein DRG11 is required for the projection of cutaneous sensory afferent fibers to the dorsal spinal cord. *Neuron* **31**, 59-73.

Croaker, G. D., Shi, E., Simpson, E., Cartmill, T. and Cass, D. T. (1998). Congenital central hypoventilation syndrome and Hirschsprung's disease. *Arch. Dis. Child* **78**, 316-322.

Cutz, E., Ma, T. K., Perrin, D. G., Moore, A. M. and Becker, L. E. (1997). Peripheral chemoreceptors in congenital central hypoventilation syndrome. *Am. J. Respir. Crit. Care Med.* **155**, 358-363.

Dauger, S., Aizenfisz, S., Renolleau, S., Durand, E., Vardon, G., Gaultier, C. and Gallego, J. (2001). Arousal response to hypoxia in newborn mice. *Respir. Physiol.* **128**, 235-240.

Dauger, S., Renolleau, S., Vardon, G., Nepote, V., Mas, C., Simonneau, M., Gaultier, C. and Gallego, J. (1999). Ventilatory responses to hypercapnia and hypoxia in Mash-1 heterozygous newborn and adult mice. *Pediatr. Res.* **46**, 535-542.

Enomoto, H., Heuckeroth, R. O., Golden, J. P., Johnson, E. M. and Milbrandt, J. (2000). Development of cranial parasympathetic ganglia requires sequential actions of GDNF and neurturin. *Development* **127**, 4877-4889.

Enomoto, H., Crawford, P. A., Gorodinsky, A., Heuckeroth, R. O., Johnson, E. M., Jr and Milbrandt, J. (2001). RET signaling is essential for migration, axonal growth and axon guidance of developing sympathetic neurons. *Development* **128**, 3963-3974.

Feldman, J. L., Mitchell, G. S. and Nattie, E. E. (2003). Breathing: rhythmicity, plasticity, chemosensitivity. *Annu. Rev. Neurosci*. **26**, 239-266.

Finley, J. C. and Katz, D. M. (1992). The central organization of carotid body afferent projections to the brainstem of the rat. *Brain Res.* **572**, 108-116.

Fode, C., Gradwohl, G., Morin, X., Dierich, A., LeMeur, M., Goridis, C. and Guillemot, F. (1998). The bHLH protein NEUROGENIN 2 is a determination factor for epibranchial placode-derived sensory neurons. *Neuron* **20**, 483-494.

Gonzalez, C., Almaraz, L., Obeso, A. and Rigual, R. (1994). Carotid body chemoreceptors: from natural stimuli to sensory discharges. *Physiol. Rev.* **74**, 829-898.

Gozal, D. (1998). Congenital central hypoventilation syndrome: an update. *Pediatr. Pulmonol.* **26**, 273-282.

Haase, G., Dessaud, E., Garces, A., de Bovis, B., Birling, M., Filippi, P., Schmalbruch, H., Arber, S. and deLapeyriere, O. (2002). GDNF acts through PEA3 to regulate cell body positioning and muscle innervation of specific motor neuron pools. *Neuron* **35**, 893-905.

Hashino, E., Shero, M., Junghans, D., Rohrer, H., Milbrandt, J. and Johnson, E. M., Jr (2001). GDNF and neurturin are target-derived factors essential for cranial parasympathetic neuron development. *Development* **128**, 3773-3782.

Honma, Y., Araki, T., Gianino, S., Bruce, A., Heuckeroth, R., Johnson, E. and Milbrandt, J. (2002). Artemin is a vascular-derived neurotropic factor for developing sympathetic neurons. *Neuron* **35**, 267-282.

Katz, D. M., Adler, J. E. and Black, I. B. (1987). Catecholaminergic primary sensory neurons: autonomic targets and mechanisms of transmitter regulation. *Fed. Proc.* **46**, 24-29.

Kraus, F., Haenig, B. and Kispert, A. (2001). Cloning and expression analysis of the mouse T-box gene tbx20. *Mech. Dev.* **100**, 87-91.

Le Douarin, N., Le Lièvre, C. and Fontaine-Perus, J. C. (1972). Recherches expérimentales sur l'origine embryologique du corps carotidien chez les Oiseaux. *Compte Rendu de l'Académie des Sciences* **275**, 583-586.

Lin, J. H., Saito, T., Anderson, D. J., Lance-Jones, C., Jessell, T. M. and Arber, S. (1998). Functionally related motor neuron pool and muscle sensory afferent subtypes defined by coordinate ETS gene expression. *Cell* **95**, 393-407.

Marshall, J. M. (1994). Peripheral chemoreceptors and cardiovascular regulation. *Physiol. Rev.* **74**, 543-594.

Morin, X., Cremer, H., Hirsch, M.-R., Kapur, R. P., Goridis, C. and Brunet, J.-F. (1997). Defects in sensory and autonomic ganglia and absence of locus coeruleus in mice deficient for the homeobox gene *Phox2a*. *Neuron* **18**, 411-423.

Natarajan, D., Marcos-Gutierrez, C., Pachnis, V. and de Graaff, E. (2002). Requirement of signalling by receptor tyrosine kinase RET for the directed migration of enteric nervous system progenitor cells during mammalian embryogenesis. *Development* **129**, 5151-5160.

Nattie, E. E. (2001). Central chemosensitivity, sleep, and wakefulness. *Respir. Physiol.* **129**, 257-268.

Pardal, R. and Lopez-Barneo, J. (2002). Low glucose-sensing cells in the carotid body. *Nat. Neurosci.* **5**, 197-198.

Patel, T. D., Kramer, I., Kucera, J., Niederkofler, V., Jessell, T. M., Arber, S. and Snider, W. D. (2003). Peripheral NT3 signaling is required for ETS protein expression and central patterning of proprioceptive sensory afferents. *Neuron* **38**, 403-416.

Pattyn, A., Morin, X., Cremer, H., Goridis, C. and Brunet, J.-F. (1997). Expression and interactions of the two closely related homeobox genes Phox2a and Phox2b during neurogenesis. *Development* **124**, 4065-4075.

Pattyn, A., Morin, X., Cremer, H., Goridis, C. and Brunet, J.-F. (1999). The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. *Nature* **399**, 366-370.

Pattyn, A., Goridis, C. and Brunet, J.-F. (2000a). Specification of the central noradrenergic phenotype by the homeobox gene *Phox2b*. *Mol. Cell Neurosci.* **15**, 235-243.

Pattyn, A., Hirsch, M.-R., Goridis, C. and Brunet, J.-F. (2000b). Control of hindbrain motor neuron differentiation by the homeobox gene *Phox2b*. *Development* **127**, 1349-1358.

Perrin, D. G., Cutz, E., Becker, L. E., Bryan, A. C., Madapallimatum, A. and Sole, M. J. (1984). Sudden infant death syndrome: increased carotid-body dopamine and noradrenaline content. *Lancet* **2**, 535-537.

Qian, Y., Fritzsch, B., Shirasawa, S., Chen, C. L., Choi, Y. and Ma, Q. (2001). Formation of brainstem (nor)adrenergic centers and first-order relay visceral sensory neurons is dependent on homeodomain protein Rnx/Tlx3. *Genes Dev.* **15**, 2533-2545.

Qian, Y., Shirasawa, S., Chen, C. L., Cheng, L. and Ma, Q. (2002). Proper development of relay somatic sensory neurons and D2/D4 interneurons requires homeobox genes Rnx/Tlx-3 and Tlx-1. *Genes Dev.* **16**, 1220-1233.

Rodman, J., Curran, A., Henderson, K., Dempsey, J. and Smith, C. (2001). Carotid body denervation in dogs: eupnea and the ventilatory response to hyperoxic hypercapnia. *J. Appl. Physiol.* **91**, 328-335.

Saito, T., Greenwood, A., Sun, Q. and Anderson, D. J. (1995). Identification by differential RT-PCR of a novel paired homeodomain protein specifically expressed in sensory neurons and a subset of their CNS targets. *Mol. Cell Neurosci.* **6**, 280-292.

Shapiro, R. E. and Miselis, R. R. (1985). The central organization of the vagus nerve innervating the stomach of the rat. *J. Comp. Neurol.* **238**, 473-488.

Spengler, C. M., Gozal, D. and Shea, S. A. (2001). Chemoreceptive mechanisms elucidated by studies of congenital central hypoventilation syndrome. *Respir. Physiol.* **129**, 247-255.

Taber Pierce, E. (1973). Time of origin of neurons in the brain stem of the mouse. *Prog. Brain Res.* **40**, 53-65.

Taraviras, S. and Pachnis, V. (1999). Development of the mammalian enteric nervous system. *Curr. Opin. Genet. Dev.* **9**, 321-327.

Tiveron, M.-C., Hirsch, M.-R. and Brunet, J.-F. (1996). The expression pattern of the transcription factor Phox2 delineates synaptic pathways of the autonomic nervous system. *J. Neurosci.* **16**, 7649-7660.

Valarché, I., Tissier-Seta, J.-P., Hirsch, M.-R., Martinez, S., Goridis, C. and Brunet, J.-F. (1993). The mouse homeodomain protein Phox2 regulates *Ncam* promoter activity in concert with Cux/CDP and is a putative determinant of neurotransmitter phenotype. *Development* **119**, 881-896.

Wang, Z. Z., Dinger, B., Fidone, S. J. and Stensaas, L. J. (1998). Changes in tyrosine hydroxylase and substance P immunoreactivity in the cat carotid body following chronic hypoxia and denervation. *Neuroscience* **83**, 1273-1281.