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PHOX2B mutations and ventilatory control

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ABSTRACT

The transcription factor *PHOX2B* is essential for the development of the autonomic nervous system. In humans, polyalanine expansion mutations in *PHOX2B* cause Congenital Central Hypoventilation Syndrome (CCHS), a rare life-threatening disorder characterized by hypoventilation during sleep and impaired chemosensitivity. CCHS is combined with comparatively less severe impairments of autonomic functions including thermoregulation, cardiac rhythm, and digestive motility. Respiratory phenotype analyses of mice carrying an invalidated *Phox2b* allele (*Phox2b*$^{+/-}$ mutant mice) or the *Phox2b* mutation (+7 alanine expansion) found in patients with CCHS (*Phox2b*$^{27Ala/+}$ mice) have shed light on the role for *PHOX2B* in breathing control and on the pathophysiological mechanisms underlying CCHS. Newborn mice that lacked one *Phox2b* allele (*Phox2b*$^{+/-}$) had sleep apneas and depressed sensitivity to hypercapnia. However, these impairments resolved rapidly, whereas the CCHS phenotype is irreversible. Heterozygous *Phox2b*$^{27Ala/+}$ pups exhibited a lack of responsiveness to hypercapnia and unstable breathing; they died within the first few postnatal hours. The generation of mouse models of CCHS provides tools for evaluating treatments aimed at alleviating both the respiratory symptoms and all other autonomic symptoms of CCHS.

1. Introduction

Respiratory control impairments occurring early during development stages may compromise brain oxygenation, thereby leading to irreversible motor and cognitive disorders. The etiology and incidence of these impairments vary considerably, from apnea of prematurity, seen in 85% of infants born before 34 weeks of gestation (Schmidt et al., 2006), to Congenital Central Hypoventilation Syndrome (CCHS or Ondine's syndrome), a rare disorder typically presenting in the newborn period (Weese-Mayer et al., 1999). The incidence was estimated at 1 per 200,000 live births based on the French cohort of patients (Trang et al., 2005) but a more accurate estimate will be obtained in the near future from on-going international epidemiological studies of CCHS populations.

Mouse studies of respiratory phenotypes in mutant newborn mice have helped to identify candidate genes for developmental respiratory control disorders (Gaultier and Gallego, 2005). Regarding CCHS, several groups (Matera et al., 2002; Amiel et al., 2003b; Weese-Mayer et al., 2003) examined the RNX gene, as RNX knockout mice exhibited respiratory control disorders, but found no mutations. Weese-Mayer et al. (2002) analyzed brain-derived neurotrophic factor (BDNF) as a potential candidate gene in CCHS after respiratory control disorders were observed in mice with *Bdnf* mutations (Erickson et al., 1996). Based on mouse studies showing cross-regulation of the *Phox2b* and *Mash1* genes and impaired ventilatory responses to hypercapnia, the human ortholog of *Mash1* (*HASH-1*) was considered an additional candidate gene for CCHS (de Pontual et al., 2003; Weese-Mayer et al., 2003).

Although rare, CCHS provides a unique opportunity to identify genetic factors involved in respiratory control development. This syndrome is characterized by sleep-related hypoventilation and apneas (especially in severe cases) with severe abnormalities in chemosensitivity, as well as by various autonomic disorders of widely variable penetrance (Weese-Mayer et al., 1999; Marazita et al., 2001; Weese-Mayer et al., 2001; Vanderlaan et al., 2004). However, a general limitation of most clinical descriptions of CCHS is the lack of genetic testing. The considerable inter-individual variability in the number and severity of associated symptoms probably reflects differences in the nature of the genetic defect (polyalanine expansion, missense or frameshift mutations, see below). Affected patients have absent or markedly reduced ventilatory responses to sustained hypercapnia (Paton et al., 1989) and, to a lesser extent, to sustained hypoxia (Paton et al., 1989). These abnormalities are generally ascribed to impaired central integration of chemosensory inputs to the brainstem, rather than to failure of the chemoreceptors, which are at least partially active (Marcus et al., 1991; Gozal et al., 1993; Spengler et al., 2001). Respiratory control disorders are

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difficult to analyze in the most severe forms of CCHS (e.g., patients who cannot breathe spontaneously even when awake). For this reason, previous studies may not reflect the entire spectrum of respiratory disorders in CCHS.

The identification of *PHOX2B* as the disease-causing gene in CCHS (Amiel et al., 2003a; Sasaki et al., 2003; Weese-Mayer et al., 2003; Matera et al., 2004) has stimulated considerable interest in the role for this gene not only in CCHS, but also in prevalent conditions such as sleep apnea. Recently, there have been several reports of adults exhibiting sleep-related hypoventilation and, in some instances, central apneas and severe hypoxemia, with *PHOX2B* mutations generally characterized by five additional alanine residues in *PHOX2B* (Matera et al., 2004; Trang et al., 2004; Trochet et al., 2005a; Weese-Mayer et al., 2005b; Antic et al., 2006; Barratt et al., 2007; Diedrich et al., 2007; Doherty et al., 2007; Trochet et al., 2008). These observations suggest that respiratory control disorders associated with *PHOX2B* mutations may be more prevalent than previously inferred from the very low incidence of CCHS in newborns. More generally, *PHOX2B* appears pivotal to the development of respiratory networks. To analyze the functional impact of *PHOX2B* mutations on breathing at the organism level, mouse models must be studied. The present review focuses on the respiratory phenotypes of mice lacking one *Phox2b* allele (*Phox2b*$^{+/-}$ mutants). The fact that *Phox2b*$^{+/-}$ pups develop normally is in striking contrast to the severe abnormalities seen in humans with CCHS. However, the purpose of this mouse model is to determine which components of respiratory control, if any, depend on *Phox2b* for normal development. This approach is critical to unraveling the pathogenesis of CCHS. The present review also describes the respiratory phenotype of mice carrying polyalanine expansions. We studied mice carrying a +7 alanine expansion (*Phox2b*$^{27Ala/+}$ mice). This genotype was the most frequent in a cohort of 188 patients with CCHS occurring either as an isolated disorder or in combination with Hirschsprung disease (HSCR) and/or tumors of the sympathetic nervous system (Trochet et al., 2005a).

2. *PHOX2B*, the main disease-causing gene for CCHS

About 20% of patients with CCHS also have Hirschsprung disease, a developmental disorder of the enteric nervous system characterized by absence of ganglion cells in the distal colon (Weese-Mayer et al., 2001; Amiel et al., 2008). This association suggested shared pathophysiological mechanisms in CCHS and HSCR. Furthermore, most patients with CCHS exhibit a variety of autonomic disorders, and CCHS is associated with tumors developed from neural crest cells (neuroblastoma), in addition to chemosensitivity disorders. Taken together, these observations suggested abnormal neural crest development as the abnormality underlying CCHS. Therefore, human genome screening and mouse model studies were conducted to investigate genes involved in the development and migration of neural crest cells. Of special interest was the gene encoding the RET receptor tyrosine kinase signaling pathway, which involves the sequential expression of *MASH1*, *PHOX2A/2B*, *RET*, and *TH* (Pattyn et al., 1999). This pathway is responsible for the development of all transiently or permanently produced noradrenergic derivatives (Pattyn et al., 1999). The gene causing CCHS was found to be the transcription-factor gene *PHOX2B* (Amiel et al., 2003a; Sasaki et al., 2003; Weese-Mayer et al., 2003; Matera et al., 2004). Over 90% of patients carry a heterozygous *PHOX2B* mutation consisting, in most cases, in a polyalanine-repeat expansion (Trochet et al., 2005b; Berry-Kravis et al., 2006). A small proportion of patients with CCHS, however, carry other *PHOX2B* mutations instead, such as missense, nonsense, or frameshift mutations, which severely disrupted *PHOX2B* function (Trochet et al., 2005a; Berry-Kravis et al., 2006). These important results pointed toward *PHOX2B* as a pivotal factor in the neurochemical control of breathing, and more generally, in autonomic nervous system disorders, Hirschsprung disease, and neuroblastoma. Several CCHS patients carry heterozygous mutations affecting seven genes generally involved in neural crest cell development (reviewed in Weese-Mayer et al., 2005a, see also www.genetests.org). However, the role in CCHS for mutations in genes other than *PHOX2B* remains unclear.

The finding that *PHOX2B* was the main disease-causing gene for CCHS was consistent with previous knowledge on the role for this gene in neurodevelopmental processes, which was reviewed recently (Brunet and Goridis, 2008). The transcription factor *Phox2b* is required for the development of most neuronal types in the central and peripheral nervous systems (Tiveron et al., 1996; Pattyn et al., 1997; Brunet and Pattyn, 2002). In the peripheral nervous system, *Phox2b* is expressed in the neurons of all autonomic and sensory ganglia of the VIIth, IXth, and Xth cranial nerves. In the central nervous system, *Phox2b* is expressed in the hindbrain branchiomotor and visceromotor neurons, all noradrenergic neurons, and neurons in the nucleus tractus solitarius (Pattyn et al., 1999, 2000a,b; Dauger et al., 2003). Despite their diverse origins, most *Phox2b*-dependent neurons are part of the visceral nervous system and connect synaptically to form medullary visceral reflex arcs (Brunet and Pattyn, 2002).

Furthermore, the specific role for *Phox2b* in central chemosensory pathways was clarified recently. In adult rats, *Phox2b* is expressed by a group of chemosensitive glutamatergic interneurons located in the retrotrapezoid nucleus (RTN) (Stornetta et al., 2006). A finding of major relevance to CCHS is *Phox2b* expression by a chain of neurons involved in the integration of peripheral and central chemoreception (Stornetta et al., 2006). The chain included the carotid bodies, chemoreceptor afferents, chemoresponsive projections of the NTS to the ventrolateral medulla, and central chemoreceptors located in the RTN (Stornetta et al., 2006).

These important findings failed, however, to explain the contrast between the pervasive role for *Phox2b* in autonomous circuit development and the relatively targeted impact of *PHOX2B* mutations on respiratory control in CCHS. Genetic mouse models were needed to clarify the role for *Phox2b* in breathing control and, in particular, to investigate the emerging genotype–phenotype relationships in humans with CCHS.

3. Ventilatory phenotype in *Phox2b*$^{+/-}$ mice

3.1. Early assessment of respiratory control in mice

To investigate the role for *Phox2b* in the development of respiratory control, the respiratory phenotype of neonatal mice with one invalidated *Phox2b* allele (*Phox2b*$^{+/-}$) was determined. Homozygous *Phox2b* knock-out mice (*Phox2b*$^{-/-}$) die *in utero* around embryonic day 14 (Pattyn et al., 2000b), whereas *Phox2b*$^{+/-}$ pups survive and are fertile. Body weight and mouth temperature were normal in 2-day-old *Phox2b*$^{+/-}$ mice (Dauger et al., 2003). At 5 days of age, however, the mutant pups had slightly lower body weights and lower temperatures than the wild-type pups, suggesting disorders of autonomic regulation such as described in patients with CCHS (Weese-Mayer et al., 1999; Marazita et al., 2001; Weese-Mayer et al., 2001; Vanderlaan et al., 2004).

Breathing control was assessed *in vivo*, in unrestrained, non-anaesthetized newborn mice. Breathing was quantified based on breath duration, inspiratory and expiratory durations, tidal volume, ventilation, and apneas. Tidal volume (V_T) and ventilation (V_E) were divided by body weight to adjust for inter- and intra-individual differences in growth, which are particularly marked