Haploinsufficiency for Phox2b in mice causes dilated pupils and atrophy of the ciliary ganglion: mechanistic insights into human congenital central hypoventilation syndrome

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Dilp1 is a semi-dominant mouse mutation that causes dilated pupils when heterozygous and is lethal when homozygous. We report here that it is caused by a point mutation that introduces a stop codon close to the start of the coding sequence of the paired-like homeobox transcription factor Phox2b. Mice carrying a targeted allele of Phox2b also have dilated pupils and the two alleles do not complement. Phox2b is necessary for the development of the autonomic nervous system and when absent one of the consequences is that all parasympathetic ganglia fail to form. Constriction of the pupil is a parasympathetic response mediated by the ciliary ganglion and we find that in Phox2b heterozygous mutants it is highly atrophic. The development of other parasympathetic and sympathetic ganglia appears to be largely unaffected indicating that the ciliary ganglion is exquisitely sensitive to a reduction in dose of this transcription factor. PHOX2B has been implicated in human disease. Mutations, principally leading to polyalanine expansions within the protein, have been found in patients with congenital central hypoventilation syndrome (CCHS), the cardinal feature of which is an inability to breathe unassisted when asleep. Additionally, some CCHS patients have ocular abnormalities, including pupillary defects, although they principally have constricted rather than dilated pupils. The apparent phenotypic differences observed between mice carrying a loss-of-function mutation of Phox2b and CCHS patients indicate that PHOX2B mutations found in CCHS patients, all of which can produce proteins with intact DNA-binding domains, are gain-of-function mutations that alter rather than abolish protein function.

INTRODUCTION

Identification of the genetic basis of mutant phenotypes or disease is an effective means of assigning function to genes. Similarities and differences between the phenotypes of mouse and human mutations of the same gene can illuminate the pathological processes underlying disease. Furthermore, in the mouse, dominant mutations can be bred to homozygosity to reveal additional phenotypes that may never, or rarely, be seen in humans and which further elucidate the biological processes involved. In order to identify genes that are involved in visual function, and to create mouse models of human genetic eye disease, we have used the powerful point mutagen N-ethyl-N-nitrosourea (ENU) to generate random mutations in the mouse genome. Screening mutagenized offspring for a tracking response to a visual stimulus, or by slit-lamp and indirect ophthalmoscopic examination, or both, we produced a collection of 25 inherited mouse mutants with a variety of eye defects (1).

Four of the inherited dominant phenotypes we found cause the pupil to be dilated and unresponsive to light. We named these Dilp1–Dilp4 (dilated pupils 1–4). These mutations

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map to at least three different loci, suggesting that this subtle phenotype can be due to changes in different genes that may impinge on a common pathway. We have now identified the underlying mutation responsible for Dilp1 to be a stop codon near the start of the paired-like homeobox gene Phox2b. This gene is essential for the formation of the medullary reflex circuits of the autonomic nervous system (2). In particular, it is required for the differentiation of all autonomic ganglionic neurons—sympathetic, parasympathetic and enteric (3)—and the first and second order visceral sensory neurons, in cranial ganglia and the nucleus of the solitary tract, respectively (4).

Mutations in the orthologous human gene PHOX2B that lead to an expansion of a polyalanine tract in the PHOX2B protein have been found to be responsible for the majority of cases of congenital central hypoventilation syndrome (CCHS) (5). However, these mutations will permit the synthesis of protein that retains DNA binding capability and patients often have constricted pupils, unlike Dilp1 mice in which heterozygous loss-of-function produces the opposite effect.

RESULTS

Dilp1 is caused by loss-of-function of the Phox2b gene

Dilp1 is one of four ENU-induced mutations found in our screen for eye and vision mutants that causes a dilated pupils phenotype when heterozygous. When the eyes of Dilp1 heterozygous animals are illuminated there is no pupillary response to light, and the iris constrictor muscles fail to act (see Fig. 2I in reference 1 for a picture of the Dilp1 phenotype). To establish whether the defect in Dilp1 animals is in the afferent or efferent pathway of the pupillary light response or in the muscles of the iris we applied the parasympathetic agonist, carbachol, to their eyes. The mutant pupils fail to constrict indicating that the defect in these animals is in the efferent pathway or the iris constrictor muscle (data not shown).

We have previously mapped the Dilp1 mutation to a region on mid-Chr 5 between the simple sequence length polymorphism (SSLP) markers D5Mit15 and D5Mit356 (1). In order to further refine the map position we backcrossed mutant animals to C57BL/6 animals and reduced the critical interval to a 3.9 Mb region between the SSLP markers D5Mit258 and D5Mit83 which contains approximately 19 genes or predicted genes (Mouse Build 30, http://www.ensembl.org). We considered one of the genes within this interval, Phox2b, to be a particularly good candidate for the Dilp1 mutation as it encodes a paired-like homeodomain-containing transcription factor which is crucial for the development of the autonomic system (2). We amplified by PCR the three exons of Phox2b and their flanking regions and determined the sequence in Dilp1 mutant mice, the parental strain Balb/cAnN and the two strains used to make and maintain the mutation, C3H/HeN and C57BL/6. We identified a heterozygous T to G nucleotide substitution in exon 1 of Phox2b in all Dilp1 mice when compared to the sequence of the three strains plus two other inbred mouse strains, 101 and CBA, and the closely related species Mus musculus castaneus (Fig. 1A and B). This T to G transversion in codon 44 introduces a premature stop codon (Fig. 1C).

Sequencing of Phox2b RT–PCR products from Dilp1 mutant mice showed that the mutant allele is transcribed, correctly spliced and not subject to nonsense-mediated decay (Fig. 1D). Translation of the mutant allele would produce a severely truncated protein lacking the homeodomain and polyalanine tracts. We predict, therefore, that Dilp1 is a loss-of-function mutation of Phox2b.

A targeted disruption of the Phox2b gene, Phox2b<sup>tm1.Lbr</sup> (hereafter Phox2b<sup>LacZ<sup>+</sup></sup>), has been made by the insertion of a LacZ transgene into the second exon of the gene and embryos homozygous for this loss of function of Phox2b die between E10 and E14 with a failure of all autonomic ganglia to form (3). Heterozygous mice were originally reported to have no obvious phenotype but subsequent analysis has found that heterozygous mutants have an altered response to hypoxia and hypercapnia as pups, but soon recover (4). To determine if mice carrying the targeted Phox2b mutation display a similar eye phenotype to Dilp1 heterozygotes we examined the eyes of Phox2b<sup>LacZ<sup>+</sup></sup> mice and found that in every case the mice had an identical bilateral dilated pupils phenotype to that seen in Dilp1/+ mice and the pupils do not constrict on application of carbachol (data not shown). The similarity between the phenotypes seen in the Phox2b<sup>LacZ<sup>+</sup></sup> and Dilp1/+ mice strongly suggests that the T to G transversion we have found in the Phox2b gene...
in the Dilp1 mice is responsible for the dilated pupils phenotype.

Dilp1 homozygotes and mice homozygous for the targeted Phox2b mutation demonstrate similar embryonic lethality (1,3). We genotyped 42 offspring from an intercross of Dilp1 heterozygous mice and identified 29 heterozygotes (all of which had the dilated pupils phenotype), 13 mice homozygous for the wild-type allele and no Dilp1 homozygotes. We went on to test for complementation of the recessive lethality Dilp1 and Phox2bLacZ mutations by mating mice heterozygous for each mutation. We typed their offspring at the Phox2b locus and examined them for the dilated pupils phenotype. Twenty animals from this cross had normal eyes and typed as wild type. The remaining 25 animals had dilated pupils, of which 13 were Dilp1/± and 12 Phox2bLacZ/+. No live offspring typed as being Dilp1/Phox2bLacZ. The probability of 0/45 of the live offspring being compound heterozygotes is 2.38 × 10⁻⁶ (0.75⁵). A chi-squared test gives a value of 14.875 with one degree of freedom, which has a probability of <0.001. We conclude that the two mutations do not complement and Dilp1 and Phox2bLacZ are allelic. The concordance between phenotype and genotype indicates that the dilated pupils phenotype is completely penetrant in the heterozygous animals. The apparent reduction in the number of heterozygotes is not statistically significant and haploinsufficiency for Phox2b does not appear to affect viability in this strain background.

The ciliary ganglion is severely atrophied in Phox2b mutant mice

Previous analysis has shown that all autonomic ganglia fail to form or subsequently degenerate in mice that completely lack Phox2b (3). Pupillary constriction is under the control of the parasympathetic ciliary ganglion, which prompted us to examine this ganglion in Phox2b mutant mice. Staining with anti-Phox2b antibody revealed severe atrophy of the ciliary ganglia of E13.5 Phox2bLacZ/+ embryos and E13.5 Phox2bDilp1+/+ embryos (Fig. 2A–F). Quantifying the number of Phox2b-positive cells present in serial sections through the ciliary ganglion from both Phox2bLacZ heterozygous and Phox2bDilp1 heterozygous embryos revealed an 80 and 87% reduction in positive cells respectively, as compared to wild-type (Fig. 3). That the degree of atrophy observed in heterozygous mutant animals was equivalent for both the targeted and Dilp1 mutations further supports the idea that Dilp1 is a loss-of-function mutation. To determine whether this decrease in the number of Phox2b-positive cells reflects a reduction in the number of neuronal cells present we also stained sections through the eye and ciliary ganglion with anti-Isl1 (Isl1) antibody. A similar decrease in the number of positively staining cells was observed in the heterozygous mutant animals (Fig. 2G and H). These data suggest that a wild-type level of expression of Phox2b is essential for the generation and/or maintenance of neuronal cells in the ciliary ganglion. It is interesting to note that there is a previously undescribed smaller group of Phox2b- and Isl1-positive cells close to the eye whose number does not appear to be reduced in the heterozygous mutant animals (asterisked in Fig. 2D and F–H).

Neurons affected in CCHS patients are unaffected in Phox2b mutant mice

Although abnormally dilated pupils have been found in CCHS patients (6,7), the incidence of constricted pupils is much more frequent (8) suggesting a defect in the superior cervical ganglion, responsible for mediating pupil dilation and part of the sympathetic nervous system. To ascertain if the presence of only one functional copy of Phox2b leads to a defect in this ganglion we stained serial sections with the anti-Phox2b and anti-Isl1 antibodies. Phox2b- and Isl1-positive cells appeared unaffected in heterozygous Phox2b mutant mice, as compared with wild-type littermates (Fig. 21–L). There is no Phox2b haploinsufficient phenotype for the development of this ganglion in mice. Human patients with CCHS, including those in which the disease is associated with mutations in PHOX2B, often also have Hirschsprung disease (HSCR), a failure of innervation of the distal gut. In addition, there has been a suggestion that haploinsufficiency for PHOX2B may cause isolated HSCR (9). Embryos that completely lack Phox2b have a failure of the enteric neurons to migrate distally through the developing gut (3). We examined whole-mount preparations of the guts of E13.5 Phox2bDilp1+/+ and wild-type embryos probed with Phox2b antibody. The innervation of the gut by enteric neurons was indistinguishable from that seen in wild-type animals (data not shown).

No other anomaly has been reported in the ganglia of Phox2bLacZ/+ mice, with the exception of the petrosal ganglion, which controls respiration in response to hypoxia, where there is a ~45% reduction in the number of tyrosine hydroxylase (Th) positive cells (4). It seems that there is a difference in the dose requirement of the Phox2b transcription factor for the normal development of different ganglia. The severe atrophy of the ciliary ganglion in heterozygous loss-of-function Phox2b mutants suggests that the development of this particular ganglion is exquisitely susceptible to reduced levels of Phox2b protein.

DISCUSSION

Dilp1 is a loss-of-function mutation of Phox2b

We report here that the underlying genetic defect responsible for the dilated pupils phenotype seen in Dilp1 mice is a nonsense mutation in the first exon of the Phox2b gene which, if translated, would result in the production of a severely truncated protein product consisting of the first 43 amino acids. The dilated pupils phenotype of Dilp1 mice is also seen in mice that carry a targeted disruption of the gene and the two alleles do not complement which implies that the Dilp1 mutation causes loss of function. Phox2b is a transcription factor essential for the successful development of the autonomic nervous system (3). Mice heterozygous for loss of Phox2b are superficially normal and are fertile. So far, only two phenotypes have been detected in these heterozygotes, the defective pupillary response to light described here and a defective response to hypoxia just after birth described in the Phox2bLacZ heterozygotes by Dauger et al. (4). The response of Phox2bDilp1 heterozygotes to hypoxia has not as yet been
investigated because the specialized plethsmography equipment for analysis of neonates is not available on the site where the mice are housed. We would nevertheless expect them to respond in a similar fashion to the Phox2bLacZ heterozygotes unless the phenotype is modified by the different genetic background of the Phox2bDilp1 mice. These two mutant phenotypes are associated with severe atrophy of the ciliary ganglion and loss of Th expression in the petrosal ganglion, respectively. The effect on the petrosal ganglion appears to be transitory as gauged by the number of Th-positive cells present in this structure; by P10 there is no statistical difference between wild-type and heterozygous mutant animals in the number of positively-staining cells, whereas there is at E16.5 (4). Other ganglia that have been examined appear to develop normally in mice that are haploinsufficient for Phox2b (Fig. 2I–L). It would appear that the development of the ciliary ganglion is particularly sensitive to a reduction in the dose of this transcription factor.

**Comparison of the phenotype of Phox2b mutant mice and human CCHS patients**

Mutations in PHOX2B are the major cause of CCHS, an autosomal dominant disorder characterized by an impairment of the autonomic of control breathing. Eighty-seven of 106 patients examined in three studies were found to have heterozygous expansions of between five and 13 alanines in a 20-amino-acid polyalanine tract close to the C-terminal end of the PHOX2B protein (position of this tract in the protein is shown in Fig. 1A) (5,10,11). Four of the remaining patients were found to have heterozygous PHOX2B mutations other than polyalanine expansions.

Consideration of the phenotype of Phox2b mutant mice is useful in elucidating the mechanism by which mutations in the PHOX2B gene bring about CCHS. There are clearly both similarities and differences between mice that are heterozygous for Phox2b loss-of-function mutations and human CCHS patients. It has already been noted that haploinsufficiency for Phox2b in mice only partially models CCHS with respect to the respiratory phenotype (4). Although the mice have an altered response to hypoxia and hypercapnia two days after birth, they recover over the next week, and older mice respond normally (4). This suggests either that the role of Phox2b in establishing control of respiration is different between human and mice, or that loss-of-function mutations in the mouse are not functionally equivalent to the alanine tract expansion, frameshift and truncation mutations found in CCHS patients.
same cohort (last two bars). In a few cases where sections were missing the cell number was interpolated. White bars, wild-type (WT); black bars, Phox2b LacZ/+ animals from the same cohort (first two bars) and wild-type (n = 6) animals from the same cohort (last two bars). In a few cases where sections were missing the cell number was interpolated. White bars, wild-type (WT); black bars, Phox2b heterozygous mutants.

In mice, the dilated pupils phenotype caused by haploinsufficiency of Phox2b is completely penetrant and we have not observed any other eye defects. Such a consistent eye phenotype is not seen in CCHS patients. One study reports that 60% of CCHS patients have ocular abnormalities, including exotropia, exotropia, ptosis, anisocoria and myopia (12). The pupils of some CCHS patients have been found to be dilated (6,7). However, Amiel et al. (5) report that of 23 patients examined, 20 had an abnormal pupillary response to light and in most cases their pupils were constricted; the opposite of what we observe in the mice. An earlier study found that 27 of 37 CCHS patients (the genetic origin of which was not determined) also had constricted pupils (8). Constricted pupils are most likely to result from atrophy of the superior cervical ganglia, rather than the ciliary ganglia, although the variable phenotype, and the fact that they tend to show a slower response to light, suggests that the latter may also be affected. However, the superior cervical ganglion is unaffected by haploinsufficiency of Phox2b in the mouse (Fig. 2I–L). In addition, CCHS patients often have a spectrum of associated abnormalities of the autonomic system. About 20–30% also have HSCR, a combination sometimes termed Haddad syndrome, many have mild generalized hypotonia and they may have tumours of neural crest origin such as neuroblastomas and ganglioneuromas. It is well established that HSCR is sensitive to genetic background, so that mutations show variable penetrance through HSCR families, and the disease phenotype is often only expressed when other genes in the developmental pathway leading to enteric innervation are also affected. With this caveat, in our study we found no defect in the gut innervation of mice heterozygous for loss of Phox2b.

**Figure 3.** Quantification of the amount of atrophy of the ciliary ganglion in Phox2b mutant mice. The number of Phox2b-positive cells present in serial 12 μm (first two bars) and 10 μm (last two bars) sections through the ciliary ganglion of E13.5 embryos stained with anti-Phox2b antibody were counted. Mean values ± standard error measures are shown for wild-type (n = 6 ganglia) and Phox2bLacZ/+ (n = 6) animals from the same cohort (first two bars) and wild-type (n = 4) and Phox2bLacZ/+ (n = 4) animals from the same cohort (last two bars). In a few cases where sections were missing the cell number was interpolated. White bars, wild-type (WT); black bars, Phox2b heterozygous mutants.

Does loss-of-function or gain-of-function of PHOX2B cause CCHS?

It is striking that to date in CCHS no mutations have been found that truncate the protein upstream of the homeodomain, nor have any missense mutations been identified, raising the possibility that the mutations seen in CCHS patients cause a change of function rather than ablate the PHOX2B protein. Supporting this notion is the observation of an individual hemizygous for a 5 Mb deletion, including the PHOX2B gene, who has developmental delay, severe hypotonia, facial dysmorphism and HSCR, but does not have CCHS (9). Ocular defects were not noted in this patient (J. Amiel, personal communication).

Is it likely that expansions of the 20-amino-acid polyalanine tract of PHOX2B act in a dominant-negative manner to cause CCHS? The study by Weese-Mayer et al. (10) suggests that they do. An association was found between length of the polyalanine expansion, the severity of associated autonomic dysfunction symptoms and the time required each day in ventilatory support. Recent *in vitro* studies have shown that transcriptional activation by Phox2b is impaired when the alanine tract is expanded by five alanines, which is the minimum expansion size found in CCHS patients so far (13). Decreasing the number of alanines in the tract by up to 13 also impaired activity, but to a lesser extent. Carriers of a five-alanine contraction have been found in the normal population (5,10) suggesting that there might be a threshold of activity only below which the mutant phenotype is manifest. Four CCHS patients have been found to have mutations in *PHOX2B* other than polyalanine tract expansions. Three have frameshift mutations downstream of the homeodomain (5,11) and one has a nonsense mutation that would lead to the production of a truncated protein lacking the whole C-terminal region, including the last three amino acids of the homeodomain (10). As paired-homeodomain proteins such as PHOX2B bind as dimers to their target sites (14) these fusion or truncated proteins, as well as PHOX2B forms with expanded polyalanine tracts, may act in a dominant-negative manner and interfere with the function of the wild-type protein. They would therefore cause a more severe phenotype than a loss-of-function allele.

Ala nine repeat expansions have been found to be associated with several genetic diseases in humans and at least two of these are thought to act in a dominant-negative fashion. Synpolydactyly is a dominantly inherited limb malformation that can be caused by expansions in a polyalanine tract in the *HOXD13* gene (15). The size of the expansion has been found to correlate with the penetrance and severity of the disorder (16). Polyalanine expansions as well as missense and nonsense mutations in the *HOXA13* gene cause hand–foot–genital syndrome (HFGS) (17–19). These mutations are thought to act in a dominant-negative way because a less severe HFGS phenotype has been found in a patient hemizygous for the *HOXA* cluster (20).

We propose that the alanine expansions, or homeobox-distal mutations, found in the *PHOX2B* gene of CCHS patients result in a protein that is capable of binding to the correct target sites in the genome, but is unable to correctly carry out its normal regulatory function. As *PHOX2B* is expressed throughout the
autonomic nervous system, disruptions of development or function of certain aspects of sympathetic, parasympathetic and enteric function are seen. By contrast, mice that have only a single functional Phox2b gene have somewhat less severe phenotype overall, with no effect on the enteric nervous system, and effects on the autonomic nervous system limited to a severe atrophy of the ciliary ganglia and transitory loss of Th expression in the petrosal ganglia.

MATERIALS AND METHODS

Animals and clinical examinations

The animal studies described in this paper were carried out under the guidance issued by the Medical Research Council in Responsibility in the Use of Animals for Medical Research (July 1993) and Home Office Project Licence nos PPL60/2242 and PPL60/3124. For carbamylcholine chloride (carbachol) in PBS was dropped into the eyes. The anterior segment of the eye was examined before treatment and 5 min after treatment using a Nikon FS-3V zoom slit-lamp biomicroscope.

Immunohistochemistry

Pregnant mice were sacrificed by cervical dislocation at E13.5. Embryos were dissected and fixed for 2 h in 4% paraformaldehyde in PBS, cryoprotected overnight in PBS with 30% sucrose, embedded in OCT (Tissue-Tek) and frozen on dry ice. Cryosections (10 µm) were thaw-mounted on Superfrost slides (BDH), dried at RT and stored at −80°C. For fluorescent immunohistochemistry, thawed sections were blocked for 1 h in 10% sheep serum, 0.1% Triton X-100 in PBS. Cells were labelled with either rabbit anti-Phox2b (21) or rabbit anti-Isl1 (22) diluted 1:500 and 1:250, respectively, in blocking buffer and incubated overnight at RT in a humidified chamber. After three washes for 5 min in PBST (PBS, 0.1% Triton X-100), slides were incubated for 2 h at RT with TXR-conjugated donkey anti-rabbit IgG (Jackson) diluted 1:800 in blocking buffer containing 0.05 µg/ml DAPI (Sigma). After three further PBST washes, slides were mounted in Vectashield (Vector), visualized using a Zeiss Axioplan 2 microscope and images captured using IPLab3.2 (Scanalytics) and processed using Adobe Photoshop 7.0 (Adobe, Inc.). Immunohistochemistry for brightfield visualization of dianaminobenzidine stained slides was carried out as described (23).

Mutational analysis of the Phox2b gene

Exons 1–3 and the immediate flanking sequences of the Phox2b gene were amplified from genomic DNA using intronic primers that were also used for subsequent sequence analysis. PCR products were purified using Millipore Multi-screen PCR 96-well filtration system on a Biomek 2000 robotic platform and sequenced directly using Big Dye™ terminator cycle sequencing. Sequences were analysed using the Sequencher™ program. Animals were genotyped for the Phox2b LacZ allele by PCR using primers specific for LacZ. The primer sequences are available on request.

RT–PCR

Total RNA was isolated from the heads of E11.5 embryos using Tri-Reagent™ (Sigma). First strand cDNA was synthesized using a SuperScript™ II Reverse Transcription kit (Invitrogen) and a primer specific for Phox2b exon 2, ex1-2R (5’-GCTCTTCCCCGTTGATGAC-3’). An aliquot was then amplified by the PCR using primers ex1-2R and ex1-2F (5’-TGAATATTTCACTCCATCTC-3’) using BIO-X-ACT (Bioline). PCR products were purified and sequenced as described above.

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