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Molecular control of ciliary neuron development: BMPs and downstream transcriptional control in the parasympathetic lineage

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SUMMARY

The generation of noradrenergic sympathetic neurons is controlled by BMPs and the downstream transcription factors Mash1, Phox2b, Phox2a and dHand. We examined the role of these signals in developing cholinergic parasympathetic neurons. The expression of *Mash1* (*Cash1*), *Phox2b* and *Phox2a* in the chick ciliary ganglion is followed by the sequential expression of panneuronal, noradrenergic and cholinergic marker genes. BMPs are expressed at the site where ciliary ganglia form and are essential and sufficient for ciliary neuron development. Unlike sympathetic neurons, ciliary neurons do not express *dHand*; noradrenergic gene expression is eventually lost but can be maintained by ectopic dHand expression. Together, these results demonstrate a common BMP dependence of sympathetic neurons and parasympathetic ciliary neurons and implicate dHand in the maintenance of noradrenergic gene expression in the autonomic nervous system.

Key words: Ciliary, Cholinergic, Noradrenergic, dHand, BMP5, BMP7, Autonomic nervous system

INTRODUCTION

The developing peripheral nervous system is an instructive model to study the generation of different neuron types from pluripotent neural crest precursor cells (Christiansen et al., 2000; Anderson, 2001). The analysis of sympathetic neuron development has revealed essential signals and mechanisms involved in the control of neuron fate and differentiation (Francis and Landis, 1999; Ernsberger and Rohrer, 1999; Ernsberger and Rohrer, 1999). Sympathetic neuron generation is initiated by signals from the midline structures when neural crest precursor cells aggregate to form sympathetic ganglion primordia. Major components of these signals are BMP family members, which are essential and sufficient to induce the generation of sympathetic neurons from neural crest cells in vitro and in vivo (Varley et al., 1995; Reissmann et al., 1996; Shah et al., 1996; Shah and Anderson, 1997; Schneider et al., 1999). BMP2/4 and 7 are produced by the dorsal aorta before and during sympathetic neuron development (Reissmann et al., 1996; Shah et al., 1996). Inhibition of BMP function by the BMP antagonist noggin abolished the expression of characteristic noradrenergic marker genes *tyrosine hydroxylase* (*TH*), *dopamine-β-hydroxylase* (*DBH*) and of pan-neuronal marker genes *SCG10* and *neurofilament* (Schneider et al., 1999). BMPs act directly on neural crest precursor cells and induce the expression of a group of transcriptional regulators, including the proneural gene *Mash1* (referred to as *Cash1* in the chick), the paired homeodomain proteins *Phox2a* and *Phox2b* and the bHLH factor *dHand* (also known as *Hand2*) (Reissmann et al., 1996; Shah et al., 1996; Howard et al., 1999; Howard et al., 2000). These transcription factors in turn control the further differentiation of sympathetic neuron precursor cells (Guillemot et al., 1993; Lo et al., 1998; Lo et al., 1999; Hirsch et al., 1998; Morin et al., 1997; Pattyn et al., 1999; Stanke et al., 1999; Howard et al., 2000). Essential functions of *Phox2b* and *Mash1* for sympathetic neuron development have been demonstrated in knockout studies, where a lack of neuronal and noradrenergic differentiation was observed (Guillemot et al., 1993; Hirsch et al., 1998; Pattyn et al., 1999). In complementary overexpression experiments Phox2b, Phox2a and dHand induced the generation of sympathetic neurons from neural crest precursor cells both in vitro and in vivo (Stanke et al., 1999; Lo et al., 1999; Howard et al., 1999; Howard et al., 2000). As Phox2a and Phox2b directly bind to the promoter of the DBH and TH genes and activate their transcription (Zellmer et al., 1995; Kim et al., 1998; Swanson et al., 1997; Lo et al., 1999; Seo et al., 2002), these studies suggested a molecular mechanism that could explain noradrenergic phenotype specification and differentiation. *Phox2a* is also thought to represent a link between noradrenergic and generic neuronal differentiation (Lo et al., 1998). In addition, there is in vitro evidence that suggests an interaction of BMP-Phox2 signaling with other signal transduction pathways in noradrenergic differentiation, in particular with pathways involving cAMP, protein kinase A or MAPK signaling (Lo et al., 1999; Swanson et al., 1997; Swanson et al., 2000; Wu and Howard, 2001).

While progress has been made in uncovering the signals and mechanisms involved in the development of noradrenergic sympathetic neurons, less is known about the strategies by

which cholinergic parasympathetic neurons are generated. Whereas sympathetic ganglia form near the neural tube, parasympathetic ganglia are generated close to their peripheral target organs by neural crest cells that migrate for longer distances. In the mature state, parasympathetic neurons provide a functionally cholinergic innervation to their peripheral targets, although there is evidence for expression of some adrenergic characteristics during development (Teitelman et al., 1985; Iacovitti et al., 1985; Landis et al., 1987). Interestingly, Mash1, Phox2a and Phox2b are not only expressed in parasympathetic ganglia but are also essential for parasympathetic ganglion development, as revealed by the severe effects in knockout mice (Guillemot and Joyner, 1993; Guillemot et al., 1993; Morin et al., 1997; Tiveron et al., 1996; Hirsch et al., 1998; Pattyn et al., 1999).

These findings brought into question, whether the expression of these genes and parasympathetic ganglion development are also dependent on BMPs. In addition, the issue was raised of how the same group of transcription factors is able to specify different neuronal fates, i.e. noradrenergic neurons in sympathetic and cholinergic neurons in parasympathetic ganglia. One possibility would be that different neuronal fates are generated by different thresholds of the same transcription factor(s). In the neural tube, the identities of neuronal progenitors are assigned by the graded action of inductive signals that are secreted by ventral and dorsal signaling centres (Jessell, 2000; Anderson, 2001). BMPs produced in the roof plate and dorsal neural tube specify different types of dorsal interneurons in a concentration-dependent manner (Lee and Jessell, 1999). The signaling of BMP family members in embryonic tissues can result in increased levels of downstream transcription factors that specify different cell fates at different thresholds (Gurdon et al., 1998; Gurdon et al., 1999; Shimizu and Gurdon, 1999). Thus, different levels of BMPs and downstream transcription factors may produce noradrenergic sympathetic or cholinergic parasympathetic neurons. There is indeed recent evidence, in vitro, that BMPs affect the decision of PNS progenitors to acquire a sensory or autonomic neuron phenotype and induce cholinergic or noradrenergic genes in autonomic precursors in a concentration-dependent manner (White et al., 2001; Lo et al., 2002). An alternative possibility would be that different environmental signals influence the neural crest precursor cells in such a way that the same network of transcription factors results in different readouts, depending on the cellular context.

This study examines the role of BMPs and transcriptional control genes for the development of parasympathetic ciliary ganglion neurons in the chick embryo. We report that BMPs are locally expressed at the site of ciliary ganglion formation and are required for the development of this ganglion. Overexpression of BMPs results in the generation of additional ciliary ganglion neurons, but does not alter the transmitter phenotype. During normal ciliary ganglion development *Cash1*, *Phox2b* and *Phox2a* are expressed with a similar timing as in sympathetic ganglia. In addition, noradrenergic genes are expressed before cholinergic marker genes but are subsequently downregulated. The transient expression of noradrenergic marker genes may be due to the absence of *dHand* expression in ciliary ganglia, since ectopic dHand expression maintains noradrenergic differentiation. Our finding lead to the following conclusions: first, there is a common dependence of sympathetic and parasympathetic ciliary neuron development on BMPs. Second, dHand is probably involved in the maintenance of noradrenergic gene expression in sympathetic neurons. Third, the specification of sympathetic and parasympathetic ciliary neurons involves unknown signals, leading to differential gene expression in their neural crest precursor cells, which determine the pattern of transcription factors induced by BMPs and their subsequent differentiation.

MATERIALS AND METHODS

Implantation of virus-producing chicken embryo fibroblast cells into chick embryos

For the infection of embryos with RCAS vectors, DF-1 or CEF cells were transfected with the RCAS-cDNAs. Cell aggregates made of infected dHand-RCAS(B) cells (Howard et al., 2000) or BMP4-RCAS(B) CEF-cells were implanted on the right side of the embryo caudal to the developing anlage of the eye at the level of the mesencephalon at stage 11. The eggs were further incubated and the embryos were staged and killed by decapitation at day 8 (dHand) or day 4.5 (BMP4). The heads were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3), kept in 15% (w/v) sucrose overnight, embedded in Tissue Tek (Sakura Finetek Europe BV, Zoeterwoude Netherlands) and sectioned. Consecutive 14 μm cryostat cross sections were separately collected from a large region of the eye around the optic nerve, including the implantation area, and were analyzed for expression of *reverse transcriptase* (*RT*), *Phox2b*, *TH*, *DBH* and *choline acetyl transferase* (*ChAT*). In general, the surrounding mesenchyme was strongly infected, whereas the ganglion displayed incomplete infection.

Injection of noggin-expressing CHO cells, CHO control cells and of virus-concentrate into chick embryos

Noggin-expressing CHO cells or CHO control cells were collected by centrifugation and resuspended in 50 μl PBS. RCAS viral stock preparation was carried out as described previously (Vogel-Höpker and Rohrer, 2002). Cell suspensions were injected into the mesenchyme caudal to the developing anlage of the eye of stage 11 embryos, using fine glass capillaries attached to an aspirator tube (Sigma A-51779). The eggs were further incubated, staged (Hamburger and Hamilton, 1951) and killed by decapitation. The heads were fixed, sectioned, and the sections were analyzed for expression of *Sox10, SCG10, Phox2b*, *VAChT* and *Cash1* by in situ hybridisation. At least three embryos were analysed for each marker.

In situ hybridization of sections

Nonradioactive in situ hybridization and preparation of digoxigenin- or fluorescein-labeled probes for chick *RT*, *TH*, *DBH*, *Phox2a*, *Phox2b*, *ChAT*, vesicular acetylcholine transporter (*VAChT*), chick high-affinity choline transporter (*CHT1*), *Cash1*, *SCG10*, *NF160*, *Sox10* were carried out as described previously (Ernsberger et al., 1997; Stanke et al., 1999). As a probe for chick *CHT1*, a 1134 bp fragment corresponding to bases 426-1559 of the human sequence was used. The *CHT1* fragment was cloned from E9 chick CG-cDNA, using degenerated PCR primers based on homologous sequences from nematode *CHO-1* and rat *CHT1*. The resulting cDNA fragment shows 80% identity to human sequence at nucleotide level and 85% identity at protein level (EMBL/GenBank/DDBJ accession number AJ11267).

Double in situ hybridization was carried out using DIG- or fluorescein-labeled probes for *VAChT* and *TH*. The first colour reaction with Fast Red was stopped by washing in PBS. Photos were taken and the antibody was stripped off by washing two times for 10 minutes with 1 ml 0.1 M glycine pH 1.8. After an additional wash for 1 hour in MABT the second colour reaction with NBT/BCIP was carried out.

Morphometric analysis

The number of *TH*-positive cells was counted on all sections infected by the virus indicated by expression of *RT* mRNA. On alternate sections the area of *Phox2b* or *ChAT* mRNA expression was quantified morphometrically, using the Metamorph Imaging System (Version 4.6, Universal Imaging Corporation). The number of *TH*-positive cells were counted in relation to the area of *Phox2b*-positive cells and expressed as cells/mm^2.

To visualize the effect of BMP treatment on ciliary ganglion development, digital images of Phox2b-stained serial sections were aligned and the resulting stacks were used for a 3D-reconstruction, using Metamorph Imaging System Software.

RESULTS

Developmental onset of pan-neuronal and subset-specific gene expression in the chick ciliary ganglion

The development of sympathetic neurons is characterized by the initial expression of BMP-induced transcription factors, followed by the expression of specific noradrenergic genes and of generic neuronal genes. The present analysis of chick ciliary ganglion development revealed a remarkably similar gene expression profile. The initial expression of characteristic marker genes was observed in ganglion primordia that form around the oculomotor nerve at stage 18. Ciliary neuron development is initiated by the expression of the proneural gene *Cash1*, followed by *Phox2b* and *Phox2a*. After the onset of *Phox2b* expression, the noradrenergic genes *DBH* and *TH* (not shown) and the panneuronal genes *SCG10, NF160* (not shown) are expressed in subpopulations of *Phox2*-positive cells (Fig. 1A; stage 19/20). In contrast, the bHLH transcription factors *dHand* and *eHand* were not detected (Fig. 1A and data not shown). Cholinergic differentiation was analysed using three different marker genes, *ChAT*, *VAChT* and *CHT1* (Okuda et al., 2000; Misawa et al., 2001; Lips et al., 2002). For all three cholinergic genes the onset of expression was after the initial *TH*/*DBH* expression (Fig. 1A; stage 19/20 vs stage 24/25; *ChAT* not shown). *TH* and *DBH* were, however, expressed only transiently, resulting in ciliary ganglia that are composed almost exclusively by cholinergic neurons at E8 (Fig. 1A; stage 24/25 vs stage 32/33; very few DBH-expressing neurons are detectable at stage 32/33; *TH* not shown). The absence of dHand and the virtually complete downregulation of *TH* and *DBH* expression thus represent major differences between ciliary and sympathetic neuron differentiation.

BMPs in parasympathetic ciliary ganglion development

As BMPs control the expression of *Cash1* and *Phox2a/Phox2b* in sympathetic ganglia (Reissmann et al., 1996; Shah et al., 1996; Schneider et al., 1999), we investigated whether BMPs are expressed in the vicinity of the forming ciliary ganglion. The analysis of *BMP4, 5* and *7* expression in the retro-orbital region demonstrates *BMP7* expression (Fig. 2) in the region where the ciliary ganglion forms. Weak, but significant mesenchymal *BMP5* expression, but no *BMP4* expression was detected (not shown). Thus, at the onset of *Cash1* and *Phox2b* expression at stage 18, both *BMP7* and *BMP5* could be detected, which is compatible with the notion that BMPs control the expression of these transcriptional regulators.

To see whether the development of ciliary neurons is also dependent on BMPs, the BMP antagonist noggin was applied in the vicinity of the developing ciliary ganglion. Cell suspensions of noggin-producing CHO cells or CHO control cells were applied at stage 10/11 unilaterally into the craniofacial mesenchyme, close to the optic stalk. The implantation of control cells did not affect ciliary ganglion development (not shown). In contrast, we observed in a large proportion of noggin-treated embryos (5 out of 6) a complete lack, unilaterally, of differentiated ciliary ganglion cells, when the embryos were analysed for the expression of the generic neuronal marker *SCG10*, the autonomic markers *Cash1*, *Phox2b* and the cholinergic marker *VAChT* at stage 24/25 (Fig. 3A-D). However, a large aggregate of cells expressing the neural crest marker *Sox10* could be observed in the region, where the ciliary ganglion would form (Fig. 3E,F). In most cases also eye development was severely affected on the noggin-treated side, resulting in lacking or rudimentary retinae (Fig. 3A-H). To address the possibility that the impaired ciliary ganglion development might be caused indirectly by the absence of the eye, we analysed ciliary ganglion development after ablation of the optic cup. The gene expression pattern of ciliary ganglia was completely unaffected by the lack of eye ablation up to stage 24/25 (*SCG10*, *Phox2b* see Fig. 3G,H; *Cash-1* and *VAChT*, not shown). This agrees with previous studies demonstrating that ciliary neuron survival was not dependent on the peripheral targets up to embryonic day 8 (Landmesser and Pilar, 1974; Lee et al., 2001).

Ectopic cholinergic ciliary ganglion neurons are induced by BMP4

To gain further insight into the role of BMPs in ciliary ganglion development, BMP4 was ectopically expressed in the ciliary ganglion and its environment, using the avian retroviral vector RCAS (B) (Reissmann et al., 1996; Howard et al., 2000). The unilateral implantation of virus-producing chick embryo fibroblast cells (CEFs) into stage 10/11 chick embryos resulted in the infection and BMP4 production of ciliary ganglion and surrounding mesenchymal cells. BMP4 overexpression resulted in a considerable enlargement of the ciliary ganglion (Fig. 4A). Using 3D reconstructions of Phox2b-stained serial sections we observed, in BMP-treated embryos, ectopic neurons in the oculomotor nerve and in postganglionic ciliary nerves. The enlarged ganglia were composed of neurons with the same characteristics as observed during normal development, i.e. many apparent cholinergic neurons that were positive for *ChAT* (Fig. 4A). To investigate whether BMP overexpression would stimulate adrenergic differentiation in parasympathetic ciliary ganglia, the number of *TH*-positive cells/section (Fig. 4C) and the areas of *ChAT*-positive and *Phox2b*-positive cells/section (Fig. 4B) were determined in control and BMP4-treated embryos. *Phox2b*, *ChAT* and *TH* increased to a similar extent (Fig. 4B,C). Thus, when *ChAT* and *TH* expression is examined in relation to the area of Phox2b expression, i.e. the total area of ciliary ganglion neurons, neither the proportion of *ChAT*-expressing cells (Fig. 4D) nor of *TH*-positive cells (Fig. 4E) was changed in response to the BMP4 treatment.

The control of noradrenergic differentiation in the parasympathetic ciliary ganglion

While mature parasympathetic neurons display a cholinergic

neurotransmitter phenotype, during development a transient expression of noradrenergic genes was observed, apparently controlled in part by the same transcription factors that lead to maintained noradrenergic gene expression in sympathetic ganglia. What causes the decrease of *TH* and *DBH* expression in parasympathetic ganglia? Two explanations are obvious, the loss of *TH*/*DBH*-positive noradrenergic neurons or a switch in the neurotransmitter phenotype from noradrenergic to cholinergic. Although ciliary ganglion cell number apparently does not change during this period (Landmesser and Pilar, 1974), recent evidence suggests that considerable cell death does occur and is compensated by the differentiation of neuron precursor cells present in the ganglia (Lee et al., 2001). The demonstration of ciliary ganglion neurons that co-express *TH* and the cholinergic marker gene *VAChT* (Fig. 5) is in agreement with the notion that cells with noradrenergic gene expression acquire a cholinergic phenotype. Owing to the transient *TH* and *DBH* expression and the graded increase in the expression of cholinergic marker genes, the relatively low proportion of double-positive cells is in line with the expectations. With respect to noradrenergic differentiation, these data thus demonstrate a decrease of *TH* and *DBH* as a major difference between ciliary and sympathetic neurons. This may either be due to the selective repression/loss of *TH*/*DBH* expression in parasympathetic ganglia or the selective maintenance of noradrenergic gene expression in sympathetic neurons.

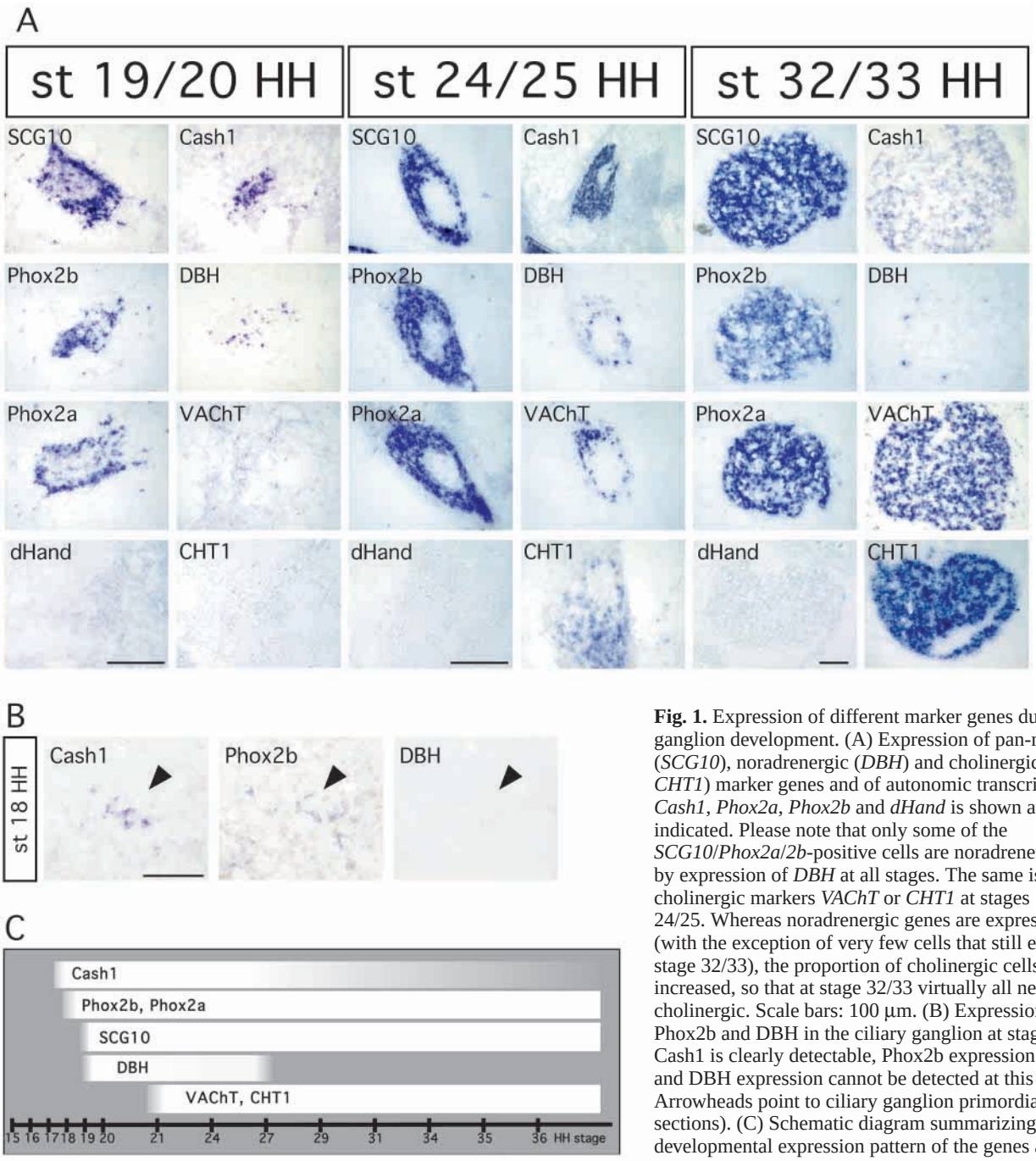

Fig. 1. Expression of different marker genes during ciliary ganglion development. (A) Expression of pan-neuronal (*SCG10*), noradrenergic (*DBH*) and cholinergic (*VAChT*, *CHT1*) marker genes and of autonomic transcription factors *Cash1*, *Phox2a*, *Phox2b* and *dHand* is shown at the stages indicated. Please note that only some of the *SCG10*/*Phox2a*/*2b*-positive cells are noradrenergic as revealed by expression of *DBH* at all stages. The same is true for the cholinergic markers *VAChT* or *CHT1* at stages 19/20 and 24/25. Whereas noradrenergic genes are expressed transiently (with the exception of very few cells that still express DBH at stage 32/33), the proportion of cholinergic cells is strongly increased, so that at stage 32/33 virtually all neurons are cholinergic. Scale bars: 100 μm. (B) Expression of Cash1, Phox2b and DBH in the ciliary ganglion at stage 18. Whereas Cash1 is clearly detectable, Phox2b expression has just begun, and DBH expression cannot be detected at this stage. Arrowheads point to ciliary ganglion primordia (consecutive sections). (C) Schematic diagram summarizing the developmental expression pattern of the genes analysed in A.

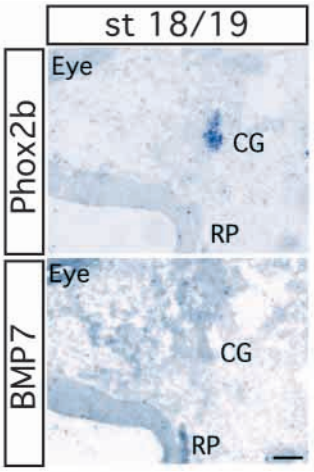

Fig. 2. Expression of *BMP7* at the site of ciliary ganglion formation. In the environment of *Phox2b*-positive ciliary ganglion cells (CG, upper panel) *BMP7* expression is detected in the retro-orbital mesenchyme (lower panel) by in situ hybridisation (stage 18/19). Stronger expression of *BMP7* can be seen in Rathke's Pouch (RP) and in the retina. The location of the eye is indicated. Scale bar: 100 μm.

dHand expression in ciliary ganglia maintains noradrenergic properties

Differential control of noradrenergic gene expression implies signals that are selectively expressed in ciliary and sympathetic ganglia, respectively. Of the transcription factors that have been shown to be directly or indirectly involved in the control of noradrenergic gene expression, only *dHand* is selectively expressed in sympathetic ganglia (Fig. 1A) (Howard et al., 1999; Howard et al., 2000), whereas *Cash1*, *Phox2a* and *Phox2b* are pan-autonomic genes. In sympathetic ganglia, *dHand* is detectable throughout development and thus could play a role in the regulation of *TH* and *DBH* at later stages. Thus, the absence of *dHand* expression in ciliary ganglia could explain the transient nature of noradrenergic gene expression.

To test this proposed role, dHand was ectopically expressed in ciliary ganglia using retroviral vectors. Interestingly, a strong increase in the number of *TH*-expressing (Fig. 6A) and *DBH*-expressing cells (not shown) was observed in E8 embryos in response to dHand expression (Fig. 6A,B). For quantification, the number of *TH*-positive cells was determined in relation to the ganglion area (*Phox2b*-positive) in sections of dHand-RCAS-infected and uninfected contralateral ganglia. The increase in the proportion of *TH*-positive cells ranged between 1.4- and 12-fold, with a mean increase of 2.6-fold (Fig. 6B), reflecting the variation in ciliary ganglion infection by dHand-RCAS. In contrast to the surrounding mesenchyme, the ciliary ganglion was only partially infected, which may be explained by the inability to infect early postmitotic ciliary neurons. The increased number of *TH*-expressing cells in response to ectopic *dHand* expression is compatible with the notion that *TH* and *DBH* expression are downregulated in ciliary ganglia because of a lack of *dHand* which would maintain *TH*/*DBH* expression in sympathetic ganglia.

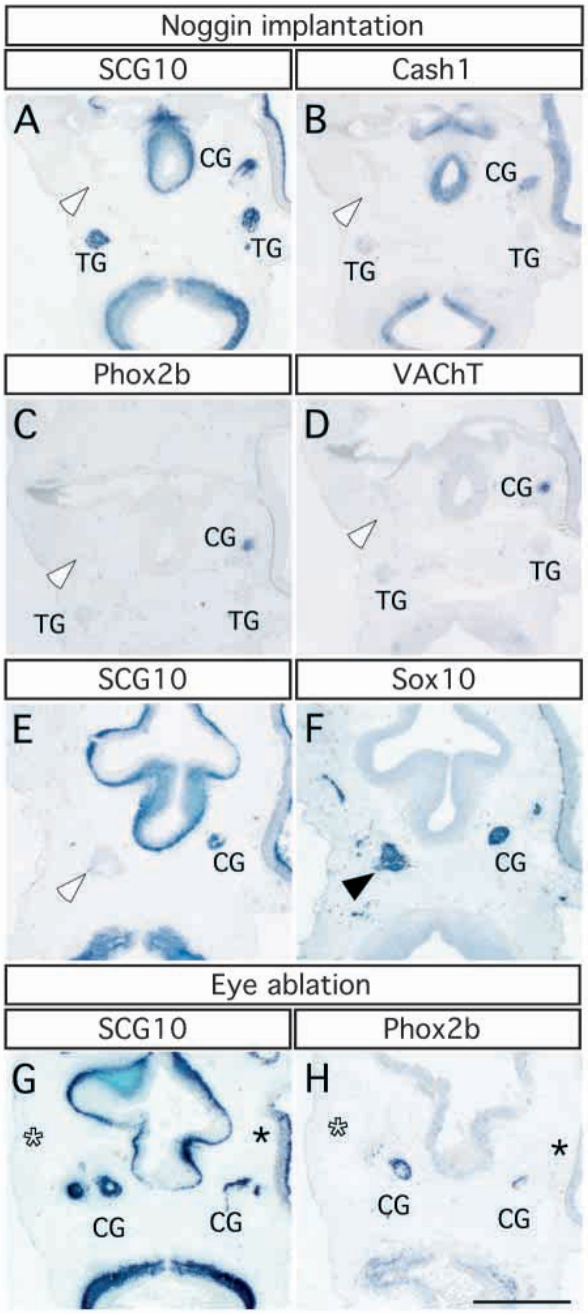

Fig. 3. The BMP inhibitor noggin prevents the development of the ciliary ganglion. After unilateral implantation of noggin-expressing CHO cells in E2 chick embryos the ciliary ganglion, as shown by expression of *SCG10* (A), *Cash1* (B), *Phox2b* (C) and *VAChT* (D), is absent at stage 24/25 (white arrowheads, A-D). In parallel, the development of the eye is strongly reduced (A-F left side; compare with normal right side). *Sox10*-positive neural crest cells aggregate at the position of the ciliary ganglion (black arrowhead, F), but remain undifferentiated (*SCG10*-negative) on the noggin-treated side (white arrowhead, E). Unilateral ablation of the optic vesicle in E2 chick embryos also prevents eye development (G,H; white asterisks; compare normal contralateral eyes, black asterisks), but does not affect ciliary ganglion formation as demonstrated by *SCG10* and *Phox2b* expression in the CG (G,H). The *SCG10*-positive structure adjacent to the CG in (G) is an enlarged oculomotor branch of the trigeminal ganglion, frequently observed in eye-ablated embryos. Scale bar: 1 mm.

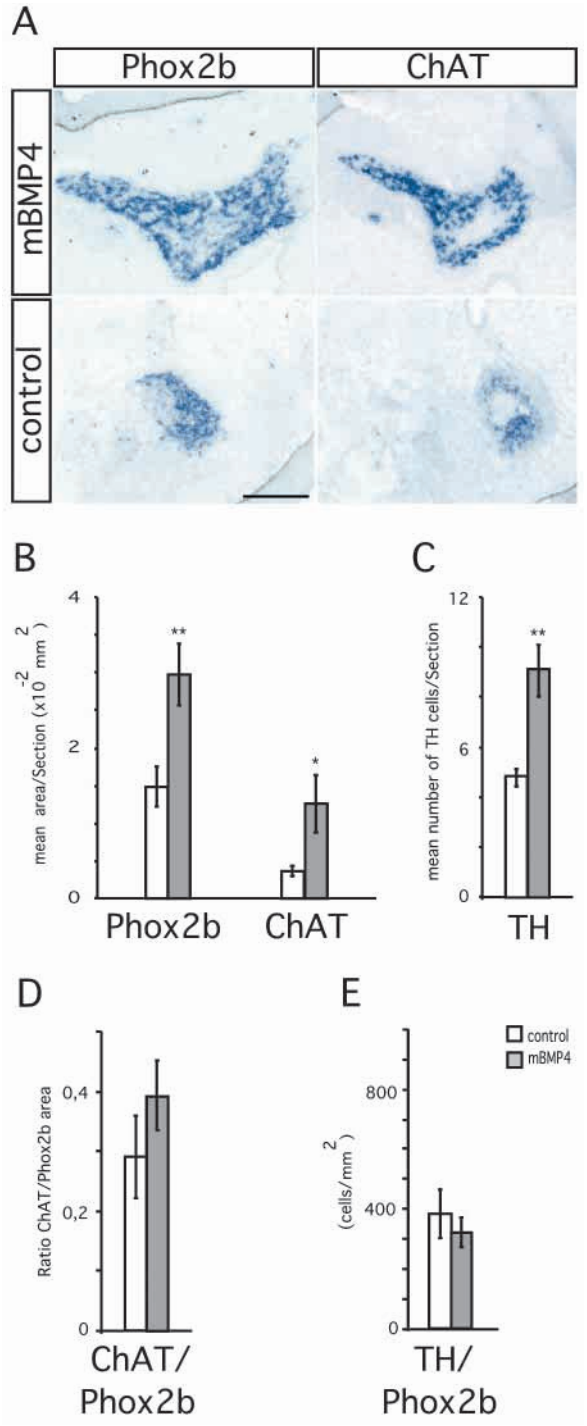

Fig. 4. BMP4 overexpression results in enlarged ciliary ganglia. Embryos, infected at E2 with mBMP4-RCAS display enlarged ciliary ganglia at stage 24/25 as detected by expression of *Phox2b* and *ChAT* (A). (B) Quantification of the area of *Phox2b* and *ChAT*-positive cells revealed a strong increase in comparison to the control side (*Phox2b*, P=0.008; *ChAT*, P=0.039; Student's t-test). Also the number of *TH*-positive cells (C) was significantly increased (P=0.002). However, the ratio between cholinergic markers and *Phox2b* (D) or noradrenergic markers and *Phox2b* (E) showed no significant difference between mBMP4 implanted and control side (P=0.051 and P=0.155, respectively), indicating that BMP4 increases ciliary neuron development but does not affect the phenotype. Scale bar: 250 μm.

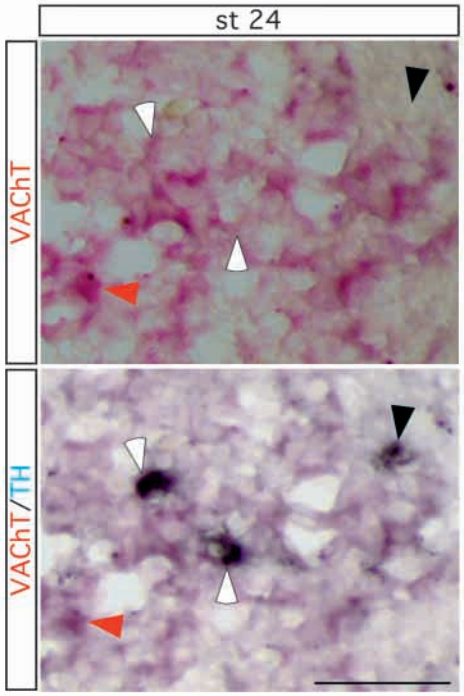

Fig. 5. Ciliary neurons transiently co-express cholinergic and noradrenergic marker genes. Double in situ hybridization for *VAChT* (red) and *TH* (black) at stage 24 shows the presence of cells that co-express *VAChT* and *TH* (white arrowheads) or only express *VAChT* (red arrowhead) or *TH* (black arrowhead). The *VAChT* signal in double-stained cells (white arrowheads) is low but significantly above background, as illustrated by comparison with cells devoid of *VAChT* expression (black arrowhead). Scale bar: 25 μm.

DISCUSSION

The present analysis of chick cholinergic ciliary neuron development revealed gene expression patterns and control mechanisms that are closely related to that of sympathetic neurons. BMPs were found to be essential and sufficient for ciliary neuron development and BMP downstream signaling involving the transcription factors Cash1, Phox2a and Phox2b, as shown previously for sympathetic ganglia. However, chick sympathetic and ciliary neurons differ with respect to the developmental expression of the noradrenergic marker genes *TH* and *DBH*. Our results suggest that this difference is due to the selective expression of the transcription factor dHand in sympathetic neurons, maintaining TH/DBH expression in this lineage (Fig. 7). Thus, the generation of different neuronal subtypes, noradrenergic sympathetic and cholinergic parasympathetic neurons, in response to a BMP signal is explained by the differential expression of transcriptional control elements, including dHand. This scenario would implicate fate-determining differences between sympathetic and parasympathetic ciliary precursor cells at the time they respond to BMPs (Fig. 7).

Ciliary neurons are derived from neural crest at the mesencephalic/metencephalic border region (Hammond and Yntema, 1958; Noden, 1975; Narayanan and Narayanan, 1978). These cells migrate rostrally towards the optic vesicle and form the ciliary ganglion primordium behind the eye, close

to the optic nerve. The ganglion was first detectable as an aggregate of *Cash-1*-positive cells at stage 18 that was wrapped around the oculomotor nerve. The onset of *Phox2b* and *Phox2a* expression was observed shortly afterwards, followed by panneuronal and noradrenergic gene expression. Cholinergic marker gene expression starts after the ganglion has been generated, i.e. after the expression of *Cash1*, *Phox2a*/*b* and *TH*/*DBH*. A previous study identified ciliary neuron precursors during migration, using an antibody against a ciliary neuron-specific cell surface antigen (Barald, 1988). As there was evidence to suggest that the antigen may be involved in high-affinity choline transport (Barald, 1988), the present analysis also includes the choline transporter. Our data demonstrate, however, that *CHT1* (Okuda et al., 2000; Misawa et al., 2001; Lips et al., 2002) is first expressed together with *ChAT* and *VAChT* after the formation of ciliary ganglion primordia and after a number of other neuronal genes have started their expression. Although our results do not exclude an earlier specification of ciliary neurons, they exclude *CHT1* as an early indicator for migrating ciliary neuron precursors.

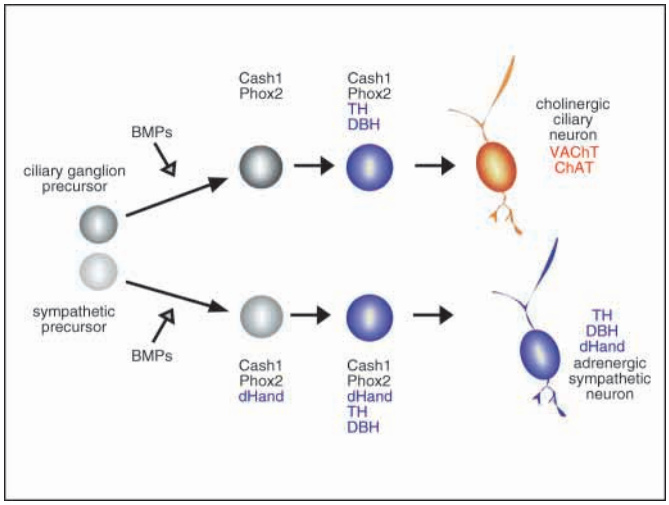

Fig. 7. Schematic diagram summarizing the role of BMPs in the development of noradrenergic sympathetic and parasympathetic ciliary neurons. BMPs are sufficient and essential for the development of both noradrenergic sympathetic (Varley et al., 1995; Reissmann et al., 1996; Shah et al., 1996; Schneider et al., 1999) and parasympathetic ciliary neurons (the present study). The differential expression of the BMP-downstream transcription factor dHand suggests that sympathetic and ciliary neuron precursors display differences that determine BMP downstream signaling and in turn neuron identity (illustrated by the different shading of sympathetic and parasympathetic precursor cells). dHand is implicated in the maintenance of noradrenergic differentiation (TH, DBH) in sympathetic neurons.

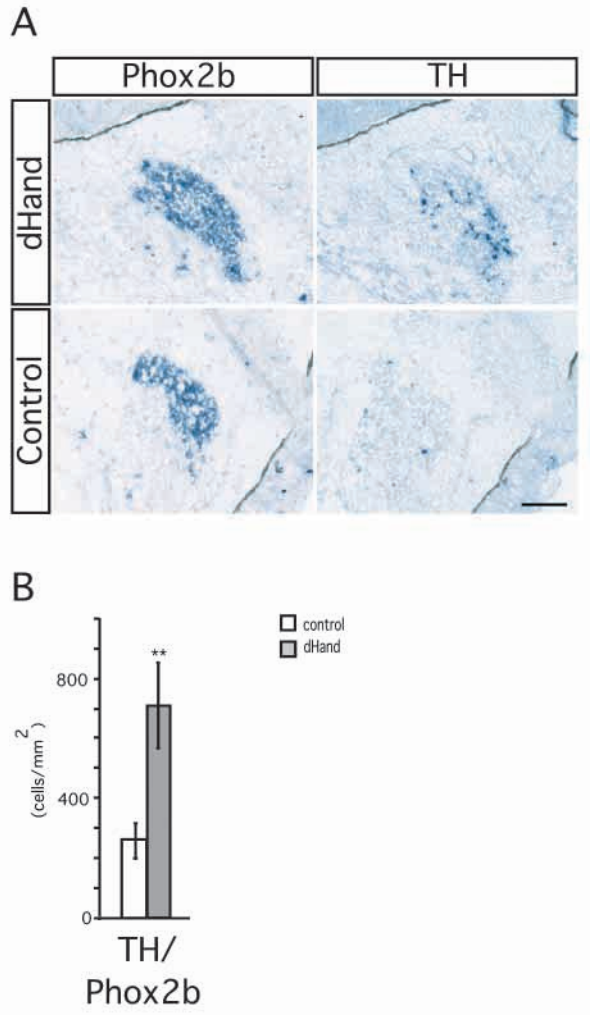

Fig. 6. The number of noradrenergic cells in ciliary ganglia is strongly increased by ectopic expression of dHand. (A) Embryos infected at E2 with dHand-RCAS display an increased number of *TH*-positive cells at E8. Phox2b and TH stainings were done on alternate sections. (B) The quantification of *TH*-positive cells per ciliary ganglion area (*Phox2b*-positive) revealed a strong increase compared to controls (n=9; P=0.009). Scale bar: 100 μm.

As sympathetic neuron generation is controlled by BMPs that are expressed in the dorsal aorta, in close vicinity to the forming primary sympathetic ganglia (Reissmann et al., 1996; Shah et al., 1996), we asked whether BMPs are also involved in ciliary ganglion development. *BMP5* and *BMP7* were found to be expressed in Rathke's pouch and in the mesenchyme around the ciliary ganglion anlage at stage 18, albeit at low levels. Whereas the expression in Rathke's pouch seems to be too far away to represent a significant source of BMPs for the ciliary neuron precursors, mesenchymal BMP7 (Luo et al., 1995; Dudley et al., 1995) is most likely involved in ciliary neuron development. However, the possibility should also be considered that ciliary ganglion precursors may encounter BMPs during their migration.

Are BMPs important for ciliary neuron development? The interference with the function of BMPs by the application of the BMP-inhibitor noggin (Zimmerman et al., 1996; Schneider et al., 1999; Vogel-Höpker and Rohrer, 2002) resulted in the complete lack of differentiated ciliary neurons, strongly suggesting that ciliary neuron development requires BMPs. The presence of aggregates of Sox10-positive cells at the site of the ciliary ganglion demonstrates that neural crest migration and aggregation are not affected by noggin. Noggin-treatment also affected the development of the eye, whereas control embryos, receiving control CHO cells, had normal sized eyes and the ciliary ganglion was present. To investigate whether the effects on ciliary neurons are an indirect result of the missing

eye, the optic vesicle was unilaterally extirpated at E2. Although the eye was virtually completely missing on the operated side, there was no difference with respect to the size and gene expression pattern of the ciliary ganglion, as expected from previous studies (Landmesser and Pilar, 1974; Lee et al., 2001). Thus, both ciliary neuron development and the development of the eye depend on BMPs. Indeed, in the BMP7 knockout mice, eye development is severely affected (Luo et al., 1995; Dudley et al., 1995). The fate of the ciliary ganglion has not been analysed in these mice. Since noggin interferes with the action of several BMP family members, the identity of the BMP(s) that are essential for ciliary ganglion development remains unclear. In view of the redundant expression of BMPs and the stronger phenotype of the BMP5/BMP7 double knockout mice as compared to single knockout mice (Solloway and Robertson, 1999), it is possible that both BMP5 and BMP7 are involved in ciliary neuron development. The proposed role of BMPs in ciliary neuron development is strongly supported by BMP overexpression experiments. Forced expression of BMP4 produced enlarged ciliary ganglia through the generation of ectopic neurons from neural crest precursor cells. These cells were generated in the oculomotor nerve and postganglionic ciliary nerve. As expected from previous BMP overexpression experiments in sympathetic ganglia and peripheral nerves (Reissmann et al., 1996; Howard et al., 2000) these cells also expressed *Phox2b*. Interestingly, the neurons generated in response to BMPs displayed a cholinergic rather than a noradrenergic phenotype, as did the neurons in the ciliary ganglion. This is in contrast to the situation in the trunk, where BMPs produce enlarged noradrenergic sympathetic ganglia and ectopic noradrenergic neurons in peripheral nerves (Reissmann et al., 1996; Howard et al., 2000; Ernsberger et al., 2000).

This result raised the question of why noradrenergic gene expression is regulated differentially in ciliary neurons and nerves as compared to sympathetic neurons and trunk precursor cells. In view of the similar expression pattern of *Mash1* and *Phox2* genes in sympathetic and parasympathetic ganglia and the *Mash1*- and *Phox2*-dependent development of cranial parasympathetic ganglia (Hirsch et al., 1998; Morin et al., 1997; Pattyn et al., 1999) it seems very likely that Mash1 and Phox2 genes are also involved in the initial onset of *TH* and *DBH* expression in the ciliary ganglion. Although noradrenergic gene expression in the mouse ciliary ganglion has not been analysed in *Mash1* and *Phox2* knockouts, *DBH* expression is controlled by *Mash1* in the parasympathetic sphenopalatine ganglion (Hirsch et al., 1998). In addition, ciliary ganglion development depends on *Mash1* (Hirsch et al., 1998) and likely also on *Phox2* genes, as other cranial parasympathetic ganglia are missing in the *Phox2a* and *Phoxb* knockouts (Morin et al., 1997; Pattyn et al., 1999). However, whereas *TH* and *DBH* are rapidly induced in all *Phox2*-positive sympathetic neuron precursors, *TH*/*DBH*-positive cells in ciliary ganglia represent at all stages only a subpopulation of *Phox2*-expressing cells. This could be explained by a lower sensitivity of parasympathetic precursor cells to BMPs, requiring higher concentrations for *TH* expression, as compared to the expression of cholinergic markers like *VAChT* (White et al., 2001). The observation that BMP overexpression does not increase the proportion of TH/DBH-expressing cells argues, however, against this notion. The presence of cells that co-express *TH* and *VAChT* at stage 24 suggests that ciliary neurons transiently express noradrenergic genes before they acquire a cholinergic transmitter phenotype. The low number of cells that are clearly double-labeled is expected if noradrenergic and cholinergic gene expression overlap only during a short time period. Although we cannot exclude that the disappearance of noradrenergic cells is partly due to the selective death of these cells (Lee et al., 2001), this possibility seems to be unlikely in view of the cells being in a transitory stage from a noradrenergic to cholinergic phenotype and the increased TH and DBH expression in response to dHand.

The selective downregulation of *TH* and *DBH* expression in ciliary as compared to sympathetic ganglia may either be due to the presence of a *TH*/*DBH* repressor in ciliary ganglia or due to the lack of a *TH*/*DBH* maintenance signal. Here, we show that the bHLH transcription factor dHand could be such a maintenance factor. *dHand* is expressed in sympathetic neurons under the control of BMPs, downstream of *Phox2b* (Howard et al., 2000). Forced expression of dHand in trunk neural crest precursors elicits the generation of noradrenergic neurons both in vitro and in vivo (Howard et al., 1999; Howard et al., 2000). The increased number of *TH*- and *DBH*-positive cells in dHand-expressing ciliary ganglia now suggests that dHand is also involved in maintaining noradrenergic differentiation. The effects of forced dHand expression in neural crest cells are compatible with the notion that dHand represents a late BMP effector, that is able to act independently of the upstream factors Cash1, Phox2b, Phox2a. This is in line with the observation that dHand is able to transactivate DBH reporter constructs (M. Howard, personal communication). However, dHand overexpression elicits the expression of both *Phox2a* and *Phox2b* genes (Howard et al., 2000) and thus, may act in combination with Phox2 transcription factors. Both possibilities are compatible with a role for dHand in the maintenance of *TH* and *DBH* expression, a role that still needs to be confirmed by loss-of-function experiments. The *dHand* knockout mice die, however, too early to investigate a role for dHand as a maintenance signal of DBH and TH expression (Yamagishi et al., 1999).

The differential expression of *dHand* has also important implications for the understanding of sympathetic and parasympathetic ciliary neuron development and the role of BMPs in this process. What is the role of BMPs in the generation of different autonomic neuronal subtypes? In the spinal cord, different types of dorsal neurons are specified by different levels of BMPs (Lee and Jessell, 1999) and our recent work on noradrenergic neuron development in the hindbrain demonstrated that the generation of noradrenergic locus coeruleus neurons depends directly or indirectly on the BMP-mediated dorsal patterning of rhombomere 1 (Vogel-Höpker and Rohrer, 2002). As *ChAT* is induced at lower BMP levels than *TH* in cultured peripheral nerve precursor cells it was proposed that parasympathetic versus sympathetic neuron generation may be specified by different BMP levels in vivo (White et al., 2001). The present demonstration that parasympathetic ciliary ganglion cells initially also display a noradrenergic phenotype is difficult to accommodate with this idea. One could argue that TH/DBH would be expressed in autonomic neurons as long as there is a high-level BMP expression in the vicinity of autonomic ganglia. Indeed, BMP7 and BMP5 expression in the retro-orbital mesenchyme

decreases between stage 18 and stage 20 (F. Müller, unpublished observation), whereas BMP4 expression in the dorsal aorta is maintained up to E9 (U. Ernsberger, personal communication). Thus, there is a correlation between BMP expression and TH/DBH expression in autonomic ganglia. However, strong evidence against a BMP-level-dependent specification of cholinergic parasympathetic and noradrenergic sympathetic neurons is the observation that BMP overexpression in the ciliary ganglion did not increase *TH*/*DBH* expression, whereas *TH*/*DBH* expression has been shown to be induced in neural crest precursors in trunk peripheral nerve (Ernsberger et al., 2000). This result could also be explained by assuming that BMP overexpression in ciliary compared with sympathetic ganglia and nerves never reached the high BMP levels required to elicit *TH*/*DBH* expression, because of differential expression of BMP inhibitors. However, there are several factors that do not substantiate this explanation. (i) Assuming that *TH*/*DBH* expression requires high BMP concentrations, the presence of *TH*/*DBH*-positive cells would imply that high BMP levels are present and can be reached in the ciliary ganglion environment. (ii) The generation of ectopic neurons by BMP overexpression demonstrates that significant increases in BMP levels were reached in vivo in both cases. (iii) The lack of *dHand* expression in *TH*/*DBH* ciliary neurons generated in response to BMPs argues for a difference between ciliary and sympathetic neuron precursors. In conclusion, the present results are more compatible with the notion of differences between autonomic precursor cells in the head region where the ciliary ganglion forms and autonomic precursor cells in sympathetic ganglia and trunk peripheral nerve. It will be interesting to investigate to what extent the present findings can be generalized to cranial and trunk parasympathetic ganglia.

What may cause a difference between sympathetic and ciliary precursors? These differences may reflect different anteroposterior positional values (Jessell and Lumsden, 1998; Rubinstein and Shimamura, 1998) since ciliary and sympathetic precursors are derived from different axial levels (Le Douarin and Kalcheim, 1999), or local signals in the environment of the forming ganglia. Heterotopic neural crest transplantation experiments demonstrated that the developmental capacities of the neural crest cells are qualitatively equivalent at all axial levels (Le Dourin et al., 1975; Le Douarin and Kalcheim, 1999), which strongly suggest that local signals specify the fate of neural crest cells following their migration. The finding that autonomic precursor cells are present in the ciliary ganglion at E4.5 and later that can differentiate to noradrenergic neurons upon heterotopic transplantation (Le Douarin et al., 1978; Dupin, 1984) seems to contradict our inability to elicit noradrenergic differentiation by BMP4 overexpression. It should, however, be noted that the potential of these cells is repressed during normal development, apparently by local signals, and is revealed and realized only after ganglia disassemble during backtransplantation (Le Douarin et al., 1978; Dupin, 1984; Schweizer et al., 1983). Thus, we propose that the local environmental, signals that define parasympathetic neuron identity in the ciliary ganglion primordium e.g. the repression of *dHand* expression, cannot be overcome by increased expression of BMPs and downstream signaling in vivo, in the ciliary ganglion environment. This may be possible in vitro or after transplantation of these cells into a different environment in vivo. Interestingly, not only neural crest precursors from the ciliary ganglion, but also ciliary neurons are able to acquire a noradrenergic phenotype upon transplantation and migration into the trunk (Coulombe and Bronner-Fraser, 1986). The finding that noradrenergic differentiation of immature ciliary neurons does not occur upon implantation into the head mesenchyme of young embryos (Sechrist et al., 1998) supports our conclusion that signals from the head mesenchyme environment prevents full noradrenergic differentiation.

What is the reason for a low number of neurons maintaining *TH* and *DBH* in the ciliary ganglion in the absence of *dHand*? The compensation by the closely related factor *eHand* (Srivastava et al., 1995; Hollenberg et al., 1995; Howard et al., 1999) is excluded as *eHand* expression was not detected in the ciliary ganglion (data not shown). We propose that *dHand* function may be required only during a relatively early developmental period and that its function can be replaced later by other signaling pathways. For dopaminergic neurons in cranial sensory ganglia, in particular in the petrosal ganglion, there is evidence for an early, transient *TH* expression, followed later by a second, sustained *TH* expression, elicited by signals from the target (Katz and Erb, 1990) and/or maintained by electrical activity (Brosenitsch and Katz, 2001). A similar scenario would account for early and late expression of *TH* and *DBH* in mammalian parasympathetic ganglia (Hirsch et al., 1998; Landis et al., 1987; Leblanc and Landis, 1989). In view of the very low number of *TH*/*DBH*-positive cells that remain in chick ciliary ganglia, this issue was not addressed in our study.

Whereas the present study revealed mechanisms involved in the control of noradrenergic differentiation in the ciliary parasympathetic ganglion versus sympathetic ganglia, the extrinsic signals and the transcription factors controlling the early, target-independent expression of cholinergic marker genes are not known (Ernsberger and Rohrer, 1999) and it remains a matter of speculation whether similar factors are involved in sympathetic and parasympathetic ganglia. Candidate cholinergic differentiation factors are ligands for the c-ret receptor, since c-ret is selectively expressed in cholinergic sympathetic neurons (Ernsberger et al., 2000) and ciliary neurons (Hashino et al., 2001) and since c-ret ligands are able to induce cholinergic marker genes in vitro (Brodski et al., 2002). Neuropoietic cytokines acting through gp130/LIFRβ receptors are involved in the expression of VIP but not of ChAT and VAChT in chick sympathetic neuron development in vivo (Geissen et al., 1998; Duong et al., 2002).

In conclusion, our results provide evidence that BMPs are essential for the generation of both parasympathetic and sympathetic neurons and suggest that BMPs act on neural crest precursor cells that display specific location-dependent differences that in turn determine the response to BMP signaling and eventually the autonomic neuron subtype. The transcription factor *dHand* is not only an indicator for this difference but may be responsible for the differential expression of the noradrenergic marker genes *TH* and *DBH* in sympathetic and parasympathetic ciliary neurons.

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