HoxB8 in noradrenergic specification and differentiation of the autonomic nervous system

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Different prespecification of mesencephalic and trunk neural crest cells determines their response to environmental differentiation signals and contributes to the generation of different autonomic neuron subtypes, parasympathetic ciliary neurons in the head and trunk noradrenergic sympathetic neurons. The differentiation of ciliary and sympathetic neurons shares many features, including the initial BMP-induced expression of noradrenergic characteristics that is, however, subsequently lost in ciliary but maintained in sympathetic neurons. The molecular basis of specific prespecification and differentiation patterns has remained unclear. We show here that HoxB gene expression in trunk neural crest is maintained in sympathetic neurons. Ectopic expression of a single HoxB gene, HoxB8, in mesencephalic neural crest results in a strongly increased expression of sympathetic neuron characteristics like the transcription factor Hand2, tyrosine hydroxylase (TH) and dopamine-beta-hydroxylase (DBH) in ciliary neurons. Other subtype-specific properties like RGS4 and Rcad are not induced. HoxB8 has only minor effects in postmitotic ciliary neurons and is unable to induce TH and DBH in the enteric nervous system. Thus, we conclude that HoxB8 acts by maintaining noradrenergic properties transiently expressed in ciliary neuron progenitors during normal development. HoxB8, HoxB9, HoxB1 and HoxD10 elicit either small and transient or no effects on noradrenergic differentiation, suggesting a selective effect of HoxB8. These results implicate that Hox genes contribute to the differential development of autonomic neuron precursors by maintaining noradrenergic properties in the trunk sympathetic neuron lineage.

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Introduction

The development of the autonomic nervous system is initiated by bone morphogenetic proteins (BMPs) acting on neural crest cells (NCC) in the primordia of both sympathetic and parasympathetic ganglia (Morikawa et al., 2009; Müller and Rohrer, 2002; Schneider et al., 1999). These extrinsic signals induce a regulatory network of transcription factors in the NCC, which in turn leads to the differentiation of sympathetic and parasympathetic neurons (Goridis and Rohrer, 2002). The network of transcription factors that are implied in sympathetic neuron development includes Ascl1, Insm1, Phox2a/Phox2b, Hand2, GAT2A/3, Sox4, Sox11 and AP2oxβ (Coppola et al., 2005, 2010; Goridis and Rohrer, 2002; Pattyn et al., 1999, 2006; Potzner et al., 2010; Schmidt et al., 2011; Stanke et al., 1999; Tsarovina et al., 2004; Wildner et al., 2008). These transcription factors are important at distinct time points of sympathetic neuron development leading to the generation of postmitotic noradrenergic, TH and DBH positive neurons (Ensberger et al., 1995; Goridis and Rohrer, 2002). The absence of Phox2b for example leads to a complete prevention of sympathetic ganglia development (Pattyn et al., 1999). The loss of Hand2 reduces the number of TH and DBH positive cells but has no influence on the generic neuron differentiation as shown by normal expression of the pan-neuronal genes TUJ1 or SCC10 (Howard et al., 1999, 2000; Lucas et al., 2006; Schmidt et al., 2009).

The development of parasympathetic ciliary neurons from mesencephalic NCC shares many features with initial sympathetic neuron generation like the expression of Ascl1 and Phox2b that are induced by BMPs (Müller and Rohrer, 2002). Although virtually all of the differentiated ciliary neurons are cholinergic, TH and DBH are also transiently expressed. At later time points, TH and DBH expression is restricted to a very small subpopulation of ciliary neurons (Lee et al., 2005; Müller and Rohrer, 2002). In contrast to sympathetic neurons, ciliary neurons do not express GATA3 and Hand2 expression is limited to the small noradrenergic cell population (Müller and Rohrer, 2002).

Also the development of enteric neurons, which are derived from the vagal neural crest is controlled by BMP signaling (Chalazonitis et al., 2004, 2008; Goldstein et al., 2005; Lo et al., 1997) with essential functions for Phox2b (Pattyn et al., 1999), Ascl1 (Blaugrund et al., 1996) and Hand2 (D’Autreaux et al., 2007; Hendershot et al., 2007; Lei and Howard, 2011; Wu and Howard, 2002). TH is expressed only transiently in Ascl1-dependent embryonic mouse enteric neurons (Baetge et al., 0012-1606/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
The developmental field of sympathetic, enteric and parasympathetic ciliary neurons may be explained by different local signals in the ganglion primordia that determine instructively the fate of these lineages together with BMPs. This hypothesis is supported by transplantation of mesencephalic neural crest and ciliary ganglia into the trunk region where they colonize sympathetic ganglia and many of them differentiate to noradrenergic neurons (Dupin, 1984; Le Douarin and Teillet, 1974; Le Lièvre et al., 1980; Lee et al., 2005). Alternatively, mesencephalic ciliary neuron progenitors, vagal enteric and trunk sympathetic neuron progenitors may be prespecified so that they respond differently to environmental signals, including the autonomic neuron inducer BMP (Müller and Rohrer, 2002). There is indeed evidence for intrinsic differences in the potential of mesencephalic and trunk neural crest to form trigeminal neurons and cartilage (Lwigale et al., 2004) and in their response to a variety of growth and differentiation signals (Abzhanov et al., 2003). In addition, sympathet- ic and ciliary neural progenitor subpopulations have been identified in migrating neural crest by functional criteria (Schmidt et al., 2011) and marker gene expression (Barald, 1989; Kasemeier-Kuljesa et al., 2010). It has also been suggested that the vagal neural crest is pre-specified to form the enteric nervous system (reviewed in Young and Newgreen, 2001).

For cranial neural crest, which contributes to the three branchial arches it has been demonstrated that the anteroposterior identity of NCC contributing to specific branchial arches is controlled by their specific Hox code (reviewed in Trainor and Krumlauf, 2000a, 2000b). Interestingly, Hox gene expression in the branchial arches is maintained by permissive environmental signals from the mesen- chyme and in the absence of these signals neural crest cells rapidly lose their identity (Schilling et al., 2001; Trainor and Krumlauf, 2000a). These results suggested that differential Hox gene expression underlies the differential response to arch-specific differentiation signals. Accordingly, forced expression of Hoxa2 in the first arch is sufficient to produce a transformation of first arch structures into second arch elements (Grammatopoulos et al., 2000; Pasqualetti et al., 2000). Loss of Hoxa2 leads to the transformation of second arch skeletal elements to- ward first arch neural crest fates (Rijli et al., 1993) and the ectopic gener- ation of facial ganglion neurons (Yang et al., 2008). Differential expression of Hox genes between mesencephalic and trunk neural crest (reviewed in McGinnis and Krumlauf, 1992; Pearson et al., 2005) is also responsible, at least in part, for the differential survival response to BMP and FGF in vitro (Abzhanov et al., 2003). However, as Hox gene expression is maintained only transiently in trunk neural crest transplanted into the mesencephalic region it remained unclear whether Hox genes can account for the strongly reduced potential of trunk neural crest to form cartilage and sensory trigeminal neurons (Lwigale et al., 2004). The contribution of Hox genes to the differential prespecification of autonomic neuron precursors in mesencephalic and trunk neural crest has not been investigated.

The Hox genes are a family of highly conserved transcription factors that are expressed very early in development and are organized in 4 clusters and 13 paralogous groups. They are expressed in a very restricted pattern along the body axis. The Hox gene expression is linked to their chromosomal organization (Kmita and Duboule, 2003; McGinnis and Krumlauf, 1992) and changes in their expression pattern lead to changes of body segment identities (Manley and Capecchi, 1998; Pearson et al., 2005; Studer et al., 1996; van den Akker et al., 2001). Also in the nervous system, anteroposterior regional identity is specified by Hox genes (Keynes and Lumsden, 1990; Narita and Rijli, 2009) with an anterior border of expression at the rostral hindbrain (Alexander et al., 2009; McGinnis and Krumlauf, 1992). In the hindbrain and spinal cord, essential roles have been identified for Hox genes in the specification of motoneu- rons (Bell et al., 1999; Dassen et al., 2003, 2005; Goddard et al., 1996; Studer et al., 1996). In the spinal cord, many Hox genes are initially coexpressed in neuronal progenitors and become restricted to exclusive expression domains during neuron differentiation, which involves cross-repressive interactions between different Hox genes soon after the neurons are born (Dasen et al., 2003, 2005). This process results in a restricted expression of Hox6 and Hox10 proteins in brachial and lumbar motor neuron columns and Hox9 proteins in thoracic motoneurons where they initiate the specification of appropriate motoneuron fates. A single HoxC gene, HoxC9, is essential and sufficient to control the generation of thoracic motoneurons by repressing more anterior Hox genes, including HoxB6 (Jung et al., 2010). Within these rostro-caudal divisions additional sets of Hox genes are involved in the generation of the diverse motoneuron sub- types that are allocated to different motoneuron pools (Dasen et al., 2005).

The role of Hox genes in the development of the autonomic ner- vous system has remained unclear. Here, we investigated Hox gene function in the different prespecification and differentiation of ciliary and sympathetic neuron precursors of mesencephalic and trunk neural crest, respectively. To address this issue we analyzed the expres- sion pattern of Hox genes in the PNS and focus on HoxB genes due to their expression at all rostrocaudal levels of the trunk. Whereas HoxB5, B6, B7, B8 and B9 are expressed in the DRG and SG they are absent in ciliary as well as in enteric ganglia. Due to its strong and main- maintained expression in trunk neural crest and SG we further concentrated on HoxB8 and its possible role in the differentiation and maintenance of the noradrenergic phenotype. The ectopic expres- sion of HoxB8 in ciliary neuron progenitors leads to a strong in- crease in the number of Hand2, TH and DBH expressing neurons in this cholinergic ciliary ganglion. In differentiated ciliary neurons and in enteric neuron progenitors HoxB8 does not induce the expression of TH and DBH. Taken together our results support the notion of an early influence of HoxB genes on the prespecification and differentiation of NCC.

Materials and methods

Animals, tissue fixation and sectioning

Chick embryos were staged according to Hamburger and Hamilton (1951). Embryos between embryonic day (E) 1.5 (HH stage 10) and E8 (HH stage 35) were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 3 h or overnight depending on the HH stage. For in situ hybridization on cryo- and vibratome sections, the formerly dehydrated sections were rehydrated by a metha-

In situ hybridization

Non-radioactive in situ hybridization on cryosections or vibratome sections and preparation of digoxigenin labeled probes for chick SCG10, Sox10, P0,2b, TH, DBH, Hand2, RT, RGS4, Ret, HoxB8, HoxB9, Meis1, Meis2 and Pbx was carried out as described previously (Ehrnsberger et al., 1997; Stanke et al., 1999) (Meis1, Meis2 and Pbx plasmids were kindly provided by D. Schulte). For the in situ hybridization on vibratome sections, the formerly dehydrated sections were rehydrated by a metha-

pH 7.5; 1.85 M NaCl; 100 mM NaH2PO4xH2O; 50 mM EDTA), 50%
formamide, 2% blocking reagent in H_{2}O) for 3 h (at 68 °C) which then was replaced by the in situ probe (diluted 1:150 in hybridization buffer 1 × salt, 50% formamide, 10% yeast RNA, 20% dextran sulfate (50% (w/v)), 1% 50 × Denhardt’s). After the overnight incubation (at 68 °C) the probe was removed followed by washing steps with 1 × SSC, 50% formamide, 0.1% Tween20 for 30 min and twice with 0.2 × SSC, 50% formamide, 0.1% Tween20 for 30 min at 68 °C. The next washing with maleic acid buffer (MABT) and the blocking step with 10% chick serum in MABT were performed at room temperature. Anti-DIG antibody was diluted 1:2500 in MABT with 1% chick serum and the sections were incubated overnight. After several washing steps with MABT, the sections were incubated twice with NTMT (100 mM NaCl, 100 mM Tris pH 9.5, 50 mM MgCl_{2}, 0.1% Tween20) for 10 min. The staining was started by adding staining buffer (4.5 μl NBT, 3.5 μl BCIP in NTMT) to the sections and stopped by adding PBS with 1% Tween20, pH 4.0. For analyzing the sections were embedded in Kaiser’s Glycerine on glass slides and covered with cover slips.

Whole-mount in situ hybridization was performed using a modified protocol from Riddle et al. (1993). After overnight fixation in 4% paraformaldehyde in 0.1 M phosphate buffer embryos were washed twice with PBT (PBS with 0.1% Tween 20) and dehydrated through an ascending methanol series in PBT like the vibratome sections described above. Embryos were rehydrated through a descending methanol washed twice in PBT and permeabilized with proteinase K (10 μg/ml) for 5–10 min. After two washing steps with PBT, the embryos were fixed with 4% paraformaldehyde for 20 min, washed again several times with PBT and once with prehybridization buffer (50% formamide, 20× SSC (pH 4.5), 10% SDS, 0.5% total yeast RNA, 0.05% Heparin) and incubated with fresh prehybridization buffer for 1 h at 68 °C. The prehybridization buffer was then replaced with hybridization buffer (prehybridization buffer with digoxigenin-labeled riboprobe at 1 μg/ml) and incubated overnight at 68 °C. Following hybridization, embryos were washed twice for 30 min at 68 °C with prehybridization buffer, and 10 min with 1:1 prehybridization buffer with MABT solution. Before preblocking for 1 h in MABT with 2% blocking reagent, embryos were washed three times in MABT at RT. The blocking step was performed with MABT, 2% blocking reagent and 20% heat inactivated CHS for 2 h at room temperature. After an overnight incubation at 4 °C with the preadsorbed antibody in the blocking buffer, embryos were washed three times for 1 h with MABT at room temperature. The buffer was exchanged by washing three times for 10 min each time with NTMT (described above). The detection reaction was performed by adding the staining solution (described above). After the detection reaction was complete, embryos were washed several times with PBS and postfixed with 4% PFA.

**Proliferation analysis and immunostaining**

Proliferating cells were detected on cryosections using the Click-IT® EdU Alexa Fluor® 488 Imaging Kit (Invitrogen, Karlsruhe, Germany) as described previously (Warren et al., 2009). To identify proliferating neuronal cells of the ciliary ganglion, immunostaining for Isl1 was additionally performed on the same sections before the detection of EdU positive cells. The control and RCASHoxB8 infected ciliary ganglia were also stained for TH and for beta tubulin III (anti TUJ1 antibody). The sections were treated with 10 mM sodium citrate buffer (pH 6.0; boiling for 10 min) for antigen retrieval, blocked with PBS containing 10% fetal calf serum, 1% bovine serum albumin and 0.2% Triton X-100 and incubated with primary antibody overnight at 4 °C. Nuclei were stained by DAPI (Sanofi Aventis, Frankfurt, Germany). The primary antibody was mouse anti Islet1 1:20 (39.4D5, Developmental Studies Hybridoma Bank, Iowa, USA), mouse anti TUJ1 1:500 (MMS-435P, Hiss Diagnostics, Freiburg, Germany) and chick anti TH 1:300 (Aves Labs, Inc., Oregon, USA). The secondary antibody was labeled with Alexa594 or Alexa 488 (dilution 1:500; Invitrogen, Karlsruhe, Germany).

**Hox/mir196 overexpression in vivo**

For construction of chick RCAS-BP(B)-HoxB8, RCAS-BP(B)-HoxC8, RCAS-BP(B)-HoxB9, and RCAS-BP(B)-HoxD10 PCR technology was used to insert a Kozak sequence linked to a SpeI flanking site, followed by the coding sequence of chick HoxB8, HoxC8, HoxB9 and HoxD10, a myc tag and a Csl flanking site. The HoxD10 plasmid was a kind gift of Cliff Tabin (Harvard Medical School, Boston). The replication-competent avian sarcoma (RCAS) vector was used and modified as described previously (Tsarovina et al., 2004). The RCAS-BP(B)-mmHoxB1 construct was kindly provided by Andrew Lumsden (Kings College, London) and the RCAS::mirt-196 construct was a gift of Jennifer Mansfield (Harvard Medical School, Boston).

Fertilized virus and pathogen-free eggs (Charles River, Sulzfeld, Germany) were incubated and infected with empty RCAS virus for control or RCASHoxB8, RCASHoxC8, RCASHoxB9, RCASHoxB1 or RCASHoxD10 virus concentration as described (Rüdiger et al., 2009). The cranial neural tube of embryos at HH stage 8/9 was infected and embryos were kept either up to HH stage 25, 29 or 35, pulsed with EdU (400 μl 10 mM EdU in PBS) for 4 h or directly fixed in 4% paraformaldehyde and embedded.

**Hox overexpression in vitro**

Ciliary ganglia from E5/E8 chicken embryos were dissected from E5/E8 embryos, dissociated to single cells (0.1% trypsin) and cells were electroporated with the Amaxa Nucleofector II (Program: Small Cell Number (SCN) #5 for E5, #2 for E8, transfection efficiency ~30%). For analyzing the transfection efficiency, 160,000 cells were transfected with 1 μg pcAGGSeGFP in nucleofector solution. 40,000 cells/well were plated on poly-ornithine/laminin coated 4 well culture dishes and cultured in MEM, 1%P,S, 1% Glut, 10% horse serum and 3% eye extract. After 2 days in culture, the cells were fixed for 15 min with 4% paraformaldehyde, washed with PBS, 1% BSA, 0.1% Triton-X (PTB1) and incubated with the Isl1 antibody (39.4DS, Developmental Studies Hybridoma Bank; diluted 1:200 in PTB1) for 1 h at room temperature. After washing with PBS, 0.1% BSA, and 0.1% Triton-X (PTB2) the cells were incubated with the Alexa594-labeled anti mouse antibody (Invitrogen, Germany; 1:500 in PTB2) and stained for DAPI (Sanofi Aventis, Germany) for 1 h. After several washing steps with PBS the cells were analyzed with a Zeiss Axiopt 2 microscope and a VisiRon systems spot RT3 camera for Isl+/GFP+ neurons. For qPCR analysis 400,000 cells were transfected with 1 μg of pcAGGSeGFP (control) or 1 μg of pcAGGS%HoxB8myc in nucleofector solution. The cells were plated on poly-ornithine/laminin coated 4 cm culture dishes and harvested after 2 days for RNA isolation RNA by using the RNeasy Mini Kit (Qiagen, Germany).

**shRNA constructs and transfection**

The shRNA 3 against Gallus gallus HoxB8 was designed using BLOCK-IT™ RNai Designer (Invitrogen, Karlsruhe, Germany) and targets the following sequence: shHoxB8 5’UUU CUU GCC GCG CUU CUG UGC UUC C3 (Invitrogen primer name: stealth_7500). Scrambled shRNA (scr-shRNA, Invitrogen, Karlsruhe, Germany) served as control condition.

Paravertebral lumbosacral sympathetic ganglia from E7 chicken embryos were dissociated (Rohner and Thoenen, 1987; Zackenfels et al., 1995) and the Amaxa Nucleofector II was used to electroporate 400,000 cells (Program: Small Cell Number (SCN) #2, transfection efficiency ~50%). 20 μM of the scrambled shRNA as control and 20 μM shHoxB8 were used for the transfection. Transfected cells were plated on poly-ornithine/laminin coated 4 cm culture dishes and cultured in MEM, 1% P/S, 1% Glut, 10% horse serum; 5% fetal calf serum. For
RNA isolation 400,000 cells were plated and harvested after 2 days. RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany).

RT-PCR

Total RNA from E7 chick SG, E5 chick DRG and E5 CG were isolated by using a RNeasy kit (Qiagen, Hilden, Germany). cDNA synthesis on total RNA was performed using the M-MLV Reverse Transcriptase Kit (Invitrogen, Karlsruhe, Germany). The cDNA was used as template for PCR amplification in a 50 μl reaction (Tsarovina et al., 2004) with the Taq Polymerase Kit (Invitrogen, Karlsruhe, Germany). The temperature profile consisted of 35 cycles (Hox genes) or 25 cycles (GAPDH) (95 °C for 15 s, 62 °C for 30 s and 72 °C for 30 s) and a final 5 minute extension at 72 °C. Ethidium bromide stained gel bands of Hox genes were visually compared to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gel band. All Hox gene PCR reactions were performed at the same time for each cDNA preparation (n=3) and the amplifications were done together. For primer sequences used, see Table S2 in supplementary materials.

qPCR analysis

Equal amounts of RNA were used to synthesize cDNA with Oligo(dT)-primers and Superscript-III-reverse-transcriptase according to manufacturer’s instructions (Invitrogen, Karlsruhe, Germany). PCR-Reaction was conducted with Abgene’s Absolute Blue SYBR-Green qPCR Mix (Abgene, Epsom, UK) in a Stratagene Mx3000p Light Cycler (Stratagene, Waldbronn, Germany). All primers (MWG Biotech AG, Ebersberg, Germany) were designed to optimally anneal at 58 °C with PerlPrimer (Marshall, 2004) (PCR Reaction: 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s repeated for 50 cycles and a subsequent dissociation curve). The primer pairs (see Table S2 in the supplementary material) were analyzed for efficiency (∼95%) and used for quantitative analysis. At least triplicates of every condition were performed in parallel. Data were normalized using either Islet1 or GAPDH as reference gene and evaluated using the delta-delta Ct-method using Microsoft Excel. Experiments were repeated independently at least three times and statistically analyzed using unpaired two-tailed Student’s t-test.

Morphometric analysis

In situ hybridization

The area of Phox2b, Hand2, TH and DBH expression in ciliary ganglia was imaged using a Zeiss Axioscope 2 microscope and a Visitron systems spot RT3 camera. The areas were quantified morphometrically using the MetaVue (version 7.1.3.0) imaging system. The measured area was manually thresholded and determined in ×10^−3 mm²/section (Buralu et al., 2004; Howard et al., 2000; Lucas et al., 2006; Schmidt et al., 2011; Tsarovina et al., 2004). Additionally, positive cells were counted per section. For both quantifications two ganglia in at least 3 embryos (4 sections per embryo) were analyzed for each stage. Results are given as mean area/mean cells per section ± s.e.m. For statistical analysis unpaired two-tailed Student’s t-test was used.

Immunostaining

The EdU/Is1 staining of ciliary ganglia and the Is1/GFP staining of ciliary neuron cultures were imaged using a Zeiss Axioscope 2 microscope and a Visitron systems spot RT3 camera. The TH/TUJ1 antibody staining of ciliary ganglia was imaged using Zeiss AxioImagerZ1 and a Zeiss AxioCam MRm. The TH expression was quantified either as percentage of TUJ1 positive area (measured with the AxioVision Rel. 4.8 imaging system) or as positive cells. Two ganglia in 3 control and HoxB8 infected embryos (4 sections per embryo) were analyzed. Results are given as mean cells per section ± s.e.m. For statistical analysis unpaired two-tailed Student’s t-test was used.

Results

Expression of the HoxB genes in embryonic chick DRG, SG and enteric neurons

The sensory dorsal root ganglia (DRG) and sympathetic ganglia (SG) arise from neural crest cells (Le Douarin et al., 2004) in the trunk region where Hox genes of the paralogous groups 5–11 are expressed. The Hox genes from groups 1 to 4 are prominently expressed in the hindbrain rhombomeres whereas groups 11, 12 and 13 are expressed very posteriorly in the trunk or tail region respectively (Lance-Jones et al., 2001; McGinnis and Krumlauf, 1992; reviewed in Pearson et al., 2005).

To address the question which Hox genes are expressed in trunk peripheral ganglia, Hox genes from groups 5 to 11 were analyzed by PCR in E5 chick DRG and E7 chick SG, dissected from the lumbosacral region. Mesencephalic neural crest derived E5 ciliary ganglia were analyzed for comparison. PCR primers were selected for all Hox genes of groups 5–11 where genomic information was available. 15 out of 20 analyzed genes are expressed in trunk peripheral ganglia but not in ciliary ganglia (Table 1). The result of the PCR analysis was confirmed by in situ hybridization for all Hox genes (not shown). The Hox cluster was exceptional as all members of the Hoxb cluster analyzed (Hoxb5, B6, B7, B8 and B9) are strongly expressed in both lumbosacral and brachial regions. At the brachial level Hoxb gene expression was examined by in situ hybridization of sections of E4–E7 chick embryos. Results are shown for Hoxb8 and Hoxb9 that are representative for all analyzed Hoxb genes (Hoxb5–9). Hoxb8 and Hoxb9 expression is detectable in SG and DRG from E4 up to E7, the latest stage analyzed (Fig. 1A–L). The similar expression pattern of Hoxb8/9 and the neuron specific marker Sccg10, together with lack of in situ hybridization signal in dorsal and ventral roots suggests a restriction of Hoxb genes to the neuronal population in SG and DRG. In the spinal cord, Hoxb expression is maintained in the ventricular region and in the majority of Sccg10 expressing neurons, but is expressed only in a small minority of LMC motoneurons (Fig. 1) (Dasen et al., 2005).

Hoxb gene expression is detectable in the chick neural tube at about HH stage 8 (Bel-Vialar et al., 2002). To identify the anterior boundary of Hoxb8 expression whole mount in situ hybridization was carried out for HH 10–12 embryos (Fig. 2A–C). The area of Hoxb8 expression extends in the trunk dorsal neural tube, which includes premigratory neural crest up to somites 5 and 6, with strong expression levels reached at...
somite 7 (Fig. 2A–C). HoxB8 expression is detectable in migrating neural crest cells and ganglion primordia marked by Sox10 and Phox2b (Fig. 2D–F) and in the neural tube throughout all analyzed stages (Figs. 1, 2). Thus, HoxB8 and the other 5' members of the HoxB complex are expressed in migrating trunk neural crest and expression is maintained in differentiated SG and DRG neurons at least up to E7, which is in agreement with a potential function in the specification and differentiation of trunk peripheral neurons.

Interestingly, HoxB5–9 are not detected in enteric neurons of E5–E7 chick embryos by in situ hybridization as shown for HoxB8 (Fig. 3B,D,F) and HoxB9 (Fig. 3H,J,L). For HoxB8 (Fig. 2) and HoxB9 (Bel-Vialar et al., 2002) this is explained by the absence or low level of expression in enteric precursors of the vagal neural crest, which extends in the post-otic hindbrain from somites 1–7 (Burns and Le Douarin, 1998; Le Douarin and Teillet, 1974). The lack of HoxB5–7 in enteric neurons may either be due to the absence of expression or to downregulation in vagal neural crest migrating to the gut.

Ectopic expression of HoxB8 in the parasympathetic ciliary ganglia changes the expression of Hand2 and the noradrenergic marker genes TH and DBH

The timing of Hox8 expression during sympathetic neuron development is compatible with a role in the prespecification of progenitors and the maintenance of noradrenergic sympathetic neuron differentiation. Due to the redundant and pleiotropic functions of different members of the Hox gene family (Alexander et al., 2009; Wellik, 2009) we decided to investigate the role of Hox8 genes in the autonomic nervous system by overexpression in Hox gene-deficient mesencephalic neural crest precursors of the ciliary ganglion rather than by knockdown in sympathetic ganglia. The development of ciliary neurons is induced by BMPs and the downstream transcription factors Phox2b, Phox2a and Ascl1 but differs from sympathetic neuron development by the only transient expression of noradrenergic marker genes, the lack of Gata2 expression and a final cholinergic neurotransmitter phenotype (Lee et al., 2005; Müller and Rohrer, 2002; Tsarovina et al., 2004). This may be due to a pre-specification of ciliary precursors that influences the response to BMP signaling and/or due to the absence of signals that maintain noradrenergic differentiation. By expressing HoxB genes in ciliary progenitors and neurons we tested the working hypothesis that HoxB genes may participate in the specification, noradrenergic differentiation and maintenance of noradrenergic properties of sympathetic neurons.

To elicit Hox gene expression in ciliary neuron progenitors, the cranial neural tube was infected at HH stage 8/9 (E2) (Hamburger and Hamilton, 1951) with a RCAS virus containing HoxB8 (RCA-SHOxB8). To analyze the infection efficiency in situ hybridization against reverse transcriptase (RT) was performed on sections of control RCA and RCASHoxB8 infected embryos at E5. Additionally, the expression of HoxB8 was analyzed in infected embryos (Fig. S1). Control and HoxB8 infected embryos show strong expression of RT throughout ciliary ganglion (Fig. S1B,F), whereas HoxB8 expression is detected only in the RCASHoxB8 embryos (Fig. S1C,G).

The HoxB8 and control infected CG were then analyzed at E5 by in situ hybridization for the expression of Phox2b (Fig. 4A,E), Hand2 (Fig. 4B,F), TH (Fig. 4C,G) and DBH (Fig. 4D,H). Hand2, TH and DBH expressing cells were quantified by determining morphometrically
the stained areas, which are referred to the total, Phox2b expressing ganglion area as described previously (Burau et al., 2004; Howard et al., 2000; Lucas et al., 2006; Schmidt et al., 2011; Tsarovina et al., 2004).

Ectopic HoxB8 expression in the ciliary neurons leads to a massive, >40 fold increase in the area of Hand2+ cells compared to control infected embryos (area of Hand2+ cells represents 42.5 ± 6.9% of the Phox2b+ area as compared to 0.5 ± 0.2% in controls; Fig. 4I). The area of cells expressing the noradrenergic marker genes TH and DBH, which represent only 0.3 ± 0.1% and 0.3 ± 0.02% of the Phox2b+ area in the control situation, respectively, is also significantly (>10 fold) increased by the HoxB8 infection (4.2 ± 1.4% and 4 ± 1%; Fig. 4J,K). An alternative quantification by counting the number of stained cells per section also demonstrated a strong increase in Hand2, DBH and TH expressing cells in ciliary ganglia (Fig. S2A–C). The smaller increase observed in the cell counts is most likely due to difficulties identifying individual cells at high cell density, resulting in an underestimation of the HoxB8 effect.

Taken together, these results show that the ectopic HoxB8 expression from E2 onwards increases the percentage of cells expressing sympathetic neuron markers in parasympathetic neurons.

These results raised the question whether the observed increase at the transcriptional level would also lead to increased protein expression. This was addressed by immunostaining for TH and demonstrated a significant increase in the number and density of TH-positive cells in response to HoxB8 (Fig. S3). However, the intensity of TH staining in the ciliary ganglion is much lower as compared to sections of E5 sympathetic ganglia (not shown), which implies differential translational control.

To investigate whether HoxB8 induces a transient or permanent change in ciliary neuron differentiation we further analyzed infected embryos at E6 and E8. The in situ hybridization analysis of E6 (Fig. S4) and E8 (Fig. 5; Fig S2) embryos revealed that the proportion as well as

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Fig. 2. Expression of HoxB8 in the neural tube and migrating neural crest cells. HoxB8 expression was analyzed by whole mount in situ hybridization at ss 11, 14 and 16 (A–C) and on cross sections at E3 (vibratome sections; D–F). The anterior border of the HoxB8 expression in the neural tube is located at somite 7 (arrowhead) at ss 11 (A), 14 (B) and 16 (C). Staining in the head region at ss 16 is background staining. The migrating neural crest cells (D; Sox10+, arrow) and the early sympathetic ganglia (SG; E; Phox2b+, arrow) at ss 30 express HoxB8 (F; arrow) (scale bar 50 μm; asterisk marks the first somite).

Fig. 3. Expression analysis of HoxB8 and HoxB9 in the enteric ganglia. Enteric neurons of the esophagus in the trunk region of chick embryos (at E5, E6 and E7) are identified by in situ hybridization against Phox2b (A,C,E,G,J,K, arrowheads). Neither HoxB8 nor HoxB9 is expressed in the enteric neurons of the esophagus at E5 (B,H), at E6 (D,I) and at E7 (F,L) (arrowheads) (scale bar 50 μm).
the numbers of Hand2, TH and DBH positive cells is strongly increased compared to control infected embryos up to E8 (Fig. S4F–K; Fig. 5F–K; Fig. S2D–F). Whereas the effect on Hand2 expression decreased with development, the proportion and number of noradrenergic TH and DBH positive cells in the ciliary ganglion remained at similar levels between E5 and E8 (Fig. 4I–K; Fig. 5I–K; Fig. S2). Thus, the HoxB8-induced increase in the proportion of Hand2, TH and DBH positive cells is maintained at least up to E8. This suggests that HoxB8 expression either elicits a permanent modification of the expression pattern in the cholinergic ciliary neurons and/or that the presence of HoxB8 maintains the transient noradrenergic phenotype.

The parasympathetic cholinergic ciliary neurons do not adopt all characteristics of sympathetic noradrenergic neurons

The strong effects of HoxB8 on the expression levels of Hand2, TH and DBH in the CG raised the question whether HoxB8 would also induce other characteristics of sympathetic noradrenergic neurons in the parasympathetic cholinergic CG. To address this aspect several additional sympathetic marker genes were analyzed in the ciliary neurons.

Regulator of G Protein 4 (RGS4) and RCadherin (RCad) have been identified in a SAGE analysis as genes that are preferentially expressed in the SG (at E7) as compared to CG (at E5) (not shown). This is confirmed by the in situ hybridization of SG at E7 (Fig. 6A–C) and in CG of control infected embryos at E5 (Fig. 6D–F). However, the overexpression of HoxB8 did not induce RGS4 or RCad in E5 ciliary neurons (Fig. 6G–I).

Another specific feature of developing sympathetic neurons is the fact that they continue to proliferate after the onset of differentiation, i.e. they obtain their noradrenergic phenotype before their last cell division (DiCicco-Bloom et al., 1990; Rohrer, 2011; Rohrer and Thoenen, 1987; Rothman et al., 1978). In contrast, the cholinergic ciliary neurons start to differentiate after withdrawal from the cell cycle and thus ciliary neurons expressing differentiation markers do not proliferate (Lee et al., 2002; Rohrer, 2011; Rohrer and Thoenen, 1987). To investigate if the HoxB8 overexpression changes neurogenesis characteristics in ciliary ganglia, proliferation was studied by EdU labeling combined with antibody staining for the neuronal marker Isl1. As shown before, only EdU−, postmitotic neurons are detected in the control situation at E5 and E6. EdU+ proliferating cells are negative for Isl1 and may represent either cells of the glial lineage or neuron progenitors (Fig. 7A–C, G–I). The overexpression of HoxB8 did not lead to neuronal differentiation of proliferating cells at E5 or E6 as shown by the lack of EdU+/Isl1+ neurons (Fig. 7D–F, J–L). In contrast, a large proportion of EdU+/Isl1+ neurons is present in E7 sympathetic ganglia (Fig. 7M–O).

Taken together, the results demonstrate that ectopic HoxB8 expression affects only a subset of the genes expressed in the sympathetic neuron lineage but does not lead to the acquisition of the full identity of noradrenergic sympathetic neurons.

The HoxB8 effect on Hand2 is critically dependent on the onset of HoxB8 expression

The increase of Hand2+, TH+ and DBH+ cells in the CG by the RCASHoxB8 infection of ciliary neuron progenitors raised the question whether the effect can only be elicited in neuron progenitors or if a later overexpression of HoxB8 in postmitotic differentiated ciliary

Fig. 4. Ectopic expression of HoxB8 in mesencephalic neural crest and ciliary neurons leads to a significant increase of Hand2+, TH+ and DBH+ neurons in E5 ciliary ganglia. In situ hybridization of cross sections from the head region of E5 RCASHoxB8 infected embryos (E–H) shows a significant increase of Hand2+, TH+ and DBH+ cells in the ciliary ganglion compared to the small subpopulation of Hand2+, TH+ and DBH+ cells (arrowheads) in the control infected embryos (A–D). Hand2, TH and DBH expression is quantified as the percentage of the Phox2b expression area [I–K] (unpaired Student's t-test; *** = p ≤ 0.001; *= p ≤ 0.05; n=3; scale bar 50 μm).
neurons does also lead to a change in the expression levels of these genes. As the RCAS expression system relies on proliferating cells, pCAGGS expression vectors for **HoxB8** were used for the transfection of postmitotic ciliary neurons. Ciliary neurons from E5 or E8 embryos were electroporated with either pCAGGSeGFP or pCAGGSHoxB8 with a transfection efficiency of about 30% as shown by the proportion GFP+/Isl1+ cells (Fig. 8A–F). After two days in culture **Hand2** and **TH** expression were analyzed by qPCR and normalized to the neuronal marker **Isl1**. **HoxB8** overexpression increased the level of **Hand2** mRNA at both ages but reaching significance only in E8 cultures (Fig. 8G,I). However, compared to the >40 fold increase in **Hand2** expression upon **HoxB8** expression in ciliary neuron progenitors, the effect in differentiated ciliary neurons is much lower even when taking the transfection efficiency into account. In addition, **TH** expression was not increased by **HoxB8** overexpression at E5 and E8 (Fig. 8H,J).

These results show that **HoxB8** is unable to induce **TH** and has only a minor effect on **Hand2** when overexpressed in differentiated ciliary neurons, suggesting that the expression of **TH** and **DBH** is affected by **HoxB8** only in ciliary neuron progenitor cells.

**HoxB8 does not induce TH and DBH in enteric neurons**

Enteric neurons in the chick embryo are devoid of **TH** and **DBH** expression (Rothman et al., 1990; Smith et al., 1977), although **Phox2b**, **Phox2a**, **Ascl1** and **Hand2** are expressed by enteric neurons (Groves et al., 1995; Wu and Howard, 2002). As enteric neurons of the esophagus were found to be devoid of **HoxB5–9** (Fig. 3), it was of interest to investigate whether ectopic **HoxB8** expression in vagal neural crest and enteric progenitors is able to elicit the expression of noradrenergic marker genes (Fig. 9A–D). **TH** and **DBH** are undetectable in **HoxB8** expressing enteric neurons (Fig. 9D; and data not shown), which demonstrates that **HoxB8** is not sufficient to induce a sympathetic neuron specification in enteric progenitors.

As Hox proteins act in concert with members of the TALE (Three Aminoacid Loop Extension) family of transcription factors (**Meis1**, **Meis2**, **Pbx1**) (Moens and Selleri, 2006) the lack of **HoxB8** effect in the enteric nervous system may be explained by the absence of essential cofactors for **HoxB8**. The analysis of **Meis1**, **Meis2** and **Pbx1** expression in sympathetic, ciliary and enteric neurons by in situ hybridization revealed that only **Meis1** is expressed in sympathetic and ciliary ganglia (Fig. S5B–D,F–H), which seems to be sufficient for **HoxB8** function. In enteric ganglia, **Meis1**, **Meis2** and **Pbx1** were not detectable (Fig. S5J–L). This result suggests that the lack or low level expression of TALE transcription factors in enteric progenitors and neurons prevents an effect of **HoxB8** on the expression of **Hand2**, **TH** and **DBH**.

**HoxC8, HoxB9, HoxB1 and HoxD10 are unable to stably induce the expression of Hand2, TH and DBH expression**

Paralogous Hox genes show a very high sequence similarity and function redundantly (Manley and Capecchi, 1998; Rossel and Capecchi, 1999; Wellik, 2009). In addition, there is also evidence for redundant
Regarding the noradrenergic marker genes, DBH but not TH expression was increased (Fig. 10C,D,G,H,K,L,O,P). HoxD10 had no influence either on the expression level of Hand2 (Fig. 10R) or on TH (Fig. 10S) or DBH (Fig. 10T). However, the effects of HoxC8, HoxB9 and HoxB1 on TH and DBH expression were only transient and could not be detected in E6 embryos (Fig. S6). At E6 Hand2 was only increased in HoxB1 infected ganglia and at a much reduced level compared to E5 (Fig. S6B,N,U; 6.7 ± 1.1% at E6 as compared to 27.9 ± 4.3% of the Phox2b+ area at E5 (Fig. 10B,N,U)). Hand2 levels were not increased significantly in E6 ciliary ganglia by HoxC8, HoxB9 or HoxD10 overexpression (Fig. S6F,J,R). The noradrenergic marker genes TH and DBH were not significantly increased in E6 ciliary neurons by overexpressing HoxC8, HoxB1, HoxB9 and HoxD10, which is in marked contrast to the effect of HoxB8 (Fig. S6G,H,K,L,O,P,S,T).

Taken together, these results show that although HoxC8 and HoxB1 also are able to influence the expression of Hand2, TH and DBH, only HoxB8 elicits a stable increase in the proportion of noradrenergic neurons. Thus, a special role regarding the differentiation of the noradrenergic phenotype can be assigned to HoxB8.

To address the question whether HoxB8 is not only sufficient but also essential for the expression and maintenance of noradrenergic characteristics HoxB8 was knocked down in cultured E7 sympathetic neurons by specific shRNA. We found that the expression of Hand2, TH and DBH revealed by qPCR was unaffected (Fig. S7B; n=6), which may be explained by the relatively small reduction of HoxB8 mRNA (Fig. S7A; 44% ± 6%; n=6) as compared to controls transfected with scrambled shRNA (scr shRNA). An in vivo knockdown of HoxB8 and HoxA7 in the limb has previously been achieved by the RCAS virus-mediated expression of mir196 (Hornstein et al., 2005; Mansfield et al., 2004). However, Hand2, TH and DBH were expressed at normal levels upon infection of trunk neural crest cells with mir196-RCAS (not shown). These results indicate that HoxB8 may not play a unique and essential role but rather that Hox8 paralogs and adjacent Hox genes like HoxB9 may participate in a combinatorial Hox code to regulate sympathetic neuron differentiation.

Discussion

Cell fate acquisition of neural crest cells involves early restrictions in developmental potential that result in the differential response of neural crest subpopulations to environmental cues. This is evident for autonomic neuron progenitor cells in mesencephalic, vagal and trunk neural crest that respond to BMPs by differentiation to parasympathetic cholinergic ciliary neurons, enteric neurons and noradrenergic sympathetic neurons, respectively. In this study we demonstrate that ectopic expression of the trunk-specific HoxB8 gene in mesencephalic neural crest results in ciliary neurons expressing sympathetic neuron markers. In contrast, noradrenergic markers are not induced by HoxB8 in the enteric nervous system. These findings imply Hox8 gene function in the different prespecification of mesencephalic and trunk neural crest and in the maintenance of noradrenergic differentiation.

Control of ciliary, enteric and sympathetic neuron development

The development of ciliary and sympathetic neurons is initiated by BMPs that are expressed in the vicinity of neural crest cells that aggregate after migration to form ganglion primordia (Müller and Rohrer, 2002; Reissmann et al., 1996; Shah et al., 1996). BMPs are also essential for the differentiation, proliferation and survival of enteric precursors (Chalazonitis et al., 2004, 2008; Goldstein et al., 2005; Lo et al., 1997). A similar set of transcription factors, including Phox2b, Phox2a and Ascl1 is initially expressed in sympathetic, enteric and ciliary neuron lineages. A major difference in the subsequent differentiation is that Hand2, TH and DBH are expressed in ciliary neuron progenitors only transiently and become restricted to a very small subpopulation of ciliary neurons, whereas these genes are expressed...
in developing and mature sympathetic neurons in the trunk (Lee et al., 2005; Müller and Rohrer, 2002). Developing enteric neurons in the embryonic avian gut express Hand2 (Wu and Howard, 2002) but not TH and DBH (Rothman et al., 1990; Smith et al., 1977). In addition, Gata2 is only expressed in sympathetic but not in ciliary and enteric ganglia (Groves et al., 1995; Tsarovina et al., 2004).

The different development of ciliary and sympathetic neuron progenitors downstream of BMP signaling has been explained by environmental factors that repress Hand2, Gata2 and noradrenergic differentiation in the ciliary ganglion (Lee et al., 2005; Müller and Rohrer, 2002). This notion is supported by transplantation of mesencephalic neural crest and ciliary ganglia into the trunk region, which resulted in the expression of Hand2 and TH in transplanted cells that have migrated to and differentiated in sympathetic ganglia (Coulombe and Bronner-Fraser, 1986; Dupin, 1984; Le Douarin and Teillet, 1974; Le Douarin et al., 1978; Le Lièvre et al., 1980; Sechrist et al., 1998). Quantitative analysis demonstrated, however, the presence of a subpopulation of mesencephalic neural crest cells that did not acquire Hand2 and TH expression in the ectopic location. In addition, in a second subpopulation of mesencephalic neural crest catecholaminergic properties were induced but not maintained (Lee et al., 2005). Conversely, trunk neural crest is intrinsically restricted in its developmental potential, reflected by the reduced number of trigeminal sensory neurons and lack of mesenchymal derivatives upon transplantation of trunk neural crest to the mesencephalic region (Couly et al., 1998; Creuzet et al., 2002; Lwigale et al., 2004). Taken together, these results demonstrate a restriction of mesencephalic neural crest cells toward a non-noradrenergic fate and raise the question as to the molecular basis for this prespecification.

**Hox genes in neural crest patterning**

Hox genes specify rostrocaudal differences in many tissues including the nervous system, best studied in hindbrain, spinal cord and cranial neural crest. For cranial neural crest gain-of-function experiments demonstrated that Hox2a is sufficient to induce the identity of branchial arch 2 in branchial arch 1 neural crest cells (Grammatopoulos et al., 2000; Pasqualetti et al., 2000). Also the different response of cultured cranial and trunk neural crest cells to BMPs and FGFs was shown to depend in part on the differential expression of Hox genes (Abzhanov et al., 2003). However, it was concluded that Hox gene expression is unlikely to account for the reduced potential of trunk neural crest to generate mesencephalic sensory neuron fates (Lwigale et al., 2004).

We have analyzed in lumbosacral sympathetic ganglia and sensory DRG the expression of all chick Hox genes by RT-PCR. Interestingly, most of the Hox genes are expressed, although at different levels. HoxB expression was also observed in the brachial region by in situ hybridization and was found to be restricted to neuronal...
lineages and absent in peripheral nerves. Genes of the HoxB cluster were strongly expressed in sympathetic ganglia and in this study we focused on the function of HoxB8. Interestingly, vagal neural crest-derived enteric neurons, which also depend on BMP signaling (Chalazonitis et al., 2004, 2008; Goldstein et al., 2005; Lo et al., 1997) and Phox2b (Pattyn et al., 1999) but are devoid of TH and DBH in the chick (Rothman et al., 1990; Smith et al., 1977) did not show HoxB gene expression. As HoxB8 and HoxB9 are initially expressed in chick dorsal spinal cord and migrating neural crest at rostrocaudal levels up to somites 5/7 and 9, respectively (Fig. 1; Bel-Vialar et al., 2002) vagal crest precursors are expected to display no or low level of HoxB8/9, which explains the lack of expression in enteric ganglia. It is unclear whether HoxB5–7 are not or transiently expressed in vagal neural crest and downregulated subsequently in enteric precursors.

The general expression of HoxB genes in sympathetic ganglia at all rostrocaudal levels as compared to the absence of HoxB genes vagal and mesencephalic neural crest resulted in a large increase in the proportion of Hand2+, TH+ and DBH+ neurons in the ciliary ganglion but not in the enteric nervous system. In the spinal cord, HoxC6 specifies the identity of brachial motoneurons in the lateral motoneuron column by its action in post-mitotic neuron, whereas thoracic motoneurons can be specified by HoxC9 only in progenitor cells (Dasen et al., 2003). Our finding that increased numbers of ciliary neurons with noradrenergic properties are only generated upon forced HoxB8 expression in mesencephalic neural crest progenitors but not upon overexpression in postmitotic ciliary neurons argues against a late function of HoxB8. We suggest that HoxB8 effects are restricted to lineages where neuron progenitors are present that express noradrenergic genes either transiently (ciliary ganglion) or permanently (SG). HoxB8 is involved in the enhancement and/or maintenance of noradrenergic characteristics but seems unable to initiate TH and DBH expression, e.g. in postmitotic cholinergic ciliary neurons and in precursors and neurons of the enteric nervous system. The lack of TH/DBH induction in the enteric nervous system may, however, also be due to the absence of TALE-cofactors as neither the expression of Meis1, Meis2 nor Pbx1 was detectable.
HoxB8 overexpression is not sufficient to induce full sympathetic neuron identity in the ciliary ganglion

Why is the HoxB8 effect on Hand2 expression in ciliary ganglia restricted to a subpopulation of neurons? The analysis of RT expression, which allows to identify RCAS-infected cells suggests a virtually complete infection at E5. As RCAS-infection requires that cells proliferate, only proliferating neural crest cells and ciliary neuron progenitors, but not postmitotic neurons will be infected and express HoxB8. It is unclear, however, to which extent ciliary ganglion cells are infected at the specific stage when HoxB8 is effective in increasing and/or preventing the downregulation of Hand2, TH and DBH expression. Thus, one can argue that the HoxB8-induced Hand2 expression in ciliary ganglia reflects the efficiency of infecting ciliary neuron progenitors and mesencephalic neural crest with RCAS virus. Alternative possibilities are that Hand2 expression is repressed or downregulated in subpopulations of mesencephalic neural crest that also do not induce Hand2 upon transplantation into the trunk region (Lee et al., 2005) or that combined actions of several Hox genes would be required to induce Hand2, TH and DBH in all cells.

Interestingly, HoxB8 overexpression results in a much larger number of Hand2 expressing cells as compared to neurons expressing TH and DBH. This is in contrast to the situation in sympathetic ganglia where TH and DBH expression is initiated shortly after Hand2 in all cells of the sympathetic neuron lineage. Thus, although about 40% of ciliary neuron progenitors respond to HoxB8 by inducing Hand2, this is not sufficient to induce TH and DBH in a large number of ciliary neurons. This is in agreement with the previously observed about 2-fold increase in TH+ ciliary neurons upon forced Hand2 expression (Müller and Rohrer, 2002). The difference to the situation in sympathetic ganglia may be explained by a requirement of additional regulatory factors that are not induced by HoxB8, in particular Gata2 that is needed for initial noradrenergic differentiation of sympathetic neurons (Lim et al., 2000; Tsarovina et al., 2004). The lack of Gata2 may also explain the absence of even transient TH and DBH expression in avian Hand2-expressing enteric neurons.

The only partial change from parasympathetic to sympathetic neuron identity in HoxB8-expressing ciliary ganglia is also reflected by the absence of RGS4 and Rcad. A differential expression of RGS4 and Rcad has been detected in a SAGE screen comparing E7 chick sympathetic and E5 ciliary ganglia, which has now been confirmed by in situ hybridization. RGS4 expression overlaps extensively with Phox2b and is regulated by Phox2b and Asc11 (Grillet et al., 2003).

Sympathetic and ciliary neuron lineages also differ in the way neurons are generated during neurogenesis. Whereas ciliary neurons are produced by differentiation of progenitors after withdrawal from the cell cycle, postmitotic sympathetic neurons are generated from cycling immature sympathetic neurons that already express differentiated properties. HoxB8-induced Hand2 expression did not alter the characteristics of neurogenesis in the ciliary ganglion although Hand2 is involved in the control of immature sympathetic neuron proliferation (Hendershot et al., 2008).

Taken together, HoxB8 overexpression results in only a partial change of ciliary neuron characteristics. The pattern of affected genes, together with the timing requirement of HoxB8 overexpression during ciliary neuron development and the lack of effects on enteric neurons suggests a role for HoxB8 in maintaining expression of TH, DBH and Hand2 genes transiently expressed in the ciliary neuron lineage.

Functional equivalence of Hox8 paralogs and other Hox genes in the regulation of noradrenergic characteristics

A combinatorial Hox code, i.e. the combination of functionally active Hox genes controls patterning and cell specification. The specification of spinal cord motoneurons depends on the action of multiple paralogs as shown for neurons of the forelimb lateral motoneuron column (Dasen et al., 2003; Vermot et al., 2005). There is also evidence for redundant functions, e.g. that the Hox6 paralogs HoxA6 and HoxC6 are sufficient to change the fate of thoracic preganglionic neurons to forelimb motoneurons (Dasen et al., 2003). The strong effects of HoxB8 in ciliary neurons raised the question of whether other Hox8 paralogs or less related Hox genes are functionally equivalent. We observed that HoxC8 increased Hand2, TH and DBH when ectopically expressed in mesencephalic neural crest and its progeny in ciliary ganglia. Similar effects were elicited by HoxB9 and even in response to HoxB1, which is not expressed in trunk sympathetic ganglia. However, only transient increases in the expression of noradrenergic marker genes were observed in response to HoxC8, HoxB9 and HoxB1 and HoxD10 showed no effect. These results suggest that a number of different Hox genes may act redundantly or cooperatively in the initial noradrenergic differentiation, whereas HoxB8 may have a specific role in the stabilization and maintenance of noradrenergic characteristics.

The question of whether HoxB8 displays an essential and unique role in the maintenance of noradrenergic characteristics can only be addressed by loss-of-function approaches. The normal expression of TH, DBH and Hand2 upon in vitro and in vivo HoxB8 knockdown may be due to a low efficiency of HoxB8 shRNA and mir196 in sympathetic ganglia. It seems more likely, however, that the partial effects of HoxC8, and HoxB9 observed upon forced expression in ciliary ganglia have to be taken as indication for a significant and redundant function in the sympathetic neuron lineage.

Although effects of the HoxB8 knockout in the mouse have not been investigated for the autonomic nervous system, there is evidence for a function in sensory neuron development. HoxB8 affects the development of cranial DRG, with a loss of C2 DRG in the HoxB8 knockout (van den Akker et al., 1999) and the maintenance of the normally degenerating C1 DRG (Forier’s ganglion) upon expanding the anterior expression boundary up to the otic vesicle by ectopic HoxB8 expression (Fanarraga et al., 1997). The restriction to C2 is in line with the finding that single gene loss-of-function Hox mutations generally result in transformations only at the most anterior limit of gene expression. Much more pronounced and extended effects are observed upon the loss of paralog function (McIntyre et al., 2007; Wellik and Capechi, 2003). Interestingly, in overexpression experiments, similar effects on C1 DRG were observed for HoxC8, HoxD8 and HoxB7 (van den Akker et al., 1999, 2001), suggesting functional
redundancy not only between paralogous genes but also between neighboring genes and non-paralogous genes in separate clusters (Davis and Capecchi, 1996; De la Cruz et al., 1999; Favier et al., 1996; Zákány and Duboule, 1996). Thus, the analysis of Hox gene function in sensory DRG neuron development supports the notion of a role for HoxB8 and functionally redundant Hox genes in sympathetic neuron differentiation.

Our study focused on the HoxB cluster, in particular HoxB8/9 that are expressed throughout the entire rostrocaudal axis in sympathetic ganglia and spinal cord (Dasen et al., 2005). It remains to be shown whether the expression of other Hox genes with differential anteroposterior expression correlates with sympathetic neuron subtypes innervating different targets and contributes to neuron subtype specification.

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References


