Essential role of Gata transcription factors in sympathetic neuron development

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Summary

Sympathetic neurons are specified during their development from neural crest precursors by a network of crossregulatory transcription factors, which includes Mash1, Phox2b, Hand2 and Phox2a. Here, we have studied the function of Gata2 and Gata3 zinc-finger transcription factors in autonomic neuron development. In the chick, Gata2 but not Gata3 is expressed in developing sympathetic precursor cells. Gata2 expression starts after Mash1, Phox2b, Hand2 and Phox2a expression, but before the onset of the noradrenergic marker genes Th and Dbh, and is maintained throughout development. Gata2 expression is affected in the chick embryo by Bmp gain- and loss-of-function experiments, and by overexpression of Phox2b, Phox2a, Hand2 and Mash1. Together with the lack of Gata2/3 expression in Phox2b knockout mice, these results characterize Gata2 as member of the Bmp-induced cluster of transcription factors. Loss-of-function experiments resulted in a strong reduction in the size of the sympathetic chain and in decreased Th expression. Ectopic expression of Gata2 in chick neural crest precursors elicited the generation of neurons with a non-autonomic, Th-negative phenotype. This implies a function for Gata factors in autonomic neuron differentiation, which, however, depends on co-regulators present in the sympathetic lineage. The present data establish Gata2 and Gata3 in the chick and mouse, respectively, as essential members of the transcription factor network controlling sympathetic neuron development.

Key words: Autonomic, Ciliary, Cholinergic, Noradrenergic, Hand2 (dhand), Bmp, Phox2b, Gata2, Gata3, Chick, Mouse

Introduction

Neural stem cells in the developing and adult nervous system have the potential to give rise to the different types of glial cells and to a large variety of distinct neuronal subtypes. Considerable progress has been made in the last few years in the identification of the molecular signals and mechanisms that control neuronal and glial specification and differentiation. In particular, the generation of autonomous neurons from neural crest stem cells was shown to be induced by an extrinsic signal (Bmps), which elicits the expression of a network of transcription factors that, in turn, control autonomic neuron differentiation (Goridis and Rohrer, 2002). This network includes Mash1 (Ascl1 – Mouse Genome Informatics), the mammalian homologue of the Drosophila achaete scute gene complex, and the paired homeodomain transcription factors Phox2b, possibly acting in concert with their coexpressed paralogue Phox2a. Phox2 proteins bind to the promoter of the subtype-specific noradrenergic marker genes tyrosine hydroxylase (Th) and dopamine-β-hydroxylase (Dbh) and activate their transcription.

Although Mash1 and Phox2b were shown to be essential and sufficient to elicit noradrenergic neuron development from neural crest precursor cells, they also function together in the generation of cholinergic autonomic neurons and several other neuron subtypes. Thus, to generate noradrenergic neurons, Mash1 and Phox2 genes must be enforced by the action of additional autonomic and noradrenergic regulators expressed in this lineage. The basic helix-loop-helix (bHLH) transcription factor Hand2 (previously known as dHand) has recently been identified as a noradrenergic co-determinant, due to its ability to elicit noradrenergic differentiation in neural crest (Howard et al., 2000) and parasympathetic precursors (Müller and Rohrer, 2002), and due to its expression in noradrenergic sympathetic but not in parasympathetic ciliary neurons (Müller and Rohrer, 2002). The effects on the expression of the noradrenergic marker gene dopamine-β-hydroxylase (Dbh) can be explained by a direct interaction with Phox2a to stimulate transcription from the Dbh promoter (Xu et al., 2003; Rychlik et al., 2003). Finally, members of the Gata family of transcription factors have been implicated in the control of noradrenergic differentiation (Groves et al., 1995; Lim et al., 2000).

The Gata transcription factors are key regulators of...
hematopoiesis (Pevny et al., 1991; Tsai et al., 1989; Maeno et al., 1996; Murphy and Reiner, 2002), cardiovascular and urogenital development (Zhou et al., 1998; Molkentin et al., 1997) and nervous system development (Pandolfi et al., 1995; Nardelli et al., 1999; Pata et al., 1999; Dasen et al., 1999; van Doorninck et al., 1999; Craven et al., 2004; Karis et al., 2001). In hematopoiesis and developing heart and liver, Gata transcription factors mediate the effects of Bmps (Maeno et al., 1996; Schultheiss et al., 1997; Rossi et al., 2001). Recent evidence suggests that Gata factors maintain Bmp expression during cardiac precursor maturation (Petterkin et al., 2003; Peterkin et al., 1996; Murphy and Reiner, 2002), cardiovascular and nervous system development (Pandolfi et al., 1995; Nardelli et al., 1999; Pata et al., 1999; van Doorninck et al., 1999; Craven et al., 2004; Karis et al., 2001). Since in the sympathetic ganglia of Gata3-deficient mice abrogation of Th and Dbh expression, but not generic neuronal differentiation, has been reported (Lim et al., 2000), Gata3 was considered to selectively control neuron subtype differentiation and to represent a noradrenergic co-determinant for Phox2a/b and Mash1. Although the Gata3 knockouts demonstrated the importance of this factor, its position in the transcriptional network specifying sympathetic neurons was not clear. Here, we have analysed the action of Gata transcription factors in autonomic neuron development in the chick and re-investigated the sympathetic neuron phenotype in Gata3-deficient mouse embryos. We demonstrate that Gata2 but not Gata3 is expressed in the avian autonomic nervous system. Gata2 expression in the chick starts after the expression of Cash1, Phox2b, Phox2a and Hand2 and is induced by overexpression of these transcription factors. Bmp-dependent expression characterizes Gata2 as an additional member of the transcriptional network acting in the sympathetic lineage downstream of Bmps. The elimination of Gata3 in the mouse and the knockdown of Gata2 in the chick result in a strong decrease in both sympathetic ganglion size and Th expression. These results, together with the effect of Gata2 overexpression demonstrate a function for Gata2/3 in the type-specific, as well as generic, differentiation of noradrenergic neurons, acting in the context of other autonomic co-determinants.

Materials and methods

Expression pattern of Gata transcription factors

Chick embryos were staged according to Hamburger and Hamilton (1951). Embryos between embryonic day (E) 3 (stage 18) and E20 (stage 45) were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer overnight. The fixative was replaced by 15% sucrose in 0.1 M sodium phosphate buffer overnight. Cryosections of 12 μm were analysed for expression of Phox2b, Hand2, Phox2a, Gata2, Gata3, Th and Dbh by in-situ hybridisation. At least three embryos were analysed for each stage.

Implantation of agarose beads loaded with noggin or BSA in chick embryos

The implantation technique used is described in detail by Schneider et al. (Schneider et al., 1999). Agarose beads (Affi-Gel blue beads; Biorad, Hercules, CA) were incubated for at least 1 hour in a small volume of loading buffer containing either 1 mg/ml noggin or bovine serum albumin (BSA). Two beads were implanted into the trunk region of 2-day-old chick embryos, placed at the last somite and 2-3 somites more rostral. The eggs were further incubated until stage 19, fixed, embedded and sectioned. Cryosections of 12 μm were collected, including from the implantation area, and assessed for expression of Sox10 and Gata2 by in-situ hybridisation. The area of Sox10 and Gata2 expression was quantified morphometrically and the areas were expressed in μm²/section. The results are given as the mean area per section±s.e.m. of at least six embryos analysed.

Expression of transgenes in vivo using retroviral replication-competent avian sarcoma (RCAS) vectors

Fertilized virus-free chicken eggs were obtained from Charles River (Sulzfeld, Germany) and incubated for 2 days. Cell aggregates of DF1 fibroblasts infected with RCASBP(B)-Hand2 (Howard et al., 2000), RCASBP(B)-Phox2b (Stanke et al., 1999), RCASBP(B)-CNS-Gata2, RCASBP(B)-CNS-dnGata2, RCASBP(B)-CNS-engrailed and RCASBP(B)-CNS-VP16-Gata2 were implanted on the right side of the embryos at brachial levels between the neural tube and the last somite formed (Reissman et al., 1996). The eggs were further incubated until E8. Embryos were killed by decapitation. The trunk and cervical region of the embryos were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer overnight, kept in 15% sucrose in 0.1 M sodium phosphate buffer overnight, embedded in Tissue Tek (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands) and sectioned. Cryosections of 12 μm were collected and analysed for expression of reverse transcriptase (RT), Th, Scg10 and Gata2 (RCASBP(B)-Hand2 and RCASBP(B)-Phox2b infections), RT, Th, Scg10, NF160, Phox2b and Cash1 (RCASBP(B)-CNS-Gata2 infections) and RT, Th, Dbh, Scg10, NF160 and Phox2b (RCASBP(B)-CNS-dnGata2, RCAS-BP(B)-CNS-engrailed and RCASBP(B)-CNS-VP16-Gata2 infections) by in-situ hybridisation. For the quantitative analysis, between 5 and 13 embryos were analysed for each of the genes investigated.

Mouse breeding, genotyping and rescue

The generation and genotyping of Phox2b mutant mice and Gata3 mutant mice have been reported (Pattyn et al., 1999; Pandolfi et al., 1995; Lim et al., 2000). Homozygous Gata3 mutants, which normally die at mid-gestation, were rescued beyond E10 with noradrenergic agonists as described for Phox2b mutants (Pattyn et al., 2000).

In-situ hybridisation on sections

Non-radioactive in-situ hybridisation on cryosections and preparation of digoxigenin- or fluoresceine-labelled probes for chick RT, Th, Scg10, NF160, Gata2, Gata3, Phox2b, Phox2a, Hand2, Cash1 and Sox10 were carried out as described previously (Ernsberger et al., 1997; Stanke et al., 1999) (Gata2 and Gata3 plasmids were generously provided by M. Zenke). For double in-situ hybridisations...
Fast Red (Roche Diagnostics, Mannheim, Germany) was used for staining the first probe. Sections were photographed and the antibody was stripped off by washing twice for 10 minutes with 1 ml 0.1 M glycine pH 1.8. After equilibration in MABT for 1 hour, the second colour reaction with Nitroblue Tetrozolium/5-bromo-Á-chloro-3-indolyl phosphate (NBT/BCIP) was carried out.

In-situ hybridisation using mouse Dbb, Gata2 (gift of J. Nardelli), Gata3 (gift of D. Engel), Hand2 (gift of Y.-S. Dai), Mash1 (gift of F. Guillemot), Ret, Sox10 (gift of K. Kulbrodt) and Th antisense riboprobes, immunohistochemistry using Phox2a, Phox2b, β-galactosidase (Cappel) antisera or Tuj1 monoclonal antibody (Covance), and combined in-situ hybridisation with immunohistochemistry were performed as previously described (Tiveron et al., 1996). Double-immunofluorescence experiments using Phox2a and Tuj1 antibodies were analysed on a Leica microscope. Pictures were superimposed in Photoshop.

Morphometric analysis
Chick
The area of Th, Dbb, Phox2b, NF160 and Sg10 expression was quantified morphometrically using the Metamorph Imaging System (version 4.6, Universal Imaging Corporation) on all sections infected by the virus, as indicated by expression of RT mRNA. Areas were expressed in mm²/section. The results are given as the mean area per section±s.e.m. of at least five embryos analysed. Student’s t-test was used for statistical analysis.

Mouse stellate ganglion
The surface of the stellate ganglion – stained with Phox2b antibody – has been calculated on sagittal sections of E13.5 embryos using the Leica Qfluoro Program. Measurements of control ganglia were considered as 100%. For each genotype, four sections were counted on four chains corresponding to two embryos.

Mouse thoracic chain
The number of Phox2b-positive cells were counted on sagittal sections at E13.5 at the thoracic level on a segment spanning three vertebrae, at the same level in the control and the mutants. For each genotype, four sections were counted on four chains corresponding to two embryos.

TUNEL analysis
TUNEL-positive cells were detected using the apoptag detection kit (Appligene) following the manufacturer’s instructions. The rostralmost part of the sympathetic chain (anterior to the fusion of the dorsal aorta) was delimited on transverse sections at E11.5 using a Sox10 in-situ hybridisation signal on adjacent sections, and cells were counted within that area. For each genotype, six sections were analysed on four chains corresponding to two embryos.

Construction of plasmid transgenes
RCAS-BP(B)-CNS-Gata2
PCR technology was used to insert a Koazak sequence linked to a Clal site and a NotI site flanking the coding sequence of ggGata2 (Yamamoto et al., 1990). Primer: sense: 5'-AGT ATC GAT GAC CAB C AT G GA GGT GGC CAC GGA TCA GC-3'; antisense: 5'-GAT CGA GCC GCC GC T TA T CCC ATG GCT GTA ACC AT-3'. (sense primer: bold, Clal site; underlined, Kozak sequence; bold+underlined, Start) (antisense primer: bold, NotI site; bold+underlined, Stop)

The PCR product was then cloned directly into the pCRII-TOPO vector (Invitrogen). After restriction analysing and sequencing, the insert was cloned into the Clal and NotI sites of the avian retroviral vector RCAS-BP(B)-CNS. The RCAS-BP(B)-CNS is a modification of the RCAS-BP(B) vector (Hughes and Kosik, 1984), inserting a unique NotI and SpeI site directly behind ClalP7029

RCAS-BP(B)-CNS-dnGata2
PCR technology was used to insert a BamHI site and an Xhol site flanking the two zinc finger domains of ggGata2 (Yamamoto et al., 1990; Yang et al., 1994). Primer: sense: 5'-CTG GGA TCC TCA GAA GGC AGA GAG TGT GTG AA-3'; antisense: 5'-TTT TCT GTT AGA TCT GTT TT CAT GGT CAG ACC CC-3'. (sense primer: bold: BamHI site; antisense primer: bold: Xhol site)

The BamHI-Xhol digested PCR fragment was ligated into the pIEP vector (pIEP vector was generously provided by C. Goridis) after eliminating the original BamHI-Xhol fragment (Phox2a DNA binding site). The original pIEP vector contains the engreled effector domain from Drosophila melanogaster (AA1-AA289) (Han and Manley, 1993) upstream from the Phox2a homeodomain, that is flanked by a BamHI site and an Xhol site. The pIEP vector also contains two myc-tags downstream from the Xbal site. PCR technology was then used to insert a Koazak sequence linked to a Clal site and a SpeI site flanking the dnGata2 coding region. The PCR product was cloned into the Clal and NotI site of the RCAS-BP(B)-CNS vector. Primer: sense: 5'-ACA ATC GAT GCC GCC A AT G GC CCT GGA GGA TCG CTG CA-3'; antisense: 5'-TCT ACT AGT TCA GAG TTC GTC CTC TCT GCT GAT CAG-3'. (sense primer: bold, Clal site; underlined, Koazak sequence; bold+underlined, start) (antisense primer: bold, SpeI site; bold+underlined, stop)

RCAS-BP(B)-CNS-VP16-Gata2
The engreled domain of RCAS-BP(B)-CNS-dnGata2 was substituted by the VP16 domain (Ala481-Gly541 + spacer of 6 AA) (VP16 vector was generously provided by C. Goridis).

RCAS-BP(B)-CNS-engrailed
The zinc finger domain of RCAS-BP(B)-CNS-dnGata2 was substituted by the spacer Ala-Gly-Gly.

Semiquantitative RT-PCR analysis
Total RNA from chick sympathetic ganglia was isolated using an RNeasy kit (Qiagen). Relative levels of Gata2 and Gata3 expression were determined by RT-PCR. Primer pairs were designed for amplification of specific cDNA fragments.

Gata2 primers: sense: 5'-CAA CTA CAT GGA ACC AGC GC-3'; antisense: 5'-AGG CTG CTG CTG TAG TCA TG-3'; Gata3 primers: sense: 5'-CTC CGT ATT ACG GCA ACT CC-3'; antisense: 5'-GCT GCA GAC AGC CTT CTC TT-3';

cDNA from total RNA was synthesised with oligo(dT) primers and Moloney murine leukaemia virus reverse transcriptase (Superscript II; Life Technologies) at 45°C for 1 hour. cDNA derived from 20-30 ng RNA was used as template for PCR amplification in a 50 µl reaction volume containing 1x PCR buffer, 0.2 mM dNTPs and 0.1 µM each primer. Hot start was performed by adding 1.5 units of AmpliTaq. The temperature profile consisted of 25-36 cycles (95°C for 15 seconds, 65°C for 30 seconds and 72°C for 30 seconds) and a final 5 minutes extension at 72°C. To achieve accurate quantification, 10 µl aliquots were collected during the PCR run at various cycle numbers. PCR products were separated by electrophoresis on 1% agarose gel and stained with Ethidiumbromide. Their fluorescence intensities were measured by using the Gel Doc 2000 (Bio Rad Laboratories). In all experiments, amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragments was run in parallel to normalize different cDNA samples (Friedel et al., 1997).

Results
Expression of Gata2 and Gata3 in the chick and mouse sympathetic lineage
In the vertebrate peripheral nervous system, Gata3 expression has been analysed only in the mouse embryo (George et al.,...
1994), whereas data on Gata2 expression are available only for autonomic neurons in the trunk of chick embryos (Groves et al., 1995). The present in-situ hybridisation analysis of Gata2 and Gata3 expression in the chick embryo demonstrated that in sympathetic ganglia analysed between E3 (stage 18) and E20 (stage 45), Gata2 but not Gata3 was detectable (Fig. 1). This result was confirmed by semiquantitative RT-PCR, which showed about 1000-fold lower levels of Gata3 mRNA compared with Gata2 mRNA in E7 sympathetic ganglia (the mean of two experiments). By contrast, Gata2 and Gata3 are coexpressed in the spinal cord (Fig. 1), as described previously (Kornhauser et al., 1994; Nardelli et al., 1999; Pata et al., 1999). This finding suggests that, in the chick, Gata2 rather than Gata3 may be involved in noradrenergic differentiation. By contrast, in the mouse embryo, Gata2 and Gata3 are expressed at equivalent levels in sympathetic ganglia (Fig. 5).

**Gata2 is expressed after the onset of Phox2a, Phox2b and Hand2 expression**

We analysed the developmental expression of Gata factors in the sympathetic lineage of the chick embryo in relation to other developmental marker genes as a first indication of their epistatic relationship. Chick embryos (stage 18-19) were sectioned in the brachial region and analysed by in-situ hybridisation on consecutive sections. Embryos were grouped according to the number of somites. In 32-somite embryos, Gata2 was not yet detected, whereas Phox2b was strongly expressed and Hand2 expression just started (Fig. 2). At this axial level, Gata2 expression in primary sympathetic ganglia was first detected in 33/34-somite embryos, i.e. after Phox2b, Hand2 and Phox2a, but before the onset of Th expression, which began in 35-somite embryos (Fig. 2). In addition to sympathetic ganglion primordia, Gata2 is also expressed in the ventral part of the dorsal aorta. The results, summarized in Table 1, show that Gata2 is expressed before the noradrenergic marker genes, which is a prerequisite for the proposed function in noradrenergic differentiation.

**Gata2 expression is dependent on Bmp signalling**

Previous studies demonstrated that the expression of Mash1, Phox2a, Phox2b (Schneider et al., 1999) and Hand2 (Howard et al., 2000) is dependent on Bmps that are expressed in the dorsal aorta, close to the developing sympathetic ganglia. To investigate whether Gata2 expression would be controlled also by Bmps or other, unknown signals, the Bmp antagonist noggin was applied in vivo in the area where the sympathetic ganglia form. Using Sox10 as a general marker for neural crest and early neural crest derivatives, only a small reduction in the number of sympathetic precursors, located dorsolaterally from the dorsal aorta, was apparent (Fig. 3). By contrast, Gata2 expression was strongly reduced or absent. The effect of the noggin treatment was restricted to the primordia of the sympathetic ganglia and did not affect Gata2 expression in the spinal cord or ventral aorta. Quantification by the

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**Table 1. Expression of Phox2b, Hand2, Phox2a, Gata2, Th and Dbh gene transcripts in the chick embryo**

<table>
<thead>
<tr>
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All sections examined correspond to the wing bud region. Data from one embryo are listed in one row.

+ sections with positive cells in primary sympathetic ganglia; –, sections devoid of positive cells in primary sympathetic ganglia.

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**Fig. 2.** The onset of Gata2 expression in sympathetic ganglia in relation to the expression of Phox2b, Hand2, Phox2a and Th. Frozen sections from the brachial region of stage 18/19 chick embryos, staged according to the number of somites, were analysed for the expression of Phox2b, Hand2, Phox2a, Gata2 and Th. Gata2 expression was first observed in 34-somite embryos, after the onset of expression of Phox2b, Hand2 and Phox2a, but before Th, which was first observed in 35-somite embryos. Black and white arrowheads indicate the presence or absence, respectively, of gene expression in primary sympathetic ganglia. Gata2 expression was additionally detected in the ventral part of the dorsal aorta (da) (I) and in the spinal cord (D, I, N). The results are summarised in Table 1.
morphometric analysis of Sox10- and Gata2-positive cells revealed a strong, 80% reduction in Gata2 expression and a non-significant reduction (24%) in Sox10 expression. Since the number of Sox10-positive cells is reduced only to a small extent, the effect on Gata2 must reflect the action of Bmps on Sox10 and Gata2-expressing cells. The mean±s.e.m. of at least three embryos is shown. ** significantly different from BSA controls (P<0.01) (Student’s t-test).

**Fig. 3.** Gata2 expression in sympathetic ganglion primordia is blocked by the Hand2 inhibitor noggin. Chick embryos were treated with BSA (A,B) or noggin (C,D) at stage 14 and analysed at stage 19 for the expression of Sox10 (A,C) and Gata2 (B,D) in sympathetic ganglion primordia close to the dorsal aorta (da). Arrowheads point to primary sympathetic ganglia. (E) Sox10 and Gata2 expression is quantified by determining the area of Sox10- and Gata2-expressing cells. The mean±s.e.m. of at least three embryos is shown.

**Epistatic relationship between Gata2, Phox2b and Hand2**

As Gata2 is expressed after the Bmp-induced factors Phox2b, Hand2 and Phox2a, it seemed very likely that the effect of Bmps is indirect, mediated by these transcription factors. To address the epistatic relationship between Gata2 and Phox2b and other Bmp downstream transcriptional regulators, both gain- and loss-of-function approaches were followed. Previous studies demonstrated that Phox2b and Hand2 expression in the brachial nerve results in the generation of ectopic Th+ and Dbh+ noradrenergic neurons (Stanke et al., 1999; Howard et al., 2004). Using this experimental paradigm, we investigated whether Gata2 would be expressed under these conditions. On consecutive sections of Phox2b-infected brachial nerve (Fig. 4) a very similar pattern of cells expressing Th (A) and Gata2 (B)

was observed, strongly suggesting that Gata2 is induced by Phox2b. Similarly, Gata2 and Th expression were induced in response to Mash1 and Hand2 (not shown), although the number of ectopically induced cells was lower compared with Phox2b. Since frequent reciprocal crosstalks in the sympathetic lineage make it difficult to rigorously establish epistatic relationships by overexpression experiments (Stanke et al., 1999, 2004; Howard et al., 2000), Gata2/3 expression was investigated in Phox2b knockout mice. The absence of Gata2 and Gata3 expression in E10.5 sympathetic ganglion primordia (Fig. 5) confirmed our conclusion that Gata2/3 acts downstream of Phox2b in the network of Bmp-induced transcription factors.

**Fig. 4.** Gata2 is induced in peripheral nerve precursors by ectopic expression of Phox2b and BMP24. Chick embryos were infected at E2 with RCAS-Phox2b and RCAS-BMP4 and analysed at E8 for the presence of ectopic neurons in the brachial nerve. In response to Phox2b and BMP24 neurons were generated that express noradrenergic properties: Th (A,C) and Gata2 (B,D). These cells also coexpress neuronal characteristics (Stanke et al., 1999; Howard et al., 2000).

**Essential role of Gata2 in chick sympathetic neuron generation**

The strong effects of the Gata3 knockout on noradrenergic gene expression in the mouse (Lim et al., 2000) raised the question whether elimination of Gata2, which in the chick sympathetic lineage appears to be functionally equivalent to mouse Gata3, would also impair the development of the noradrenergic phenotype. To interfere with the action of endogenous Gata2, a repressive form of Gata2, in which the engrailed repressor domain is fused to Gata2 zinc finger domains, was expressed in sympathetic neuron precursor cells. Previous studies have demonstrated that the engrailed repressor does not function by titrating promoter binding sites but rather interferes with transcription initiation (Han and Manley, 1993; Jaynes and O’Farrell, 1991). Indeed, a similar engrailed-Gata2 fusion protein has very recently been constructed and was shown to act as dominant-negative Gata2 (dnGata2) (Craven et al., 2004). DnGata2 was expressed unilaterally using RCAS retroviral vectors, so that the sympathetic ganglia of the contralateral side could be used as internal control. As additional controls, ganglia were infected with RCAS vectors expressing only the engrailed repressor domain or a VP16-Gata2 variant, where the transactivating N-terminal region of Gata2 is replaced by the VP16 activation domain. The expression of dnGata2 in sympathetic neuron precursors resulted in a strong (50%) reduction of Th expression (Fig. 6),
and smaller effects on Scg10 (Stmn2 – Mouse Genome Informatics) (35%), Dbh (41%) and Phox2b (31%) (Fig. 6). The expression of neurofilament (NF 160) was also strongly reduced (not shown). All reductions were highly significant (P<0.01) with respect to the control side. Th expression was also significantly reduced compared with Phox2b (P<0.05; n=11) and Scg10 (P<0.05; n=11), whereas Dbh was not significantly more reduced than Phox2b and Scg10. Control infections with the engrailed RCAS virus or RCAS-VP16-GATA2 affected neither Th nor Scg10 expression (Fig. 6), excluding the possibility that dnGata2 would act in an unspecific manner by titrating transcription factors unrelated to endogenous Gata2. In conclusion, these results demonstrate a dual effect of Gata2 knockdown: a reduction in sympathetic ganglion size, reflected by a smaller area of Phox2b- and SCG10-expressing cells, and an additional effect on Th expression in the remaining cells. These effects suggest a more general role in sympathetic neuron development than reported for murine Gata3 (Lim et al., 2000).

**Gata3 knockout mice display strong defects in sympathetic neuron development**

To resolve the different results obtained by interfering with Gata2 and Gata3 in the chick and mouse, respectively, the development of the sympathetic lineage was re-investigated in Gata3–/– mice. In the Gata3 knockout, sympathetic ganglion development of the sympathetic lineage was re-investigated in Gata2 and Gata3 in the chick and mouse, respectively, the general role in sympathetic neuron development than reported for endogenous Gata2. In conclusion, these results demonstrate a dual effect of Gata2 knockdown: a reduction in sympathetic ganglion size, reflected by a smaller area of Phox2b- and SCG10-expressing cells, and an additional effect on Th expression in the remaining cells. These effects suggest a more general role in sympathetic neuron development than reported for murine Gata3 (Lim et al., 2000).

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**Fig. 5.** Lack of Gata2 and Gata3 expression in Phox2b–/– mice. The expression of Gata2 and Gata3 was studied in E13.5 Phox2b+/+ and Phox2b–/– mouse embryos. In the Phox2b knockout Gata2 (B) and Gata3 (D) were not detectable, by contrast to the heterozygotes (A,C). Sympathetic ganglion precursors could be detected at the dorsal aorta in both Phox2b+/+ (E) and Phox2b–/– (F) embryos by LacZ staining.

**Fig. 6.** The expression of a dominant-negative variant of Gata2 results in a reduction of Th, Scg10, Dbh and Phox2b expression. Chick embryos were infected unilaterally at E2 with either RCAS-dnGata2 or RCAS-_engrailed and RCAS-VP16-Gata2 as controls and analysed at E8 for the expression of Th (A), Scg10 (B), Dbh (C) and Phox2b (D) in infected sympathetic ganglia. The areas of Th-, Scg10-, Dbh- or Phox2b-expressing cells were quantified on alternate sections, both on the infected and the contralateral uninfected side. For the RCAS-engrailed and RCAS-VP16-Gata2 controls, only data of the infected ganglia are shown for simplicity; the data of the uninfected side are identical. The data represent the mean±s.e.m. of at least nine embryos. (A) * significantly different from engrailed-infected side, VP16-Gata2-infected side and dnGata2-infected side (P<0.001, P<0.005, P<0.005, respectively). (B) * significantly different from engrailed-infected side, VP16-Gata2-infected side and dnGata2-infected side (P<0.001, P<0.01, P<0.01, respectively). (C) * significantly different from dnGata2-uninfected side (P<0.005). (D) * significantly different from dnGata2-uninfected side (P<0.01) (all Student’s t-tests).
shown) were detectable in Gata3−/− embryos by analysing Phox2b expression (Fig. 8). At more caudal levels, the effect on ganglion size was less dramatic, but the thoracic chain was still reduced by 60% in size. In the remaining cells in the

Fig. 7. Gene-expression pattern in E10.5 sympathetic ganglia of Gata3−/− mice. The expression of Phox2b (A,B), Mash1 (C,D), Phox2a/TuJ1 (E,F), Hand2 (G,H), Ret (I,J) and Dbh (K,L) is not affected in Gata3-deficient mice. By contrast, the ganglia are devoid of Gata2 expression (O,P) and display a significant reduction in Th expression levels (M,N).

Fig. 8. Reduction in ganglion size and Th expression and increased apoptosis in sympathetic ganglia of Gata3−/− mice. (A,B) Immunostaining for βIII-tubulin combined with in-situ hybridisation for Sox10 reveal a reduced ganglion size in mutant (B) compared with control (A) embryos at E11.5. (C,D) Cell death analysis by TUNEL staining showing an increased number of apoptotic cells. (E) Quantification (P<0.01; n=4). (F-O) Reduction in sympathetic ganglion size and Th expression in E13.5 sympathetic ganglia of Gata3−/− mice. In Gata3−/− mice the sympathetic stellate ganglion (G) is strongly reduced in size compared with wild type (F), as revealed by immunostaining for Phox2b antibody. The reduction, quantified in (H) is by about 88% (P<0.001; n=4). Also at thoracic levels (I,J,L,M) a strong reduction in ganglion size is evident on Phox2b immunostains (I,J), quantified in (K) at around 60% (P<0.01; n=4). While Dbh expression was somewhat reduced (L,M), Th expression was almost undetectable in the mutants (N,O).
ganglion rudiments, Th expression was practically abolished, and Dbh expression was now reduced. In conclusion, we found a much more global phenotype in the Gata3−/− mouse embryos than previously reported (Lim et al., 2000), which is also more in agreement with the effect of dnGata2 in chick sympathetic ganglia.

**Gata2 overexpression reveals a preferential action on generic neuron differentiation rather than on noradrenergic differentiation in peripheral nerve precursor cells**

The loss-of-function approaches revealed an essential role of Gata2/3 in sympathetic neuron development but did not reveal the mechanism of action. The loss of sympathetic ganglion cells in Gata2/3-deficient ganglia may be explained by a selective control of cell survival by Gata2/3 or by effects on differentiation that indirectly lead to apoptotic cell death. To begin to address these issues, the action of Gata2 was studied in gain-of-function experiments. Using the chick embryo as a model to overexpress transcriptional control genes in neural crest precursor cells (Stanke et al., 1999; Howard et al., 2000; Stanke et al., 2004), we observed that Gata2 induced the generation of ectopic neurons in peripheral nerve precursors (Fig. 9). These neurons expressed NF160 (not shown). Interestingly, the great majority of these ectopic neurons were devoid of the autonomic markers Phox2a and Phox2b. Only a small subpopulation of the Sg10-positive cells displayed properties of noradrenergic neurons, Phox2b, Th, Dbh (not shown) and Cash1 (Fig. 9). Cash1 expression was very low, which is expected from its transient expression during normal development. Double in-situ hybridisation for Sg10 and Th demonstrated the very small proportion of the Sg10-positive cells that coexpressed Th. In addition to Th+ neurons, there was also a small number of Th− cells devoid of neuronal properties. Infection of peripheral nerves with control RCAS virus [engrailed-RCAS-(BP)B] was unable to induce ectopic neurons (not shown), as expected from previous control RCAS infections (Reissmann et al., 1996; Stanke at al., 1999).

These data suggest a role for Gata2 and Gata3 in the neuronal differentiation of sympathetic precursor cells in the chick and mouse, respectively. The preferential generation of non-autonomic neurons in response to Gata2 in the peripheral nerve implies that the action of Gata2 depends on the cellular context, i.e. that the effect on Th expression, revealed by the loss-of-function approaches, seems to be dependent on coregulators present in the sympathetic neuron lineage but not in peripheral nerve precursor cells.

**Gata2 expression in parasympathetic ganglia and the locus coeruleus**

The importance of Gata2/3 in sympathetic neuron development raised the issue whether Gata2/3 would play a similar role in other autonomic ganglia and/or in central noradrenergic neurons. The parasympathetic chick ciliary ganglion was found to completely lack Gata2 and Gata3 expression at all stages analysed by in-situ hybridisation between stage 20 (E3) and stage 35 (E8) (Fig. 10). The result from the in-situ hybridisation was confirmed by semiquantitative RT-PCR, which revealed in E5 ciliary ganglia 1000-fold and 200-fold lower levels of Gata2 and Gata3 mRNA, respectively, compared with Gata2 mRNA in E7 sympathetic ganglia. Low-level signals for Gata2 and Gata3 by RT-PCR can be explained by Gata2 expression in cells of the retro-orbital mesenchyme, contaminating to some extent the ganglion preparation. Also the chick parasympathetic sphenopalatine ganglion was devoid of Gata2 expression (Fig. 10). However, Gata2 was detectable in the submandibular ganglion (Fig. 10), and strong Gata2 expression was present in trunk parasympathetic ganglia, i.e. cardiac ganglia (Fig. 10) and the Remak ganglion (Groves et al., 1995). From all chick ganglia investigated, Gata3 was detected only in the cardiac ganglia (not shown). In the mouse, the sphenopalatine, otic and submandibular ganglia were devoid of Gata3 expression at E13.5, with low, but detectable expression in cardiac ganglia (not shown). Interestingly, low-level Th expression was present in the chick parasympathetic sphenopalatine, submandibular, cardiac (Fig. 10) and the Remak ganglion (Cantino et al., 1982; Suzuki et al., 1994), whereas in the ciliary ganglion only very few Th-positive cells remained (Müller and Rohrer, 2002) (Fig. 10). Dbh expression paralleled Th expression, both with respect to ganglion type and expression levels (not shown). The expression of a variable subset of noradrenergic traits in cholinergic parasympathetic neurons has been described previously in several species (Grzanna and Coyle, 1978; Landis et al., 1987; Leblanc and Landis, 1989; Baluk and Gabella, 1990; Hardebo et al., 1992).

As Gata2/3 are essential for the initiation and/or maintenance of Th and Dbh in noradrenergic sympathetic neurons it was of interest whether central noradrenergic neurons also depend on Gata2/3 for their differentiation. The major noradrenergic centre of the central nervous system, the locus coeruleus, lacked Gata2 and Gata3 expression in mouse (Fig. 10).
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and chick embryos (not shown) and Gata3 knockout mice had an intact locus coeruleus (Fig. 11). These results, together with the findings in parasympathetic ganglia, demonstrate that there is no strict correlation between Gata2/3 expression and noradrenergic differentiation.

Discussion

Here, we demonstrate that Gata transcription factors, Gata2 in chick and Gata3 in mouse, are members of the network of transcription factors controlling the development of noradrenergic sympathetic neurons from neural crest precursors. Gata2 is expressed in a Bmp-dependent manner, immediately before the onset of noradrenergic differentiation, but after Mash1, Phox2b, Hand2 and Phox2a. The Phox2b-dependent expression of Gata2 and Gata3, evident in the Phox2b knockout, suggests a similar timing of expression in the mouse. Loss-of-function experiments demonstrate the importance of Gata3 in the mouse and Gata2 in the chick for sympathetic neuron development and for the onset of Th expression. Gata2 overexpression in the chick embryo elicits the generation of ectopic neurons from neural crest precursor cells, which implicates a function of Gata2/Gata3 in the expression of generic and subtype-specific neuronal traits. The action of Gata2/Gata3 depends, however, on the cellular context, as the vast majority of Gata2-induced ectopic neurons in the peripheral nerve display a non-autonomic, non-noradrenergic phenotype, whereas in sympathetic neurons Th expression and the maintenance (but not the onset) of Dbh expression are dependent on Gata2/Gata3. Thus, the effects on Th and Dbh may depend on additional co-regulators present in the sympathetic lineage.

Gata factors in relation to other Bmp-induced transcriptional determinants of the sympathetic lineage

The members of the Gata family of zinc finger transcription factors have selective effects on various aspects of tissue and organ development. Of the six family members, only Gata2 and Gata3 are expressed in the vertebrate nervous system and were shown to control the development and differentiation of specific neuronal subpopulations (Yamamoto et al., 1990; Ko and Engel, 1993; Whyatt et al., 1993; Zhou et al., 2000; Karunaratne et al., 2002; Dasen et al., 1999; van Doorninck et al., 1999; Craven et al., 2004; Lim et al., 2000; Karis et al., 2001). In general, Gata2 and Gata3 are coexpressed in the central nervous system and Gata3 is downstream of Gata2 (Nardelli et al., 1999; Pata et al., 1999). The expression of these factors in the peripheral nervous system is much less clear, as

Fig. 10. Expression of Gata2 and Th in chick parasympathetic ganglia. Ciliary (A-C), sphenopalatine (D-F), submandibular (G-I) and cardiac (J-L) ganglia were analysed for the expression of Phox2b, Gata2 and Th. Whereas Gata2 is not detectable in the cranial ciliary and sphenopalatine ganglion, Gata2 is expressed at very low levels in submandibular ganglia, and strong expression is evident in cardiac ganglia. Low-level Th expression is detectable in the sphenopalatine ganglion, submandibular and cardiac ganglia and in a small number of cells in the ciliary ganglion (Müller and Rohrer, 2002), but not in the section shown in C. Expression was analysed on parallel sections.

Fig. 11. Gata2 and Gata3 do not control the central noradrenergic phenotype. (A-C) The anlage of locus coeruleus (LC) visible in the dorsal metencephalon by anti-Phox2a immunohistochemistry (A) expresses neither Gata2 (B) nor Gata3 (C) at E10.5. (D-G) At E13.5 the anlage of the LC, visible by in-situ hybridisation for Dbh (F) expresses neither Gata2 nor Gata3 (arrowhead in D and E, respectively) and is intact in Gata3 mutants (G).
data are often available for only one factor in a single species and tissue (Groves et al., 1995; Nardelli et al., 1999; George et al., 1994). We demonstrate here that Gata3 is not expressed in chick sympathetic ganglia, whereas Gata2 is detectable throughout development. The onset of Gata2 expression in the chick was found to occur after the sequential expression of Mash1, Phox2b, Hand2 and Phox2a.

The timing of Gata2 expression and its proposed role in noradrenergic differentiation raised the question whether Gata2 expression is dependent on Bmps, which have been shown to control the expression of Mash1, Phox2a/b and Hand2 in sympathetic neuron precursors at the dorsal aorta (Schneider et al., 1999; Howard et al., 2000) and which control Gata factor expression in other developmental contexts (Maeno et al., 1996; Schultheiss et al., 1997; Rossi et al., 2001; Patient and McGhee, 2002). Alternatively, Gata2 expression might be induced by additional, independent signals. The present data firmly establish that Gata2 expression is prevented by the Bmp inhibitor noggin and is expressed in Bmp-induced ectopic neurons. Thus, Gata2 represents an additional member of the group of transcription factors induced by Bmps. The epistatic relationship between Gata2 and the other factors has been addressed by overexpression of Phox2b, Hand2 and Mash1. The induction of Gata2 expression by each of these factors is in agreement with the timing of expression and suggests that Gata2 may be directly or indirectly controlled by these transcription factors. The notion that Gata2 is expressed downstream of Phox2b is confirmed by the lack of Gata2/3 expression in the Phox2b knockout mice and, conversely, the initial presence of cells expressing Phox2b, Phox2a and Hand2 in the absence of Gata3.

**Generic and subtype-specific role of Gata factors in the sympathetic lineage**

A major problem in the control of neurogenesis is how and when the expression of neuron subtype-specific properties and generic neuronal characteristics are coordinated. For sympathetic neuron development this issue is still unclear. The loss of Mash1 and Phox2b does affect both noradrenergic and pan-neuronal gene expression (Guillemot et al., 1993; Hirsch et al., 1998; Pattyn et al., 1999) and in gain-of-function experiments no selective effects were observed for Phox2a, Phox2b and Hand2 (Stanke et al., 1999; Howard et al., 2000). Under certain in-vitro conditions, Mash1 was able to induce properties of autonomic neurons, but nor noradrenergic differentiation (Lo et al., 1998). Also in vivo, Mash1 overexpression in peripheral nerve precursors results in the preferential generation of non-adrenergic neurons, suggesting a major role of Mash1 in the control of generic neuronal traits (Stanke et al., 2004). The previous analysis of Gata3-deficient mice suggested that Gata3 may selectively control the noradrenergic phenotype in this lineage (Lim et al., 2000). To define the role of Gata2 in chick sympathetic neurons, a dominant-negative variant of Gata2 was expressed in developing sympathetic ganglia. The Gata2 knockout resulted in reduced Th expression and in a smaller size of sympathetic ganglia, identified by Phox2b and Scg10. In agreement with this action of dnGATA2 on ganglion size, we found a virtually complete loss of the superior cervical and stellate ganglia and the strong atrophy of thoracic sympathetic chain ganglia in Gata3–/– mice, not reported in the first description of these mutants (Lim et al., 2000). In the cells of the rudimentary sympathetic ganglia the expression of Th is almost abolished and that of Dbh diminished.

In Gata3–/– embryos, expression of Phox2b, Mash1 and Dbh were initially normal, while Th expression was already substantially reduced and that of Gata2 virtually absent. The lack of Gata2 expression is surprising, as Gata2 has been shown to be upstream of Gata3 in several systems. However, reciprocal crossregulations between Gata2 and Gata3 have also been observed in the spinal cord (Karunaratne et al., 2002) and hindbrain (Craven et al., 2004). The strong reduction in Th expression at E10.5 suggests a role for Gata3 in the establishment of high-level Th expression. Already at E11.5 the sympathetic ganglion size is reduced and, in parallel, the number of apoptotic cells is strongly increased. The continued cell death is thought to result in the rudimentary sympathetic ganglia observed at E13.5.

These findings raise the question of how Gata2/3 functions in sympathetic precursor cells and why the cells die in the absence of Gata2/3. Gata2/3 factors could specifically control sympathetic neuron survival (but not differentiation) or, alternatively, control sympathetic neuron differentiation in a more general way. In the latter case, immature cells would be generated in Gata3–/– ganglia that subsequently die since they are deficient in many properties, including survival signalling. We favour the latter possibility, since in gain-of-function experiments Gata2 acts as a differentiation factor rather than as a survival factor inducing the production of neurons in peripheral nerves, devoid of neurons during normal development.

The ectopic expression of Phox2a, Phox2b and Hand2 in neural crest precursor cells elicits the generation of noradrenergic neurons (Stanke et al., 1999; Howard et al., 2000). This is explained by the strong crossregulations among these factors, resulting in the induction of the complete network by each individual factor. Thus, it was expected that Gata2 may be able to induce the corresponding set of co-regulators required for noradrenergic and generic neuronal differentiation. The present results do not support this possibility and reveal for Gata2 a potential to control generic neuronal differentiation. This indicates that the function of Gata2 is dependent on the interaction with co-regulators, resulting in the induction of noradrenergic genes in the context of Mash1, Phox2a/b and Hand2 in sympathetic precursors, while non-autonomic neurons are generated in peripheral nerve precursors. Whereas overexpression of Phox2a and Hand2 induce upstream members of the transcriptional network involved in sympathetic neuron differentiation, Gata2, perhaps as the most downstream factor, has only a very weak crossregulating activity with respect to Phox2a, Hand2, Phox2b and Cash1.

What is the reason for the generation of a small population of noradrenergic, autonomic neurons and of some Th-positive cells devoid of Scg10 in Gata2-infected nerves? The most likely explanation is that peripheral nerve precursors represent a mixture of cells at different stages of commitment and differentiation. Only in a minor fraction of the cells Gata2 may be able to induce Phox2a and additional upstream transcription factors that would elicit, together with Gata2, noradrenergic neuron development. It should be noted that peripheral nerve precursors are biased towards autonomic neuron differentiation
(White et al., 2001). *Th*-positive, *Seg10*-negative cells might be explained by the very low *Cash1* expression in Gata2 overexpression experiments.

**Gata function and the noradrenergic phenotype**

In the autonomic nervous system, Mash1 and Phox2 transcription factors are essential for the generation of both sympathetic and parasympathetic ganglion neurons, i.e. functionally noradrenergic and cholinergic phenotypes (for the most part). Therefore, additional regulators have to be hypothesized which modify Phox2 and Mash1 action and are selectively expressed in noradrenergic or cholinergic neurons. There is evidence that the bHLH transcription factor Hand2 is such a factor: it is expressed selectively in sympathetic neurons and capable, upon ectopic expression, of inducing adrenergic differentiation in neural crest precursors and to maintain the normally transient *Th* expression of parasympathetic ciliary neurons (Müller and Rohrer, 2002). The present observations identify another such factor in the form of Gata2/3, also absent from ciliary and sphenopalatine parasympathetic ganglia, and which, in combination with Hand2 (and possibly in direct interaction with it (Dai et al., 2002)) may contribute to the continued expression of *Th* and *Dbh* in sympathetic neurons. However, investigation on a larger scale shows that Gata2/3 function is not associated with noradrenergic properties per se. Although Gata2 expression in the parasympathetic cardiac, submandibular and Remak’s ganglion (Groves et al., 1995) correlates with the presence of noradrenergic gene expression, Gata2 and Gata3 are not expressed in the chick and mouse sphenopalatine ganglion, also containing considerable numbers of neurons expressing *Th* and/or *Dbh* (Fig. 10). It should be mentioned in this context that variable aspects of noradrenergic traits are expressed in cholinergic parasympathetic neurons, often transiently, and never resulting in a functionally noradrenergic phenotype (Grazzana and Coyle, 1978; Landis et al., 1987; Leblanc and Landis, 1989; Baluk and Gabella, 1990; Hardebo et al., 1992). Finally, Gata2/3 are absent in both chick and mouse from the major noradrenergic centre of the brain, the locus coeruleus, and the development of the locus coeruleus does not depend on Gata3. The selective function of Gata2/3 in the development of noradrenergic sympathetic but not LC neurons illustrates differences in the molecular control of the noradrenergic phenotype in different lineages, after the initial, common dependence on Mash1 and Phox2a/b.

In conclusion, Gata2/3 have been identified as members of the group of Hand2-induced transcription factors that are essential for the generation and differentiation of sympathetic neurons. Among the sympathetic phenotypic traits that were tested to date, Gata2/Gata3 displays a preferential role in the expression of *Th*, a function that depends, however, on the presence of additional co-regulators present in the sympathetic neuronal lineage. It will be interesting to investigate whether Phox2a/b and Hand2 and/or unknown co-regulators are physically interacting with Gata2/3 and to identify the target genes controlled by Gata2/3 in the sympathetic lineage.

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