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The transcription factor GATA3 is a downstream effector of *Hoxb1* specification in rhombomere 4

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SUMMARY

In this paper, we show that the transcription factor GATA3 is dynamically expressed during hindbrain development. Function of GATA3 in ventral rhombomere (r) 4 is dependent on functional GATA2, which in turn is under the control of *Hoxb1*. In particular, the absence of Hoxb1 results in the loss of *GATA2* expression in r4 and the absence of GATA2 results in the loss of *GATA3* expression. The lack of *GATA3* expression in r4 inhibits the projection of contralateral vestibuloacoustic efferent neurons and the migration of facial branchiomotor neurons similar to *Hoxb1*-deficient mice. Ubiquitous expression of *Hoxb1* in the hindbrain induces ectopic expression of *GATA2* and *GATA3* in ventral r2 and r3. These findings demonstrate that *GATA2* and *GATA3* lie downstream of *Hoxb1* and provide the first example of *Hox* pathway transcription factors within a defined population of vertebrate motor neurons.

Key words: Hindbrain, Mouse, Rhombomere 4, Motor Neurons, *Hoxb1*, *GATA2*, *GATA3*, Regulation

INTRODUCTION

GATA3 is a member of the family of transcription factors that have recently emerged as key regulators in various developmental processes (reviewed in Simon, 1995; Molkentin et al., 1997; Koutsourakis et al., 1999). The GATA family proteins are characterised by conserved C_4-type zinc finger domains that mediate binding to a DNA motif centred around the nucleotide sequence GAT (Yamamoto et al., 1990; Ko and Engel, 1993; Whyatt et al., 1993). The GATA family comprises six vertebrate members that are expressed in distinct and spatiotemporally restricted domains. Gene ablation studies have demonstrated the vital and non-redundant role of GATA factors for normal embryonic development (Pevny et al., 1991; Tsai et al., 1994; Pandolfi et al., 1995; Molkentin et al., 1997; Koutsourakis et al., 1999). *GATA3*-deficient mice, produced by gene targeting, die at 10-11.5 dpc (days post coitum) and exhibit severe deficits in hematopoiesis, abdominal hemorrhaging, retardation of the lower jaw, abnormal morphology of the central nervous system (CNS) (Pandolfi et al., 1995) and a block of T lymhpocyte differentiation (Ting et al., 1996; Hendriks et al., 1999). In *GATA3*$^{-/-}$ chimaeras, the cytoarchitecture of serotonergic neurons of the caudal raphe nuclei is affected and, as a result, they show a serious defect in their locomotor performance on a rotorod (van Doorninck et al., 1999). Altogether these findings suggest that GATA3 is involved in multiple, even unrelated, functions during development. Among all *GATA* genes, *GATA2* and *GATA3* appear to be the only family members expressed in the CNS (Yamamoto et al., 1990; Kornhauser et al., 1994; Lieuw et al., 1995) where their expression patterns overlap extensively (George et al., 1994; Kornhauser et al., 1994; Lakshmanan et al., 1999; Nardelli et al., 1999). However, at 10.5 dpc, *GATA3* expression is most prominent in rhombomere 4 in the hindbrain (Nardelli et al., 1999; van Doorninck et al., 1999).

In vertebrates, the developing hindbrain becomes rostrocaudally segmented into lineage-restricted units, termed rhombomeres (r), which are crucially involved in specifying the architecture of the future brainstem. The primary candidates for conferring rhombomeric identity are *Hox* genes (reviewed in Lumsden and Krumlauf, 1996). *Hox* genes are usually expressed in domains that have diffuse posterior limits, but sharp anterior limits coincident with rhombomeric boundaries. An exception to this common profile is the rostral

Hoxb1 expression in r4 with sharp anterior and posterior boundaries (Murphy et al., 1989; Murphy and Hill, 1991). Differences in *Hox* expression profiles have their functional correlates in hindbrain development in vivo as revealed by gene targeting studies. Loss of function of *Hoxa1* results in hindbrain segmentation defects, where r4 is partially deleted but its identity remains unchanged (Carpenter et al., 1993; Dolle et al., 1993). On the contrary, in the absence of *Hoxb1*, segmentation remains unaffected but r4 identity is lost resulting in abnormal migratory behaviour of r4-specific motor neurons (Goddard et al., 1996; Studer et al., 1996). Similarly, misexpression of *Hoxa1* and *Hoxb1* results in altered hindbrain motor neuron organisation and in homeotic transformation of rhombomeres (Zhang et al., 1994; Bell et al., 1999).

A deeper understanding of the regulatory circuits that guide neuronal specification is limited by the lack of information about interactions between *Hox* genes and their target genes expressed in subsets of neurons. The restricted expression of *GATA3* in the developing CNS led us to suspect that *GATA3* might be a component of the *Hoxb1*-dependent genetic hierarchy specifying neuronal identities of r4. In this study, we show that *GATA2* and *GATA3* are expressed specifically in the developing r4 efferent system in a *Hoxb1*-dependent manner. Temporally, *GATA2* expression precedes that of *GATA3* and is required to initiate *GATA3* expression in r4 efferent neurons. We show that *GATA3* mutant mice have defects in r4-derived motor neurons similar to those found in *Hoxb1* mutants, providing evidence that *Hoxb1* exerts part of its function through *GATA2* and *GATA3*. This is the first example of *Hox* pathway transcription factors within a defined population of vertebrate neurons that is not represented by any other *Hox* gene.

MATERIALS AND METHODS

Mutant mice

The *GATA2* and *Hoxb1* mutants used in these studies were previously generated by targeted disruption of the respective genes (Tsai et al., 1994; Studer et al., 1996). Generation of the *GATA3*^{nlslacZ} and *GATA3*^{taulacz} mice strains was as described (Hendriks et al., 1999).

For staging embryos, noon of the day of the vaginal plug observation was designated as 0.5 days post coitum (dpc). The wild-type embryos used for in situ hybridisation analysis were the F_1 progeny obtained from CVA×NMRI intercross.

Whole-mount β-galactosidase procedure

Embryos were collected in phosphate-buffered saline (PBS) and fixed for 1-2 hours in 4% paraformaldehyde/PBS (4% PFA) on ice. Embryos were then rinsed twice in PBS and washed 3× 10 minutes in β-galactosidase buffer (0.1 M phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 0.02% NP-40, 0.01% deoxycholate). After this, embryos were incubated in β-galactosidase buffer supplemented with 5 mM ferricyanide, 5 mM ferrocyanide and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) overnight at room temperature, with gentle rocking and protected from light. Embryos were subsequently washed 3× 5 minutes in PBS and postfixed in 4% PFA overnight at 4°C. For double-labelling with β-galactosidase and in situ hybridisation or immunohistochemistry, β-galactosidase activity was visualised before these procedures. Embryos were collected and processed through β-galactosidase staining as above, except that incubation time in staining solution was kept minimal (3-4 hours at 37°C).

In situ hybridisation and immunohistochemistry

Whole-mount in situ hybridisation was performed as described (Wilkinson, 1993) with a few modifications, using DIG-labelled RNA probes synthesized from mouse *GATA2*, *GATA3* and *Hoxb1* cloned DNAs. For double in situ hybridisation, *GATA3* probe was synthesized with digoxigenin-UTP and *Hoxb1* probe with fluorescein-UTP. The probes were added together to the hybridisation buffer and revealed sequentially. The anti-DIG conjugate was developed with NBT/BCIP (Boehringer), whereas the anti-FITC conjugate was revealed with Fast Red (Sigma). For photography, hindbrains were dissected out, flattened on the microscope slide and mounted with 70% glycerol in PBS. Whole-mount immunohistochemistry using the anti-neurofilament light chain antibody (2H3, Developmental Studies Hybridoma Bank) was done as described (Mark et al., 1993).

In vivo retinoic acid (RA) treatment

Pregnant wild-type females mated to *GATA3*^{nlslacZ} heterozygous males, were administered RA at 7.5 dpc essentially as described (Conlon and Rossant, 1992; Marshall et al., 1992). All-*trans* retinoic acid (Sigma) was dissolved at 25 mg/ml in DMSO and stored frozen in aliquots under argon. Just before use, a RA aliquot was diluted one in twenty in vegetable oil and 0.2-0.3 ml delivered by gavage for a final dose of 10 mg/kg of maternal body weight. The control mice received the same mixture without RA. Embryos were recovered at 10.5 dpc and processed for whole-mount β-galactosidase staining or in situ hybridisation as above.

Generation of transgenic embryos

The *Hoxb1/β-actin* construct used in this study is the same as already described in Pöpperl et al. (1995). The DNA construct was linearised with *Bam*HI and microinjected into fertilized mouse eggs generated from crosses of F_1 hybrids (CBA/CA × C57BL/10). Transgenic embryos were identified by PCR using a sense oligo specific to human *β-actin* (5′-TATTCTCGCAGGATCAGTCG-3′) and an antisense oligo specific to mouse *Hoxb1* (5′-GAGAGTGCTGGGTTCTGACG-3′).

Retrograde labelling

10.5-10.75 dpc embryos were dissected in PBS and fixed in 4% PFA. Embryos were injected with the carbocyanine dye DiI (Molecular Probes) at a final concentration of 6 mg/ml in dimethylformamide. Extensive tracer injections were made in the second branchial arch and at the r4 exit point under a dissecting microscope using a micromanipulator and a pressure microinjector. Injected embryos were kept in the dark at room temperature for one week and subsequently hindbrains were dissected free of adjacent tissue and mounted flat with the pial side uppermost. Labelling was viewed under a confocal microscope (BioRad) using a rhodamine filter. A projection of a z-series of optical sections was obtained and contrasting false colours were given using Photoshop (Adobe).

RESULTS

Spatiotemporal expression pattern of *GATA3* and *GATA2* in the developing hindbrain

In situ hybridisation showed that the first *GATA3*-expressing cells in the developing CNS appeared at 9.0 dpc, about 20-somite stage, when they were confined to the ventral neural tube of the r4 territory (Fig. 1H). The ventral location of the signal suggested that these cells might correspond to the progenitors of the prospective r4 efferent neurons. *GATA3* expression in the r4 domain increased during the next days of development (Fig. 1I,J). Additionally, another domain of *GATA3*-expressing cells emerged, which was located dorsal to

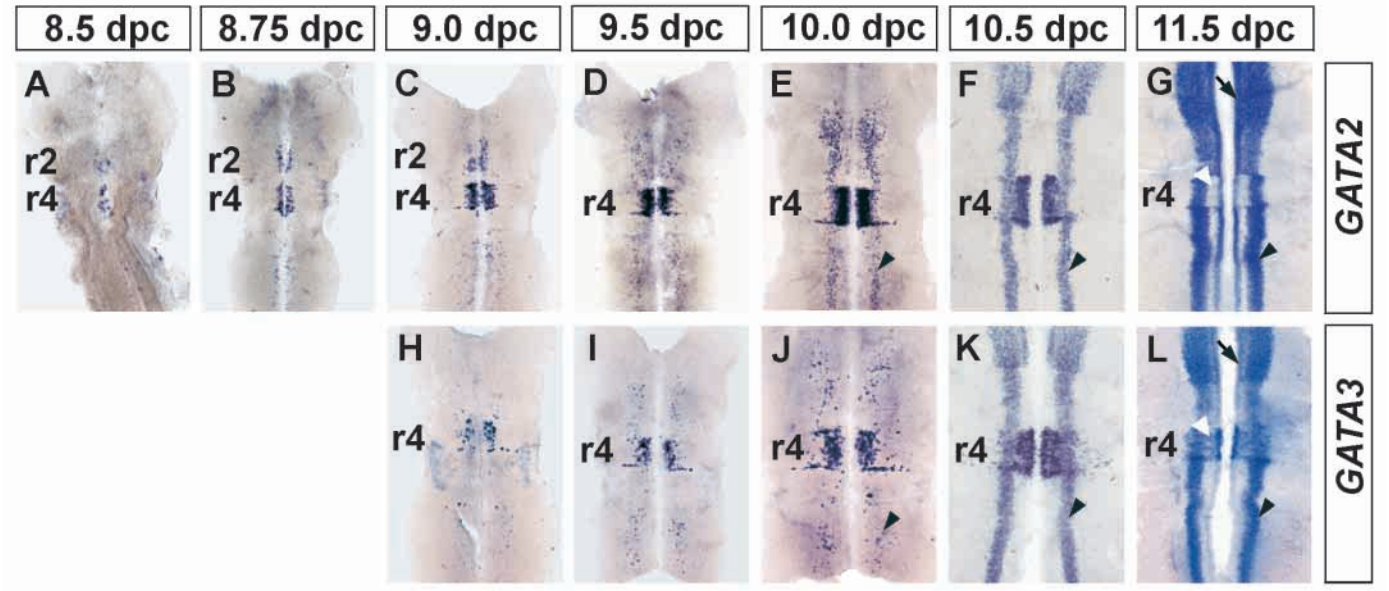

Fig. 1. Whole-mount in situ hybridisation for *GATA2* (A-G) and *GATA3* (H-L). Flat mounts of mouse hindbrain preparations from different embryonic stages (as indicated by dpc) are shown. Rhombomeric positions are indicated (r2, r4). Both *GATA2* (A-C) and *GATA3* (H) are first expressed in the ventral part of r4, with *GATA2* expression preceding that of *GATA3*. Note that *GATA2* is expressed also in r2 during 8.5-9.0 dpc. (D-F, I-K) Changes in *GATA2* (D-F) and *GATA3* (I-K) expression during 9.5, 10.0 and 10.5 dpc. Both GATA factors are expressed in the lateral columns that span the entire hindbrain (E,F,J,K, black arrowheads), where *GATA2* expression (E,F) also precedes *GATA3* (J,K). (G,L) At 11.5 dpc, *GATA2* (G) and *GATA3* (L) expression is found in the contiguous ventral stripes running along the floorplate throughout the hindbrain (black arrows). Both GATA factors are expressed at high levels in the lateral columns (black arrowheads). Note that *GATA3* expression persists in the ventral r4 domain (L, white arrowheads), whereas *GATA2* expression is largely lost (G, white arrowheads).

the r4 *GATA3* domain (Fig. 1J,K, black arrowhead). This population was not restricted to r4, but extended rostrally from the midbrain/hindbrain junction caudally to the end of the neural tube. The position and generation time of this domain was coincident with the differentiation of ventral interneurons (Ericson et al., 1997; Matise and Joyner, 1997). At 11.5 dpc, *GATA3* expression had additionally appeared in two ventral stripes that ran on either side of the floor plate through the hindbrain into the cervical spinal cord (Fig. 1L, black arrow). In r4, *GATA3*-expressing cells concentrated into two narrow stripes facing the floor plate on either side whereas the rest of *GATA3*-expressing cells were scattered along the lateral aspect

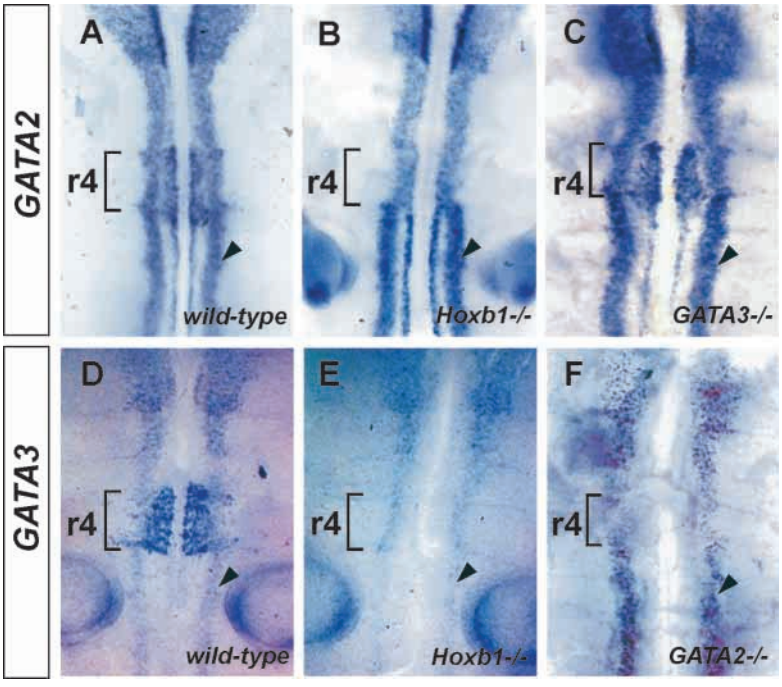

Fig. 2. Relationship of *Hoxb1*, *GATA2* and *GATA3* expression in the hindbrain. Flat-mount hindbrain preparations (rostral is up) of whole-mount in situ hybridisations to mouse embryos are shown. Rhombomere 4 position is indicated (r4, bracket) to show the presence or absence of the *GATA*-positive ventral r4 domain. Arrowheads point to the *GATA*-positive interneuron region. (A-C) Expression of *GATA2* at 10.75 dpc in wild-type (A), *Hoxb1*$^{-/-}$ (B) and *GATA3*$^{-/-}$ (C) mouse embryos. (D-F) Expression of *GATA3* at 10.5 dpc in wild-type (D), *Hoxb1*$^{-/-}$ (E) and *GATA2*$^{-/-}$ (F) mouse embryos. (B,E) Expression of both GATA factors is lost in ventral r4 of *Hoxb1*$^{-/-}$ mutants, while it persists in the interneuron region (arrowheads). (C) Expression of *GATA2* is maintained in *GATA3*$^{-/-}$ hindbrain. (F) *GATA3* expression is completely lost in ventral r4 and severely reduced in the interneuron region (arrowheads) in *GATA2*$^{-/-}$ hindbrain.

of the ventral neural tube (Fig. 1L, white arrowhead). Soma of these cells were orientated mediolaterally, indicative of ventral-to-lateral migration.

Since *GATA2* and *GATA3* have partially overlapping expression patterns in the CNS (Kornhauser et al., 1994; Lieuw et al., 1995), we also examined *GATA2* expression in the hindbrain by whole-mount in situ hybridisation. Fig. 1 shows that *GATA2* expression was very similar to that of *GATA3*, with a few important differences. Firstly, *GATA2* expression temporally preceded that of *GATA3* at virtually all sites in the hindbrain. *GATA2* transcript could already be detected in the ventral r4 domain at 8.5 dpc, just after the embryo had completed turning (12-13 somite stage) (Fig. 1A). Secondly, at early stages, *GATA2* was expressed in a similar domain in r2 corresponding to the area that forms the trigeminal motor nucleus (Fig. 1A-C). However, during 9.5-10.5 dpc, *GATA2* expression was reduced to barely detectable levels in the r2 domain, whereas in r4 its level increased dramatically (Fig. 1D-F). By 11.5 dpc, *GATA2* expression was downregulated in ventral r4, which continued, however, to express *GATA3* (compare Fig. 1G,L). *GATA2* expression also preceded that of *GATA3* in the longitudinal lateral columns and in the ventral stripes adjacent to the floor plate. In contrast to the r4 domain, these sites fully retained *GATA2* expression at 11.5 dpc (Fig. 1G) and at later stages of development (data not shown). Thus, it appears that *GATA2* and *GATA3* are expressed in a precisely controlled temporal manner. The expression domains can be classified as those being rhombomere-specific and those spanning the entire hindbrain. These domains differ in the maintenance of *GATA2* expression at early stages of neuronal development: *GATA2* expression is specifically lost in r4, whereas retained in the other domains. These results are in good accordance with those recently published by Nardelli et al. (1999), except that we observed earlier onset of expression for *GATA2* as well as *GATA3*.

GATA3 expression in r4 is associated with CVA neurons

Transverse sections through the r4 neural tube at 10.5 dpc showed that *GATA3* was expressed in the lateral aspect of the ventral neural tube (Fig. 4A). To define more precisely the neuronal populations expressing *GATA3*, double-labelling with antibodies directed against GATA3 and LIM-homeodomain transcription factors was performed. These studies revealed that the ventral r4 GATA3$^+$ cells co-expressed the motor neuron marker Isl1 whereas the GATA3$^+$ longitudinal columns were negative for Isl1 and formed a column of Lim1/2$^+$ ventral interneurons (J. B. and I. P., unpublished; Nardelli et al., 1999). In the chick hindbrain, double-labelling of fluorescent dextran and LIM-HD markers showed that in r4 the lateral population of Isl1/2$^+$ cells labelled the facial and vestibular motor neurons (Varela-Echavarria et al., 1996). Thus, the GATA3$^+$/Isl1/2$^+$ population in r4 might represent a distinct subpopulation of motor neuron precursors. Lineage tracing studies have revealed that rhombomere 4 gives rise to two major motor neuron populations: the facial branchiomotor (FBM) neurons that innervate the muscles derived from the second branchial arch and the inner ear efferent neurons (also known as contralateral vestibuloacoustic, or CVA neurons) that lead the efferent supply to the sensory fields in the inner ear. Both populations have a common origin and derive from a single motor neuron pool (Simon and Lumsden, 1993; Auclair et al., 1996; Bruce et al., 1997). During differentiation, however, the two groups display distinct migratory behaviours. The FBM neurons, which represent the largest group, migrate caudally parallel to the floor plate to form the facial motor nucleus in rostral r6, hence their perikarya are orientated rostrocaudally (Auclair et al., 1996; Studer et al., 1996; Bruce et al., 1997). Inner ear efferent neurons orientate their processes predominantly mediolaterally, migrate laterally away from the floorplate and project ipsilaterally or contralaterally across the floorplate (Fritzsch et al., 1993; Simon and Lumsden, 1993; Bruce et al., 1997).

Expression of *GATA3* in r4 begins at 9.0 dpc before neuronal differentiation occurs suggesting that *GATA3* might be expressed in the progenitor population of the r4 motor neurons (Fig. 1H). However, at 10.5 dpc, a subset of the ventrally located r4 *GATA3*$^+$ population might correspond to inner ear efferents. To test this hypothesis, we took advantage of the *GATA3*^{lacZ} mice that were generated by gene targeting (Hendriks et al., 1999). In the *GATA3*^{taulacZ} and the *GATA3*^{nlslacZ} mouse strains, β-galactosidase (*E.coli lacZ* gene product) is expressed under the control of the *GATA3* elements providing a lineage tracer for cells expressing *GATA3* and, at the same time, creating a *GATA3* null allele. The two strains differ in the cellular localisation of the reporter protein: in the *GATA3*^{nlslacZ} mice, β-galactosidase accumulates in the nucleus of cells whereas, in the *GATA3*^{taulacZ} mice, it is transported to axons and allows the visualization of axonal projections. The β-galactosidase expression pattern in the heterozygous embryos fully recapitulated that of endogenous *GATA3* gene as confirmed by in situ hybridisation and immunocytochemistry (data not shown). At 10.5 dpc, β-galactosidase-positive neurons in r4 of *GATA3*^{taulacZ} heterozygous mice formed both ipsilateral and contralateral projections, thus verifying the identity of these cells as inner ear efferents (Fig. 5A). Similar to the GATA3 antibody staining results, β-galactosidase expression was not observed in the FBM population that had already started its caudal migration into r5, suggesting that *GATA3* is not directly involved in the migration of facial branchiomotor neurons. In summary, *GATA3* is first expressed in neural progenitors located in ventral r4 and is subsequently maintained in the otic efferent subpopulations when they are fully differentiated (A. K. et al., unpublished data), suggesting that *GATA3* might play a role in both, specification and differentiation of r4 efferent neurons.

GATA3 expression is induced via *GATA2* as a result of *Hoxb1* expression in ventral r4

The restricted expression of *GATA2* and *GATA3* in ventral r4 during hindbrain segmentation when *Hox* genes are expressed in rhombomere-specific patterns led us to hypothesize that *GATA* genes might act downstream of *Hox* genes in specifying defined population of neurons. *Hoxb1* is initially expressed rostrocaudally along the neural tube until the presumptive r3/4 boundary and becomes restricted to r4 via an autoregulatory loop just before *GATA2* is upregulated in r4 (Pöpperl et al., 1995; Studer et al., 1998). The temporal delay of *GATA3* expression compared to *GATA2* also suggests that *GATA2* might regulate *GATA3* in ventral r4.

We first asked whether *GATA2* and *GATA3* expression in r4 was dependent on *Hoxb1* by analysing *GATA* expression in

Hoxb1-deficient embryos generated by conventional gene targeting (Studer et al., 1996). In situ hybridisation showed that expression of both GATA factors were exclusively lost in ventral r4 of *Hoxb1*$^{-/-}$ embryos (Fig. 2B,E) when compared to wild-type controls (Fig. 2A,D). However, their expression was maintained in the interneuron region (indicated by a black arrowhead) and in the ventral columns adjacent to the floorplate, i.e. in the expression domains that are not restricted to r4. This result demonstrates that the ventral r4 expression of *GATA2* and *GATA3* is under the direct or indirect control of Hoxb1.

One prediction of such a genetic hierarchy would be that ectopic expression of *Hoxb1* should induce *GATA* expression in the hindbrain anterior to r4. To test this hypothesis, we first used an in vivo treatment with retinoic acid (RA) known to ectopically induce *Hoxb1* expression in r2/r3 (Conlon and Rossant, 1992; Marshall et al., 1992) and next we globally overexpressed *Hoxb1* in transgenic mice. We gavaged RA to wild-type females mated to *GATA3*^{nlslacZ} heterozygous males at day 7.5 of pregnancy and analysed the *GATA3*-driven β-galactosidase expression in embryos at 10.5 dpc. Fig. 3 shows that RA treatment indeed induced *GATA3* expression in the ventral r2/r3 territory (Fig. 3B), a domain that normally never expresses *GATA3* at this stage of development (Fig. 3A). The fact that *GATA3* expression pattern was controlled precisely in the same manner as *Hox* genes upon the RA treatment, suggests that these genes lie on the same genetic pathway. RA treatment, however, alters the expression pattern of a number of regulatory genes and therefore *GATA3* might also respond directly to RA via as yet unknown regulatory elements. We therefore asked whether ectopic expression of *Hoxb1* alone would be sufficient to drive *GATA3* expression in the same rhombomeres. It has been shown that global overexpression of *Hoxb1* in transgenic mice can induce its own ectopic expression in r2/r3 via an autoregulatory loop (Pöpperl et al., 1995). We therefore ubiquitously expressed *Hoxb1* under the control of the human β-actin promoter in transgenic embryos, and analysed *GATA2* and *GATA3* expression at 9.5 and 10.5 dpc, respectively. Fig. 4B shows a wild-type hindbrain with *Hoxb1* expression in red restricted to r4 and *GATA3* expression in blue in ventral r4 and in a dorsal longitudinal column as previously described. After ubiquitous expression of *Hoxb1* in the hindbrain, ectopic patches of *GATA3* expression were detected in ventral r2 and r3 (Fig. 4C, arrows). Similarly, overexpression of *Hoxb1* induced higher levels of *GATA2* expression in r2, which expanded laterally along the r2/r3 boundary, a feature seen exclusively in the endogenous r4/r5 boundary expression of *GATA2* (Fig. 4E, arrows). The patchy ectopic pattern of *GATA* expression observed in these experiments might result either from insufficient levels of ectopic Hoxb1 to induce high levels of *GATA2* and *GATA3* expression in r2/r3 or from the absence of other factors required to work in synergy with Hoxb1. Thus, in agreement with the *Hoxb1* loss-of-function data, *Hoxb1* overexpression studies confirm that GATA factors are downstream of *Hoxb1* in ventral r4, and define a regulatory cascade leading from *Hoxb1* to *GATA* genes.

We next assessed the epistatic relationship of *GATA2* and *GATA3* genes in r4 and analysed *GATA2* expression in *GATA3*$^{-/-}$ embryos and *GATA3* expression in *GATA2*$^{-/-}$ embryos. Fig. 2C and F show that *GATA2* expression was maintained in the absence of GATA3, while *GATA3* expression was lost in the absence of GATA2 in the r4 ventral domain. These results are in agreement with the pattern of the onset and timing of expression of the genes and strongly suggest that *GATA2* regulates *GATA3* in ventral r4 confirming recently published data (Nardelli et al., 1999). Contrasting the results in the report mentioned above, *GATA3* expression was still observed in the interneuron region of *GATA2*$^{-/-}$ mutant embryos, albeit at a reduced level (Fig. 2F, arrow). Thus, although *GATA2* expression also precedes *GATA3* in the interneuron region, *GATA3* functions, at least in part, independently of *GATA2* in these cells. Taken together, our data demonstrate a regulatory cascade in ventral r4 that leads from *Hoxb1* to *GATA2* to *GATA3*.

The migration of r4 efferent neurons is affected in *GATA3*$^{-/-}$ embryos

The first major defect in *Hoxb1*$^{-/-}$ mutant embryos is a lack in the migration of FBM neurons into r5/r6 and of CVA neurons into the contralateral side (Goddard et al., 1996; Studer et al., 1996). Instead, motor neurons located in r4 express the motor neuron marker *Isl1* and migrate into ectopic positions (Studer et al., 1996). If *GATA2* and *GATA3* genes are downstream effectors of *Hoxb1* in r4 efferent neurons, then *GATA2* and *GATA3* mutant embryos should reproduce at least part of the *Hoxb1* mutant phenotype. A recent report on *GATA2*-deficient embryos (Nardelli et al., 1999) shows variable neurogenesis defects in the hindbrain including a striking reduction, but not a complete absence, of FBM neurons migrating into r5. No description was made about the behaviour of CVA neurons.

To assess whether *GATA3* might be involved in the specification and/or migration of FBM and CVA neurons similarly to *Hoxb1*, we examined the organisation of r4 motor nuclei and their trajectories in *GATA3* heterozygous and homozygous mutant embryos at 10.5 dpc. We first looked at *GATA3*^{nlslacZ} and *GATA3*^{taulacZ} heterozygous and homozygous hindbrain preparations to follow the CVA trajectory. We subsequently retrograde labelled wild-type and *GATA3*$^{-/-}$ mutant embryos with DiI to trace CVA and FBM trajectories.

There was no gross reduction in cell numbers and size of β-gal-positive cells in *GATA3*^{nlslacZ} and *GATA3*^{taulacZ} homozygous embryos, indicating that *GATA3* might not be required for the early differentiation and survival of CVA neurons. However, at this early stage, there was a very severe reduction in the number of contralateral projections in *GATA3*$^{-/-}$ mutants (Fig. 5B,D, arrowhead) when compared to their heterozygous littermates (Fig. 5A,C), using embryos from either the *GATA3*^{taulacZ} strain with β-galactosidase detection, or from the *GATA3*^{nlslacZ} strain with anti-neurofilament antibody detection. Thus, as in *Hoxb1*$^{-/-}$ embryos, the absence of *GATA3* also leads to a reduction of contralateral projections in r4 (Studer et al., 1996), supporting a functional relationship between *Hoxb1* and *GATA3*.

To determine whether the FBM caudal pathway was also affected in *GATA3*$^{-/-}$ mutant embryos, we retrogradely labelled the facial nerve by extensively injecting DiI in the second branchial arch along the nerve and at the r4 exit point in wild-type and *GATA3*$^{-/-}$ mutant embryos.

In 10.5 dpc wild-type embryos (Fig. 6A), branchiomotor cell bodies generated in ventral r4 initiated their caudal migration into r5 (black arrowheads), whereas CVA neurons projected

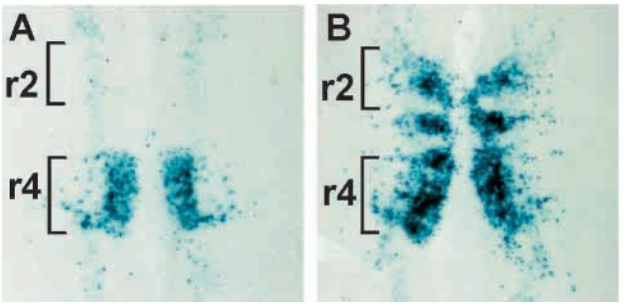

Fig. 3. Changes of *GATA3* expression in the hindbrain following RA treatment. Flat-mount hindbrain preparations are shown (r2, r4 and brackets indicate rhombomere positions). β-galactosidase staining to *GATA3*^{nlslacZ} heterozygous embryos at 10.5 dpc treated in vivo with RA (B) or vehicle (A). Note that RA induces ectopic *lacZ* expression in ventral r2/r3 (B).

across the floor plate to the contralateral side (white arrows). In *GATA3*$^{-/-}$ homozygous mutant embryos, no facial motor neurons were seen caudal to r4 along the floor plate (Fig. 6B). In contrast, individual cell bodies were seen migrating laterally towards their exit points (white arrowheads) instead of caudally and/or contralaterally, a behaviour reminiscent of the affected facial neurons in *Hoxb1*$^{-/-}$ mutant embryos (Studer et al., 1996). As observed in *GATA3*$^{-/-}$ embryos by β-galactosidase staining, fewer CVA neurons compared to wild-type and heterozygous embryos could be traced in homozygous mutant embryos. We also observed a reduced number of facial visceromotor neurons, normally generated in r5, albeit at variable levels (Fig. 6B, asterisk), a defect already observed in *GATA2* mutant embryos (Nardelli et al., 1999).

Taken together, these results show a defect in the migration of two r4 efferent neuron populations, the facial branchiomotor neurons migrating into r5 and the vestibuloacoustic efferent neurons in the contralateral r4. However, whereas the FBM migratory behaviour was completely abolished in *GATA3*$^{-/-}$ mutants, the CVA projections were strongly reduced, but not eliminated. This is different from *Hoxb1*$^{-/-}$ mutants where both populations were totally affected and suggests that either other factors are involved in this process or that GATA2 partially compensates for GATA3 function.

DISCUSSION

In this report, we have undertaken a functional analysis of the spatiotemporal expression pattern of the transcription factor GATA3 in the developing hindbrain. Our results confirm a recent report (Nardelli et al., 1999) that *GATA3* is activated in ventral r4 after the expression of *Hoxb1* and *GATA2*. However, our data show that in r4, *GATA3* is the last gene in a cascade that starts with *Hoxb1* and includes *GATA2*. We demonstrate that expression of both GATA factors is induced ectopically in *Hoxb1* gain-of-function embryos and abolished in *Hoxb1* loss-of-function embryos. In addition, *GATA3* expression in r4 is absent in *GATA2* mutant embryos (our data and Nardelli et al., 1999).

Relationship between *GATA2* and *GATA3* in the developing hindbrain

The functions of GATA factors have been most intensively studied in hematopoiesis. GATA2 appears to be required for proliferation of hematopoietic progenitor cells and in primitive erythropoiesis, whereas GATA1 has a role in definitive erythropoiesis (Pevny et al., 1991; Tsai et al., 1994). GATA3 is essential for T cell development and Th2 cell differentiation (Ting et al., 1996; Zheng and Flavell, 1997; Hendriks et al., 1999). Since *GATA2* is not expressed in mature hematopoietic cells (Nagai et al., 1994) and overexpression of *GATA2* in an avian erythroid cell line inhibits terminal differentiation (Briegel et al., 1993), downregulation of *GATA2* appears to be essential for proper hematopoietic differentiation. In contrast, studies on *GATA* gene expression in chick midbrain revealed that *GATA2* and *GATA3* were expressed in identical spatiotemporal pattern in both developing and adult optic tectum, and that variations were detected only in the relative amounts of *GATA2* and *GATA3* mRNAs between different cellular layers (Kornhauser et al., 1994). These data suggested that the mode of action of GATA2 in the brain might be different from that in the hematopoietic system. However, our results in

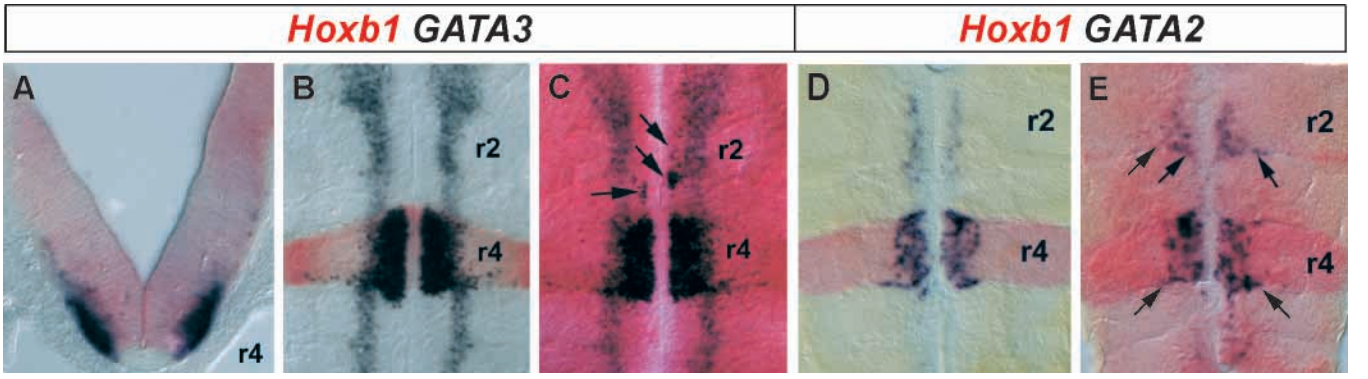

Fig. 4. Overexpression of *Hoxb1* in transgenic mice. Transverse section at r4 level (A) and flat-mount hindbrain preparations viewed from the pial surface (B-E) of 10.5 dpc double-labelled *Hoxb1/GATA3* wild-type (A,B) and transgenic embryos (C) and 9.5 dpc double-labelled *Hoxb1/GATA2* wild-type (D) and transgenic embryos (E) are shown. *Hoxb1* is stained in red, while *GATA3* and *GATA2* are visualised in dark blue. (C) Ectopic patches of *GATA3*-positive cells are visible in ventral r2 and r3 as indicated by arrows and (E) higher expression of *GATA2* than normal is seen in r2. Note in E ectopic *GATA2* expression expanding laterally along the boundary, a feature reminiscent to the endogenous r4 expression (arrows).

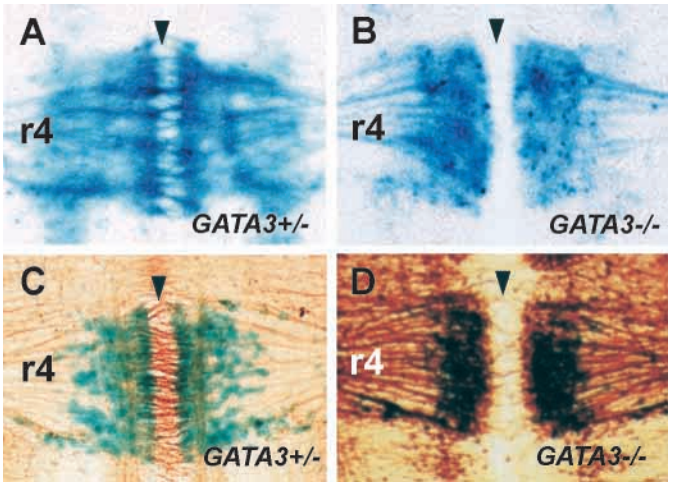

Fig. 5. Analysis of contralateral projections of vestibuloacoustic neurons in *GATA3*$^{-/-}$ mutant embryos. r4 position is indicated, arrowheads point to the floorplate. (A,B) β-galactosidase staining in the hindbrains of *GATA3*^{taulacZ} embryos at 10.5 dpc. β-galactosidase-positive CVA neurons show numerous projections across the floorplate in heterozygotes (A, arrowhead), whereas only a few projections are observed in homozygous mutants (B). (C,D) β-galactosidase staining (green) and anti-neurofilament immunochemistry (brown) in hindbrains of *GATA3*^{nlslacZ} embryos. (C) *GATA3*^{nlslacZ} heterozygotes, showing normal nuclei of β-galactosidase-positive cells and axonal projections in r4. (D) The β-galactosidase positive nuclei appear in similar quantities, but the number of r4 contralaterals is decreased in *GATA3*^{nlslacZ} homozygotes.

the hindbrain and at earlier phases in development, demonstrate that *GATA* expression in the developing brainstem follows a similar sequence of events as in hematopoietic lineages: *GATA2* expression temporally precedes that of *GATA3* and, at least in the r4 efferent neurons, *GATA2* is subsequently downregulated. GATA2 function in r4 cannot be solely ascribed to the control of cellular proliferation, since GATA2 is also found in newly formed postmitotic neurons defined by Isl1/2 expression (Nardelli et al., 1999). Thus, *GATA2* may be also involved in initial neuron maturation in r4. It remains currently unknown what mechanisms cause the downregulation of *GATA2* in r4, and how this phenomenon is related to neuron subtype specification. It would be tempting to speculate that *GATA2* is downregulated by GATA3 and necessary to progress with the differentiation of r4 efferent neurons, similar to the paradigm for GATA2 function in erythropoiesis. The murine *GATA3* promoter contains several consensus GATA-factor binding sites (Lieuw et al., 1997), suggesting that GATA2 might act directly on *GATA3* expression in r4. In turn, GATA-sites are also present in the *GATA2* promoter indicating that the transcriptional activation of *GATA2* and *GATA3* genes may involve crisscross-regulatory mechanisms.

Persistance of *GATA2* expression in the CNS domains other than ventral r4 during later embryonic stages (Fig. 1G, and data not shown) suggests that *GATA2* might have distinct roles in r4 and in interneuron/midbrain neuronal differentiation. Requirement of GATA2 for *GATA3* expression also differs between these two classes of domains. In the absence of GATA2, no *GATA3* expression is seen in the r4 ventral domain, whereas *GATA3* is present at significantly reduced but still recognisable level in the interneuron region. At 10.5 dpc, *GATA3*-positive cells in ventral r4 are postmitotic, since they coexpress motor neuron marker Isl1/2. Similarly to *GATA3* expression in the chick optic tectum (Kornhauser et al., 1994) we did not observe a decline in *GATA3* expression during mouse hindbrain development.

GATA2 and *GATA3* as mediator genes of *Hoxb1* function in r4

The observation that *GATA* genes were expressed in a rhombomere-restricted fashion after the onset of *Hox* genes raised the possibility for Hox proteins to be candidate upstream regulators of *GATA* genes. *GATA2* expression in r4 starts at 8.5 dpc just in a few ventral cells and *GATA3* expression follows at 9.0 dpc, whereas *Hoxb1* is upregulated in r4 at 8.25 dpc. Among several *Hox* genes expressed in r4, high levels of *Hoxb1* are exclusive to r4 (Murphy and Hill, 1991). *Hoxb1* inactivation results in altered migratory behaviour of r4 motor neurons, leaving the hindbrain segmentation intact (Goddard et al., 1996; Studer et al., 1996). FBM neurons in *Hoxb1*$^{-/-}$ mutants initially form, but instead of migrating into r6 they remain in r4 and undergo a lateral migration reminiscent of that of the trigeminal motor nucleus. CVA neurons, in *Hoxb1*$^{-/-}$ mutants, do not form contralateral projections. These features indicate that, in *Hoxb1*$^{-/-}$ mutants, r4 might have partially adopted r2 identity (Studer et al., 1996). This study presents for the first time *GATA2* and *GATA3* as mediator genes of these defects. Their expression profile is specific to r4 at the appropriate time and, as shown by loss- and gain-of-function

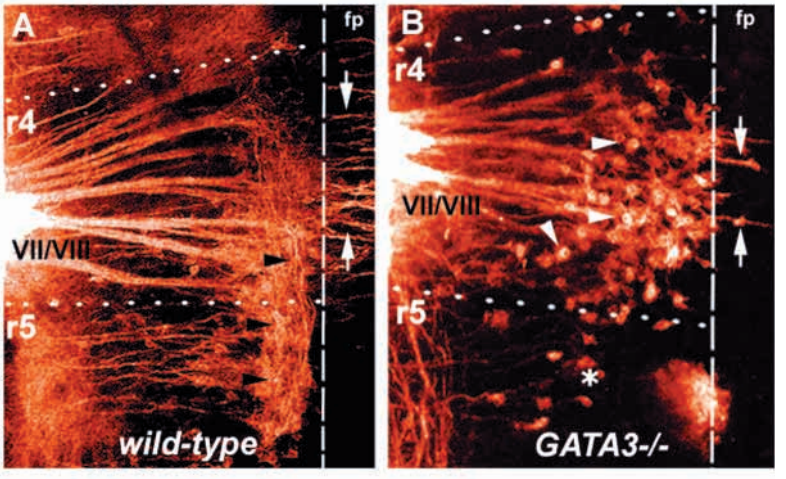

Fig. 6. Retrograde DiI labelling of the facial motor nerve in 10.5 dpc wild-type (A) and *GATA3*$^{-/-}$ homozygous (B) mutant mice. DiI was injected at the VII/VIII exit point and hindbrain preparations are viewed from the pial surface. Rostral is to the top and the floor plate (fp) is to the right. (A) At 10.5 dpc wild-type facial branchiomotor neurons (FBM) are seen in r4 and r5 (black arrowheads) and contralateral projections (CVA) have crossed the r4 floor plate (white arrows). *GATA3*$^{-/-}$ homozygous embryos (B) show a complete absence of FBM migrating caudally. The majority of cell bodies in r4 migrate instead laterally towards their exit point (white arrowheads). Note the dramatic reduction of both CVA crossing the floor plate (white arrows) and r5 facial visceromotor neurons (asterisk) compared to wild type.

studies (*Hoxb1*$^{-/-}$ mutant analysis and RA induction/*Hoxb1* overexpression experiments, respectively), r4-restricted expression of GATA factors is under the control of *Hoxb1*. In its early phase of expression, *GATA2* is also weakly expressed in an equivalent domain in r2. The reason for this may lie in the notion that initially r2 and r4 are patterned in a very similar manner, and r4 adopts its specific identity with some temporal delay after the restriction of *Hoxb1* expression to r4 (Studer et al., 1996). To date, there is no evidence that Hoxb1 directly regulates *GATA2*. Hox/Pbx r4-specific consensus binding sites (Pöpperl et al., 1995) were not found in the human and mouse *GATA2* promoter regions, for which the nucleotide sequence was available (~600 bp for mouse and 2200 bp for human *GATA2* gene; GenBank accession numbers AB007371 and U79137, respectively). Obviously, not all the regulatory sequences of the *GATA2* locus have been defined and, therefore, the issue remains open for future studies. Alternatively, other genes involved in neurogenesis and/or neural specification might be upstream of *GATA* genes and be direct targets of *Hoxb1*. *Hoxb2*$^{-/-}$ embryos have similar facial motor nerve defects to *Hoxb1*$^{-/-}$ mutants (Barrow and Capecchi, 1996; Davenne et al., 1999) and, in addition, *Hoxb2* is under the direct control of *Hoxb1* (Maconochie et al., 1997). Therefore, *Hoxb2* could be placed either between *Hoxb1* and *GATA2* or alternatively in a parallel pathway. In the light of these results, it would be interesting to see whether GATA factors are affected in *Hoxb2* mutants. Interestingly, *GATA2* expression is also upregulated after global overexpression of *Hoxb1* in chick embryos (Bell et al., 1999), suggesting that *GATA* genes are evolutionary conserved targets of *Hoxb1*.

Altered migratory behaviour of r4 motor neurons in *GATA3*$^{-/-}$ mutants, similar to those observed in *Hoxb1*$^{-/-}$ mutants, provides evidence for the role of *GATA* genes in mediating the segmental patterning clues to r4 motor neurons. However, the formation of contralateral projections of CVA neurons is only reduced in *GATA3*$^{-/-}$ mutants, instead of completely eliminated as appears to be the case in *Hoxb1*$^{-/-}$ mutants. One explanation could be that the two GATA factors, which have similar DNA-binding properties, could play temporarily redundant roles. In *GATA3*$^{-/-}$ mutants expression levels of *GATA2* are normal in ventral r4, suggesting that GATA2 could partially compensate for the loss of GATA3 in CVA precursors. This would be similar to what has been observed in hematopoiesis, where GATA1 and GATA2 can counterbalance each other (Pevny et al., 1995). Alternatively, other factors than GATA3 may also contribute to the CVA identity.

In contrast to contralateral neurons, *GATA3* appears not to be directly involved in the migration of facial branchiomotor neurons, as it is clearly not expressed in the mature FBM population during caudal migration. Instead, *GATA3* appears to have a role at earlier stages in FBM development, in a precursor cell population. If r4 progenitor cells committed to migrate posteriorly are not properly differentiated in the absence of GATA3, then they will not be able to respond to cues responsible for migration. It is even possible that maturation of the CVA provides signals to the FBM to acquire proper specification and undergo caudal migration. However, we can not exclude that the altered migration of r4 motor neurons in *GATA3*$^{-/-}$ embryos might be due to a secondary defect, resulting from abnormalities in the CNS development not found in these studies. Clarification of these issues requires further studies, including generation of conditional knockouts, *GATA2/GATA3* double knockouts and identification of *GATA* target genes in r4.

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