Role of *Hoxa*-2 in axon pathfinding and rostral hindbrain patterning

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SUMMARY

Segmentation plays an important role in neuronal diversification and organisation in the developing hindbrain. For instance, cranial nerve branchiomotor nuclei are organised segmentally within the basilar plates of successive pairs of rhombomeres. To reach their targets, motor axons follow highly stereotyped pathways exiting the hindbrain only via specific exit points in the even-numbered rhombomeres. Hox genes are good candidates for controlling this pathfinding, since they are segmentally expressed and involved in rhombomeric patterning. Here we report that in *Hoxa*-2−/− embryos, the segmental identities of rhombomere (r) 2 and r3 are molecularly as well as anatomically altered. Cellular analysis by retrograde dye labelling reveals that r2 and r3 trigeminal motor axons turn caudally and exit the hindbrain from the r4 facial nerve exit point and not from their normal exit point in r2. Furthermore, dorsal r2-r3 patterning is affected, with loss of cochlear nuclei and enlargement of the lateral part of the cerebellum. These results point to a novel role for *Hoxa*-2 in the control of r2-r3 motor axon guidance, and also suggest that its absence may lead to homeotic changes in the alar plates of these rhombomeres.

Key words: rhombomere, axon guidance, segmentation, cerebellum, EGL cell, hindbrain, patterning

INTRODUCTION

The patterning of the vertebrate hindbrain proceeds through a segmentation process that has been highly conserved in vertebrate evolution (Lumsden, 1990; Keynes and Krumlauf, 1994; Wilkinson, 1995). This region of the neural tube is transiently divided, along the anteroposterior axis, into a series of 7-8 lineage-restricted compartments (Fraser et al., 1990; Birgbauer and Fraser, 1994; Wingate and Lumsden, 1996). This metameric organisation is reflected in the organisation of the associated cranial nerves. Retrograde labelling, using fluorescent lipophilic dyes, of mouse and chicken hindbrain motor nuclei has shown the existence of a two rhombomere-repate organisation (Lumsden and Keynes, 1989; Marshall et al., 1992; Carpenter et al., 1993). The trigeminal (Vth) nerve collects axons from motor nuclei lying in rhombomere (r) 2, r3 and a few in r1, exits the brain from r2 and innervates the first branchial arch. Conversely, the facioacoustic (VIIth/VIIIth) and glossopharyngeal (IXth) nerves collect axons from motor nuclei lying in r4/r5 and r6/r7 respectively, and exit the brain from r4 (VIIth/VIIIth) and r6 (IXth) innervating the second and the third branchial arches, respectively. Within the hindbrain, motor axons follow highly stereotyped pathways but very little is known about the genetic control of this pathfinding.

Rhombomere-specific production of neural crest cells is observed along the dorsal part of the hindbrain, resulting in a segmental pathway of migration (Lumsden et al., 1991; Sechrist et al., 1993). The even-numbered rhombomeres generate, together with r1, the vast majority of hindbrain crest cells whereas r3 and r5 are massively depleted from crest cells through apoptosis (Graham et al., 1993, 1994), generating small subpopulations migrating rostrally and caudally in the arches (Sechrist et al., 1993, Köntges and Lumsden, 1996). The hindbrain neural crest cells migrate ventrally in three distinct streams at the axial levels of rhombomeres 2, 4 and 6, and give rise to cranial sensory ganglia and mesenchyme that will populate the three branchial arches and contribute to the formation of muscular, skeletal and vascular structures (Le Lievre and Le Douarin, 1975; Noden, 1983; Bockman and Kirby, 1984; Couly et al., 1993; Köntges and Lumsden, 1996). A late-migrating subset of neural crest cells, derived exclusively from even-numbered rhombomeres, populate the prospective exit points of the cranial nerves and may thus provide chemoattractive signal(s) for growing axons (Niederländer and Lumsden, 1996).

Clues to the genetic control of hindbrain and neural crest segmentation and patterning came from the finding that several classes of genes display segment-restricted expression patterns, including transcription factors such as *Krox-20* and Hox genes at the 3′ ends of the *Hoxa* and *Hoxb* clusters, growth factors, receptor tyrosine kinases (RTK) and their ligands and genes involved in the cellular response to retinoic acid (reviewed in Wilkinson, 1995; Lumsden and Krumlauf, 1996). Functional inactivation by gene targeting of several of the above genes allowed the investigation of their roles in hindbrain segmentation, neuronal specification, and patterning of neural crest-derived structures.
The Hox genes appear to be good candidates to establish rhombomeric segmental identities, as suggested by the results of the Hoxa-1 and Hoxb-1 knockouts (Lu�in et al., 1991; Chisaka et al., 1992; Mark et al., 1993; Carpenter et al., 1993; Dollê et al., 1993; Goddard et al., 1996; Studer et al., 1996). The Hoxa-1 null mutant mice are not viable and exhibit defects in the formation of the inner ear, cranial nerves and certain bones of the skull. Dye injections, morphological criteria and molecular markers showed a complete or near-complete deletion of r5 and a severe reduction of r4 suggesting a role in maintenance and/or generation of segments, more than conferring a specific fate to hindbrain neurons (Lu�in et al., 1991; Chisaka et al., 1992; Mark et al., 1993; Carpenter et al., 1993; Dollê et al., 1993). In contrast, the targeted inactivation of its paralog, Hoxb-1, did not produce any segmentation defect, but resulted in failure to form the motor component of the facial nerve due to inability of r4 motor neuron cell bodies to migrate properly (Goddard et al., 1996; Studer et al., 1996). Underlying this defect is the formation of an r4 that may be partially homeothermically transformed towards an r2 identity (Studer et al., 1996).

Another transcription factor, Krox-20, plays an important role in hindbrain segmentation and patterning (Schneider-Maunoury et al., 1993, 1997; Swiatek and Gridley, 1993). Krox-20 is specifically activated in the presumptive r3 and r5 territories before the appearance of any rhombomeric boundaries (Wilkinson et al., 1989). In the Krox-20 mutants, the r3 and r5 territories are normally formed, at early stages, but fail to be maintained and are completely eliminated. This results in shortening of the hindbrain, with fusion of the Vth with the VIIth/VIIIth and the IXth with the Xth ganglion in the periphery, and perinatal death (Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993). As a consequence of the segmentation defect, the facial and the trigeminal exit points are brought closer, the trigeminal motor axons fasciculate with the facial motor axons and are subsequently misrouted towards the second arch (Schneider-Maunoury et al., 1997). Interestingly, it was shown that Krox-20 is an upstream regulator of the Hoxb-2 (Sham et al., 1993) and the Hoxa-2 (Nonchev et al., 1996) genes in r3 and r5 suggesting that at least some of the abnormalities observed in the Krox-20 mutants may be accounted for by the lack of expression of these genes in those rhombomeres.

Hoxa-2 is the most anteriorly expressed Hox gene, with an expression boundary coinciding with the r1/r2 border (Krumlauf, 1993; Prince and Lumsden, 1994). In addition, Hoxa-2 is the only Hox gene expressed in r2 and, in r3, is coexpressed only with its paralog, Hoxb-2 (Krumlauf, 1993; and refs. therein). The targeted inactivation of Hoxa-2 resulted in lethality at birth and in a homeotic transformation of the second arch neural crest-derived skeletal elements into first arch derivatives (Gendron-Maguire et al., 1993; Rijli et al., 1993), demonstrating that Hoxa-2, acting as a Drosophila selector gene, modifies in the second branchial arch a skeletogenic ground patterning program common to the neural crest of (part of) the first and second arches (Rijli et al., 1993). However, the Hoxa-2 knockout did not result in any apparent segmentation defect at 9.5 dpc, both molecularly and morphologically (Gendron-Maguire et al., 1993; Rijli et al., 1993).

The recent finding that the expression of MDK1, an Eph receptor tyrosine kinase (RTK), was abolished in r3 and altered in r2 and r4 of Hoxa-2 mutant embryos (Taneja et al., 1996) prompted us to further investigate the segmentation pattern, at later stages than previously investigated, using both molecular and morphological criteria. In this report, we demonstrate that Hoxa-2 is required for normal axial pathfinding of r2 and r3 motor neurons. In its absence, a significant proportion of these motor neuron axons turn caudally and exit through the r4 exit point, thus innervating the transformed second branchial arch, instead of the first. Furthermore, we show that the lack of Hoxa-2 results in the loss of the r1/r2 boundary, reduction of alar r2 and r3 territories and a concomitant expansion of the r1 territory, both by molecular and morphological criteria. These hindbrain defects lead to an enlargement of the cerebellum and to a corresponding reduction of pontine structures, suggesting that fate changes may have occurred in the alar plates of the Hoxa-2 mutant fetuses.

MATERIALS AND METHODS

In situ hybridisation, immunostaining and histological analysis

Whole-mount in situ hybridisation was performed as described (Dup£ et al., 1997). The Sek-1 probe was derived by PCR amplification of cDNA from total P19 cell RNA, using the specific primers 5’GGAGC-TACGGATATTTATGGG3’ and 5’CTTCTGTGTATAAACC-GAGCC3’ derived from the available Sek-1 sequence (Gilardi-Hebenstreit et al., 1992). The resulting fragment, spanning nucleotides 2411-3450, was subcloned into Bluescript (Stratagene). The Sax-1 (Schubert et al., 1995), PLZF (Cook et al., 1995), Otx2 (Simeone et al., 1992) and En-2 (Davis and Joyner, 1988) probes were kind gifts from P. Gruss, A. Zelent, S.-L. Ang and A. Joyner, respectively. Whole-mount immunostaining using the anti-neurofilament monoclonal antibody 2H3 (Developmental Studies Hybridoma Bank) was performed as described (Mark et al., 1993). Mouse fetuses were fixed in Bouin’s fluid and brains were dissected out, photographed and subsequently embedded in paraffin for histology. Serial sections 7 µm thick were stained with Mallory’s trichrome.

Retrograde labeling

Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline and injected with the carbocyanine tracers DiI and DiO (Molecular Probes). Flat-mount preparations and analysis under a confocal microscope of retrogradely labeled specimens were as described (Studer et al., 1996).

RESULTS

Molecular and morphological changes in the hindbrain of Hoxa-2 mutant embryos

Initial characterization of the expression patterns of Krox-20, Hoxb-2, Hoxb-1 and Hoxa-3 in the hindbrain of 8.5-9.5 day post coitum (dpc) Hoxa-2 mutant embryos did not reveal any obvious alterations (Gendron-Maguire et al., 1993; Rijli et al., 1993). Furthermore, the segmentation pattern and gross morphology of rhombomeres at 9.5 dpc did not appear to be affected (Gendron-Maguire et al., 1993; Rijli et al., 1993). However, the finding that the expression of MDK1, an Eph receptor tyrosine kinase (RTK), was abolished in r3 and altered in r2 and r4 of Hoxa-2 mutant (Taneja et al., 1996; see also Fig. 5) prompted us to further investigate the segmentation pattern, at both the molecular and morphological levels.

In 9.0 dpc wild-type (WT) embryos, the Eph RTK Sek-1 is expressed at high levels in r3 and r5 and at a lower level dorsally in r2 (Nieto et al., 1992; Figs 1A, 5). In Hoxa-2 mutant embryos, Sek-1 expression is selectively abolished in r2 (arrow
in Fig. 1B), whereas the remainder of its expression domain is not affected. This result shows that Hoxa-2 is required to maintain Sek-1 expression in r2 and suggests a possible change in the r2 segmental identity, since Hoxa-2 is the only Hox gene expressed in r2 (Krumlauf, 1993; Prince and Lumsden, 1994; Fig. 5).

The overall size of the r1-r3 territory and the position of the mid-hindbrain border do not appear to be significantly affected in Hoxa-2−/− embryos (compare Fig. 1E with 1F) as inferred by double whole-mount in situ hybridisation of WT and homozygous 9.0 dpc embryos with Hoxb-1, which marks r4 (Frohman et al., 1990), and Otx2, which has a sharp caudal expression boundary at the mid-hindbrain border (Simeone et al, 1992; arrowheads in Fig. 1E,F). However, double in situ hybridisation of WT and Hoxa-2−/− 9.0 dpc embryos with Hoxb-1 and En-2, which is expressed in the mid-hindbrain domain, including r1, (Davis and Joyner, 1988) and is involved in cerebellar development (Millen et al., 1994), reveals a caudal expansion of the r1 expression domain of En-2 in mutant, as compared to WT embryos (compare the expression domains demarcated by the arrowheads in Fig. 1E,F).

To examine r1 features at later stages, we analysed the expression pattern of the homeobox gene Sax-1 (Schubert et al., 1995) which, in WT 10.5 dpc embryos, is selectively expressed in the ventral portion of r1 with a sharp caudal boundary at the r2 border and a narrow stripe of cells next to the floor plate extending further caudally (Fig. 1G). In Hoxa-2−/− embryos, the sharp r1/r2 boundary does not exist and the broad Sax-1 expression domain extends posteriorly into the r2 and, possibly, r3 territories, as inferred by comparing the gaps between the expression domains of Hoxb-1 and Sax-1 in double staining of WT and Hoxa-2−/− 10.5 dpc embryos (compare Fig. 1G,H).

The described molecular changes in the hindbrain of Hoxa-2−/− embryos prompted us to analyse the segmentation pattern of Hoxa-2−/− embryos at 10.5 dpc, i.e. later than previously analysed (Gendron-Maguire et al., 1993; Rijli et al., 1993). Comparison of flatmount WT and mutant hindbrains (Fig. 2A,B) shows an apparent loss of the r1/r2 border (compare Fig. 2A,B). This finding was further confirmed by examining the expression pattern of the PLZF gene (Cook et al., 1995) which, in WT 10.5 dpc embryos, is strongly expressed at rhombomeric bound-

Fig. 1. Changes in r2 molecular identity of Hoxa-2−/− embryos. (A,B) Dorsal views of WT (A) and mutant (B) 9.0 dpc embryos probed with Sek-1 for r2, r3 and r5. The arrow in B indicates the lack of expression in mutant r2 (r2*). (C,D) Lateral view of WT (C) and Hoxa-2−/− (D) embryos at 9.0 dpc probed with Otx-2 and Hoxb-1, as a marker for r4. The arrowheads denote the mid-hindbrain boundary. (E,F) Lateral view of WT (E) and Hoxa-2−/− (F) embryos at 9.0 dpc probed with En-2 and Hoxb-1. Note the caudal expansion of the metencephalic (r1) expression domain of En-2 in the mutant (F) as compared to WT (E), as denoted by the arrowheads. (G,H) 10.5 dpc WT (G) and Hoxa-2−/− (H) hindbrains probed with Sax-1 and Hoxb-1, as markers for r1 and r4 segmental identities, respectively. Arrowheads in H indicate the caudal spreading of Sax-1 expression domain in the mutant hindbrain. ot, otocyst. Bar, 570 μm (A,B; G,H), 580 μm (C,D) and 560 μm (E,F).
Pathfinding of motor axons is altered in Hoxa-2⁻/⁻ embryos

The above rhombomeric abnormalities and molecular changes observed in the mutants may be expected to have consequences on the patterning of rostral hindbrain structures. Therefore, we investigated the development and axonal projections of the trigeminal (V) and facial (VII) motor nuclei by retrograde DiO and DiI labelling, respectively, of 10.5 dpc WT (Fig. 3A-C) and Hoxa-2⁻/⁻ (Fig. 3D-I; and data not shown) embryos.

In WT embryos, DiI injection at the VIIth nerve exit point in r4 labels neurons only in r4 and r5 (Fig. 3A), whereas DiO injection at the Vth nerve exit point in r2 labels neurons only in r2, r3 and r1 (Fig. 3B). Motor axon trajectories are highly stereotyped: neurons in r2 and r4 project their axons dorsally (laterally) to reach their respective exit points located in the alar plate, whereas motor axons in r3 and r5 first project in a dorsal direction and then turn rostrally to exit the hindbrain via the r2 and r4 exit points, respectively (Fig. 3A,B). Strikingly, DiI injection at the r4 VIIth nerve exit point of the Hoxa-2⁻/⁻ mutant in Fig. 2D reveals that all of the r3 and some of the r2 motor axons drastically change their pathfinding, turning caudally and exiting the hindbrain with r4 and r5 facial motor axons (arrow). DiO retrograde labelling of axons exiting at the r2 Vth nerve exit point confirms the lack of r3 axons exiting from r2, whereas fewer axons are labeled in r2 than in WT (Fig. 3E). Labelling of five additional Hoxa-2⁻/⁻ mutants not only confirmed these results, but revealed that, in some embryos, r3 motor axons, similarly to those of r2, displayed mixed trajectories, some exiting at the r2 and others at the r4 exit points (Fig. 3G,H; and data not shown). In the case of r2 axons, it is noteworthy that a few migrate a long way caudally to reach the r4 exit point, instead of going out at the closest exit point in r2 (arrows in Fig. 3D,G). Accordingly, whole-mount immunostaining by an anti-neurofilament antibody reveals that the thickness of the axon bundle at the r2 exit point is reduced and the presence of an additional nerve branch innervating the second branchial arch of Hoxa-2⁻/⁻ 10.5 dpc embryos (bracket and arrow in Fig. 4B, respectively).

Cerebellar and pons defects in Hoxa-2⁻/⁻ fetuses

The alar plate of the rostral metencephalon (r1 + isthmic region) generates the majority of the cells in the cerebellar cortex, with a mesencephalic contribution restricted only to a mediiodorsal territory (Martinez and Alvarado-Mallart, 1989; Hallonet et al., 1990; Hallonet and Le Douarin, 1993). Fate-mapping studies in chick have also shown that there is a very limited cerebellar contribution from alar r2 and that the border between the cerebellum and the longitudinal column of cochlear nuclei, originating from the alar plates of more posterior rhombomeres, coincides exactly with the r2/r3 boundary (Marin and Puelles, 1995). Thus, with anterior homeotic changes within the r2-r3 domain of the Hoxa-2⁻/⁻ mutants, expansion of the cerebellar territory and reduction of the cochlear nuclei may be expected.

Fig. 4C shows the brain of a 18.5 dpc WT fetus. In all five 18.5 dpc homozygous mutants examined (Fig. 4D, and data not shown), the cerebellum appears consistently extended along its rostrocaudal and mediolateral axis (compare Fig. 4C,D). Horizontal sections through different levels of the cerebellum and comparison between WT and mutant fetuses confirm this phenotype (compare Fig. 4E,F and G,H; and data not shown). Similar results were obtained with transverse and sagittal sections (data not shown). Interestingly, in horizontal sections of the ventral part of the Hoxa-2⁻/⁻ cerebellum, the external
germinal layer (EGL) appears thicker than in WT (compare Fig. 4E,F), with a dramatic accumulation of cells in a specific posteroventral region (open arrowhead) which may correspond to the proliferating ‘germinal trigone’ (Altman and Bayer, 1978) (compare Fig. 4G,H). In addition, horizontal sections through the dorsal pons of the Hoxa-2+/- fetus reveals a striking loss of the anterior portion of the cochlear nuclei (co) column, as compared to WT (compare Fig. 4J). Altogether these results suggest that the alar plates of r2 and r3 may be partially transformed to a more anterior cerebellar phenotype in the hindbrain of Hoxa-2-/- mutants.

**DISCUSSION**

In this report, we show that the functional inactivation of Hoxa-2 leads to molecular and morphological changes in the hindbrain of the mutant embryos (summarised in Fig. 5), demonstrating that Hoxa-2 does not only act as a selector gene for second arch mesenchymal neural crest cell (NCC) morphogenetic identity (Rijli et al., 1993; Gendron-Maguire et al., 1993) but it also plays a fundamental role in rostral hindbrain patterning by establishing r2 and, to a lesser extent, r3 segmental identities and by contributing in the genetic control of r2-r3 motor neuron axon pathfinding.

**r2 and r3 molecular and morphological segmental identities are altered in Hoxa-2 mutants**

Previous loss- and gain-of-function studies (Chisaka and Capecchi, 1991; Lufkin et al., 1991; Chisaka et al., 1992; Carpenter et al., 1993; Mark et al., 1993; Zhang et al., 1994; Studer et al., 1996; Goddard et al., 1996; Barrow and Capecchi, 1996; Dupé et al., 1997) have investigated the functional role of Hox genes in the specification of segment identity. The emerging picture is consistent with a role of these genes in establishing and maintaining the segmental identity of rhombomeres, although the interpretation of the mutant phenotypes may have been hampered by the existence of other Hox genes displaying expression patterns overlapping that of the investigated gene. Hoxa-2 is the only Hox gene expressed in r2 (Krumlauf, 1993; Prince and Lumsden, 1994; Fig. 5), therefore providing a unique situation to investigate its role in rhombomere patterning in the absence of expression of any other Hox gene. In initial studies (Gendron-Maguire et al., 1993; Rijli et al., 1993), the molecular identities of rhombomeres (as assessed by the expression patterns of Krox-20, Hoxb-2, Hoxb-1, and Hoxa-3) appeared unmodified in Hoxa-2 mutants, and hindbrain alterations were not detected at the stage analysed (9.5 dpc), using standard histological techniques. However, the aberrant expression pattern of MDKI (Taneja et al., 1996; Fig. 5) suggested the existence of abnormalities in the hindbrain of the mutants that may be morphologically evident at later stages than previously investigated. In the present study, we find patterning defects restricted to the rostralmost expression domain of Hoxa-2, namely in r2 and r3, in the hindbrain of 10.5 dpc Hoxa-2 mutant embryos (Figs 1 and 2). The molecular changes in the expression patterns of Sek-1, En-2 and Sax-1 in the r1-r2 region of the mutants (Fig. 1B,F,H), may be consistent with a switch in cell fate of r2 towards an r1 identity and the corresponding morphological phenotype observed in the mutants (Figs 2B,D and 4D,F,H,J; see below) may further support this hypothesis. Accordingly, the r1/r2 boundary is missing in Hoxa-2 mutants, even though some r2 features seem to be conserved since an r2/r3 border is still, at least partially, formed and the r2 exit point is present (Fig. 2B), suggesting that specification of the remaining r2 territory may not be under the control of Hox genes (see also below). Interestingly, r3 and its derivatives also appear to be molecularly and morphologically affected by the Hoxa-2 inactivation (Figs 1 to 5; see also

![Fig. 3](image-url). r2 and r3 motor axon trajectories are altered in Hoxa-2-/- embryos. (A-C) Retrograde DiI and DiO labelling of facial (A) and trigeminal (B) motor nerves, respectively, and a composite image of the two patterns (C) in a 10.5 dpc WT embryo. The r4 exit point (VII) collects axons only from r4 and r5 (A), and the r2 exit point (V) only from r2, r3 and a few from r1 (B); the arrow in B shows the pathway of r3 motor axons. (D-I) Retrograde DiI and DiO tracing at the r4 (D,G) and the r2 (E,H) exit points of two 10.5 dpc Hoxa-2-/- embryos, respectively, and composite images of the patterns (F,I). The mutant in D shows DiI labelling of all of the r3 and some of the r2 motor axons and the arrow indicates their altered migration pathways; accordingly, DiO injection in E labels only r2 motor axons. The mutant in G-I has been bilaterally injected. Note that r3 motor axons display mixed trajectories, with some correctly exiting through r2 (H), and others through r4 (G). The arrow in G indicates the trajectories of some r2 motor axons exiting at r4. Bar, 210 μm (A-C), 260 μm (D-F) and 525 μm (G-I).
Taneja et al., 1996), although to a lesser extent than r2, since segment boundaries can still be detected and the r3 territory appears only dorsally reduced (Fig. 2B). Since in r3 Hoxa-2 is only coexpressed with its paralog Hoxb-2 (Krumlauf, 1993; and refs. therein), one possibility is that Hoxb-2 may partially compensate for the loss of Hoxa-2. The analysis of compound Hoxa-2/Hoxb-2 mutants will reveal whether these genes genetically interact for r3 patterning. In this respect, it is noteworthy that the functional inactivation of Krox-20, an upstream regulator of both Hoxa-2 (Nonchev et al., 1996) and Hoxb-2 (Sham et al., 1993), leads to the complete elimination of r3 and r5, while the rest of hindbrain segmentation is maintained (Schneider-Maunoury et al., 1997).

**Hoxa-2 is required for normal pathfinding of r2-r3 motor axons**

The experiments presented here indicate that Hoxa-2 is required to control r2-r3 motor axon trajectories, providing the first evidence for the involvement of a Hox gene in axon guidance. In the mutants, axons from the r2/r3 region exit, incorrectly, from r4 and innervate the transformed second branchial arch. Changes in the neural crest of the second branchial arch, in the neuroepithelium of the hindbrain and in the identity of the motor neurons themselves could be invoked to interpret these results.

The misrouting of the r2/r3 axons into the second branchial arch is consistent with the homeotic transformation of the second arch neural crest-derived skeletal elements into first arch derivatives (Gendron-Maguire et al., 1993; Rijli et al., 1993) since an anteriorly transformed second arch may be able to attract axons bearing matching positional information. However, this is not sufficient to explain the axon routes, since axons exiting from the r2 exit point do not turn caudally to innervate the transformed second

**Fig. 4. Cranial nerve and cerebellar alterations in Hoxa-2-/- mutants.** (A,B) Lateral views of anti-neurofilament staining of 10.5 dpc WT (A) and Hoxa-2-/- (B) embryos. Note in B that the trigeminal axon bundle is markedly reduced at the exit point (compare brackets in A,B) and that an additional nerve branch (arrow) innervates the second branchial arch of the mutant (BA2*). (C,D) Whole-mount preparations of WT (C) and Hoxa-2-/- (D) newborn brains. The mutant cerebellum (cb) in D is extended along its rostrocaudal and mediolateral axis (compare arrows in C and D). (E-J) Horizontal histological sections of the WT (E,G,I) and mutant (F,H,J) brains shown in C and D, respectively, at the level of the cerebellum (E-H) and pons (I-J). Note that, in the lateral part of the ventral-most portion of the mutant cerebellum (F), the external germinal layer (EGL) (arrow) is thicker than in the WT (E). At a slightly more dorsal level, increased EGL cell accumulation (open arrowhead) is observed in mutant (H), as compared to WT (G). Conversely, the cochlear nuclei column (co) (between arrows) is markedly reduced in the mutant pons (J), as compared to WT (I). BA1, BA2, first and second branchial arch, respectively; mb, midbrain; cp, choroid plexus. Bar, 770 μm (A,B), 970 μm (C,D) and 300 μm (E-J).
rhombomeres, as assessed by PLZF staining and morphological observation (see Fig. 2), are presented with dashed lines. For simplicity, the Hoxa-2-/- r1/r2 boundary is shown nearly absent and the dorsal reduction of r3 (see Fig. 2) is not shown. The r2/r3 boundary is also presented with a dashed line, although it appears to be partially altered as well in Hoxa-2-/- mutants (see Fig. 2). Less or more intense colour shading represents lower or higher levels of gene expression, respectively. Note that, in the mutant, dorsal r2 Sek-1 expression (light-green) is lacking (at 9.0 dpc) and En-2 (grey-shaded bar) as well as Sax-1 (yellow) expression domains have spread caudally into at least the r2* territory (at 9.0 and 10.5 dpc, respectively) (see Fig. 1). Note also the changes in MDK1 expression in Hoxa-2-/- (red) 9.0 dpc embryos (Taneja et al., 1996). Rhombomeres in which a molecular and/or anatomical change has been observed in Hoxa-2-/- mutants are marked with an asterisk. BA1, BA2, first and second arch, respectively; BA2*, homeotically transformed Hoxa-2-/- second arch (Rijli et al., 1993; Gendron-Maguire et al., 1993); ov, otic vesicle; gV, gVII, trigeminal and facial ganglia, respectively.

Beside chemoattraction from the exit points, a limited degree of guidance cues might also reside in the neuroepithelium, as suggested by r3 rotation experiments (Guthrie and Lumsden, 1992). Members of the Eph family of RTKs have been implicated in axon repulsion (e.g. Cheng et al., 1995; Drescher et al., 1995; Wang and Anderson, 1997; Varela-Echavarria and Guthrie, 1997 and refs. therein). The detected changes in the expression of two Eph family RTKs in the hindbrain of Hoxa-2-/- embryos suggest that changes in the identity of the r2 and r4 microenvironment and pathfinding signals emanating from these rhombomeres could also account for the misrouting of the r2/r3 axons. The Sek-1 and MDK1 expression profiles of r2 and r4, which are dissimilar in the WT become identical in the Hoxa-2-/- embryos (Fig. 5), providing a possible molecular basis for the inability of r3 and r2 axons to distinguish between r2 and r4. Extension of Sax-1 and En-2 expression into r2 may also result in alterations of pathfinding signals from this territory. The lack of MDK1 expression in the r3 of Hoxa-2-/- embryos (Tanega et al., 1996; Fig. 5) may withdraw a ‘barrier’ allowing some r2 and r3 axons to reach r4. It is noteworthy that r2-r3 motor axons can ‘choose’ to exit either at the r2 or the r4 exit points (Fig. 3). This variability (Fig. 3D-I; and data not shown) may reflect a variable reduction in the r2 territory and its exit point among different mutants. This reduction would alter quantitatively and/or qualitatively the chemoattractive signals from r2, thus allowing some axons to be attracted by the r4 territory.

A further possibility is that some r4 facial branchiomotor neurons, which normally migrate into r5 (Studer et al., 1996),
may also aberrantly migrate into r2/r3 territories and send their axons through the r4 exit point. This could result from the elimination of inhibitory cues, emanating from r2 and/or r3 and would be consistent with the molecular analysis. Indeed, as assessed by retrograde labelling, there appears to be an occasional paucity of VIIth nerve motor neurons in the r4 of the mutants (Fig. 3).

Alternatively, r2 and r3 trigeminal motor neurons may partially change their fate to a VIIth nerve identity. r2-r3 motor neuron axonal trajectories similar to those shown in this study have been reported in mouse embryos treated with high doses of retinoic acid at midgastrulation (Marshall et al., 1992; Kessel, 1993). Those results were consistent with a morphological transformation of the trigeminal motor nerve towards a facial identity, accompanied by anteriorised expression of more posterior markers such as Hoxb-1 and Hoxb-2, which indeed have been recently shown to control aspects of facial motor nucleus identity during normal development (Goddard et al., 1996; Studer et al., 1996; Barrow and Capecchi, 1996). Since Hoxb-1, Hoxb-2, Hoxa-3 and Hox-3 are not ectopically expressed in Hoxa-2 mutant r2-r3 territories (Gendron-Maguire et al., 1993; Rijli et al., 1993; and data not shown), we consider the above hypothesis less likely.

Finally, it may be argued that, in the absence of Hoxa-2, r2-r3 motor neurons adopt a ‘generic’ motor neuron fate with no anteroposterior specification such that they respond equally to the r2 and r4 chemoattractive signals extending axons towards either exit point. In the absence of an available trigeminal neuron axonal trajectories similar to those shown in this study have been reported in mouse embryos treated with high doses of retinoic acid at midgastrulation (Marshall et al., 1992; Kessel, 1993). Those results were consistent with a morphological transformation of the trigeminal motor nerve towards a facial identity, accompanied by anteriorised expression of more posterior markers such as Hoxb-1 and Hoxb-2, which indeed have been recently shown to control aspects of facial motor nucleus identity during normal development (Goddard et al., 1996; Studer et al., 1996; Barrow and Capecchi, 1996). Since Hoxb-1, Hoxb-2, Hoxa-3 and Hox-3 are not ectopically expressed in Hoxa-2 mutant r2-r3 territories (Gendron-Maguire et al., 1993; Rijli et al., 1993; and data not shown), we consider the above hypothesis less likely.

In summary, we favour the idea that it is not a switch in the identity of r2/r3 motor neurons but changes in axon pathfinding signals emanating from the r2-r4 region that result in the aberrant axonal projections. In contrast, there may be a switch in cell fate identity in the alar plates of r2 and r3, as suggested by the molecular and morphological analysis presented (see also below). This apparent dichotomy can be obviated by not requiring Hoxa-2 to specify the fate of all cells within a rhombomere but assuming that Hoxa-2 acts to specify the fate of subsets of cell populations in these segments. In this respect, it is noteworthy that Hoxb-1 appears to be acting in a comparable manner, since its targeted inactivation led to selective loss of the facial somatomotor nucleus due to the misspecification of r4 branchiomotor neurons (Goddard et al., 1996; Studer et al., 1996).

The cerebellar and pons defects observed in Hoxa-2/- mutant fetuses may indicate cell identity changes in the rhombic lip

Fate-mapping studies have demonstrated that the cerebellum is mostly derived from the alar plate of the metencephalic brain vesicle, with a limited mesencephalic contribution (Martinez and Alvarado-Mallart, 1989; Hallonet et al., 1990; Hallonet and Le Douarin, 1993). In particular, while r2 may contribute to deep cerebellar nuclei (Marin and Puelles, 1995), granule neuron precursors of the cerebellar cortex originate from the proliferating edge of the neuroepithelium of rostral metencephalon (r1+ isthmic region), within the ‘rhombic lip’ (Hatten and Heintz, 1995 and refs. therein), and migrate anteriorly and medially covering the entire developing cerebellum to form the external germinal layer (EGL) (Hallonet and Le Douarin, 1993). The limit between cerebellar and pontine structures coincides with the r2/r3 boundary, since r3 does not normally form any part of the cerebellum (Tan and Le Douarin, 1991; Marin and Puelles, 1995). The Hoxa-2 mutant mice lack any Hox expression in both r1 and r2, and the only Hox gene expressed in mutant r3 is Hoxb-2 (Rijli et al., 1993; Gendron-Maguire et al., 1993). Can cerebellar derivatives be now induced in the mutant r2-r3 region? We speculate that the enlargement of the cerebellar surface in the Hoxa-2 mutants may be due to increased EGL cell number (Fig. 4F,H) resulting from abnormal recruitment of dorsal, rhombic lip-derived, r2 and r3 cells. Analysis of granule neuron-specific markers (Alder et al., 1996; Yang et al., 1996) will be required to address this point. This hypothesis is indirectly supported by the striking loss of the anterior portion of the cochlear nuclei (co) column (Fig. 4I) which originates from the alar plates of r3 and more posterior rhombomeres (Marin and Puelles, 1995). In this respect, it is interesting to note that a fate change in r2 and r3, leading to development of ectopic cerebellar structures including EGL cells, could be induced by grafting prospective isthmocerebellar neuroepithelium from a quail or a mouse embryo into the hindbrain of a host chick embryo (Martinez et al., 1995). Interestingly, induction of ectopic cerebellar structures occurred only in the alar plates of the rhombomeres immediately contacting the graft, and only when the graft was not adjacent to the host interrhombomeric boundaries (Martinez et al., 1995). These findings suggested that rhombomere alar plates can be transformed to a cerebellar fate in the absence of segment boundaries, which therefore may act as barriers to morphogenetic signal(s) spreading from the graft. It is tempting to speculate that the lack of the r1/r2 boundary in Hoxa-2 mutant embryos may also withdraw a barrier to putative signal(s) spreading from the isthmic region.

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