

Retinoic acid synthesis and hindbrain patterning in the mouse embryo

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SUMMARY

Targeted disruption of the murine retinaldehyde dehydrogenase 2 (*Raldh2*) gene precludes embryonic retinoic acid (RA) synthesis, leading to midgestational lethality (Niederreither, K., Subbarayan, V., Dollé, P. and Chambon, P. (1999). *Nature Genet.* 21, 444-448). We describe here the effects of this RA deficiency on the development of the hindbrain and associated neural crest. Morphological segmentation is impaired throughout the hindbrain of *Raldh2*^{-/-} embryos, but its caudal portion becomes preferentially reduced in size during development. Specification of the midbrain region and of the rostralmost rhombomeres is apparently normal in the absence of RA synthesis. In contrast, marked alterations are seen throughout the caudal hindbrain of mutant embryos. Instead of being expressed in two alternate rhombomeres (r3 and r5), *Krox20* is expressed in a single broad domain, correlating with an abnormal expansion of the r2-r3 marker *Meis2*. Instead of forming a defined r4, *Hoxb1*- and

Wnt8A-expressing cells are scattered throughout the caudal hindbrain, whereas r5/r8 markers such as *kreisler* or group 3/4 *Hox* genes are undetectable or markedly downregulated. Lack of alternate *Eph* receptor gene expression could explain the failure to establish rhombomere boundaries. Increased apoptosis and altered migratory pathways of the posterior rhombencephalic neural crest cells are associated with impaired branchial arch morphogenesis in mutant embryos. We conclude that RA produced by the embryo is required to generate posterior cell fates in the developing mouse hindbrain, its absence leading to an abnormal r3 (and, to a lesser extent, r4) identity of the caudal hindbrain cells.

Key words: Anteroposterior patterning, Mouse, Nervous system, Neural crest, *Raldh2*, Retinaldehyde dehydrogenase, Retinoids, Rhombomeres

INTRODUCTION

Regional diversity in the vertebrate hindbrain (rhombencephalon) is achieved through the generation of seven to eight neuroepithelial compartments or rhombomeres. This segmentation is required to define the periodic organization of hindbrain neurons and the migration pathways of neural crest cells (NCC) that colonize the branchial arches (reviewed by Lumsden and Krumlauf, 1996). Several *Hox* genes from paralogy groups 1-4 display spatially restricted expression domains in the developing hindbrain (e.g. Wilkinson et al., 1989), and knockout studies have shown that these genes are important to enable the formation and/or provide positional identity to specific rhombomeres (reviewed by Rijli et al., 1998). Two transcription factors, *Krox20* and *Kreisler*, which are critically required for the development of rhombomeres (r) 3 and 5 (Schneider-Maunoury et al., 1997) and r5 and 6 (McKay et al., 1994), respectively, are direct activators of *Hox* genes in these rhombomeres (Sham et al., 1993; Nonchev et al., 1996; Manzanares et al., 1999).

Assignment of anteroposterior positional values in the vertebrate nervous system involves both 'vertical' signaling from the mesoderm to the overlying neurectoderm and 'planar'

signals diffusing in the neurectoderm itself (reviewed by Beddington and Robertson, 1999). Experiments performed in *Xenopus* support an 'activation-transformation' model which proposes that the newly induced neural plate assumes an anterior identity, and acquires more posterior fates in response to additional ('transforming') signals (Nieuwkoop et al., 1985; reviewed in Kolm and Sive, 1997). Retinoic acid (RA) treatment of neurula stage *Xenopus* embryos leads to reduction of anterior (forebrain, midbrain) neural tissue and expansion of posterior (hindbrain, spinal cord) structures, thus suggesting that endogenous RA may represent such a 'transforming' signal (Durstont et al., 1989; Ruiz i Altaba and Jessell, 1991). Further experiments performed on *Xenopus*, mouse and zebrafish embryos revealed more specific RA effects within the rostral hindbrain, suggesting respecification of anterior rhombomeres towards a more posterior identity (reviewed by Conlon, 1995; Marshall et al., 1996). Conversely, overexpression of dominant negative retinoic acid receptors (DN-RARs) partially anteriorizes the posterior rhombomeres of *Xenopus* embryos (Blumberg et al., 1997; Kolm et al., 1997; Van der Wees et al., 1998). Direct evidence that endogenous retinoids are required for hindbrain patterning was found in quail embryos, where a complete dietary retinoid deficiency

misspecifies the caudal hindbrain region or myelencephalon, which normally gives rise to r4-r8 (Maden et al., 1996; Gale et al., 1999).

The extent to which endogenous retinoids control anteroposterior hindbrain patterning in mammalian species is less clear, because complete vitamin A deficiency (VAD) results in maternal infertility. Thus, the characteristic rodent fetal VAD syndrome (Wilson et al., 1958), which corresponds to a partial deficiency, does not include early hindbrain defects. However, supplementing fully VAD rats with low doses of RA can yield embryos with no posterior cranial nerves and hypoplastic branchial arches (White et al., 1998). Targeted disruptions of murine RAR genes also revealed partly overlapping functions during craniofacial development (reviewed in Mark et al., 1998). Interestingly, the combined disruption of RAR α and RAR β results in an expansion of r5 and abnormal r5/6/7 boundaries (Dupé et al., 1999). These hindbrain alterations are milder than those of VAD quail embryos, possibly because the RA signal can still be transduced to some extent by the remaining receptors (see Discussion).

We have used the genetic approach to create a murine model for embryonic RA deficiency. Retinaldehyde dehydrogenase 2 (Raldh2) was characterized as a NAD-dependent dehydrogenase with high substrate specificity for retinaldehyde, the intermediate product of vitamin A (retinol) oxidation into RA (Zhao et al., 1996; Wang et al., 1996). During development, Raldh2 expression correlates both temporally and spatially with the sites of RA production (Niederreither et al., 1997; Berggren et al., 1999). We disrupted the mouse *Raldh2* gene and found that homozygous mutant embryos, which die at approx. 10.5 days post-coitum (dpc), lack detectable RA production except in the developing eye and display impaired body turning and heart looping, shortening of the trunk region and absence of limb buds (Niederreither et al., 1999). They also exhibit hypoplastic otic vesicles and lack of externally visible second and third branchial arches. Instead of being expressed in r5 and r6 as in wild-type embryos, *Fgf3* is expressed at abnormally low levels throughout the caudal hindbrain of mutant embryos (Niederreither et al., 1999). These observations prompted us to analyze in more detail the rhombencephalon and associated neural crest in *Raldh2* mutants.

MATERIALS AND METHODS

Embryos

The *Raldh2* targeting construct and the generation of *Raldh2* mutant mice has been described previously (Niederreither et al., 1999). In the present study, litters from *Raldh2*^{+/-} breedings were collected between 7.5 and 9.5 dpc. Embryos were genotyped by PCR analysis of their extraembryonic membranes. For RA-rescue experiments, all-trans RA (Sigma) was suspended in ethanol, diluted in sunflower oil and administered orally to pregnant females (2.5 mg/kg body weight) at 12 hour intervals from 6.75 dpc (evening) to 8.25 dpc (morning). The embryos were collected 4 hours after the last gavage.

Morphology and histology

Both coronal and sagittal sections of 9.5 dpc embryos were analyzed after hematoxylin/eosin staining (Mark et al., 1993). For flatmount analysis, embryos fixed in 4% paraformaldehyde and stored in

methanol were rehydrated in phosphate-buffered saline (PBS). The hindbrain region was opened dorsally, separated from the underlying mesenchyme and examined as a flatmount in PBS between a glass slide and coverslip.

Molecular analyses

Whole-mount in situ hybridization was performed as described by Décimo et al. (1995), using probes from template plasmids produced in our laboratory or kindly provided by G. Barsh (Stanford University: *kreisler*), E. Bober (TU Braunschweig: *NKx5.1*), P. Charnay (ENS, Paris: *Krox20*), P. Gruss (MPI, Göttingen: *Pax2*), R. Krumlauf (NIMR, London: *Hoxa2/b1/b3/b4*), G. Martin (UCSF, San Francisco: *Fgf8*), A. McMahon (Harvard University: *Fgf3*, *En2*), A. Nieto (Cajal Institute, Madrid: *snail*) and D. Wilkinson (NIMR, London: *EphA2/A4*). Immunohistochemistry using the 2H3 neurofilament-specific antibody (Developmental Studies Hybridoma Bank) was as described (Mark et al., 1993). TUNEL assays were performed according to Conlon et al. (1995).

RESULTS

Defective rhombomeric segmentation

Rhombomeric segmentation was analyzed in 9.5 dpc *Raldh2*^{-/-} embryos. Scanning electron micrographs of the floor of the 4th ventricle revealed that the characteristic wild-type rhombomeric undulations were absent in mutant embryos (data not shown). Histological analysis of coronal (not shown) and sagittal sections showed that the *Raldh2*^{-/-} hindbrain neuroepithelium lacked rhombomeric morphology (compare Fig. 1A and B). Flatmounts of the dissected hindbrain neuroepithelium (Schneider-Maunoury et al., 1997) also failed to reveal the normal rhombomeric boundaries in *Raldh2*^{-/-} mutants (compare Fig. 1C and D). Their hindbrain was characterized by a rostral sulcus (Fig. 1B,D, open arrows) and an ill-defined caudal sulcus (filled arrows), which may be remnants of the primary constrictions (preotic and otic sulci) seen at earlier stages in wild-type embryos (e.g. Fig. 4A,C). Ill-defined undulations could be seen rostrally to the putative preotic sulcus (Fig. 1D, arrowheads), while the caudal hindbrain lacked rhombomeric segmentation and was progressively reduced in size towards the spinal cord.

Altered segmental gene expression in the *Raldh2*^{-/-} hindbrain

Molecular markers were used to further characterize the hindbrain defects of *Raldh2*^{-/-} mutants. The *Krox20* gene is normally expressed, shortly before and during hindbrain segmentation, in two alternate rhombomeres, r3 and r5 (Fig. 2A). In 8.5 dpc mutants, *Krox20* was expressed in a single broad domain throughout the caudal hindbrain, with a poorly defined posterior boundary (Fig. 2B,C). The *Hoxb1* gene, which is selectively expressed in the developing r4 of 8.5 dpc wild-type embryos (Fig. 2D), was expressed in the mutants in a patchy fashion, in a region encompassing the caudal portion of the abnormal *Krox20* expression domain (Fig. 2E). *Hoxb1* expression was apparently normal in the caudal neural plate, but was downregulated in the foregut epithelium of the mutants (compare Fig. 2D and E). Expression of the *Kreisler* gene, which marks r5-r6 and the associated neural crest cells (NCC) in wild-type embryos (Fig. 2F), was undetectable in *Raldh2*^{-/-} embryos (Fig. 2G).

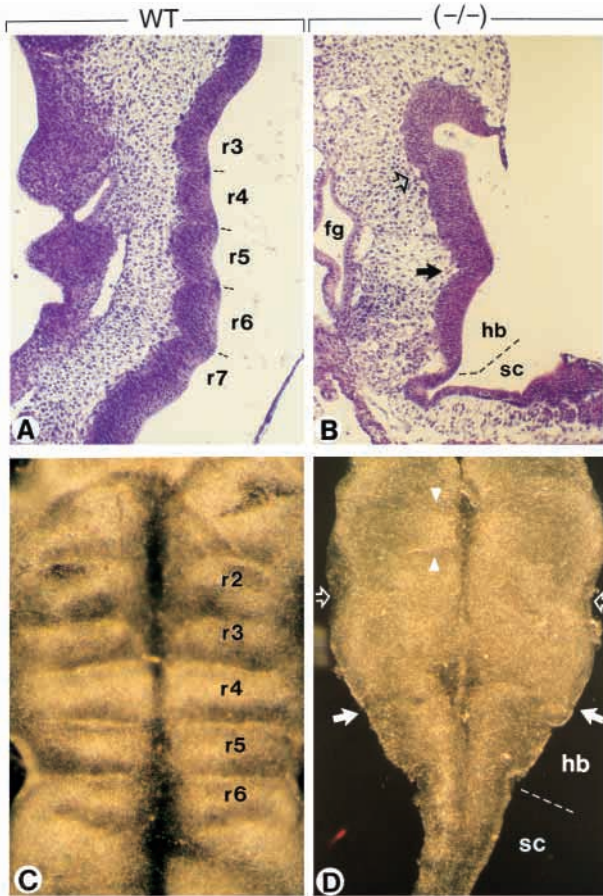


Fig. 1. Abnormal rhombomeric segmentation in the *Raldh2*^{-/-} mutant embryos. (A,B) Parasagittal sections through the hindbrain of 9.5 dpc wild-type (A) and *Raldh2*^{-/-} (B) embryos. Hematoxylin-eosin staining. (C,D) Flatmounts of the dissected hindbrain neuroepithelium of 9.5 dpc wild-type (C) and *Raldh2*^{-/-} (D) embryos, viewed at the same magnification under dark-field illumination. Open arrows in B and D point to the putative preotic sulcus, and filled arrows, to the ill-defined putative otic sulcus. Arrowheads show poorly defined rhombomere boundaries in the rostral hindbrain. Note the progressive size reduction of the mutant hindbrain, from the putative preotic sulcus to the spinal cord. fg: foregut; hb: hindbrain; r: rhombomeres; sc: spinal cord.

Hoxb1 expression had retreated caudally in the neuroepithelium, but was selectively maintained in a sharp band of hindbrain cells (pre-r4) in wild-type embryos (Fig. 3A). This expression pattern was abnormal in *Raldh2*^{-/-} littermates, in which *Hoxb1* transcripts always exhibited a diffuse distribution in the prospective hindbrain (Fig. 3B).

The *Wnt8A* gene is transiently expressed, in 8.25 dpc wild-type embryos, in the prospective r4 and in a few more rostral cells (Fig. 3C). These domains were not properly established in *Raldh2*^{-/-} embryos, where *Wnt8A* transcripts were present in a single, ill-defined hindbrain region (Fig. 3D). Note also the abnormal persistence of *Wnt8A* transcripts in the trunk region of the mutant embryos (Fig. 3D).

Altered expression of *Hox3* and *Hox4* gene paralogs

In 8.5 dpc wild-type embryos, *Hoxa3* is strongly expressed in the caudal hindbrain up to the prospective r4/r5 boundary, with decreasing transcript levels towards the cervical spinal cord (Fig. 3E). Only a few ventrally located cells weakly expressed *Hoxa3* in the hindbrain of *Raldh2*^{-/-} embryos, with no sharp anterior boundary (Fig. 3F). The paralogous gene *Hoxb3*, which is also expressed up to the r4/r5 boundary, is specifically upregulated in r5 of wild-type embryos (Wilkinson et al., 1989). *Hoxb3* was weakly expressed in the caudal hindbrain of *Raldh2*^{-/-} embryos, but no upregulation was detected towards its anterior boundary (data not shown).

Early molecular defects

We analyzed *Hoxb1* expression prior to 8.5 dpc, as expression of the paralogous gene *Hoxa1* is downregulated in the anterior neuroectoderm of 7.75 dpc *Raldh2*^{-/-} embryos (Niederreither et al., 1999). Expression of *Hoxb1* did not appear to be affected in 7.5/7.75 dpc mutant embryos (data not shown). By 8.25 dpc,

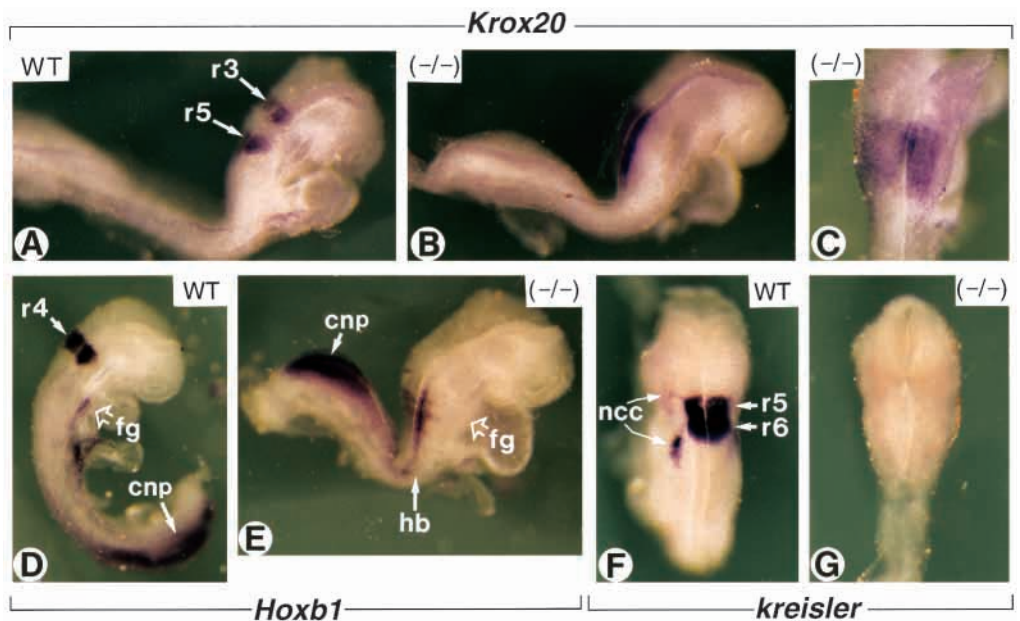


Fig. 2. Molecular alterations of the developing myelencephalon of *Raldh2*^{-/-} embryos. Whole-mount in situ hybridizations were performed on 8.5 dpc wild-type (A,D,F) and *Raldh2*^{-/-} (B,C,E,G) embryos using digoxigenin-labelled probes for *Krox20* (A-C), *Hoxb1* (D,E) or *kreisler* (F,G) gene transcripts. (A,B,D,E) Profile views; (C,F,G) dorsal views. r3-r6, rhombomeres; pos, preotic sulcus; cnp, caudal neural plate; fg, foregut; hb, hindbrain; ncc, neural crest cells.

The *Hoxa4*, *Hoxb4* and *Hoxd4* genes have similar neuroepithelial expression boundaries near the r6/r7 boundary (Fig. 3G and data not shown). As for most other *Hox* genes, their expression in the neuroepithelium extends more rostrally than in the somitic mesoderm (see Fig. 3G). Expression of these genes did not extend into the hindbrain region of *Raldh2*^{-/-} embryos; thus, their neuroepithelial and mesodermal expression boundaries were aligned (Fig. 3H and data not shown). In contrast, their expression appeared normal along the mutant spinal cord and somitic mesoderm. However, the strong expression of *Hoxa4* seen in the gut mesoderm of wild-type embryos was lacking in *Raldh2*^{-/-} embryos (data not shown).

Rostral hindbrain patterning

We next defined the rostral extent of the phenotypic alterations, as expression of the forebrain marker *Otx2* was found to be unaffected in *Raldh2*^{-/-} embryos (Niederreither et al., 1999). The restricted expression patterns of several genes including *Fgf8*, *Pax2*, *Gbx2* (not shown), *Engrailed2* (*En2*, Fig. 4A) or *Meis2* (Fig. 4B), are thought to be defined by an organizing center at the mid/hindbrain boundary (Joyner, 1996, and refs. therein). These genes appeared to be expressed normally in that region of mutant embryos (Fig. 4A,B, and data not shown), suggesting that mid/hindbrain organizer function does not require RA. The *Meis2* gene is also specifically expressed in r2 and r3 of wild-type embryos (Fig. 4B). In mutant embryos, *Meis2* exhibited a sharp and well positioned rostral expression boundary, but transcripts extended posteriorly throughout the caudal hindbrain (Fig. 4B).

The *CRABPI* gene has a complex expression pattern in the hindbrain of 8.25 dpc wild-type embryos, its expression being high in pre-r2, absent from pre-r3, high in pre-r4/6 and weaker in pre-r7 (Fig. 4C; Maden et al., 1992). The *CRABPI* pattern was altered in both the rostral and caudal hindbrain of *Raldh2*^{-/-} embryos (Fig. 4D). Rostral to the preotic sulcus, the *CRABPI*-labelled area (pre-r2) was larger than in wild-type embryos, whereas the adjacent transcript-free area (pre-r3) was not sharply defined and extended caudally within the myelencephalon (compare Fig. 4C and D). Posteriorly, there was no decrease in *CRABPI* expression in the area corresponding to wild-type pre-r7. Thus, the *Raldh2*^{-/-} phenotypic defects extend up to the presumptive r2 territory, which appears enlarged and does not establish a sharp boundary with the prospective r3. Note, in this respect, that the *Hoxa2* (Fig. 5D) and *CYP26* (data not shown) expression patterns in *Raldh2*^{-/-} mutants also reveal an enlargement of the r2 territory.

Lack of segmental *Eph* expression

Members of the Eph/ephrin receptor/ligand system are likely to mediate the cell-sorting events leading to compartmentalization of rhombomeric territories (Xu et al., 1999). In wild-type embryos, the *EphA4* receptor gene is expressed in prospective r3 and r5 cells (Fig. 4E), whereas *EphA2* is expressed in the

intervening pre-r4 territory (Fig. 4G). The *Raldh2*^{-/-} embryos failed to establish these alternate *Eph* expression domains. In 8.25 dpc mutants, *EphA4* was expressed as a single broad domain spanning most of the myelencephalic region, with poorly defined anterior and posterior boundaries (Fig. 4F), and *EphA2* was weakly expressed in an ectopic caudal domain (compare Fig. 4G and H), which was already seen in younger (presomite-stage) *Raldh2*^{-/-} embryos (data not shown). These altered distributions of Eph receptors might not allow appropriate cell segregations in the mutant hindbrain, and therefore account for the observed segmentation defects.

Alterations of neural crest

During hindbrain segmentation, neural crest cells (NCC) delaminate from specific rhombomeres and migrate along segmental pathways to colonize the branchial arches. While the first branchial arch receives a contribution from both mesencephalic and metencephalic NCC, the 2nd arch is colonized by r4-derived NCC and the 3rd and 4th arches by r6/7-derived NCC (Trainor and Tam, 1995, and refs. therein). The lack of second and third branchial arches in 9.5 dpc *Raldh2*^{-/-} embryos (Niederreither et al., 1999) suggested possible defects of the hindbrain-derived NCC, which were therefore analyzed with various molecular markers. We found that *AP2γ* (*AP-2.2*) gene expression, which labels the premigratory NCC at 8.0 dpc (Chazaud et al., 1996), was not altered in *Raldh2*^{-/-} embryos (data not shown). *AP2α* (data not

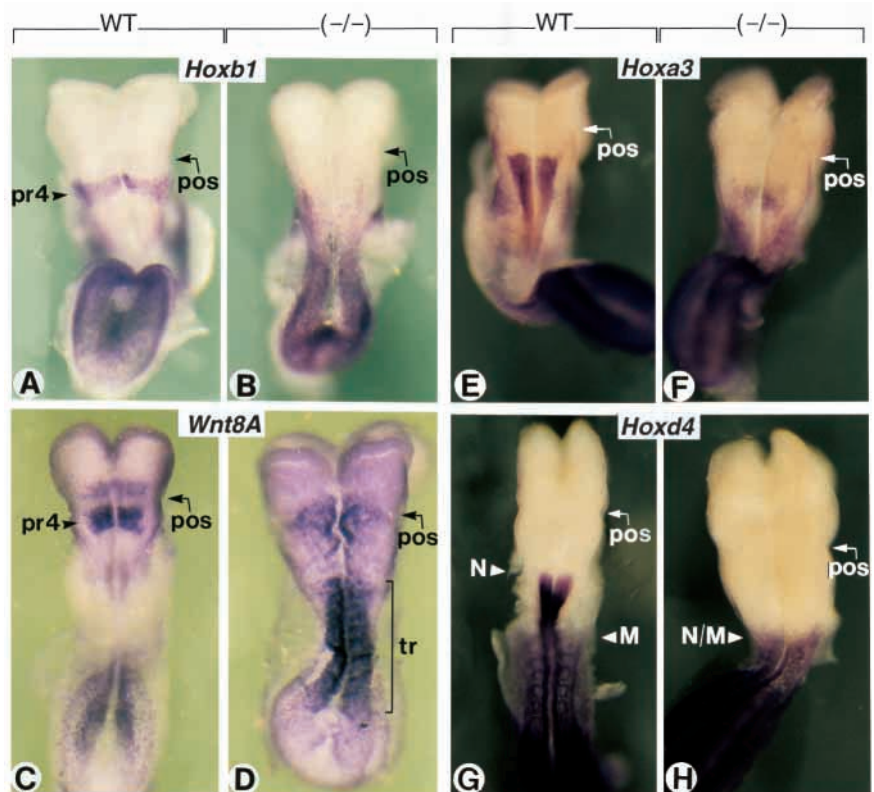


Fig. 3. Early alterations of *Hoxb1*, *Wnt8A*, *Hoxa3* and *Hoxd4* expression in the developing hindbrain of *Raldh2*^{-/-} embryos. Comparison of dorsal views of *Hoxb1* (A,B), *Wnt8A* (C,D) *Hoxa3* (E,F) and *Hoxd4* (G,H) transcript distributions in 8.25 dpc wild-type (A,C,E,G) and *Raldh2*^{-/-} (B,D,F,H) embryos. pr4, prospective fourth rhombomere; N/M, neuroectodermal and mesodermal expression boundary, respectively; pos, preotic sulcus; tr, trunk region.

shown) and *snail* (Fig. 5A,B) gene transcripts are good markers of migrating NCC (Mitchell et al., 1991; Nieto et al., 1992). In 8.5 dpc wild-type embryos, the first arch was strongly labelled by *snail*, while the 2nd and 3rd/4th arch NCC appeared as two less intensely labelled cell groups (Fig. 5A). Whereas the first arch was clearly labelled in *Raldh2*^{-/-} littermates, putative NCC were distributed more caudally as a continuous sheet near the hindbrain neuroepithelium (Fig. 5B, bracket). *Hoxa2* transcripts specifically marked NCC migrating towards the 2nd arch in 8.5 dpc wild-type embryos (Fig. 5C). In *Raldh2*^{-/-} littermates, *Hoxa2*-labelled cells did not form a defined migratory stream (Fig. 5D).

Raldh2^{-/-} embryos were also analyzed at 9.5 dpc for *CRABPI* transcripts, which are strongly expressed in the NCC accumulating in arches 2 to 4 of wild-type embryos (Maden et al., 1992). The first arch NCC, as well as the frontonasal region containing forebrain and midbrain-derived NCC (Trainor and Tam, 1995), were labelled to the same extent in *Raldh2*^{-/-} and wild-type embryos (compare Fig. 5E and F). In contrast, only few migratory cells were labelled along the myelencephalic region, and these appeared to follow aberrant migratory routes (Fig. 5F and data not shown).

Partial RA-rescue of the *Raldh2*^{-/-} hindbrain phenotype

As some abnormalities of the *Raldh2*^{-/-} embryos can be rescued by maternal administration of subteratogenic doses of RA (Niederreither et al., 1999), we investigated whether maternally supplied RA could also affect the *Raldh2*^{-/-} hindbrain phenotype. Four doses of RA were given from 6.75 to 8.25 dpc and the expression of the r4-specific marker *Hoxb1* was analyzed at 8.5 dpc. Wild-type and *Raldh2*^{+/-} embryos from RA-treated litters exhibited normal *Hoxb1* expression in r4, without (Fig. 6A) or with discrete ectopic expression rostrally to r4 (Fig. 6B). Most of the RA-treated *Raldh2*^{-/-} embryos exhibited a partial rescue of r4 expression. In some embryos, *Hoxb1* expression was almost restricted to the length of a single rhombomere, but was mosaic-like and lacked a sharp posterior boundary (Fig. 6E). Other embryos exhibited abnormal caudal expansion of *Hoxb1* expression (Fig. 6F, and data not shown). Note also some ectopic anteriorisation of *Hoxb1* transcripts, that may correspond to a RA teratogenic effect (Fig. 6E,F).

Experiments performed with *Krox20* (data not shown) and *Hoxa2* (Fig. 6C,G) revealed that the RA-induced phenotypic rescue was more effective in rostral than in caudal hindbrain. While r2 and r3 were well delineated, the prospective r4 was abnormally wide and the putative r5 was very poorly defined in RA-treated *Raldh2*^{-/-} embryos

(compare Fig. 6C and G). Analysis of *Fgf3* transcripts confirmed the poor phenotypic rescue of posterior myelencephalon. Instead of the normal r5/6-restricted expression pattern seen in RA-treated control littermates (Fig. 6D), *Raldh2*^{-/-} embryos exhibited widely deregulated *Fgf3* expression towards more caudal neuroepithelium (Fig. 6H).

We conclude from these experiments that there is an anterior to posterior gradient in the rescue efficiency of hindbrain segmental defects by exogenously administered RA, as the patterning of anterior rhombomeres could be significantly improved, whereas this rescue was incomplete for r4 and the least efficient in the caudal myelencephalon.

Neuronal differentiation

Neuronal differentiation can be assessed by the immunological detection of neurofilaments. At 9.5 dpc, these are essentially

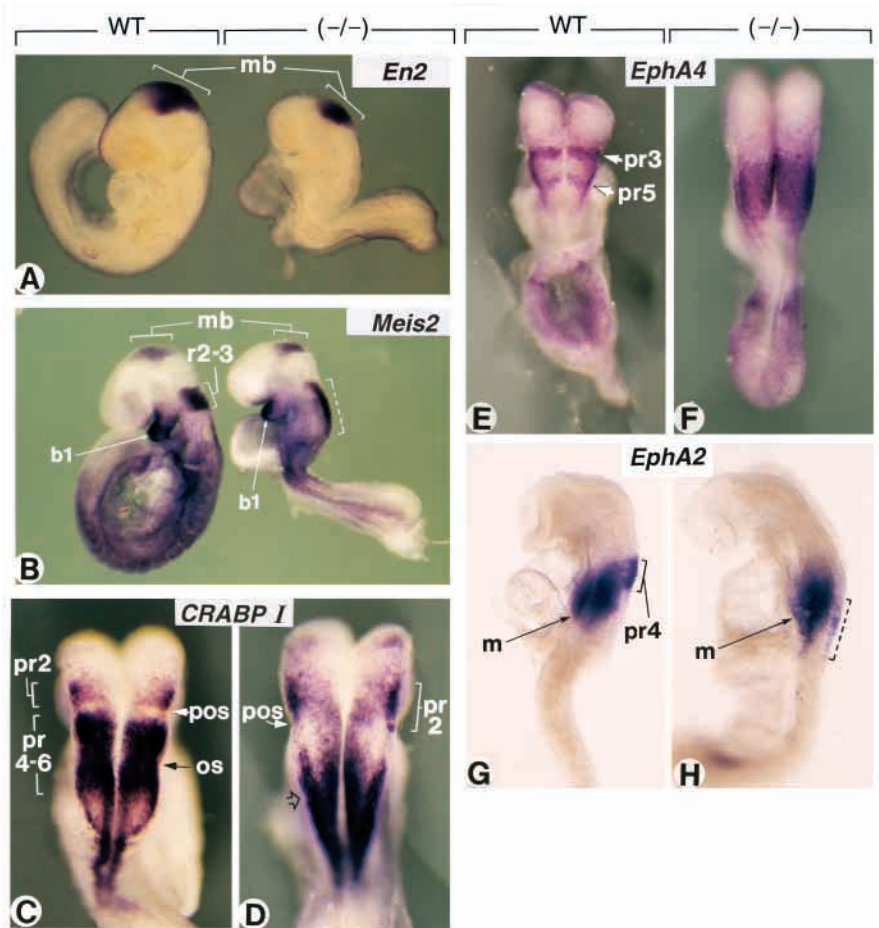


Fig. 4. (A-D) Mes/metencephalic patterning in *Raldh2*^{-/-} embryos. (A) Profile views of *Engrailed2* (*En2*) transcript distribution in the midbrain region of 8.75 dpc wild-type (left) and *Raldh2*^{-/-} (right) embryos. (B) Profile views of *Meis2* transcript distribution in 8.75 dpc wild-type (left) and *Raldh2*^{-/-} (right) embryos. *Meis2* expression abnormally extends throughout the caudal hindbrain in the mutant embryo (dashed bracket). (C,D) Dorsal views of *CRABPI* transcript distribution in the hindbrain of 8.25 dpc wild-type and *Raldh2*^{-/-} embryos, respectively. (E-H) Expression patterns of *EphA4* (E,F, dorsal views) and *EphA2* (G,H, profile views) in 8.25 dpc wild-type and *Raldh2*^{-/-} embryos. Note the weak and abnormally posterior *EphA2* expression domain in the mutant hindbrain neuroepithelium by comparison with the mesenchymal domain, which is not altered by the mutation. b1, first branchial arch; m, mesenchymal expression; mb, midbrain; os, otic sulcus; pos, preotic sulcus; pr2-6, prospective rhombomeres; r2-3, rhombomeres.

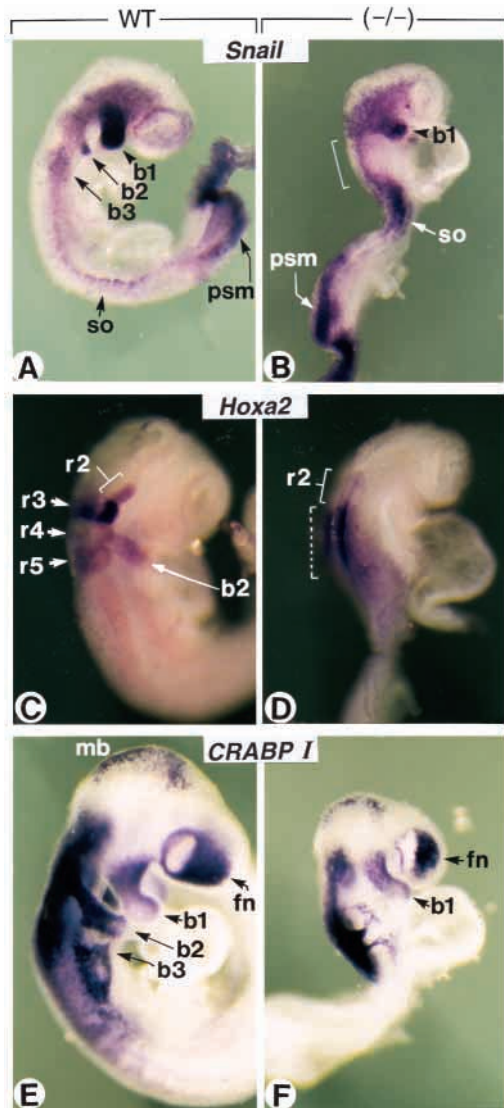


Fig. 5. Abnormalities of the rhombencephalic neural crest in *Raldh2*^{-/-} embryos. (A,B) *Snail* transcript distribution in 8.5 dpc wild-type (A) and *Raldh2*^{-/-} (B) embryos. The bracket shows the abnormal distribution of putative NCC along the hindbrain neuroepithelium in the mutant. Note also the strong labelling in the abnormal somitic region. (C,D) *Hoxa2* transcript distribution in 8.5 dpc wild-type (C) and *Raldh2*^{-/-} (D) embryos. Strong *Hoxa2* expression extends abnormally along the caudal hindbrain neuroepithelium (dashed bracket), and the putative NCC do not form a defined migratory stream. (E,F) *CRABP I* transcript distribution in 9.5 dpc wild-type (E) and *Raldh2*^{-/-} (F) embryos. b1-b3, branchial arches or prospective branchial arch NCC; fn, frontonasal mesenchyme; mb, midbrain; psm, presomitic mesoderm; r2-5, rhombomeres; so, somites.

detected in the developing neurons of the midbrain and the Vth (trigeminal) and VIIth (facial) cranial nerves, whose developing sensory ganglia and proximodistal fiber organisation can be seen in control embryos (Fig. 7A). Neurofilament immunoreactivity was detected in the midbrain and cranial nerve ganglia of *Raldh2*^{-/-} embryos, with a particularly intense labelling of the putative facial nerve ganglion (Fig. 7B). However, no fasciculated fiber tracts were observed along these ganglia (Fig. 7B), suggesting that, although sensory neurons

differentiated, their axonal outgrowth was impaired. Dorsal views also revealed some labelled fibers in the hindbrain of control embryos. These were not detected in the *Raldh2*^{-/-} embryos, possibly due to their segmentation and patterning defects (data not shown).

Abnormal patterns of cell death

The hindbrain defects of VAD quail embryos have been correlated with a wave of cell death occurring in the mesoderm and, subsequently, the neuroepithelium of the caudal hindbrain region at early somite stages (Maden et al., 1997). To investigate whether abnormal apoptosis may contribute to the *Raldh2*^{-/-} hindbrain defects, whole-mount TUNEL assays were performed on 3-4 to 8-10 somite stage (8.25-8.5 dpc) embryos. In agreement with previous reports (McKay et al., 1994; Conlon et al., 1995), little cell death was observed in the hindbrain region of control embryos. The youngest embryos (approx. 4 somites) exhibited limited cell death along the dorsal edges of the hindbrain (prospective roof plate) (Fig. 8A). Apoptotic cells were mostly restricted to the anterior hindbrain (r2) of 8- to 10-somite embryos (Fig. 8C).

At the 3- to 4-somite stage, *Raldh2*^{-/-} embryos exhibited increased cell death, both within and along the dorsal edges of the hindbrain neuroepithelium (compare Fig. 8A and B). Exaggerated cell death remained conspicuous throughout the hindbrain neuroepithelium and the adjacent mesenchyme of 8- to 10-somite stage mutants (Fig. 8D, and data not shown).

Altered otocyst regionalization

Several lines of evidence indicate a role for RA in the developing inner ear (Choo et al., 1998; and refs. therein). Thus, we investigated whether region-specific gene expression patterns are established in the *Raldh2*^{-/-} otocysts. Two regulatory genes, *NKx5.1* and *Pax2*, are expressed in almost complementary domains within the otocyst epithelium of 9.5 dpc wild-type embryos (Rinkwitz-Brandt et al., 1995; see Fig. 9A). *Pax2* transcripts were not expressed in the otocyst epithelium of 9.5 dpc *Raldh2*^{-/-} embryos, but were expressed at normal levels in other embryonic areas (Fig. 9B). In contrast, *NKx5.1* transcripts were distributed throughout the otocyst epithelium of *Raldh2*^{-/-} embryos (Fig. 9C). These results show that, in addition to its altered growth, regional patterning of the otocyst is affected by *Raldh2* disruption.

DISCUSSION

Retinoic acid synthesis and 'posteriorization' during hindbrain patterning

As summarized in Fig. 10, the *Raldh2*^{-/-} mutation, which results in lack of RA production during early mouse embryogenesis, alters the segmentation, growth and patterning of the rhombencephalon. Molecular analyses indicate that the mid/hindbrain 'organizer' region is functional and specification of the anteriormost rhombomeres does occur in *Raldh2*^{-/-} embryos. In contrast, patterning of the putative r3-r8 region is profoundly altered. Instead of being expressed in two stripes defining r3 and r5, the transcription factor *Krox20* is expressed in a single domain spanning the whole caudal hindbrain of mutant embryos. *Krox20* controls the segmental expression of several target genes including *Hoxa2*, *Hoxb2* and

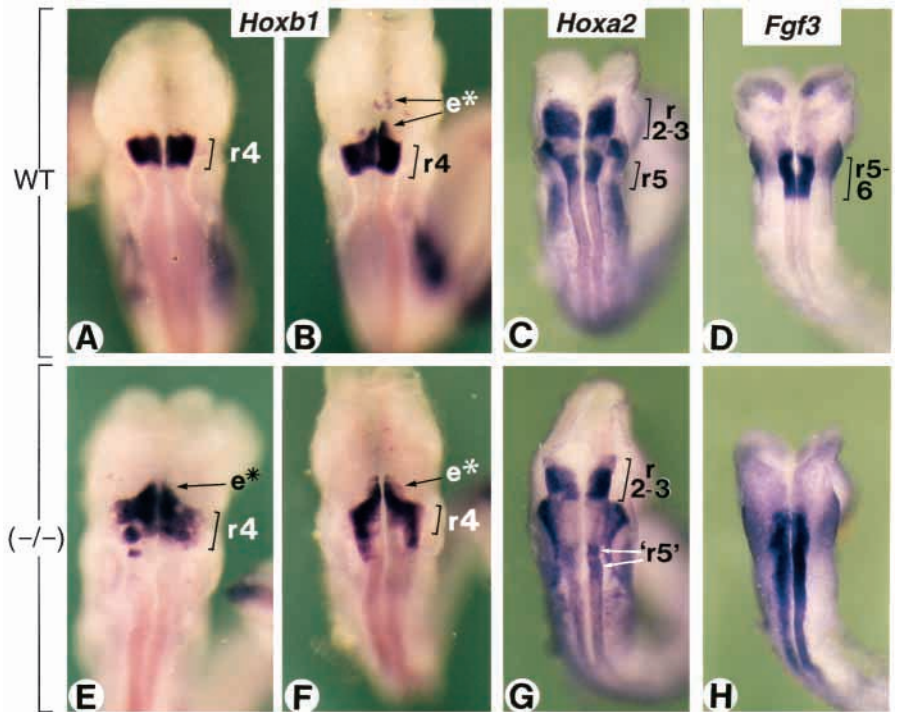


Fig. 6. Partial rescue of the *Raldh2*^{-/-} hindbrain phenotype by maternal RA supplementation. *Hoxb1* (A,B,E,F), *Hoxa2* (C,G) and *Fgf3* (D,H) transcript distributions were analyzed in 8.5 dpc embryos whose mothers received four RA doses (2.5 mg/kg) from 6.75 to 8.25 dpc. (A-D) Wild-type embryos; (E-H) *Raldh2*^{-/-} embryos. r2-6, rhombomeres; e*, ectopic rostral *Hoxb1* expression.

Epha4 (Sham et al., 1993; Nonchev et al., 1996; Theil et al., 1998). Accordingly, all these genes exhibit abnormal expression patterns resembling that of *Krox20* in mutants. Furthermore, expression of the *Meis2* gene, which is normally restricted to r2 and r3, also extends along the posterior hindbrain of *Raldh2*^{-/-} embryos. The abnormal *Hoxb1* and *Wnt8A* expression patterns indicate that cells with r4 characteristics do not form a defined segmental domain, but instead are scattered throughout the caudalmost portion of the mutant hindbrain. On the other hand, the expression of the transcription factor *Kreisler*, which normally defines r5 and r6, is absent in *Raldh2*^{-/-} embryos. Furthermore, various genes normally expressed in the r5-r8 region, including group 3 and 4 *Hox* genes (this study) and *Fgf3* (Niederreither et al., 1999), are markedly downregulated in the *Raldh2*^{-/-} hindbrain region.

Altogether, the caudal hindbrain region of *Raldh2*^{-/-} embryos appears to be anteriorized, as its cells abnormally express r3 and, to a lesser extent, r4 molecular combinations, while the expression of r5 to r8 gene markers is either lacking or downregulated. Our data clearly implicate RA as an endogenous signal required to 'posteriorize' the developing mouse hindbrain caudally to r3. These data are essentially consistent with the previously reported VAD quail hindbrain phenotype (Maden et al., 1996; Gale et al., 1999). We note, however, that *Hoxb1* expression was not detected in the VAD quail hindbrain, in contrast to the *Raldh2*^{-/-} mutation. Gale et al. (1999) concluded that specification of the posterior hindbrain is lost in VAD quails, its cells participating in the formation of an enlarged anterior hindbrain. Accordingly, we suggest that the abnormal 'anterior transformation' of this hindbrain region leads to its progressive size reduction in *Raldh2*^{-/-} embryos. Our data differ from those of Van der Wees et al. (1999), who analyzed the effects of DN-RAR overexpression in *Xenopus* and concluded that the posterior hindbrain is partly transformed into an r4/r5 identity. However,

the DN-RAR used in that study did not appear to fully inhibit RA signaling.

The early appearance of the molecular alterations in the *Raldh2*^{-/-} hindbrain indicates that embryonic RA acts shortly after (or during) neurulation. According both to the distribution of *Raldh2* transcripts and protein (Niederreither et al., 1997; Berggren et al., 1999), and to recent findings on RA distribution in the chick embryo (Maden et al., 1998), RA is likely to act as a 'vertical' signal produced by the presomitic mesoderm and, later, the rostral somites. *Raldh2* is expressed in the early embryonic mesoderm up to a boundary located at the level of the first developing somite, which is adjacent to the prospective r7/8 region, while the RA-metabolizing enzyme CYP26 is oppositely expressed in the anteriormost head

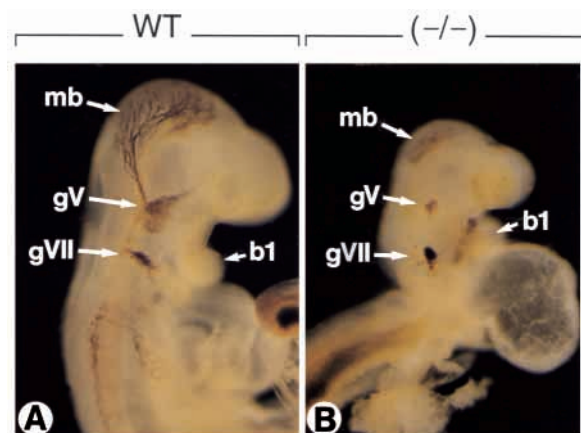


Fig. 7. Neurofilament differentiation in *Raldh2*^{-/-} embryos. Whole-mount immunohistochemistry was performed with an anti-neurofilament antibody on 9.5 dpc wild-type (A) and *Raldh2*^{-/-} (B) littermate embryos. b1, first branchial arch; mb, midbrain; gV/VII, cranial nerve ganglia.

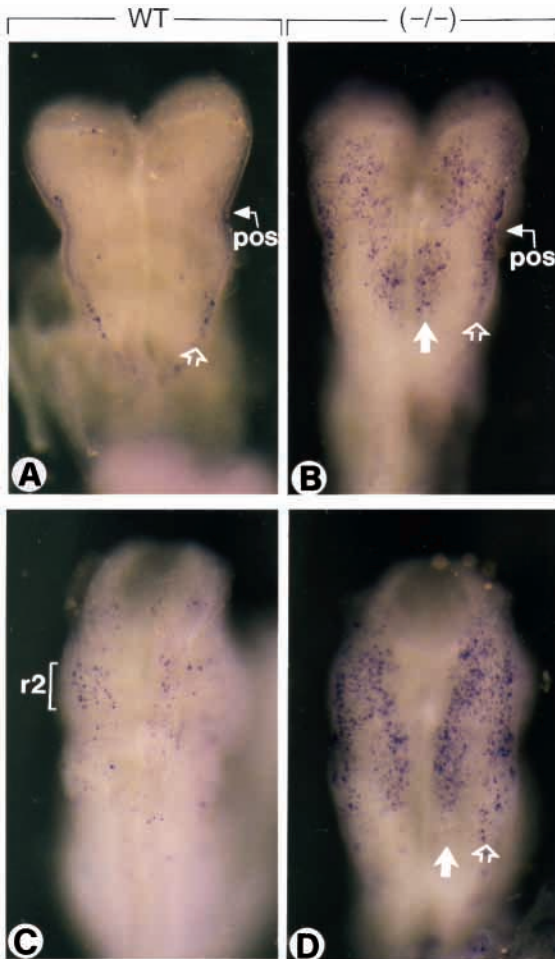


Fig. 8. Abnormal patterns of cell death in the hindbrain and putative neural crest cells of *Raldh2*^{-/-} embryos. Whole-mount TUNEL assays were performed on wild-type (A,C) and *Raldh2*^{-/-} (B,D) embryos at the 3- to 4-somite stage (A,B) and the 8- to 10-somite stage (C,D). Note the increased number of labelled cells, both within the hindbrain neuroepithelium (filled arrows) and along its dorsal edges (open arrows). Other regions such as the frontonasal mesenchyme or the posterior region of the embryo, were normally labelled in *Raldh2*^{-/-} embryos, thus excluding an overall excess of cell death. pos, preotic sulcus; r2, rhombomere 2.

but abnormally expands in the posterior hindbrain of *RARα/RARβ* mutants. *Krox20* exhibits normal expression in r3, but abnormal posterior expansion of its r5 domain in *RARα/RARβ* embryos (Dupé et al., 1999). These differences strongly suggest that *RARγ* can transduce the early RA signaling events which are impaired in the *Raldh2*^{-/-} mutants, and that the *RARα/RARβ* null phenotype reflects a subsequent function of embryonic RA, required for posterior restriction of *kreisler* and *Krox20* expression domains and, thus, definition of the r5/6 and r6/7 boundaries. Clearly, this function cannot be mediated by *RARγ*, which is not expressed in the developing rostral neuroepithelium, in contrast to *RARα* and *RARβ* which are coexpressed in this tissue up to the posterior hindbrain level (Dupé et al., 1999, and refs. therein).

Alterations of the neural crest

The *Raldh2*^{-/-} embryos exhibit cranial NCC alterations correlating with the level of the hindbrain defects. Indeed, the frontonasal region and first branchial arch, which receive contributions from the midbrain and r1/r2 levels (Trainor and Tam, 1995; and refs. therein), are normally colonized by NCC in *Raldh2*^{-/-} embryos. In contrast, more caudal hindbrain NCC

mesoderm and later, in r2 (Fujii et al., 1997). These complementary expression patterns may define a gradient of RA availability throughout the hindbrain region. Interestingly, CYP26 overexpression by mRNA injection in *Xenopus* embryos induces dose-dependent anteriorisation of the posterior rhombomeres (Hollemann et al., 1998). We found that the *Raldh2*^{-/-} hindbrain defects can be partly reverted by maternal RA supplementation, but the efficiency of this RA rescue decreases within the hindbrain in a rostral to caudal direction, suggesting two possibilities: (i) as proposed by others (Grapin-Botton et al., 1998; Gould et al., 1998), determination of posterior rhombomeric fates may require RA concentrations, which were not reached in our rescue experiments, or (ii) normal hindbrain patterning requires continuous RA synthesis, which cannot be mimicked by maternal administration.

The *Raldh2*^{-/-} hindbrain phenotype is clearly distinct from that of the *RARα/RARβ* compound mutant embryos, which have apparently normal anterior rhombomeres (r1-r4), but exhibit an enlarged r5 and altered r5/6/7 boundaries (Dupé et al., 1999). The major molecular difference concerns *kreisler*, whose expression is abolished in *Raldh2*^{-/-} embryos,

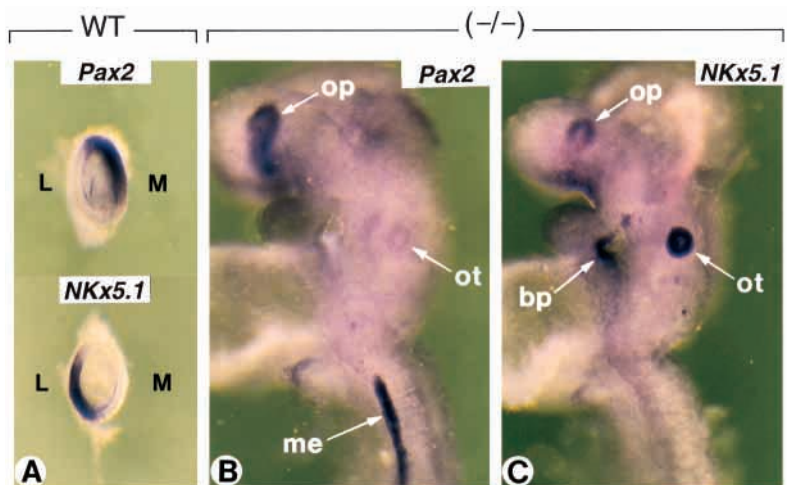
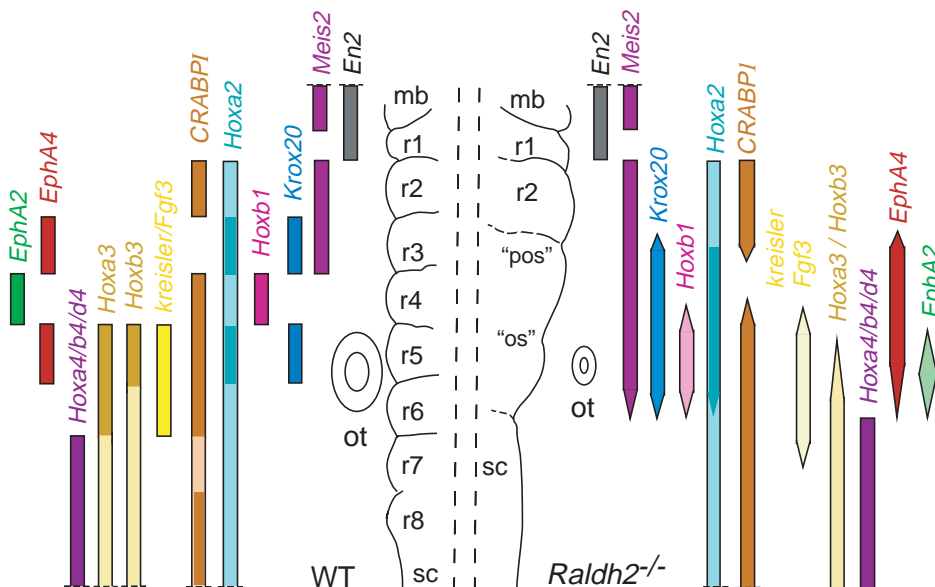


Fig. 9. Altered gene expression in the otocysts of *Raldh2*^{-/-} embryos. (A) Complementary expression patterns of the *Pax2* and *NKx5.1* genes in the otocyst epithelium of 9.5 dpc wild-type embryos. (B) *Pax2* transcript distribution in a 9.5 dpc *Raldh2*^{-/-} embryo. *Pax2* transcripts are strongly expressed in the mesonephros and optic vesicle, but are not detected in the mutant otocyst. (C) *NKx5.1* transcript distribution in a 9.5 dpc *Raldh2*^{-/-} embryo. Note that the transcripts are expressed throughout the mutant otocyst. bp, branchial pouch; L, lateral side; M, medial side; me, mesonephros; op, optic vesicle; ot, otocyst.

Fig. 10. Scheme of the morphological and molecular hindbrain alterations seen in the *Raldh2*^{-/-} mutant embryos. Although the morphological hindbrain defects are depicted at 9.5 dpc, the molecular patterns were analyzed at 8.25/8.5 dpc, prior to the appearance of these defects (see the main text for details). Gene expression patterns are shown as bars, with dark and light colours representing high and low expression levels, respectively, and tapered ends depicting the lack of a sharp expression boundary. mb, midbrain; r1-r8, rhombomeres; os, otic sulcus; ot, otocyst; pos, preotic sulcus; sc, spinal cord; WT, wild type.



tend to remain near the neuroepithelium and/or follow aberrant migratory routes in mutants. Furthermore, high numbers of dying cells are seen along the edges of the hindbrain neuroectoderm and in the adjacent mesenchyme, suggesting that a number of pre- or early migratory NCC are eliminated by apoptosis. These NCC alterations, which provide a basis for the lack of morphological development of caudal branchial arches, may result from the misspecification of hindbrain cells in the mutant embryos. Exaggerated apoptosis may result from the inappropriate r3-like identity of the caudal hindbrain, as r3-derived NCC are known to undergo selective apoptosis during normal development (Graham et al., 1996). Maden et al. (1997) previously reported abnormal apoptosis in the hindbrain neuroepithelium and mesenchyme of VAD quail embryos, although in a more restricted AP location in comparison to *Raldh2*^{-/-} embryos.

Retinoic acid and control of *Hox* gene expression

Several 'anterior' (3'-located) *Hox* genes harbor RARE(s) in their regulatory sequences. The *in vivo* contribution of these RAREs has been studied in the context of *lacZ* transgenes or targeted disruptions within the endogenous loci. The analysis of *Raldh2* mutant embryos provides another means to investigate which aspects of *Hox* gene expression depend on endogenous RA synthesis. Headfold-stage *Raldh2*^{-/-} embryos display altered *Hoxa1* expression (Niederreither et al., 1999) which is similar to the effect of a targeted disruption of the *Hoxa1* RARE (Dupé et al., 1997). The paralogous *Hoxb1* gene contains three interspersed RAREs which control distinct aspects of its regulation. Disruption of the proximal 3' RARE affects *Hoxb1* early expression in the neuroectoderm and, to a lesser extent, the mesoderm of headfold-stage embryos (Studer et al., 1998), while mutation of the distal 3' RARE selectively extinguishes later expression in the foregut region (Huang et al., 1998). *Hoxb1* expression was selectively lacking in the foregut region of 8.5 dpc *Raldh2*^{-/-} embryos, but was not obviously altered at the headfold stage. Further experiments, including a comparison of *Hoxb1* expression levels between age-matched *Hoxb1*^{3'RARE} (Studer et al., 1998) and *Raldh2*^{-/-}

embryos, are required to clarify this discrepancy. The 5'-located *Hoxb1* RARE appears to function as a hindbrain repressor element, preventing *Hoxb1* expression in r3 and r5 cells (Studer et al., 1994). The lack of restriction of *Hoxb1* expression in the *Raldh2*^{-/-} hindbrain could result from lack of repressing activity of the 5' RARE. It will be interesting to analyze the expression of *lacZ* reporter constructs containing this repressor element (Studer et al., 1994) in a *Raldh2*^{-/-} background.

RAREs have also been identified in three *Hox* genes from group 4. Mutation of the *Hoxa4* RARE has no apparent effect on expression in neural tube or somitic derivatives (prevertebrae), but abolishes expression in the splanchnopleure-derived mesoderm of various organs, including the lung and gut (Packer et al., 1998). Likewise, the *Raldh2*^{-/-} embryos exhibit a lack of *Hoxa4* expression in the gut mesoderm. The *Hoxb4* RARE was characterized as an early neural enhancer required for the establishment of the correct rhombomeric expression boundary at approx. 8.25 dpc (Gould et al., 1998). An adjacent neural enhancer, required for sustained expression at later stages, appears to function as a *Hox* responsive element mediating auto- and/or cross-regulatory interactions. *Raldh2*^{-/-} embryos exhibit a selective downregulation of *Hoxb4* expression in the rostralmost portion of its neuroectodermal expression domain, consistent with an altered function of the RARE-containing neural enhancer. Gould et al. (1998) used various approaches, including the co-culture of hindbrain and somite explants, to demonstrate that the activation of the *Hoxb4* early neural enhancer requires endogenous RA signaling, as well as another somite-derived factor. However, their experiments did not determine whether the active retinoid signal was released from the somites or from the neuroectoderm as a response to a somitic signal. *Raldh2* mutant embryos may be useful to define the source of the RA signal required for *Hox* gene regulation and hindbrain patterning.

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