

## Key roles of retinoic acid receptors alpha and beta in the patterning of the caudal hindbrain, pharyngeal arches and otocyst in the mouse

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### SUMMARY

Mouse fetuses carrying targeted inactivations of both the  $RAR\alpha$  and the  $RAR\beta$  genes display a variety of malformations in structures known to be partially derived from the mesenchymal neural crest originating from post-otic rhombomeres (e.g. thymus and great cephalic arteries) (Ghyselinck, N., Dupé, V., Dierich, A., Messaddeq, N., Garnier, J.M., Rochette-Egly, C., Chambon, P. and Mark M. (1997). *Int. J. Dev. Biol.* 41, 425-447). In a search for neural crest defects, we have analysed the rhombomeres, cranial nerves and pharyngeal arches of these double null mutants at early embryonic stages. The mutant post-otic cranial nerves are disorganized, indicating that RARs are involved in the patterning of structures derived from neurogenic neural crest, even though the lack of  $RAR\alpha$  and  $RAR\beta$  has no detectable effect on the number and migration path of neural crest cells. Interestingly, the double null mutation impairs early developmental processes known to be independent of the neural crest e.g., the initial formation of the 3rd and 4th branchial pouches and of the 3rd, 4th and 6th arch arteries. The double mutation also results in an enlargement of rhombomere 5,

which is likely to be responsible for the induction of supernumerary otic vesicles, in a disappearance of the rhombomere 5/6 boundary, and in profound alterations of rhombomere identities. In the mutant hindbrain, the expression domain of *kreisler* is twice its normal size and the caudal stripe of *Krox-20* extends into the presumptive rhombomeres 6 and 7 region. In this region, *Hoxb-1* is ectopically expressed, *Hoxb-3* is ectopically up-regulated and *Hoxd-4* expression is abolished. These data, which indicate that retinoic acid signaling through  $RAR\alpha$  and/or  $RAR\beta$  is essential for the specification of rhombomere identities and for the control of caudal hindbrain segmentation by restricting the expression domains of *kreisler* and of *Krox-20*, also strongly suggest that this signaling plays a crucial role in the posteriorization of the hindbrain neuroectoderm.

Key words: Retinoic acid, Endoderm, Cranial mesoderm, Rhombencephalon, Neural crest, *kreisler*, *Krox-20*, *Hox*-genes, Morphogen

### INTRODUCTION

The effects of retinoids, the biologically active derivatives of vitamin A, are transduced by nuclear receptors, the RARs ( $\alpha$ ,  $\beta$  and  $\gamma$ ) and the RXRs ( $\alpha$ ,  $\beta$  and  $\gamma$ ). RXR:RAR heterodimers and RXR homodimers bind cognate regulatory DNA sequences (response elements), and act as ligand-responsive transcriptional regulators (Chambon, 1996). Simultaneous knockout of two RAR isotypes, or of RXR $\alpha$  and a given RAR isotype ( $\alpha$ ,  $\beta$  or  $\gamma$ ), leads to numerous developmental defects which, altogether, recapitulate the fetal vitamin A deficiency (VAD) syndrome, thus demonstrating that these receptors transduce the retinoid signal during embryonic development (Lohnes et al., 1994; Mendelsohn et al., 1994b; Kastner et al., 1994, 1995, 1997; Ghyselinck et al., 1997; Mascres et al., 1998). When examined at late developmental stages (i.e. E14.5 and E18.5), fetuses lacking all  $RAR\alpha$  isoforms and either all

$RAR\beta$  isoforms or only the  $RAR\beta 2$  isoform ( $RAR\alpha^{-/-}/RAR\beta^{-/-}$  and  $RAR\alpha^{-/-}/RAR\beta 2^{-/-}$  fetuses), as well as  $RAR\alpha 1^{-/-}/RAR\beta^{-/-}$  and  $RAR\alpha^{-/-}/RAR\gamma^{-/-}$  fetuses, show numerous defects in structures derived from the 3rd, 4th and 6th pharyngeal arches, namely malformations of the hyoid bone and the thyroid cartilage, abnormal arrangement of the great cephalic arteries and agenesis or ectopias of the thymus and parathyroid glands (Ghyselinck et al., 1997; Lohnes et al., 1994; Mendelsohn et al., 1994b; Luo et al., 1996). The aim of the present work was to gain insights into the role of retinoid signaling in the ontogenesis of these three caudal pharyngeal arches. As similar abnormalities can be generated in chick embryos by ablation of neural crest cell (NCC) progenitors present in the post-otic hindbrain (Kirby and Waldo, 1990), we have examined the hindbrain, migrating NCC and pharyngeal arches arteries and nerves of RAR double null mutants at early developmental stages (i.e. E8.5-E10.5), looking for morphological abnormalities and

alteration in the expression of *Krox-20*, *kreisler* and of different *Hox* genes known to be involved in hindbrain and pharyngeal arch patterning. We choose to study  $RAR\alpha^{-/-}/RAR\beta^{-/-}$  double null mutants because their spectrum of abnormalities is more severe than that of  $RAR\alpha^{-/-}/RAR\beta^{2-/-}$  mutants (Ghyselinck et al., 1997). Moreover, as opposed to the  $RAR\alpha^{-/-}/RAR\gamma^{-/-}$  mutants, those lacking  $RAR\alpha$  and  $RAR\beta$  never display exencephaly which can be responsible for secondary alterations of pharyngeal arch morphogenesis (Lohnes et al., 1994; Ghyselinck et al., 1997).

## MATERIALS AND METHODS

### Mouse lines, mating and genotyping

$RAR\alpha^{+/-}/RAR\beta^{+/-}$  mice (Lufkin et al., 1993; Ghyselinck et al., 1997) were intercrossed to generate  $RAR\alpha^{-/-}/RAR\beta^{-/-}$  mutant embryos that were recovered at a frequency of 1/16. Mice were mated overnight

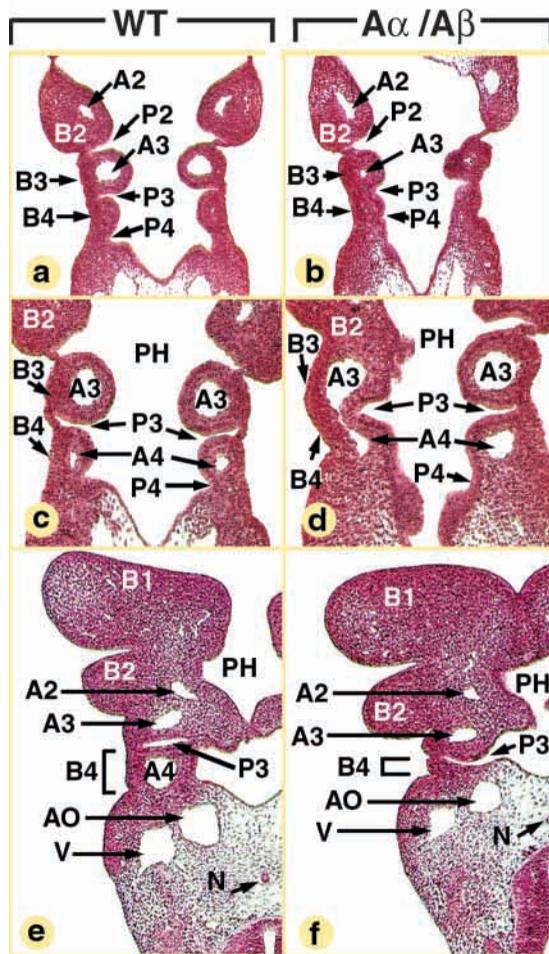
and the next morning was considered 0.5 days post-coitum (embryonic day 0.5, E0.5). Genotyping was performed by PCR on genomic DNA from yolk sac (conditions available upon request).

### Histology, whole-mount immunohistochemistry and in situ hybridization

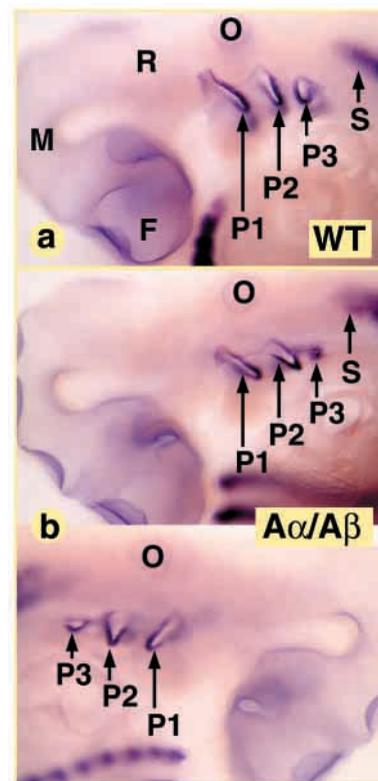
Serial histological sections of E9.5-E11.5 embryos fixed in Bouin's fluid were stained with Groat's hematoxylin and Mallory's trichrome (Mark et al., 1993). For immunohistochemistry, embryos were fixed in 4% paraformaldehyde for 4 hours at 4°C, rinsed in phosphate-buffered saline, and stored in 70% ethanol at 4°C. Whole-mount immunohistochemistry using the 2H3, neurofilament-specific, antibody and the Islet-1 antibody (Developmental Studies Hybridoma Bank) was as described by Mark et al. (1993). Whole-mount in situ RNA hybridization was carried out as described by Décimo et al. (1995) using the following probes: CRABPI (Dollé et al., 1990), *Hoxb-1* and *Hoxb-3* (Hunt et al., 1991), *Hoxd-4* (Featherstone et al., 1988), *Hoxb-4* (Graham et al., 1988), *kreisler* (Cordes and Barsh, 1994), *Krox-20* (Wilkinson et al., 1989) and *pax-1* (Deutsch et al., 1998).

## RESULTS

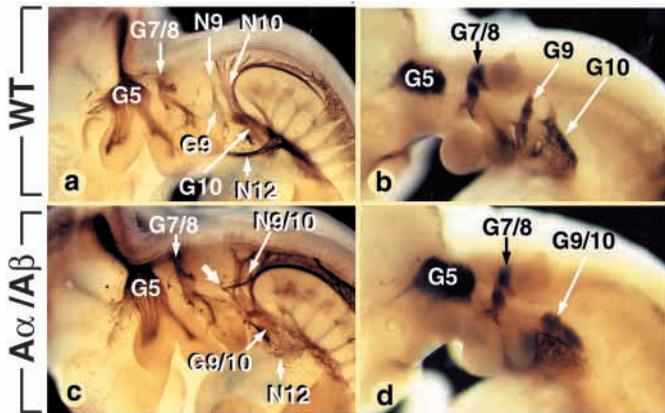
For the sake of simplicity, homozygous  $RAR\alpha$  and  $RAR\beta$  null mutants are designated hereafter as  $A\alpha$  and  $A\beta$ , the '-/-' sign indicating homozygosity being omitted.  $RAR\alpha^{-/-}/RAR\beta^{-/-}$  mutants are referred to as  $A\alpha/A\beta$  mutants. At least three  $RAR\alpha^{-/-}$  and  $RAR\beta^{-/-}$  single mutants



**Fig. 1.** Frontal histological sections at comparable levels of the pharyngeal arches of WT and  $A\alpha/A\beta$  mutants at E9.5 (a-d) and E10.5 (e,f). a, b, c and d are from four different embryos. A2, A3 and A4, 2nd, 3rd and 4th arch arteries; AO, dorsal aorta; B1, B2, B3 and B4, pharyngeal (branchial) arches one to four; N, notochord; P2, P3 and P4, 2nd, 3rd and 4th branchial pouches; PH, lumen of the pharynx; V, anterior cardinal vein. Magnifications:  $\times 40$  (a,b),  $\times 50$  (c,d) and  $\times 45$  (e,f).



**Fig. 2.** Whole-mount in situ analysis of *pax-1* expression in the branchial pouches of E9.5 WT (a) and  $A\alpha/A\beta$  embryos (b). Both sides of the mutant embryo are displayed to show the asymmetrical hypoplasia of the 3rd pouch. F, forebrain; M, midbrain; O, otocyst; P1, P2 and P3, 1st, 2nd and 3rd branchial pouches; R, rhombencephalon; S, somite.



**Fig. 3.** Comparison of the cranial nerves and ganglia of E10.5 WT and  $A\alpha/A\beta$  mutants immunostained with monoclonal antibodies directed against the  $155 \times 10^3 M_r$  neurofilament (a,c) and Islet-1 (b,d). G5 and G7/8 trigeminal and facial-acoustic ganglia, respectively; G9 and G10, distal ganglia of the glossopharyngeal (IX) and vagus (X) nerves, respectively; G9/10 abnormal fusion of the distal ganglia of cranial nerves IX and X; N9, N10 and N12, glossopharyngeal, vagus and hypoglossal nerves; N9/10, abnormal fusion of cranial nerves IX and X. The large arrow in c points to aberrant axonal projections.

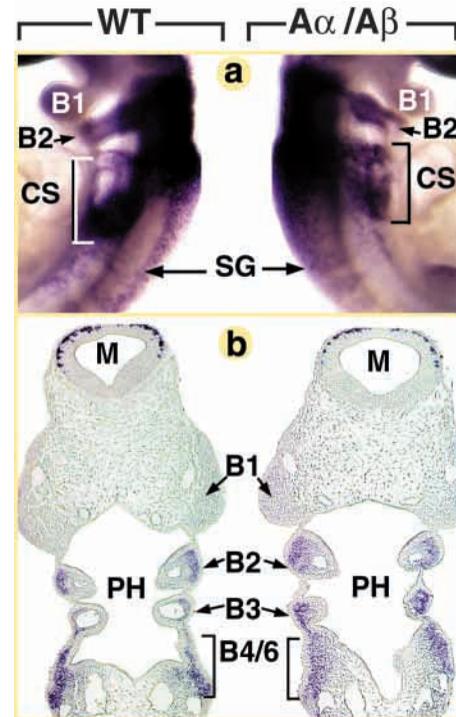
were examined histologically and analyzed using immunohistochemistry and in situ hybridization techniques: the histological abnormalities and alterations in gene expression patterns detected in the  $A\alpha/A\beta$  mutants were never observed in the single null mutants, which were indistinguishable from wild type (E9.0 to E11.5) embryos in all respects (data not shown).

#### Early defects of post-otic pharyngeal arches and disorganization of post-otic cranial nerves

Wild-type (WT) embryos at E9.5 possess 4 pharyngeal arches of which the bulges of the first 3 are visible externally. The 3rd and 4th branchial pouches (P3 and P4 in Fig. 1a and c), which are outpocketings of the pharyngeal endoderm, are well defined. The 3rd pouch contacts the surface ectoderm (P3, Fig. 1a and c). Only the bulges of the first two pharyngeal arches were visible in E9.5 ( $n=8$ )  $A\alpha/A\beta$  embryos (data not shown). Of the 3 embryos analyzed histologically at this developmental stage, 2 exhibited a fusion of the mesenchyme of the 3rd and 4th pharyngeal arches (B3 and B4), a failure of the 3rd pouch (P3) to contact the ectoderm and a marked decrease in the size of the 4th pouch (P4) (compare Fig. 1a and c with 1b and d). The hypoplasia of the 3rd pharyngeal pouch in E9.5  $A\alpha/A\beta$  embryos was confirmed by whole-mount in situ hybridisation using a *pax-1* antisense probe which specifically labels the endoderm of the first three branchial pouches (P1-P3, Fig. 2).

In WT embryos, the 3rd arch arteries form between E9.0 and E9.5 and the 4th and 6th arch arteries between E9.5 and E10.5; all these vessels are bilaterally symmetrical until E11.5. In E9.5  $A\alpha/A\beta$  embryos, the 3rd arch arteries were hypoplastic, absent or fused with the 4th arch arteries (compare A3 in Fig. 1a and c with 1b and d; Table 1). At E10.5, the 3rd, the 4th and/or the 6th arch arteries were absent on one or both sides of the mutant embryos (compare B4 in Fig. 1e and f; Table 1).

Whole-mount immunostaining of E10.5  $A\alpha/A\beta$  ( $n=4$ )



**Fig. 4.** Distribution of CRABPI transcripts in E9.5 WT and  $A\alpha/A\beta$  embryos. (a,b) Lateral views and frontal sections of the same embryos. B1, B2 and B3, pharyngeal (branchial) arches one to three; B4/6, presumptive 4th and 6th pharyngeal arches; CS, caudal stream of cranial neural crest cells emanating from rhombomeres 6 and 7; M, mesencephalon; PH, lumen of the pharynx; SG, first spinal ganglion.

embryos using anti-neurofilament antibodies revealed a completely penetrant disorganization of post-otic cranial nerves, which was not observed in the corresponding single null mutants (Ghyselinck et al., 1997, and data not shown). The fibers of nerves IX (glossopharyngeal; N9 in Fig. 3a) and X (vagus; N10 in Fig. 3a) formed a single bundle (N9/10 in Fig. 3c) displaying aberrant projections towards the 2nd pharyngeal arch (large arrow in Fig. 3c). The distal ganglia of these nerves were fused, as confirmed by anti-Islet-1 immunostaining at E10.5 and histological sections at E10.5 and E11.5 (G9/10; compare Fig. 3b and d and Fig. 5e and f). Moreover, the fibers of cranial nerve XII (hypoglossal) were not fasciculated (compare N12 in Fig. 3a and c) and the diameter of this nerve was reduced at E11.5 (see brackets in Fig. 5e and f). These early defects of hypoglossal nerve formation likely account for the complete absence of the hypoglossal nerve foramen in the basioccipital bone of  $A\alpha/A\beta$  fetuses (Ghyselinck et al., 1997).

All components of the first two pharyngeal arches were apparently normal (Fig. 1a, b, e and f; Figs. 2 and 3). Thus,  $A\alpha/A\beta$  mutants selectively display fusion of the 3rd and 4th pharyngeal arches and other regionally restricted abnormalities affecting the 3rd and 4th endodermal pouches, as well as the 3rd, 4th and 6th pharyngeal arch arteries and nerves.

#### Absence of obvious alterations in the number and migratory paths of neural crest cells

The issue of NCC migration was investigated by whole-mount in situ hybridisation with a probe recognizing cellular retinoic

acid binding protein I (CRABPI) transcripts. In E9.5 WT embryos, CRABPI is strongly expressed in two broad subectodermal sheets of NCC arising from rhombomere 4 (r4) and from r6 and r7 (Fig. 4a; Maden et al., 1992); these NCC colonize the periphery of the 2nd and of the 3rd and 4th pharyngeal arches respectively, enveloping groups of CRABPI-negative mesenchymal cells derived from the paraxial mesoderm (Fig. 4b; Trainor and Tam, 1995). We did not detect any significant alteration in the number and migratory paths of NCC in the pharyngeal arches of E9.5  $\alpha/\beta$  embryos after labeling with the CRABPI probe (Fig. 4).

### Morphological defects of the otocysts and alterations of segmentation and segmental identities in the post-otic hindbrain

Histological sections through the hindbrain of E9.0 ( $n=1$ ), E9.5 ( $n=3$ ) and E10.5 ( $n=3$ )  $\alpha/\beta$  embryos revealed a marked enlargement of r5 and an absence of the morphological boundary between r6 and r7 (Fig. 5a-d). In contrast, the first four rhombomeres (r1-r4) were indistinguishable from their WT homologs (Fig. 5a-d and data not shown). At E9.5 and E10.5, the mutant otocysts were of normal size and shape, but they were facing the enlarged r5 instead of lying close to the r5/r6 boundary as is the case in WT embryos (O in Fig. 5c and d and data not shown). In 3 out of 5 mutant embryos a small unilateral vesicle lined by epithelial cells was present lateral to the caudal, unsegmented, portion of the hindbrain (O2 in Fig. 5d). In the  $\alpha/\beta$  embryo analysed at E9.0, the otic pits were enlarged and apparently partially duplicated (O and O2; compare Fig. 5a and b). Altogether, these observations indicate that in  $\alpha/\beta$  embryos, the position of the normal otocyst is shifted rostrally and that the otic placode gives rise to supernumerary otocysts.

With the aim of further investigating the alterations of the post-otic hindbrain segmentation in  $\alpha/\beta$  mutants and their molecular basis, the in situ hybridisation expression pattern of different axial molecular markers was analysed. *Kreisler* expression in the hindbrain precedes the appearance of rhombomere boundaries and is required for the formation of r5 (Manzanares et al., 1999b) or of r5 and r6 (McKay et al., 1994; Cordes and Barsh, 1994; Moens et al., 1996). Whole-mount in situ hybridizations were carried out with the *kreisler* probe on two E8.5 embryos at the 7 and at the 10 somite stages. In WT embryos at the 7 somite stage, *kreisler* is expressed in a single domain corresponding to the prospective r5 and r6 (Fig. 6a); at the 10 somite stage, an additional domain appears in the roof plate of the anterior hindbrain (arrow in Fig. 6b), as previously described in the chick embryo (Eichmann et al., 1997). Strikingly, in  $\alpha/\beta$  embryos at the 7 and 10 somite stages, the expression domain of *kreisler* corresponding to the prospective r5/r6 territory was twice the size of the WT domain (Fig. 6a and b). Moreover, at the 7 somite stage, the sharp caudal limit characteristic of the WT expression domain was missing (Fig. 6a). *Kreisler* expression in the roof plate of the pre-otic rhombomeres was not affected in the  $\alpha/\beta$  embryo analyzed at the 10 somite stage (Fig. 6b).

Inactivation of *Krox-20* has revealed its crucial

role in the maintenance of the hindbrain territories corresponding to presumptive r3 and r5 (Schneider-Maunoury et al., 1993, 1997; Swiatek and Gridley, 1993). *Krox-20* expression was analysed at E9.25, i.e. at a stage when it is normally expressed simultaneously in two non-adjacent, sharply defined, stripes coinciding with r3 and r5 (Fig. 6c). In  $\alpha/\beta$  mutants, the *Krox-20* expression in r3 was not affected (r3 in Fig. 6c and data not shown). In contrast, the caudal stripe of *Krox-20* expression was enlarged and its posterior limit did not coincide with the r5/r6 morphological boundary but extended to the middle of r6. Furthermore, patches of *Krox-20*-expressing cells were detected throughout the presumptive r6 territory (Fig. 6c).

Genes in the 3' region of the *Hox* clusters are segmentally expressed in the embryonic hindbrain with sharp anterior expression limits coinciding with rhombomere boundaries. A direct correlation exists between *Hox* gene expression and commitment to a specific rhombomere fate (Rijli et al., 1998). In the neural tube of E9.5 WT embryos, *Hoxb-1* is expressed exclusively in r4 (Fig. 7a; Murphy et al., 1989); *Hoxb-3* expression has an anterior limit at the r4/r5 boundary and is specifically up-regulated in r5 (Fig. 7b; Hunt et al., 1991); *Hoxd-4* transcripts are detected in the neurectoderm posterior to the r6/r7 boundary and at a much lower level in the paraxial mesoderm posterior to the somites 5/6 junction (Fig. 7c and d and data not shown; Morrison et al., 1997 and references therein). In E9.5  $\alpha/\beta$  embryos, *Hoxb-1* was expressed at a high level in r4 and at a lower level in an ectopic domain showing an anterior limit at the prospective r6/r7 boundary (Fig. 7a); the domain of stronger *Hoxb-3* expression was enlarged as it comprised both r5 and r6 (Fig. 7b); *Hoxd-4* expression in the neural tube was abolished except in the most caudal region of the embryo (Fig. 7c,d). Note that *Hoxd-4* expression was not down-regulated in the forelimb bud (L in Fig. 7d), which therefore provided an in situ hybridisation internal positive control. Interestingly *Hoxb-4* expression was not altered in E9.5  $\alpha/\beta$  embryos.

In WT embryos, the level of CRABPI transcripts are high in r4 to r6 and gradually decreases in r7 and more posterior regions of the neural tube (Fig. 4a; Ruberte et al., 1991; Maden

**Table 1. Abnormal patterns of the pharyngeal arch arteries in individual  $\alpha/\beta$  embryos**

Arch artery	Individual $\alpha/\beta$ embryos												Normal derivatives of arch arteries
	E9.5						E10.5						
	a		b		c		d		e		f		
	L	R	L	R	L	R	L	R	L	R	L	R	
2	+	+	+	+	+	+	NA <sup>‡</sup>						Stapedial arteries
3	-	+	*	+	+/-	+/-	+	+	+	-	+	+	Common carotids
4	NA <sup>§</sup>						-	-	+	+	-	-	Arch of aorta or subclavian artery
6							-	+	-	-	-	-	Pulmonary arteries

a-f individuals analysed on serial histological sections. L and R, left and right sides, respectively; +, - and +/-, artery is of normal diameter, lacking and hypoplastic, respectively; \*artery is fused with its caudal counterpart; NA<sup>‡</sup>, not applicable as this artery is regressing at E10.5; NA<sup>§</sup>, these arteries are not yet formed or are being formed at E9.5.

et al., 1992). In  $\text{A}\alpha/\text{A}\beta$  mutants, the CRABPI domain of stronger expression had expanded caudally, reaching the level of the first spinal ganglion (SG in Fig. 4a).

## DISCUSSION

### A localized and partial state of functional retinoic acid deficiency in hindbrain and pharyngeal arches of $\text{RAR}\alpha/\text{RAR}\beta$ null embryos

In  $\text{A}\alpha/\text{A}\beta$  mutants, the morphology of the pouches, arteries and nerves of the two rostral pharyngeal arches is normal; the molecular identities of the first 4 rhombomeres also appears normal as judged from the expression patterns of *kreisler* in the rostral hindbrain, of *Krox-20* in r3 and of *Hoxb-1* in r4. Altogether, these data indicate that this double null mutation selectively alters the development of the 3rd and 4th pharyngeal structures and of the 6th arch artery, as well as the segmentation and molecular addresses of r5, r6 and r7. Thus, the analysis of  $\text{A}\alpha/\text{A}\beta$  mutant embryos strongly suggests that RA signaling plays an essential role in patterning of the post-otic hindbrain and caudal pharyngeal arches. However, most probably because of the extensive functional redundancy between the 3 RAR isotypes (Kastner et al., 1995, 1997; Mascrez et al., 1998), the defects observed in  $\text{A}\alpha/\text{A}\beta$  embryos only partially recapitulate those generated in these structures by a full RA deficiency (Maden et al., 1996; White et al., 1998). It is also noteworthy that the abnormalities found in  $\text{A}\alpha/\text{A}\beta$  mutants are confined to the region which co-expresses  $\text{RAR}\alpha$  and  $\text{RAR}\beta$  in WT embryos, with an anterior limit that corresponds to the level of the presumptive otocyst (Ruberte et al., 1991; Ang and Duester, 1997; Mendelsohn et al., 1994a). In contrast, the study of mice lacking *RALDH2*, a major RA synthesizing enzyme (Niederreither et al., 1999), has shown that the development of the second pharyngeal arches also requires RA.

### RA signaling is required for the specification of the otogenic field

In  $\text{A}\alpha/\text{A}\beta$  mutants, small ectopic otic vesicles are formed from an enlarged otic pit that also gives rise to a much larger orthotopic otocyst. Supernumerary otic vesicles are also seen in retinoid-deficient rat embryos (White et al., 1998) and in mouse embryos lacking both  $\text{RXR}\alpha$  and  $\text{RXR}\beta$  (Wendling et al., 1999). Altogether, these data establish a key role for RA-activated  $\text{RXR}/\text{RAR}$  heterodimers in the specification of the otic placode from the ectoderm. Several lines of evidence suggest that the morphogenesis of the otocyst is critically dependent on signal substances secreted by neuroepithelial cells at the r5/r6 level (for references see Mark et al., 1993). Therefore, the systematic association of ectopic otic vesicles with a marked enlargement of r5 in  $\text{A}\alpha/\text{A}\beta$  embryos (as well as in  $\text{RXR}\alpha^{-}/\text{RXR}\beta^{-}$  embryos; O. W., P. C. and M. M., unpublished results), strongly suggests that these two abnormalities are causally related. Surprisingly, the putative otogenic signals emanating from the neural tube after the formation of the otocyst are apparently not disturbed, as E14.5 and E18.5  $\text{A}\alpha/\text{A}\beta$  fetuses have normal inner ears, and ectopic inner ears are never maintained at the fetal stages of development.

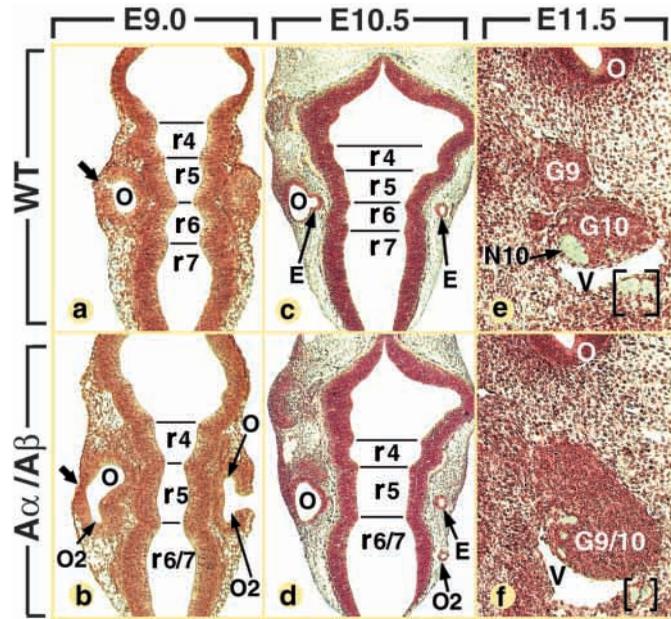
### Independent effects of RA signaling on the development of neural crest, arch arteries and pharyngeal endoderm

All  $\text{A}\alpha/\text{A}\beta$  fetuses faithfully recapitulate the spectrum of abnormalities affecting the thymus and parathyroid glands as well as the great cephalic arteries and the aorticopulmonary septum observed in the chick following surgical ablation of large portions of post-otic NCC premigratory precursors (Kirby and Waldo, 1990; Ghyselinck et al., 1997). Therefore, we have previously proposed that a massive, generalized, deficit in NCC emigrating from r5 and more caudal regions of the rhombencephalon could account for this spectrum of defects in the  $\text{A}\alpha/\text{A}\beta$  mutants. Our analysis of CRABPI transcript distribution indicates that this is not the case, as the production and migration pattern of NCC are apparently normal in  $\text{A}\alpha/\text{A}\beta$  embryos. The morphological analysis of  $\text{A}\alpha/\text{A}\beta$  embryos also demonstrates that impaired RA signaling affects the formation of endodermal (3rd and 4th branchial pouches) and mesodermal (endothelial lining of the arch arteries) structures. NCC are indispensable for the maintenance of the arch arteries, but not for their initial formation (Bockman et al., 1989; Waldo et al., 1996). The necessity of RA signaling for the formation of the 3rd, 4th and 6th arch arteries suggests therefore that RA acts either on the migration of angioblasts or on the assembly of cords of endothelial cells (Noden, 1991). Likewise NCC play apparently no role in the patterning of the branchial pouches (Bockman et al., 1989). As the thymus and parathyroid glands are largely derived from the endoderm of the 3rd branchial pouches (Le Douarin, 1982 and refs therein), the present hypoplasia of these pouches in  $\text{A}\alpha/\text{A}\beta$  embryos is sufficient to account for the glandular defects observed at later stages in  $\text{A}\alpha/\text{A}\beta$  fetuses (Ghyselinck et al., 1997; Mark et al., 1998; Peters et al., 1998). Delayed formation of the 3rd pouches in  $\text{A}\alpha/\text{A}\beta$  embryos may also be responsible for the observed fusions between structures belonging to the 3rd and 4th pharyngeal arches (i.e. fusions of the 3rd and 4th arch arteries, of the IXth and the Xth nerves and of their distal ganglia), as well as for the fusion of the (3rd arch-derived) greater horns of the hyoid bone and the (4th arch-derived) thyroid cartilage seen in almost all  $\text{A}\alpha/\text{A}\beta$  fetuses (Ghyselinck et al., 1997).

### RA signaling controls caudal hindbrain segmentation by restricting the expression domains of *kreisler* and *Krox-20*

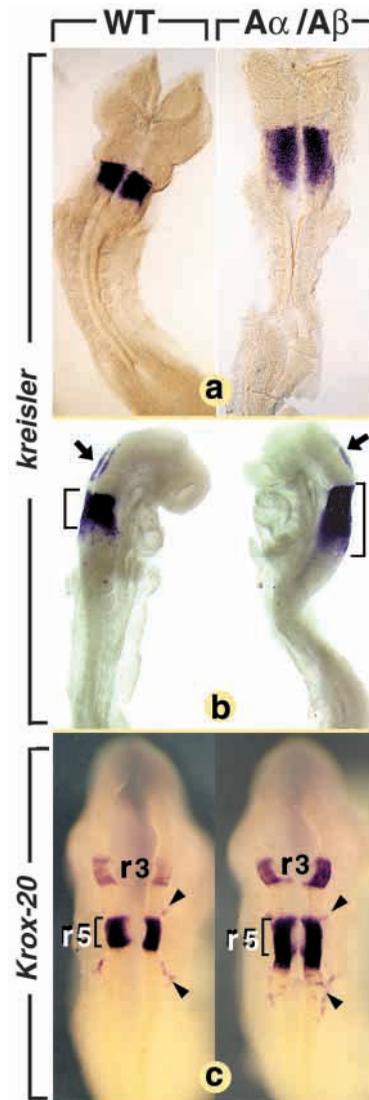
In  $\text{A}\alpha/\text{A}\beta$  embryos, the enlargement of r5 and loss of the r6/r7 morphological boundary is spatially correlated with and preceded by ectopic expression of *kreisler* in the caudal hindbrain (Fig. 8). This finding adds to the notion that *kreisler* has a crucial role in segmentation (McKay et al., 1994; Cordes and Barsh, 1994; Moens et al., 1996; Manzanares et al., 1999a). Previous studies have popularized the idea that expression of *Krox-20* and acquisition by a given cell of r3 or r5 identities are equivalent (reviewed by Guthrie, 1996). However, the fact that in  $\text{A}\alpha/\text{A}\beta$  mutants, the caudal expression domain of *Krox-20* extends beyond the r5/r6 boundary indicates that the posterior limit of *Krox-20* expression is not sufficient to define a r5/r6 boundary (Fig. 8).

Excess RA can inhibit *kreisler* expression in the chick and mouse embryo hindbrain (Grapin-Botton et al., 1998; O. W., P. C. and M. M., unpublished results) and studies on different



**Fig. 5.** Frontal histological sections at comparable levels of the hindbrain of WT and  $A\alpha/A\beta$  mutants at E9.0 (a,b), E10.5 (c,d) and E11.5 (e,f). E, endolymphatic duct of the otocyst; G9 and G10, distal ganglia of the glossopharyngeal and vagus nerves respectively. G9/10, abnormal fusion of the distal ganglia of the glossopharyngeal and vagus nerves; N10, vagus nerve; O, orthotopic otocyst; O2, ectopic otocyst or ectopic portion of the otic placode; r4 to r7, rhombomeres 4 to 7; r6/7, presumptive rhombomere 6 and 7 region in the mutant hindbrain; V, anterior cardinal vein. The brackets encompass the hypoglossal nerve and the unlabelled large arrow indicates the point of closure of the otic placode. Magnifications:  $\times 40$  (a,b),  $\times 20$  (c,d),  $\times 65$  (e,f).

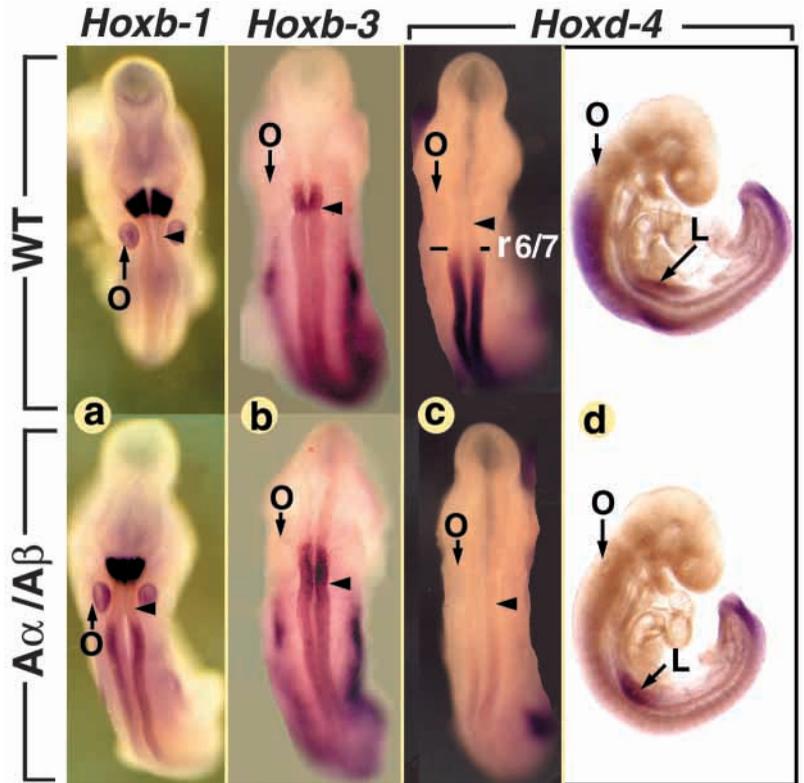
species have shown that RA imbalance disturbs *Krox-20* expression (Godsave et al., 1998; Gale et al., 1996; Wood et al., 1994; Van der Wees et al., 1998; Maden et al., 1996). Interestingly, the enlargement of the caudal stripes of *kreisler* and *Krox-20* expression in  $A\alpha/A\beta$  mutants reflects a state of functional RA deficiency, as they are similarly observed in mouse embryos cultured in the presence of a pan-RAR antagonist (O. W., P. C. and M. M., unpublished results). Thus, our present results support the conclusion that RA is required for the establishment of the restricted expression of *Krox-20* and *kreisler* in r5 and r5-r6 respectively, and strongly suggests that endogenous RA is a negative regulator of *kreisler* and *Krox-20* expression in the caudal hindbrain of mouse embryos. As it has been suggested that *kreisler* acts upstream of *Krox-20* in the control of hindbrain segmentation (Frohman et al., 1993; McKay et al., 1994), the increase of *Krox-20* expression in  $A\alpha/A\beta$  mutants might be secondary to the up-regulation of *kreisler*. Interestingly, the r5/r6 expression domain of *kreisler* is also enlarged in  $RXR\alpha/RXR\beta$  double null mutants (O. W., P. C. and M. M., unpublished results). Altogether, our data demonstrate that RA signaling through  $RAR\alpha$  and/or  $RAR\beta$ , most probably in the form of heterodimers with RXRs (Kastner et al., 1997; Mascres et al., 1998), acts upstream of *kreisler* and *Krox-20* and consequently at the top of a genetic hierarchy involved in the control of caudal hindbrain segmentation.



**Fig. 6.** Distribution of *kreisler* (a,b) and *Krox-20* (c) transcripts in WT and  $A\alpha/A\beta$  embryos. (a) E8.5 (7-somite stage), dorsal view; (b) E8.5 (10-somite stage), lateral view; (c) E9.25, dorsal view. r3 and r5, rhombomeres 3 and 5. The arrows and brackets in b indicate the anterior and posterior domains of *kreisler* expression, respectively; the arrowheads in c point to neural crest cells expressing *Krox-20*.

#### Alteration of the 'Hox code' in $RAR\alpha^{-1}/RAR\beta^{-1}$ mutants indicates a posteriorizing function of RA signaling in caudal hindbrain patterning

The combination of expressed *Hox* genes ('Hox code') is altered in the r6/r7 region of  $A\alpha/A\beta$  mutants (Fig. 8). *Hoxd-4*, whose promoter contains a typical consensus RA response element (RARE) (Morrison et al., 1997 and references therein) is not expressed. The loss of *Hoxd-4* anterior expression at the cervical, thoracic and lumbar levels of the neural tube of  $A\alpha/A\beta$  mutants provides direct evidence that RA signaling is indispensable for either its induction or maintenance. The expression of *Hoxb-3* in the  $A\alpha/A\beta$  r6 is increased and 'anteriorized' to a level similar to that observed in WT r5. This is probably a direct consequence of the enlargement of the *kreisler* expression domain (Fig. 8), as *kreisler* is directly

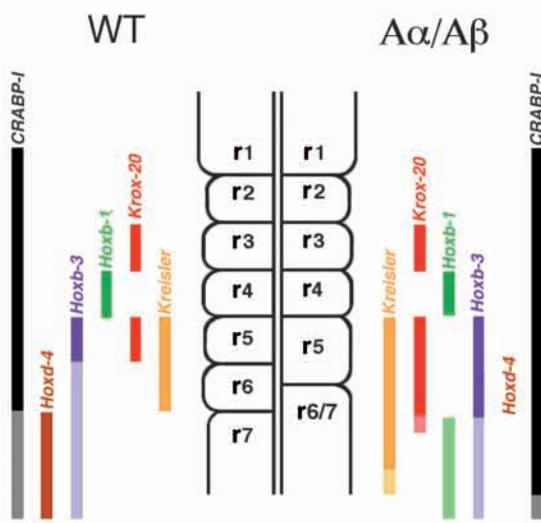


**Fig. 7.** Distribution of *Hox* gene transcripts on dorsal (a-c) and lateral (d) views of E9.5 WT and  $A\alpha/A\beta$  embryos. L, forelimb bud; O, otocyst; r6/7, boundary between rhombomeres 6 and 7. The arrowhead indicates the boundary between rhombomeres 5 and 6. Note that the signal observed in the otocyst with the *Hoxb-1* probe is an artefact.

responsible for the relatively stronger expression levels of *Hoxb-3* in WT r5 (Manzanares et al., 1999a). In WT, *kreisler* also directly up-regulates the expression of *Hoxa-3* in r5 and r6 and *Krox-20* controls directly the up-regulation of *Hoxa-2* and *Hoxb-2* in r3 and r5 (see references in Manzanares et al., 1999a). Thus, in addition to *Hoxb-3*, several ‘anterior’ *Hox* genes may be over-expressed in the presumptive r6 region of  $A\alpha/A\beta$  mutants, in response to the marked enlargement of the

*kreisler* and *Krox-20* expression domains. *Hoxb-1* is ectopically expressed in the  $A\alpha/A\beta$  r6/r7 region. Three RAREs have been identified in the regions flanking the murine *Hoxb-1* gene, which are conserved in the chick (Marshall et al., 1994; Huang et al., 1998; Studer et al., 1994). Two of these RAREs exhibit enhancer properties, while the third one has a silencer activity that restricts *Hoxb-1* expression to r4 at E9.5. Interestingly, in E9.5  $A\alpha/A\beta$  embryos, the ectopic expression domain of *Hoxb-1* in the neurectoderm caudal to the presumptive r6/r7 boundary is a normal feature of *Hoxb-1* expression in the chick embryo at a comparable developmental stage (Sundin and Eichele, 1990). Thus, another yet undiscovered, silencer containing a RARE may exist in the mouse *Hoxb-1* locus, with no counterpart in the chick.

There is increasing evidence implicating RA as a posteriorizing factor in the hindbrain. Excess RA treatment leads to an anterior shift in the expression domains of many *Hox* genes and concomitant posterior transformations of rhombomere identities (reviewed by Conlon, 1995). Exogenous RA can mimic the effects of graded endogenous posteriorizing signals generated by the somites on *Hox* genes and *kreisler* expressions in the hindbrain (Gould et al., 1998; Grapin-Botton et al., 1997, 1998; Itasaki et al., 1996). Binding sites for RXR/RAR heterodimers which are functional in vivo are present in several of the *Hox* genes expressed in the rhombencephalon (Gould et al., 1998 and references therein; Dupé et al., 1997; Huang et al., 1998; Gavalas et al., 1998), arguing that these heterodimers may directly regulate *Hox* genes in real life. We show here that a state of RAR deficiency induces ectopic posterior expression or overexpression of *kreisler*, *Krox-20*, *Hoxb-1* and *Hoxb-3*, which corresponds to an anterior transformation of the molecular identities of rhombomeres 6 and 7 (Fig. 8). Thus, our results support the



**Fig. 8.** Diagram of the expression patterns of *Kreisler* (E8.5), *Krox-20* (E9.25), *Hoxb-1*, *Hoxb-3*, *Hoxd-4* and *CRABPI* (E9.5) in the normal and  $A\alpha/A\beta$  mutant rhombencephalon. The extent of the expression domains of the different genes is denoted by vertical lines. Fainter colors indicate a weaker expression level. r1-r7, rhombomeres 1 to 7; r6/7presumptive rhombomere 6 and 7 region.

view that RA is a transformer signal responsible for the anteroposterior patterning in this region of the central nervous system (Conlon, 1995).

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