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Vitamin A-deficient quail embryos have half a hindbrain and other neural defects

Malcolm Maden*, Emily Gale*, Igor Kostetskii† and Maija Zile†

Background: Retinoic acid (RA) is a morphogenetically active signalling molecule thought to be involved in the development of several embryonic systems (based on its effect when applied in excess and the fact that it can be detected endogenously in embryos). Here, we adopt a novel approach and use the vitamin A-deficient (A^-) quail embryo to ask what defects these embryos show when they develop in the absence of RA, with particular reference to the nervous system.

Results: We have examined the anatomy, the expression domains of a variety of genes and the immunoreactivity to several antibodies in these A^- embryos. In addition to the previously documented cardiovascular abnormalities, we find that the somites are smaller in A^- embryos, otic vesicle development is abnormal and the somites continue up to and underneath the otic vesicle. In the central nervous system, we find that neural crest cells need RA for normal development and survival, and the neural tube fails to extend any neurites into the periphery. Using general hindbrain morphology and the expression patterns of *Hoxa*-2, *Hoxb*-1, *Hoxb*-4, *Krox*-20 and FGF-3 as markers, we conclude that segmentation in the myelencephalon (rhombomeres 4–8) is disrupted. In contrast, the dorsoventral axis of the neural tube using *Shh,* islet-1 and *Pax*-3 as markers is normal.

Conclusions: These results demonstrate at least three roles for RA in central nervous system development: neural crest survival, neurite outgrowth and hindbrain patterning.

Addresses: *Developmental Biology Research Centre, King's College London, 26–29 Drury Lane, London WC2B 5RL, UK. †Department of Food Science and Human Nutrition, Michigan State University, East Lansing, Michigan 48824, USA.

Correspondence to: Malcolm Maden
E-mail address: udbl124@uk.ac.kcl.cc.bay

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Background

Before the role of any molecule in development can be fully established three criteria need to be fulfilled. Firstly, the molecule must be shown to be present in the embryo at the correct time and in the correct location. Secondly, the phenotype of the embryo should be altered when the molecule is present ectopically or at excessive levels. And thirdly, the phenotype of the embryo should be altered when the molecule is absent.

With regard to retinoic acid (RA) — a vitamin A derivative and morphogenetically active compound in development which is considered to be an important signalling molecule — two of these three criteria have been well established. RA has been detected endogenously in the embryos of all of the major vertebrate groups (fish, amphibians, birds and mammals) except reptiles. Within a given group, RA has also been detected endogenously in different embryonic fields. It is present during gastrulation in zebrafish [1] and *Xenopus* [2,3] embryos and in Hensen's node of the chick embryo [4], and RA is synthesized from retinol in the node of the mouse embryo [5]. These observations suggest that RA may be involved in the establishment of the initial anteroposterior axis of the embryo. Slightly later in development, RA has been detected in the developing central nervous system (CNS) of the day 10.5 mouse embryo at levels which are graded from the anterior to the posterior: in the forebrain and midbrain, very low levels of RA are observed; in the hindbrain slightly higher levels are found; and the spinal cord contains the highest levels [6]. Slightly later still, RA has been detected in the limb bud of both chick and mouse embryos [7,8] and with higher levels on the posterior side than the anterior side [7]. The same differential distribution is seen in the other limb system commonly studied, the regenerating amphibian limb [9].

The second criterion, that the embryonic phenotype should be altered by administration of RA has been widely reported and many embryonic systems are affected by RA. An RA implant can establish a secondary axis in chick embryos [10] and RA treatment at gastrulation or later stages induces ectopic activation of several *Hox* genes followed by the appearance of localized abnormalities in the hindbrain and associated branchial arches [11–16]. Most interestingly, some of the individual units of the hindbrain, the rhombomeres (see below) can be respecified by RA administration at these early stages — in both mouse

and zebrafish embryos, rhombomere 2 (r2) takes on some of the characteristics of a r4 phenotype [12,16]. RA also affects heart development [17,18], and in developing and regenerating limbs it induces complete duplication, so that two limbs are produced instead of one [19,20]. Thus, the embryonic systems which have been found to contain RA also seem to be affected by excessive levels of RA.

The third criterion, that the embryonic phenotype should be altered when the embryo is devoid of RA, is the subject of the work reported here, using the vitamin A-deficient quail as a model system. Previous studies using quails or chickens in this way have demonstrated that the most obvious defect is in the cardiovascular system [21–23]. The heart develops, although abnormally, but there is no circulatory system connecting the heart to the blood islands. Here we concentrate on defects in the central nervous system (CNS) of these vitamin A-deficient embryos by examining their anatomy and the expression domains of a variety of patterning genes, particularly the *Hox* genes.

Hox genes are known to be activated by RA both in culture [24] and *in vivo* [11–16]. The principles of activation — genes located at the 3′ end of the cluster are activated at lower concentrations of RA than those at the 5′ end, and 3′ genes are activated before 5′ genes — which were established in culture also apply *in vivo*. The region of the embryo which has become a focus of interest in the study of *Hox* genes is the hindbrain, as the anterior limits of expression of certain *Hox* genes coincide with the boundaries of the individual units of the hindbrain, the rhombomeres. During early development of the CNS, the initially smooth, cylindrical, neural tube develops periodic undulations within the hindbrain to form eight segmental units called rhombomeres [25,26]. These rhombomeres are lineage-restricted cellular compartments [27] and the odd and even rhombomeres have different adhesive properties [28]. Neuronal differentiation follows a segmental pattern, with neurons in even-numbered rhombomeres differentiating before those in odd-numbered ones [29]. It is particularly striking therefore that the patterns of expression of *Hox* genes in the hindbrain follows this two segment periodicity to a significant degree [30].

The coincidence between RA effects on the hindbrain, *Hox* gene studies centering on the hindbrain and the effects of RA on *Hox* genes leads us to hypothesize that endogenous RA is involved in the normal development of the hindbrain. An additional reason for believing this is that certain *Hox* genes contain specific upstream sequences known as RA-response elements (RAREs) to which the retinoic acid receptors (RARs) bind and activate transcription. So far, three *Hox* genes have been identified which contain such sequences, *Hoxa*-1 [31], *Hoxb*-1 [32,33] and *Hoxd*-4 [34], and we would therefore expect endogenous levels of RA to be critical in the expression of these genes.

The work reported here supports these hypotheses on the role of RA. We demonstrate that, in the vitamin A-deficient quail embryo, the myelencephalon of the hindbrain (r4–8) fails to develop normally. Anteroposterior organization of the neural tube is abnormal, therefore, but we show that its dorsoventral organisation is not affected. Two other neural defects are described: neural crest cells migrate mostly as normal, but then they appear to undergo cell death; and the neural tube fails to extend any neurites into the periphery. These results demonstrate multiple roles for RA in the development of the CNS.

Results

The vitamin A-deficient quail embryo

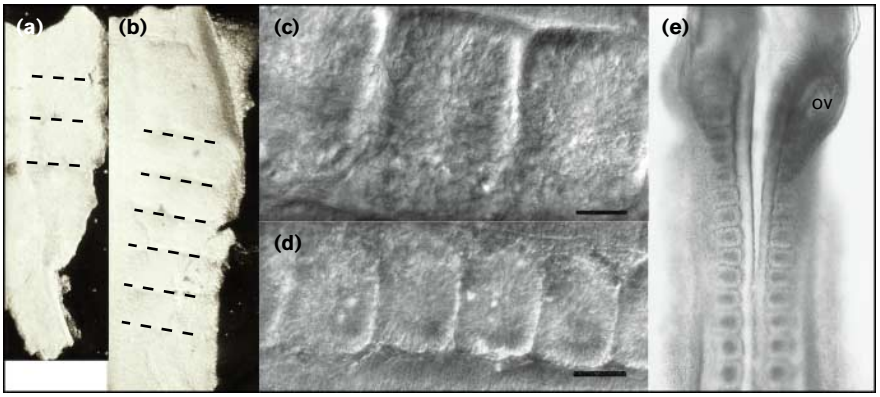
Vitamin A-deficient eggs and embryonic tissues have been shown to be lacking in detectable retinoids by several methods. Firstly, using high-pressure liquid chromatography (HPLC), no retinoids can be detected in the yolk or albumin, the former being white [35]. Secondly, using HPLC and the F9 reporter cell system, there are no detectable retinoids in the embryonic tissues [35,36]. Thirdly, there is no immunoreactivity in embryonic tissues with a specific anti-RA monoclonal antibody [37]. Fourthly, the embryos develop the characteristic cardiovascular abnormalities described before in vitamin A-deficient chicks [21] and quails [22]. Fifthly, the stage 5–8 embryo does not express RARβ2 [38], the RA-inducible form of the RARs [39]. And lastly, the cardiovascular defects can be rescued by the administration of various retinoids within the first 24 hours of development [23]. On the basis of this evidence, we conclude that these embryos are retinoid-deficient and that the defects described are due to this deficiency. They are referred to in the following text as A^- embryos.

The A^- embryo at stage 19/20

Embryos up to 72 hours of incubation (stage 19/20) were examined both externally and in serial sections. In general, A^- embryos were much smaller than controls, being about half the head-to-tail length of controls, but were very closely comparable in stages. In addition to the failure of the cardiovascular system to develop correctly (single closed distended ventricle, *situs inversus* and failure of omphalomesenteric veins and vitelline arteries to form) as has been well documented [21–23,36,37], abnormalities in the CNS, somites and the otic vesicle were clearly apparent in the A^- embryos.

External examination of the CNS showed no morphological evidence of the myelencephalon (r4–8). In contrast, the gross morphology of the telencephalon, mesencephalon and metencephalon appeared normal, including normally located constrictions of the neuroepithelium both between and within these brain segments (Fig. 1a,b). In the normal quail embryo, a flatmounted hindbrain showed six rhombomere boundaries (Fig. 1b), whereas a

Figure 1



(a) Flatmount of the hindbrain from a stage 16 A⁻ quail embryo showing that there are only 3 rhombomere boundaries (broken lines). **(b)** Flatmount of a similarly staged normal quail embryo at the same magnification as in (a), showing the presence of 6 rhombomere boundaries (broken lines). The hindbrain is clearly much shorter in A⁻ embryos. **(c)** Nomarski micrograph of cervical somites from a normal 19 somite quail embryo at 400× magnification. Bar = 100 μm. **(d)** Nomarski micrograph of cervical somites from an A⁻ embryo at the same stage and at the same magnification as a showing a considerable reduction in size. Bar = 100 μm. **(e)** Nomarski micrograph of a 19 somite A⁻ embryo showing that the somites continue up to and underneath the otic vesicle (ov).

similarly staged A⁻ embryo showed only three such boundaries (Fig. 1a) and the narrowing of the flatmount where the spinal cord began was much further rostral than normal. The detailed structure of the hindbrain is currently being studied by electron microscopy and the results will be presented elsewhere. The vibratome sections in Figure 4b,d show the essential features of the missing rhombomeres.

Although the number of somites in control and A⁻ embryos was roughly comparable (A⁻ embryos tended to have only two or three somites fewer than controls), the size of individual somites was very different (Fig. 1c,d). Control somites measured 230–250 μm in the rostrocaudal dimension, whereas the somites of A⁻ embryos were only 150–170 μm. Thus, there was a reduction of 30–40 % in somite length, giving a reduction of 75 % in somite volume. This must make a major contribution to the reduced overall size of the A⁻ embryos. Instead of the fragmentation of occipital somites which occurs in normal embryos posterior to the otic vesicle, the somites in A⁻ embryos continued up to and underneath the otic vesicle (Fig. 1e). Furthermore, the otic vesicle, although formed and located in the appropriate place, remained open. It failed to close and sink ventrally beneath the ectoderm (Fig. 2b).

Neural crest and neural tube development

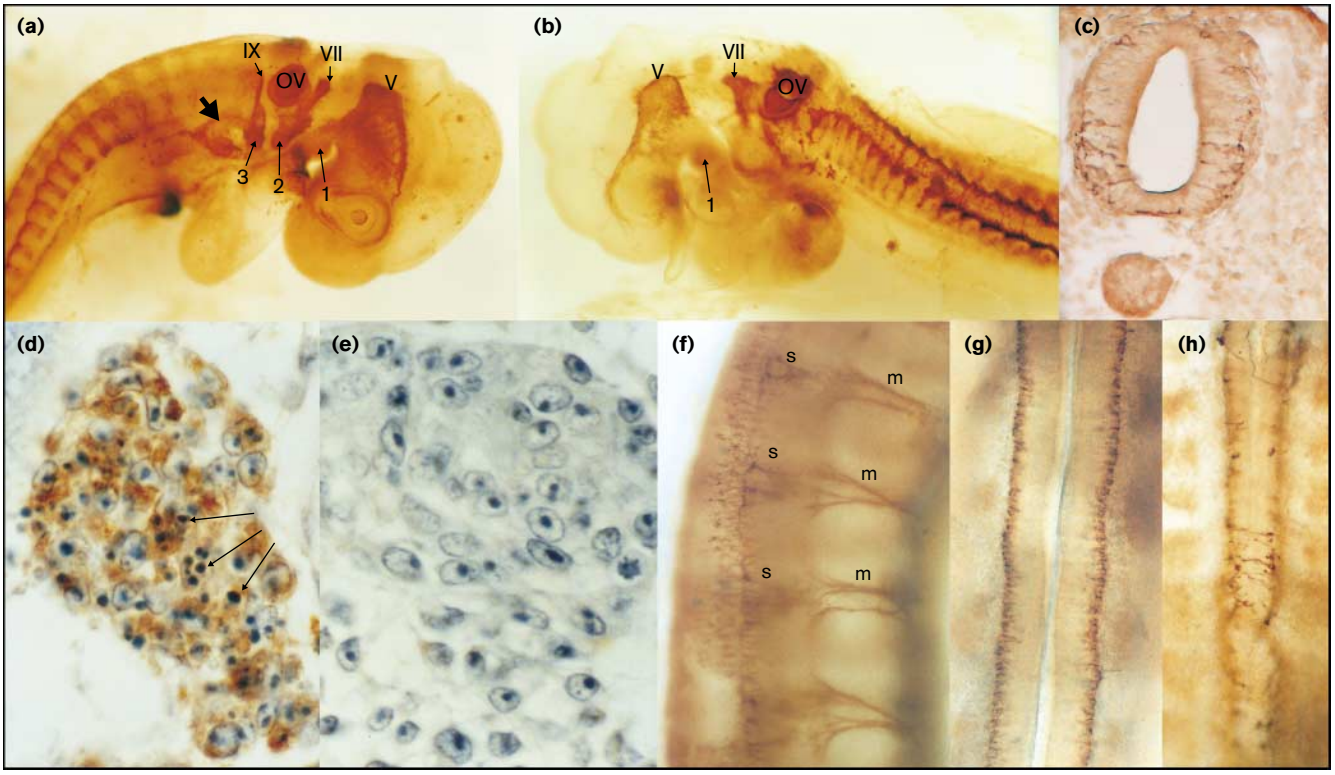
To examine the progress of neural crest development and neuronal differentiation we used HNK-1 and anti-neurofilament (anti-NF) antibodies both in wholemounts and sections.

HNK-1 wholemounts of normal embryos revealed the typical pattern of neural crest migration (Fig. 2a). The crest from r2 migrated anteriorly around the eye and ventrally into the first arch. The crest from r4 emerged beside the otic vesicle and migrated into arch 2. The post otic crest from r6,7 migrated into arch 3 and in a loop into the heart. More caudally, neural crest was seen in repeating stripes migrating through the anterior halves of each somite (Fig. 2a). In A⁻ embryos, the neural crest streams from r2 and from r4 were both fairly normal in direction although they seemed less intensely stained than might be expected (Fig. 2b). Post otically, however, the normally organized stream of crest from r6,7 into branchial arch 3 and a loop of crest into the heart (Fig. 2a) was completely absent and the crest pattern resembled that of the trunk (Fig. 2b).

In control embryos, the first three brachial arches were clearly visible as they were filled with HNK-1 positive cells (Fig. 2a). In A⁻ embryos, only the first arch was visible (Fig. 2b), arches 2 and 3 were not discernable as there were no branchial grooves, no accumulating HNK-1 cells were seen, and internally the aortic arches were fused to form one large blood vessel.

The abnormal internal anatomy was confirmed on sectioned material which also revealed two other features. Firstly, in the trunk, migration of the neural crest was not always normal. Instances of apparent inhibition of migration were seen with HNK-1 positive cells accumulating dorsal to the neural tube. Secondly, there was extensive cell death in A⁻ embryos, particularly in the streams of neural crest emanating from the neural tube at all rostrocaudal levels. Instead of the healthy crest cells which stained with HNK-1 in sections of normal embryos, pycnotic nuclei were present in a clump of HNK-1 positive cells in the region of the dorsal root ganglion of an A⁻ embryo (Fig. 2d). Pycnotic nuclei were very rarely seen in normal quail embryos, only their prominent nucleoli being noticeable (Fig. 2e). Sections of the head and branchial arches of A⁻ embryos revealed a dearth of mesenchymal cells which was presumably caused by the death of neural crest cells and which may also explain the

Figure 2



(a) HNK-1 stained normal 24 somite embryo, showing a typical pattern of neural crest migration pathways and branchial arches. The crest from r2 (V) migrates around the eye and also into the first arch. The crest from r4 (VII) moves ventrally beside the otic vesicle (ov) into arch 2. The post otic crest (IX) from r6,7 moves into arch 3 and in a loop into the heart (arrow). More caudally, neural crest can be seen in repeating stripes migrating through the anterior halves of each somite. Branchial arches 1, 2 and 3 (marked 1,2,3) can clearly be seen as they are filled with HNK-1 positive cells. **(b)** HNK-1 stained A⁻ embryo at 25 somites, showing a normal pattern of migration of crest from r2 (V) and crest from r4 (VII). The otic vesicle (ov) is open onto the surface of the embryo and has a tongue-like flap extending ventrally. Post otically there is no sign of any stream of crest into arch 3 (see IX in (a)), nor of a crest stream into the heart (see arrow in (a)). Instead, the pattern resembles that of the repeated stripes of crest migrating through the somites (although the somites are smaller – see Fig. 1). Thus, there seems to be no post otic hindbrain in A⁻ embryos. Furthermore, only brachial arch 1 (marked as 1) can be seen externally, the remaining arches are not present. **(c)** Section through a stage 17 A⁻ embryo stained with a neurofilament antibody showing some NF-positive neurites within the neural tube, but none extending into the periphery. **(d)** High-power view of a group of HNK-1 positive cells in an A⁻ embryo in the location of the dorsal root ganglia. There is a great deal of cell death here, as evidenced by the appearance of dark spheres with no cell membranes round them (arrows). **(e)** Cells in a similar location to (d) in a normal quail embryo showing the absence of any evidence of cell death. **(f)** Side view of a neurofilament stained wholemount of a normal stage 17 quail embryo showing neurites in the periphery forming motorneurons (m) and sensory neurons (s) pathways. **(g)** Dorsal view of a normal embryo as in (f). Here, the peripheral location and high density of NF-positive neurons can clearly be seen. **(h)** Dorsal view of a NF-stained wholemount of an A⁻ stage 17 quail embryo showing far fewer numbers of neurons which are positive. Also, no neurites are present in the periphery, and the course of the neurites is chaotic compared to the organized regularity of controls.

paler and less extensive staining of A⁻ embryos in HNK-1 wholemounts (Fig. 2b).

Staining with anti-NF antibodies [40] revealed that the development of the neural tube was severely affected in A⁻ embryos compared to controls, despite somite numbers and external stages being comparable between the two. Control embryos had developed a typical picture of normal development by stage 19/20, with motorneuron axons extending from the ventral neural tube into the periphery and neural crest-derived dorsal root ganglia extending axons back to the neural tube and out to the periphery (Fig. 2f). NF-positive neurons were located in the mantle layer of the neural tube where differentiation was taking place, as can be readily seen in dorsal views of wholemounts (Fig. 2g). In contrast, sections through A⁻ embryos of a similar stage revealed that there were no axons extending into the surrounding sclerotome (Fig. 2c) and the neural crest cells which had begun to accumulate in the position of dorsal root ganglia did not express NF proteins. There were, however, some neurons within the neural tube which expressed NF immunoreactivity, although they were far fewer in number than normal. Wholemounts of these embryos revealed that the NF-positive neurites

often followed chaotic trajectories — for example, in Figure 2h a neurite can be seen to have crossed the midline and turned rostrally, which was never observed in controls at this stage (Fig. 2g).

Anteroposterior patterning in the neural tube

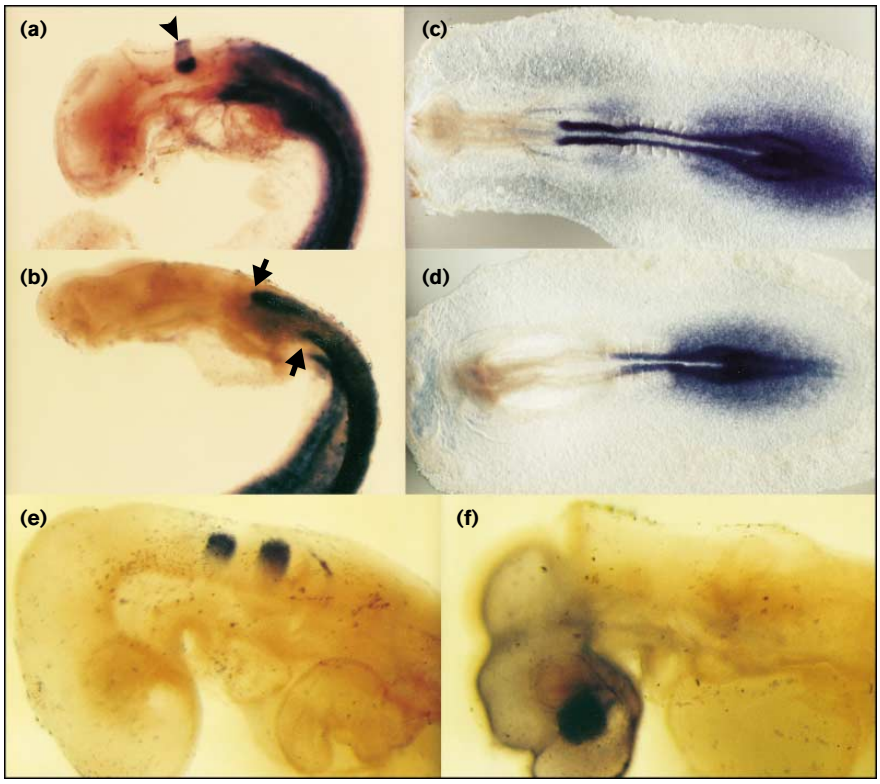
On the basis of this histological analysis, we hypothesized that the myelencephalon (r4–8) was missing in the A⁻ embryos, although from morphology alone no numbers could be assigned to those rhombomeres remaining or those missing. To identify rhombomeres more precisely, we used several markers in wholemount *in situ* hybridizations: *Hoxb*-1, *Hoxb*-4, *Krox*-20, *Hoxa*-2 and FGF-3.

Hoxb-1 was expressed as a single stripe in r4 and then posteriorly from the r6/7 border in both the CNS and mesenchyme of a normal 19 somite quail embryo (Fig. 3a), as it is in chick embryos [41]. From the posterior border of expression at r6/7, expression in the CNS and mesenchyme were in synchrony. In A⁻ embryos, the r4 stripe of *Hoxb*-1 was absent (Fig. 3b) and the posterior expression in the mesenchyme was out of synchrony with the CNS, the latter expressing further rostrally than normal. Examination of *Hoxb*-1 expression at an earlier stage of development (stage 8) revealed that, in the A⁻ embryo, expression did not extended so far anteriorly as in controls (Fig. 3c,d). This *Hoxb*-1 data suggests that, at the very least, r4 was missing and that the remaining CNS had moved rostrally to fill the gap. However, the rostral displacement was greater than the size of r4, suggesting that more neural tissue than just r4 had been lost or respecified.

Krox-20 was expressed in r3 and r5 in the normal quail embryo (Fig. 3e), as it is in the chick embryo [42]. In chicks and quails, expression of *Krox*-20 in both rhombomeres continues for a much longer time than in mouse embryos. In the A⁻ embryos at stage 16, no expression of *Krox*-20 was detected (Fig. 3f). This suggests that, in addition to r4, r3 and r5 also fail to be normally specified in A⁻ embryos, or that *Krox*-20 expression is dependent upon the presence of RA. We are currently examining further stages to determine whether *Krox*-20 expression appears and then fades, or never appears at all.

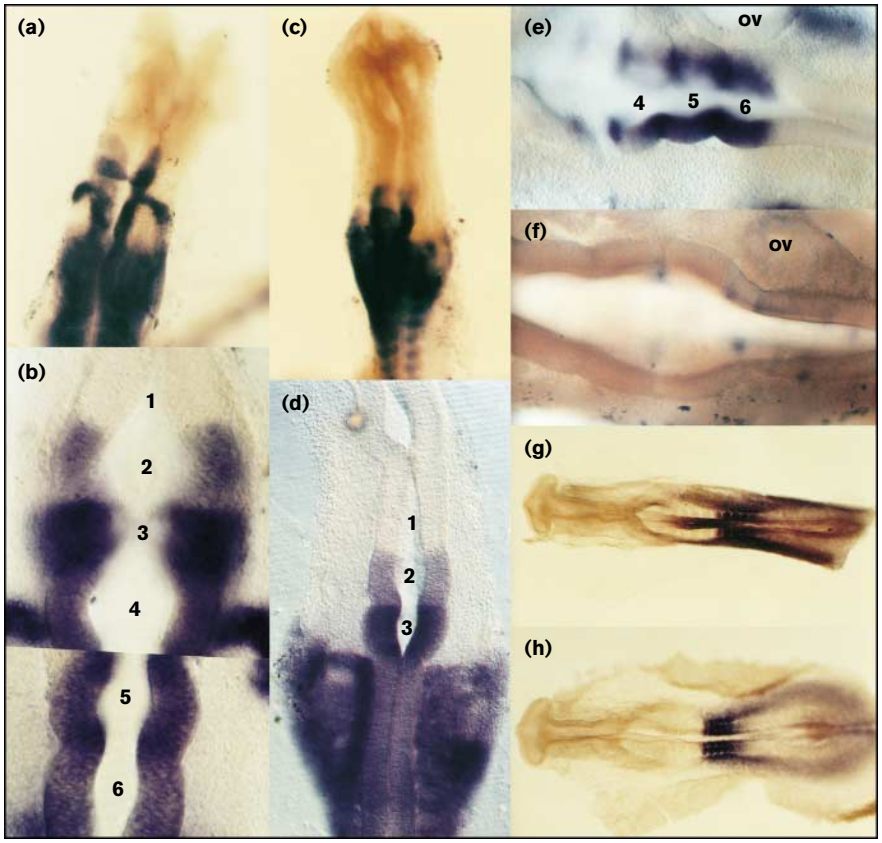
We next analyzed *Hoxa*-2, which is expressed both in the CNS and neural crest. It is expressed in the chick from the r1/2 border caudally and in the neural crest from r4 and r6 caudally [43]. This is a very useful gene for the identification of hindbrain units because of the variation in expression levels between rhombomeres as well as in neural crest. The 13 somite normal quail embryo showed the same expression pattern, including rhombomeric variations, as the chick: *Hoxa*-2 was not expressed in r1, was expressed weakly in r2, not in the crest from r2, strongly

Figure 3



Wholemount *in situ* hybridizations of normal and A⁻ quail embryos. **(a)** Stage 13 normal embryo showing *Hoxb*-1 expression domains as a stripe in r4 (arrowhead) and then throughout the posterior of the embryo from the r6/7 border. Note that the neural and mesenchymal expressions are in register from the posterior border backwards. **(b)** Stage 13 A⁻ embryo showing altered *Hoxb*-1 domains. The r4 stripe has gone and the neural and mesenchymal domains in the posterior of the embryo are now out of register. Upper arrow shows the rostral extent of the CNS domain; lower arrow shows rostral extent of the mesenchymal domain. **(c)** *Hoxb*-1 expression in a 5 somite normal embryo showing that the expression in the CNS extends a considerable distance rostrally from the first somite. **(d)** *Hoxb*-1 expression in a 5 somite A⁻ embryo showing that the expression in the CNS does not extend rostrally from the first somite, unlike the normal embryo in (c). **(e)** *Krox*-20 expression in a normal stage 15 embryo showing expression in r3 and r5. **(f)** *Krox*-20 expression in a stage 16 A⁻ embryo showing a complete absence of expression despite overexposure to DIG antibody which has reacted non-specifically with the anterior end of the embryo.

Figure 4



Wholemount *in situ* hybridizations of normal and A⁻ quail embryos. **(a,b)** *Hoxa*-2 expression in a normal stage 12 embryo, (a) wholemount and (b) sectioned through the hindbrain to show rhombomeric and neural crest patterns; r1–6 are marked in (b). *Hoxa*-2 is not expressed in r1, it is expressed weakly in r2, strongly in r3, less strongly in r4, in the migrating neural crest from r4 (arrows), strongly in r5, less strongly in r6, and in the neural crest from r6 posteriorly. **(c,d)** *Hoxa-2* expression in a stage 12 A⁻ embryo, (c) wholemount and (d) sectioned through the hindbrain. As in the control, r1 does not express *Hoxa*-2, r2 expresses it weakly and r3 expresses it strongly; more caudal to r3, there are no more rhombomeres and the mesenchymal expression becomes one large domain, as found in the normal embryo caudal to r7 (a). There is no stream of r4 neural crest, as in normal embryos. Rhombomeres 1–3 are marked. **(e)** FGF-3 expression in r4–6 of the hindbrain of a normal stage 14 embryo; ov, otic vesicle; 4, 5, 6 denote rhombomeres. **(f)** FGF-3 expression in the hindbrain of a stage 14 A⁻ embryo. Here, no FGF-3 expression can be detected in the neuroepithelium, confirming the absence of r4–6; ov, otic vesicle. **(g)** *Hoxb*-4 expression in a normal stage 11 quail embryo showing mesenchymal expression beginning at about somite 7 and CNS expression extending further rostrally to the spinal cord/hindbrain junction. **(h)** *Hoxb*-4 expression in an A⁻ stage 11 quail embryo showing that the CNS and mesenchymal expression are now in register because of the loss of specification of a segment of CNS (the myelencephalon).

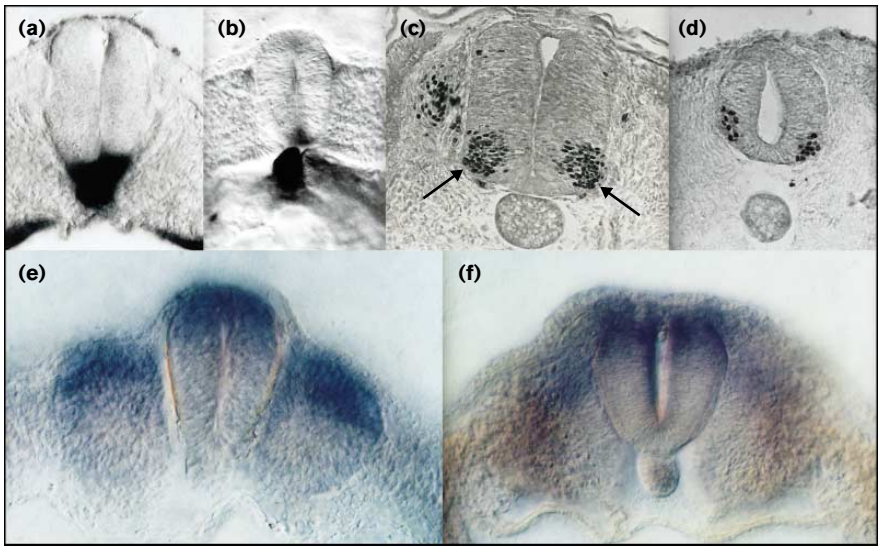
in r3, weakly in r4, strongly in the crest from r4 and strongly in r5 and more posterior rhombomeres (Fig. 4a,b). The A⁻ embryo, however, showed an aberrant expression of *Hoxa-2* (Fig. 4c,d). The absence of expression in r1, low expression in r2, lack of expression in crest from r2, and stronger expression in r3 seemed normal in these A⁻ embryos, but then the individual stream of r4 crest was lost in heavy expression normally associated with the area caudal to r6 (Fig. 4c). Sections of these *in situ* hybridizations confirmed that the rhombomeres posterior to r3 were missing in A⁻ embryos (Fig. 4d). On the basis of variations in levels of *Hoxa-2* expression, we conclude that the rhombomeres present in A⁻ embryos are r1–3, although the absence of *Krox*-20 expression does not fully concur with the presence of r3.

In order to confirm the loss of posterior rhombomeres, we examined the expression of fibroblast growth factor 3 (FGF-3). FGF-3 is expressed in r4–6 in the chick embryo [44]. In the normal quail embryo, the same expression pattern was found in r4–6 (Fig. 4e), but in the A⁻ embryo there was no FGF-3 expression in the hindbrain neuroepithelium (Fig. 4f). Finally, we examined the expression of *Hoxb*-4 which is normally expressed in the neural tube up to the r6/7 border [45] and in the normal quail was found in a similar location (Fig. 4g), although the precise borders of expression were stage-dependent. At stage 10, the expression of *Hoxb*-4 in the CNS extended further rostrally than the mesenchymal expression, the latter being at the level of somite 7 (Fig. 4g). In an A⁻ embryo of a similar stage, the CNS and mesenchymal expression were in synchrony, suggesting that a piece of CNS expressing *Hoxb*-4 anterior to somite 7 may have been lost (Fig. 4h). This loss was combined with a widespread decrease in *Hoxb*-4 expression in the CNS, while expression in the somites remained unchanged, indicating a general loss of control of *Hoxb*-4 expression in the CNS.

Dorsoventral patterning in the neural tube

As the anteroposterior patterning of the hindbrain was clearly abnormal in the A⁻ embryos, we also asked whether the same was true of the dorsoventral patterning. Dorsoventral CNS patterning is controlled by the notochord via the floorplate and is manifest in the differentiation of motorneurons in the ventral half of the neural tube [46,47]. To examine these aspects, we used *Sonic hedgehog*

Figure 5



(a,b) Expression of *Shh* in stage 14 (a) normal and (b) A⁻ embryos. The notochord and the floor plate are expressing *Shh.* In the normal embryo and in the A⁻ embryo, the notochord strongly expresses *Shh,* but the floor plate expression is considerably weaker than normal. **(c,d)** Expression of islet-1 in stage 19 (c) normal and (d) A⁻ embryos. In the normal embryo, immunoreactivity marks the location of motorneurons (arrows) and islet-1 positive cells are also present in the dorsal root ganglia. In the A⁻ embryo, a reduced number of islet-1 positive cells are present in the neural tube. In addition, there is no sign of a dorsal root ganglion and a large gap between the notochord and the neural tube is present. **(e,f)** Expression of *Pax*-3 in stage 14 (e) normal and (f) A⁻ embryos. In both cases, *Pax*-3 is expressed in the dorsal third of the neural tube and the dorsal part of the somites.

(*Shh*) expression as a marker for the notochord and floorplate, islet-1 expression as a marker for motorneurons, and *Pax-3* expression as a dorsal marker.

Shh was expressed in the notochord and floorplate of the 19 somite normal quail embryo (Fig. 5a), as it is in chick embryos [48]. A similar expression pattern was seen in the A⁻ embryo, although expression in the floor plate was not as strong as normal (Fig. 5b) — perhaps due to the decreased size of A⁻ embryos compared with controls. In addition, there was occasionally a large gap between the notochord and floor plate (visible in Figs 2c and 5d), whereas in normal embryos the two tissues were always touching (Fig. 5c), and this may have also been partly responsible for the decreased expression levels of *Shh* in the floor plate.

Using the antibody directed against islet-1 on sections of stage 19/20 quail embryos revealed a similar distribution to that described in the chick [49], namely in motorneurons, but also in cells of the dorsal root ganglia (Fig. 5c). In A⁻ embryos, cells expressing islet-1 were also present in the correct location, although in reduced numbers compared with normal embryos (Fig. 5d). Again, this may have been simply due to the lag in development (although somite numbers were comparable between controls and A⁻ embryos) or it could have been due to the gap between the notochord and the neural tube referred to above.

As a final marker for dorsoventral polarity, we examined the expression of *Pax-3*. After neural tube closure, *Pax-3* is expressed in the dorsal third of the chick neural tube [50], and the same was true in the normal quail embryo (Fig. 5e). No difference in expression could be detected in the A⁻ embryo (Fig. 5f).

Despite differences in expression levels or cell numbers, which may be due to other factors, these results suggest that the basis of dorsoventral polarity is preserved in embryos which develop in the absence of vitamin A.

Discussion

RA is considered to play an important role in the development of the CNS (for review, see [51]). It induces the formation of neurites in neuroblastoma cells and embryonal carcinoma cells, and induces neurites in explanted or dissociated dorsal root ganglia and spinal cord. It is present endogenously in the developing CNS and, when applied in excess to embryos, it deletes or respecifies anterior rhombomeres of the hindbrain. It inhibits the migration of neural crest cells in culture [52] and respecifies neural crest migratory pathways *in vivo* [53]. We may therefore summarize the presumed targets of RA action on the CNS as: pattern specification, neurite outgrowth and the neural crest.

Here, we describe a crucial test of the role of RA in embryogenesis by examining the development of the vitamin A-deficient quail embryo. By all experimental criteria, the yolk and embryonic tissues of these animals are truly retinoid-deficient, and the cardiovascular defects can be rescued by RA administration at the appropriate time during development [23]. The CNS of these A⁻ embryos is deficient in three aspects: pattern specification, neurite outgrowth and the neural crest, justifying the conclusions reached from previous studies.

Neural defects

Neural crest cells were produced in apparently normal numbers in A⁻ embryos and initially followed normal pathways. After migration, however, many neural crest cells

exhibited a morphology typical of cell death (which we have confirmed in other unpublished studies). This resulted in a failure of the branchial arches to form or fill up with crest cells; the head was also sparsely populated with mesenchymal cells and the dorsal root ganglia failed to differentiate and express neurofilament proteins. These observations strongly support the hypothesis, derived from studies *in vitro*, that RA supports the survival and proliferation of crest-derived neuronal precursor cells [54], and suggest an interaction of RA with the genes of the apoptosis pathway.

The neural tube of A$^-$ embryos was considerably less well developed than normal and its walls were thinner. The use of an anti-NF antibody revealed that far fewer neurons than normal were expressing this protein, and that some of those that did so projected their neurites in highly abnormal directions. Furthermore, no neurons extended neurites outside the neural tube into the surrounding mesenchyme. This supports the many studies showing that RA induces neuronal differentiation, neurite extension and neurite elongation in a variety of explants, primary cultures and cell lines (for review, see [51]). It further suggests that a mesenchymal source of RA may be responsible for neurite extension into the periphery, and that certain aspects of neurite guidance in the CNS are disrupted in A$^-$ embryos.

Patterning of the CNS was also affected in the A$^-$ embryos, showing an intriguing differential effect on the anteroposterior axis *versus* the dorsoventral axis. The dorsoventral axis of the neural tube seemed relatively normal, as assessed by the expression of *Shh* in the notochord and floor plate, the expression of islet-1 in the motorneuron precursors and the expression of *Pax-3* in the dorsal neural tube. In contrast, anteroposterior patterning was highly abnormal because the posterior rhombomeres of the hindbrain failed to develop, indicating a disruption of the segmentation of the myelencephalon. The forebrain, midbrain and spinal cord seemed normal, but the hindbrain consisted of only three rhombomeres instead of the normal eight. Because the trigeminal neural crest pattern was normal and the expression of *Hoxa-2* was normal for the anterior hindbrain, we suggest that these rhombomeres are numbers 1, 2 and 3. However, this does not entirely accord with the patterns of gene expression, because the third rhombomere does not express *Krox-20* as it should if it were a normal r3. Thus, some other features of segmentation must also have been disrupted in these A$^-$ embryos.

In an attempt to identify which rhombomeres were abnormal, the expression patterns of *Hoxb*-1, *Hoxb*-4, *Krox*-20 and FGF-3 were examined. The r4 stripe of *Hoxb*-1 was absent and the CNS expression was more rostral than normal, *Krox*-20 was absent both in r3 and more caudally in r5, FGF-3 which is normally in r4–6 was completely missing, and a section of CNS expressing *Hoxb*-4 was absent, resulting in an apparent caudal shift in the CNS domain. Some of these features suggest that r4–8 are absent, implying that they were not specified during CNS development. Alternatively, the tissue may still be present without any visible signs of physical or molecular segmentation. Indeed, certain features of the gene-expression patterns were disrupted in a complex way, apparently unrelated to the simple loss of tissue such as the loss of the rostral stripe of *Krox*-20 and the overall down-regulation of *Hoxb*-4 in the CNS. Furthermore, gene-expression patterns are dynamic and change significantly throughout development, so future studies will examine the progression of expression domains in these A$^-$ embryos from early stages in order to distinguish between a failure of specification and a failure of segmentation.

It is striking that excess RA affects anterior rhombomeres [15,55], and that a deficiency of RA causes posterior rhombomeres to be affected (these results). The juxtaposition between these two phenomena seems to be at the r3/4 border — the metencephalon/myelencephalon border that coincides with the first morphological segment to appear within the hindbrain. This observation could suggest that RA is involved in early axial segment determination during gastrulation, as implied by the ideas about RA synthesis in the node [5]. However, why disturbing a gradient of RA with a high point at the posterior end should result in a missing segment towards the anterior end is not immediately obvious. Alternatively, the observation of a disrupted myelencephalon could suggest that RA is involved in a later segmentation event during neurulation. Perhaps RA acts to set the levels of expression of Hox genes and other patterning genes which are expressed in a segment-specific manner in the hindbrain, as recent work on the RAREs in the upstream sequences of the gene *Hoxb*-1 has emphasized [32,33].

Other embryonic defects

There were other defects in these A$^-$ embryos in addition to the problems of neural development. As described previously, there are gross abnormalities in the cardiovascular system. The heart has a single closed distended ventricle, there is *situs inversus*, and the omphalomesenteric veins and vitelline arteries fail to form [21–23]. Here, we show that, although there were a similar number of somites in control and A$^-$ embryos, the A$^-$ somites were smaller. This suggests a role for RA in overall size control of the body axis, as these observations are reminiscent of experiments in which small *Xenopus* embryos were generated by removing a large amount of tissue from the blastula, but the resulting somite number in the small embryos was the same as normal [56]. In both cases, each somite must be composed of fewer cells. Perhaps RA controls body size during gastrulation, as it has already been proposed that the node is source of RA which acts to set up the head-to-tail axis of the embryo [5].

A third defect is in the otic vesicle, which fails to close and internalize. Although this could indicate a role for RA in otic development, this is more likely to be a secondary effect due to the loss of neuroepithelium, which expresses FGF-3. The A⁻ disrupted otic phenotype is remarkably similar to the effect of treating the otic region of the chick embryo with antisense oligonucleotides or an antiserum to FGF-3 [57], supporting the postulated role for FGF-3 from the adjacent neuroepithelium in otic development.

Conclusions

RA is involved in the development of several embryonic systems. In the nervous system, neural crest cells need RA to survive and the neural tube fails to extend neurites into the periphery without RA. In such vitamin A-deficient embryos, anteroposterior patterning of the neural tube is abnormal, but the dorsoventral patterning is not. The abnormal anteroposterior patterning, as revealed by the absence or loss of segmentation of the myelencephalon, possibly develops due to an abnormality in the specification of mesoderm at gastrulation because these vitamin A-deficient embryos also have smaller somites which extend rostally underneath the otic vesicle. RA may therefore be involved in the establishment of pattern during gastrulation. The ability to rescue defects in the A⁻ embryo with the judicious administration of RA presents us with a valuable opportunity to discover more about the genetic control of pattern in the vertebrate CNS.

Materials and methods

Japanese quail (*Coturnix coturnix japonica*) were raised at the Michigan State University Poultry Research Farm. Normal fertilised eggs were obtained from quail hens fed a normal ration. Vitamin A-deficient eggs were obtained from quail hens fed a semipurified diet adequate in all nutrients except that 13-*cis*-retinoic acid (10 mg kg^{-1} of diet) was added as the only source of vitamin A. For a detailed description of the diet and the deficiency phenotype see Dersch and Zile [23]. Eggs were incubated at 38 °C and the embryos staged according to Hamburger and Hamilton [58]. Embryos were fixed at 4 °C overnight in 4 % paraformaldehyde, transferred to 100 % methanol and stored at −20 °C until use in *in situ* hybridization or transferred to PBS and stored at 4 °C until use in immunocytochemistry. Wholemount *in situ* hybridization and immunocytochemistry was performed as described previously [41,51].

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