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A novel role for retinoids in patterning the avian forebrain during presomite stages

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SUMMARY

Retinoids, and in particular retinoic acid (RA), are known to induce posterior fates in neural tissue. However, alterations in retinoid signalling dramatically affect anterior development. Previous reports have demonstrated a late role for retinoids in patterning craniofacial and forebrain structures, but an earlier role in anterior patterning is not well understood. We show that enzymes involved in synthesizing retinoids are expressed in the avian hypoblast and in tissues directly involved in head patterning, such as anterior definitive endoderm and prechordal mesendoderm. We found that in the vitamin A-deficient (VAD) quail model, which lacks biologically active RA from the first stages of development, anterior endodermal markers such as *Bmp2*, *Bmp7*, *Hex* and the Wnt antagonist *crescent* are affected during early gastrulation. Furthermore, prechordal mesendodermal and prospective ventral telencephalic markers are expanded posteriorly, *Shh* expression in the axial mesoderm is reduced, and *Bmp2* and *Bmp7* are abnormally expressed in the ventral midline of the neural tube. At early somite stages, VAD embryos have increased cell death in ventral neuroectoderm and foregut endoderm, but normal cranial neural crest production, whereas at later stages extensive apoptosis occurs in head mesenchyme and ventral neuroectoderm. As a result, VAD embryos end up with a single and reduced telencephalic vesicle and an abnormally patterned diencephalon. Therefore, we propose that retinoids have a dual role in patterning the anterior forebrain during development. During early gastrulation, RA acts in anterior endodermal cells to modulate the anteroposterior (AP) positional identity of prechordal mesendodermal inductive signals to the overlying neuroectoderm. Later on, at neural pore closure, RA is required for patterning of the mesenchyme of the frontonasal process and the forebrain by modulating signalling molecules involved in craniofacial morphogenesis.

Key words: Retinoids, VAD embryos, Forebrain, Avian

INTRODUCTION

The precise nature of signals and mechanisms underlying the process of AP patterning is not entirely understood. At present, two main models have been put forward to explain how these events work at a molecular level in the early vertebrate embryo. Whereas Spemann and collaborators proposed separate organizer centres for inducing different regions of the central nervous system (CNS) (Spemann, 1931; Spemann, 1938), Nieuwkoop postulated that during gastrulation, induction and patterning of the nervous system occur in two steps (Nieuwkoop et al., 1952; Nieuwkoop and Nigtevecht, 1954). During the first step or 'activation', neural fate is induced and the forebrain is specified, whereas later in development, some cells receive other signals that cause them to acquire a more caudal character ('transformation or caudalization step'). Recently, Stern and collaborators have proposed a modification of Nieuwkoop's model that consists of three successive steps (Foley et al., 2000; Stern, 2001). The first step occurs before gastrulation in which a labile, pre-neural and pre-forebrain state is induced in the overlying epiblast ('activation step'). This is mainly based on the observation that, in chick, the hypoblast, an early endodermal layer present before gastrulation, can transiently induce expression of early neural markers when transplanted in contact with competent epiblast (Foley et al., 2000). However, to maintain a forebrain or even a neural phenotype, cells must receive stabilizing signals ('stabilization step'). This maintenance function might be provided by prechordal plate mesendoderm and/or anterior head process, both of which emerge from the organizer region during gastrulation (Ang and Rossant, 1993; Foley et al., 1997; Pera and Kessel, 1997; Schier et al., 1997; Dale et al., 1999; Rowan et al., 1999; Camus et al., 2000). Finally, the 'transformation' step caudalizes the nervous system through transforming signals emanating from more posterior structures (reviewed by Wilson and Rubenstein, 2000). What are the

signalling molecules involved in patterning the early CNS? Candidate signals include fibroblast growth factors (FGFs), and components of the Nodal and Wnt pathway and/or their antagonists. Most of these molecules are expressed transiently before gastrulation in the chick hypoblast or in its mouse equivalent, the anterior visceral endoderm (AVE), and during gastrulation in node-derived structures (Varlet et al., 1997; Foley et al., 2000; Streit et al., 2000; Lawson et al., 2001; Chapman et al., 2002), suggesting conserved molecular mechanisms during the various steps of early AP patterning.

Like FGF and Wnt proteins, retinoic acid (RA) is mainly considered to be a posteriorizing factor with a high concentration posteriorly and a decreasing gradient toward the anterior end of the embryo (Chen et al., 1994; Maden et al., 1998; Maden, 1999). Retinoids comprise various isoforms, including all-trans-RA and 9-cis-RA, which are generated from vitamin A in a two-step oxidation process involving a group of enzymes belonging to the family of alcohol dehydrogenases (Duester, 2000; Ross et al., 2000). Retinoids act via specific nuclear receptors belonging to the steroid superfamily of ligand-activated transcription factors, which play fundamental roles in embryological morphogenetic processes (reviewed by Zile, 2001). The effects of retinoid signalling on the CNS have been analysed in gain- and loss-of-function experiments, which have generally revealed an involvement of RA in AP patterning. By adding exogenous RA or constitutively activating retinoic acid receptors (RAR) at early stages of development, the forebrain becomes dramatically reduced, whereas the hindbrain is expanded (Durston et al., 1989; Sive et al., 1990; Simeone et al., 1995; Avantaggiato et al., 1996; Zhang et al., 1996). To reproduce a loss-of-function of retinoid signalling, various strategies have been employed: vitamin A has been depleted in pregnant mothers of quails or rodents (Maden et al., 1996; Gale et al., 1999; White et al., 2000), dominant negative RARs have been injected into *Xenopus* embryos (Blumberg et al., 1997; Kolm et al., 1997; van der Wees et al., 1998) or, more recently, antagonists to RARs have been added in a temporally and spatially controlled fashion (Wendling et al., 2000; Dupé et al., 2001). These, and the inactivation of the retinaldehyde dehydrogenase *Raldh2* in mouse and zebrafish (Niederreither et al., 1999; Begemann et al., 2001; Grandel et al., 2002) have pointed to defects restricted mainly to the hindbrain and spinal cord regions. Thus, it remains unclear whether retinoids have an endogenous role in forebrain patterning.

Two sites of localized retinoid activity have been described in the developing forebrain at later stages. One site is localized in the rostrolateral head mesenchyme and has been visualized with RA-reporter mice; at this site, activity overlaps with a transient expression of *Raldh2* (Rossant et al., 1991; LaMantia et al., 1993; Wagner et al., 2000). A second source of RA synthesis is represented by the expression of *Raldh3* in the surface ectoderm and in a large part of the ventral rostral head at later stages (Li et al., 2000; Mic et al., 2000; Smith et al., 2001). It has been suggested that the ventral ectoderm RA source, which is also present in the chick embryo, might be involved in sustaining the outgrowth of the frontonasal process and forebrain (Schneider et al., 2001). This and other reports have therefore confirmed an essential role for retinoids in the maintenance of a proper craniofacial structure, mainly through the survival and proliferation of head mesenchyme (Smith et al., 2001). However, a direct role for RA in the early events of anterior neural patterning remains unclear.

In this report, we show for the first time that, in avian embryos, RA is synthesized in the hypoblast before gastrulation, and in anterior definitive endoderm and prechordal mesendoderm during gastrulation. As a first attempt to challenge the role of RA in anterior patterning, we used the well-established vitamin A-deficient quail model (VAD), which has been considered to be similar to an ‘RA knockout’ (Zile, 2001). We show that VAD embryos have altered molecular properties in the anteriormost endoderm, which comprises hypoblast and definitive endoderm, during the process of axial mesoderm specification. Furthermore, prechordal mesendodermal and prospective telencephalic markers are expanded posteriorly, and in early somite embryos increased apoptosis is detected in ventral neuroectoderm and foregut endoderm. At later stages, VAD quail embryos show a single undivided telencephalic vesicle, increased cell death in ventral mesenchymal and neuroectodermal cells and abnormal dorsoventral patterning in the diencephalon. Thus, we hypothesize that, in the forebrain, retinoids have a biphasic activity that reflects differential functional contexts of RA signalling in anterior regions: an early phase at gastrula stages, during which RA refines the rostrocaudal identity of the anterior neural plate by modulating signalling involved in axial mesoderm specification (this study); and a late phase at neural pore closure, during which RA is involved in the survival of the frontonasal mesenchyme mass and forebrain neuroectoderm (this study) (Schneider et al., 2001). We propose that during the early phase, RA might be one of the signalling molecules involved in the ‘stabilization step’ of the model proposed by Stern and colleagues (Foley et al., 2000; Stern, 2001).

MATERIALS AND METHODS

Embryo preparation and staging

Fertilized chick and quail eggs were incubated at 38°C until the required developmental stages. Incubation times and staging were as per Hamburger and Hamilton (Hamburger and Hamilton, 1951). Normal and VAD embryos were staged based on as many parameters as possible; however, in some cases, VAD embryos looked more differentiated than their normal counterparts despite having the same stage or somite number (differences in stage matching are indicated in the legends). VAD quail embryos were obtained from Japanese quail (*Coturnix coturnix japonica*) hens fed on a semi-purified diet with 13-cis-retinoic acid as the only source of vitamin A, as described in detail by Dersch and Zile (Dersch and Zile, 1993), whereas normal quail embryos were obtained from the same quail hens fed on a normal diet. Embryos for whole mount in situ hybridization and immunohistochemistry were fixed in 4% PFA/PBS at 4°C, whereas embryos for histology were fixed in Bouin’s solution (0.9% picric acid, 9% formaldehyde).

In situ hybridization and histological procedures

Whole-mount in situ hybridization with digoxigenin-labelled riboprobes was performed as previously described (Grove et al., 1998). Chick riboprobes were used in quail embryos after careful comparison with their equivalent published chick expression profiles. The *Gsc* riboprobe differs from the original version (Izpisua-Belmonte et al., 1993) by covering most of the 5′ part of the cDNA sequence (50 bp of 5′ UTR and 738 bp of coding sequence; see also

GenBank Accession Number X70471). Embryos for sectioning were embedded in 20% gelatine and fixed in 4% PFA/PBS for at least 2 days. Sections were cut at 30 μm on a vibratome and mounted in 90% glycerol before being photographed using a digital camera. For histology, embryos were embedded in Fibrowax and sectioned at 10 μm on a microtome. After the dewaxing and rehydration procedures, sections were stained with Haematoxylin and Eosin for 2 minutes.

Immunohistochemistry

Free-floating 30 μm gelatine-embedded sections were washed in PBS followed by three 1 hour washes in a blocking solution consisting of 5% goat serum and 1% Triton X-100. Sections were then incubated overnight with anti-phospho-Histone H3 (Upstate, USA) or anti-HNK1 (Zymed Laboratories, USA) at a concentration of 1:500. After three 1 hour PBS washes, sections were incubated overnight with Cy3 (Jackson IRL, USA) at a concentration of 1:100. For the TUNEL method we used the In Situ Cell Death Detection, POD kit (Roche), with some modifications. Free-floating sections were first washed in 1% Triton X-100 in PBS and, subsequently, incubated in the TUNEL-reaction mix provided in the kit for 3 hours at 37°C. Sections were then washed in 1% Triton X-100/PBS and incubated at 4°C overnight in the converter POD provided in the kit. All sections were mounted in 90% glycerol and viewed under a confocal microscope (BioRad, MRC-600).

RESULTS

The RA-synthesizing enzymes *Raldh2* and *Raldh3* are expressed in anterior tissues of pre-gastrula and gastrula chick embryos

To establish whether there are sources of retinoids in anterior regions, we looked at the expression of RA-synthesizing enzymes in the early chick embryo. We found that *Raldh2* starts to be expressed at stage XIV-2 in the posterior region where the primitive streak is formed (data not shown). By stage 3, *Raldh2* is expressed posteriorly in the elongating primitive streak and more anteriorly in the migrating primary hypoblast (Fig. 1A). By stage 4– the hypoblast expression is displaced further anteriorly into the forming germinal crescent, which extends bilaterally and caudally (Fig. 1B). In addition, *Raldh2* is expressed posteriorly in most of the primitive streak and in non-axial mesoderm as previously described (Berggren et al., 1999). At the definitive primitive streak stage, *Raldh2* expression is detected rostral to the node in the anteriormost endoderm, which is composed of hypoblast and anterior definitive endoderm (Fig. 1C; inset) (Vesque et al., 2000). At head-process stages, anterior *Raldh2* is expressed in the characteristic fan-shaped structure corresponding to prechordal mesendoderm and at lower levels in anterior endoderm (Fig. 1D; see Fig. 3A) (Seifert et al., 1993). Transverse sections along the rostrocaudal axis show strong *Raldh2* expression in prechordal mesendoderm cells and in non-axial mesoderm, whereas expression in the node is weak (Fig. 1D′-D′′). At early somite stages, anterior *Raldh2* expression is restricted to prechordal mesoderm and foregut endoderm, with a sharp posterior boundary at the level of the notochord (arrows in Fig. 1E,E′). Interestingly, we also found that *Raldh3* (previously known as *Aldh6*) is transiently expressed at stage 4+/5– in the node and in migrating mesodermal cells, as clearly shown in mid-sagittal sections (Fig. 1F,F′). This expression is quickly downregulated and, as already described, *Raldh3* expression becomes confined to the oral ectoderm at later stages (data not shown) (Suzuki et al., 2000). No expression of *Raldh1* has been

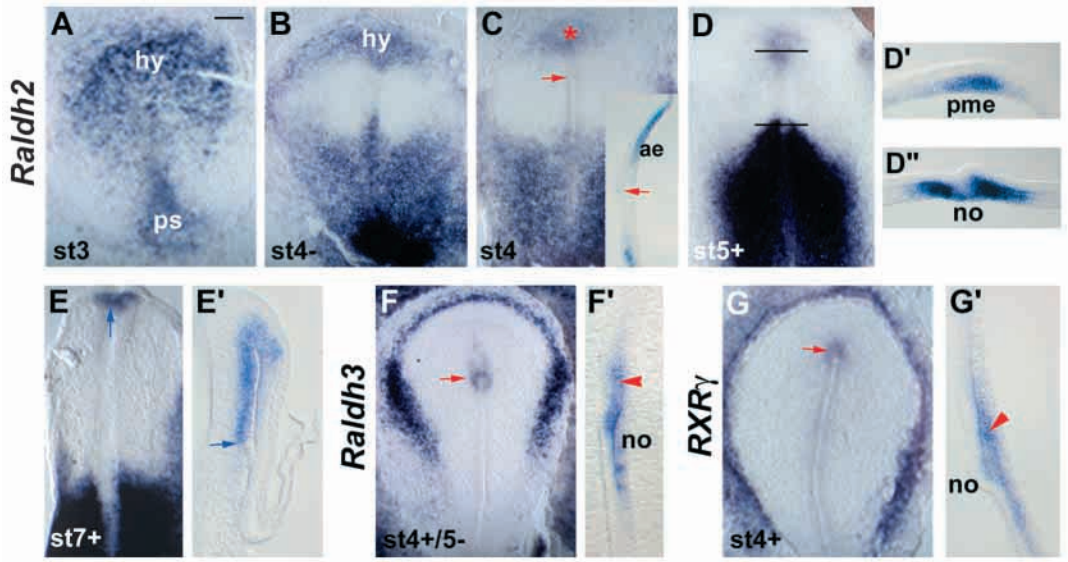

Fig. 1. Retinoids are synthesized in tissues involved in early anterior patterning. (A-G′) Dorsal views and sections of stages 3-7+ whole-mount chick embryos hybridized with the markers indicated laterally. (A) In anterior regions, *Raldh2* is expressed in the hypoblast (hy), and it remains confined (B,C) to the lower layer in anterior endoderm (ae in mid-sagittal section in inset). Asterisk in C indicates anterior endoderm. (D) At head process stages, anterior *Raldh2* expression is mainly localized in prechordal mesendoderm (pme), as seen in transverse sections (D′). Lines (D) indicate the position of the transverse sections (D′,D′′). (E) At two-somite stages, *Raldh2* is maintained in prechordal mesoderm and foregut endoderm with a sharp posterior boundary at the level of the notochord (blue arrow in mid-sagittal section in E′, dorsal is left). (F,F′) Expression of *Raldh3* in the node (no) and in ingressing mesodermal cells (red arrowhead). Strong expression is also visible in the marginal zone surrounding the embryo. (G,G′) Stage 4+ embryos hybridized with RXRγ have a restricted expression in mesodermal cells in the node region (G′, red arrowhead). Red arrows indicate the position of the node. Scale bar in A: 260 μm in A; 400 μm in B,C,D,F,G; 220 μm in E; 200 μm in D′-D′′′,E′,F′,G′; 270 μm in inset in C.

detected at these developmental stages (data not shown) (Godbout, 1992).

To determine the sites of RA action in the early embryo, we analyzed the distribution of various isoforms of retinoic acid and retinoid X receptors (RAR and RXR, respectively), when both *Raldh2* and *Raldh3* show localized anterior expression. We found that most RAR isoforms are either ubiquitously expressed in the embryo or localized at the anterior and lateral marginal zone, whereas RXR isoforms show a more restricted expression pattern (data not shown). RXRγ expression is restricted to mesodermal precursors located in the node region (Fig. 1G,G′), suggesting that RXRγ could be the ideal partner in the specification and migration of mesodermal cells during gastrulation.

In summary, we found that two RA-synthesizing enzymes are expressed in anterior structures involved in early forebrain induction and patterning. Whereas *Raldh2* expression is located in the hypoblast in pre-gastrula embryos and in prechordal mesendoderm during gastrulation, *Raldh3* is transiently expressed in the node and in axial mesodermal precursors emerging from the node.

Altered expression of anterior endodermal markers in early VAD embryos

The striking expression of *Raldh2* in anterior endoderm at a stage when this structure provides signals necessary for axial mesoderm specification (Vesque et al., 2000) suggests an endogenous role of RA signalling in patterning early endodermal and mesodermal properties. To functionally test this hypothesis, we took advantage of the VAD quail model, which lacks biologically active RA throughout development. We first assessed whether TGFβ family members were altered in anterior endoderm, which at stage 4/5 is composed of hypoblast and anterior definitive endoderm (Fig. 2A-A″,C-C″) (Vesque et al., 2000). Surprisingly, in VAD embryos *Bmp2* and *Bmp7* have stronger levels of expression in hypoblast cells when compared with normal embryos (*n*=6; asterisks in Fig. 2B,B′,D,D′), which suggests an upregulation of BMP signalling in the hypoblast. Furthermore, the anterior definitive endoderm domain of *Bmp2*, and to a lesser extent of *Bmp7*, expression is laterally expanded, although levels of transcripts do not change (arrowheads in Fig. 2B,D,B″,D″).

Next, we tested whether the homeobox gene *Hex*, a necessary component for anteriormost axial patterning (Martinez-Barbera et al., 2000) and expressed in hypoblast and anterior definitive endoderm (Fig. 2E,E′,E″) (Yatskievych et al., 1999; Chapman et al., 2002), was affected in VAD embryos. At stage 5, VAD embryos show a dramatic downregulation of *Hex* expression in anterior regions (arrow in Fig. 2F). Transverse sections indicate that the decreased expression is mainly localized in anterior definitive endoderm (*n*=5; Fig. 2F″), whereas the hypoblast layer has only slightly reduced expression levels (Fig. 2F′). Furthermore, expression of the Wnt inhibitor *crescent* (*Crs*) (Pfeffer et al., 1997) is also dramatically downregulated in anterior and lateral definitive endoderm of stage 6– VAD embryos (*n*=3; Fig. 3E-F″), whereas the prechordal mesodermal expression is expanded posteriorly (arrows in Fig. 3F; see also next section).

Altogether, these data suggest an endogenous role for retinoids in modulating anterior endodermal signalling, which has been shown to be required in forebrain development. However, whereas BMP genes have altered expression levels mainly in primitive endoderm of VAD embryos, the expression of *Hex* and *Crs* is affected more in anterior definitive endoderm, suggesting distinct roles for RA in patterning anterior endodermal properties.

Abnormal specification of axial mesodermal and ventral neural midline cells in early VAD embryos

Considering the fundamental role attributed to anterior endoderm (hypoblast and anterior definitive endoderm) in the

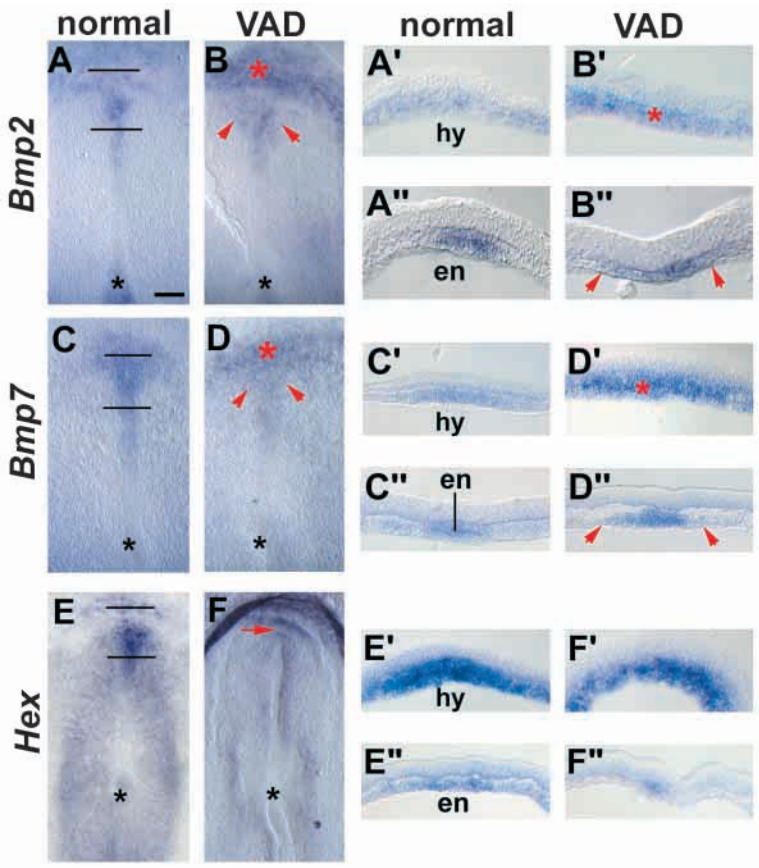

Fig. 2. Endodermal properties are altered in VAD embryos. Anterior dorsal views and transverse sections of normal and VAD whole-mount quail embryos hybridized with the markers indicated. (A-D″) Anterior expression of *Bmp2* and *Bmp7* in stage 4/5 normal and VAD embryos. The panels show only expression anterior to the node (asterisks). Lines (A,C,E) indicate the position of the transverse sections shown at hypoblast (hy) (A′,C′,E′) and anterior definitive endoderm (en) (A″,C″,E″) levels. Note that at stage 4/5 *Bmp2* and *Bmp7* expression is detected at higher levels in hypoblast cells of VAD embryos (asterisks in B,B′,D,D′) than of normal embryos (A,A′,C,C′). Furthermore, VAD embryos have an expanded expression domain of *Bmp2* and to a lesser extent of *Bmp7*, in anterior definitive endoderm (arrowheads in B,B″,D,D″). (E-F″) Anterior endodermal expression of *Hex* in stage 5 normal and VAD embryos. Note the dramatic downregulation of *Hex* expression in anterior endoderm (arrow in F), in particular in definitive endoderm (F″), whereas expression in hypoblast is only partially reduced (F′). Scale bar in A: 100 μm in A-F; 40 μm in A′-F″.

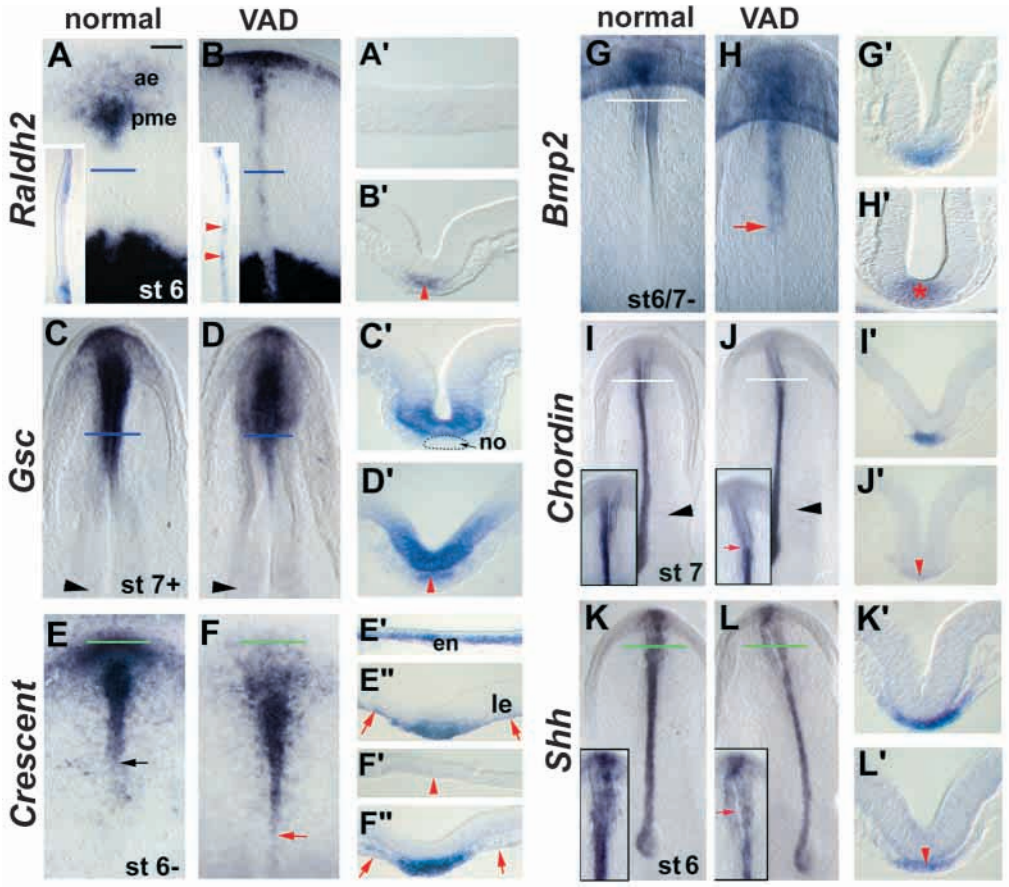

Fig. 3. Altered specification of midline properties in the absence of retinoids. Dorsal views and transverse sections of normal and VAD whole-mount quail embryos hybridized with the markers indicated. (A-B′) Compared with normal embryos where *Raldh2* expression is localized in prechordal mesendoderm (pme) and at lower levels in anterior endoderm (ae), VAD embryos have patches of ectopic expression along the whole length of anterior axial mesoderm (B, arrowheads in inset and in B′). (C-D′) Ectopic *Gsc* expression is detected in presumptive notochord (no) cells (arrowhead in D′). (E-F″) Axial mesodermal *Crs* expression is found more posteriorly in VAD embryos than in normal embryos (compare arrows in E,F). Transverse sections shown (E′-F″) indicate absence of *Crs* expression in anterior (en) (arrowhead in F′) and lateral embryonic endoderm (le) (arrows in F″) of VAD embryos. (G-H′) Similar to the previous markers, *Bmp2* expression in prechordal mesoderm is posteriorly expanded in VAD embryos (arrow in H). In addition, ectopic expression is detected in ventral neuroectodermal cells (asterisk in H′). (I-J′) The notochord marker *chordin* shows a posteriorly displaced anterior expression boundary in VAD embryos when compared with normal embryos (arrow in inset in J). Transverse sections at the level of presumptive notochord indicate diminished expression of *chordin* in VAD embryos (arrowhead in J′). (K-L′) The axial mesodermal marker *Shh* is expressed at lower levels in the most anterior region (arrow in inset in L and arrowhead in L′). Coloured lines designate the AP position of the transverse sections shown laterally. Arrowheads in C,D,I,J indicate the position of the somites. Note that the embryo in H is slightly older than the embryo in G. Scale bar in A: 100 μm in A,B,E,F,G,H; 125 μm in C,D; 150 μm in I-L; 50 μm in A′-L′.

specification of axial mesoderm (Vesque et al., 2000), we were interested in establishing whether prechordal mesoderm and notochordal markers were perturbed in VAD quail embryos.

As described above, anterior *Raldh2* expression at stage 5/6 is specifically upregulated in prechordal mesendoderm but is absent from notochord cells (Fig. 1; gap of expression in Fig. 3A and inset). Surprisingly, we found that stage 6 VAD embryos have patches of ectopic *Raldh2* expression along the whole anterior axial mesoderm (*n*=2; Fig. 3B, arrowheads in inset). Transverse sections at the level of presumptive rostral notochord indicate the lack of *Raldh2* expression in normal embryos and ectopic mesodermal expression in VAD embryos (Fig. 3A′,B′). Next, we looked at the expression of the transcription factor gene *goosecoid* (*Gsc)* and at the late expression of *Crs*, *Bmp2* and *Bmp7* in prechordal mesodermal cells (Fig. 3C,E,G; data not shown). Transverse sections reveal no *Gsc* expression in the notochord of normal embryos (Fig. 3C′), whereas abnormal ectopic expression is detected in VAD embryos (*n*=5; Fig. 3D′), and *Crs* and *Bmp2* midline expression is expanded towards the node when compared with normal embryos (*n*=7; arrows in Fig. 3F,H). Next, to ascertain whether the extension of the anterior notochord was altered in VAD embryos, we used *chordin* as a molecular marker for notochord cells in stage 7 embryos (Fig. 3I,I′) (Vesque et al., 2000). In VAD embryos the chordin-free region is posteriorly expanded, and at presumptive anterior notochord levels, expression is mostly undetectable (Fig. 3J, inset, J′). In summary, these data indicate abnormal patterning of axial mesoderm, i.e. prechordal mesodermal markers are expressed more posteriorly, in presumptive anterior notochord.

Furthermore, and surprisingly, transverse sections at all rostrocaudal levels reveal ectopic *Bmp2* and *Bmp7* expression in the ventral neural midline of VAD embryos (*n*=8; Fig. 3H′; data not shown), which suggests that BMP signalling is also altered in the ventral neural tube. It has been reported that the coordinate action of TGFβ family members and SHH is required for the correct induction of anterior properties of ventral midline cells at prospective forebrain levels (Dale et al., 1997; Dale et al., 1999; Ericson et al., 1995). Thus, to ascertain whether the abnormal distribution of BMP genes accompanied altered *Shh* expression, we looked at *Shh* distribution at stage 6 of normal and VAD embryos (Fig. 3K-L′). Fig. 3L shows that, in VAD embryos, overall levels of *Shh* expression are slightly decreased along the rostrocaudal axis; however, lower levels are more evident in anterior regions (*n*=4; inset in Fig. 3L).

In summary, our molecular analysis suggests an endogenous role for retinoids in the AP specification of axial mesoderm into prechordal mesoderm and anterior notochord.

In addition, abnormal BMP gene expression in the ventral neural tube and lower levels of *Shh* expression in prechordal mesoderm suggest an altered balance of signalling molecules involved in anterior ventral neural patterning.

The prospective telencephalic territory posteriorly expanded in early VAD embryos

Heterotopic grafting and tissue recombination experiments have demonstrated that prechordal mesendoderm is a potent anterior neural inducer (reviewed by Kiecker and Niehrs, 2001). Therefore, the posterior expansion of prechordal mesendoderm markers observed in VAD embryos could result in the induction of neural tissue with a more anterior character. To test this hypothesis, we looked at the expression of various anterior neural markers in stage 6-11 VAD embryos. The transcription factor *Otx2* is a marker for presumptive fore- and midbrain regions in all vertebrate species examined, and its expression is reduced in presence of excess RA (Bally-Cuif et al., 1995; Simeone et al., 1995; Swindell et al., 1999). Concordantly, we observed an AP enlargement of the *Otx2* expression domain in the absence of RA (*n*=3; Fig. 4B), which suggests an expansion of prosencephalic and mesencephalic tissue. To assess the degree of expansion at the level of the prosencephalon, we used *Pax6* as a presumptive diencephalic marker (Bell et al., 2001). At stage 7+, *Pax6* is expressed in the caudal half of the prosencephalon but is excluded from the most anterior region of the embryo (Fig. 4C). In VAD embryos the posterior *Pax6*-positive region is not affected, whereas the most anterior *Pax6*-negative region has an increased AP length (*n*=3; Fig. 4D). To verify that the affected region corresponds to the future telencephalon, we used *Nkx2.1* as a ventral regional marker (Crossley et al., 2001) and *Bf1* (*qin*) as a prospective ventral telencephalic marker (Bell et al., 2001). At stage 9, the *Nkx2.1* expression domain encompasses mainly the hypothalamic primordium (Fig. 4E; inset). In VAD embryos the posterior expression boundary is clearly shifted caudally, whereas the anterior boundary has not changed (*n*=5; Fig. 4F; arrowhead in F′). Furthermore, the caudomedian expression boundary of *Bf1* (red line in Fig. 4G) has shifted towards the prospective diencephalon at stage 11+ (*n*=4; Fig. 4H; inset). Thus, our analysis with regional forebrain-specific markers shows an expansion of telencephalic tissue in the absence of retinoids, and suggests a role for RA in maintaining

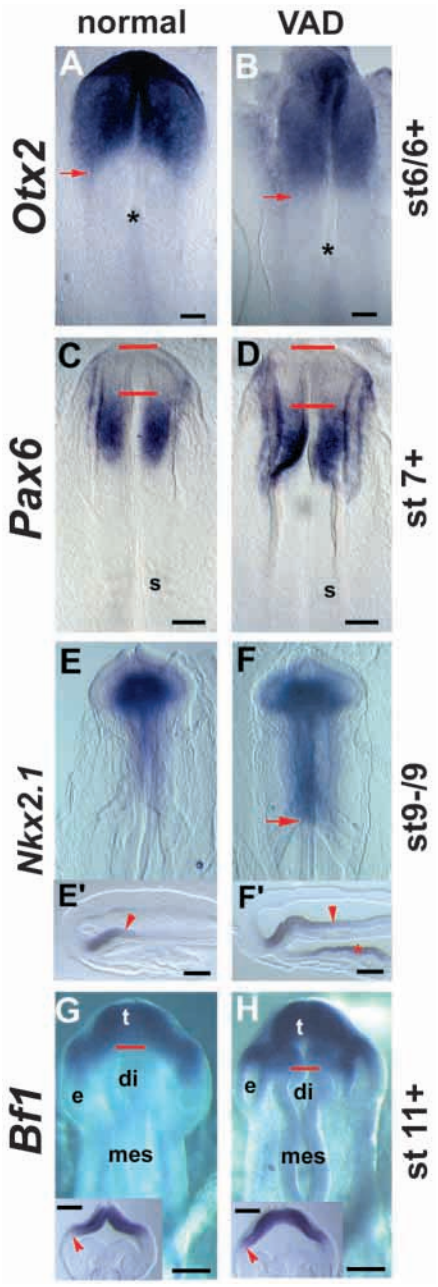

Fig. 4. Posterior expansion of prospective telencephalic tissue. Dorsal views and sections of normal and VAD whole-mount quail embryos hybridized with the markers indicated. (A,B) The general fore- and midbrain marker *Otx2* shows an expanded anterior expression in VAD embryos (see arrows), whereas (C,D) the *Pax6*-negative region indicates that the affected expanded territory corresponds to the future telencephalon (compare AP length of region marked with red lines in C,D). Note that the *Pax-6*-positive domain is not changed between normal and VAD embryos. (E-H) The markers *Nkx2.1* and *Bf1* confirm that the expansion of anterior territories corresponds to the presumptive ventral telencephalon, which is maintained until stage 11+ (arrow in F and red lines in G,H). Note that only the posterior *Nkx2.1* expression border is shifted (arrowheads in mid-sagittal sections E′,F′), whereas the anterior boundary has not changed. In VAD embryos, ectopic expression of *Nkx2.1* is also detected in the ventral foregut (asterisk in F′). Arrows in insets in G,H indicate a caudal shift of *Bf1* expression in horizontal section at ventral levels. The asterisks (A,B) indicate the position of the node. Note that the embryos in B and F are slightly older than the embryos in A and E. s, somite; t, telencephalon; di, diencephalon; mes, mesencephalon; e, eye. Scale bars: 100 μm in A,B; 200 μm in C-H.

a proper boundary between the future telencephalon and diencephalon.

Increased cell death is first detected in ventral neuroectoderm and foregut endoderm of VAD quail embryos

Next, we investigated whether the above-described molecular changes in presomite and early somite VAD embryos precede putative cellular and morphological abnormalities at later stages. We first assessed the presence of apoptotic cells using the TUNEL technique (Gavrieli et al., 1992). In anterior regions, stage 10 normal embryos show localized cell death at the anterior neural pore (Fig. 5A) (Maden et al., 1997) and a few apoptotic cells in ventral regions (Fig. 5A; inset). In VAD embryos of similar stages we noticed an increase in the rate of apoptotic cells in ventral neuroectoderm and in foregut endoderm (*n*=4; Fig. 5F; inset), whereas the extent of apoptotic cells at the anterior neural pore shows no obvious change. To ascertain whether the rate of dying cells was maintained at later stages, frontal and horizontal sections of stage 12-20 VAD quail embryos were examined for the presence of apoptotic cells (Fig. 5B,C,G,H; data not shown). At stage 13/14, a dramatic increase of dying cells is evident in the ventral neural tube and in head mesenchymal cells, which in many cases invade the forebrain vesicle (*n*=5; Fig. 5G). Furthermore, high levels of programmed cell death are retained in head mesenchyme and in ventral neuroectoderm until stage 19/20 (*n*=2; Fig. 5H). To establish whether the dying mesenchymal cells are of neural crest origin, the HNK1 antibody (Luider et al., 1992) was used in adjacent para-sagittal sections (Fig. 5D,E,I,J). Whereas at stage 10, HNK1-positive crest cells are normally distributed in VAD embryos (*n*=3; Fig. 5I), their presence decreases dramatically at later stages, consistent with an increased incidence of apoptotic cells (*n*=6; Fig. 5E,J). In summary, our data indicate that the absence of retinoids induces a first wave of programmed cell death in the ventral neuroectoderm and foregut endoderm, whereas the migration of forebrain neural crest cells is not affected. However, at later stages a second and more dramatic wave of cell death occurs within the neural crest-derived head mesenchyme, whereas the high rate of apoptotic cells is maintained in ventral neuroectoderm.

VAD quail embryos have abnormal forebrain morphogenesis at later stages

It has been previously reported that VAD embryos die at ~4 days of development because of cardiovascular defects (Twal et al., 1995; Chen et al., 1996; Maden et al., 1996; Gale et al., 1999). Up to stage 14, VAD and normal embryos are morphologically indistinguishable; however, after 72 hours of incubation (stage 19/20), VAD embryos are about three quarters the head-to-tail length of normal embryos, they develop no body torsion but at a morphological level are comparable in stages with normal embryos (Maden et al., 1996; Zile, 1998). At the level of the rostral brain, we found that 100% of stage 20 VAD embryos lack a cephalic flexure, and have a single and reduced telencephalic vesicle, which is variable in size and placed either centrally or slightly laterally (Table 1; Fig. 6D,E). However, all VAD embryos develop two eyes, although they can be different sizes (see Table 1). We classified VAD embryos as having a moderate (12%), severe (77%) and very severe (11%) phenotype according to the degree of telencephalic reduction (Table 1). Moreover, we found that 70% of VAD embryos have an enlarged diencephalon and/or develop ectopic transverse ridges within the diencephalon (Fig. 6B), whereas 100% of VAD embryos lack a morphological zona limitans intrathalamica (zli) as seen by histological and molecular analysis (A.H. and M.S., unpublished). Coronal sections at anterior levels confirm the presence of a smaller and single vesicle with no interhemispheric fissure and an abnormally thick

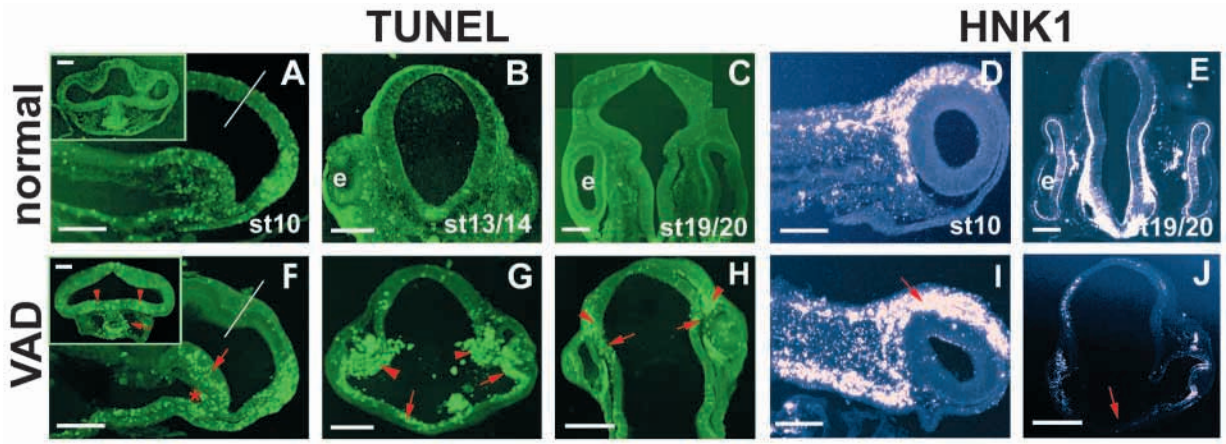

Fig. 5. VAD embryos show increased cell death in ventral neuroectoderm and head mesenchyme. Apoptotic cells in sections of normal (A-E) and VAD (F-J) quail embryos detected using the TUNEL assay (A-C,F-H), and neural crest cells detected using an HNK1 antibody (D,E,I,J). (A,F,D,I) Mid-sagittal and para-sagittal sections, (B,G,E,J; insets in A,F) frontal and (C,H) horizontal sections. Stage 10 VAD embryos show an increased rate of apoptotic cells in ventral neuroectoderm (arrow in F and arrowheads in inset) and foregut endoderm (asterisk in F and arrow in inset), but no change in the distribution of neural crest cells (D,I). VAD embryos (arrow in I) have an apparent difference of HNK1-positive cells because the section is more lateral. (B,G) In stage 13/14 VAD embryos, ventral neuroectodermal (arrows in G) and mesenchymal cells around the eye regions (arrowheads in G) show a dramatic increase of programmed cell death. (C,H,E,J) At stage 19/20 the dramatic increase in the rate of dying cells is still detected in neuroectodermal (arrows in H) and head mesenchymal tissues (arrowheads in H). Moreover, the absence of HNK1-positive cells in ventral regions (arrow in J) indicates a lack of neural crest-derived cells. Lines in A and F designate the level of the frontal section showed in the insets. Scale bars: 100 μm in A,F,D,I; 200 μm in B,C,E,G,H,J.

Table 1. Representation of the different forebrain phenotypes observed in stage 19/20 VAD quail embryos

Phenotype	Embryos with phenotype (%)
Single uncleaved vesicle	100
Lack of cephalic flexure	100
Telencephalon	
Absent or very severely affected*	11
Severely affected†	77
Moderately affected‡	12
Normal	0
Diencephalon	
No zli	100
Ectopic ridges§	70
Enlarged§	70
Normal	30
Eyes	
No eyes	1
Right eye smaller	53
Left eye smaller	23
Normal eyes	23
Nasal pits	
None	15
No right	48
No left	29
Normal	8

*Indicates reduction of more than 80%.
†Indicates reduction between 50% and 80%.
‡Indicates reduction of 50% or less than 50%.
§These two abnormalities can be detected in different embryos.
Sixty-six VAD embryos were examined.

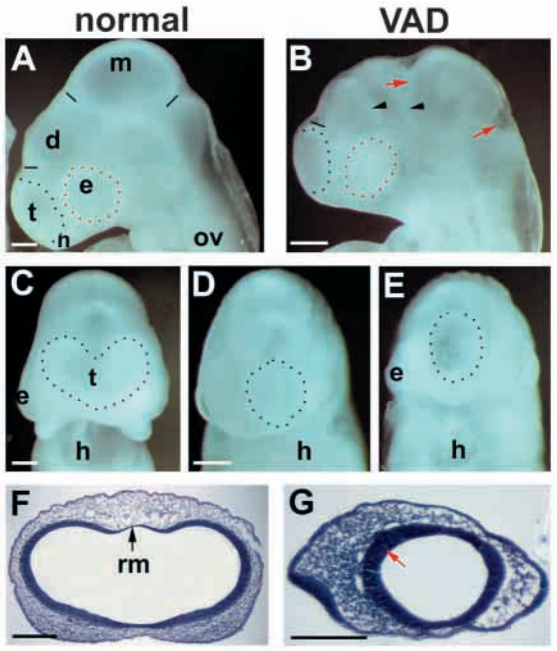

Fig. 6. Abnormal forebrain morphogenesis in the absence of retinoids. Lateral (A,B) and frontal (C,D,E) views of normal (A,C) and severely affected VAD (B,D,E) whole-mount stage 20 embryos. VAD embryos develop a smaller and single telencephalic vesicle circled in (D,E), which can be located laterally (D) or centrally (E) instead of two telencephalic (t) vesicles (A,C). Note (B) the ectopic morphological borders within the diencephalon (black arrowheads) and pronounced constrictions (red arrows) between vesicles. (F,G) Frontal sections of normal (F) and VAD (G) quail embryos at the level of the telencephalon/nasal pit. Note the lack of a rostral midline (rm) and the presence of a thick neuroectoderm in the single anterior vesicle of VAD embryos (arrow in G). e, eye; n, nasal pit; ov, optic vesicle; h, heart. Scale bars: 200 μm (bar in D also applies to E).

neuroectoderm (Fig. 6G); at more posterior levels the neuroectoderm is dramatically reduced (see also Fig. 5J; data not shown). Thus, at the level of the forebrain, VAD embryos have a single telencephalic vesicle but retain two eye fields and have an abnormally patterned diencephalon.

DISCUSSION

RA signalling is involved in patterning anterior endodermal cells

It is well established that the primitive (or extra-embryonic) and definitive (or embryonic) endoderm are required for forebrain development; however, little is known about the molecular mechanisms and signalling pathways involved in this process.

In the mouse, the AVE is necessary for the development of anterior structures (Thomas and Beddington, 1996); however, its transplantation does not generate an ectopic forebrain (Tam and Steiner, 1999; Kimura et al., 2000). In the chick, the hypoblast can only transiently induce early anterior and neural markers (Knötgen et al., 1999; Foley et al., 2000). Therefore, the role of the AVE and the hypoblast remain unclear. One hypothesis is that the hypoblast might pre-pattern the naïve epiblast, before it becomes responsive to additional stabilizing signals secreted by the node. Alternatively, both the AVE and the hypoblast could be involved in regulating cell movements, as suggested by fate mapping in mouse and transplantation studies in chick (Foley et al., 2000; Kimura et al., 2000). Finally, removal of the hypoblast in chick causes the formation of ectopic primitive streaks, which suggests that the hypoblast could position the primitive streak by emitting an inhibitor of axis formation (Bertocchini and Stern, 2002). Interestingly, it has been shown that local application of RA can cause a pronounced curving of the primitive streak, away from the retinoid source (Knezevic and Mackem, 2001), in a manner reminiscent to that observed with hypoblast rotation (Foley et al., 2000); however, no presence of retinoids has been documented in pre-gastrula stage embryos so far. We found that *Raldh2* is highly expressed in the expanding hypoblast and thus it is conceivable that retinoids participate in the early events in which the hypoblast is implicated. We propose that one of the roles of RA in the hypoblast might be to provide a proper developmental environment for migrating cells, a role that has already been suggested in branchial arch patterning (Wendling et al., 2000; Zile et al., 2000).

During early gastrulation the anteriormost endoderm, comprising the hypoblast and anterior definitive endoderm, is required for proper forebrain development. In particular, it has been shown that BMP2 and BMP7 can partially mimic the action of anterior endodermal cells in suppressing notochord and in inducing prospective prechordal mesoderm characteristics in the axial mesoderm (Vesque et al., 2000). Our study shows that during axial mesodermal specification, retinoid synthesis is present in anterior endoderm, as seen by

the expression profile of *Raldh2*. Therefore, RA could freely diffuse from the endoderm into the overlying anterior neural plate and towards the emerging axial mesoderm. However, the presence of the RA-degrading enzyme CYP26 in anterior neuroectoderm from stage 3+/4– onwards (data not shown) (Swindell et al., 1999) excludes a vertical action of RA directly into the neural plate at this stage, and favours instead a direct or indirect role of RA in axial mesoderm specification (see model in Fig. 7). Accordingly, our functional data demonstrate that in the absence of retinoids, *Bmp2* and *Bmp7* expression is upregulated in the hypoblast, and axial mesodermal cells acquire altered prechordal mesoderm properties. Thus, we propose that retinoids cooperate in refining the extent of prechordal mesododerm characteristics by modulating adequate levels of BMP signalling in the hypoblast (Vesque et al., 2000). The need to maintain precise levels of both RA and BMP signalling has been already described in other systems (Schultheiss et al., 1997; Yatskievych et al., 1997; Andree et al., 1998; Ladd et al., 1998; Ghatpande et al., 2000; Schlange et al., 2000). In the head, low levels of BMP proteins and higher levels of RA are necessary to pattern the frontonasal mesenchyme in chick embryos (Lee et al., 2001). Our data validate that precise levels of RA and BMP proteins are also a requisite for early anterior patterning, and add further insight into the regulation of BMP proteins by retinoids.

Finally, it has been demonstrated that removal of anterior definitive endoderm during stages 4+ and 5 leads to dramatic forebrain reductions and concomitant gene expression alterations, such as abolishment of the homeobox gene *Hex* and the Wnt antagonist *Crs* (Withington et al., 2001). Furthermore, *Hex* deficiency (Martinez-Barbera et al., 2000) and overactivation of Wnt signalling produced by abolishing Wnt antagonism (reviewed in Yamaguchi, 2001) will lead to different degrees of forebrain malformations, which suggests an essential role for these genes in anterior patterning. Accordingly, our data show that *Hex* and *Crs* anterior definitive endoderm expression is severely reduced in the absence of RA, whereas expression of *Bmp2*, and to a lesser extent *Bmp7*, is laterally expanded, which suggests abnormal characteristics of the anterior definitive endoderm cells in the absence of RA. Our data are in agreement with a recent study in zebrafish, which demonstrates that RA signalling plays an important early role in regionalizing the zebrafish endoderm (Stafford and Prince, 2002), and therefore we suggest that retinoids might act independently on anterior endodermal cells thereby modulating instructive signals necessary for proper head patterning.

In summary, we propose that among the various signalling molecules involved in endodermal and early anterior CNS patterning, RA is an essential component that might act at the level of primitive and definitive anterior endoderm thereby modulating levels of expression of various target genes. More studies need to be performed in order to characterize the precise role of retinoids in the genetic cascade of endodermal signalling.

Retinoids are required in the specification and regionalization of the developing prosencephalon

Various reports have shown that prechordal mesoderm is a potent anterior inducer when transplanted ectopically, and is absolutely necessary for maintaining and/or reinforcing anterior neural plate specification and, thus, head structures (reviewed in Kiecker and Niehrs, 2001). Moreover, in chick, signals provided by axial mesendoderm refine the rostrocaudal character of the overlying neuroectoderm (Rowan et al., 1999). Therefore, accurate AP specification of prechordal mesoderm is a prerequisite for correct regionalization of the overlying anterior neuroectoderm. Our data are in accordance with these findings and show that an extension of prechordal mesendoderm caused by lack of retinoids induces a reciprocal expansion of the anterior neural plate. In *Xenopus*, injection of a dominant-negative retinoid acid receptor that can inhibit RA signalling leads to an expansion of anterior structures, as observed by the increased expression of the forebrain/midbrain marker *Otx2* (Blumberg et al., 1997). Conversely, in mouse and chick, RA exposure abolishes *Otx2* expression in midline mesendoderm, and induces various degrees of forebrain malformations in mouse embryos (Bally-Cuif et al., 1995; Simeone et al., 1995). Our data not only confirm the endogenous requirement of retinoids in restricting *Otx2* expression in avian embryos, but also identify the ventral telencephalon as the main target subregion within the developing prosencephalon that is affected by the absence of retinoids. Therefore, the early endogenous role of RA might be to restrict the AP extent of the ventral telencephalon. However, our data cannot distinguish between a posterior to anterior fate conversion or an expansion of the future telencephalon. The maintenance of a normal diencephalic *Pax6* domain prompts us to opt for the latter possibility; however, studies involving tissue explants with the use of regionalized molecular markers in the presence or absence of RA would help solve this issue.

Finally, the expansion of the posterior expression boundary of *Nkx2.1*, which normally marks the border between

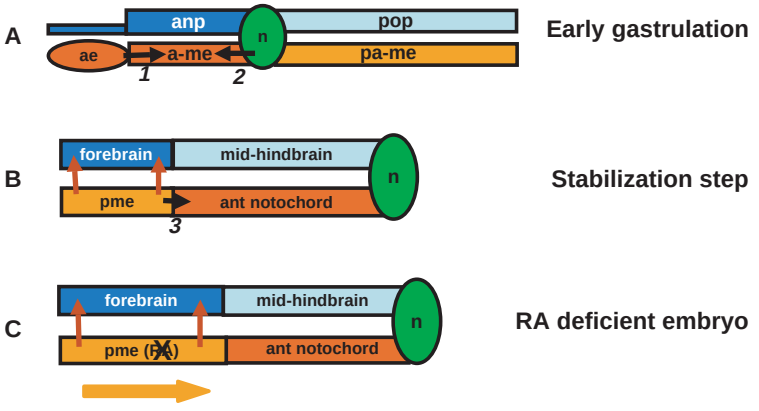

Fig. 7. RA action during presomite stages in anterior regions. (A) At early gastrula stages, RA signals from the anterior endoderm (ae) and node (n) to specify axial mesodermal cells emerging from the node (arrows 1 and 2). (B) During the stabilization step RA signals from the prechordal mesendoderm (pme) to restrict the extent of posterior pme (arrow 3); during this stage other signalling pathways pattern the overlying forebrain primordium (red arrows). (C) In the absence of RA, pme is extended posteriorly (yellow arrow) and altered signals are transferred to the overlying neuroectoderm (red dashed arrows). ae, anterior endoderm; pme, prechordal mesendoderm; a-me, axial mesoderm; pa-me, paraxial mesoderm; anp, anterior neural plate; pop, posterior neural plate; n, node.

prechordal and epichordal neural plate (Shimamura et al., 1995), could imply a specific role of retinoids in maintaining the correct position of morphological boundaries within the prosencephalon. Various observations are in agreement with this hypothesis. First, VAD embryos fail to develop a morphological zli boundary, as seen in histological preparations and in the absence of *Shh* expression in the zli (A.H. and M.S., unpublished). Second, VAD embryos present ectopic constrictions and/or ridges in the dorsal diencephalon (Fig. 6B). Finally, in the hindbrain, local application of RA causes the disappearance of posterior hindbrain boundaries (Nittenberg et al., 1997) and, conversely, treatment with a retinoid receptor antagonist fails to specify gene expression borders involved in boundary formation (Dupé et al., 2001). Thus, as in the hindbrain, the endogenous role of RA in anterior regions might be to restrict the expression domains of regulatory genes involved in regionalizing the developing prosencephalon during a crucial time-window.

A dual role of RA action in patterning the forebrain

Although VAD embryos show an expansion of anterior neural tissue at early stages, the telencephalic vesicles are not separated and are dramatically reduced at later stages. How are these events correlated? We propose various hypotheses to explain this discrepancy. First, we found a reduction of *Shh* expression in prechordal mesoderm before the appearance of an increased rate of cell death in ventral neuroectoderm and foregut endoderm. SHH has been shown to act as an anti-apoptotic factor and might therefore be responsible for the increased ventral cell death seen in VAD embryos (see also Ahlgren and Bronner-Fraser, 1999; Britto et al., 2002; Charrier et al., 2001). Second, VAD embryos show abnormal expression of *Bmp2* and *Bmp7* in ventral neural midline, which might induce, together with reduced *Shh* expression, altered rostrocaudal properties in the overlying neuroectoderm. Therefore, mis-specification of axial identity in the mesenchyme and neuroepithelium could lead to programmed cell death, a phenomenon referred to as positional apoptosis and already described in the hindbrain (Maden et al., 1997). Third, decrease of retinoid signalling in the frontonasal mass, either through inactivation of RAR at the gene level or through RAR antagonists, generates various degrees of forebrain malformations and increased death of head mesenchymal cells (Lohnes et al., 1994; Schneider et al., 2001). The study by Schneider et al. (Schneider et al., 2001) has shown that, in stage 10 chick embryos, retinoids might be involved in maintaining a proper growth rate of head mesenchymal cells, and thus an adequate outgrowth of the frontonasal process and forebrain structures. Our study demonstrates that, at stage 10, VAD embryos have already increased cell death in ventral neuroectoderm and endoderm but migration of cranial neural crest cells is normal. Subsequently, from stage 12 onwards, VAD embryos show a dramatic increase in programmed cell death of mesenchymal and neuroectodermal cells. Thus, in contrast to the findings of Schneider and collaborators, we identified apoptotic cells in ventral neuroectoderm before the appearance of dying cells in neural-crest derived cells, which suggests that retinoids might have different roles at different stages in patterning the developing forebrain. We propose, therefore, that the overall increase of cell death observed in VAD embryos at stage 20, is derived from an additive effect between an early event restricted mainly to the ventral neuroectoderm and foregut endoderm, and a later event confined to the head mesenchyme.

Conclusion

In summary, this study has unravelled a novel role for RA signalling during gastrulation in anterior patterning that has not been reported previously (see model in Fig. 7). We speculate that RA synthesized in hypoblast cells might be involved in providing a proper developmental environment for cell motility and/or regulating primitive streak formation. Subsequently, during the process of axial mesoderm specification into prechordal mesoderm and notochord, RA is synthesized from two sources, one located in the node and another in anterior endoderm, which is composed of remnants of hypoblast and anterior definitive endoderm. Whereas the node source might be involved in the differentiation of axial mesodermal precursors, the anteriormost endodermal source might be required in modulating regulatory genes and signalling molecules during the specification of axial mesodermal cells (Fig. 7A). In particular, RA would restrict the AP extent of prechordal mesoderm, thereby maintaining and stabilizing anterior neural plate characteristics in conjunction with other signalling pathways, in accordance with the 'stabilization step' proposed by Stern and collaborators (Stern, 2001) (Fig. 7B). Therefore, altered vertical signals that are due to the absence of retinoids and to abnormal AP specification of prechordal mesoderm would fail to maintain a proper forebrain anlage (Fig. 7C). Previous data obtained from *Xenopus* and mouse embryos with exogenous doses of RA had suggested a direct action of RA on axial mesodermal cells (Ruiz i Altaba and Jessell, 1991; Bally-Cuif et al., 1995; Simeone et al., 1995); however, no endogenous sources of RA synthesis had been described at that time to justify such a hypothesis.

Finally, a late source of retinoids localized in the head mesenchyme and involved in sustaining proper forebrain outgrowth would be responsible for the late phase of cell death observed in VAD and antagonist-treated embryos (this study) (Schneider et al., 2000). Thus, although presumptive telencephalic structures are more widely induced in early VAD embryos, these cannot be maintained at later stages.

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