Retinoic acid causes abnormal development and segmental patterning of the anterior hindbrain in *Xenopus* embryos

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Summary

Retinoic acid is a very potent teratogen and has also been implicated as an endogenous developmental signalling molecule in vertebrate embryos. One of the regions of the embryo reliably affected by exogenously applied RA is the hindbrain. In this paper, we describe in detail the hindbrain of *Xenopus laevis* embryos briefly treated with various levels of RA at gastrula stages. Such treatments lead to development of embryos with loss of anterior structures. In addition, RA has a general effect on rhombomere morphology and specific effects on the development of the anterior rhombomeres. This effect is demonstrated using neurofilament antibodies, HRP staining and *in situ* hybridisation using a probe for expression of the *Xenopus Krox-20* gene. Anatomically it is evident that the development of the hindbrain normally anterior to the otocyst (rhombomeres 1—4) is abnormal following RA treatment. Sensory and motor axons of cranial nerves V and VII form a single root and the peripheral paths of V and VII and IX and X are also abnormal, as is the more anterior location of the otocyst. These anatomical changes are accompanied by changes in the pattern of expression for the gene *XKrox-20*, which normally expresses in rhombomeres 3 and 5, but is found in a single band in the anterior hindbrain of treated embryos which standardly fail to generate the normal external segmental appearance. The results are discussed in terms of both the teratogenic and possible endogenous roles of RA during normal development of the central nervous system. We conclude that low doses of RA applied during gastrulation have specific effects on the anterior *Xenopus* hindbrain which appear to be evolutionarily conserved in the light of similar recent findings in zebrafish.

Key words: retinoic acid, *Xenopus*, hindbrain, *Krox-20*, cranial nerves.

Introduction

Retinoic acid (RA) belongs to a family of related compounds, the retinoids, that exhibit diverse effects on cell differentiation, metabolism and growth, both in vivo and in vitro (reviewed in Lotan, 1980). RA, a potent metabolite of retinol (vitamin A), has also been shown to have teratogenic effects on vertebrate embryogenesis (reviewed in Geelen, 1979). In addition, RA has a potential endogenous morphogen, since it has been shown to have striking effects on pattern formation in several systems. In the regenerating amphibian limb, RA causes duplications in the proximo-distal axis (Maden, 1982, 1983; Thoms and Stocum, 1984) and in the developing chick limb bud, it mimics the action of or induces a zone of polarising activity (ZPA) by causing duplication of digits along the anterior-posterior (A-P) axis (Tickle et al. 1982, Wanek et al. 1991). In *Xenopus* and zebrafish embryos, exogenous RA affects development along the A-P embryonic axis (Durston et al. 1989; Sive et al. 1990; Ruiz i Altaba and Jessell, 1991a,b; Holder and Hill, 1991). Furthermore, endogenous RA has been detected in developing chick limb buds and in whole *Xenopus* embryos, adding support to the notion that it may be an endogenous morphogen (Thaller and Eichele, 1987, 1988; Durston et al. 1989). The elucidation of the molecular mechanisms by which RA exerts its effects will undoubtedly lead to a better understanding of vertebrate development. The search for molecular targets for RA has led to the identification of families of cytoplasmic binding proteins and nuclear retinoid receptors (reviewed in Brockes, 1989). The role of cytoplasmic binding proteins is unclear, but they show interesting patterns of expression in the vertebrate CNS as do the nuclear receptors (see for example Maden et

The downstream genes that are involved in mediating the effects of RA are largely unknown, but one family, Hox homeobox genes, have received particular attention. Vertebrate homeobox genes have been implicated in the process of regionalization or positional specification along the anterior–posterior axis of the vertebrate embryo on the basis of sequence similarity to Drosophila genes, and their pattern of expression in the embryo (Kessel and Gruss, 1990; Graham et al. 1989; Duboule and Dollé, 1989; Wilkinson and Krumlauf, 1990). Hox genes also play an important role in the patterning of cranial neural crest (Hunt et al. 1991a,b). Several experiments performed in vitro have shown that homeobox gene expression is regulated during RA-induced differentiation of cultured cells. The properties of the in vitro response of these genes to RA have led to the suggestion that homeobox-containing genes are among the primary targets of RA (LaRosa and Gudas, 1988; Papalopulu et al. 1991). In addition, in parallel to the colinear expression of homeobox genes in the mouse embryo (Graham et al. 1989; Duboule and Dollé, 1989), it has been shown that the response of the human Hox-2 homeobox gene cluster to RA is colinear with their chromosomal order in terms of their degree of induction and their temporal activation (Simeone et al. 1990, 1991).

Whole-mount staining of Xenopus embryos

Xenopus tadpoles (stage 43–45) were stained with an anti-neurofilament and an anti-muscle antibody. The anti-neurofilament antibody was a mouse monoclonal antibody (3A10), against the 68×10^3 M, subunit of chicken neurofilament (a gift from Professor Andrew Lumsden). The protocol used was a compromise of the protocols described in Dent et al. (1989) and in Lumsden and Keynes (1989). Embryos were fixed in Dent’s fix (20 % v/v DMSO, 80 % v/v methanol) for a minimum of 2 h and were bleached for 1 to 2 days in Dent’s fix + 10 % hydrogen peroxide. Embryos were dissected free of epidermis over the area of interest, as this was found to improve the penetration of antibodies. Bleached embryos were washed 3×20 min with TBST [10 mM Tris-HCl pH 7.0, 150 mM NaCl, 1 % (v/v) Tween-20], with 20 % FCS added in the last wash to block non-specific binding. Embryos (5–10 tadpoles/tube) were incubated in 200 μl of neat 3A10 containing 20 % (v/v) FCS for 3–4 days at 4°C with gentle rocking. Embryos were then washed 3×90 min in 15 ml TBST and were incubated in 500 μl FITC- or HRP-conjugated rabbit anti-mouse secondary antibody (Sigma) diluted 1:100 in TBST containing 20 % FCS. After an overnight incubation at 4°C, at least three washes were carried out before.

With HRP-coupled antibodies, a staining reaction was carried out. Embryos were incubated for 2 h in the dark in TBST containing 0.5 mg ml^{-1} DAB (Sigma) and then transferred to 1 ml TBST containing 0.5 mg ml^{-1} DAB and 0.02 % hydrogen peroxide. The staining reaction was allowed to proceed in the dark until an optimal signal was obtained (10–15 min). Both HRP- and FITC-stained embryos were then washed 2×5 min in 100 % methanol. For confocal microscopy, FITC-labelled embryos were at this stage dissected and isolated hindbrains were transferred to DABCO mounting solution (BDH) in depression slides. HRP-stained embryos were observed under a dissecting microscope.
The protocol used for the anti-muscle mouse monoclonal antibody 12/101 (Kintner and Brockes, 1985; a gift from Dr P. Vize) was essentially the same as above, but the time of incubation with the first antibody was reduced to overnight and no detergent was included in the incubation or washing steps. The rabbit anti-mouse secondary antibody was conjugated to alkaline phosphatase (Sigma) and was used in 1:100 dilution. The alkaline phosphatase substrate was purchased from Vector Labs (Kit II: SK5200) and the staining was performed according to the manufacturer’s instructions, using levamisole (Sigma) to block endogenous phosphatase activity. A number of embryos were sectioned after whole-mount staining. These were dehydrated, wax-embedded, sectioned and stained with 0.02% toluidine blue: FITC-labelled sections were mounted in DABCO and viewed under the fluorescence microscope, prior to toluidine blue staining.

**HRP analysis**

Embryos of the required stage were anaesthetized in MS 222 and a small area of skin removed to allow access to the application site. HRP was recrystallized onto the tips of sharpened forceps, which were then used to crush cranial ganglia and nerves. Embryos were allowed to recover for 2–3 h before being re-anaesthetized and fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (PB – pH 7.4) for 1–2 h. The brain and rostral spinal cord were then dissected free, with care being taken to keep the otocyst and surrounding nerve roots intact. The tissue was then washed in PB and soaked in 0.04% DAB (Sigma) for 15 min prior to addition of 0.03% hydrogen peroxide for a further 10–15 min. The brains were washed in PB, dehydrated, and cleared and mounted in DPX between coverslips for viewing as whole-mounts. Some specimens were sectioned on a vibratome prior to staining.

Information on controls was collated from 24 stage 39–46 embryos, 7 of which had the preotic ganglia of V and VII as well as the roots of IV and VI filled with HRP. A set of 14 embryos had the tracts and nuclei of IX and X filled by a postotic ganglion HRP application and the remaining three embryos had both preotic and contralateral postotic ganglia HRP applications. The description of the relevant neuroanatomy of control embryos was derived from complete whole-mounted specimens and horizontally or transversely sectioned embryos. The RA data is derived from 24 treated embryos of comparable stages to controls. Of these, 11 were preotic HRP applications, 5 were postotic and 8 were combination fills with preotic on one side and postotic on the other. 16 of these specimens were sectioned (horizontally or transversely) and viewed as whole-mounts. Some specimens were sectioned on a vibratome prior to staining.

**Xenopus Krox-20 in situ hybridization**

_in situ_ hybridization on stage 20 control and embryos treated with 0.5 μM RA was performed as described for mouse embryos by Wilkinson and Green (1990), except that sections were bleached in 2% H2O2 in PBS for 5 h at room temperature prior to pretreatments.

**Results**

**Dose-dependence of RA phenotype at tadpole stage**

One of the most dramatic effects of RA at this stage is the stunting of the body axis. To a large degree this is due to the dose-dependent truncation of the tail. A dose-dependent truncation of structures is also evident rostrally (Fig. 1). The effects range from microcephalic embryos with small eyes to complete absence of all anterior neural structures up to the spinal cord. Eye, cement gland and heart formation are progressively impaired as the dose of RA increases, as has been previously reported (Durston et al. 1989; Sive et al. 1990). Pronephros is present even in the most severely affected embryos, but occupies progressively more anterior positions in the body and the pronephric tubules appear less tightly packed. Even at high RA concentrations that completely abolish head formation, myotomes are present on either side of the notochord and, in these cases, myotomes are consequently situated closer to the new rostral end of the embryo (Fig. 3).

**Early appearance of the RA phenotype**

Following treatment with RA at concentrations of 0.5, 1, 2.5, 5 and 10μM, the early neurula stage was the earliest at which abnormal development could be externally recognized. Observation of a large number of embryos in separate experiments revealed that the RA-treated embryos are consistently narrower and more elongated than sibling controls and, viewed externally, the neural folds appear thicker and this is shown for the 0.5μM concentration in Fig. 2A. Therefore, RA appears to interfere at some step of the process of elevation, folding and fusion of the neural folds. Formation of cement gland is also impaired in

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**Fig. 1.** Dose-dependence of RA phenotypes. *Xenopus* embryos at stage 9 were treated for 30 min with increasing concentrations of RA and allowed to develop to stage 40. There is a truncation of anterior pattern and concurrent failure of tail development which increases with the dose of RA. The embryo treated with 0.5μM has two small eyes and the least apparently altered morphology, while the embryo at 1μM has a single median cyclopic eye. Eyes do not develop in embryos treated with 2.5μM RA or higher concentrations, and development of the heart and visceral arch skeleton is also impaired.
RA-treated embryos (Fig. 2B) in a dose-dependent manner. This is an early sign of truncation of anterior pattern, since the cement gland is an ectodermal organ that normally forms anterior to the brain.

In order to investigate RA-induced alterations in the CNS, in particular the hindbrain, we chose to analyse embryos with the least severe phenotype where both the midbrain and the entire hindbrain region was represented in the pattern. This is important as it is difficult to examine alterations to patterning of specific structures if there has been gross deletion of large parts of the brain, as occurs at high RA concentrations; therefore we focussed our analysis on embryos that had been treated with 0.5 and 1 µM RA. Such embryos had two eyes in most cases, but some had only one fused eye at the rostral end of the reduced anterior extreme of the CNS (Fig. 1).

The size of the hindbrain in RA-treated embryos

At low concentrations of RA (0.5 and 1 µM), forebrain and eye formation is reduced but there is a morphologically distinct midbrain–hindbrain boundary (isthmus) (Figs 3 and 4). Judging by the position of mesodermal organs and spinal nerves in relation to the posterior end of the hindbrain, we have obtained no evidence that the neural tube fails to close (although this may be the case at higher concentrations; not shown). Therefore we were able to identify with confidence anatomical features in the posterior hindbrain region; specifically the region of narrowing of the fourth ventricle (obex), the hindbrain external surface and the position of the first spinal nerve. We were thus able to measure the distance between the isthmus and obex. This distance was not significantly different in RA-treated embryos as compared to controls (Table 1). However, in sections and whole-mount material, the RA-hindbrain appears broader than the control (Figs 3 and 4).

**Altered rhombomeres and expression of XKrox-20**

Horizontal sections of control tadpoles reveal a regular pattern of seven rhombomeres (see Lumsden and Keynes, 1989; Fraser et al. 1990 and Fig. 4). Experimental animals exposed to low doses of RA (0.5 and 1 µM) display an irregular rhombomeric pattern. The seven evenly spaced rhombomeres appear to be replaced with fewer, apparently larger and generally less regular bulges (Fig. 4B, D). There are no apparent rhombomeres at high (5 and 10 µM) concentrations of RA (not shown). Despite the absence of overt rhombomeres, it is possible that there is underlying segmental organisation. Therefore, we examined the expression of the zinc-finger-containing gene Krox-20 which displays a segmentally restricted pattern of expression in the mouse. The expression domains of the Xenopus homologue, XKrox-20, in normal and RA-treated embryos, support the view that rhombomeric structure is disturbed in experimental embryos, but that there is still some underlying molecular segmentation. In situ hybridisation shows that, as in the mouse, the XKrox-20 is expressed in two domains that later correspond to rhombomeres 3 and 5 in normal embryos (Bradley et al. in preparation) at stage 20. In serial sections of RA-treated embryos, only a single band of expression is present at the anterior end of the hindbrain, in contrast to two domains in the normal control embryo (Fig. 5). However, it is not possible to establish clearly whether one band has completely disappeared or whether the two expression domains have become compressed together in the RA-treated embryos.

**Peripheral abnormalities in RA-treated Xenopus embryos—otic cyst and cranial nerves**

In normal development, the centre of the otocyst is found opposite rhombomere 5 (Fig. 6). In RA-treated embryos, it is found in a more anterior position opposite the midbrain–hindbrain boundary (Fig. 6 and see summary Fig. 9), as described by Durston et al. (1989). The roots of the cranial nerves occupy distinct positions in the hindbrain in relation to the rhombomeres (Lumsden and Keynes, 1989). The trochlear nerve root lies in rhombomere 1, the trigeminal in rhombomere 2, the facial and acoustic nerve roots lie in rhombomere 4, the abducent in 5, the glossopharyngeal
Control

+RA

in 6 and the combined roots of the vagus and accessory in rhombomere 7. The exact locations of the roots relative to each other and to the otocyst in normal embryos can be described from neurofilament- and HRP-stained preparations. The root of the trochlear nerve (IV) exits dorsolaterally at the midbrain–hindbrain junction (Figs 7 and 9). The root of the trigeminal (V) and facial (VII) are both evident rostral to the otocyst. The Vth root enters the hindbrain more rostrally and slightly ventral to the VIIth root (Figs 7 and 8) and the root of the abducent (VI) runs caudally to enter the brain ventrally beneath the otocyst. The acoustic nerve (root VIII) can be clearly seen in neurofilament-stained preparations (Fig. 7A–C) but it was rarely filled by HRP applications to preotic or postotic roots. The roots of IX and X form a close set and enter the brain together caudal to the otocyst (Figs 7, 8 and 9).

The root pattern in RA-treated embryos is clearly different from normal. The anterior displacement of the otocyst to the hindbrain–midbrain border is coupled with the formation of aberrant root positions and misplaced paths for the peripheral nerves and ganglia associated with the relevant cranial roots (Figs 7 and 8).

Two consistent features of these aberrant patterns are: (i) that the roots of V and VII form at the same rostrocaudal level, although, as in normals, the sensory axons of VII enter more dorsally than those of V (Fig. 8B); and (ii) that the peripheral placements of the ganglia and nerves of V, VII, IX and X are always aberrant, a point clearly seen in Figs 7 and 11, where axons from V and VII run caudally and IX and X cranially around the medial and lateral walls of the otocyst. In a number of cases, the input of each of these nerves formed as small misplaced rootlets on the lateral or dorsolateral wall of the hindbrain. These aberrant patterns of nerves are important in interpreting the structure of motor nuclei revealed in the hindbrain in HRP-filled specimens (see below).

RA-induced abnormalities in the hindbrain and midbrain regions — nuclei and axon tracts

Control and RA-treated embryos were analysed by HRP tracing at stages 39–46. Experimental embryos (at 0.5 or 1 µM RA) which had mostly two or occasionally a single fused eye at the anterior extent of the CNS were selected for analysis in an attempt to standardize the phenotype examined. HRP was applied to the cranial ganglia rostral or caudal to the otocyst to reveal motor nuclei and sensory projections of the relevant cranial roots described above. Individual cranial nerve roots are difficult to see within the surrounding mesenchyme, therefore HRP crushes were aimed at the ganglia positioned just anterior and posterior to the otic vesicle. No attempt was made to label the Vth and VIIth ganglia separately. In control embryos, preotic crushes thus
Control

+ RA

Fig. 5. Molecular segmentation in the hindbrain of RA-treated embryos. In situ hybridization of control (A–C) and 0.5 μM RA-treated embryos (D–F) at stage 20 using a Xenopus Krox-20 probe. (A,D) shows a low power bright-field sagittal sections of the embryos. Bright-field (B,E) and dark-ground (C,F) of respective embryos. Anterior is to the left and ventral to top in all panels. Control embryos show two strong stripes of Krox-20 expression which correspond to the future rhombomeres r3 and r5. In RA-treated embryos, only a single stripe of Krox-20 expression is detected throughout all serial sections. This illustrates that despite the absence of overt morphological segments, some aspects of segmental organization are preserved in the hindbrain of RA-treated embryos.

labelled the Vth and VIIth ganglia and roots, and the extraocular motor nerves III, IV and VI which run close to these ganglia. Postotic crushes labelled the IXth and Xth ganglia and their roots. However, in RA-treated embryos due to the abnormal locations and fusions of preotic and postotic nerves within the mesenchyme surrounding the otic vesicle, the positions of axons and cells labelled in the CNS were less predictable than in controls. A summary presenting the principal features of the arrangement of central nuclei and central projection pathways for cranial roots III, IV, V, VI, VII, IX and X is shown in Fig. 9 (see also Fig. 12).

(i) Preotic labelling
The Vth root is more anterior and enters the brain slightly ventral to that of the VIIth nerve (Figs 7 and 8). The principal sensory projections within the hindbrain are also clearly separable. That of V extends rostrally to the midbrain border and caudally as a dorsolateral tract to the spinal cord. The projection of VII is separate from and more dorsal to that of V, extending as far rostrally, but not as far caudally, finishing within the hindbrain. The motor nuclei of V and VII are not clearly separable and we could not identify distinct lateral line or parasympathetic neurons projecting to the anterior lateral line and VII roots, respectively (Will, 1982; Matesz and Szekely, 1973). The motor nuclei of V and VII lie close to the site of root entry of both nerves and are situated at the same dorsoventral and mediolateral position (Figs 9 and 10). The Vth motor nucleus is more rostral than that of VII.

Preotic ganglion HRP applications also resulted in retrograde labelling of the IIIrd, IVth and VIIth nerves and their central nuclei (Figs 9, 10 and 12). The IIIrd motor nucleus lies in the ventral midbrain and has both an ipsilateral and a contralateral component (Figs 9 and 10). Axons from the contralateral neurons cross the midline ventrally to exit together with the ipsilateral axons. The IVth motor nucleus lies at the midbrain–hindbrain border, contralateral to its exit point (Figs 9, 10 and 12). Its axons loop dorsally through the caudal tectum to exit contralaterally at the midbrain–hindbrain border. The Vth motor nucleus lies close to the midline posterior and ventral to the Vth and VIIth motor nuclei, and projects its axons anteriorly to exit via a ventrally placed root opposite the otocyst.
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Fig. 6. RA alters regionalization in the hindbrain. Sections of stage 45 tadpoles through the hindbrain of control (left) and 0.5 μM RA-treated embryos (right, +RA) stained with anti-neurofilament antibody. White arrows mark the three exit points of the Vth, VIIth/VIIIth and IXth cranial nerves from top to bottom respectively in control embryos. Only two discrete exit points are observed in RA-treated embryos (right). From these sections it is not possible to determine if one of the nerves is missing or is superimposed upon another. However, the distance from the most posterior exit point and the first spinal nerve is identical in the two embryos. Note the more anterior position of the otic vesicle (OV) spanning the isthmus (i, open arrow) between the midbrain (mb) and hindbrain (hb).

In RA-treated embryos, axons entering or leaving the brain rostral to the otocyst usually did so in a single root. Our data do not allow us to identify this root as either V or VII but the single root contained axons that project into both the central tracts of V and VII, which are positioned normally. The Vth projection extended to the dorsal spinal cord and the VIIth was more dorsal but less extensive caudally (Fig. 8). As described above, in some cases, small individual nerves were seen entering close to the main root, or aberrantly placed small groups of axons projected caudally around the outer surface of the anteriorly placed otocyst or between the otocyst and the brain (Fig. 11). In these cases, axons entered the brain at abnormal caudal positions, forming variable numbers of small rootlets.

A single column of neurons, in the position of motor nuclei V and VII, was filled as in normal embryos, but when the hindbrain–midbrain border was evident this row of cells appeared to be closer to it than normal and often more compact (Fig. 10). The motor axons from these cells exit in a single root (Fig. 8). In several preotic applications, cells were filled more caudally than normal. On close inspection, the axons of these cells were seen to leave the brain via a single root or via numerous small rootlets at a position caudal to the otocyst at a level corresponding to the normal location of the main root of IX and X. This subset of axons then moves rostrally around the otocyst towards the preotic HRP application site. It seemed, therefore, that these were axons from the IXth and Xth roots, which were misdirected peripherally.

As in normals, preotic HRP applications also labelled motor neurons in the IIIrd, IVth and VIth extraocular motor nuclei. The relative positions of these nuclei in
Fig. 7. Confocal analysis of RA-induced cranial nerve alterations in whole-mount embryos. The same embryos stained with anti-neurofilament antibody in Fig. 4 were examined by optical sections in lateral views. Anterior is to the right and posterior to the left in all panels. The RA-treated embryo is shown in A–C, and the control in D–F. OV marks the otic vesicle. In the control (D), the VIIth nerve appears as a flat band in front of the otocyst and the serial sections (D–F) clearly define the Vth, VIIth, VIIIth and IXth/Xth nerve complexes. In the RA-treated embryos, only the VIIth, VIIIth, IXth/Xth nerves are apparent and no staining for the distinctive Vth nerve (trigeminal) is observed. h hindbrain; m midbrain.

(i) Postotic HRP applications
The postotic HRP applications in normal embryos filled the IXth and Xth central nuclei. The roots of these cranial nerves lie together, posterior to the otocyst and a characteristic set of central axonal projections are revealed by HRP (Fig. 12D). These extend rostrally and caudally as laterally placed tracts from the level of the root. The central nuclei of IX and X form a continuous column of neurons running from the rostrocaudal level of the combined root input to the caudal hindbrain (Figs 9 and 10). In some cases, spinal primary and secondary motor neurons were filled, presumably as a result of inadvertent HRP application to the rostral myotome, although these could be spinal accessory neurons (see Matesz and Szekely, 1978).

In RA-treated embryos following postotic HRP application, the axon tracts caudal to those of the root entry were normal for the IXth and Xth nerves, as were the positions of the motor nuclei (Figs 9, 10 and 12). However, nuclei at more cranial locations were also filled with HRP, a situation never seen in normal embryos. On close inspection of whole-mounted and sectioned embryos, it was apparent that these labelled neurons were filled via aberrantly placed rootlets entering the brain at variable positions medial and rostral to the anteriorly placed otocyst and by axons...
Retinoic acid and hindbrain patterning

VII

Fig. 8. Camera-lucida drawings of facial and trigeminal cranial nerve root pattern in control and RA-treated embryos. (A) Ventrolateral view of a whole-mounted, control Xenopus brain. HRP was applied to the preotic ganglion and has labelled the roots and sensory tracts of the trigeminal (V) and facial (VII). The Vth root is more rostral than the VIIth root, and extends further caudally. Outlines of the positions of motor nuclei of III, IV, V, VI and VII are shown dotted. The ventral midline is dashed. (B) Ventrolateral view of a whole-mounted, RA-treated Xenopus brain. HRP was applied to the preotic ganglia as in A. Most of the axons entering the Vth and VIIth sensory tracts now do so at the same rostrocaudal level; a few are seen to enter the VIIth tract more rostrally. The dorsoventral position and rostrocaudal extent of the tracts are normal. Dotted outlines show position of labelled motor neurons in the positions of nuclei III, V, VII and VI. Midbrain–hindbrain border (mh); scale bar 100 μm.

entering the combined Vth and VIIth root (Fig. 11). These rostrally situated cell bodies are, most likely, normally located motor neurons of the Vth or VIIth nuclei filled via abnormally situated peripheral axons of the Vth and VIIth roots, which were previously identified following preotic HRP applications (see above).

In an attempt to clarify the exact relationship of central nuclei in RA-treated embryos, measurements were taken between various landmarks within the hindbrain in whole-mounted HRP specimens. These data for overall length of the hindbrain from midbrain border to obex, the rostral end of the IX/X motor nucleus to the obex and the caudal extent of the VII motor nucleus to the midbrain border are presented in Table 1. Although none of the measurements were significantly different from control values, the hindbrain in RA-treated specimens is invariably shorter than controls from obex to midbrain border, and this reduction is due to an apparent shortening in the rostral region of the hindbrain, between the caudal extent of the VII nucleus and the midbrain border.

Fig. 9. Summary drawing of the motor nuclei and root positions of cranial nerves III, IV, V, VI, VII, IX and X in the Xenopus tadpole hindbrain. Normal (right) and RA-treated (left) specimens. Information on controls was collated from 24 stage 39–46 embryos, 7 of which had the preotic ganglia of V and VII as well as the roots of IV and VI filled with HRP. A set of 14 embryos had the tracts and nuclei of IX and X filled by a postotic ganglion HRP application and the remaining three embryos had both preotic and contralateral postotic ganglia HRP applications. The description of the relevant neuroanatomy of control embryos was derived from complete whole-mounted specimens and horizontally or transversely sectioned embryos. The observations are in agreement with previous neuroanatomical descriptions based on architectonics and neuronal tracing (see Matesz and Szekely, 1978; Will, 1982; Nikunwe and Nieuwenhuys, 1983). The RA data is derived from 24 treated embryos of comparable stages to controls. Of these, 11 were preotic HRP applications, 5 were postotic and 8 were combination fills with preotic on one side and postotic on the other. 16 of these specimens were sectioned (horizontally or transversely) and 8 were viewed as whole-mounts.

Spinal cord HRP applications
When HRP is crushed into the rostral spinal cord, cells in the brain with descending axons are filled. These groups include a number of nuclei that provide useful markers with which to assess further the structure of the brain of RA-treated embryos and allow quantification of filled cells. Such applications in normal larvae at stages 40–45 fill large numbers of reticulospinal neurons
including, in some cases, the largest such cell, Mauthner’s neuron. The vestibulospinal nucleus located in the hindbrain close to the level of the otocyst and the ventral midbrain nucleus of the longitudinal fasciculus at the level of the oculomotor nucleus are also readily identifiable (Fig. 13).

In normal embryos, many cells are filled in the hindbrain and midbrain regions and some cells are labelled in the forebrain (Table 2). In RA-treated embryos (Table 2), the number of cells in the midbrain is reduced, although a clear ventral nucleus of the Table 1. Quantitative analysis of hindbrain dimensions in normal and RA-treated HRP whole-mounted specimens

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Hindbrain</th>
<th>Midbrain</th>
<th>Forebrain</th>
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<tr>
<td>controls</td>
<td>128±11 (9)*</td>
<td>53±9 (7)</td>
<td>59±9 (6)</td>
</tr>
<tr>
<td>RA treated</td>
<td>104±16 (6)</td>
<td>56±3 (3)</td>
<td>40±14 (5)</td>
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Note: *n given in brackets.

Fig. 10. Camera lucida drawing of the central patterning of cranial nerve nuclei in control and RA-treated Xenopus brains. (A) Horizontal section showing the normal positions of cranial nerve motor neurons. The neurons of nuclei III, IV, V, VI and VII were filled from a preotic HRP application, and the neurons of the IXth/Xth complex from a postotic application. Data from two specimens have been superimposed. (B) Horizontal section through a RA-treated brain. HRP was applied to the preotic ganglion. Neurons occupying the positions of Vth and VIIth nuclei are seen to have a common exit point for their axons. Neurons of the VIIth nucleus occupy their normal position just rostral and medial to the VIIth motor neurons. One neuron in the contralateral IVth nucleus, and three cells in the IXth/Xth complex were also labelled. (C) Horizontal section through a RA-treated brain. HRP was applied to the postotic ganglia on one side and to the preotic ganglia on the other. The preotic fill has labelled only a few neurons in the IIIrd, Vth and IXth/Xth nuclei. Many more cells were labelled by the postotic application and most of these occupy the normal position of IXth/Xth neurons. A few neurons in the VIIth and one in the IXth nucleus are also labelled. (D) Ventral view of a whole-mounted, RA-treated Xenopus brain. HRP was applied to the preotic ganglia on one side only. This specimen had only one median eye and was clearly compressed in the rostrocaudal axis. Nonetheless the positions of neurons in the nuclei of III, V, VI, IX and X are relatively normal. The neurons within the IXth/Xth nuclei and the few within the contralateral Vth and VIIth nuclei were labelled due to disrupted peripheral pathways of their neurons. Midline is dashed throughout. m/h is the midbrain–hindbrain border. Scale bar is 100 μm.

longitudinal fasciculus was evident in the majority of cases (Fig. 13). In the hindbrain, the number of filled cells is also reduced; reticulospinal and vestibulospinal neurons are present (Fig. 13), but Mauthner’s neurons were only rarely labelled (Fig. 13D). Thus, it appears that there is an overall reduction in the descending projecting cells of the brain in RA-treated embryos. We do not know whether some specific neuron groups are affected more than others.

Table 2. Number of brain cells filled by a rostral spinal and HRP crush

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Hindbrain</th>
<th>Midbrain</th>
<th>Forebrain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>1</td>
<td>211</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>214</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>214</td>
<td>–</td>
</tr>
<tr>
<td>mean</td>
<td>507*</td>
<td>161</td>
<td>14.5</td>
</tr>
<tr>
<td>RA-treated</td>
<td>1</td>
<td>52</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>42</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>28</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>29</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>49</td>
<td>–</td>
</tr>
<tr>
<td>mean</td>
<td>203*</td>
<td>36</td>
<td></td>
</tr>
</tbody>
</table>

* significantly different at 5% level by t-test.
Retinoic acid and hindbrain patterning

Fig. 11. Photomicrograph of a horizontal section through an otocyst in a RA-treated *Xenopus*. HRP was applied to a preotic ganglion (just off bottom left of micrograph). Note how axons (arrows) are wrapped around the otocyst and peripheral neurons lie adjacent to its lateral wall (curved arrows). These abnormal axon and neuron positions in RA-treated animals result in the anomalous labelling of postotic neurons from preotic HRP applications, and vice versa. Scale bar is 50 μm.

Discussion

Our general description of *Xenopus* embryos treated with RA are in accord with those of Durston *et al.* (1989) and Sive *et al.* (1990) showing a clear dose-dependent loss of anterior structures. Detailed neuroanatomy using neurofilament antibody and HRP staining reveals that, in addition to this loss of anterior neural structures, RA causes a more subtle but reproducible lesion in the anterior hindbrain region. Durston *et al.* (1989) originally concluded that RA causes a respecification of neural fate resulting in an expansion of posterior neural structure, specifically the hindbrain, at the expense of anterior forebrain and midbrain regions. Our results indicate that RA has additional effects on the anterior rhombomeres of the hindbrain, which may be more easily interpreted as region-specific reductions in neural tissue rather than as respecification. The evidence for reduction or compression of anterior rhombomeres comes directly from HRP retrograde labelling of cranial motor nuclei, anterior

Fig. 12. Photomicrographs of HRP-labelled cranial motor neurons in RA-treated brains. These transverse sections show that this dorsoventral position of the motor neurons and the sensory tracts is indistinguishable from normal. (A) Section through midbrain showing the normal dorsal looped trajectory of trochlear axons (curved arrows) and a single ipsilateral oculomotor neuron (III). Some neurons in the HRP-labelled preotic ganglion are also seen in this section. (B) Section through anterior hindbrain showing neurons of the Vth/VIIth complex and a few VI neurons. HRP was applied preotically on the right and postotically on the left. (C) Ventromedial abducent neurons (VI) are labelled from a preotic HRP application, together with the sensory tracts of V and VII, and the contralateral IXth/Xth tract is labelled from a contralateral postotic application. (D) Neurons of the IXth/Xth complex are labelled together with their associated sensory tracts on the same side. The contralateral Vth tract has also been labelled. Scale bar is 100 μm.
Fig. 13. Photomicrographs of neurons labelled from rostral spinal cord HRP applications. (A) Horizontal section through midbrain and hindbrain of a normal Xenopus larva. Ventral midbrain cells and vestibulospinal neurons are clearly labelled. Some more medial hindbrain reticulospinal neurons are more faintly labelled. (B) Horizontal section through midbrain and hindbrain of a RA-treated Xenopus. Eyes are present at the truncated anterior midbrain. As in controls, ventral midbrain cells and vestibulospinal neurons are labelled, but the distance between these two cell groups is reduced. Some reticulospinal neurons are also apparent. (C–E) Transverse sections of RA-treated embryos. (C) TS through midbrain showing large ventral midbrain neurons. The ectopically placed otocysts are apparent in this section. (D) TS showing one Mauthner’s neuron (arrowed), and two Mauthner’s axons (curved arrow) crossing the ventral floor plate at the level of the rostral vestibulospinal neurons. (E) TS through vestibulospinal complex, showing its characteristic location dorsolateral to the reticulospinal neurons. vm, ventral midbrain neurons; vsp, vestibulospinal neurons; rsp, reticulospinal neurons; oto, otocyst. Scale bar is 100μm.

Displacement of the otocyst, from measurements of hindbrain dimensions and from the distribution of Xkrox-20 expression. The motor nuclei of cranial roots III, IV, V, VI, VII, IX and X are found in defined locations in the stage 40–45 tadpole brain (Fig. 9). RA-treated embryos show apparently normally located nuclei for roots VI, IX and X, which are derived from rhombomeres 5 to 8 (Keynes and Lumsden, 1990). Cells occupying positions of the IIIrd, IVth, Vth and VIIth motor nuclei are clearly present but those of V and VII appear to be compressed, and located closer to the midbrain border than normal. We cannot exclude the possibility that all of the cells in this location are either V or VII because we did not examine target specificity of their projections. A lesion to the anterior rhombomeres is also strongly supported by the abnormal
rhombomeric anatomy in the hindbrain and altered expression domain of \(XKrox-20\). In RA-treated embryos, only a single stripe of \(Krox-20\) expression is observed in a relatively anterior hindbrain position. The stripe appears of normal width and size, so we cannot determine whether rhombomere 3 or 5 has been lost or whether fusion of these rhombomeres has occurred. The fact that \(Krox-20\) expression occurs indicates that segmentation has not been totally disrupted, despite the lack of morphological evidence for rhombomeres in RA-treated embryos. This conclusion is also supported by the apparently relatively normal positions of the 1Vth, 1VIth, 1Xth and 1Xth cranial nuclei and also possibly the 1Vth and 1VIIth. The fact that the 1VIIth motor nucleus and the vestibulospinal nucleus are present suggests that the specification of rhombomeres 4 and 5 is normal (Lumsden and Keynes, 1989).

The evidence from previous studies (Durston et al. 1989; Sive et al. 1990; Ruiz i Altaba and Jessell, 1991a), and from the current experimental results, shows that relatively low concentrations of RA cause reductions in forebrain and midbrain structures and that the anterior hindbrain is specifically affected. One possibility is that the reduction in anterior neural tissue (forebrain and anterior midbrain) as compared to the hindbrain is mediated by a separate effect of RA. Two lines of evidence show that at least two midbrain nuclei are found in RA-treated embryos: the oculomotor nucleus and the nucleus of the longitudinal fasciculus. Secondly, analysis of brains of mouse (Morriss-Kay et al. 1991) and zebrafish (Holder and Hill, 1991) embryos that have been treated with RA at comparable gastrula stages show the remarkably consistent result that anterior rhombomeres are reduced, but in these species the forebrain and anterior midbrain are apparently unaffected, suggesting that the effects of RA on the development of these two regions of the CNS can be effectively uncoupled. The common effect of RA on zebrafish, \(Xenopus\) and mouse embryos is, therefore, that on the anterior hindbrain and associated structures: the loss of the more anterior forebrain and midbrain structures appears to be unique to \(Xenopus\). Another possibility, originally suggested by Durston et al. (1989) is that RA causes respecification of anterior into posterior brain regions in \(Xenopus\). We feel that this interpretation is highly unlikely because, in addition to arguments about multiple mechanisms of RA action described above, if there is a posteriorization and remodelling of anterior neural structures, there must have been a global respecification leading to the normal spatial arrangement of cranial nerve nuclei and spinal nerves, because the relative positions of all of these are maintained in RA-treated embryos. Furthermore, our evidence regarding the descending spinal projections indicates that although the RA-treated hindbrain is broader, it contains fewer neurons, suggesting that the increase in volume reported by Durston et al. (1989) is most likely due to an increase in extracellular space, the number of glial cells or neurons of an unspecified class.

It remains unclear, therefore, what is the primary site of RA action. Indeed, it probably affects more than one tissue. RA can affect the CNS directly (Durston et al. 1989; Ruiz i Altaba and Jessell, 1991b), but there is increasing evidence that, in association with inducing growth factors, it can affect specification of mesoderm in \(Xenopus\) (Cho and De Robertis, 1990; Cho et al. 1991; Ruiz i Altaba and Jessell, 1991a). In the case of embryos treated during gastrulation, effects on the CNS may be secondary, caused by abnormal inductive interactions between an altered mesoderm and the overlying ectoderm. It is known, for example, in RA-treated mouse embryos, that the amount of mesenchyme in the head region is reduced (Morriss, 1975), and that expression of homeobox genes in the CNS can be regulated by the underlying mesoderm (Hemmatti-Brivanlou et al. 1990; Cho and De Robertis, 1990). This interpretation is consistent with the classical observations that the mesoderm is involved in specifying neural fate during some phase of neural induction. This conclusion focusses attention on the origin and distribution of mesoderm in the head and implies that the cranial mesoderm may be differentially distributed or differentially affected by RA in vertebrate embryos. However, in the head, the final pattern is a result of interactions between multiple tissues including the neural epithelium, surface ectoderm, mesoderm and pharyngeal endoderm, and it is too much to expect that the alteration of only one component of this network by RA would generate a simple transformation.

We wish to thank Peter Bayley for help with confocal microscopy and Malcolm Maden and Jonathan Cooke for many fruitful discussions. JDWC was supported by Action Research and NH acknowledges financial support from the SERC; NP was supported by the Greek State Scholarship Foundation.

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