Retinoic acid modifies the pattern of cell differentiation in the central nervous system of neurula stage *Xenopus* embryos

A. RUIZ i ALTABA and T. M. JESSELL

Howard Hughes Medical Institute, Center for Neurobiology and Behavior, Department of Biochemistry and Molecular Biophysics, Columbia University, New York, NY 10032, USA

Summary

Neural cell markers have been used to examine the effect of retinoic acid (RA) on the development of the central nervous system (CNS) of *Xenopus* embryos. RA treatment of neurula stage embryos resulted in a concentration-dependent perturbation of anterior CNS development leading to a reduction in the size of the forebrain, midbrain and hindbrain. In addition the overt segmental organization of the hindbrain was abolished by high concentrations of RA. The regional expression of two cell-specific markers, the homeobox protein *XhoX3* and the neurotransmitter serotonin was also examined in embryos exposed to RA. Treatment with RA caused a concentration-dependent change in the pattern of expression of *XhoX3* and serotonin and resulted in the ectopic appearance of immunoreactive neurons in anterior regions of the CNS, including the forebrain. Collectively, our results extend previous studies by showing that RA treatment of embryos at the neurula stage inhibits the development of anterior regions of the CNS while promoting the differentiation of more posterior cell types. The relevance of these findings to the possible role of endogenous retinoids in the determination of neural cell fate and axial patterning is discussed.

Key words: axial patterning, central nervous system, embryo, retinoic acid, *Xenopus laevis*.

Introduction

The development of the embryonic vertebrate central nervous system (CNS) begins with the induction and patterning of neural ectoderm. In *Xenopus* embryos, induction of neural tissue from undifferentiated dorsal ectoderm is mediated by signals that derive from adjacent dorsal mesoderm during gastrulation (Spermann, 1938; Hamburger, 1988; Dixon and Kintner, 1989). Axial mesoderm of different anterior–posterior (A–P) character induces neural tissue of similar axial character (Mangold, 1933; Ruiz i Altaba and Melton, 1989) providing evidence that early regional differentiation along the A–P axis of the neural plate is controlled, in part, by dorsal mesoderm. A signal spreading through the ectoderm may also contribute to A–P patterning of the nervous system (Ruiz i Altaba, 1990).

Recently, the development of the A–P axis of *Xenopus* embryos has been shown to be sensitive to RA (Durston *et al.* 1989; Sive *et al.* 1990; Cho and De Robertis, 1990; Ruiz i Altaba and Jessell, 1991). Application of RA to blastula and gastrula stage embryos to RA has been suggested to result from an anterior-to-posterior transformation of the CNS that is mediated by a direct action of RA on neural ectoderm (Durston *et al.* 1989). RA has, indeed, been shown to inhibit the development of anterior ectodermal structures such as the cement gland (Sive *et al.* 1990). However, RA can also affect directly the differentiation of anterior-dorsal mesoderm (Ruiz i Altaba and Jessell, 1991). This finding suggests that the neural defects observed after RA treatment of pre-neurula stage embryos result, at least in part, from an earlier perturbation of mesodermal differentiation.

The pattern of cell differentiation that is observed when RA is applied at later stages, after the involution of mesoderm and the formation of the neural plate may provide a more direct indication of the effects of RA on the differentiation of neural ectoderm. In preliminary observations, we found that application of RA to neurula stage embryos resulted in less extreme axial deficiencies (Ruiz i Altaba and Jessell, 1991). For example, headless embryos were never observed and the cement gland and eyes still appeared in anterior positions. To characterize in more detail the effects of late application of RA on the pattern of cellular differentiation in the developing CNS, we have examined the development of distinct regions of the CNS and the appearance of specific cell types using
antibody markers. We show that the marked impairment of anterior neural development is accompanied by a reduction in the A–P extent of the hindbrain region and a loss of the overt segmental pattern of the hindbrain. In addition, RA causes the ectopic, anterior appearance of cell types characteristic of more posterior regions of the CNS. These findings provide evidence that RA modifies the pattern of cell differentiation in the developing CNS by a direct action on neural ectoderm. Retinoids may therefore regulate the development of the embryonic A–P axis in Xenopus embryos by modifying the pattern of cellular differentiation first in the mesoderm and later in the neural ectoderm.

Materials and methods

Embryo manipulations

Xenopus eggs were obtained by injecting female frogs with human chorionic gonadotropin. Embryos were obtained by fertilizing eggs in vitro with testis homogenates. Embryos were staged according to Nieuwkoop and Faber (1967). All-trans retinoic acid was purchased from Sigma and kept as a stock at $5 \times 10^{-3} \text{M}$ in DMSO at $-20^\circ\text{C}$. Appropriate dilutions were made directly into 0.1x MMR (10 mM NaCl, 0.2 mM KCl, 0.1 mM MgSO$_4$·7H$_2$O, 0.2 mM CaCl$_2$·2H$_2$O, 0.5 mM Hepes pH 7.6, 0.01 mM EDTA). Early neurula stage embryos (stages 14–16) were treated continuously with retinoic acid at concentrations ranging from $10^{-10}$ M to $10^{-7}$ M. Embryos were allowed to develop to the tadpole stage for assessment of morphology and antigen expression. The phenotypes observed after RA treatment were detected in about 70% of the embryos from each batch (20–40 embryos), with the exception of changes in the pattern of serotonin expression evoked by low concentrations of RA (see Results). Each experiment was performed at least three times using different batches of embryos.

Antibodies

The antibodies used were (1) mAb 5A5, which recognizes the α2,8 polysialyl side chain on N-CAM (N-CAM-PSA) (Dodd et al. 1988); (2) mAb HNK-1, which recognizes a carbohydrate epitope (Abo and Balch, 1981); (3) a polyclonal antiserum against serotonin, kindly provided by H. Tamir; (4) a polyclonal antiserum against the N-terminal region of the XhoX3 homeobox protein (Ruiz i Altaba et al. 1991). A detailed description of the normal pattern of XhoX3 protein expression in the developing CNS is described elsewhere (Ruiz i Altaba et al. 1991). Monoclonal antibodies were used as culture supernatants diluted 1:1. The serotonin antiserum was used at 1:1000 and the XhoX3 antiserum at 1:100.

Whole-mount immunohistochemistry

The protocol used for whole-mount immunohistochemistry is essentially that of Dent et al. (1989). Embryos were fixed in 3.7% formaldehyde, 0.1 mM MOPS pH 7.4, 1 mM magnesium sulfate and 2 mM EGTA (Patel et al. 1989), transferred to methanol and stored at $-20^\circ\text{C}$. Before staining, the embryos were transferred in steps into PBS containing 0.1% Triton X-100. Non-specific binding was reduced by incubation in 1% BSA, 1 mM magnesium sulfate in PBS plus 0.1% Triton X-100. Antibodies were diluted in PBS containing Triton X-100 with 10% heat-inactivated fetal calf serum and applied to the fixed embryos overnight at 4°C. Secondary antibodies coupled to horseradish peroxidase (Fab fragments, Boehringer Mannheim) were used at 1:100. After each antibody incubation, embryos were extensively washed in PBS containing Triton X-100 for at least 4h with 4–6 changes of buffer. Embryos were incubated for 30 min with diaminobenzidine alone in PBS plus Triton X-100 before reaction of the horseradish peroxidase with diaminobenzidine (0.05 mg ml$^{-1}$) in the presence of hydrogen peroxide (0.003 %). The reaction was terminated by transferring the embryos to methanol. Embryos were then cleared with benzyl alcohol/benzyl benzoate and photographs taken with a dissecting or a Zeiss axiophot microscope using Ilford Pan F film and a green filter to enhance contrast. Histological sections (10 μm) of whole-mount preparations were cut in a microtome after embedding in paraffin.

Results

Identification of cell types in the CNS by expression of antigenic markers

We monitored the effects of RA on cell differentiation in the embryonic Xenopus CNS using antibodies directed against tissue- and cell-specific antigens. Mab 5A5 recognizes the highly sialylated form of N-CAM (N-CAM-PSA) and was used to delineate the overall features of cell differentiation in the embryonic CNS, in particular the segmental organization of rhombomeres and cranial nerves in the hindbrain. A mAb against the HNK-1 carbohydrate epitope was used to identify Rohon–Beard neurons in the embryonic dorsal spinal cord (Nordlander, 1989). Rabbit antisera directed against serotonin and the Xenopus homeobox protein XhoX3 were used as markers of distinct classes of neurons located at defined positions along the A–P axis of the embryonic CNS. The expression of these four antigens in normal tadpole-stage embryos is shown at low magnification in Fig. 1.

Retinoic acid treatment of neurula stage embryos perturbs the development of the anterior CNS

Application of RA to blastula and gastrula stage embryos has been shown to cause severe anterior truncations including the complete loss of heads (Durston et al. 1989; Sive et al. 1990; Ruiz i Altaba and Jessell, 1991). Application of RA at the early neurula stage (stages 14–16) also results in the development of tadpole stage embryos with defects in the CNS (Ruiz i Altaba and Jessell, 1991). Examination of the overall morphology of embryos treated with low concentrations of RA ($10^{-10}$–$10^{-9}$ M) revealed minor defects in anterior structures, primarily a decrease in the size of the forebrain. Treatment of embryos with intermediate ($10^{-8}$ M) and high ($10^{-7}$ M) concentrations of RA resulted in a progressive reduction in the size of the brain and a decrease in the A–P extent of the body axis (Ruiz i Altaba and Jessell, 1991; see Fig. 2 for overall brain defects). However, in contrast to the effects of RA applied to early embryos, high concentrations of RA applied at the neurula stage did not cause the deletion of all anterior ectodermal derivatives; for example, the cement glands and eyes were always present (see below).

To extend these morphological observations on the
Xenopus recognize subsets of homeobox gene \textit{Xhox3} to study in more detail the effects of RA on neural hindbrain sialylated N-CAM is recognized by the monoclonal antibody 5A5 (Dodd \textit{et al.} 1988), and is expressed exclusively in the developing neural tube. The monoclonal antibody HNK-1 (Abo and Balch, 1981) recognizes a variety of neural and non-neural cell types, including Rohon-Beard sensory neurons in the dorsal region of the spinal cord (Nordlander, 1989). Antisera against the Xenopus homeobox gene \textit{Xhox3} recognize subsets of neurons in the midbrain, hindbrain and spinal cord (Ruiz \textit{et al.} 1991). Antisera against the neurotransmitter serotonin (5-HT) label a small population of cells in the ventral region of the anterior hindbrain (van Mier \textit{et al.} 1986). Arrows point to relevant regions of antigen expression. In all cases, anterior is to the left and the dorsal side is upwards. Scale bar=1 mm.

Overall structure of the CNS, we examined the distribution of N-CAM-PSA in the CNS of embryos exposed to RA at the early neurula stage. In untreated tadpole stage embryos, N-CAM-PSA was expressed at high levels in the forebrain and anterior midbrain but the caudal region of the midbrain and spinal cord was not affected by RA. Expression of N-CAM-PSA thus defines a marked reduction in the A-P extent of the hindbrain region, the pattern of rhombomeres delineated by the different levels of N-CAM-PSA expression was also disrupted (Fig. 2B) and boundaries between individual rhombomeres were indistinct (Fig. 2B and data not shown). Treatment of embryos with high concentrations of RA abolished overt hindbrain segmentation and N-CAM-PSA was expressed at nearly uniform levels along the A-P axis in this region of the CNS (Fig. 2C). By the late tadpole stage, embryos treated with high concentrations of RA at the neurula stage exhibited a complete loss of cranial nerves as determined by N-CAM-PSA expression (Fig. 2E). There were no obvious changes in the organization of the spinal cord revealed by N-CAM-PSA expression after treatment of neurula stage embryos with RA (Fig. 2C).

An additional, albeit indirect, indication of a reduction in the A-P extent of the hindbrain region after RA treatment of neurula stage embryos was provided by examining the spatial relationship between the eye and the otic vesicle. The position of the otic vesicle and other neural cell groups (see below) was determined: (1) in relation to that of the eye, which was always located in the anterior region of the CNS and (2) with reference to the anterior-most extent of the neural tube. The otic vesicle appeared in more anterior positions in tadpole stage \textit{Xenopus} embryos treated with low concentrations of RA (Figs 2, 3A–B). High concentrations of RA completely abolished the development of the otic vesicle (Figs 2C, 3C). Exposure of mouse embryos to RA has previously been reported to cause the development of the otic vesicle in more...
Fig. 2. Expression patterns of N-CAM-PSA in the CNS of normal and RA-treated embryos shown at the early (stage ~32–36; A–C) and late (stage ~45; D, E) tadpole stages after application of RA at the early neurula stage. (A) Overall staining pattern of N-CAM-PSA showing the subdivisions of the developing CNS. Note the differential labelling within the major brain regions, especially the hindbrain where alternating levels of N-CAM-PSA in adjacent rhombomeres reveal the segmental organization of the hindbrain. The transition at the midbrain–hindbrain junction from cells expressing high levels of N-CAM-PSA to cells expressing low levels is also pronounced (arrow). (B) N-CAM-PSA immunoreactivity in the CNS of an embryo treated with an intermediate concentration of RA shows a reduction in the size of the anterior regions of the CNS including a compressed region with intense labelling which corresponds to the hindbrain (bracket). Fewer segmental subdivisions can be detected in the hindbrain. (C) Pattern of labelling of mAb 5A5 in an embryo treated with a high concentration of RA. Note the small anterior regions of the CNS and the reduced hindbrain (bracket). In all cases, the differential expression of N-CAM-PSA at the midbrain–hindbrain junction is maintained (arrows in panels A–C). (D) Normal expression of N-CAM-PSA in the anterior region of a late tadpole stage (stage ~45) embryo. Note the reduced expression of N-CAM-PSA in the rhombomeres and midbrain when compared with the embryo shown in panel A. At this stage, N-CAM-PSA is also expressed in the cranial nerves. (E) Expression of N-CAM-PSA in the CNS of a late tadpole (stage ~45) embryo treated at the neurula stage with an intermediate concentration of RA, showing the absence of divisions and the reduced size of the brain. Cranial nerves appear to be absent, as judged by the lack of N-CAM-PSA labelling. C, cerebellar anlage; FB, forebrain; HB, hindbrain; MB, midbrain; N, normal untreated control embryos; o, otic vesicle; RA, embryos treated with RA at intermediate (I-RA) or high (H-RA) concentrations; SC, spinal cord; arabic numbers indicate the approximate positions of the different rhombomeres; roman numerals refer to the different cranial nerves; brackets depict domains of intense labelling corresponding to the hindbrain. Scale bar=0.5 mm. Scale bars in panels A and D are valid for panels A–C and D–E, respectively.

anterior positions (Sulik et al. 1988). The ectodermal precursor of the otic vesicle, the otic placode, is thought to be induced by a signal localized to the medial hindbrain (Yntema, 1950). The change in the position of the otic vesicle that we observed after RA treatment could result from a change in the regional inductive properties of the hindbrain. Alternatively, this change in the position of the otic vesicle could result from a direct effect of RA on placodal ectoderm.

The progressive loss of differential N-CAM-PSA
 Neural patterning by retinoic acid

Fig. 3. Expression patterns of the homeobox protein Xhox3 in control and RA-treated tadpole stage embryos. (A) At the early tadpole stage, Xhox3 is expressed in the dorsal–anterior hindbrain, mainly in rhombomere 1 and in a more ventral column of cells in the spinal cord and hindbrain that extends rostrally up to the boundary between rhombomeres 1 and 2. Within this ventral column, the expression of Xhox3 is highest in rhombomeres 5 and 6 (arrowhead in panel A). (B) Treatment of early neurula stage embryos with low concentrations of RA results in the reduction of the size of the hindbrain. This is manifest as a reduction in the distance between the eye and the otic vesicle (in focus), the eye and cells expressing Xhox3 dorsally in the anterior hindbrain and the eye and cells expressing Xhox3 at high levels normally found in rhombomeres 5 and 6 (slightly out of focus). (C) In older tadpoles, Xhox3 is also expressed in a small group of cells in the lateral midbrain. (C,E) Embryos treated with high concentrations of RA lack the expression of Xhox3 in the midbrain as well as any dorsal expression in the hindbrain but show ectopic expression of Xhox3 in the ventral anterior regions of the CNS, including the forebrain. Treatment with high concentrations of RA causes a reduction in the size of the CNS although the forebrain, midbrain and hindbrain subdivisions can still be distinguished. High magnifications of the brains of control (D) and RA-treated embryos (E) show the changes in morphology and Xhox3 immunoreactivity in the anterior regions of the CNS. Only a few positive nuclei can be observed in panel E since at this magnification, few cells are in the plane of focus. E, eye; FB, forebrain; HB, hindbrain; MB, midbrain; N, untreated control embryos; o, otic vesicle; RA, embryos treated with low (L-RA) or high (H-RA) concentrations of RA; SC, spinal cord; arrowheads point to regions or cells showing nuclear Xhox3 labelling; numbers identify rhombomeres. Brackets depict the hindbrain in panels A–C. Scale bar=0.5 mm. Scale bar in panels A and D are valid for panels A–C and D–E, respectively.

expression among individual rhombomeres, the loss of overt segmentation and the change in relative position of the otic vesicle suggest that RA disrupts hindbrain development in a concentration-dependent manner.

Retinoic acid causes ectopic expression of Xhox3 in the forebrain

We next examined whether the perturbation of forebrain, midbrain and hindbrain development was accompanied by changes in the position at which defined classes of neurons appeared along the A–P axis of the embryonic nervous system. The first marker of neural cell position that we examined was the Xenopus homeobox gene Xhox3. Xhox3 is expressed in the mesoderm in gastrula and neurula stage embryos, but exhibits a second period of expression in the developing
CNS that begins at the neurula stage (Ruiz i Altaba and Melton, 1989a; Ruiz i Altaba, 1990). At the early tadpole stage, Xhox3 protein was expressed in the dorsal–anterior hindbrain, mainly in rhombomere 1, and in a column of cells located more ventrally in the hindbrain extending anteriorly up to the boundary between rhombomeres 1 and 2. Within this column, cells located in rhombomeres 5 and 6 expressed higher levels of Xhox3 (Fig. 3A) and were located more laterally than the cells in other rhombomeres (not shown) suggesting that they may represent a distinct cell type. This column of cells extended throughout the spinal cord although caudally, neurons expressed Xhox3 at lower levels (Figs 3A, 4A). This pattern of Xhox3 expression was maintained in older tadpoles (stage ~40). In addition, at later stages a group of cells in the ventral hindbrain expressed Xhox3 at high levels (Figs 3D, 4B). Thus, three classes of neural cells with different locations can be defined by expression of XhoX3: (1) cells in the anterior-dorsal hindbrain, (2) cells in more posterior and ventral regions of the hindbrain and in the spinal cord, and (3) a late developing set of cells in the ventral midbrain (Figs 3D, 4A,B). Importantly, XhoX3 was not detected in the forebrain of normal embryos at any developmental stage (Fig. 3A–D; Ruiz i Altaba et al. 1991).

RA administration to neurula stage embryos changed the position at which XhoX3 immunoreactive cells were found. Low concentrations of RA decreased the number of neurons expressing XhoX3 in the dorsal region of rhombomere 1 (Fig. 3B). Treatment of embryos with intermediate or high concentrations of RA completely abolished expression of XhoX3 in this region and also in the midbrain (Fig. 4C–D). The laterally displaced cells expressing XhoX3 in rhombomeres 5 and 6 also disappeared (Figs 3C,E, 4). In contrast, expression of XhoX3 by the ventromedial column of hindbrain and spinal cord cells was unaffected by low, intermediate and high concentrations of RA. Moreover, treatment of embryos with high concentrations of RA resulted in the appearance of XhoX3-immunoreactive cells in more anterior regions of the CNS, including the forebrain. Moreover, treatment of embryos with high concentrations of RA resulted in the appearance of XhoX3-immunoreactive cells in more anterior regions of the CNS, including the forebrain (Figs 3C; 4E,F; 5A,D). Cells that expressed XhoX3 in these anterior–ventral regions were continuous with the column of cells found in the posterior hindbrain and spinal cord (Figs 3C,E; 4E,F; 5D,F). These results show that administration of RA to neurula stage embryos results in ectopic expression of the XhoX3 homeobox protein in anterior regions of the CNS, including the forebrain.

**Retinoic acid changes the expression of serotonin in the developing hindbrain**

Several different classes of cells in the CNS appear to express XhoX3. To determine whether the position of a better defined class of neurons changes after application of RA at early neurula stages, we examined the distribution of cells expressing the neurotransmitter serotonin. In normal tadpole-stage embryos, serotonin is expressed by a small number of neurons in the ventral region of rhombomere 1 (Fig. 6A,B; see also van Mier...
Fig. 5. Xhox3-immunoreactive cells in normal and RA-treated embryos. Panels A–C show cross sections of a normal tadpole stage (stage ~36) embryo at the forebrain (A), hindbrain (B) and spinal cord (C) levels. Panels D–F show cross sections at the forebrain (D), hindbrain (E) and spinal cord (F) levels of an embryo treated with a high concentration of RA. Xhox3 immunoreactivity in cell nuclei is denoted by arrowheads. Note the absence of Xhox3 expressing cells in the dorsal hindbrain (E) and the ectopic expression of this homeobox gene in the forebrain (D) in RA treated embryos. FB, forebrain; HB, hindbrain; N, normal embryo; RA, embryo treated with a high concentration of RA (H-RA); SC, spinal cord. Scale bar=10 μm.

The immunoreactive axons of these neurons projected both anteriorly into the forebrain, and posteriorly into the caudal hindbrain and spinal cord (Fig. 6B). Application of low concentrations of RA resulted in an expansion of the A–P region over which serotonergic neurons were detected in about 20–30 % of treated embryos (Fig. 6C–E). Although a minority of embryos showed such an expansion, this result was observed consistently with different batches of embryos. In one representative experiment, the number of serotonergic neurons in untreated embryos was 20.5±0.9 (mean±s.e.m., n=12) neurons per side. In embryos treated with low concentrations of RA, the number of neurons per side was increased to 30.3±2.3 (mean±s.e.m., n=6) in the subset of embryos that exhibited the expansion in the A–P domain of expression. The detection of the phenotype in a minority of the embryos may result from the fact that higher concentrations of RA decrease the number of serotonergic neurons (see below). Variations among individual embryos in access of or sensitivity to RA would result in only a minority of embryos in any given batch being exposed to a concentration of RA that falls within the range required to increase the number of serotonergic neurons.

In affected embryos, the expansion in the A–P extent of the domain of serotonin expression resulted in the appearance of serotonergic neurons in more anterior regions of the CNS as determined by their position in relation to the eyes and the anterior end of the neural tube (Fig. 6C–E). Indeed, in about 10 % of the treated embryos, serotonergic neurons were found at the most anterior extreme of the CNS, anterior to the eyes (Fig. 6E). The localization of serotonin neurons at levels coincident with and anterior to the eyes was never observed in untreated embryos. As mentioned above, the increase in number and the anterior localization of serotonergic neurons in the CNS was observed only with low concentrations of RA. Exposure of embryos to intermediate concentrations of RA resulted in a decrease in the number of serotonergic neurons (Fig. 6F). Moreover, the axons of the remaining neurons projected exclusively in a caudal direction (Fig. 6G). Exposure of embryos to high concentrations of RA resulted in the complete loss of serotonergic neurons (Fig. 6H).
Fig. 6. Expression of serotonin (5-HT) in normal and RA-treated embryos. All embryos shown are at the tadpole stage (stage ~32–34). Panel A shows the localization of serotonergic neurons in untreated embryos. Small groups of cells in the skin are also labelled. This labelling is not detected in the skin of the forehead. The axons of these neurons project both anteriorly and posteriorly. Panel B shows a high magnification view of the normal pattern of serotonin expression in the brain of tadpole stage embryos. The arrow denotes the midbrain–hindbrain junction. Early neurula stage embryos treated with low concentrations of RA show the presence of additional serotonergic neurons in more anterior regions of the CNS (C–E). Panel E shows a high magnification of the embryo shown in D. At intermediate concentrations of RA, the embryos display a reduced number of serotonergic cells (F, G). There is also a lack of axons projecting anteriorly (F). Embryos treated with high concentrations of RA show a complete absence of serotonergic cells in the CNS (H). In all cases anterior is to the left and dorsal side is upwards. E, eye; FB, forebrain; HB, hindbrain; MB, midbrain; N, normal untreated control embryos; o, otic vesicle; RA, embryos treated with low (L-RA), intermediate (I-RA) or high (H-RA) concentrations of RA; SC, spinal cord; arrows point to sites of staining; brackets depict the A–P extent of the region containing serotonergic neurons; numbers identify rhombomeres. Scale bar in panel A=0.5 mm, valid for panels A, C–D and F–G. Scale bars in panels B and E=0.1 mm.
Thus, as observed with Xhox3 expression, RA at appropriate concentrations causes the appearance, in anterior regions, of serotonergic neurons that normally are located in a more posterior region of the CNS.

**Rohon-Beard primary neurons are not affected by application of retinoic acid at the neurula stage**

To test further the idea that RA treatment might cause cell types characteristic of posterior regions of the CNS to appear in more anterior regions, we examined the location of Rohon-Beard primary sensory neurons which are present in spinal levels but not in the hindbrain (Hughes, 1957; Figs 7A, 8A, B). Rohon-Beard neurons can be identified by their characteristic morphology, their location in the dorsal region of the spinal cord and by their expression of the HNK-1 epitope (Fig. 7A; Nordlander, 1989). Treatment of embryos with low, intermediate or high concentrations of RA at the neurula stage, however, did not appear to alter the development of Rohon-Beard neurons in the spinal cord and these neurons were not detected in the hindbrain (Figs 7B, 8C,D). The determination of Rohon-Beard neurons occurs during gastrulation (Lamborghini, 1980). The failure of RA treatment at the neurula stage to change the position of Rohon-Beard cells therefore suggests that RA modifies the fate of neural cells only at times before their determination.

**Discussion**

The results presented here demonstrate that treatment of neurula stage *Xenopus* embryos with RA changes the pattern of cell differentiation in the developing CNS. The changes in cell pattern observed suggest three general features of the response of *Xenopus* neural ectoderm to RA.

First, the effects of RA are concentration-dependent, with cells in more anterior positions exhibiting greater sensitivity to RA (Fig. 9). In support of this, low concentrations of RA have marked effects on forebrain development without detectable actions on the hindbrain. Intermediate and high concentrations of RA affect the forebrain, midbrain and hindbrain without apparently affecting the spinal cord. In addition, the domain of expression of serotonergic neurons, which is normally confined to the ventral region of rhombomere 1, is expanded anteriorly by lower concentrations of RA than that required to expand the ventral column of Xhox3 neurons which is located more caudally.

Second, RA causes the ectopic appearance in anterior positions of cell types normally located in posterior regions of the CNS. With increasing concentrations of RA, cells types in progressively more posterior regions are found anteriorly, at the expense of cells types in more anterior positions. Thus, high concentrations of RA eliminate cell types characteristic of the hindbrain, for example the dorsal Xhox3 neurons and the ventral serotonergic neurons in rhombomere 1 and the lateral Xhox3 neurons in rhombomeres 5 and 6. At the same concentrations, the more ventral column of Xhox3 neurons in the posterior hindbrain and spinal cord appears to persist and to expand into more anterior regions, including the forebrain.

Third, RA changes the pattern of cell differentiation only for cells whose fate has not already been determined. For example the eye primordia and Rohon-Beard neurons do not appear to be affected by RA treatment at the neurula stage.

These observations raise several questions. First, to what extent are changes in cell pattern evoked by RA application at the neurula stage mediated directly on neural ectoderm, or indirectly by actions on axial mesoderm? Second, does RA treatment result in a complete posterior transformation of the CNS? Third, do these observations on the actions of exogenous RA provide an insight into the role of endogenous retinoids in neural patterning? We discuss these three points below.

**Evidence that retinoic acid application to neurula stage embryos has direct effects on neural ectoderm**

Application of RA to blastula and gastrula stage...
No

Fig. 8. Histological sections of normal and RA-treated embryos showing the location of HNK-1-labelled Rohon-Beard cells. Panels A and B show cross sections of a normal embryo at the tadpole stage (stage ~36) at the level of the hindbrain (A) and spinal cord (B). Panels C and D show cross sections at the level of the hindbrain (C) and spinal cord (D) of an embryo at the tadpole stage (stage ~36) treated with a high concentration of RA. In normal embryos, HNK-1 labels the cell body and axonal membranes of Rohon-Beard cells and also a small compact region within the cell body (B). The dorsal position of Rohon-Beard neurons in the spinal cord and the absence of Rohon-Beard neurons in the hindbrain are not altered by RA treatment. Arrowheads point to Rohon-Beard neurons. Fp, floor plate; N, normal embryo; No, notochord; RA, embryo treated with a high (H-RA) concentration of RA; S, somites. Scale bar=10 \( \mu m \).

\textit{Xenopus} embryos has been shown to result in the complete absence of anterior neural structures (Durst \textit{et al.} 1989; Sive \textit{et al.} 1990; Ruiz i Altaba and Jessell, 1991), a phenotype that is similar but considerably more extreme than that obtained after application of RA at the neurula stage. At the blastula and gastrula stages, RA can affect directly the axial character of mesoderm, with anterior–dorsal mesoderm exhibiting the greatest sensitivity to RA (Ruiz i Altaba and Jessell, 1991). The defects detected in the CNS when RA is administered before gastrulation are therefore likely to be secondary to the actions of RA on axial mesoderm.

The changes in neural cell pattern observed after application of RA at the neurula stage could result from direct effects on neural ectoderm or from later effects of RA on axial mesoderm. Two lines of evidence suggest that neural ectoderm has attained a high degree of autonomy by the late gastrula/early neurula stage. First, transplantation studies in amphibian embryos have shown that neural ectoderm from neurula stage embryos can develop independently of axial mesoderm when grafted into the ventral side of a host embryo (Spemann, 1918; see Saxen and Toivonen, 1962; Hamburger, 1988). In addition, experiments in which regions of the amphibian neural plate have been rotated by 180° along the A–P axis have demonstrated that the pattern of neural cell types that emerges conforms to the original polarity of the tissue (Roach, 1945; Jacobson, 1964). Thus, in neurula stage amphibian embryos, neural cell fate along the A–P axis appears no longer to be dependent on signals derived from the underlying axial mesoderm. These observations provide support for the idea that the changes in neural cell pattern observed after application of RA at the neurula stage result from direct effects on neural ectoderm. However, we cannot entirely exclude that RA has some effects on late aspects of mesodermal differentiation.

\textit{Retinoic acid treatment at the neurula stage does not cause a complete posterior transformation of the CNS} RA applied at neurula stages caused a marked reduction in the size of the anterior region of the CNS.
Neural patterning by retinoic acid

Fig. 9. Summary of the effects of RA on the expression pattern of different neural markers. RA causes a concentration-dependent inhibition of normal anterior CNS differentiation, and the ectopic, anterior expression of cells normally found in more posterior regions of the CNS. The drawing at the left shows the generalized morphology of the anterior CNS and head in untreated and RA-treated embryos. The table at the right summarizes the expression pattern of the midbrain expression of Xho3 (X3-M), the expression of Xho3 in the dorsal-anterior hindbrain (X3D-H), the expression of serotonin (5-HT) in the anterior hindbrain, and the expression of Xho3 in the more ventral posterior hindbrain and spinal cord (X3-H/SC) located at different positions along the A-P axis of the CNS. A single + refers to the normal pattern. +/− indicates partial reduction in cell number, ++ refers to an increase in the number and a change in position of the positive cells, and − refers to the absence of positive cells. A, anterior; P, posterior.

and the appearance of neurons expressing Xho3 and serotonin in more anterior regions. However, there was not a complete posterior transformation of anterior regions of the CNS. For example, eyes still developed in the forebrain and Rohon-Beard neurons were not detected ectopically in anterior regions of the CNS in RA-treated embryos. One reason for the incomplete acquisition of posterior neural properties by the anterior CNS is likely to be the timing of addition of RA. Neural cells whose fate is determined prior to neurula stages, for example the eye primordia and primary sensory (Rohon-Beard) and primary motor neurons, are likely to be refractory to the actions of RA applied at the early neurula stage. In contrast, neural cell types that are determined at the early neurula stage or later, for example serotonergic neurons (van Mier et al. 1986) and most of the Xho3 immunoreactive neurons, would therefore be sensitive to the effects of RA with the threshold for sensitivity varying according to the A-P position of the neuron.

Application of RA at gastrula stages could result in a more complete acquisition of posterior character by anterior regions of the CNS. However, this possibility is difficult to test because application of RA at earlier stages has pronounced effects on dorsal mesoderm (Ruiz i Altaba and Jessell, 1991), which in turn is likely to affect the development of the neural ectoderm.

Involvement of retinoic acid in neural patterning

The present results raise the possibility that RA has a role in neural patterning. RA has been detected in gastrula stage Xenopus embryos (Durston et al. 1989); however, its regional distribution remains unknown. The finding that anterior neural development is markedly suppressed by low concentrations of exogenous RA suggests that endogenous RA may be absent from anterior regions of the axial mesoderm and neural plate during normal development. One possible source of RA in neural tissues is the floor plate, a specialized cell group at the ventral midline of the posterior neural tube. Studies in chick embryos have shown that the floor plate can synthesize retinoids and mimic the effects of RA in respecifying digit pattern in the chick wing bud (Wagner et al. 1990). If the floor plate is a relevant source of morphogenetically active retinoids, its absence from the forebrain area (Kingsbury, 1930) would provide a cellular basis for the idea that anterior regions of the CNS are not exposed to high concentrations of retinoids, at least from sources within the neural tube.

The ectopic appearance of Xho3 neurons in the forebrain after RA treatment is also consistent with the idea that the anterior regions of the CNS are normally not exposed to RA. Indeed, other homeobox genes that are known to be regulated by RA, such as those in the Hox clusters (Simeone et al. 1990), are not expressed in anterior regions of the CNS (Holland and Hogan, 1988). It is important to note, however, that the differential sensitivity of anterior and posterior regions of the CNS to exogenous RA could also reflect regional differences in the expression of RA receptor subtypes within the developing neural tube (Ruberte et al. 1990, 1991; Ellinger-Ziegelbauer and Dreyer, 1991; Smith and Eichele, 1991).

The observation that RA affects both the A-P domain of Xho3 homeobox gene expression and hindbrain segmentation in Xenopus embryos raises the possibility that these two phenomena are related. Although it remains to be determined whether RA can alter the A-P pattern of expression of other homeobox genes and whether the changes in homeobox gene
expression in the hindbrain mediate the effects of RA on the cellular organization of the hindbrain, our results are consistent with the possibility that RA affects hindbrain pattern and segmentation by regulating of the A–P domains of expression of different homeobox genes (Wilkinson and Krumlauf, 1990; Keynes and Lumsden, 1990). Indeed, several homeobox genes exhibit distinct boundaries of expression that correspond to rhombomere boundaries (for example Wilkinson et al. 1989; Graham et al. 1989; Sundin and Eichele, 1990), and the expression of some of these genes is responsive to RA in teratocarcinoma cells (Simeone et al. 1990).

Many previous studies have examined the interactions underlying the establishment of A–P pattern in the amphibian neural ectoderm (see Hamburger, 1988). The acquisition of A–P polarity by the embryonic CNS may result from an initial induction of neural ectoderm of anterior character mediated by the prechordal plate mesoderm as it involutes under the dorsal ectoderm, followed by an anterior-to-posterior transformation of neural ectoderm in posterior regions mediated by signals from the later involuting chordamesoderm (Nieuwoop et al. 1952, 1985; Toivonen and Saxen, 1968; Sive et al. 1989). Alternatively, A–P polarity has been suggested to result from the actions of two distinct inducing signals responsible, independently, for the differentiation of anterior and posterior neural ectoderm (Saxen and Toivonen, 1961, 1962). RA could have a role in the differential induction of anterior and posterior neural ectoderm. RA does not induce neural differentiation in competent ectoderm when applied alone (Ruiz i Altaba and Melton, 1989b; Durston et al. 1989; Sive et al. 1990) but could promote posterior neural differentiation by modifying the character of induced neural ectoderm. The possibility that RA present within the chordamesoderm is responsible for promoting the differentiation of posterior neural ectoderm was suggested initially by Durston et al. (1989). However, this interpretation was based on an analysis of neural phenotype in embryos exposed to RA at the blastula and gastrula stages when many of the defects in neural development are likely to result from direct effects of RA on axial mesoderm (Ruiz i Altaba and Jessell, 1991). It is also possible that the floor plate of the neural tube, which is coextensive with the notochord, provides a posterior source of RA within the neural tube (Wagner et al. 1990).

The source and actions of the neural-inducing signals are unknown at present. Anterior axial (prechordal) mesoderm induces neural ectoderm of anterior character (brain) whereas posterior axial mesoderm (chordamesoderm) induces posterior neural tissue (spinal cord) (Mangold, 1933; Leussink, 1970; Ruiz i Altaba and Melton, 1989b). The difference in A–P character of neural ectoderm induced by prechordal mesoderm and chordamesoderm could be achieved in two different ways. First, both types of mesoderm could contain the same inducer, which directs the differentiation of anterior neural ectoderm, with the chordamesoderm also providing a signal, possibly RA, that promotes posterior differentiation. Second, a general neural inducer may promote the differentiation of neural ectoderm of indeterminate A–P character. Additional instructive signals from the prechordal plate mesoderm may be required for the differentiation of anterior neural structures (forebrain). In this case, the ground state of neural ectoderm would not be anterior in character. As described above, the differentiation of posterior neural ectoderm would be dependent on signals, possibly RA, that derive from the chordamesoderm or floor plate. Exposure of neural ectoderm to different concentrations of RA could also be involved in the development of A–P pattern in the posterior CNS. Regardless of the origins and actions of the neural-inducing signals, the principle by which RA patterns neural ectoderm may be similar to that involved in the earlier patterning of the embryonic mesoderm where RA appears to regulate the axial character of mesoderm by modifying the response of ectodermal cells to mesoderm-inducing peptide growth factors (Ruiz i Altaba and Jessell, 1991).

RA may also affect the D–V pattern of the neural tube. In RA-treated embryos, we observed that Xhox3-labelled cells in the dorsal area of rhombomere 1 disappeared before serotonergic neurons in the ventral area of the same rhombomere and before the appearance of Xhox3-labelled neurons in the ventral area of rhombomere 2 (Figs 4C–D, 6; and data not shown). Thus, at the neurula stage, prospective dorsal neural cells appear to be more sensitive to RA than prospective ventral cells.

Evidence that endogenous RA has a role in cell patterning has derived primarily from analyses of the effects of exogenous RA on the axial pattern of the developing limb (e.g. Tickle et al. 1982; Maden, 1983; Kim and Stocum, 1986; Eichele, 1989) and the detection of endogenous RA in the chick wing bud (Thaller and Eichele, 1987). The marked effects of exogenous RA on mesodermal (Ruiz i Altaba and Jessell, 1991) and neural patterning, together with the presence of RA in Xenopus embryos (Durston et al. 1989) suggest that RA may have a more general role in the patterning of early embryonic tissues. Moreover, in the three systems in which axial patterning is sensitive to retinooids, the limb, the axial mesoderm and the neural ectoderm, RA may have a consistency in its actions, promoting posterior and ventral fates by acting as a concentration-dependent modifier of the response to inductive signals.

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