Retinoic acid modifies development of the midbrain–hindbrain border and affects cranial ganglion formation in zebrafish embryos

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Summary

Considerable evidence now suggests that retinoic acid (RA) is an important modulator of patterning events in early neuronal development in vertebrates. In this paper, we describe the effects of exogenously applied RA on early neural development in the zebrafish embryo. Neural anatomy is assessed by immunocytochemical and histochemical analysis of the developing embryo in whole mounts at 24 h post-fertilization. RA was applied for one hour at concentrations ranging from $10^{-9}$ to $10^{-6}$M to embryos at 50% epiboly, the midgastrula stage, and at $10^{-7}$M to embryos at early and late gastrula stages.

The neuroanatomical analysis shows that $10^{-7}$M RA causes a defined lesion to the developing central nervous system which corresponds to a loss of a region of the brain in the caudal midbrain–rostral hindbrain area, the precursor of the cerebellum and associated neural structures. The region that fails to develop corresponds to the cranial expression domain of the engrailed protein as assessed by the monoclonal antibody 4D9 (Patel et al. 1989: Expression of engrailed proteins in arthropods, annelids and chordates. Cell 58, 955–968). Structures caudal to rhombomere 4 are unaffected by $10^{-7}$M RA, as are the cranial midbrain and forebrain: $10^{-7}$M RA also affects the development of cranial ganglia, principally the Vth, anterior lateral line and VIIIth ganglia, suggesting that RA affects normal development of the cranial neural crest. Effects of RA at stages immediately prior to and after gastrulation show some similar and some distinct features.

Results are discussed in terms of the possible role of RA as an endogenous moderator of normal head development.

Key words: retinoic acid, zebrafish, engrailed, cranial ganglia.

Introduction

Retinoic acid (RA) is a candidate developmental signalling molecule which may play a role in pattern-forming events in various regions of the vertebrate embryo (reviewed by Brookes, 1989). In the developing and regenerating limb, RA respecifies positional values, and endogenous retinoids are present in the limb at appropriate stages and in patterns consistent with its role as a positional signalling molecule. Recently, Durston et al. (1989) demonstrated that exogenously applied RA dramatically affects development of the anterior neural structures of Xenopus embryos following exposure of the gastrula for brief periods. The effect of RA is dose-dependent, the most severe phenotype being loss of forebrain and midbrain and apparent increase in hindbrain tissue mass. At lower doses the midbrain and forebrain structures are more complete (see also Sive et al. 1990), but a distinct lesion is present in the anterior hindbrain affecting the development of the first three rhombomeres (Papalopulu et al. 1991). Endogenous levels of RA consistent with those seen in other regions of vertebrate embryos are found in the early Xenopus embryo (Durston et al. 1989). Further evidence for a role for RA in the specification of neural development comes from experiments with the teratocarcinoma cell line HT2/D1, which expresses genes of the Hox-2 cluster in an ordered sequence dependent upon the inducing concentration of RA (Simeone et al. 1990, 1991). The Hox-2 genes are expressed in a temporal and spatial sequence in the CNS of the developing mouse and are thought to be directly involved in specification of neuronal pattern (reviewed by Wilkinson and Krumlauf, 1990). Most recently, Papalopulu et al. (1991) and Krumlauf et al. (1991) have shown that exogenously applied RA alters the expression pattern of homeobox genes and the zinc finger protein Xen-Krox 20 in developing Xenopus embryos. In addition, the existence of nuclear RA receptors and cytoplasmic binding proteins for retinoids in the CNS supports a controlling role for RA in neuronal specification (Maden et al. 1990, 1991; Ruberte et al. 1991). Most recently, RA has also been implicated in a second phase of neural development,
the control of axon outgrowth (Hunter et al. 1991; Wagner et al. 1990).

In this paper, we describe the effects of RA on neural development in the zebrafish embryo which provides an excellent vertebrate model system because of its rapid development, the transparency of the embryo, and the extensive knowledge of the early neuroanatomy of the brain (see, for example, Kimmel et al. 1982, 1985; Trevarrow et al. 1990; Wilson et al. 1990) and spinal cord (Myers et al. 1986; Kuwada et al. 1990). Following exposure to RA at various stages of development and at a range of concentrations, we establish that resulting embryos bear lesions specifically in the caudal midbrain and cranial hindbrain and in a number of peripheral cranial ganglia. At appropriate concentrations the forebrain, caudal hindbrain and spinal cord and trunk are unaffected. The sensitivity of the caudal midbrain/rostral hindbrain to RA coincides with the anterior site of expression of the engrailed protein (Patel et al. 1989; Hatta et al. 1991), and we demonstrate that this expression domain is reduced in extent in a dose-dependent manner by RA. However, RA treatment does not alter the caudal segmental expression of engrailed in myotomal cells. RA also affects development of the cranial ganglia in a stage- and concentration-specific manner. These results are discussed in terms of the possible role of RA as a positional signalling molecule affecting the anterior regions of the central and peripheral nervous systems.

Materials and methods

General

Zebrafish embryos were collected from our laboratory colony by natural spawning using methods described previously (see, for example, Stewart et al. 1988). The breeding fish were maintained at 28.5°C, on a 14 h light/10 h dark cycle and natural spawning was assumed to occur at 'fish dawn', taken as 0 h post-fertilization (hpf) (this is considered to be accurate to within approximately ±30 min, see Wilson et al. 1990). Embryos were raised in Hank's saline (137 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 1.0 mM MgSO4, 0.25 mM Na2HPO4, 4.2 mM NaHCO3) at 10% v/v in a humidified incubator at 28.5°C. Age post-fertilization was calculated to the point when embryos were removed from the incubator. Prior to fixation the chorions were removed from the embryos with fine forceps. All embryos were fixed at 24 hpf for staining with Zn-1 and 4D9 antibodies, we used the method of Hannemann et al. (1988). For staining with monoclonal antibodies HNK-1 and MZ15, we used a modification of the method described by Wilson et al. (1990).

Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C. After two washes in 1% Triton X-100 in PBS, non-specific binding sites were blocked by incubation for 30 min in 10% normal goat serum (NGS)/1% Triton X-100 in PBS at room temperature. Primary antibody was applied as neat supernatant and the embryos were incubated overnight at 4°C. After washing six times in 1% NGS/1% Triton X-100 in PBS (in which the secondary antibody was also diluted), the embryos were incubated in secondary antibody overnight at 4°C. The embryos were then washed six times in PBS and reacted for peroxidase activity as described below. Throughout this procedure, the pH was maintained at 7.4. The secondary antibody for all immunohistochemical methods described was goat anti-mouse IgG conjugated to peroxidase (Sigma), used at a concentration no greater than 1:200. Peroxidase was demonstrated in histochemical preparations by reaction with 0.1 M diaminobenzidine (DAB); following a pre-soak period of 20 min, hydrogen peroxide was added. Development time was usually between 10 and 15 min.

Acetylcholinesterase histochemistry

We used the method of Karnovsky and Roots (1964). The embryos were placed into a reaction medium containing 5 mM trisodium citrate, 3 mM copper sulphate, 0.5 mM potassium ferriyanide and 0.5 mg ml⁻¹ substrate (acetyltiocholine iodide) in 0.1 M maleate buffer (pH6.0) and incubated on ice in the dark. We found that agitation of the specimens resulted in very even staining.

After whole-mount staining with antibodies and acetylcholinesterase (AChE), the yolk sacs were dissected from the embryos using fine forceps. The embryos were then dehydrated through a series of alcohols, cleared in Histoclear (National Diagnostics Ltd) and mounted on glass slides or between glass coverslips (which allowed the embryos to be examined through the microscope). Embryos were then dehydrated through a series of alcohols, cleared in Histoclear (National Diagnostics Ltd) and mounted on glass slides or between glass coverslips (which allowed the embryos to be examined through the microscope).

Fig. 1. (A) Lateral view of the head of a control 24 h embryo stained for acetylcholinesterase, showing the rhombomeres (numbered 1 to 7), the first myotome (myo), the ventral midbrain nucleus of the medial longitudinal fasciculus (nm), the epiphysis (e) and the diencephalic (d) nuclei separated by the optic fissure (curved arrow). The large arrow indicates the fold at the hindbrain–midbrain border and the otocyst is outlined by the dashed line. (B) Similarly stained embryo following treatment with 10⁻¹⁹ M RA at 50% epiboly. Labels as for (A). Note the anterior displacement of the otocyst (outlined by dashed lines) and the normal structure of the forebrain. Scale bars=75 μm.

Fig. 2. (A) Lateral view of an HNK-1-stained control embryo in the trunk focused on the segmental primary motor neurons, the axons of which are arrowed. Rohon-Beard cells (rb) are located dorsally. (B) Lateral view of the head of an HNK-1 stained control embryo showing the cranial ganglia evident at 24 hpf and the medial (mlf) and lateral (llf) longitudinal fasciculi. tri, trigeminal ganglion; alg, anterior lateral line ganglion; ac, acoustic (VIIIth) ganglion; plg, posterior lateral line ganglion; dashed lines, otocyst. (C) Dorsal view of an HNK-1 stained 24 hpf embryo treated with 10⁻²⁵ M RA at 50% epiboly revealing the normal development of Rohon-Beard cells in the trunk region. Scale bars in A and C=10 μm; in (B)=75 μm.

Fig. 3. Dorsal view of the midbrain and hindbrain of a control embryo stained with the MZ15 antibody which reveals the otocysts (ot) and the notochord (arrowed), the cranial extent of which lies at the anterior otocyst border. e, eye. Scale bar=100 μm.
viewed from both sides) in DPX. Preparations were examined using Nomarski Interference Contrast (NIC) optics with an Olympus BH2 system microscope.

Retinoic acid treatment
RA was purchased as a powder from Sigma, and a suspension made in DMSO at a stock concentration of $10^{-4} \text{ M}$, which was kept frozen at $-20^\circ \text{C}$. For working concentrations the stock was further diluted in Hanks' saline. Embryos from defined stages were selected and exposed to the RA/1% DMSO/10% Hanks' saline solution for one hour. Controls were treated with 1% DMSO/10% Hanks' alone. The embryos were then washed thoroughly in several changes of 10% Hanks' saline and allowed to develop until 24 hpf. Embryos were treated in two groups. In the first, embryos at 50% epiboly (5.2 hpf) (stages named according to Hisaoka and Battle, 1958) were exposed to a range of RA concentrations varying from $10^{-9}$ to $10^{-6}$ M. In the second group, embryos were exposed to $10^{-7}$ M RA for one hour at either dome stage (4.3 hpf) or at 90% epiboly (8.5 hpf). For reference, gastrulation begins at 5.2 hpf (Warga and Kimmel, 1990).

Results
(A) The effect of RA concentration on zebrafish development
In order to establish which concentration of RA had the most marked effects on pattern formation, embryos at 50% epiboly were exposed for one hour to RA concentrations ranging from $10^{-9}$ to $10^{-6}$ M. The anatomy of resulting embryos was analysed, using a range of methods, at 24 hpf. This is a stage when sufficient structural markers have differentiated to allow a detailed assessment of the effects of treatment.

RA treatment was observed to affect the development both of the brain structures derived from the neural tube and of peripheral neural structures in the head derived from neural crest and placodes. For clarity, the structure of the central and peripheral nervous systems will be described separately.

(1) Anatomical markers for development of neural tube-derived structures
Prior to describing RA effects, the principal anatomical features of the CNS used for the subsequent quantitative analysis of RA action will be described for the 24 h embryo. Control embryos used for this analysis were treated exactly as for experimental embryos with the exception that DMSO was exogenously applied to the 50% epiboly embryo in the absence of RA. A number of staining methods were used to assess the relative locations of structures in the control 24 h embryo. For all categories described, at least 10 specimens were examined.

(i) The relative position of the otocyst.

The otocyst provides an excellent anatomical landmark for the location of neural tube-derived structures. The otocyst can easily be visualised by NIC optics, and is conveniently stained, along with the notochord, by the antibody MZ15 (Fig. 3). For the quantitative analysis of otocyst position, a series of measurements was made, defining its location relative to the first myotome caudally and the posterior aspect of the eye cranially (refer to summary Fig. 8). In order to be certain of the position of the first myotome, AChE-stained preparations were used; the otocyst and eye being clearly visible in such specimens with NIC optics.

The location of the otocyst relative to the eye and first myotome is shown in Table 1. These data indicate that the otocyst is some 82 \mu m in length and is approximately twice as far from the eye as from the first myotome. The position of the otocyst was also assessed relative to the rhombomeres of the hindbrain and the notochord. The rhombomeres are readily visualised in AChE preparations (Fig. 1) and, as previously described by Hannemann and Westerfield (1988 – see also Hannemann et al. 1987; Trevarrow et al. 1990), the otocyst lies adjacent to rhombomeres 4, 5 and 6, with rhombomeres 4 and 6 at its cranial and caudal borders, respectively. MZ15 immunocytochemistry clearly illustrates that the cranial extent of the notochord lies at the anterior margin of the otocyst (see Figs 3, 7).

(ii) Anatomy of neural differentiation in the brain and spinal cord.

For a detailed description of the anatomy of the neural structures of the 24 h embryo, the reader is referred to Wilson et al. (1990), Trevarrow et al. (1990), Hannemann et al. (1988), Myers et al. (1986), and Metcalfe et al. (1990). For our current purpose, it is necessary to highlight specific landmarks used for quantitative analysis of RA effects, which are summar-
Table 1. Quantification of anterior structure from acetylcholinesterase-stained 24 h embryos following retinoic acid treatment

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>$10^{-5}$ M RA</th>
<th>$10^{-8}$ M RA</th>
<th>$10^{-7}$ M RA</th>
<th>90% epiboly</th>
</tr>
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<tbody>
<tr>
<td>Anterior otocyst to eye</td>
<td>246±1.0</td>
<td>227±6.2</td>
<td>202±11.0</td>
<td>110.9±3.7*</td>
<td>56.4±5.1†</td>
</tr>
<tr>
<td>Posterior otocyst to myotome</td>
<td>123±4.1</td>
<td>110±3.0</td>
<td>102±3.2</td>
<td>79.2±2.8</td>
<td>76.7±2.5</td>
</tr>
<tr>
<td>Otocyst length</td>
<td>82±2.5</td>
<td>80±3.8</td>
<td>90±2.0</td>
<td>79.2±2.8</td>
<td>76.7±2.5</td>
</tr>
<tr>
<td>Totals (microns)</td>
<td>465.4</td>
<td>419</td>
<td>395</td>
<td>328.5</td>
<td>277.1</td>
</tr>
</tbody>
</table>

*Significant from control at 0.1% by t-test.
†Significant from $10^{-7}$ M RA at 50% epiboly at 5% by t-test.
Units±s.E.

Fig. 7. Camera lucida drawings of MZ15-stained embryos showing the relative positions of the otocyst and the notochord at 24 hpf. (A) Control embryo, with the anterior extent of the notochord lying at the anterior end of the otocyst. (B and C) $10^{-7}$ M RA-treated embryos from the 50% epiboly group showing variations in the anterior extent of the notochord which now lies at the middle or posterior margin of the otocyst. Scale bar=100 μm.

Fig. 8. Summary diagram of the key neural structures examined with AChE and 4D9 staining: left half is a control and the right side an RA-treated embryo. Labels as for Fig. 1. The hatched area in the midbrain and hindbrain represents 4D9-positive cells that are absent in the RA-treated embryo. The arrows on the left side indicate the measurements taken for the initial analysis of brain structure shown in Table 1.

Our neuroanatomical analysis was performed on specimens stained with AChE, HNK-1 and Zn-1. In such stained preparations, forebrain neural complexes are seen in the telencephalon and diencephalon, separated by the optic fissure; the ephysis is clear in the dorsal caudal forebrain; the ventral midbrain complex, which represents the early differentiation of the nucleus of the medial longitudinal fasciculus, is evident. Rhombomeres are clearly differentiated by AChE histochemistry, with the first seven being readily distinguishable. The caudal rhombomeres are less easily discernible (see also Hannemann et al. 1988). In the spinal cord, two sets of neurons are clearly stained by HNK-1; the dorsal sensory Rohon-Beard cells (Metcalfe et al. 1990) and the segmental primary motor neurons (Myers et al. 1986). These are shown in Fig. 3, and were quantified to give an accurate measure of normal spinal cord structure. The results are presented in Table 2. The position of the most cranial Rohon-Beard cell was also a reasonably constant feature: it generally appeared just caudal to the location of the posterior lateral line ganglion (described below).

In addition to the location of neural cell bodies in the
Retinoic acid and zebrafish development

In Fig. 9, summary diagrams of normal (A) and RA-treated (B) embryos stained with HNK-1. Labels as for Fig. 1 and 2B.

CNS, HNK-1 also revealed principal axon tracts within the brain and spinal cord. In the brain, these tracts have been described in detail by Wilson et al. (1990): those that are most useful for this study are the anterior and posterior optic commissures; the descending tract of the medial longitudinal fasciculus; the sensory descending tract of the trigeminal input to the rostral hindbrain (described below), its path down the spinal cord and its relation to the central projection from the Rohon-Beard cells. These are summarised in part in Fig. 9 and illustrated in Figs 2A–C.

(iii) The expression domains of the engrailed antibody 4D9. As an additional marker for normal development of the neural tube, 4D9 expression was used. The epitope for 4D9 expresses at the caudal midbrain–cranial hindbrain junction at the site of future development of the cerebellum (Patel et al. 1989; Hatta et al. 1991). In normal embryos, the midbrain–hindbrain border is evident in NIC optics (Fig. 1A) and the 4D9 expression domain is quantifiable in its cranio-caudal extent (Fig. 4A and B). In controls, this distance is 90±3.7 μm (s.e.) (see Table 3). As expected for an anti-homeoprotein antibody, 4D9 stains nuclei, and a few nuclei are also stained in each of the first three cranial rhombomeres (Fig. 4B), as described by Hatta et al. (1991).

In addition to the cranial expression domains, 4D9 also reveals small groups of cells in the segmental myotomes (see Fig. 4A and Patel et al. 1989; Hatta et al. 1991). This expression is also quantifiable, providing an additional measure for normal development of the trunk in RA-treated embryos. In controls, a mean of 23±0.7 μm such myotomal units were revealed (Table 3) in 24 h embryos. As a further measure of trunk development, the position and form of the pronephros was noted in HNK-1-stained embryos (see Metcalfe et al. 1990).

(2) The effects of varying RA concentrations on development of neural tube derived structures

(i) General considerations. Varying concentrations of RA exogenously applied to embryos at 50 % epiboly for one hour show a clear-cut progression of effects on embryos at 24 h of development. These are described in detail below, but outlining general trends will clarify this subsequent analysis. In general, 10^{-9} M RA has little, if any, effect on the developing embryo by 24 hpf, whereas 10^{-8} and 10^{-7} M RA treatments show progressively more severe abnormalities. These abnormalities are clearly identifiable and affect preferentially head development, with minor effects on the tail and little effect on the trunk. 10^{-6} M RA severely disrupts normal development and is likely to be toxic, although again trunk structures appear most refractory to its action.

The effects of 10^{-8} and 10^{-7} M RA are principally focussed on the development of the caudal midbrain–rostral hindbrain border, the normal cranial expression domain of the engrailed protein revealed by the 4D9 antibody. This point is illustrated in Fig. 4A–C and quantitative data relevant to CNS development is presented in Tables 1, 2 and 3.

(ii) The effect of 10^{-9} M RA. As mentioned above, this concentration of RA has little effect on normal development. All quantitative measures of CNS development, position of the otocyst relative to the eye and first myotome (Table 1) (and the rhombomeres and notochord), number of Rohon-Beard and primary motor neurons (Table 2) and extent of cranial 4D9 expression (Table 3) are indistinguishable from normal. The forebrain, midbrain and hindbrain neuron com-
plexes and relative positions of the principal axon tracts are also normal, as are the number of 4D9-positive myotome cell groups in the trunk (Table 3).

(iii) The effects of $10^{-8}$ and $10^{-7}$ M RA. The effects of these two concentrations will be described together because they are basically similar, but with a more dramatic effect at the higher concentration.

The relative position of the otocyst with respect to the eye is altered (but not with respect to the first myotome – Table 1). Its position relative to the notochord is more variable; in some cases at $10^{-7}$ M RA it moves more anteriorly, but never more than the length of the normal otocyst (Fig. 7). The otocyst itself remains the same size, indicating that its development from the otic placode is unaffected. At $10^{-7}$ M RA the distance between the eye and the anterior margin of the otocyst is more than halved (from $246\pm1$ to $110.9\pm3.7\mu m$), a difference that is highly significant (0.1%, t-test).

Constancy in the distance of the otocyst from the first myotome, and the minor alteration in position relative to the cranial extent of the notochord, indicates that RA at these concentrations does not significantly affect development of the midbrain and caudal hindbrain. In this regard, the location of the otocyst relative to the rhombomeres becomes an important point. AChE preparations from these two experimental groups show that rhombomeres at the level of the otocyst and immediately caudal to it are clearly formed, as in normal embryos, but the rhombomeres cranial to it are abnormal in the majority of cases. Of a total of 32 embryos stained with AChE, seven showed identifiable anterior rhombomeres, and in each case three were present, showing those opposite the otocyst to be the normal rhombomeres for this relative location; 4, 5 and 6. In the remaining 25 specimens, the anterior hindbrain neuron groups were joined and were therefore not identifiable as separate rhombomeric units (see Fig. 1B and summary Fig. 8).

At the cranial end of the hindbrain, the anterior extent of the rhombomeres was often continuous with the medial ventral midbrain neuron complex which normally overlaps the posterior margin of the laterally placed eye (Fig. 1B and summary Fig. 8). In all cases examined, this complex, which is predominantly the nucleus of the medial longitudinal fasciculus, is present, a result confirmed by examination of 34 HNK-1-stained embryos from these treatment groups. Structures anterior to this ventral midbrain complex appeared normal (Figs 1A and B). The epiphysis was invariably present in the dorsal, caudal forebrain, as were the telencephalic and diencephalic neuron complexes normally placed above and below the optic fissure. HNK-1-stained embryos also revealed the anterior and posterior optic commissures and the medial longitudinal fasciculus, the latter running in its normal ventral medial location through the hindbrain.

More caudally, quantification of Rohon-Beard and primary motor neuron numbers indicates that no alteration had occurred in the trunk regions (Fig. 2C), the data being indistinguishable from controls.

**Table 2. Quantification of principal neural structures stained by HNK-1 in 24h embryos following retinoic acid treatment**

<table>
<thead>
<tr>
<th>Structure</th>
<th>Control (n=10)</th>
<th>10^{-8} M RA (n=10)</th>
<th>10^{-7} M RA (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vth ganglion</td>
<td>24.3±1.1</td>
<td>22.4±1.1</td>
<td>19.2±1.1</td>
</tr>
<tr>
<td>anterior lateral line</td>
<td>14.5±1.6</td>
<td>13.4±0.8</td>
<td>12.6±0.4</td>
</tr>
<tr>
<td>VIIth ganglia</td>
<td>24.1±1.1</td>
<td>24.1±1.1</td>
<td>22.4±1.1</td>
</tr>
<tr>
<td>posterior lateral line</td>
<td>7.5±0.5</td>
<td>7.2±0.5</td>
<td>6.9±0.4</td>
</tr>
<tr>
<td>posterior colliculus</td>
<td>89.7±2.6</td>
<td>91.9±3.0</td>
<td>88.4±3.1</td>
</tr>
<tr>
<td>Rhombomeric cells</td>
<td>12.8±0.5</td>
<td>11.1±0.6</td>
<td>10.6±0.5</td>
</tr>
<tr>
<td>Primary motor neurons</td>
<td>89.7±2.6</td>
<td>91.9±3.0</td>
<td>88.4±3.1</td>
</tr>
</tbody>
</table>

* Significant from control at 1% by t-test.
+ Significant from control at 5% by t-test.
(Table 2). This result is strengthened by the quantification of segmental 4D9 myotome staining which is also essentially identical to controls (Table 3) and the pronephros was also present and essentially normally formed in HNK-1-stained specimens.

Overall, these results indicate that the only region of the CNS affected by 10⁻⁶ and 10⁻⁷ M RA lies between the levels of the otocyst and the eye, with the forebrain, ventral midbrain and all structures including and lying caudal to the otocyst developing normally. For this reason, the cranial expression domain of 4D9 becomes crucially important because this region lies in the area where it appears to be absent by all previous criteria. On analysis of 4D9 staining, 10⁻⁷ M RA essentially abolishes the cranial staining of this antibody, although the caudal segmental myotomal staining is unaffected (Fig 4C). This abolition was complete in 70% of 10⁻⁷ M RA-treated specimens (Table 3) and in the remaining 30% only a few 4D9-positive nuclei were seen in small patches appearing in either dorsal or ventral regions of the normal expression. In 10⁻⁸ M RA-treated specimens, the expression was present in the majority of cases but was again very patchy and limited in its extent. In neither category was expression clear enough to be able to measure its cranio-caudal extent. Of the 26 4D9 stained specimens examined in these treatment groups, no staining of 4D9 was evident in the three cranial rhombomeres.

(iv) The effects of 10⁻⁶ M RA. The effects of 10⁻⁶ M RA are much more severe than those for lower concentrations. No structures could be discerned within the CNS in the head, with forebrain, midbrain and hindbrain neurons not clearly differentiated. In AChE specimens, positive cells were spread throughout an expanded neural structure at the anterior end of the embryo. Eyes and otocysts were not formed so no analysis of pattern was possible. The tail was also wasted. The only body region to show identifiable staining was the trunk, where 4D9 segmental myotome staining was evident (Table 3). In occasional specimens, some Rohon-Beard cells and primary motor neurons differentiated. These results, combined with the observation that AChE-stained preparations occasionally revealed myotomes, show that the trunk is remarkably refractory to RA action when compared to the anterior and posterior regions of the embryo.

| Table 3. Analysis of 4D9 expression in control and retinoic acid-treated embryos |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                 | Control | 10⁻⁶ M RA | 10⁻⁷ M RA | 10⁻⁶ M RA | 10⁻⁷ M RA | 10⁻⁸ M RA |
| Myotome segments (number)      | 23.0±0.7 | 25.5±0.5 | 20.3±0.8 | 8.1±1.45 | 17.5±0.6 | 18.7±1.1 |
| Cranial stripe µm              | 50±3.7  | 87±5.4 | -      | -      | -      | -      |
| Percentage with cranial stripe | 100     | 100     | 30.7   | 0      | 25     | 25     |

(3) Anatomical markers for the development of peripheral neural populations in the head. RA treatment at varying concentrations was also assessed with respect to peripheral cranial neural populations derived from the neural crest and placodes. The otocyst has already been described as an important landmark for the analysis of RA effects on the development of the neural tube and it is evident that this structure forms normally after treatments of RA at 10⁻⁹ to 10⁻⁷ M. The only alteration with respect to the otocyst is its relative position, although, like all other cranial structures, it does not develop following 10⁻⁶ M RA treatment.

The peripheral cranial ganglia clearly stained by HNK-1 at 24 h in control embryos are the trigeminal (Vth) ganglion, the anterior lateral line ganglion, the acoustic (VIIth) ganglion and the posterior lateral line ganglion. The anatomy of these structures has been described previously by Metcalfe et al. (1990), so their normal location and central projections are described in summary only (Fig. 2 and summary Fig. 9). The Vth ganglion is the largest and most cranial, lying as a flattened palm of neurons extending fine branches of axons cranially and ventrally beneath the skin over the head. Caudally the central projection from this ganglion enters the CNS and runs dorso-laterally to the spinal cord via the lateral longitudinal fasciculus, where it forms a tract coincident with the central projections of Rohon-Beard cells (Figs 2B, 10A). In controls, there is a mean of 24 neurons in this ganglion (Table 2). The anterior lateral line and VIIth ganglia form a close complex of neurons at the anterior margin of the otocyst with the anterior lateral line ganglion extending cranially and that of the VIIth ganglion extending close to the surface of the otocyst (Fig. 2B). Because of the close apposition of these ganglia, it was not possible to quantify the number of neurons that constitute them individually so the data are presented for the whole complex. There are fewer neurons in this complex (approximately 14 neurons – see Table 2) than in the adjacent Vth ganglion. Lying caudal to the otocyst is the posterior lateral line ganglion, connecting centrally via a cranially projecting tract of axons and sending axons caudally in the periphery to connect to the lateral line neuromasts differentiating in the lateral skin of the embryo. At 24 h growth cones of these peripheral, caudally projecting axons are evident in many HNK-1 specimens (see Metcalfe et al. 1990). In control embryos, this ganglion has a characteristic sausage shape (Figs 2B, 12), and comprises a mean of 7 neurons (Table 2).

The vagal (Xth) ganglion is not stained by HNK-1 until 48 h of development (Metcalfe et al. 1990) and was not included in the study at this stage.
Fig. 10. Camera-lucida drawings of the location of the trigeminal ganglion and the distribution of peripheral superficial axons. (A) Control 24 hpf embryo. Ilf, lateral longitudinal fasciculus; tri, trigeminal ganglion; ot, otocyst. (B) Embryo at 24 hpf following treatment with $10^{-7}$ M RA at 50% epiboly. The normal ganglion is reduced in cell number and two anterior 'ganglia' are present. The additional drawings of these illustrate the centrally projecting axons of each of the aberrant 'ganglia'. (C) The most extreme effect of RA treatment ($10^{-7}$ M RA at 50% epiboly) is the dramatic reduction in ganglion cell numbers and distribution of individual cells over the lateral-anterior region of the head. Scale bars=50 $\mu$m.

(i) The effects of $10^{-9}$ M RA. As was the case with the neural tube derived structures, $10^{-9}$ M RA has virtually no identifiable effect on the cranial ganglia evident at 24 h. The data shown in Table 2 indicate that the Vth, anterior lateral line and VIIth complex and posterior lateral line ganglion have neuronal numbers indistinguishable from control values.

(ii) The effects of $10^{-8}$ M and $10^{-7}$ M RA. The first effects of RA action are evident at $10^{-8}$ M, with a reduction in the number of Vth ganglion neurons. Although this reduction was not significant compared to controls, it is clearly a midpoint between controls and the drastic reduction in neuronal numbers seen in this ganglion at $10^{-7}$ M RA. In this case, the fall in numbers (to a mean of about 6) is significantly different at the 1% level from control values (Table 2). The effect on differentiation of the Vth ganglion is not simply a reduction in numbers, as at both $10^{-8}$ M and $10^{-7}$ M the neurons are not neatly clustered into a single ganglion. At both concentrations, the large and easily identifiable neurons typical of cranial ganglia are spread in positions at or anterior to their normal location (Fig. 5A); often present singly or in small groups as far anterior as the lateral telencephalon (Fig. 10). When this spreading is seen, fine axons are evident connecting superficially between groups of neurons, and in some cases centrally projecting axons could be seen from aberrantly positioned ganglion cell groups (Fig. 10B).

Reduction in numbers of neurons constituting the anterior lateral line and VIIth ganglia is also evident at $10^{-7}$ M RA and this is significant at the 5% level (Table 2). Unlike the Vth ganglion, however, there is no reduction in ganglion neuron numbers at $10^{-8}$ M. When present at $10^{-7}$ M RA, the cells appear in small groups at the anterior margin of the otocyst and have short, centrally projecting axons at the same level as they do in controls.

In all cases examined, the posterior lateral line ganglion appeared normal in its relative position caudal...
and lateral to the otocyst and comprised the normal number of neurons (Table 2). The central cranially directed projection and the peripheral caudally directed axons were also normal in appearance. Growth cones of the caudally extending axons were also evident in the trunk region of many experimental embryos.

(B) The effect of embryonic stage on treatment of RA at $10^{-7} \text{M}$

In order to assess the importance of embryonic stage at which embryos were exposed to RA on subsequent development two stages in addition to 50% epiboly were selected for study using a constant concentration of $10^{-7} \text{M}$ RA. The same analysis was performed in these cases as has already been described above, and the results will again be presented with respect first to neural tube and secondly to peripheral neural structures in the head.

1. $10^{-7} \text{M}$ RA treatment at dome stage

Treatment at dome stage with $10^{-7} \text{M}$ RA results in more severe abnormalities than comparable treatment at 50% epiboly. In general, neural tube differentiation in the head is similar to the effects of $10^{-6} \text{M}$ RA at the later stage in that the neural complexes of the forebrain, midbrain and hindbrain are not recognisable at 24 h of development. The eye is usually absent but the otocyst is present. The results clearly differ, however, with respect to the trunk and tail where differentiation is almost normal following treatment at dome stage. With $10^{-5} \text{M}$ RA at 50% epiboly the tail is wasted and the trunk has only a few myotomes and associated Rohon-Beard and primary motor neurons. In sharp contrast, quantification of the trunk structures; Rohon-Beard and primary motor neurons and 4D9 staining of myotome segments are indistinguishable from controls (Tables 2 and 3). The result with an HNK-1 stained preparation is a striking embryo which has a severely affected head with essentially no visible staining, with a posterior half-embryo normal in every measurable respect. Such an embryo is shown in Fig. 6.

Despite the dramatic effects on the internal differentiation of the anterior neural structures, peripheral ganglia form in the head region, albeit abnormally. As with treatment at 50% epiboly, both the Vth and anterior lateral line and VIIIth ganglia are dramatically reduced, and to a similar level (Table 2). The neurons of the Vth ganglion show a diffuse distribution with neurons developing in aberrant positions anterior and lateral in the anterior head region.

The distinctive result with respect to peripheral ganglia is a clear cut increase in neurons composing the posterior-lateral line ganglion. Table 2 shows the mean number of neurons in this ganglion to rise almost threefold over controls, a result significant at the 5% level. That the enlarged structure is clearly the posterior lateral line ganglion is shown by the central and peripheral projections of the neurons and the location of the ganglion posterior to the otocyst (Fig. 11B). The peripheral axons project posteriorly and laterally as they do in controls and growth cones are evident on the extending axons in the lateral trunk region beneath the skin. Some neurons in these aberrant ganglia project anteriorly (Fig. 11B).

2. $10^{-7} \text{M}$ RA treatment at 90% epiboly

Embryos developed following this treatment show a range of characteristics which clearly vary from those seen following treatment at dome stage. The principal differences lie in development of the neural tube, where the eye forms in all cases. Measurement of the relative positions of the otocyst relative to the eye and first myotome show an even more clear-cut reduction in the distance between the otocyst and the eye than is seen with $10^{-7} \text{M}$ RA at 50% epiboly (Table 1). This distance is reduced by approximately 200 µm compared with controls. As with the earlier described cases, however, the anterior end of the notochord still lies at the level of the otocyst, although AChE-stained preparations showed the anterior rhombomere units to be condensed together and continuous with the ventral-medial midbrain neural complex. The forebrain neural complexes, including the epiphysial neurons, were present, although staining was often weaker than in controls. Thus the overall pattern is similar to that seen in $10^{-7} \text{M}$ RA embryos treated at 50% epiboly but is generally more severe. Consistent with this conclusion, cranial 4D9 staining was absent in 75% of cases.

In the trunk region and tail, development was essentially normal, with Rohon-Beard and primary motor neurons present in normal numbers, the pro-
nephros was present and the number of 4D9-stained segmental myotome groups was also normal.

With respect to the peripheral ganglia of the head, the Vth ganglion had fewer neurons and they were spread diffusely, as with other 10^{-7} M RA treatments. In contrast to other such treatments, however, the anterior lateral line and VIIIth ganglia were totally absent except for one or two neurons in one or two cases.

On close inspection of the dorsal and lateral regions of the hindbrain immediately beneath the skin, many individual flattened HNK-1-positive cells were seen in half of the 13 preparations in this group so stained. These cells are not seen in control 24 h embryos but were evident to a more limited extent in 10^{-7} M RA embryos treated at 50 % epiboly. A typical example is shown in Fig. 5B. The identity of these cells is unknown, but they may be neural-crest-derived cells that are misplaced due to abnormal migration.

Discussion

The results demonstrate that RA causes defined lesions to the central and peripheral nervous systems in the head during development between gastrula and 24 hpf. This result is most clearly described following RA treatment at 50 % epiboly, a stage when gastrulation has just begun but is not yet complete (see Warga and Kimmel, 1990). At this stage, by all criteria of quantitative anatomy, labelling of embryos with the panel of antibodies and AChE staining, 10^{-8} M RA has little or no effect on embryo development. However, 10^{-8} and 10^{-7} M RA given at this stage cause defined and reproducible alterations to the developing head, and 10^{-5} M RA produces a severely affected phenotype, indicating a clear concentration-dependent effect.

The most striking effect of RA is on the anterior neural tube, where a distinct lesion is evident, affecting the midbrain–hindbrain border and the rostral rhombomeres. This effect is localized and does not involve the most anterior neural structures, which develop normally. This result is similar to that seen in RA-treated mouse embryos (Morris Kaye et al. 1991), and apparently contrasts with the effects of RA on Xenopus embryos, in which there is a dramatic loss of anterior neural structures (Durston et al. 1989; Sive et al. 1990). Two recent lines of evidence show, however, that the hindbrain–midbrain region common to zebrafish and mouse also occurs in RA-treated Xenopus. Firstly, if Xenopus embryos are exposed to RA at earlier neurula stages, anterior structures, including the eye and cement gland, are maintained whereas more caudal alterations in brain structure are still evident (Ruiz i Altaba and Jessell, 1991b). Secondly, a detailed analysis of hindbrain neuroanatomy in Xenopus embryos treated with RA at gastrula stages reveals a clear lesion in the anterior rhombomeres which also affects expression of the regulatory gene Xen-Krox-20 in this region (Papalopulu et al. 1991; Krumlauf et al. 1991). It is evident, therefore, that the lesion of the hindbrain–midbrain region is a common feature of zebrafish, Xenopus and mouse embryos treated with RA and that the loss of anterior structures is unique to Xenopus.

The most convincing proof that the hindbrain–midbrain border of the CNS is missing in 10^{-7} M RA treated embryos comes from the use of the anti- engrailed antibody 4D9 which labels neural cells in this area in normal embryos (Hatta et al. 1991; Patel et al. 1989). At 10^{-8} M the cranial expression domain of 4D9 is normal (Table 3), but at 10^{-8} M the staining is markedly reduced in intensity and in distribution. In the majority of cases treated with 10^{-7} M RA (Table 3), expression of 4D9 is totally absent in this region (Fig. 4C). 4D9 staining is also evident in normal embryos in a few cells in each of the first three rhombomeres (Hatta et al. 1991, and see Fig. 4B). It is interesting to note that AChE-stained embryos show, in cases treated with 10^{-7} M RA, abnormal development of these rhombomeres. In the majority of these cases, neurons anterior to the anterior otocyst boundary and rhombomere 4 are present but are not clearly separated into segmental units, as they are in controls (cf Figs 1A and B). In the remaining cases, these rhombomeres were clearly demarcated, indicating that they truly represent the cranial three rhombomeres. In both types of embryo, the neurons of these rhombomeres overlap in the rostro-caudal axis the ventral midbrain neurons which extend anterior to the posterior margin of the eye. It can be suggested, therefore, that RA at 10^{-8} M affects all cells normally expressing 4D9 engrailed antigen in this anterior expression domain. With regard to 4D9 expression, RA has an increasing effect progressing from 10^{-9} to 10^{-7}. It is presently unclear whether the cells that normally express 4D9 in the cranial stripe are present or absent in RA-treated embryos, but the clear shortening of the brain in such embryos suggests that these cells are not present. Further work is necessary, therefore, to clarify the effect of RA on engrailed expression. At present, the regulation of normal engrailed gene expression in vertebrates in general and the zebrafish in particular (see Hatta et al. 1990, 1991) is not clear. The possibility must exist, however, that one of the normal roles of RA in head development is the moderation, directly or indirectly, of the expression of engrailed and other neural control genes during regionalisation of the neural tube. The effect on engrailed expression in zebrafish is also complicated by the differential effect of RA on the cranial as compared with the segmental myotomal expression, the latter being remarkably refractory to RA action: it is the only clear staining seen at 10^{-6} M RA at 50 % epiboly (Table 3). The loss of cranial engrailed expression following RA treatment also occurs in Xenopus (Sive et al. 1990), but this effect appears to be due to the loss of anterior structures in this embryo. The cranial engrailed expression domain is only absent in Xenopus embryos that are also obviously lacking forebrain and midbrain structures. These differences may be due to subtle variations in the response of developmental mechanisms to RA action. In Xenopus the susceptibility of specification of anterior
structures at gastrulation to RA may be more acute and the effects on *entrained* may be masked by this separate RA effect. This is supported by the observation that RA action in zebrafish causes loss of the hindbrain–midbrain border and affects the most rostral rhombomeres with no effect on brain development at positions anterior or posterior to these areas. Loss of the eye and severe disruption of overall brain development only occurs at $10^{-8}$ M RA, a concentration that is likely to be highly toxic. The finding that the RA effect is coincident with the loss of cranial *entrained* homeoprotein expression is also of interest because of the recent studies of Hemmati-Brivanlou et al. (1990) which conclude that the notochord is involved in inducing the cranial expression in *Xenopus*. It will be of interest to establish the role of RA in mesodermal induction because the possibility exists that the RA effect on *entrained* in zebrafish is mediated through the mesoderm. In zebrafish, however, it must not involve the notochord because this mesodermal component has its anterior border at the level of the otocyst which is considerably caudal to the midbrain–hindbrain border. It may be the case that the mesodermal component affected by RA is not the notochord but the more anteriorly placed prechordal plate mesoderm, the fate of which is poorly understood in all vertebrates. The link between a primary effect of RA on the mesoderm, possibly mediated at the process of mesodermal induction, is strengthened by the recent demonstration by Ruiz i Altaba and Jessell (1991a) that RA causes defects in the anterior region of *Xenopus* embryos if it is given exogenously in mesodermal induction assay experiments and by the demonstration, also in *Xenopus*, that RA may cooperate with growth factors in determining anterior–posterior mesodermal patterning (Cho and DeRobertis, 1990).

The effects of altering the stage of development at which RA was administered gave some minor alterations to the eventual structures which differentiated as compared with 50% epiboly. The same basic effect, with loss of the midbrain–hindbrain border and disruption of cranial ganglia, was evident. In general terms, treatment at dome stage was more disruptive in the head than treatment at later stages, but at all stages the head was clearly more sensitive than the trunk, which formed essentially normally, supporting the view that the trunk is refractory to RA treatment. There were two points worthy of further comment about treatment of dome-stage embryos at 90% epiboly. The first of these is the clear-cut increase in the number of posterior lateral line ganglion neurons in the dome-staged group (Fig. 11). A significant, approximately threefold, increase in neuron number was evident, and the structure of the ganglion was clearly abnormal. In addition to the normal central and peripheral projection of axons from this ganglion, other aberrant anteriorly projecting peripheral axons were seen. The posterior lateral line ganglion is thought to be placodal in origin, forming from a postauditory placode (see Metcalfe, 1985), indicating that RA can affect placoidly derived, as well as neural-crest-derived, structures in the head.

This alteration by RA treatment is the only one in which a structure is increased, suggesting some respecification, and it contrasts with the clear-cut loss of structure at the midbrain–hindbrain border. The second observation worthy of comment is the appearance beneath the skin, in embryos treated at 90% epiboly, of cells which may represent misplaced neural crest-derived cells (Fig. 5B). Such cells are never evident in controls in the head at 24 hpf, but are occasionally seen in $10^{-7}$ M RA embryos treated at 50% epiboly. It is also possible that these cells arrested in migration are crest cells, and this may be the reason why the Vth, VIIth and VIIIth ganglia are so sensitive to RA treatment. It is interesting in this regard that cells of the cranial neural crest in chick and mouse embryos strongly express cellular retinoic acid-binding protein during their migration. It has been suggested by a number of authors (Maden et al. 1990; Dencker et al. 1990) that CRABP-positive cells are primary targets for RA action in normal development.

It is a pleasure to thank Liz Delaney for her early work on this project and for her efforts in establishing the zebrafish colony; and Chuck Kimmel for all his encouragement and practical help over the past two years. Thanks also to Malcolm Maden, Jon Clarke, Chuck Kimmel and Robb Krumlauf for their discussions and comments on the manuscript. Antibodies were kindly provided by Chuck Kimmel (4D9 and Zn-1); Fiona Watt (MZ15); and Claudio Stern (HNK-1). The work was supported by a SERC project grant to NH.

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(Accepted 8 August 1991)