Rostral Truncation of a Cyclostome, Lampetra japonica, Induced by All-Trans Retinoic Acid Defines the Head/Trunk Interface of the Vertebrate Body

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ABSTRACT The effect of all-trans retinoic acid on embryogenesis was studied in a cyclostome, Lampetra japonica. Treatment with 0.05–0.5 μM retinoic acid on early gastrula and early neurula resulted in loss of the pharynx and in the rostral truncation of the neural tube. The mouth, pharynx, esophagus, heart, endostyle, and rostral brain were missing with graded severity. In the severest case, the embryo consisted only of trunk segments, especially myotomes that extended to the rostral end of the axis. The effect appeared to be dose- and stage-dependent: Rostral pharyngeal arches were more vulnerable to a lower amount of retinoic acid, and earlier treatment resulted in severer defects. The initial protrusion of the anterior axis started equally in control and retinoic acid-treated embryos, implying that the head morphogenesis is omitted in treated embryos. By identifying the number of myotomes based on the differentiation of hypobranchial muscles, there seemed to be no myotomes lost by retinoic acid-induced truncation. The rostral truncation, therefore, was not simply a limitation of the anterior axis but was restricted to the ventral portion; only the branchial arches disappeared with normally developing myotomes dorsally. The absent region can be defined as the vertebrate head in a morphological sense, including the branchiomeretic and preotic paraxial regions as well as the heart. The results suggest the presence of distinct programs between somitic and branchiomeric portions of the body, providing a developmental basis for the dual-metamerical body plan of vertebrates. Dev. Dyn. 1998;211:35-51. © 1998 Wiley-Liss, Inc.

Key words: branchial region; metamerism; cranial nerves; neural crest; all-trans retinoic acid; lampreys

INTRODUCTION The vertebrate body is characterized by possession of segmentally arranged units along the anteroposterior axis: myotomes, pharyngeal arches and peripheral nerves. All of these characters are also present in amphioxus, a close relative of vertebrates, and probably comprise the most basic body plan of the common ancestor of cephalochordates and craniates. Among the classes of vertebrates, lampreys belong to a sister group of gnathostomes. Fossil records suggest that the dichotomy of these two major lines may have taken place quite early in the vertebrate evolution (for review, see Janvier, 1993). Regardless of the specific characteristics evolved during their own history, lampreys share common morphological features with gnathostomes, i.e., anteroposterior as well as dorsoventral dissociation of branchiomerism and somitomerism and transient neuromeres in the rostral neural tube (Kuratani, 1997; Kuratani et al., 1997a). These synapomorphies are apparently missing in amphioxus, in which the two metamerisms are separated only dorsoventrally.

The metamerical body plan becomes distinct through the organized and hierarchical processes of cell growth and differentiation, for which spatiotemporally regulated gene expression is a prerequisite. Alternatively, this body plan is profoundly related to such gene expression patterns. All-trans retinoic acid (RA) has been shown to affect diverse aspects of cellular development (for review, see Lotan, 1980) as well as the patterning processes of vertebrates (for review, see Hofmann and Eichele, 1994): In several different species, administration of RA results in the truncation of the rostral brain and posteriorization of structures caudal to a certain level of the hindbrain (Holder and Hill, 1991; Morriss-Kay et al., 1991; Papalopulu et al., 1991; for reviews, see Morriss-Kay, 1993; Osumi-Yamashita, 1996). Advances in molecular genetics have shed light on regulatory gene functions in embryonic patterning. In this respect, the effect of RA on axial development has drawn the attention of molecular embryologists, because this molecule appears to alter the axial value of the body by shifting the genetic code, as exemplified by Hox gene expression pattern: Morphological changes depending on RA dosage roughly coin...
To investigate the evolutionary process of the vertebrate body plan, comparative analyses are essential not only by looking at the normal developmental processes but also by examining the effects of shifts in patterning programs. The effects of RA have been examined in a variety of gnathostomes and amphioxus (Holland and Holland, 1996), but nothing has been reported on agnathan animals. It is intended here to evaluate the morphological change in lamprey embryos after the administration of RA, thereby comparing the developmental plan of this animal with that of gnathostomes and cephalochordates. By controlled treatment with various concentrations of RA, we successfully obtained embryos with altered patterns. Here, we demonstrate that RA affects the lamprey development in a similar but distinct way to that shown by the experiments in gnathostomes. Long-term viability after RA treatment and the many branchial arches possessed by this animal helped to elucidate some previously unrecognized aspects of vertebrate body architecture.

**RESULTS**

**Experiment 1**

**External features.** Development of the embryos treated with 1 µM and 5 µM RA at early gastrula stopped within the first 2 hours. Other embryos treated with lower concentrations of RA survived. No substantial difference was detected morphologically between the control embryos and those treated with 0.01 µM RA. On the second day of treatment, the anterior protrusion began to appear in both control and RA-treated embryos (Fig. 1A–C). The protrusions of 0.1 µM RA-treated embryos were more pointed at the tip than the...
control embryos, and, on day 3, the head portion was more slender (Fig. 1D–F). There were no apparent differences between the control and 0.01 µM RA-treated embryos (Fig. 1D,E). By 4 days of treatment (around stage 25), control and 0.01 µM RA-treated embryos had developed normally, whereas 0.1 µM RA-treated embryos showed a truncated head and strong ventral bending of the body axis, which lacked the pharynx (Fig. 1G–I).

At 5 days, roughly corresponding to stage 26, melanocytes were first seen in the embryos. Normally, they appeared around the otocyst, forming a couple of cell streams both rostral and caudal to the otocyst (Fig. 2) that corresponded to rhombomere 4 (r4) on the neuraxis (Kuratani et al., 1997b). In the 0.1 µM RA-treated embryos, the melanocyte also appeared and was distributed in the rostralmost portion of the body (Fig. 2C). In 5 of 16 embryos of this group at all stages, an epithelial cyst that was reminiscent of an otocyst was associated with the melanocytes, showing that the neuraxis of these embryos was truncated at the level of the anterior hindbrain.

**Anatomical features.** At day 3 of RA treatment, when the control embryo had grown to stage 21 (Fig. 3B), nervous tissue was detected by using the monoclonal antibody (MAb), T-6793, which recognizes the acetylated tubulin. In the neural tube of control and 0.01 µM RA-treated embryos, Rohon-Beard cells formed the dorsolateral fasciculus (DLF), which had a rostral tip that reached the caudal hindbrain, and commissure neurons grew axons ventrally to form the ventrolateral fasciculus (VLF); in the midiencephalic region, the interstitial nucleus, the anlage of the medial longitudinal fasciculus, was observed (Fig. 3B; Kuratani et al., 1997b). The otocyst was already present, and the DLF reached a level caudal to the otocyst (Fig. 3B).

In all of the 0.1 µM RA-treated embryos (3 of 3), the DLF and VLF were found up to the anterior end of the neural tube (Fig. 3A,C). An otocyst-like cyst was often observed nearby (see below). The interstitial nucleus, on the other hand, could not be identified.

Consistent with the RA-induced changes at stage 21, older embryos also lacked a number of head-specific structures in the nervous system. At day 3.5 of RA treatment, when control embryos grew to stage 24, the posterior commissure and some neuromeres were recognized in the neural tube in control and 0.01 µM RA-treated embryos. The eye primordium was visible, and the maxillomandibular and facial nerve roots were present. Around the eye stalk, supraoptic and postoptic tracts were developing. In the anterior head ectoderm, olfactory epithelium had appeared by a compact sheet of cells showing strong acetylated tubulin (AT) immunoreactivity (not shown). In the 0.1 µM RA-treated embryos, on the other hand, no neuronal structures rostral to the otocyst could be identified, including the eye, the cranial nerve roots, the nerve tracts, or the olfactory epithelium (2 of 2 embryos).

Defects of the peripheral nervous system (PNS) in 0.1 µM RA-treated embryos were correlated with the truncation of the central nervous system (CNS) as well as with the absence of the pharynx. By day 9 of RA administration, the control and 0.01 µM RA-treated embryos had grown to stages 27–28, and all of the branchiomeric nerves were present (Fig. 4); in contrast, the 0.1 µM RA-treated embryos apparently lacked the branchiomeric nerves (Fig. 5B), i.e., all of the peripheral nerves were developing either between or within myotomes, a feature of the spinal nerves of this animal.
Of those, three anteriormost nerves passed the intermyotomic space and fasciculated distally to supply the gut and the putative pharyngeal region (Fig. 5B).

Concomitant with the loss of the head, the rostral end of the notochord bent ventrally and reached the rostral end of the body (Figs. 5B, 6). The pharyngeal pouches were never visible, but endodermal tissue extended rostrally to the rostral tip of the notochord, where two tissues were mixed (Figs. 5B, 7A). No mouth opening or stomodaeum was apparent. Myotomes in the lamprey developed caudal to the otocyst and grew secondarily in a rostral direction (Fig. 4), whereas, in the 0.1 µM RA-treated embryo, the rostralmost one developed slightly caudal to the rostral end of the body (Figs. 6, 7). The space between the otocyst and the first myotome was filled with the rostral endodermal tissue, which, by this stage, had become greenish, similar to the tint of the liver primordium in the normal embryo of this stage (not shown). In the histological sections, the heart was lacking in 0.1 µM RA-treated embryos, although pericardium as well as pronephric ducts were present (Fig. 7A,B; see also Fig. 3A). The number of the ducts varied in each RA-treated embryo. In the total of 18 embryos treated with 0.1 µM RA, 15 embryos lacked the entire pharynx as well as the esophagus, three embryos possessed only the esophagus and the posterior portion of the pharynx, and one embryo developed a normal endodermal configuration (Table 1).

For the analyses of muscle morphology, embryos were stained with CH-1 MAb, which recognizes tropomyosin. In normal development, the first postotic myotome becomes the infraoptic myotome, and the second becomes the supraoptic myotome (Fig. 4). Caudally, a certain number of occipital level myotomes were involved in the formation of the hypobranchial muscle, which is homologous with gnathostome tongue muscles (Fig. 4; Kuratani et al., 1997a). The myotomic origin of the hypobranchial muscle could not always be determined in the normal embryos due to the dorsocaudal expansion of the pharynx. In the RA-treated embryos, the first myotome was found slightly caudal to the rostral tip of the notochord (3 of 3), and the ventral portions of myotomes 7–12 were detached and had migrated ventrally, apparently forming the hypobranchial and hypaxial muscles (Fig. 6A).

**Experiment 2**

In the second series of experiments, RA treatment was performed at the early-neurula stage (Tahara's stage 18). The morphology of the treated embryos showed a graded series of severity, i.e., more extensive loss of the head structures with a higher dose of RA. The nervous system was the main focus of the analyses.

The development of the nervous system at stages 26 and 27 was consistent with regression of the pharynx (Figs. 8, 9). In the control embryos at stage 26, pharyngeal pouches 2–7 were present, and the first pouch was made vestigial by the modification of the second pharyn-
The notochord rostrally ended at the level of diencephalon. In the PNS, each pharyngeal arch was innervated by a branchiomeric nerve branch. When it was stained with T-6793 antibody, the olfactory epithelium was seen at the tip of the head, and the endostyle was darkly stained at the floor of the pharynx at stage 27 (Figs. 8A).

In some of the 0.05 \( \mu \text{M} \) RA-treated embryos (Figs. 8B, 9C,D), three rostral nerves, the ophthalmic, maxillomandibular, and facial nerves, were developmentally altered, but others showed normal PNS morphology. The ophthalmic nerve was apparently lost or fused with the trigeminal nerve, and the development of the facial nerve was arrested. The endostyle was always present (4 of 4 embryos; Fig. 9C). In 2 of 4 embryos, the lower lip failed to differentiate (Fig. 9D), whereas, in the other two embryos, the endodermal portion corresponding to the first pharyngeal pouch was missing (not shown). At stage 26, the vagal nerve root grew more caudally on the hindbrain than in the control (Figs. 8B). The eye was lacking in 1 of 4 embryos. Olfactory epithelium was always present.

The pharyngeal defects of embryos treated with 0.1 \( \mu \text{M} \) RA extended caudally to the third pharyngeal arch (1 of 3 embryos; Figs. 6B, 8E,F). The second pouch was usually absent (3 of 4). Concomitantly, the facial and glossopharyngeal nerves were fused distally. The second pharyngeal arch usually failed to develop, and several irregular connections were seen between the glossopharyngeal nerve and the vagus nerve. The olfactory epithelium, heart, and otocyst were always present (3 of 3), but the endostyle was missing (Fig. 8E).
In the 0.5 µM RA-treated embryos, most of the pharynx as well as the endostyle were missing (3 of 4), except for the presence of a few pharyngeal arches belonging to the vagal segment in one embryo (Figs. 8D, 9G). The esophagus could be seen in 4 of 5 embryos. Due to the similar appearances of the postotic arches, the numbering of these pouches was not always possible (see Table 1). Nevertheless, the nerve innervating these pouches resembled the vagus of the control embryo, in that the nerve root formed an arch rostral to myotomes after issuing from the hind brain (Fig. 8D). The vagal branch was distributed on the dorsal aspect of the gut, like the intestinal branch of the vagus nerve (Kuratani et al., 1997a). The rostral end of the notochord was...
found almost at the rostral tip of the body, and there was no olfactory epithelium except in 1 of 4 embryos. The otocyst was missing in some embryos. The pericardium and pronephros were present in all of the embryos, but the heart was missing in 1 of 3 examined.

**DISCUSSION**

Many studies have described the developmental changes caused by exogenous all-trans RA in various embryos. The consensus on how RA affects embryogenesis can be summarized as rostral truncation of the axis and concomitant posteriorization of segmental values. Typically, this phenomenon is realized by homoeotic transformation of segments (e.g., vertebrae), which accompanies the anteriorly shifted Hox code (Kessel, 1992; Marshall et al., 1992; for review, see Marshall et al., 1996). Ectopic expression of a Hox gene in a more rostral region can actually mimic the RA effect in the head of zebrafish (Alexander et al., 1996). Furthermore, several models of Hox gene regulation mediated by endogenous RA have been proposed (Dekker et al., 1993; Hogan et al., 1992; Simeone et al., 1990, 1991; for reviews, see Hofmann and Eichele, 1994; Langston and Gudas, 1994). It must be noted, however, that the above-proposed scheme inherently tends to deal with the vertebrate body too simply, as though it had only a single metamerical body axis. To circumvent the idiosyncrasy obtained from a rather compact group of animals (gnathostomes), it is necessary to examine a sister group of gnathostomes, the lamprey.

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Fig. 6. Changes in myotomes (experiment 1). **A:** Illustration of a 0.1 µM RA-treated embryo 9 days after the treatment. The rostral head, as well as the entire pharynx are missing. Note the positions of the rostral tip of the notochord (double arrows) and the otocyst (ot) with those structures. Myotomes are numbered from rostral to caudal direction. Seventh through twelfth myotomes are releasing myotubules ventrally (small arrows; compare with Fig. 4B). They appear to represent the autonomous development of the HBM in the absence of the pharynx. The line indicates the plane of section shown in Figure 7A. **B:** Microphotograph of the same embryo as A. Wholemounted and stained with the antibody, CH-1. Hypobranchial muscle-like myotubules are indicated by arrowheads. Scale bar = 100 µm.
RA Eliminates the Rostral Identity

With a high dose of RA at the time of gastrulation, the rostral neural tube of the lamprey embryo was lost. The apparent rostral extension of the notochord in these embryos is consistent with the rostral truncation. Based on the frequent development of the otocyst and melanocyte distribution pattern, the caudal limit of the truncation seemed to fall close to r4.
The rostral truncation of the CNS is apparently analogous with the results in gnathostome species. The severest phenotype obtained in the lamprey resembles the zebrafish embryo treated with 9-cis RA (Zhang et al., 1996); only a mild truncation can be obtained in the zebrafish by all-trans RA (Hill et al., 1995; Holder and Hill, 1991). In mouse and Xenopus, similar truncation has been obtained with RA (Morriss-Kay et al., 1991; Papalopulu et al., 1991) in which the brain rostral to the anterior hindbrain is truncated.

RA has been shown to down-regulate some rostrally expressed genes, resulting in the loss of anterior brain identities (Bally-Cuif et al., 1995; Simeone et al., 1995; Taira et al., 1994). Being expressed in the chordal- and mesodermal substrate, Otx2 appears to be one such gene responsible for anterior specification of the neur ectoderm (Ang et al., 1994). Actually, homozygous disruption of the Otx2 gene results in the rostral truncation of the CNS at a similar neuraxial level (Acampora et al., 1995; Ang et al., 1996; Matsuo et al., 1995). In the disruption of Lim1 as well, a similar level of the rostral head is lost (Shawlot and Behringer, 1995). Obviously, the next step is to examine the normal and shifted expression patterns of these anteriorly expressed regulatory genes.

The heart of a lamprey embryo was often lost by a rather high concentration of RA (Table 1). In gnathostomes as well, the heart is included in the target of teratogenic effects of retinoids or vitamin A deficiency (Wilson and Warkany, 1949), involving the malformation of mesoderm-derived myocardium and aorticopulmonary septation to which the neural crest contributes (Kirby and Waldo, 1990; Kirby et al., 1983). The heart is known to be affected by exogenous RA in the chick and zebrafish (Osmond et al., 1991; Stainier and Fishman, 1992). In the mouse, retinoid receptor-mediated pathways have been shown to be prerequisite for normal cardiogenesis (Sucov et al., 1994; for review, see Osumi-Yamashita, 1996). The intimate relationships between the heart and the pharynx during embryogenesis (for review, see Kirby and Waldo, 1990) might explain the codistribution of defects in the two structures in the lamprey embryos. Also, the pharyngeal endoderm, which is sensitive to RA in the lamprey (see below), has been suggested to be responsible for myocardial differentiation (Sugi and Lough, 1994).

RA Induces the Loss of Pharyngeal Arches

Most intriguing was the graded loss of the pharynx; the more rostral part tends to disappear with lower concentration of RA (Figs. 8, 9), indicating that more anterior pharyngeal pouches are more sensitive to RA. Pharyngeal pouches in the lamprey develop from anterior to posterior, as in gnathostomes (Damas, 1944; Kuratani et al., 1997b; Veit, 1939). The treatment at early stages seemed to have more impact than a higher concentration given at later stages; the former phenotype was more severe than the latter (Figs. 5, 6, 8, 9). In particular, the heart was usually present in 0.5 µM RA-treated embryos in the second series, whereas it was totally absent from the 0.1 µM RA-treated embryos in the first series (Figs. 5, 7). Therefore, the sensitivity of the pharyngeal arches and also of the heart to RA is stage-dependent as well as dose-dependent (Fig. 10).

The possible homeotic transformation of pharyngeal arches has been observed in gnathostomes (Lee et al., 1995; Marshall et al., 1992). Unlike vertebrates, branchial skeletons are hard to transform homeotically by RA; they are more likely to disappear (see, e.g., Mallo, 1997). In the lamprey, a similar change would only be found, if present, in the second arch; it failed to expand as the lower lip in a few specimens with low-dose

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### TABLE 1. Results Obtained in Experiments 1 and 2

<table>
<thead>
<tr>
<th>RA-affected site</th>
<th>Experiment 1: Tahara's stage 12 (early gastrula)</th>
<th>Experiment 2: Tahara's stage 18 (early neurula)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 0.01 µM 0.1 µM</td>
<td>Control 0.05 µM 0.1 µM 0.5 µM</td>
</tr>
<tr>
<td>Olfactory placode</td>
<td>10/10 8/8 1/16</td>
<td>2/2 4/4 3/3 1/4</td>
</tr>
<tr>
<td>Eye</td>
<td>5/5 4/4 0/18</td>
<td>2/2 3/4 0/3 0/4</td>
</tr>
<tr>
<td>Otoxyst</td>
<td>6/6 4/4 5/16</td>
<td>2/2 4/4 3/3 0/2</td>
</tr>
<tr>
<td>Pronephric duct</td>
<td>6/6 4/4 8/8</td>
<td>2/2 4/4 3/3 4/4</td>
</tr>
<tr>
<td>Heart</td>
<td>11/11 8/8 2/17</td>
<td>2/2 4/4 3/3 1/3</td>
</tr>
<tr>
<td>Mouth</td>
<td>11/11 8/8 1/18</td>
<td>2/2 4/4 0/4 0/4</td>
</tr>
<tr>
<td>Pharynx</td>
<td></td>
<td></td>
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<tr>
<td>Endostyle</td>
<td>11/11 8/8 1/18</td>
<td>2/2 4/4 1/4 0/4</td>
</tr>
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<td>Pouch 1</td>
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</tr>
</tbody>
</table>

*The table shows the ratio of embryos among all specimens in which the presence or absence of each structure was able to be determined. Not all structures were able to be observed in each embryo. RA, retinoic acid.

aPresence of pouches 5 and 6 was assumed in one embryo in which two posterior pouches were innervated by the vagus nerve. No pharyngeal pouch was found in the other embryos at this dose.
treatment at the late stage (Fig. 9D). Even if it was the case, the developmental window and the RA concentration that allow the transformation would be very narrow.

It has not yet been demonstrated in any experiment using gnathostomes that pharyngeal arches disappear as dramatically as they do in the lamprey. This may be
partly because gnathostome embryos cannot survive long enough to develop the pharyngeal arch. Lee et al. (1995) found that RA treatment at the neural plate stage of the rat results in the fusion of the first and second arches. The developmental stage at which they administered RA was similar to experiment 2 of the present study. The two results, however, are probably of a different nature: The fused branchial arch of the rat still contains a pharyngeal pouch. The loss of endodermal pharyngeal pouches has never been reported in gnathostomes, although it has been reported in the amphioxus (see below).

The branchiomeric nerve anomalies (Figs. 8, 9) might be secondary to those of the pharynx. Among gnathostomes, the RA treatment at midgastrula results in the partial posteriorization of the branchiomotor neurons in the mouse, with no substantial changes in the peripheral morphology (Keess, 1993; Marshall et al., 1992). In Xenopus (Papalopulu et al., 1991) and zebrafish (Holder and Hill, 1991), however, a disturbed pattern of the PNS has been illustrated. Especially in Xenopus, cranial nerves tended to fuse with each one another, as in the lamprey, although the relationship between the nerve and the pharyngeal arches has not been reported (Papalopulu et al., 1991).

The morphology of the PNS depends largely on the distribution pattern of neural crest cells in amniotes (Kuratani and Eichele, 1993; Loring and Erickson, 1987; Noden, 1975) and also in the lamprey (Kuratani et al., 1997a,b). In the pharynx and hindbrain region, cephalic crest cells in the chick embryo adhere to even-numbered rhombomeres, providing the bases for the nerve root metamerism (Kuratani and Eichele, 1993). In the lamprey, a similar, shared pattern seems to be present; the rhombomere develops only transiently around stage 23, and nerve roots arise on even-numbered rhombomeres (Kuratani et al., 1997b). RA treatment at stage 10 chick embryo results in the disturbed migration of both crest cells and cranial nerve morphology (Gale et al., 1996). A similar alteration may be involved in the RA-treated lamprey embryos, especially in those cranial nerves that were associated with the lost pharyngeal arches (Fig. 8).

**Myotomes Do Not Disappear but Shift Rostrally**

The initial rostral protrusion took place similarly in control and RA-treated embryos (Fig. 1), but the protrusion contained neural elements of quite different axial natures (Fig. 3). It might be because of the so-called posteriorization of embryonic materials that RA treatment leads to the elimination of anterior identities, in the sense that trunk-type specification is executed by skipping the accomplishment of the head.

In the preotic region of cyclostomes as well, there is the cephalic mesoderm, which is not segmented and does not develop into myotomes (Damas, 1944; Veit, 1939; our unpublished data on the lamprey; see Fig. 11A). This mesoderm, as a result of a posteriorizing effect, appears specifically to be truncated by a high concentration of RA (Figs. 5B, 6A). On the other hand, as indicated by the hypobranchial muscle formation, the postotic elements are likely to be simply posteriorized by RA treatment.

The first myotome, as the result of rostral truncation of the lamprey, was found near the rostral end of the head, as has been observed in RA-treated gnathostome embryos (Morriss-Kay et al., 1991; Sundin and Eichele, 1992; Sundin et al., 1993). This raises the question of what numbers of segments were actually shifted rostrally. In the trunk of the lamprey, myotomes go through developmental modifications in a segmental, level-specific manner; myotomes 1 (m1)–m3 grown secondarily into the preotic region, and some occipital level myotomes differentiate into hypobranchial muscles (Fig. 4).

The numbers and levels of myotomes involved in the hypobranchial muscle are not clear in the control embryos due to the expansion of the pharynx, and observation of slightly younger normal embryos did not allow us to see clearly the developing hypobranchial muscles. In light of the number of spinal motor nerves forming the hypoglossal nerve, myotomes involved in the hypobranchial muscle formation probably range from m5 through m10 in normal development (Kuratani et al., 1997a,b). Importantly, the segmental levels of myotomes involved in the putative hypobranchial muscle in RA-treated embryos are very close to those of the control embryo (Fig. 11). Therefore, the rostral
Fig. 9. Development of the cranial nerves (experiment 2). Photomicrographs of the control (A,B), 0.05 µM RA-treated (C,D), 0.1 µM RA-treated (E,F), and 0.5 µM RA-treated (G,H) embryos correspond to stage 27 of normal development. Myotomes are illuminated by the polarized lighting in B, F, and H. A,B: The first myotome (arrow in B) develops ventral to the otocyst (asterisk in A). Note the development of the olfactory epithelium (ol) and the endostyle (es). The mouth opens between the upper (ulp) and the lower (llp) lips (arrow in A). C,D: The lower lip is often obliterated (D), and the mouth (arrow in D) opens widely caudal to the upper lip (ulp). E–H: The morphology of 0.1 µM and 0.5 µM RA-treated embryos can be very similar. In both groups, only the vagus nerve is seen in the head (arrows in E and G). In G, the vagus nerve roots are arranged metamERICALLY with the rostral myotomes (arrows; compare with F). Only two pharyngeal pouches (p) are present in E, whereas none are seen in G. The distal portion of the vagus nerve in G innervates the putative pharyngeal region. In both embryos, the anteriormost myotome develops close to the rostral end (single arrow in F,H). The rostral tip of the notochord is indicated by double arrows (F,H). Note the absence of endostyle and otocyst in these embryos. Scale bar = 100 µm.
myotomes seem to develop normally in RA-treated embryos, whereas the branchial arches disappear ventrally at the same axial levels.

In summary, RA induces what is known as rostral truncation in the lamprey, but it is not a simple posteriorization of segmental values. The RA-induced anomalies are not merely axis-specific but, rather, are body part-specific, i.e., even when all of the pharyngeal pouches disappeared, all myotomes were present, and there appeared to be none clearly lost by the RA treatment. The anomalies in the pharyngeal structures, on the other hand, are more likely to be the simple disappearance of endodermal pharyngeal pouches. Thus, the developmental disturbance of segmental units is not equally coextensive for the paraxial and pharyngeal structures, but RA appears to affect different axial levels in each system. It seems more appropriate to postulate that independent developmental programs may exist for paraxial and endodermal systems that are isolated from one another.

**RA and the Vertebrate Body Plan**

Some comparative morphologists and embryologists have assumed a complete set of head segments, each possessing one branchiomere and one somitic metamere (Balfour, 1878; Bjerring, 1977; Goodrich, 1930; J arvik, 1980; van Wijkhe, 1882). Others have realized, however, that the branchiomeric and somitomeric do not develop in a coordinated manner but are shifted away from each other in actual developmental processes (Hatschek, 1892; Damas, 1944; de Beer, 1922). The distribution pattern of crest cells as well as the actual morphology of paraxial mesoderm are consistent with the latter hypothesis, the dual metamerism put forth by Romer (1972; see also J efferies, 1986; Kuratani, 1997; Starck, 1975).

The dual-metamerical model is well reflected in the developmental pattern of the PNS in vertebrate embryos as well, i.e., the branchiomeric nerves are associated segmentally with rhombomeres and pharyngeal arches, whereas each spinal nerve is associated with somite derivatives. Different embryonic environments are shown experimentally to function as distinct constraints in patterning of each group of nerves (Detwiler, 1934; Keynes and Stern, 1984; Keynes et al., 1991; Kuratani and Eichele, 1993; Rickmann and Fawcett, 1985; Teillet et al., 1987). The early developmental pattern of the lamprey PNS has shown that the dual-metamerical scheme is also applicable in this animal (Kuratani et al., 1997a). Interestingly, in this dual-metameric model, it is the head of the lamprey that was almost selectively lost by RA treatment (Fig. 11A,B). The interface between the affected and nonaffected regions corresponds roughly, if not exactly, to the head/trunk interface based on the distribution pattern of cephalic crest cells (Fig. 11C; Kuratani, 1997).

The evolutionary origin of the dual-metamerical developmental plan is an intriguing subject for speculation. In this context, tunicate embryos have been treated with RA, and rostral truncation has been observed in the tadpole larvae (Katsuyama et al., 1995). Because the possible somitomeric and branchiomeric segmentations are found in this organism (Wada et al., 1996; for review, see Gee, 1996), it would be interesting to know whether there would be a condition in which only the endodermal derivatives were affected.

The configuration of the notochord in RA-treated embryos (Figs. 4, 5, 8E–H) as well as the apparent absence of nerves with typical branchiomeric features are reminiscent of the anatomy of amphioxus (Fig. 6; Franz, 1927; Hatschek, 1892). The development of the amphioxus PNS is not well known, although the adult anatomy has shown the absence of distinction between the branchiomeric and spinal nerves, and all of the segmental dorsal nerves pass along the intermyotomic pathway (for reviews, see Franz, 1927; Fritzsch and Northcutt, 1993; J effries, 1986). Moreover, the brain of this animal is reported to have some features common to the vertebrate rostral brain (Lacalli et al., 1994) that are clearly lost by RA treatment (Fig. 11A,B). Therefore, the resemblance of the RA-treated lamprey embryo to the amphioxus larva may be no more than superficial and may be caused merely by truncation of the unsegmented head mesoderm.

The idea of selective loss of the morphological head by RA is strengthened by a similar experiment made on the amphioxus. Holland and Holland (1996) have shown that, in this animal, RA induces the absence of anterior endodermal structures, the mouth and pharyngeal slits, a result that is reminiscent of the present study. Although the pharyngeal and mouth formation processes differ greatly between the two animals (Willey,
both depend on normal pharyngeal arch development. Due to the small amount of yolk, RA-treated, mouthless amphioxus larvae cannot live longer than 4 days, and there is no knowing how normally their somitic part can develop or whether there is dose-dependent sensitivity at each pharyngeal level. Nevertheless, the apparent specific loss of pharyngeal arch development.}

**Fig. 11.** Diagram summarizing the effects of RA on lamprey development. A: Pharyngula stage of normal embryo. On the neural tube, the posterior commissure (pc) divides the forebrain and midbrain, and the midhind brain border (prm) is located between midbrain (m) and hindbrain. The notochord extends rostrally to the level of diencephalon. The dorsolateral fasciculus (DLF) terminates rostrally at the level of the rostral hindbrain. The rostral end of the forebrain is associated with the olfactory epithelium (olep). Paraxial mesoderm is segmented as myotomes caudal to the otocyst but is not clearly segmented preotically (phm). Myotomes caudal to m7 are likely to differentiate into hypobranchial musculature (HBM). Endodermal pharyngeal pouches (solid circles) penetrate the pharyngeal wall, resulting in nine pharyngeal arches. The rostralmost pouch is vestigial. The endostyle (es) develops on the floor of the pharynx. The heavy dashed line indicates the border between the truncated and unaffected regions after RA treatment (myotomes 1–3 and HBM secondarily grow rostrally beyond the border). ep, epiphysis; oc, oral cavity; ot, otocyst; phm, preotic head mesoderm. B: RA-treated embryo, representing a generalized severest case. The dorsolateral fasciculus is associated with almost the entire length of the neural tube. The otocyst can be present at the rostral tip of the head, close to the rostral tip of the notochord. The notochord is associated rostrally with the detached endoderm (arrow). The pharynx and esophagus are missing. The paraxial space of this embryo is occupied by myotomes, the seventh and caudal of which differentiate into presumptive HBM. The pericardium develops but does not contain the heart. C: A pharyngula embryo of a shark (modified from Goodrich, 1930). There appears to be an S-shaped “head/trunk interfaces” (dashed line) along the caudal limit of the cephalic crest cell distribution in this animal as well as in other gnathostome embryos (Kuratani, 1997). Note that the dashed line resembles the border shown in A.
structures commonly observed in the lamprey and amphioxus might represent shared mechanisms of dual-metamerical patterning, like the synplesiomorphies that may be obscured in many gnathostomes.

**EXPERIMENTAL PROCEDURES**

**Embryos**

Adult male and female lampreys (Lampetra japonica) were collected in Miomote River (Niigata, Japan) during the breeding season (late May through June) in 1995. They were brought into the laboratory, where eggs were artificially fertilized and kept in fresh water at 18°C. Embryos were fixed either with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PFA/PBS) for immunohistochemistry or with Bouin’s fixative for histological preparations. For the staging of embryos, developmental sequence of a brook species, L. reissneri (Tahara, 1988), was applied.

**RA Administration**

A stock solution of the all-trans RA (R-2625, Sigma, St. Louis, MO) was made at a concentration of 0.01 M in dimethylsulfoxide (DMSO) and was stored at −20°C in the dark. The solution was diluted with DMSO, and the same amount of RA/DMSO solution was added to 50 ml of Steinberg’s solution (Steinberg, 1957) diluted 1/10, into which the lamprey embryos were transferred. For the control treatment, the same amount of DMSO alone was added. Embryos were left in the solution for 1 hour at 23°C in the dark. After extensive washing with 1/10 Steinberg’s solution, they were allowed to grow at room temperature in the same buffer. Two series of experiments were made. In the first, embryos at Tahara’s stage 12 (early gastrula) were treated with different concentrations of RA. Concentrations were 0.01 µM, 0.1 µM, 1 µM, and 5 µM. The second experiment was made at stage 18 (early neurula) with concentrations of 0.05 µM, 0.1 µM, and 0.5 µM.

**Wholemount Immunostaining**

Wholemount embryos were prepared as described by Kuratani et al. (1997a) with minor modifications. After fixation with PFA/PBS at 4°C for 1 day, embryos were washed in 0.9% NaCl/distilled water, dehydrated in a graded series of methanol (50%, 80%, 100%), and stored at −20°C. The samples to be stained were placed on ice in 2 ml DMSO/methanol until they sank. Five tenths of a milliliter of 10% Triton X-100/distilled water was added, and the embryos were incubated for another 30 minutes at room temperature. After washing in Tris-HCl-buffered saline (20 µM Tris-HCl, pH 8.0; 150 µM NaCl; and 0.1% Triton X-100; TST), the samples were sequentially blocked with aqueous 1% periodic acid and with 5% dry nonfat milk in TST (TSTM).

For wholemount immunostaining of the nervous system, a MAB raised against acetylated tubulin (monoclonal antibody) was used. Embryos were incubated in primary antibody (diluted 1/1,000 in spin-clarified TSTM containing 0.1% sodium azide and 5% DMSO) for 2–3 days at room temperature while being gently agitated on a shaking platform. The secondary antibody used was horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Zymbad Laboratories, San Francisco, CA) diluted 1/200 in TSTM. After washing in TST, the embryos were preincubated with peroxidase substrates 3,3'-diaminobenzidine (DAB; 100 mg/ml) in Tris-HCl-buffered saline (TS) for 1 hour and allowed to react in TS with the same concentration of DAB and hydrogen peroxide dissolved at 0.01% for 20–40 minutes. After stopping the reaction with TS, some embryos were clarified in 30% glycerol containing 0.5% potassium hydroxide (KOH). These embryos were stored in 60% glycerol/water. Others were dehydrated in a graded series of methanol and cleared in benzyl alcohol/benzyl benzoate mixture (1:2). Embryos of both groups were mounted on depression slide glass for observation under the light microscope with Nomarski optics. Another MAB, CH-1 (purchased from Developmental Studies Hybridoma Bank, Iowa City, IA) raised against tropomyosin, was used to stain myotomes in wholemount embryos.

**Histology**

Embryos fixed with Bouin’s fixative were dehydrated and embedded in paraffin. Sections were cut at 5 µm and stained with Gill’s hematoxylin and eosin.

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**REFERENCES**


