Stereotyped Axonal Bundle Formation and Neuromeric Patterns in Embryos of a Cyclostome, Lampetra japonica

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ABSTRACT

Early embryonic development of the nervous system of a lamprey, Lampetra japonica, was studied by using immunohistochemical techniques and by scanning electron microscopy. The earliest appearance of axons was detected at Tahara's stage 21, when dorsolateral and ventral longitudinal fasciculi were present in the hindbrain and spinal cord regions. The branchiomeric nerve roots began to appear at stage 22; the fibers were joined to the dorsolateral fasciculus proximally and also extended distally into each pharyngeal arch. The anterior neural tube was divided into several neuromeres; the mid-hindbrain sulcus became apparent first, then the portion rostral to this sulcus was subdivided into two portions by the syn-parencephalic boundary. In the hindbrain around stage 23, rhombomeres developed transiently, of which, rhombomere 4 was the most distinctive. Putative crest cells forming the octavofacial nerve root anlage were selectively adhering to rhombomere 4, whereas no crest cells were found on rhombomere 3. The assignment of the crest-derived nerve anlage to rhombomeres is conserved between gnathostomes and L. japonica. The neuromerical scheme of the neural tube of L. japonica is also mostly in accordance with that in gnathostomes, sharing the basic developmental patterning of axon bundles at early developmental stages. The most distinct difference between these two groups is the topographical relationships between the hindbrain neuraxis and pharyngeal arches, as well as the otic placode. J. Comp. Neurol. 391:99–114, 1998.

Indexing terms: rhombomeres; cranial nerves; neural crest; lampreys


The attention of embryologists has been devoted to the segmentation of the vertebrate neural tube since its discovery by von Baer (1828) in a 3-day-old chick embryo. The metamerism of the nervous system attracts reawakened interest from recent developmental biology providing a new background of molecular biology and developmental neurology, in that the early distribution pattern of pioneer neurons is known to correspond to the neural segmentation, thereby providing an important factor that results in discrete, precise, and stereotyped axon bundle development (reviewed by Kimmel, 1993). The segmentation of the brain and early axonogenesis is, therefore, regarded as a prepattern of the complicated architecture of the vertebrate brain.

The segmental bulges of the neural tube were first called neuromeres by Orr (1887), who recognized in lizard embryos several common histologic features shared by bulges in the hindbrain, the rhombomeres. These features are (1) rhombomeres are bounded by internal and external sulci on the neurepithelium, (2) each rhombomere has neuropilial cells arranged in a fan-shaped pattern, (3) no single cell spans two successive rhombomeres, (4) each rhombomere has its center of proliferation in its middle part, and (5) it develops symmetrically on either side of the hindbrain. It has recently been shown that rhombomeres are established through the restriction of cell lineages; once the boundary is determined on the neuraxis, cells in neighboring segments will not intermingle over the sulci (Fraser et al., 1990; also see Birgbaur and Fraser, 1994).
Although some authors regarded neureomers as artifacts or paid little attention to their morphologic importance (Balfour, 1881; Froriep, 1891, 1892; Streeter, 1933), others considered these structures to be a profound segmental plan of the vertebrate axis (Locy, 1895; Hill, 1899, 1900; also see Neal, 1896).

Of the neureomers, rhombomers are especially curious because they are associated with cranial nerve roots which are metameral in the sense that the iterative nature of branchial arches constitutes the branchiomerism. In many vertebrate embryos, branchiomeric nerve roots emerge from even-numbered rhombomeres, thereby being associated with the head metamerism (Remak, 1855; Beranek, 1884; Kuhlenbeck, 1935; Lumsden and Keynes, 1989; Kuratani, 1991; Kuratani and Eichele, 1993). Isolation of homeobox-containing genes in vertebrates provided the above scheme for mechanical interpretations of head patterning. Of those, Hox genes in amniotes are expressed in a nested pattern; each expression domain is limited by rhombomeric sulci (reviewed by McGinnis and Krumlauf, 1992, and by Krumlauf, 1993). Not only the hindbrain, but also the branchiocomponents such as pharyngeal ectoderm, endoderm and the mesenchyme express the genes in the same order, thereby establishing the Hox code of the embryonic head (reviewed by McGinnis and Krumlauf, 1992, and by Mark et al., 1995).

As Romer (1972) has postulated, the vertebrate body plan seems to possess two distinct types of metamers; branchiomerism in the head and somitomerism in the trunk (in this context, the occipital somites are regarded as trunk components; see Kuratani, 1997). It remains to be solved where this unique plan emerged in the evolutionary path leading to vertebrates. In amphioxus, a sister group of vertebrates, Hox gene cognates are expressed in a segment-specific fashion along the axis, as in vertebrates (Holland et al., 1992; Holland and García-Fernández, 1996). However, there is no known expression pattern restricted by a cell lineage-based compartment; nor are there any rhombomeres in this animal.

In the rostral brain, a series of neureomers are also known to exist in a number of vertebrates. Interestingly, these bulges share some traits with rhombomeres and also express a number of regulatory genes in patterns restricted by segmental sulci (reviewed by Figdor and Stern, 1993; Price, 1993; Boncinelli et al., 1993; Rubenstein et al., 1994; Shimamura et al., 1995). These bulges are also unique to vertebrates, and it is not known when and how segmental organization of the central nervous system (CNS) arose in chordate evolution.

Among vertebrates, it is mostly in Gnathostoma species that the CNS development has been examined in detail. It is a prerequisite in the evolutionary context that a species distantly related to gnathostomes should be examined. The present paper is intended to provide a precise description of the early neurogenesis and differentiation of the neural tube in a cyclostome, Lampetra japonica. The authors previously described the late embryonic development of cranial nerves in this animal (Kuratani et al., 1997), but no rhombomere-like structures were seen in those embryos. In the present study, the morphology of the nervous system in younger lamprey embryos at prehatching stages were examined in an attempt to search for rhombomeres. By observing the distribution of acetylated tubulin (AT)-like immunoreactivities and three-dimensional morphology of the neural tube by scanning electron microscopy (SEM), we found that obvious segmentation in the developing hindbrain, albeit faint, appeared before the completion of cranial nerve root patterning.

**MATERIALS AND METHODS**

**Embros**

Adult male and female of Lampetra japonica were collected in a tributary of the Miomote River, Niigata, Japan, during the breeding season (late May through June) in 1995 and 1996. The eggs were artificially fertilized and kept in fresh water at 18°C or in 10% Steinberg's solution (Steinberg, 1957) below 23°C. Embryos were fixed either with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PFA/PBS), pH 7.6, for immunostaining or with 0.1 M phosphate-buffered 2.5% glutaraldehyde for scanning electron microscopy and plastic sectioning. Some embryos were also fixed with Bouin's fixative for lectin staining. Because embryonic development was easily changed by temperature, they were staged morphologically according to the table of Tahara (1988) for L. rassneri, a brook species of L. japonica. The animals used in this study were treated under protocols approved by Kumamoto University guidelines for animal experiments.

**Whole-mount immunostaining**

Whole-mount embryos were prepared as described previously (Kuratani et al., 1997) with slight modifications. After fixation with PFA/PBS at 4°C for 1 day, embryos were washed in normal saline, dehydrated in a graded series of methanol (50, 80, 100%), and stored at -20°C. The samples to be stained were placed in 20% dimethyl sulfoxide (DMSO)/methanol with 10% (v/v) hydrogen peroxide solution and gently agitated on a shaking platform. After washing in Tris-HCl-buffered saline (TST) (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Triton X-100) containing 5% (v/v) DMSO (TST/DMSO), the samples were sequentially blocked with 5% dry non-fat milk in TST/DMSO (TSTM/DMSO) overnight.
For whole-mount immunostaining of cranial nerves, monoclonal antibody (Mab) raised against \(\alpha\)-acetylated tubulin (monoclonal anti-acetylated tubulin antibody, No. T-6793, Sigma Chemical Company, St. Louis, MO) was used. Embryos were incubated in primary antibody (diluted 1/1,000 in spin-diluted TSTM/DMSO containing 0.1% sodium azide) for 2 to 4 days at room temperature while being gently agitated. Horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (ZYMED Laboratories Inc., San Francisco, CA) diluted 1/200 in phosphate-buffered saline, pH 7.6.

...immunoreactive cells were more abundantly accumulated in the neuropil of the rostral to the boundary (Fig. 1A).

Slightly later, at stage 21, the mid-hindbrain boundary is clearly detected in the neural tube, approximately at the level dorsal to the first pharyngeal pouch (Fig. 1C). An accumulation of AT-like immunoreactive products was distributed in a neuromeric pattern, showing the establishment of some primitive neuromeres (Fig. 1C). Of those, the caudal two bulges are isolated from each other by a rather clear boundary, the mid-hindbrain boundary, that delineates the caudal midbrain from the rostral hindbrain dorsal to the first pharyngeal pouch (see below). The rostral end of the DLF terminated in the midst of the third bulge, which is clearly detected in the neural tube, approximately at the level of the midbrain. There were a few neurons whose axons extended caudally (Fig. 1C). These neurons correspond to the interstitial nucleus that forms the anlage of the medial longitudinal fasciculus (MLF). Commisuratory axons developing in the posterior hindbrain and the spinal cord increased in number and formed the ventrolateral fasciculus on the ventral aspect of the neural tube.

Stage 22

The second pharyngeal pouch and the otic placode had appeared by stage 22 (Fig. 2A). The number of RB cells and commissure axons increased in the posterior hindbrain and the spinal cord. The otic placode was located lateral to the new subdivision of the hindbrain stained darkly compared with the adjacent boundary stripes in the neural tube (Fig. 2B). This rhombomere arose at the neuraxial level dorsal to the second pharyngeal pouch, and corresponds to r4 of gnathostomes because it possesses the octavofacial nerve root. The bulge was not detected in histologic sections (not shown). The neuromeres were more heavily stained toward the apical surface, and even more at the center of each segment. The most heavily stained portion along the brain axis was the posterior midbrain (Fig. 2); staining of this portion lasted until stage 24.

The octavofacial nerve root developed first. It consisted of a few irregularly oriented axon bundles; they extended rostroventrally and passed along the medial side of the otic placode (Fig. 2). The bundle was traced proximally to the DLF, although the cell bodies were not found. In some embryos, there was another axonal bundle arising from the DLF rostral to the octavofacial nerve root associated with the second pharyngeal arch; it grew caudal to the first gill pouch (Fig. 2). This bundle represents an accessory nerve of the octavofacial nerve, probably belonging to the anterior lateral line nerve that eventually merges into the octavofacial nerve trunk (Kuratani et al., 1997; see below), but the placodal origin of these neurons has not yet been determined. Some of the cells in the ectoderm covering the forebrain had differentiated into the AT-positive olfactory neurons, some sending processes toward the forebrain (Fig. 2B).

Stage 23

The olfactory epithelium became a compact plate of epithelium beneath the forebrain showing high levels of AT-like immunoreactivity (Fig. 3). Dorsal to the olfactory epithelium there were independent accessory olfactory neurons whose processes also grew toward the forebrain transiently (Fig. 3B). The staining of their cell bodies was...
Fig. 1. Head region of L. japonica embryos at stages 21 and 21. Immunohistochemically stained with the anti-acetylated tubulin Mab. A: Stage 21 embryo. Arrows indicate the rostral swelling of the neural tube that shows accumulation of peroxidase-reaction products. B: Higher magnification of the box in A. Arrows indicate the commissure neurons growing axons ventrally and then rostrally. The dorsolateral fasciculus (DLF) is located dorsal to the commissure neuron cell bodies. C: Stage 21 embryo. The rostral neural tube shows weak constrictions (an arrow and a broken line) dividing the tube into three portions. The arrow marks the position of the mid-hindbrain boundary (prm). The DLF has become thicker, and its rostral end is seen within the third segment that corresponds to the rostral hindbrain. Arrowheads indicate the initial development of the medial longitudinal fasciculus. The position of the first pharyngeal pouch (p1) is marked by a broken circle. Scale bars = 100 µm.
Fig. 2. Photomicrographs of a stage 22 embryo of *L. japonica* focused on different depths. A: Focused on the developing neurons. Ventral spinal nerves (sv) are seen at the level of the spinal cord. The medial longitudinal fasciculus (MLF) consists of axon bundles that extend caudally from cell bodies (ISN, interstitial neurons) in the transitional area between the rostral two neuromeres. Pharyngeal pouches (p1, p2) are shown by solid lines. Note the development of the octavofacial nerve root (VII) medial to the otocyst (broken circle). The otocyst develops lateral to r4 (compare with B).

B: Focused on a deeper level to show the neuromeres. The equivalent borders are shown by the same line and arrow as in Figure 1C. The fourth rhombomere (r4, demarcated by two small arrows) is more darkly stained than the rest of the adjacent neural tube with the anterior and posterior sulci that show only low levels of staining. Olfactory neurons are seen in the ectoderm covering the forebrain (arrowheads) sending axons toward the forebrain. For abbreviations, see list. Scale bar = 100 µm (applies to A,B).
Fig. 3. Whole-mount *L. japonica* embryos at stage 23. The third pharyngeal pouch (p3) is developing. 

**A:** Early axonogenesis is shown. The trigeminal nerve root (V) is present. Arrowhead indicates an accessorional nerve associated with the octavofacial nerve (VII). Rohon-Beard cell bodies (RB) are heavily stained.

**B:** Enlargement of the rostral-most part of another embryo at the same stage. Arrowheads show a cluster of commissure axons in the vicinity of the future trigeminal nerve root. The large arrow indicates the mid-hindbrain boundary, and small arrows indicate rhombomeric sulci. Postoptic tract (POT) is seen caudal to the optic stalk (OS). The olfactory epithelium (olep) is now seen as a solid AT-positive portion of the surface ectoderm. Dorsal to the definite olfactory epithelium, a few ectopic neurosensory cells (eo) are seen in the ectoderm, and these also send processes toward the forebrain. For abbreviations, see list. Scale bars = 100 µm.
similar to younger olfactory cells (Fig. 2), but the fibers did not form a fascicular appearance as olfactory axons (Fig. 3B). The ectopic neurons disappeared by stage 24. In the forebrain, interstitial neurons had increased in number (Fig. 3B). Caudal to the optic stalk, supra-, and postoptic tracts first developed (Fig. 3B). Optically, the axons of the olfactory nerve were closely associated with the supraoptic tract. 

The trigeminal nerve root first appeared at this stage (Fig. 3A). As in the case of the octavofacial nerve, the root arose from the DLF and the nerve bundle grew ventrally into the cheek process representing the mandibular arch (Fig. 3A). A few additional axon bundles were associated with this definite trigeminal nerve root; they always developed caudal to the latter (Fig. 3A). They represented accessorital roots of the octavofacial nerve, similar to the ones seen at stage 22 (Fig. 2A). These roots were fasciculated as a solid octavofacial nerve root by stage 25 (see Kuratani et al., 1997).

Rhombomeres were most clearly visible at this stage, although the neuromeric bulges were very faint compared with those reported in amniote embryos. Histologic sections showed stronger immunostaining at the luminal side of r4 neurectoderm (Fig. 4A) and boundaries were visible on the luminal surface (Fig. 4A,B). These boundaries were restricted to the dorsal portion of the neurectoderm, and ventrally no indication of rhombomeric patterning was detected. The neurectodermal cells of r4 showed a fan-shaped configuration as reported in gnathostomes (Fig. 4A).

As seen in the whole-mounts of a slightly older stage from the dorsal view (Fig. 5), the luminal distribution of AT-like immunoreactive products was the most distinct in
r4 to r6; each segment was separated by AT-negative boundary regions (Fig. 5A). In some precocious embryos, the ophthalmic nerve developed in the r1+2 region (Fig. 5B). This nerve possessed an independent root at the level rostral to the trigeminal nerve root. On the ventral aspect of the hindbrain, the commissure axons were present, extending toward the contralateral sides (Fig. 5A). The trajectory of these axons showed no sign of the transverse scaffold associated with the rhombomeric boundaries. Unlike these boundaries, the mid-hindbrain boundary was a rather clear indentation on the dorsal surface, which stayed visible throughout the development.

The rhombomeric sulcus was also vaguely detectable in the skinned embryo when observed by SEM (Fig. 6). The sulci detected were only those at levels of mid-hindbrain, r2/3, r3/4, and often r4/5. To be noted was the putative crest cell population adhering specifically to r4, extending ventrally medial to the otocyst (Fig. 6). This cell population represented the octavofacial nerve anlage. Proximally, the cell covered the entire dorsal surface of r4. Another cell

Fig. 5. Rhombomeres in a stage 23 embryo of L. japonica. Dorsal views of whole-mount specimens. The broken line indicates the syn-parencephalic border, and the large arrows indicate the mid-hindbrain border. A: Epiphysis is seen (ep). The photograph is focused on the ventral aspect of the hindbrain to show commissure axons (arrowheads). Note that the axons do not extend in a transverse direction. B: Enlargement of the same embryo focused on a different depth. The contour of the otocyst (ot) is encircled by a solid line. The earliest anlage of the ophthalmic nerve (oph) is seen in this specimen. The mid-hindbrain boundary is indicated by a large arrow, and rhombomeric sulci are indicated by small arrows. Note in each rhombomere that AT-like immunoreactive products are accumulated toward the luminal side of the neurepithelial cells (asterisks), and the accumulation is strongest in the posterior end of the midbrain (double asterisks). For abbreviations, see list. Scale bars = 100 µm in A, 50 µm in B.
population representing the anlage of the maxillomandibular nerve root was seen on the caudal part of r1-2 (Fig. 6). No such cell mass was associated with r3 or r5.

**Stages 24 and 25**

At these stages, RB cells caudal to the posterior hindbrain stained heavily with the anti-AT antibody (Fig. 7A,B). The mid-hindbrain boundary had become more conspicuous than in previous stages. Some of the embryos had developed the earliest anlage of the vagus nerve root, and in a few cases, the glossopharyngeal nerve as well. The vagus nerve root was directed ventrally into the fourth branchial arch (Fig. 7B). By stage 24, AT-positive commissure axons had accumulated in the hindbrain, roughly corresponding to the sites of cranial nerve roots (Fig. 7B). These neurons were not seen in the rostral-most portion of the hindbrain or in the midbrain (Fig. 7A,B).

The definite anlage of the epiphysis appeared at stage 24, at the level corresponding to the anterior-most sulcus observed at stage 21 (Fig. 7C). The posterior commissure was clearly stained at the site of the dien-mesencephalic border (Kuratani et al., 1997). All the branchiomeric nerves had developed by stage 25 (Kuratani et al., 1997), whereas in the hindbrain, rhombomeric sulci had disappeared (Figs. 7, 8); they could not be found on the external surface by SEM either (not shown). This condition lasted throughout the developmental stages observed (to stage 31).

**DISCUSSION**

In the present study, the early axonogenesis and segmentation of the brain were described in a series of embryos of an extant jawless craniate, _L. japonica_. By using whole-
mount immunostaining and by SEM, segmental bulges similar to amniote rhombomeres were described in lamprey embryos for the first time. Comparison with the developmental pattern in gnathostomes poses several interesting questions as to the establishment of the vertebrate body plan.

**Fig. 7.** Stage 24 embryos of *L. japonica*. Stained with the anti-AT monoclonal antibody. 

**A:** Lateral view of a whole-mount embryo. The fourth pharyngeal pouch (p4) is beginning to form. Mid-hindbrain boundary (large arrow) and rostral and caudal sulci of r4 (small arrows) are indicated on the neural tube. Note the extensive accumulation of interstitial neurons (ISN) in the syn-mesencephalic regions. Rohon-Beard cells (RB) are heavily stained. Arrowheads show accessory nerves of the octavofacial nerve. 

**B:** Another embryo of the same stage, slightly older than A. The vagus nerve root (X) has developed. Note that commissure neurons are heavily stained (arrowheads) and are lacking in the posterior midbrain. Also note the spatial relation between the epiphysis (ep) and the syn-encephalic border (broken line). For abbreviations, see list. Scale bar = 100 µm (applies to A, B).
In the hindbrain of amniotes, cephalic crest cell populations adhere to even-numbered rhombomeres in the preotic region (r2 and r4) and to r6 as well, thereby the crest cells develop into cranial nerve anlage (Kuratani, 1991; Kuratani and Eichele, 1993; Niederländer and Lumsden, 1996). Such bi-rhombomeric repetition would probably be associated with the metamerism of the branchial arch, comprising developmental units of the vertebrate head (Köntges and Lumsden, 1996). This morphologic scheme confirms the mechanism of the segmental specification in terms of molecular biology, i.e., a series of homeotic selector genes, the Hox genes, are expressed along the neuraxis. The expression domains of each Hox gene shifts its anterior border by a bi-rhombomeric length, thereby establishing the nesting pattern of gene expressions, the Hox code (reviewed by McGinnis and Krumlauf, 1992; and Graham, 1992).

The existence of rhombomeres is not widespread in all chordates. For example, no neuromere-like structures have been found in amphioxus (Hatschek, 1881; Willey, 1891; Franz, 1927; Lacalli et al., 1994). Neal (1918) and Neal and Rand (1936) pointed out that rhombomeres apparently become clearer in embryos of more advanced vertebrates. Although evolutionary grades cannot always be measured between sister groups, it is true that amniote rhombomeres are much more distinguishable than those in known anamniotes. Rhombomeres must have evolved somewhere in the evolutionary lineage to vertebrates, and it is therefore important to determine whether or not they have developed in lampreys that have long been dichotomized from the ancestor of gnathostomes (reviewed by Janvier, 1993).

Compared with the hindbrain in amniotes, the rhombomeric boundaries in L. japonica were very faint and lasted only for a short period during development. They were most clearly segmented near the r4 level, and dissociation of r1 and r2 was not determined through the stages observed (summarized in Fig. 9). Importantly, semi-bi-rhombomeric patterning seems to exist also in L. japonica, as shown in relation to crest cells and cranial nerve roots: the ophthalmic and trigeminal nerve arise from r1+2, octavofacial root from r4, and glossopharyngeovagal root from r6 (Figs. 5, 6, 8A). In the chick embryo, the adhesion of crest cells on even-numbered rhombomeres plays an important role in nerve root formation (Moody and Heaton, 1983; Kuratani and Eichele, 1993; Niederländer and Lumsden, 1996). In L. japonica, the crest cells representing the anlage of the octavofacial nerve covered the entire
length of r4, whereas no crest cells were adhering to r3 (Fig. 6). More precise distribution pattern of crest cells will be described elsewhere together with the development of cranial ganglia. So far, there have been no descriptions of agnathan neuromeres clear enough to compare with modern data in gnathostomes. For example, segmental bulges were illustrated in Bdellostoma by von Kupffer (1900), which are almost the only data obtained so far in myxinoids. However, the morphology of the brain by their descriptions suggests that the developmental stage is rather too late to observe the neurepithelial bulge, and the segments are dubious as real rhombomeres. Nor is there any information about cranial nerve roots in this embryo (Dean, 1899). In lampreys also, the observed embryos were too old to detect the real rhombomeres (von Kupffer, 1895). Polygonal subdivisions or neuromeres were described in rather old larvae of Petromyzon by Bergquist and Källén (1953a, 1953b). It is unlikely that these segments represent true developmental compartments, because at least in L. japonica, the real rhombomeres totally disappear long before the stage corresponding to the embryos observed (Fig. 8C). In addition to the bi-rhombomeric registration of the cranial nerves to the hindbrain, the lamprey rhombomeres in the present observation share some features in common with gnathostomes (Orr, 1887): the dorsal part of rhombomeres at early stages exhibit a fan-shaped arrangement of neurepithelial cells, and the apical side of the cells is constricted in the middle of each rhombomere (Fig. 4). This neurepithelial configuration is reported to occur in amniote rhombomeres also (Stunkard, 1922; Tuckett and Morriss-Kay, 1985; Heyman et al., 1993) and it has been explained by the formation of rhombomeres being induced by cytoskeletal elements in the neurepithelial cells (Tuckett and Morriss-Kay, 1985). It remains to be studied whether the accumulation pattern of acetylated tubulin-like immunoreactive products has any relation to the neurepithelial compartmentalization.

Bi-rhombomeric repetition appears to be partly dependent on alternating cell adhesion for even- and odd-

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**Fig. 9.** Schematic representation of the developing nervous system of L. japonica. Neurons and neuromeres are shown in the scheme. Neuromerical swellings are shaded. Due to complexity, neuromeres in stage 24 embryo are not shaded except for r4. Three rostral bulges at stage 22 correspond to, from rostral to caudal direction, parencephalon + secondary prosencephalon, syencephalon + mesencephalon, and rostral hindbrain segment (probably r1 +2), respectively (see insert). Rhombomeres are most clearly seen at stage 23 around the level of r4. No rhombomeres caudal to r6 were found. Note that the topographical relationship between neuromeres and pharyngeal pouches is constant throughout the developmental stages. Circle indicates the optic stalk. Inset: Hypothetical diagram of the neuromerical plan of the brain of L. japonica. The rhombencephalon probably contains 6 rhombomeres (r1–6), of which the division of r1 and r2 is unclear. The neural tube caudal to r6 is defined as the spinal cord. The forebrain is to be subdivided into at least three portions. Division of the telencephalon (t) and position of the posterior commissure (pc, dots) are from Kuratani et al. (1997). Cranial nerve roots are indicated by small circles. Post, posterior; ant, anterior; paren, parencephalon; mesen, mesencephalon. For other abbreviations, see list.
Compared with gnathostome pharyngula, the position of the whole pharyngeal system of lamprey is also shifted rostrally in relation to the neuraxis, and the second pharyngeal pouch arises ventral to the otic placode or r4 (Fig. 10). This topographical situation is similar to that in Petromyzon as described by Veit (1939) and Damas (1944), which also resembles some fossil osteochordans, close relatives of L. japonica (Janvier, 1996). The cranial nerve roots in these animals are thus oriented rostrally after issuing from the brainstem (Fig. 10; also see Kuratani et al., 1997).

The early phase of axonal growth in the embryonic brain has been described for a variety of gnathostome species (Windle and Austin, 1936; Windle and Baxter, 1936; Herrick, 1937; Ströer, 1956; Lyser, 1966; Windle, 1970; Keyser, 1972; Wilson et al., 1990; Easter et al., 1993; Hartenstein, 1993; Figdor and Stern, 1993; Burrell and Easter, 1994). As is generally accepted, the early axonal trajectories, especially those along the circumferential path, depend on the neurepithelial segmental boundaries (Figdor and Stern, 1993; reviewed by Wilson et al., 1993).

In the chick hindbrain, accumulation of neurofilament is seen at the rhombomeric boundary regions (Lumsden and Keynes, 1989; Keynes and Lumsden, 1990; Guthrie and Lumsden, 1991). In the lamprey embryos, however, no such accumulation was observed at any stage of development, nor did the commissure axons on the ventral aspect of the hindbrain (Figs. 5, 8) follow any segmental pattern. Likewise, the site of the posterior commissure that first becomes apparent at stage 25 (Kuratani et al., 1997) is not preceded by any visible boundaries on the neur ectoderm, which delineate the anterior border of the definite midbrain.

It has been recognized in the zebrafish development that reticulospinal neurons are arranged in a metameric pattern; the same sets of neurons are distributed in each rhombomere (Kimmel et al., 1982, 1985; Metcalfe et al., 1986; Mendelson, 1986a, 1986b). Such a feature may be widespread in teleost embryos because a similar phenomenon is reported in the goldfish (Lee et al., 1993), but has never been seen in other vertebrates (Tello, 1923; Manns and Fritzsche, 1992; Lumsden et al., 1994). The branchial nerve efferent neurons are arranged in a pseudo-metameric pattern in some vertebrates but are not always clearly limited by rhombomeric boundaries (Bok, 1915; Tello, 1923; reviewed by Gilland and Baker, 1993). In larvae or adult lampreys and hagfish, there is also no apparent metameric arrangement of particular neurons recognized in the hindbrain (Saito, 1928; Schober, 1964; Niewenhuis, 1972; Rovainie et al., 1973; Ronan, 1989; Swain et al., 1993, 1994; J acobs et al., 1996). At the 1-cm stage of L. japonica, retrograde labeling of reticulospinal neurons by 3,000-molecular weight dextran amines showed the accumulation of labeled neurons at levels of cranial nerve roots (unpublished observation). This pattern was essentially similar to the accumulation of commissure neurons that starts at stage 24 when rhombomeres begin to disappear (Fig. 7), and the accumulation never coincided with rhombomeric boundaries. It remains to be tested by Dil-tracting method whether any homologous set of reticulospinal neurons develops in each rhombomere in earlier embryos.

The initial axons developing in the brain and the spinal cord are thought to function in guiding late developing axons, thereby forming constant anatomical neuronal

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**Fig. 10. Topographical relationships between branchiomeric nerves and pharyngeal pouches of the lamprey and amniote embryos.** The whole pharynx of L. japonica embryo is located more rostrally to the hindbrain than that in the amniote embryos. Concomitantly, the branchiomeric nerve roots are oriented rostrally after issuing from the hindbrain. The otic placode in the lamprey is also located more rostrally (r4 level) than that in amniotes (r5 to r6). For abbreviations, see list.
tracts (Shiga and Oppenheim, 1991; Chitnis et al., 1992; reviewed by Kimmel, 1993). Such pattern is also dependent on the neuromerical configuration of the neuroderm, which is expected to be rather constant among vertebrates (reviewed by Kimmel, 1993). The early brain of the lamprey embryo showed rather a conserved pattern of tract formation. For example, the interstitial nuclei or the precursor of MLF is one of the earliest neurons to develop in gnathostomes (Windle and Austin, 1936; Windle and Baxter, 1936; Lyser, 1966; Windle, 1970; Easter et al., 1993).

The mesencephalic trigeminal neurons also develop quite early in some gnathostome embryos (Kuratani et al., 1988; Covell and Noden, 1989; Easter et al., 1993). These neurons are believed to arise from the mesencephalic neural crest (Piatt, 1945; Covell and Noden, 1989) and resemble R8 cells in that their cell bodies remain in the neuroderm. The mesencephalon of the lamprey showed no sign of axons arising from the mid-sagittal line of the mesencephalic roof from stages 20 through 30 (until the completion of ammocoete larva), which should cover the period of neurogenesis in the neural crest. Probably, unlike gnathostomes, the lamprey mesencephalic crest has no potential for Rohon-Beard cell-like neurogenesis. This notion is consistent with the absence of the mesencephalic trigeminal nucleus in the adult lamprey (reviewed by Sarnat and Netzky, 1981). The dorsal medial neurons that have been suggested to be homologous with the gnathostome mesencephalic trigeminal nucleus (Northcutt, 1979; but see Finger and Rovainen, 1982, and Koyama et al., 1987) were not found at any developmental stages observed by the methods used in the present study.

There have been various opinions as to the common segmental plan of the vertebrate brain. The position and number of the neuromeres is still an intriguing issue, which is profoundly connected to the position of the neuraxis (or the site of sulcus limitans and/or the floor plate) and the rostral end of the neural tube (reviewed by Kimmel, 1993). Such pattern is also dependent on the neuromerical configuration of the neurectoderm. The mesencephalon of the lamprey showed rather a conserved pattern of tract formation in the developing chick hindbrain. Development 120:1347–1356.


