Segmental development of reticulospinal and branchiomotor neurons in lamprey: insights into the evolution of the vertebrate hindbrain

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Summary

During development, the vertebrate hindbrain is subdivided along its anteroposterior axis into a series of segmental bulges called rhombomeres. These segments in turn generate a repeated pattern of rhombomere-specific neurons, including reticulospinal and branchiomotor neurons. In amphioxus (Cephalochordata), the sister group of the vertebrates, a bona fide segmented hindbrain is lacking, although the embryonic brain vesicle shows molecular anteroposterior regionalization. Therefore, evaluation of the segmental patterning of the central nervous system of agnathan embryos is relevant to our understanding of the origin of the developmental plan of the vertebrate hindbrain. To investigate the neuronal organization of the hindbrain of the Japanese lamprey, *Lethenteron japonicum*, we retrogradely labeled the reticulospinal and branchial motoneurons. By combining this analysis with a study of the expression patterns of genes identifying specific rhombomeric territories such as *LjKrox20*, *LjPax6*, *LjEphC* and *LjHox3*, we found that the reticular neurons in the lamprey hindbrain, including isthmic, bulbar and Mauthner cells, develop in conserved rhombomere-specific positions, similar to those in the zebrafish. By contrast, lamprey trigeminal and facial motor nuclei are not in register with rhombomere boundaries, unlike those of gnathostomes. The trigeminal-facial boundary corresponds to the rostral border of *LjHox3* expression in the middle of rhombomere 4. Exogenous application of retinoic acid (RA) induced a rostral shift of both the *LjHox3* expression domain and branchiomotor nuclei with no obvious repatterning of rhombomeric segmentation and reticular neurons. Therefore, whereas subtype variations of motoneuron identity along the anteroposterior axis may rely on Hox-dependent positional values, as in gnathostomes, such variations in the lamprey are not constrained by hindbrain segmentation. We hypothesize that the registering of hindbrain segmentation and neuronal patterning may have been acquired through successive and independent stepwise patterning changes during evolution.

Key words: Evolution, Hindbrain, Rhombomere, Reticulospinal neuron, Branchiomotor neuron, Lamprey, Hox genes

Introduction

The vertebrate developmental plan is characterized by segmental organization along the anteroposterior axis, which underlies the serial homology of structures in the vertebrate body. Furthermore, a series of cell lineage-restricted bulges, called neuromeres, appear along the anteroposterior axis of the neural tube during development (von Baer, 1828; Bergquist and Källén, 1953; Vaage, 1969; Fraser et al., 1990; Figdor and Stern, 1993; Puelles and Rubenstein, 1993). These neuromeres serve as centers for cell proliferation, migration and neuronal differentiation. The neuromeres in the hindbrain, or rhombomeres (r) (Orr, 1887; Vaage, 1969), have attracted the attention of developmental biologists for their topographical relationships with peripheral nerves and segmental boundary-related gene expression patterns.

Rhombomeres appear as repetitive units of neuronal development and as a prepattern for the spatial deployment of the neural crest-derived ectomesenchyme in the craniofacial region (Lumsden and Keynes, 1989; Clarke and Lumsden, 1993; Kuratani and Eichele, 1993; Köntges and Lumsden, 1996) (reviewed by Santagati and Rijli, 2003). The homeobox-containing transcription factors of the Hox gene family display nested, segmentally restricted, expression patterns with sharp anterior boundaries mapping to the rhombomeric borders, and are involved in position-dependent specification of the rhombomeres and pharyngeal arch derivatives (e.g. Lufkin et al., 1991; Chisaka et al., 1992; Gendron-Maguire et al., 1993; Mark et al., 1993; Carpenter et al., 1993; Rijli et al., 1993; Studer et al., 1996; Goddard et al., 1996; Bell et al., 1999; Gavalas et al., 1997; Gavalas et al., 1998; Rossel and Capecchi, 1998).
Hox code) appear to be conserved in the expression patterns (r2 and r3, and that of the facial nerve in r4 and r5. As a general rule, the trigeminal motor nucleus is generated in existence of this 2:1 rhombomere:pharyngeal arch relationship (Keynes, 1989; Noden, 1991; Gilland and Baker, 1993). The association with specific pairs of rhombomeres and innervate gnathostomes. Branchiomotor nuclei are generated in knowledge about rhombomeres is restricted to the evolutionary developmental biology. In vertebrates, our possession a region homologous to the vertebrate hindbrain, but that the segmental organization appeared after the divergence of the vertebrate lineage.

Therefore, the evolutionary origin of rhombomeres, and its relationship to a metameric developmental program of neuronal patterning, poses an intriguing question for evolutionary developmental biology. In vertebrates, our knowledge about rhombomeres is restricted to the gnathostomes. Branchiomotor nuclei are generated in association with specific pairs of rhombomeres and innervate a single branchial arch (Tello, 1923; Neal, 1896; Lumsden and Keynes, 1989; Noden, 1991; Gilland and Baker, 1993). The existence of this 2:1 rhombomere:pharyngeal arch relationship has been considered the basic developmental unit for patterning of the vertebrate head (reviewed by Kuratani, 2003). As a general rule, the trigeminal motor nucleus is generated in r2 and r3, and that of the facial nerve in r4 and r5. Hox gene expression patterns (Hox code) appear to be conserved in the gnathostome hindbrain (Hunt et al., 1991a; Hunt et al., 1991b; Lumsden and Krumlauf, 1996), with minor modifications in teleosts, which have experienced additional Hox cluster duplication (Amores et al., 1998; Naruse et al., 2000; Schilling and Knight, 2001; McClintock et al., 2002; Prince, 2002). Loss- and gain-of-function experiments indicate that Hox genes are involved in providing branchiomotor subtype positional identity along the anteroposterior axis (Studer et al., 1996; Goddard et al., 1996; Gavalas et al., 1997; Gavalas et al., 1998; Davenne et al., 1999; Jungbluth et al., 1999; Gauf et al., 2000; Dominguez del Toro et al., 2001; McClintock et al., 2002; Pattyn et al., 2003).

Unlike branchiomotor neurons, the spatial patterns of interneurons are less conserved among gnathostomes. In avians and teleosts, rhombomeres initially generate similar repeated sets of neurons belonging to distinct classes (Metcalfe et al., 1986; Mendelson, 1986a; Mendelson, 1986b; Kimmel et al., 1988; Lee et al., 1993; Hanneman et al., 1988; Kimmel, 1993; Clarke and Lumsden, 1993). Thus, each rhombomere seems to represent in its composition a serial homologue of a repeated repertoire of reticular neurons. In another divergent group, the amnieres, such segmental repetition is less obvious, and reticular neurons assume a rather columnar distribution with modulations or interruptions, so that each rhombomere is instead identified in terms of the particular reticular neuron class it produces (e.g. reticulospinal or vestibulospinal) (Auclair et al., 1999).

These observations raise the question of whether hindbrain segmentation and metameric neuronal patterning are intimately linked or independently established. To address this question, the developmental patterns of regulatory genes and neuronal specification, as well as neuroepithelial compartmentalization, should be systematically compared among the phylogenetic tree of the chordates. In particular, the lamprey, an agnathan (jawless) vertebrate, may provide useful insights to partly fill the gaps in our knowledge, because it appears to have diverged early in the vertebrate lineage.

The extant agnathan animals, including the lamprey and hagfish, are thought to form a monophyletic group (Mallatt and Sullivan, 1998; Kuraku et al., 1999; Kuratani et al., 2003) as a sister group of the gnathostomes. Segmentation in the hindbrain has been identified in agnathan embryos (Kuratani et al., 1998; Horigome et al., 1999; Pombal et al., 2001; Kuratani et al., 2001). In a series of detailed embryological observations of a Japanese lamprey, Lethenteron japonicum, conserved topographical relationships between rhombomeres, cephalic crest cell populations and cranial nerve roots were identified (Kuratani et al., 1997; Horigome et al., 1999). The lamprey also has reticulospinal neurons, and their activity appears to be involved in swimming behavior (Nieuwenhuys and Nicholson, 1998). The neuronal patterning sequence of this animal, however, has not yet been elucidated. In this study, we analyzed the patterns of motor and reticular neuron development and of regulatory gene expression in relation to the rhombomere pattern in the lamprey hindbrain. Our data suggest that registering of rhombomeric segmentation, neuronal patterning, and the Hox code may have occurred through successive independent evolutionary changes in the vertebrate lineage.

**Materials and methods**

**Embryos**

Mature male and female lampreys, Lethenteron japonicum, were collected in a tributary of the Miomote River, Niigata, Japan, during the breeding season (early June). The eggs were artificially fertilized and maintained in 10% Steinberg solution (Steinberg, 1957) at 20°C. Embryonic stages were assessed morphologically according to the sequence established by Tahara (Tahara, 1988) for L. reissneri, a brook lamprey species closely related to L. japonicum.

**Retrograde labeling of hindbrain neurons**

Rhodamine- or fluorescein-conjugated dextrans (Sigma, St Louis, MO) were injected into the spinal cord or pharyngeal arches of the embryo to label reticulospinal or branchial motoneurons, according to the method described by Glover (Glover, 1995). The injected embryos were incubated at room temperature for 30 minutes to allow the dextran to label neurons retrogradely. Embryos were then washed with 10% Steinberg solution, and fixed in 4% paraformaldehyde and 1% methanol in 0.1 M phosphate-buffered saline (PFAM/PBS). The fixed specimens were dehydrated, and clarified with a 1:2 mixture of benzyl alcohol and benzyl benzoate (BABB). Labeled neurons were then examined using a fluorescence microscope.

**Isolation of cDNA clones of lamprey genes**

A partial cDNA clone of the lamprey Krox20 gene was isolated from a L. japonicum stage 24-26 embryo cDNA library, using a previously described low stringency hybridization protocol (Pasqualetti et al., 2000). We used a probe derived from a mouse Krox20 cDNA polymerase-chain-reaction (PCR) product amplified with the specific mouse primers: 5’-ATCCGTAATTTACACTTGGGGGG-3’ (sense) and 5’-CGATGCTGCAAGCACTCGCA-3’ (antisense) respectively.
Development of reticulospinal neurons in the lamprey. (A-D) Dextran labeling of the reticular neurons. (A) Stage 23.5 embryo. Isthmic (I) and medial inferior reticulospinal (mir) neurons are labeled, whereas bulbar (B) and Mauthner (Mth) neurons are not yet detected. (B) Stage 24.5 embryo. Mth neuron and the B neuron cluster are observed at the level of the otic vesicle (OV). (C) The same embryo as in B, focused at a more dorsal hindbrain level. Crossing axons of Mth neurons are visible (arrow). (D) Stage 28 larva. I and B neurons have increased in number, and I1 neuron has appeared. Most of the reticulospinal components of the lamprey are formed at this stage. Anterior is towards the top.

Fig. 1.

Neuronal labeling in combination with in situ hybridization

After neuronal labeling, whole-mount in situ hybridizations were performed as previously described (Murakami et al., 2001). Stained embryos were fixed in PFAM/PBS, and incubated with streptavidin-horseradish peroxidase (HRP, Vector) in Tris-buffered saline (TST; diluted 1:500) at 4°C overnight. The specimens were washed five times for 60 minutes each in TST at room temperature, then HRP activity was detected using the peroxidase substrate, 3,3′-diaminobenzidine (DAB, 100 mg/ml), in TST with 0.01% hydrogen peroxide.

Retinoic acid treatment of lamprey embryos

Retinoic acid (RA) treatment was performed according to the protocol described previously (Kuratani et al., 1998b). In the present study, embryos from stages 12 through 18 were treated with 0.01 μM, 0.05 μM, or 0.1 μM all-trans RA. As a negative control, only the dimethyl sulfoxide (DMSO) was applied.

Results

Developmental sequence of lamprey reticulospinal neurons

To analyze the temporal sequence of reticular neuron development in the lamprey, rhodamine-conjugated dextrans were applied to embryonic and larval spinal cords. In stage 23.5 embryos, medial inferior group (mir) neurons appeared (Fig. 1A). By stage 24.5 embryos, four of the bulbar (B) neurons as well as the Mauthner (Mth) neuron were labeled at the level adjacent to the otic vesicle (Fig. 1B). At this stage, axons of the Mth neurons crossed the midline of the hindbrain and descended the neural tube to form the lateral longitudinal fasciculus (Fig. 1C). Such axonal growth pattern coincides with the adult anatomy of this animal group (reviewed by Nieuwenhuys and Nicholson, 1998).

The lamprey Hox3 fragment was PCR amplified from cDNA of L. japonicum using a set of degenerate primers directed against two highly conserved regions of the EphA4 molecule. A sense primer (5′-ATGATGATACGGAAGGTAGGGAAGA-3′) and an antisense primer (5′-CTTTCCAGGTCGAGTCAGAGGTT-3′) were used. We isolated several clones, and these sequences were significantly homologous to lamprey EphC, which had been cloned previously from the lamprey species L. reissneri (Suga et al., 1999). We therefore named our Eph clones LjEphC (Lethenteron japonicum EphC).

The lamprey Hox3 fragment was PCR amplified from cDNA of L. japonicum using a set of degenerate primers directed against two highly conserved regions of the Hox3 molecule: a sense primer (5′-CGGAATTCGCGGCCGC-3′) and an antisense primer (5′-CACGCTACCCGAGATTTTCT-3′). Underlines indicate restriction enzyme sites. This short Hox3 fragment allowed the design of 3′-RACE primers, as well as appropriate nested primers for marathon cDNA amplification (Marathon™ cDNA Amplification Kit, Clontech, Palo Alto, CA). The primers were: 3′-RACE primer, CCTCTGCCGCGGCTCAGCAGGT, and 3′-RACE nested primer, AATGGCCAACCTACTTAAACCTC. The PCR led to the isolation of a lamprey Hox3 fragment. This fragment was used as the probe to screen a lamprey cDNA library. Screening was performed under low-stringency conditions and isolated clones were sequenced. The deduced protein sequence was significantly homologous to Hox3, therefore we named our Hox clone LjHox3 (L. japonicum Hox3). The partial sequences have been assigned the DDBJ/EMBL/GenBank Accession Number AB125270.
adult animal could be identified (Fig. 1D) (Jacobs et al., 1996).

**Rhombomere-specific distribution of lamprey reticulospinal neurons**

To determine the anteroposterior level of each neuron in the embryonic hindbrain, we visualized the expression patterns of lamprey *Pax6* (LjPax6), *Krox20* (LjKrox20), *EphC* (LjEphC) and *LjHox3* transcripts as positional markers by whole-mount in situ hybridization. To visualize developing neurons, we analyzed the expression pattern of the neurofilament subunit gene obtained from the lamprey EST clone (DDBJ/EMBL/GenBank Accession Number, AB107053). Alternatively, we labeled the reticulospinal tracts with biotin-conjugated dextran and stained them with streptavidin-HRP after detection of the gene expression patterns.

The expression domain of *LjKrox20* corresponded to r3 and r5, as indicated by the position of the labeled domains relative to the morphological rhombomere boundaries and the trigeminal and facial nerve roots, which were stained with anti-acetylated tubulin antibody (Fig. 2A,B) (see also Kuratani et al., 1998; Horigome et al., 1999). The *LjEphC* domain also seemed to correspond to r3 and r5, because of the relative position of the otic vesicle, which is lateral to r4 in the lamprey (Kuratani et al., 1998; Horigome et al., 1999) (Fig. 4A,B). Based on this mapping, the cluster of B cells and the Mth neuron could be positioned in r4 (Fig. 3A,B, Fig. 4C-F). The Mth neuron appeared in the middle of r4 at stage 26 and corresponded to the domain of lowest *LjPax6* expression and to the rostral limit of *LjHox3* expression (Fig. 2D, Fig. 3C,D) (Murakami et al., 2001). Reticulospinal neurons, including I and B cells, were found lateral to the *LjPax6*-expressing domain along the anteroposterior neuraxis, as seen from the dorsal view of the same stage embryo (Fig. 2C). The auxiliary Mauthner neuron (Mth') developed in r5 (Fig. 3A,B). The I4 neuron was located in r3 (Fig. 3A,B), whereas the I3 neuron was positioned anterior to the r2/3 boundary (Fig. 3B). The topographical relationships between neuron position and gene expression described above did not change throughout the later stages analyzed.

**Fig. 2. Identification of rhombomeres in the lamprey.** (A) *LjKrox20* expression (blue) in relation to the localization of acetylated tubulin (brown) in a stage 25 embryo (anterior is towards the left). (B) Cranial nerve roots (arrowheads) and rhombomere boundaries (arrows) are visualized with the anti-acetylated tubulin antibody. Note that *LjKrox20* expression domain corresponds well to the morphological rhombomere boundaries (compare arrows in A and B). (C,D) *LjPax6* expression (blue) and reticulospinal neuron (brown) localization. (C) Dorsal view of a stage 26 embryo. Reticular neurons including I, B and mir have developed lateral to the *LjPax6* expression domain at the hindbrain midline. (D) Lateral view of the same embryo. Mth is located in r4, where *LjPax6* is expressed at low levels. B, bulbar neurons; I, isthmic reticular neurons; MHB, mid-hindbrain boundary; mir, medial inferior reticulospinal neurons; Mth, Mauthner neuron; Mth’, auxiliary Mauthner neuron; V1, profundus ganglion; V2/3, trigeminal ganglion; VII, facial ganglion

**Trigeminal and facial branchiomotor nuclei are not in register with rhombomere boundaries**

To simultaneously identify the relative positions of the reticular and branchiomotor neurons, we injected rhodamine-conjugated dextrans into the spinal cord and fluorescein-conjugated dextrans into branchial arches or vice versa. Branchial motor nuclei could be labeled after stage 26, and both trigeminal and facial nuclei were located adjacent to the Mth neuron. The posterior limit of the trigeminal motor nucleus was positioned just rostral to the Mth neuron, and the anterior limit of the facial motor nucleus just caudal to the Mth neuron (Fig. 5A-C). Thus, the trigeminal-facial nuclei transition was found at the mid-r4 level (Fig. 5A,B). Even at stage 30, this topography had not changed (Fig. 5D). The glossopharyngeal and vagus nuclei had also developed by this stage (Fig. 5E). Of these, the glossopharyngeal nucleus was detected caudal to putative r5, indicating that this nucleus was located in r6 (Fig. 6C).
To further determine the positions of motoneurons, the trigeminal and facial motor roots were labeled with biotin-conjugated dextran and visualized by streptavidin-HRP. Comparison with the \textit{LjKrox20} expression domain confirmed that the trigeminal motor nucleus extended caudally into \textit{r4} (Fig. 6A,B). Moreover, the anterior boundary of the facial motor nucleus was also located in \textit{r4} (Fig. 5B, Fig. 6A,B), showing that this motor nucleus developed in both \textit{r4} and \textit{r5}. Thus, the boundary between the trigeminal and facial nuclei is not in register with a specific rhombomere border, as is the case in all the gnathostomes studied so far (Fig. 6A,B; see Fig. 8) (Lumsden and Keynes, 1989; Gilland and Baker, 1993). Interestingly, the border between the trigeminal and facial nuclei corresponded to the anterior limit of \textit{LjHox3} expression (Fig. 6D), with the trigeminal motor nucleus positioned just rostral to this limit (Fig. 6D).

\textbf{Branchiomotor but not reticular neuron identities are altered by retinoic acid}

The observations described above and the comparison with gnathostome patterns suggest that, in the vertebrate lineage, the relative positions of the reticular and branchiomotor neurons may be established independently and uncoupled from segmentation. To further test this idea, we applied 0.01 \textmu M, 0.05 \textmu M or 0.1 \textmu M all-trans RA to the lamprey embryo from stages 12-18. In gnathostomes, the application of RA shifts the Hox code, transforms rhombomere identity, and causes both branchiomotor subtype changes and Mth neuron duplications (Kessel, 1992; Lopez et al., 1993).
Interestingly, we could not detect ectopic Mth neurons in r2 at any RA concentration, although this has been observed in some gnathostomes (Fig. 7A-C; n=60). Moreover, the position of the Mth neuron did not change in relation to the LjKrox20 expression domains (Fig. 7D-F). However, the application of 0.1 μM RA resulted in an obvious anterior shift of the trigeminal motor nucleus beyond the mid-hindbrain boundary (compare Fig. 7J,N), as visualized by the expression of Ljfgf8/17 (Fig. 7LJ). Furthermore, the boundaries of the branchiomotor nuclei became undetectable (compare Fig. 7K with L). This phenotype was paralleled by the rostral expansion of the LjHox3 expression limit, as assessed by its position relative to the Mth neuron (Fig. 7G,H). Thus, unlike in gnathostomes, RA signaling did not appear to alter the developmental pattern of the lamprey reticulospinal neurons. By contrast, it did affect the Hox code and branchial motoneurons in a coordinated manner, as seen in gnathostome embryos.

Discussion

In this study, we used molecular markers and retrograde labeling techniques to investigate the development of reticulospinal and branchiomotor neurons, and their projections in the hindbrain of the Japanese lamprey, L. japonicum. We mapped the relative positions of these neuronal groups in the context of the segmental organization of rhombomeres. Lamprey reticulospinal and branchiomotor neurons were sequentially positioned along the anteroposterior axis of the hindbrain, and displayed fixed relationships to each other and to gene expression domains throughout embryonic and early larval development. When we compared these relationships with the gnathostome pattern, we found evolutionary positional shifts between branchiomotor neurons and reticular neurons that were not in register with rhombomere segmentation. Moreover, the exogenous application of RA induced selective shifts in branchiomotor, but not in reticular, neuron positions along the anteroposterior axis. As amphioxus lacks clear segmentation of the brain vesicle, our data suggest that the lamprey represents an ancestral developmental patterning program of the vertebrate hindbrain and that registering of hindbrain segmentation and neuronal patterning may have been acquired through successive and independent stepwise patterning changes during evolution.

Developmental marker genes and rhombomere segmental identities in lamprey

A number of genes have been implicated in the control of hindbrain segmentation and of odd-even rhombomere segregation in gnathostomes, including the kleiser/lvalentino gene (Frohman et al., 1993; Moens et al., 1996; Moens et al., 1998), members of the ephrin/leph gene family (Xu et al., 1995;
and LjKrox20 markers to identify specific rhombomeres. rhombomere borders, and anterior limits of Hox expression domains precisely map to et al., 1998; Schilling and Knight, 2001). In gnathostomes, the (Hunt et al., 1991a; Hunt et al., 1991b; Krumlauf, 1993; Rijli et al., 1996) expression patterns are generally regarded as markers for r3 and r5 EphA4 (Schneider-Maunoury et al., 1997). Of these, LjKrox20, LjEphC, and LjHox3 (D) expression domains (blue). (A) Lateral view of a stage 26 larva. (B) Dorsal view of the same larva. Note that the trigeminal (Vm) nucleus expands posteriorly into the LjKrox20-negative region, i.e. the presumptive r4 (white arrows). The facial (VIIm) nucleus also partially maps in the presumptive r4 domain (black arrow). (C) The glossohypoglossal motor (IXm) nucleus is just posterior to the r5 domain of LjKrox20. (D) The rostral boundary of LjHox3 expression maps between the Vm and VIIm nuclei. The inset clearly shows that the trigeminal motor nucleus is located just rostral to the LjHox3 expression domain.

Fig. 6. Comparison of branchial motor neurons and regulatory gene expression domains. Branchial motor neurons (brown) are labeled in combination with LjKrox20 (A-C) or LjHox3 (D) expression domains (blue). (A) Lateral view of a stage 26 larva. (B) Dorsal view of the same larva. Note that the trigeminal (Vm) nucleus expands posteriorly into the LjKrox20-negative region, i.e. the presumptive r4 (white arrows). The facial (VIIm) nucleus also partially maps in the presumptive r4 domain (black arrow). (C) The glossohypoglossal motor (IXm) nucleus is just posterior to the r5 domain of LjKrox20. (D) The rostral boundary of LjHox3 expression maps between the Vm and VIIm nuclei. The inset clearly shows that the trigeminal motor nucleus is located just rostral to the LjHox3 expression domain.

Wilkinson, 2001) and KroX20 (Wilkinson et al., 1989; Schneider-Maunoury et al., 1997). Of these, KroX20 and EphA4 are generally regarded as markers for r3 and r5 segmentation in gnathostomes. The specification of rhombomere segmental identities and of neurons also depends on the highly organized expression patterns of the Hox genes (Hunt et al., 1991a; Hunt et al., 1991b; Krumlauf, 1993; Rijli et al., 1998; Schilling and Knight, 2001). In gnathostomes, the anterior limits of Hox expression domains precisely map to rhombomere borders, and Hox patterns are generally used as markers to identify specific rhombomeres.

In lamprey embryos, the expression patterns of both LjKrox20 and LjEphC and their localization to r3 and r5 appear to be conserved relative to their gnathostome cognates (Figs 2, 3). By contrast, the rostral boundary of the expression of LjHox3 is found between the rostral and caudal expression domains of both LjKrox20 and LjEphC (Figs 3, 4). Therefore, even though the precise location of the LjHox3 rostral limit could not be defined in the present study, it definitely mapped within r4 and did not correspond to a specific rhombomere boundary (Figs 3, 4). This pattern is apparently not conserved, as the main transcripts of gnathostome Hox paralog group 3 genes (e.g. Hoxa3 and Hoxb3) have rostral expression boundaries that map precisely to the r4/r5 border (reviewed by Carroll et al., 2001). However, in the larval zebrafish, the Hoxb3 homologue is expressed in r4 (Prince et al., 1998). Moreover, at least three types of Hoxb3 endogenous transcripts were described in the mouse, differing in size, splicing patterns, and expression domains (Sham et al., 1992). One such transcript displays a rostral expression domain extending into r4 and even into r3 segments, which is rather a feature of the expression pattern of the adjacent Hoxb2 than of Hoxb3. Thus, transcriptional control mechanisms similar to those in gnathostomes may apply to the regulation of LjHox3 expression in the lamprey hindbrain.

Evolution of the vertebrate reticular neurons

One important question in evolutionary biology concerns the identification of serial homology, because it implies the presence of metameric developmental mechanisms (reviewed by Kuratani, 2003). The present analysis allowed us to gain insights into the evolution and homology of reticular neurons in the vertebrate lineage. Reticulospinal neurons are arranged corresponding to rhombomeres in several vertebrate species, including teleosts (Metcalfe et al., 1986; Lee et al., 1993; Hanneman et al., 1998) and the rat (Auclair et al., 1999). In particular, in the aquatic vertebrates studied so far, Mth neurons always develop in r4 (Metcalfe et al., 1986; Lee et al., 1993). Based on the r3 and r5 expression of LjKrox20 and LjEphC (see above), the lamprey Mth neuron was also localized in r4, suggesting that the developmental program to generate the Mth neuron in r4 has been conserved through vertebrate evolution.

In teleosts, developing reticulospinal neurons have been classified into several families based on shared morphological features, and it has been suggested that each rhombomere develops basically the same set of neurons as the others. Serial homologies have been recognized in the Mauthner, MiD2 and MiD3 neurons of zebrafish. The axons of these neurons cross the midline and descend the spinal cord along the medial longitudinal fascicle (MLF, Fig. 8) (Metcalfe et al., 1986). In the lamprey, in addition to the Mth neuron in r4, a similar neuron called the Mth’ neuron develops in r5. The Mth’ neuron in the lamprey is similar to the zebrafish MiD2, as discussed by Kimmel et al. (Kimmel et al., 1982). In the adult animal, the axon of this neuron crosses the midline and descends to the spinal cord, like the Mth neuron (Swaim et al., 1993). Thus, the Mth and Mth’ neurons possibly represent serial homologues.

As for the isthmic reticular group, the I4 neuron is located in r3, and the I3 neuron in r2, as assessed from the LjKrox20 and LjEphC expression domains. These two neurons are of similar size, both grow axons along similar pathways, and occupy the same relative dorsoventral position within the neural tube (Jacobs et al., 1996) (Fig. 8). These neurons thus appear to represent another serial homology group. With respect to its
position and axon projection pattern, the I4 neuron in the lamprey also seems to be homologous to RoM3 of the zebrafish, and I3 to RoM2 (Figs 7, 8) (Metcalfe et al., 1986).

From the above discussion, this rhombomere-related serial homology of selected neuronal subtypes (e.g. the Mauthner neuron) appears to be a shared feature in the vertebrate lineage. This supports the idea that a metameric pattern of neuronal development was already present in the 'hindbrain region' of the vertebrate common ancestor. However, some cell types appear to be present only in the lamprey, as seen in the bulbar neurons with ipsilateral axons (Jacobs et al., 1996) (Fig. 8), which are restricted to r4, whereas in the zebrafish, two pairs of Mth homologues, MiD2 and MiD3, are located in r5 and r6, respectively (Fig. 8). Finally, whereas teleosts and lampreys have large identifiable reticular neurons, this is not the case in ammioites, whose small reticular neurons are clustered in each rhombomere. Thus, various forms of diversifications have arisen independently in each lineage of animal groups.

Based on these results, we propose an evolutionary scenario in which the vertebrate common ancestor possessed a basic metameric pattern of serially repeated sets of reticular neurons. In the lineage leading to lampreys, specific cell types, such as additional Mauthner neurons, were lost from each metamere, resulting in the anteroposteriorly differentiated neuronal pattern of the present lamprey hindbrain (Figs 8, 9). In ammioites, large identifiable neurons may have been lost secondarily as well, possibly because of the shift to the terrestrial life and related loss of a lateral line-mediated reflex system. An alternative option is that the ancestral hindbrain was already specialized anteroposteriorly as observed in the lamprey. In teleosts, each rhombomere would then have secondarily acquired a similar pattern of interneurons. The latter possibility, however, makes it difficult to explain the origin of such anteroposterior specification in the vertebrate hindbrain. Observation of hindbrain development in the hagfish might therefore provide key information to distinguish among such possibilities.

**Specification of lamprey branchiomotor neurons**

We found that, in the lamprey, the trigeminal motor nucleus...
Segmental development of the lamprey hindbrain extends posteriorly to the level of the Mth neuron, in the middle of r4 (Figs 5, 8) (Fritsch, 1998). In situ hybridization analysis using the \textit{LjKrox20} probe led to a similar conclusion (Fig. 6). Interestingly, a rostral extension of the trigeminal motor nucleus almost up to the mid-hindbrain boundary was also observed, indicating that it extends into r1. Thus, the anteroposterior extent of the lamprey trigeminal motor nucleus is unusually wide compared with that of other vertebrates. This is in keeping with the finding that the anteroposterior pattern of branchiomotor nuclei varies among vertebrate groups (see the comparison of vertebrate branchiomotor neuron patterns in Fig. 8). Nonetheless, specification of r2 and r3 as trigeminal motoneuron may be regarded as synplesiomorphic with all the vertebrate species. As noted by Song and Boord (Song and Boord, 1993), neuronal organization of the rostral hindbrain is linked to muscle innervation via the trigeminal nerves in vertebrates. This raises the possibility that the lamprey-specific distribution pattern of trigeminal motoneurons may be associated with agnathan oral movement and the specific patterning of the perioral mesenchyme (see Kuratani et al., 2001; Shigetani et al., 2002). The facial motor nucleus of the lamprey, however, is located in r4 and r5. In some gnathostomes, such as zebrafish (Chandrasekhar et al., 1997; Higashijima et al., 2000; Bingham et al., 2002) and mouse (Studer, 2001), the equivalent nucleus migrates caudally to r6 during development. In the lamprey, the facial nucleus does not show such migration (Fig. 8), indicating that its migratory properties were acquired secondarily in gnathostome evolution. It is noteworthy that the boundary...
between the trigeminal and facial nuclei did not correspond to the r3/r4 boundary, which is unlike any gnathostome analyzed so far (see Fig. 8 for a comparison).

Together, these observations suggest that variations in motoneuron identity along the anteroposterior axis of vertebrate embryos are not constrained by hindbrain segmentation. This is particularly apparent from this analysis of lamprey branchiomotor neuron development, and from accurate comparisons with the development of the branchiomotor nuclei in gnathostomes (Fig. 8). Importantly, the shift between trigeminal and facial motoneuron identities corresponded well with the anterior boundary of LjHox3 expression, as inferred from retrograde labeling of motor axons and in situ hybridization. In gnathostomes, there is mounting evidence that Hox genes control the identity of motoneurons along the anteroposterior axis. In the developing hindbrain, Hoxa and Hoxb genes are involved in the specification of cranial motor neurons, their projections, and migratory properties (Studer et al., 1996; Goddard et al., 1996; Gavalas et al., 1997; Bell et al., 1999; Davenne et al., 1999; Jungbluth et al., 1999; Gauf et al., 2000; McClintock et al., 2002; Pattyn et al., 2003; Guidato et al., 2003; Gauf et al., 2003). Interestingly, Hox genes are also involved in spinal motoneuron positional specification and innervation, despite the absence of neuromeric compartments in the developing spinal cord [Hoxa10 (Rijli et al., 1995), Hoxc genes (Tiret et al., 1998; Dasen et al., 2003), Hoxd10 (de la Cruz et al., 1999; Lance-Jones et al., 2001)]. Therefore, we speculate that the relationship between Hox gene expression domains and motoneuron identity may be an ancestral feature conserved throughout the anteroposterior axis of the central nervous

system (hindbrain and spinal cord), and which is evolutionarily as well as developmentally independent of neuromeric compartments and of the segmentation process.

Selective effect of retinoic acid on lamprey hindbrain development

It is well established that exogenous RA administration in gnathostomes causes abnormal development of the brain (Morris-Kay et al., 1991; Conlon and Rossant, 1992; Marshall et al., 1992; Durston et al., 1989; Papalopulu et al., 1991; Holder and Hill, 1991). Morphological changes correlate with shifts of Hox gene as well as of other regulatory gene expression patterns (Kessel, 1992; Lopez et al., 1995; Marshall et al., 1992; Morris-Kay et al., 1991). In the hindbrain, RA treatment results in complete changes in the segmental developmental program. For example, the r2 segmental identity is transformed into that of r4, resulting in both the duplication of the Mth neuron and the switch in fate from trigeminal to facial branchiomotor neuron (Hill et al., 1995; Marshall et al., 1992). Similar phenotypes occur with the ectopic expression of the paralogue group 1 genes, Hoxa1 or Hoxb1, in r2 (Bell et al., 1999; Alexandre et al., 1996; McClintock et al., 2001; McClintock et al., 2002), demonstrating that RA-induced repatterning is mediated through Hox gene function. Thus, in the gnathostomes, rhombomere identity, Hox codes, and neuronal patterning along the anteroposterior axis are intimately linked and appear to rely on RA-dependent developmental mechanisms.

In the lamprey, RA treatment also affects axial patterning (Kuratani et al., 1998). In this study, we showed that RA causes a rostral shift in the LjHox3 expression domain, concomitant with the rostral shift of branchiomotor neurons (Fig. 7). As in gnathostomes, the positional specification of lamprey branchiomotor neurons seems to be under the control of RA-dependent and Hox-mediated mechanisms. Interestingly,
however, lamprey reticulospinal neurons, including the Mth and bulbar cells, did not change their positions along the neuraxis following RA treatment, nor was the apparent segmental organization of the hindbrain itself altered (Fig. 7). Furthermore, we did not observe duplication of the Mth neurons (Fig. 7).

These data support the idea that neuronal patterning and segmental identity may be independently regulated in the lamprey, and that the dependence of these processes on RA signaling could be distinct in agnathans and gnathostomes.

**Evolution of the vertebrate hindbrain: a hypothetical scenario**

Based on our findings, we speculate that the gnathostome-like hindbrain organization may have developed through independent mechanisms of anteroposterior patterning during evolution (Fig. 9).

As noted above, an ancestral anteroposterior program of neuronal patterning would have already been present at the prevertebrate, amphioxus-like stage, dependent upon the position-specific expression of regulatory genes. In addition to this pattern, rhombomere-like compartments of neuroepithelial cells giving rise to repeated sets of serially homologous reticulospinal neurons were also established in the common ancestor of vertebrates. Morphological segmentation of the rhombomeres would have appeared subsequently in the vertebrate lineage, perhaps to reinforce such a metameric pattern. Partial support for this idea comes from studies of amphioxus, which also possesses interneurons similar to vertebrate reticulospinal neurons (Fritzsch and Northcutt, 1993; Fritzsch, 1996) and anteroposterior molecular regionalization of the neural tube, while lacking a bona fide segmented hindbrain.

An independent mechanism would have instead involved the anteroposterior specification of branchiomotor neurons, via a rostrocaudally regulated Hox code. Indeed, anteroposterior restriction of Hox expression has been demonstrated in amphioxus (Holland et al., 1992). Moreover, conservation of cis regulatory mechanisms that allow Hox expression in the neural tube has been demonstrated between amphioxus and vertebrates (Manzanares et al., 2000). Because the Hox code is generally in register with rhombomere boundaries in gnathostomes, the rhombomere-dependent and Hox code-dependent neural specification programs have not been distinguished from one another. Our present work, however, raises the possibility that these two distinct developmental programs were not acquired simultaneously, but may have been established as distinct evolutionary events that were secondarily integrated in the lineage of gnathostomes after the split from agnathans [Fig. 9; for the concept of integration, see Hall (Hall, 1998)]. Integration and registering of the two mechanisms in the gnathostome lineage might have relied partly on the elaboration of cis regulatory elements at Hox loci. Comparison of Hox regulatory sequences in the lamprey and gnathostomes may help to test this hypothesis further.

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