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Review

Evolution of the brain developmental plan: Insights from agnathans

Yasunori Murakami[a,*], Katsuhisa Uchida[b], Filippo M. Rijli[a], Shigeru Kuratani[b]

[a]*Institut de Génétique et de Biologie Moléculaire et Cellulaire, UMR 7104, CNRS/INSERM/ULP, Illkirch Cedex, CU de Strasbourg, France*
[b]*Evolutionary Morphology Research Team, Center for Developmental Biology (CDB), RIKEN, Kobe, Japan*

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Abstract

In vertebrate evolution, the brain exhibits both conserved and unique morphological features in each animal group. Thus, the molecular program of nervous system development is expected to have experienced various changes through evolution. In this review, we discuss recent data from the agnathan lamprey (jawless vertebrate) together with available information from amphioxus and speculate the sequence of changes during chordate evolution that have been brought into the brain developmental plan to yield the current variety of the gnathostome (jawed vertebrate) brains.

Keywords: Brain; Evolution; Vertebrates; Hindbrain; Cerebellum; Telencephalon; Amphioxus; Lamprey; Regulatory genes; *Hox* genes

Evolutionary origin of the vertebrate brain

It is still unclear which of tunicates and amphioxus are more closely related to the vertebrates (Kuraku et al., 1999; Kuratani et al., 2003; Mallatt and Sullivan, 1998). Although the basic partition of the vertebrate neural tube resembles more that of tunicates (Wada and Satoh, 2001), this review will assume cephalochordates as the closest animal group of vertebrates based on recent molecular phylogenetic analyses (Kuraku et al., 1999; Mallatt and Sullivan, 1998). This hypothesis has also been adopted in the comparison of vertebrate and amphioxus brains (Lacalli, 2001).

In amphioxus, the central nervous system is a simple tube that lacks overt partition into fore- or midbrain as seen in vertebrates. Fritsch, however, identified several regions in the amphioxus neural tube equivalent to specific anatomical domains in vertebrate brains (Fritzsch, 1996). For instance, the vertebrate ventral diencephalon generates the hypothalamus which functions as a major endocrine center in cooperation with the hypophysis, the anterior part of the pituitary gland, located just ventral to the hypothalamus (Fig. 1). In the amphioxus brain, the presence of a hypothalamus-like structure has been reported associated with the ventrally located Hatschek's pit, the hypothetical hypophysial homologue (Fig. 1) (Gorbman et al., 1999; Uchida et al., 2003). It is thus conceivable that a hypothalamus-like structure originally involved in endocrine functions may have already been present before the establishment of vertebrates (Nieuwenhuys, 1998). The analysis of expression patterns of molecular markers may provide further support to this idea. In fact, some transcription factor-encoding genes are expressed in the embryonic amphioxus brain, with patterns partly comparable to those in vertebrates (Fig. 1) (Mazet and Shimeld, 2002; Wada and Satoh, 2001). For example, the expression domain of *Nkx2.1* in the amphioxus is restricted to the ventral part of the rostralmost portion of the neural tube (Fig. 1) (Ogasawara, 2000; Venkatesh et al., 1999). Similarly, in vertebrates *Nkx2.1* is specifically expressed and functionally required in the hypothalamus (Fig. 1) (Kimura et al., 1996; Lazzaro et al., 1991). In other brain domains as well, equivalent neuronal elements have been identified between amphioxus and vertebrates, including reticulospinal and motor neurons (Fig. 1) (Fritzsch, 1996;

* Corresponding author. Fax: +33 3 8865 3201.
E-mail address: yasu@igbmc.u-strasbg.fr (Y. Murakami).

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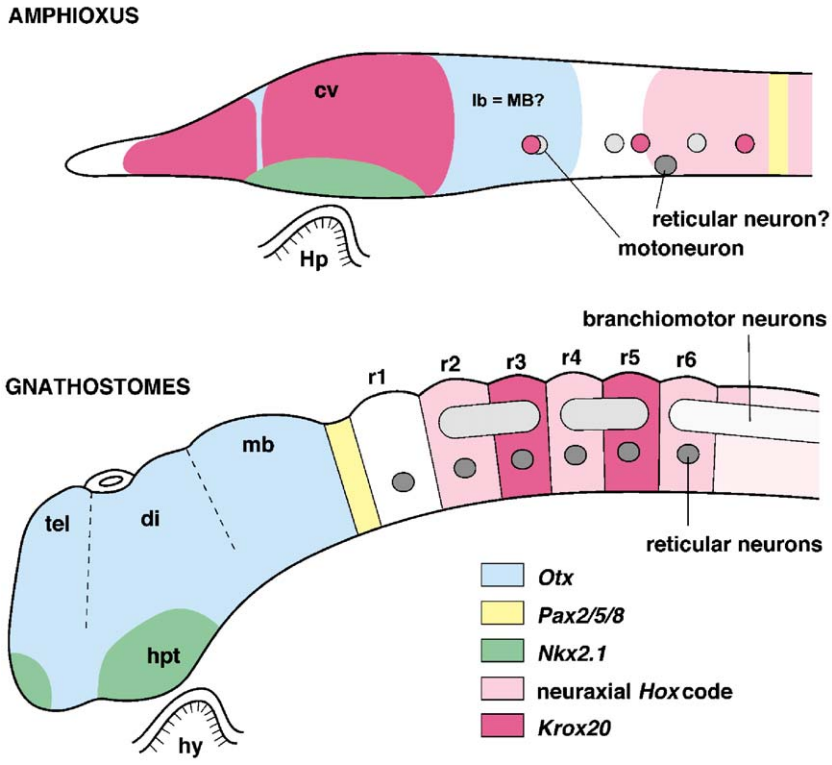

Fig. 1. Comparison of amphioxus and gnathostome brains. The amphioxus brain (top: compiled from Jackman and Kimmel, 2002; Lacalli, 2001; Venkatesh et al., 1999; Wada and Satoh, 2001) is basically a simple neural tube with no overt segmental compartments, whereas the gnathostome brain (bottom: compiled from Hauptmann and Gerster, 2000; McClintock et al., 2002; Rohr et al., 2001) is divided into several domains such as telencephalon, diencephalons, mesencephalon, and rhombencephalon. The rhombencephalon consists of a series of bulges called rhombomeres. In both the brain primordia, homologous set of regulatory genes are developmentally regulated in comparable and non-comparable regions. As examples of the comparable expression domains, *Otx* genes are upregulated in the rostralmost part, *Nkx2.1* domain restricted in the rostroventral part, and *Hox* genes expressed in the caudal region of the brain as well as in the rest of the neural tubes. No clear topographical relationships are found between positions of neuronal types and gene expressions between these animals, and no gnathostome-like rhombomeres are recognized in amphioxus neural tube. Anatomically comparable regions have been identified in both the brains, and the position of the hypophysial homologues (Hatschek's pit in amphioxus, hypophysis in gnathostomes) indicates the diencephalic domain of both the brains. Abbreviations: cv; cerebral vesicle, lb; lamellar body, mb; mesencephalon, Hp; Hatschek's pit, tel; telencephalon, di; diencephalons, r1–6; rhombomeres, hpt; hypothalamus, hy; hypophysis.

Fritzsch and Northcutt, 1993; Knight et al., 2000; Lacalli, 2001).

Neuromeres, regulatory genes, and evolution of brain developmental plan

'Neuromeres' refer to a series of embryonic segmental units, or compartments, from which differentiate the different parts of the vertebrate brain. Since the discovery by von Baer in 1828 (von Baer, 1828), neuromeres have been identified in several species of vertebrate embryos, and they are now regarded to reflect the basic developmental plan of the vertebrate brain (Bergquist and Källén, 1953; Figdor and Stern, 1993; Vaage, 1969). In the forebrain, midbrain, and hindbrain, the segmental unit is called prosomere, mesomere, and rhombomere, respectively (Puelles and Rubenstein, 1993).

In vertebrates, the rhombomeres have been intensively studied. Each rhombomere can be identified by specific sets of branchiomotor and reticulospinal neurons (Fig. 1) (Gilland and Baker, 1993; Lumsden and Keynes, 1989; Metcalfe et al., 1986; Neal, 1896; Noden, 1991; Tello, 1923) and develops as a compartment with boundaries that inhibit movement of cells into adjacent segments (Fraser et al., 1990). The establishment of rhombomeric boundaries appears to depend on cell surface molecules including the ephrin/eph families (Flanagan and Vanderhaeghen, 1998; Klein, 2004; Wilkinson, 2001), and rhombomeric identities are specified by a number of transcription factor-encoding genes such as *Hox* genes and *Krox20* (e.g. Barrow et al., 2000; Bell et al., 1999; Carpenter et al., 1993; Davenne et al., 1999; Gavalas et al., 1997, 1998; Goddard et al., 1996; Hunt et al., 1991; Lumsden and Krumlauf, 1996; Mark et al., 1993; McClintock et al., 2002; Rossel and Capecchi, 1999; Schilling and Knight, 2001; Schneider-Maunoury et al., 1993, 1997; Studer et al., 1996). For instance, the expression of *Krox20* is invariantly associated to rhombomeres (r) 3 and 5. Moreover, in these segments, *Krox20* is a direct regulator of *Hox* genes (Maconochie et al., 2001;

Manzanares et al., 2002; Nonchev et al., 1996) which, in turn, control segmental patterning.

As noted above, the amphioxus neural tube appears to contain domains that could reflect homology to the vertebrate brain. Recent studies indicate that amphioxus embryos may possess a vertebrate hindbrain-like region posterior to the cerebral vesicle, as supported by the expression patterns of several regulatory genes including the *Hox* genes (Holland et al., 1992; Jackman and Kimmel, 2002; Knight et al., 2000; Wada et al., 1999). In addition, the amphioxus *islet* gene displays iterated expression, as in vertebrates (Jackman et al, 2000). However, unlike in vertebrates, *AmphiKrox20* expression is not present in a striped pattern in the hindbrain-like region (Knight et al., 2000), although it partially overlaps with *AmphiHox* gene cognate expression domains at the level of three bilateral pairs of expressing cells (Jackman and Kimmel, 2002) (Fig. 1). Moreover, truly segmented domains have not been recognized in this animal (Lacalli et al., 1994; Wada and Satoh, 2001).

In the lineage of vertebrates, agnathans are the earliest group that exhibits neuromeres; rhombomeres can be clearly identified in lamprey embryos and larvae (Bergquist and Källén, 1953; Horigome et al., 1999; Kuratani et al., 1998). Therefore, the developmental program to generate neuromeres may have already been pre-existing in the common ancestor of lampreys and gnathostomes. Since the earliest agnathan has been found from the Cambrian era (540 mya) (Shu et al., 1999), the origin of the vertebrate-type segmented brain appears even older. To which extent, then, are the lamprey and gnathostome neuromeres alike in terms of neuronal patterning and developmental specification? In other terms, what would have the common ancestral hindbrain looked like? As already noted, rhombomeres are specified in gnathostomes by the expression of such genes as *Krox20* and *Hox*, and similar sets of interneurons develop in each rhombomere at early stages (Clarke and Lumsden, 1993). Some reticular neurons, which extend their axons into spinal cord, are readily identifiable in invariant positions of the gnathostome hindbrain (Fig. 2) (Hanneman et al., 1998; Metcalfe et al., 1986). For example, in fish, the Mauthner neuron, which is involved in the escape response by stimulating contralateral motor activity, always develops in r4 (Fig. 2) (Metcalfe et al., 1986). Moreover, r5 and r6 develop unique reticular neurons called MiD2 and MiD3, respectively (Fig. 2) (Metcalfe et al., 1986). These neurons share a common developmental program in terms of developmental timing as well as in their axonal growth patterns (Kuratani, 2003; Metcalfe et al., 1986; Murakami et al., 2004), thus representing serial homologues arising in distinct rhombomeres. The lamprey hindbrain also appears to follow a segmental plan of neuronal patterning. In the lamprey hindbrain, gene cognates of *Krox20* and *Eph* are expressed in r3 and r5, similar to the gnathostome pattern (Fig. 2). Furthermore, lamprey reticular neurons also develop in association with rhombomeres and are involved in rythmic motor activity (Fig. 2). For example, the Mauthner neuron (Mth) develops in r4 as in gnathostomes (Fig. 2), and a neuron called Mth′ specifically arises in r5, with axonal growth pattern and morphology similar to the Mauthner neuron (Jacobs et al., 1996; Nieuwenhuys et al., 1998; Swain et al., 1993). The Mth′ neuron most likely represents a serial homologue of the Mauthner neuron (Fig. 2).

Homology of reticulospinal neurons can be extended between agnathans and gnathostomes. Based on their rhombomeric positions, developmental sequence as well as axonal growth patterns, it appears clear that Mauthner neurons are homologous between these animal groups. Moreover, lamprey I3 and I4 neurons share the same location of and are most likely homologous to the RoM2 and RoM3 neurons in zebrafish, localized in r2 and r3 respectively (Fig. 2) (Kimmel et al., 1982). Therefore, a conserved metamerical program of neuronal differentiation is present in vertebrates. It is conceivable that the program for Mauthner neuron differentiation has arisen before the split of gnathostomes and agnathans and so is the origin of a basic segmental program of neuronal development. In each lineage of the vertebrate groups, however, distinct regional specification and functional differentiation may have subsequently arisen (Figs. 2, 3B). It is the case, for instance, for the zebrafish MiD2 and MiD3 neurons that find no correspondence in lamprey. Moreover, in amniotes, developing reticular neurons are normally clustered. Anatomical studies performed in rodents and chick using retrograde labeling have not led yet to the identification of single unclustered neurons (Auclair et al., 1999; Glover, 1993). Loss of giant identifiable neurons, including the Mauthner, might be linked to the transition from aquatic to terrestrial life (Fig. 5).

Evolution of the coupling of segmentation, neuronal specification, and *Hox* expression in the hindbrain

In gnathostomes, the motor nuclei of the cranial nerves are generated in association with rhombomeres, each motor root innervating a single branchial arch (Lumsden and Keynes, 1989; Murakami et al., 2004; Neal, 1896; Tello, 1923). Similarly, lamprey cranial motor nerve roots innervate individual branchial arches. However, unlike in gnathostomes, lamprey motor nuclei, in particular trigeminal (V) and facial (VII) motor neurons, are not in register with rhombomere boundaries (Fig. 2) (Murakami et al., 2004). While in gnathostome embryos, the transition between trigeminal and facial motoneurons invariably occurs at the r3–r4 border, in lamprey, it occurs instead in the middle of r4 where it coincides with the rostral expression boundary of *LjHox3* (Fig. 2) (Fritzsch, 1998;

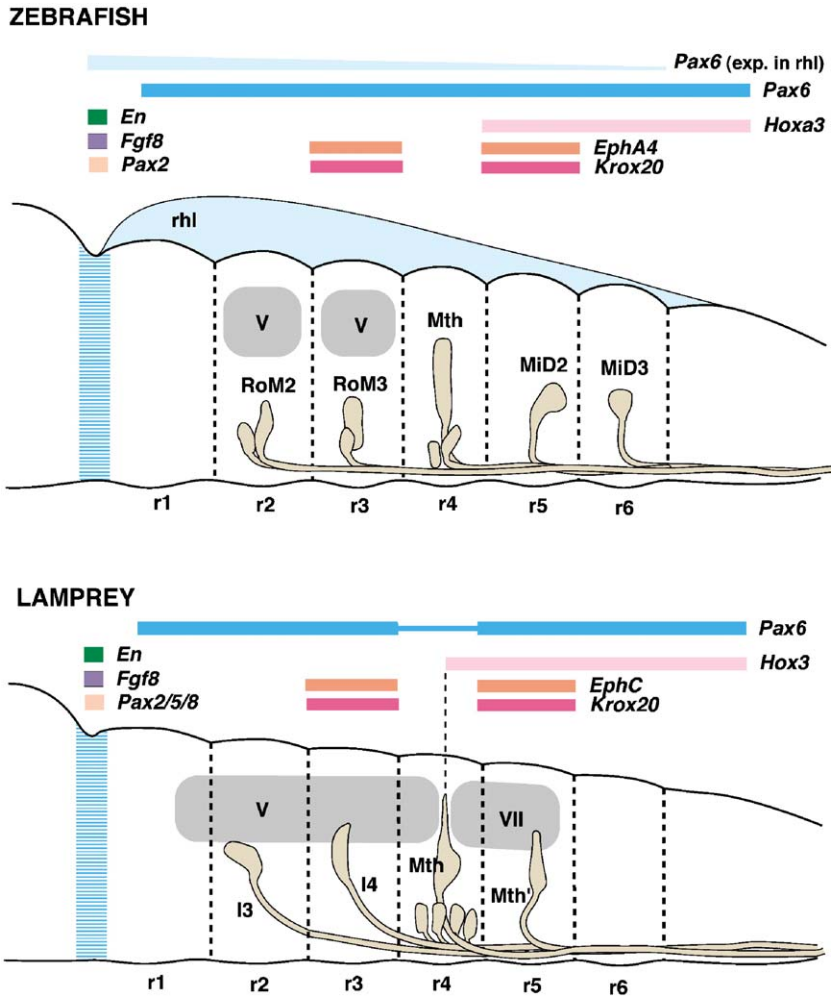

Fig. 2. Comparison of neuronal developmental patterns in the hindbrain between gnathostomes and lampreys. In the gnathostome hindbrain (zebrafish as an example: compiled from Hauptmann and Gerster, 2000; McClintock et al., 2002; Metcalfe et al., 1986; Wullimann and Rink, 2001), *En*, *Fgf8*, and *Pax2* are expressed in the MHB (shaded by blue lines), and *Pax6* in both neural tube and rhombic lip (rhl). Expression of *EphA4* and *Krox20* is restricted in r3 and r5. *Hox* gene expression is also limited rostrally by the rhombomeric boundary. Branchiomotor and reticulospinal neurons also develop in a segmental pattern in rhombomeres. Although lamprey cognates of *En*, *Fgf8*, and *Pax2/5/8* are also expressed in MHB, no *Pax6*-expression domain is seen in the rhombic lip (see Murakami et al., 2001). Consistent with the expression of lamprey *EphC* and *Krox20* genes in r3 and r5, reticulospinal neurons (I3, I4, Mth, and Mth′) develop from specific rhombomeres (Murakami et al., 2004). The developmental positions of trigeminal (V) and facial (VII) motor neurons in the lamprey do not correlate with the rhombomeric segmentation, but with *Hox3* expression boundary in the middle of r4 (Murakami et al., 2004).

Murakami et al., 2004; Takio et al., 2004). Furthermore, exogenous administration of all-*trans* retinoic acid (RA) to the lamprey embryo induced a rostral shift of *LjHox3* and posteriorization of branchiomotor neuron identity (Murakami et al., 2004), similar to gnathostomes (Fig. 3A) (Kessel, 1992; Marshall et al., 1992), albeit without rhombomere segmental changes. Thus, in lamprey, variations of *Hox*-dependent branchiomotor neuron identity along the anteroposterior axis do not appear to be constrained by hindbrain segmentation. A detailed analysis of the anterior expression borders of *Hox* genes from other paralogue groups will tell whether in lamprey the *Hox* code was not integrated in hindbrain segmentation or whether *Hox3* is an evolutionarily diversified case. Interestingly, this situation is reminiscent of the spinal cord of gnathostomes where *Hox* genes are required for motoneuron positional specification and innervation, despite the absence of neuromeric compartments (e.g. Dasen et al., 2003; Rijli et al., 1995; Tiret et al., 1998). Finally, it is noteworthy that RA-treated lamprey embryos did not show reticular neuron repatterning as well (Murakami et al., 2004), unlike in gnathostomes (Fig. 3A) (Alexandre et al., 1996; Hill et al., 1995).

The above findings imply that, in the lamprey, at least two independent programs are at work in the hindbrain. The first is involved in segment compartmentalization and segmental reticulospinal neuronal patterning, and the other in *Hox* gene-dependent branchiomotor neuron specification (Fig. 3B). In gnathostomes, in contrast, both programs have been put in register and integrated into a single one.

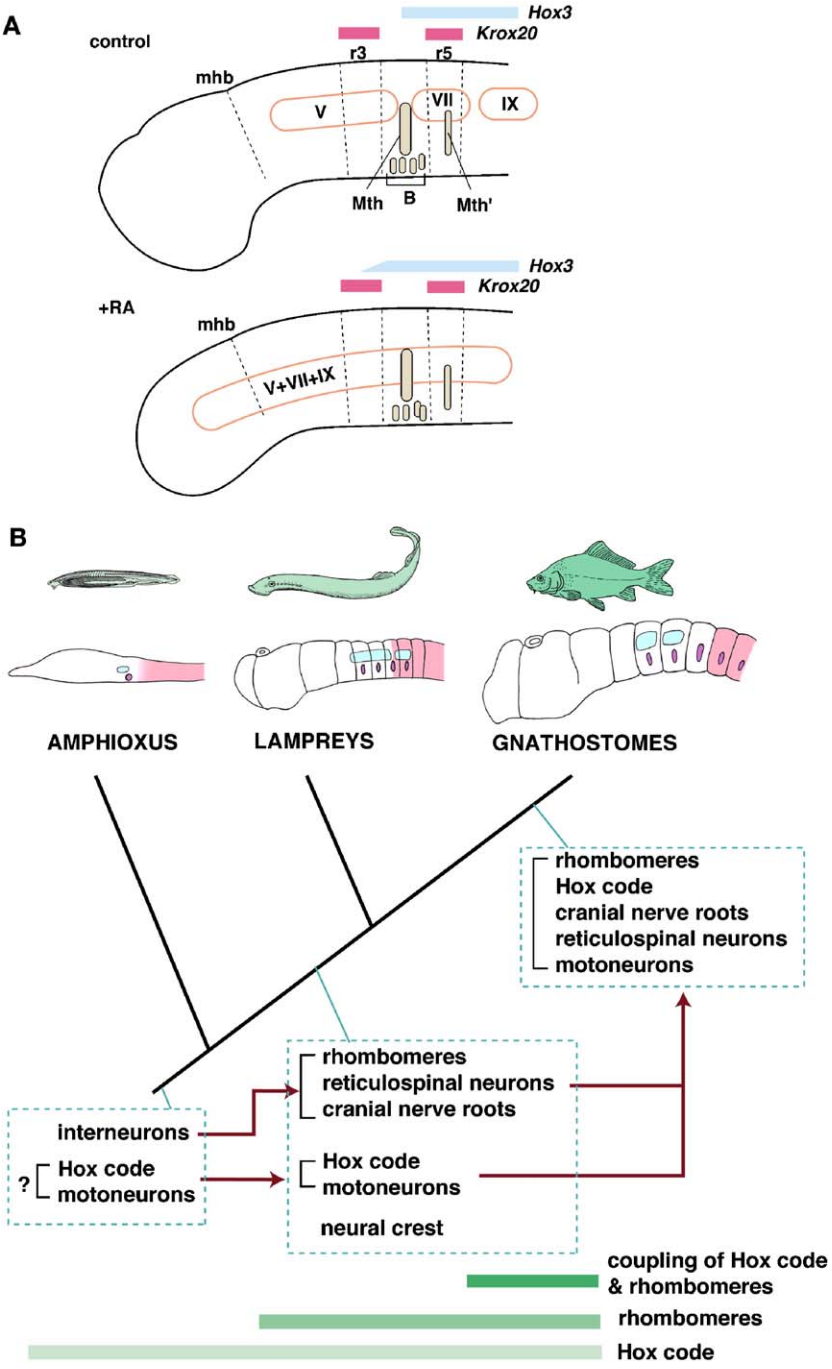

Fig. 3. Integration of developmental programs in the hindbrain evolution. (A) Comparison of the hindbrain developmental patterns between the control lamprey larva and all-*trans* retinoic acid (RA)-treated larva. With respect to the relative position of the mid-hindbrain boundary (mhb), positions of r3 and r5 as indicated by *Krox20* expression, and individual reticular neurons have not changed in the RA-treated larva. Note that only the *Hox3* expression domain and branchiomotor nuclei are shifted rostrally, and the Mth neuron never duplicates by the RA. Boundaries of motor nuclei in the RA-treated larva have become obliterated secondarily. (B) Hypothetical scenario for the hindbrain evolution. Boxes are the derived characters in the hindbrain developmental plan recognized at each segment of the evolutionary lineage. Red arrows indicate the changes of developmental programs based on the ancestral program as exaptation. Green bars indicate the sequentially introduced changes in developmental programs.

Colinear expression of *Hox* genes along the rostrocaudal axis of the neural tube is already present in the amphioxus (Holland et al., 1992; Wada et al., 1999). Thus, we speculate that *Hox* gene-dependent regional specification of motor neuron identity may be an ancestral conserved feature of vertebrates that is evolutionarily as well as developmentally independent of the segmentation process. In gnathostomes, the secondary registering of rhombomeric patterns onto the *Hox* code-regulated motoneuron patterning system required integration of ancient and newly

acquired mechanisms through stepwise patterning changes during evolution (Fig. 3B).

Evolution of the cerebellum

In many respects, the lamprey and gnathostome mesencephalon and diencephalon are comparable, including their gene expression patterns during development (Murakami et al., 2001). In gnathostome development, it is well known that the boundary between the midbrain and hindbrain (mid-hindbrain boundary: MHB) functions as an organizing center of morphogenetic patterning (Joyner et al., 2000; Martinez et al., 1991,1995; Simeone, 2000; Wurst and Bally-Cuif, 2001). Several regulatory genes are expressed in MHB, which are involved in the normal anteroposterior patterning of midbrain and cerebellum (Fig. 2) (Nakamura, 2001). For instance, FGF8 is involved in the patterning of the gnathostome cerebellum (Meyers et al., 1998), a structure mainly derived from r1 (Wingate, 2001). Lampreys also have an MHB, expressing a similar repertoire of regulatory gene cognates as in gnathostomes (Fig. 2). However, although the lamprey possesses a region comparable to the cerebellum and display expression of *LjFgf8/17* at the MHB, it does not have Purkinje cells and cerebellar nuclei, as well as components of the rhombic lip-derived cerebellar and precerebellar systems (Nieuwenhuys et al., 1998). It is noteworthy that the latter structures require specific expression of *Pax6* in the rhombic lip of the gnathostome hindbrain (Engelkamp et al., 1999). Interestingly, the lamprey rhombic lip does not express *Pax6* (Fig. 2). Thus, it is tempting to speculate that in vertebrate evolution the rostral hindbrain is incapable of differentiating into the cerebellum before the co-option of *Pax6* in that region. In other words, cerebellum has been brought about as an evolutionary innovation in gnathostomes, based on exaptation (Gould and Vrba, 1982) of MHB, rhombic lip, and some regulatory gene expression already present in the vertebrate common ancestor (Fig. 5).

Evolution of the telencephalon

Lastly, we will consider the evolution of the vertebrate telencephalon. Although the telencephalon is particularly enlarged in mammals, its relative size and shape vary in each lineage of vertebrate groups (Butler and Hodos, 1996). In teleosts, the roof plate of the telencephalon expands laterally due to a phenomenon called ‘eversion’ (Butler and Hodos, 1996; Wullimann and Mueller, 2004). The telencephalon in amniotes has developed a layered pattern, and, in mammals, the neocortex is differentiated into six layers. In archosaurians such as turtles, crocodiles, and birds, a structure called the dorsal ventricular ridge has appeared to receive inputs from the thalamus, an analogous function of mammalian cortex (Butler and Hodos, 1996; Puelles, 2001). From an evolutionary standpoint, the telencephalon is the most recent brain structure: the amphioxus does not have this structure as a morphological entity (Fritzsch, 1996). Overt telencephalon is present in the hagfish and lamprey to receive numerous input fibers from various parts of the CNS, similar to gnathostomes (Nieuwenhuys et al., 1998).

Recently, Puelles and colleagues have put forth a model for telencephalon compartments in gnathostomes (Puelles, 2001; Puelles and Rubenstein, 2003; Puelles et al., 2000; Redies and Puelles, 2001). Interestingly, their scheme is partly applicable to the lamprey brain as well, as seen in the expression domains of *Pax6* and *Emx*, suggesting that lamprey also possesses pallial structures comparable to gnathostomes (Murakami et al., 2001). These data strongly imply that the lamprey telencephalon is more complex and highly organized than was presumed. Some authors suggested that the lamprey telencephalon even possesses a region comparable to the gnathostome limbic system (Northcutt and Wicht, 1997). As far as the relative size of *Pax6/Emx* coexpression domain is concerned, however, the cortex and hippocampus in the lamprey, if present, are expected to be only very poorly developed (Fig. 4A). Consistently, lampreys have a very small dorsal thalamus sending fibers to the cortex (Puelles, 2001).

In the ventral part of the lamprey telencephalon, or subpallium, only *LjDlx1/6*, though not *LjNkx2.1*, is expressed (Fig. 4A) (Murakami et al., 2001). The equivalent domain in gnathostomes is located in the rostralmost part of the telencephalon from which differentiates the medial ganglionic eminence (MGE). Recent studies revealed that in gnathostomes the MGE generates GABAergic interneurons (Marín and Rubenstein, 2001; Marín et al., 2000); these neuroblasts migrate dorsally to the neocortex (Fig. 4A). In fact, most of the GABAergic interneurons found in the cortex appear to originate from the MGE (Marín et al., 2000). A similar migration has also been described in avian development (Cobos et al., 2001). In teleosts as well, the ventral telencephalon expresses *Nkx2.1b* whereas the dorsal part contains GABAergic interneurons (Wullimann and Mueller, 2004; Wullimann and Rink, 2002). In the mammalian neocortex, pyramidal cells connect with neighboring pyramidal cells directly or indirectly by means of GABAergic interneurons to form local, or microneural, circuits. In the *Nkx2.1*-deficient mouse, the MGE fails to develop (Sussel et al., 1999). It is interesting to speculate that the *Nkx2.1* knock-out mouse can be seen as a phenocopy of the agnathan state; the lamprey does not seem to develop an MGE nor it expresses *LjNkx2.1* in the ventral telencephalon (Figs. 4A, B). Moreover, the pallidum, an MGE-derivative, is thought to be lacking in the lamprey (Nieuwenhuys et al.,

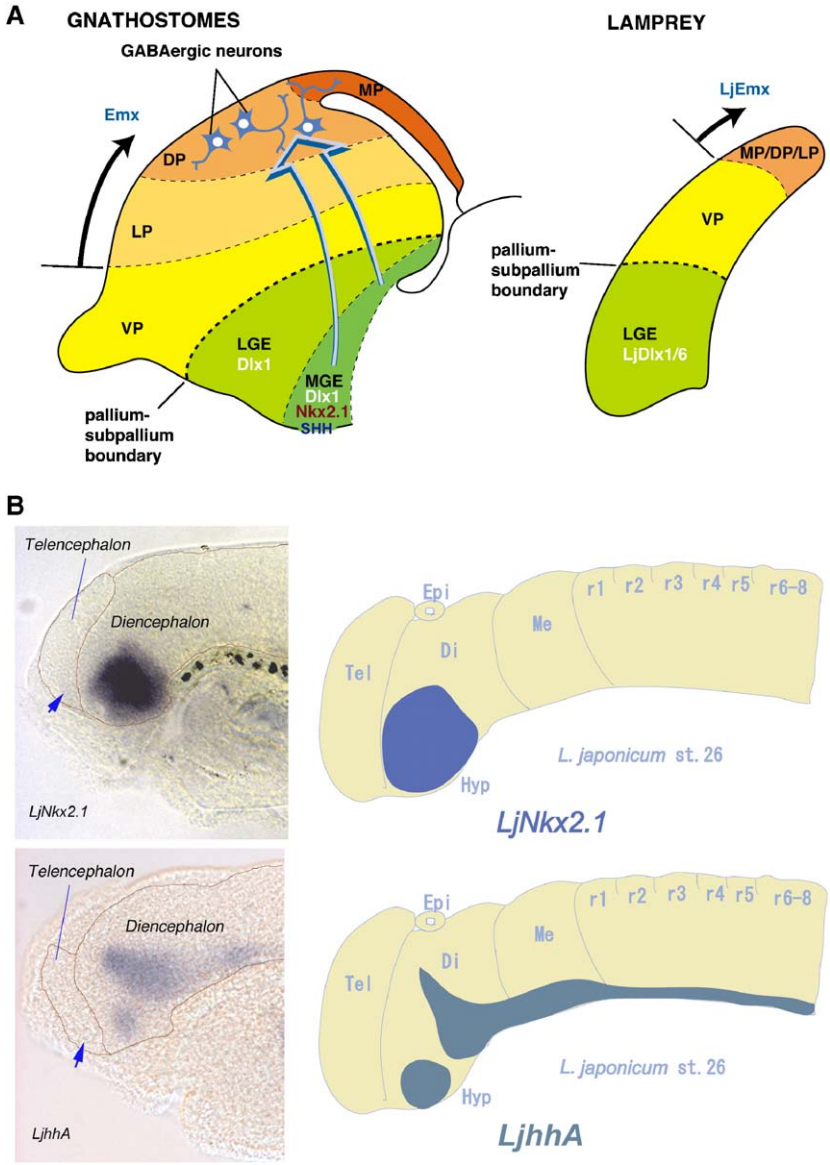

Fig. 4. Comparison of developmental plans of vertebrate telencephalon. (A) The gnathostome telencephalon is divided into pallium and subpallium by the pallium–subpallium boundary (see Puelles et al., 2000). The pallium is further divided into medial (MP)-, dorsal (DP)-, lateral (LP)-, and ventral pallium (VP). The VP is a domain of the pallium that does not express *Emx* genes. In the subpallium, lateral and medial ganglionic eminences (LGE and MGE) are recognized. MGE is specified by co-expression of *Dlx1* and *Nkx2.1*, and it originates GABAergic neurons, the precursors of cortical interneurons (see Marín and Rubenstein, 2001). In the lamprey telencephalon, pallium–subpallium boundary arises, and the pallium is identified. However, it does not have *Nkx2.1*-expression domain in the ventral part nor does it produce GABAergic neurons (see Melendez-Ferro et al., 2002; Murakami et al., 2001). (B) Expression patterns of *LjNkx2.1* and *LjhhA* genes. The *LjNkx2.1* expression domain in the lamprey telencephalon marks the hypothalamus homologue. This gene is not expressed in the subpallium of the telencephalon where pallidum homologue is expected to develop (arrow). *LjhhA*, the putative upstream gene of *LjNkx2.1*, is not expressed in the pallidum, either (arrow).

1998). Consistently, Melendez-Ferro and colleagues have recently shown that there are no GABAergic neurons in the lamprey dorsal telencephalon (Melendez-Ferro et al., 2002). In this respect, co-option of *Nkx2.1* expression in the ventro-rostral part of the telencephalon might have been a pre-requisite for the appearance of the pallidum, a key innovation to allow further acquisition of GABA neurons migrating to the cortex, in the lineage of gnathostomes (Fig. 4A). This might have allowed an explosive evolution of the telencephalon in gnathostomes, as the highest integrative center of the vertebrate central nervous system.

What could be the molecular basis for the postulated co-option of *Nkx2.1*? It has been suggested that *Sonic hedgehog* (*shh*) in the ventral telencephalon could be upstream of *Nkx2.1* (Rallu et al., 2002; Rohr et al., 2001). We therefore analyzed the expression pattern of *LjhhA*, the lamprey homologue of *shh*, in the embryonic lamprey brain and found that it was not expressed in the telencephalic primordium (Fig. 4B) (Uchida et al., 2003).

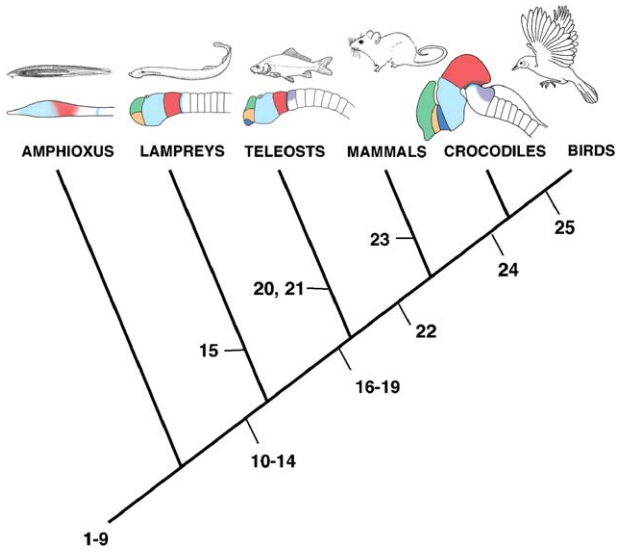

Fig. 5. Evolutionary scenario of the developmental patterning in vertebrate nervous system. On each segment of the phylogenetic tree, sequentially added developmental changes are placed as synapomorphic character states such as (1) neural tube; (2) ancestral expression pattern of regulatory genes along the anteroposterior neuraxis (*Pax6*, *Pax2/5/8*, *En*, *Fgf8/17*, *Otx*, *Emx*, *Dlx1/6*, *Nkx2.1*, etc); (3) reticular neurons?; (4) motoneurons; (5) diencephalons (shown in cyan); (6) mesencephalon? (shown in red); (7) rhombencephalon; (8) eye; (9) neuraxial *Hox* code; (10) neuromeres (establishment of neurepithelial compartments as serial homologues); (11) *Hox* code-dependent specification of branchiomotor neurons; (12) telencephalon (pallium (shown in green) + LGE (shown in orange)); (13) neural crest (peripheral ganglia); (14) paired eyes; (15) lamprey-specific serial homologues in reticulospinal neurons (B neurons); (16) integration of *Hox*-dependent- and rhombomere-dependent specification programs; (17) MGE (shown in blue) and migrating GABAergic interneurons; (18) sympathetic trunk; (19) cerebellar system (shown in gray); (20) teleost-specific serial homologues in reticulospinal neurons; (21) eversion of the telencephalon; (22) loss of serial homologues from reticulospinal neurons; (23) neocortex with six layers in mammals; (24) dorsal ventricular ridge; (25) loss of layers from the dorsal pallium.

Thus, the de novo expression of *shh* could be a key upstream factor for *Nkx2.1* expression in the gnathostome telencephalon.

Conclusions and perspectives

We speculated stepwise evolutionary changes of the developmental plan of the vertebrate brain. Some important changes appear to have been acquired around the split of vertebrates from ancestral chordates. This event involved acquisition of neuromeres as the most fundamental developmental program of the vertebrate central nervous system, together with the sensory placodes and the neural crest as the sources of the peripheral nervous system (Fig. 5). Another large-scale change was introduced at the split of gnathostomes from agnathans, as seen in a series of changes that organized the gnathostome-type cerebellum and telencephalon (Fig. 5). The latter event would be the key innovation that allowed the following radiation of gnathostomes.

In some cases, changes in brain patterning program is associated with de novo expression domains of regulatory genes, namely, co-option of gene expression patterns, not necessarily by gene duplication or invention of new genes. Most of the brain-related genes in vertebrates have homologues in the amphioxus, where they are regulated in the brain primordium (Holland et al., 1992; Jackman and Kimmel, 2002; Knight et al., 2000; Wada et al., 1999). Thus, the study of the elaboration of gene *cis*-regulatory elements will provide important insights into evolutionary mechanisms. Another important domain of study is to investigate the molecular logic of the integration and registering of distinct morphogenetic programs, as proposed for hindbrain evolution. The nervous system provides a highly sophisticated model for evolutionary studies, in which function and morphology are tightly linked on developmental mechanisms. All these changes should involve environmental and behavioral factors as a logic of selection. Thus, the Evolutionary Developmental Biology of the vertebrate brain should integrate various aspects and viewpoints of biological fields, and it is now growing to become one of the most exciting research fields.

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