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REVIEW

# The lamprey in evolutionary studies

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**Abstract** Lampreys are a key species to study the evolution of morphological characters at the dawn of Craniates and throughout the evolution of the craniate's phylum. Here, we review a number of research fields where studies on lampreys have recently brought significant and fundamental insights on the timing and mechanisms of evolution, on the amazing diversification of morphology and on the emergence of novelties among Craniates. We report recent example studies on neural crest, muscle and the acquisition of jaws, where important technical advancements in lamprey developmental biology have been made (morpholino injections, protein-soaked bead applications or even the first transgenesis trials). We describe progress in the understanding and knowledge about lamprey anatomy and physiology (skeleton, immune system and buccal secretion), ecology (life cycle, embryology), phylogeny (genome duplications, monophyly of cyclostomes), paleontology, embryonic development and the beginnings of lamprey genomics. Finally, in a special focus on the nervous system, we describe how changes in signaling, neurogenesis or neuronal migration patterns during brain development may be at the origin of some important differences observed between lamprey and gnathostome brains.

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## Why study lampreys?

The phylum Chordata includes three major taxa, the Cephalochordates (e.g. the amphioxus), the Urochordates (e.g. the ascidians) and the Craniates. Until recently, the Cephalochordates were considered the closest relatives of Craniates, but studies have provided strong support for the existence of a monophyletic group composed by the Urochordates and the Craniates (Delsuc et al. 2006). Many characteristics thought, at a time, to be exclusive of a certain group were later detected in other groups. Two examples are the cranial motoneurons (Dufour et al. 2006) and neural crest-like cells (Jeffery et al. 2004), believed to be craniate novelties and now detected also in ascidians.

The question of the emergence of Craniates, of the novelties which are associated to them (e.g. "true" brain, skull, paired external sensory organs, pharyngeal skeleton) and of their evolution and diversification within the clade cannot be tackled without including in the study all of the main Craniate taxa. However, the great majority of the so-called model species among Craniates belong to the Osteichthyans, a subgroup of Gnathostomes which includes ray-finned fishes and tetrapods. Two comparatively much less-studied key groups among the Craniates are the Chondrichthyans (cartilaginous fishes), which are the sister group of the Osteichthyans, and the Cyclostomes, whose representative species are lampreys and hagfishes (Fig. 1a).

Until very recently, hagfish embryos were impossible to obtain, posing obvious problems to study embryonic development in this group. From the end of the 19th century, where three independent researchers had procured

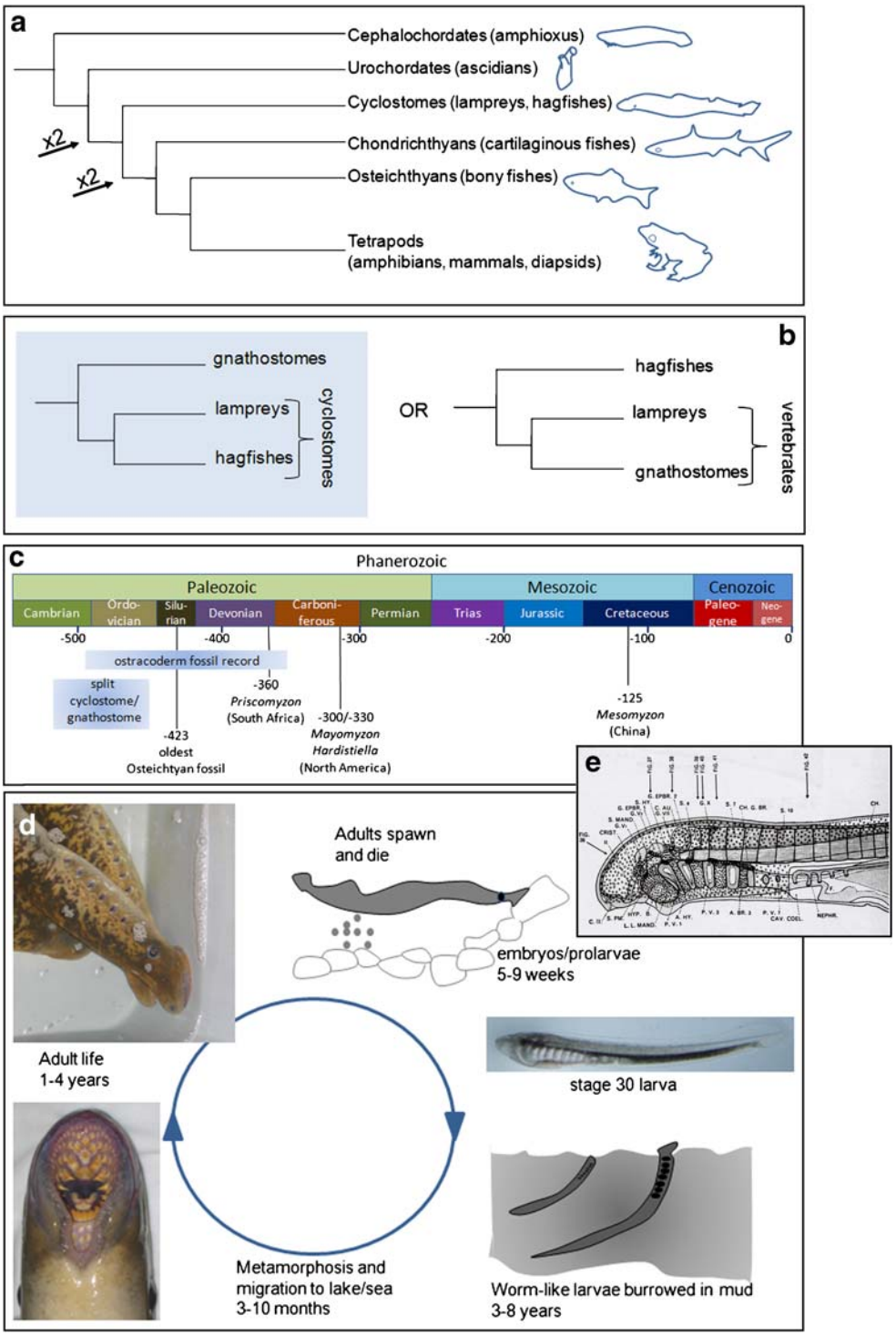


**Fig. 1** Introducing lampreys: **a** A phylogeny of chordates, where the major groups discussed in this review are indicated; ×*2* indicates the two suspected rounds of genome duplication. **b** Two possibilities for Craniate phylogeny are drawn. The "Cyclostome" hypothesis (*shaded*) currently receives more support than the "Vertebrate" hypothesis. **c** Recent paleontological breakthrough is indicated on a schema of the Phanerozoic. See text for details. **d** The life cycle of lampreys is peculiar. Major steps are indicated and illustrated by drawings or photographs (*pictures* are from *Petromyzon marinus*). **e** A reproduction from an illustration by H. Damas of a stage 24 *Lampetra fluviatilis* embryo

and studied some hagfish embryos, only three fertilised eggs were obtained during the whole 20th century, the latest in 1969. It was only in 2007 that the feat was repeated (Ota et al. 2007; for a comprehensive review of the history of hagfish embryology, see (Ota and Kuratani 2006)). For more than 100 years, lampreys have, thus, remained the only non-gnathostome Craniate species where an evolutionary and developmental approach was possible.

## Phylogenetics and genomics

### Lampreys and the search for the ancestral Craniate

The correct assessment of the phylogenetic relationships between the three groups of Craniates—lampreys, hagfishes and gnathostomes—is still a matter of debate. The opinions diverge towards two possible evolutionary scenarios,

schematised in Fig. 1b. In the first scenario, the ancestral jawless craniate would have given rise to two sister groups, one being the gnathostomes (craniates with jaws; "gnathos" means "jaw" in Greek) and the other the cyclostomes (the name means "round mouth"), the latter group including the extant groups of lampreys (Hyperoartia, Petromyzontidea) and hagfishes (Hyperotreti, Myxinoidea) and many fossil groups (Forey and Janvier 1993). In the second hypothesis, based mainly on morphological and paleontological studies, lampreys and gnathostomes would be more closely related and, therefore, would both belong to the group Vertebrata, with the hagfish as an out-group. Molecular analyses of mitochondrial, nuclear ribosomal RNA genes and nuclear protein-coding genes (Delarbre et al. 2000; Delarbre et al. 2002; Kuraku and Kuratani 2006; Lee and Kocher 1995; Mallatt and Sullivan 1998; Mallatt and Winchell 2007; Stock and Whitt 1992) have strengthened the first hypothesis, which we favour in this review. The difficulty to resolve the phylogenetic relationships among Craniates is related to the long evolutionary age of the split between Cyclostomes and Gnathostomes (about 500 mya, (Kuraku and Kuratani 2006)) and to the fact that hagfish and lamprey lineages have diverged shortly after that split (470–390 mya, (Kuraku and Kuratani 2006)). The recent contribution of Mallatt and Winchell (2007) on deuterostome phylogeny, which included two lampreys (*Petromyzon* and *Geotria*) and two hagfishes (*Eptatretus* and *Myxine*) ribosomal sequences, provides a very robust support for the cyclostomes as a monophyletic group.

The successive remodelling steps of the Craniate phylogenetic tree gave origin to different choices of nomenclature, which may be problematic when studying the subject. The lack of jaws being an ancestral character, it cannot be used per se to define a monophyletic group, and some authors object to the use of the term "agnathan" (note that the same might also hold true for the term "cyclostome"). Another source of potential confusion is the name "vertebrate", adequate to describe both gnathostomes and lampreys and with the advantage of its widespread use in common and scientific language but which may be awkward when discussing hagfish. Here, we use the terms "cyclostomes" (for lampreys plus hagfishes) and "craniates" (for cyclostomes plus jawed vertebrates).

## Tracing back lamprey fossils

The global fossil record for lampreys is quite meagre, and the year 2006 was particularly rich for lamprey-interested palaeontologists (Fig. 1c). *Mesomyzon,* a beautifully conserved modern lamprey specimen from the Cretaceous (−125 mya) was found in China (Chang et al. 2006). This finding completed the record of two older species which were previously found in North American Carboniferous deposits (−300 and −330 mya), respectively called *Mayomyzon* (Bardack and Zangerl 1968) and *Hardistiella* (Janvier and Lund 1983). As reviewed and explained by Janvier, and due to this fossil distribution, cyclostomes were long thought as "degenerate" descendants of ostracoderms (that is armoured jawless fishes), which have a rich fossil record in the Devonian period (Janvier 2006). However, last year, Gess and colleagues (Gess et al. 2006) found in South Africa a fossil lamprey they called *Priscomyzon*, which dates from the late Devonian period ("myzon" means "sucker" in Greek). *Priscomyzon* looks strikingly similar to modern lampreys, and the finding was interpreted as follows: lampreys (and hagfish) would have diverged from other Craniates before the ostracoderms, implicating that ostracoderms, although jawless, are closer to jawed vertebrates than to cyclostomes. Year 2006 was, thus, the year when DNA or RNA data and paleontological data were reconciled to suggest that cyclostomes diverged from gnathostomes about 535 to 462 mya.

## Lampreys enter the genomic era

As first proposed by Ohno (1970), one or two rounds of whole genome duplication (WGD, 1R or 2R hypothesis) are thought to have taken place in an ancestral Craniate. As opposed to smaller scale events such as tandem duplications, WGD generates enormous amounts of genetic raw material which is susceptible to acquire novel functions and, with the possibility that large sets of duplicated genes co-evolve more efficiently, leading to the generation of new gene networks used for biological innovations. Although the subject of intense debate for 35 years—both about the actual existence of WGD and about their timing—the 2R hypothesis is increasingly supported by genome-wide analysis of key chordate species (recently reviewed in (Kasahara 2007)). Partial available data from lampreys is quite contrasted, as the study of their Hox clusters has led some authors to support the 2R hypothesis (Irvine et al. 2002) while others suggest that lampreys (and hagfishes) have experienced lineage-specific WGD (Fried et al. 2003; Stadler et al. 2004). On the other hand, a survey sequencing of the elephant shark genome (a chondrichthyan, see Fig. 1a) showed that it possesses four Hox clusters like mammals (Venkatesh et al. 2007). The genome of the sea lamprey *Petromyzon marinus* is currently being assembled (Genome Sequencing Centre), and its analysis will be of primary importance to resolve the issue of the number and position in evolution of these duplications. The current version of the 2R hypothesis postulates two successive rounds of WGD, one before and one after the split between cyclostomes and jawed vertebrates ("×2" on Fig. 1a). Lamprey genome analysis will also provide information on the fate of coding and regulatory regions after gene

duplication, which may be related to morphological changes within the Craniate taxon. In particular, it will hopefully shed light into the genetic mechanisms underlying the generation of novelties in the development of the head and nervous system, especially those related to the patterning of the anterior-most neural tube region, where striking differences are evident when comparing Cephalochordates, Urochordates and Craniates (see below).

The size of lamprey genomes varies from 40% to 70% of the human genome size, depending on the species considered and the methods of analysis used. Hagfish genomes tend to be larger, from 65% to 130% of the human genome size (Animal Genome Size Database, www.genomesize.com). This gives some practical advantage to the choice of *Petromyzon marinus* as the first cyclostome genome to be sequenced (the preliminary assembly of the *P. marinus* genome is available at Pre!Ensembl http://pre.ensembl.org/Petromyzon_marinus/index.html). However, the genome organisation may be quite different between lampreys and gnathostomes: lamprey genomes, contrarily to hagfish genomes, have a much higher guanine–cytosine content (Kuraku and Kuratani 2006), and some evidence suggests that the introns may be, in general, much larger (Marc Ekker, personal communication), which increases the difficulty of genomic and genetic studies.

## Ecology and embryology

The multi-volume book *The Biology of Lampreys* (Hardisty and Potter, 1971a), which remains to this day an important reference on lamprey studies, includes chapters on lamprey ecology, embryology and neurophysiology. Lampreys are aquatic animals, with eel-like bodies. There are 38 species of lampreys living exclusively in the temperate zones of both hemispheres. Many, but not all, adult lampreys are predacious, using their round, sucking mouth to attach to the bodies of fish. Then, they rasp the tissue with a tongue-like structure to open a wound through which they can suck the blood and tissue fragments from their prey. Predacious lamprey species are typically anadromous, with a fresh water larval stage, where they are filter feeders, and a salt water post-metamorphic stage. However, species like the brook lamprey (*Lampetra planeri*) never prey upon fish, reproducing and dying in fresh water shortly after metamorphosis (see (Hardisty and Potter 1971b; Nieuwenhuys and Nicholson 1998) for more detailed descriptions).

The typical life cycle of anadromous lampreys is shown in Fig. 1d. During the spawning season, adult lampreys migrate to shallow water streams; they build a nest using their sucking mouths, reproduce and die. The eggs develop into larvae, which are so different from the adults that they were once seen as a separate species, *Ammocoetes branchialis*, and lamprey larvae are sometimes still called ammocoetes for this reason. After an initial "prolarval" stage, during which they absorb the yolk, they become filter feeding larvae. The larval period is very long, usually not less than 5 years and may last up to 18 years (Beamish and Potter 1975). Larvae then suffer a metamorphosis, which involves important remodelling of the cephalic region (including the eyes) and of the digestive apparatus accompanying the transformation of a filter-feeder into an external parasite. After metamorphosis, the adult period starts, and may last for 1 to 4 years.

The embryonic development of the lamprey is relatively long—at least when compared to developmental biology widely used fish or amphibian models, and this may prove an advantage when deciphering ontogenetic events. Most researchers use either the developmental table of Piavis (Piavis 1971) or that of Tahara (Tahara 1988), built for *Petromyzon marinus* and for *Lampetra reissneri*, respectively, to stage embryos of other species. Piavis divided the developing lampreys into pre-hatching embryos and post-hatching prolarvae. Tahara stage 24 corresponds to Piavis prolarval stage 1, both corresponding to hatching, an event which generally occurs after 11 days of development at 15°C. A classical detailed histological description of lamprey embryonic development is given in the beautiful atlas drawn by H. Damas (see a reproduction of one of his drawings on Fig. 1e for an example (Damas 1944)).

## Lampreys and gnathostomes: what is similar, what is different?

### Short portrait of a lamprey

Lampreys are unique in having a single median dorsal "nostril" (the naso-hypophyseal opening) in the head. Their skin is naked and slimy, and they have seven gill openings extending behind the eyes. The sucker which surrounds the mouth is strengthened by a ring-shaped annular cartilage and bears numerous horny denticles. The eyes possess a lens but no intrinsic eye muscles for accommodation. The extrinsic eye muscles are as in extant gnathostomes, except for the superior oblique muscle, which is attached posteriorly in the orbit, instead of anteriorly. The labyrinth of the ear has two vertical semicircular canals, a blind endolymphatic duct and a number of large ciliated sacs which play a role in equilibrium (Philippe Janvier, Tree of Life project; http://tolweb.org/tree/; see also Hammond and Whitfield 2006). Lampreys have dorsal and caudal unpaired fins, which are strengthened by numerous, thin cartilaginous radials associated with radial muscles. The brain has a very poorly developed cerebellum but large optic lobes. The spinal cord is flattened, almost ribbon-shaped, yet thicker

than that of hagfish. The organisation of the brain in the embryonic and adult lamprey will be the subject of the last section of this review. Here, we have chosen three examples which reflect the particular phylogenetic position of lampreys and their interest in molecular evolutionary studies. The first example shows how some of the mechanisms involved in the formation of skeletal tissues may be more conserved than previously thought; the second concerns the immune system of cyclostomes, which illustrates how very different adaptive immunity strategies were generated through the course of evolution; the third deals with recent findings on lamphredin, a secretion from the buccal gland of lampreys, which may share properties with some components of snake venoms.

## Collagen, *Sox9* and the skeleton

Lampreys are devoid of a mineralised skeleton, although traces of globular calcified cartilage may occur in the endoskeleton. The skull of lampreys is, like that of hagfish, made up of cartilaginous plates and bars, but it is more complex and includes a true cartilaginous braincase. The gills, although enclosed in muscularised pouches in the adult, are supported by unjointed gill arches, which form a "branchial basket". The gill arches lie externally to the gill filaments and associated blood vessels. Lampreys possess, like hagfish, a very large notochord. In addition, they also have small cartilaginous dorsal arcualia (basidorsals and interdorsals), which are metameric endoskeletal elements aligned along the notochord and flanking the spinal cord, and which form the vertebral column (P. Janvier, Tree of Life).

In the cartilage of jawed vertebrates, the major extracellular matrix proteins are type I and type II collagen, and this was long considered as a hallmark of the gnathostome skeleton. Indeed, the cartilaginous skeleton of lampreys and hagfishes was described as non-collagenous, containing instead the elastin-like proteins lamprin and myxinin, respectively (Wright et al. 2001). However, recent results have shown that two type II collagen genes (*Col2α1*) are expressed in lamprey (Zhang et al. 2006)—and also in hagfish (Zhang and Cohn 2006)—cartilages, indicating that type II collagen-based cartilage evolved earlier than previously recognised. Interestingly, lamprey *Sox9* (a HMG-box containing transcription factor) is co-expressed with *Col2α* during skeletal development, indicating a conservation of the genetic pathway for chondrogenesis in lampreys and gnathostomes (Zhang et al. 2006). The authors have also shown that amphioxus possesses an ancestral clade A fibrillar collagen (*ColA*) gene that is expressed in the notochord (Zhang and Cohn 2006). Their results suggest that the duplication and diversification of *ColA* genes at the chordate–craniate transition may underlie the evolutionary origin of craniate skeletal tissues, and that a collagen-based cartilage is a unifying character of Craniates.

## Immunity in lampreys: no MHC/TCR but VLR

Lampreys have long been considered as keys to understand the emergence of adaptive immunity. They possess overt immune anatomical structures which undergo considerable changes through their life cycle (Amemiya et al. 2007), yet they lack immunoglobulin, T cell receptor (TCR) or major histocompatibility complex (MHC) genes. Recent studies on lamprey and hagfish immune systems have brought about a spectacular example that highlights the evolution of different adaptive defence mechanisms among Craniates. It was discovered that both species have a unique form of anticipatory immunity, remarkably different from the recombination-activating gene (RAG)-mediated adaptive immunity of gnathostomes (for a review on the mechanisms of immunity across animals, see (Litman et al. 2005)). Lampreys (Pancer et al. 2004) and hagfish (Pancer et al. 2005) have variable lymphocyte receptors (VLR) composed of leucine-rich repeats (LRR), which are generated by somatic rearrangement from a large number of LRR modules. It is likely that the cyclostome VLR receptors derive from an ancestral gene, which was later duplicated in the hagfish lineage (Pancer et al. 2005). VLR rearrangements, as RAG-mediated rearrangements of gnathostome antibody genes, lead to a very high number of possible different combinations: a repertoire of $10^{14}$ (thus, similar to the mammalian antibody repertoire) unique receptors may be generated (Alder et al. 2005). Lampreys respond to immunisation with anthrax spores with increasing levels of soluble anthrax-specific VLRs, and lymphocytes are clonally selected upon antigen stimulation (Alder et al. 2005). Thus, two remarkably different forms of antigen recognition have evolved independently in the two Craniate sister groups and represent a striking example of convergent evolution of totally different strategies for generating anticipatory immunity. The authors' prediction that an array of 1,500 to 2,400 diverse LRR modules in the genome provides the source of VLR diversity will be testable soon, after the completion of *Petromyzon* genome assembly.

## CRISP in the lamprey buccal gland

Craniate parasites are quite rare, vampire bats and parasitic lampreys being the only true ectoparasite representatives. In fact, adult parasitic lampreys (19 out of the 38 described species of lampreys) may remain attached to their fish prey or host during as much as 10 days, a long time during which the fish must remain alive and during which

lampreys secrete anticoagulant to prevent clotting of the host's blood. This secretion was originally termed "lamphredin" and, emanates from the buccal glands, paired oral structures which develop at metamorphosis from invaginations of the oral epithelium. The anticoagulant and hemolytic properties of lamphredin are known since 1927 (Gage and Gage-Day 1927) but only very recently has its biochemical components been identified. Two laboratories have independently isolated two major components of lamphredin (Ito et al. 2007; Xiao et al. 2007). The first is a 160-kDa protein with fibrinogenolytic activity and corresponds to plasma albumin (Ito et al. 2007), which surprisingly functions here as a $Ca^{2+}$ and $Mg^{2+}$-dependent metalloprotease (Xiao et al. 2007). The second is a 26-kDa protein which belongs to the *c*ysteine-*r*ich *s*ecretory *p*rotein (CRISP) superfamily, and functions as an L-type $Ca^{2+}$ channel blocker, most probably acting as a vasodilator. Protein alignment of the 26-kDa lamprey protein with numerous venom CRISP proteins from snakes and lizards shows 100% conservation of all their 16 cysteine residues, with several short insertions in lamprey CRISP (Ito et al. 2007). Interestingly, a transcriptome analysis of snake and lizards toxin types has recently shown that, contrarily to previously thought, venomous function (including CRISPs, which block various ion channels and are regarded as neurotoxins) arose only once in squamate evolution, about 200 mya (Fry et al. 2006). The finding of lamprey CRISP as a secreted factor from the buccal gland may suggest that the ancestral CRISP protein originates even earlier in evolution, at the dawn of craniates. It also illustrates how hematophagous animals have evolved a number of strategies to facilitate blood sucking.

## The lamprey as an evo-devo model

The study of lampreys has started to attract the attention of evo-devo biologists more than a decade ago, and the promises offered by the lamprey as an evo-devo model were the subject of a review by Kuratani (Kuratani et al. 2002). Gene expression, immunocytochemical and sequence analysis are frequently performed techniques, while more functional studies are now at their beginnings. This difficult start has been mainly due to the limited period in which live lamprey embryos are available, only a few weeks per year (although different species reproduce at different times). While an exhaustive list of the major contributions to the field is beyond the scope of this review, we have chosen a few examples of studies where promising technical advances were made. Studies concerning evolution and development of the nervous system will be specifically discussed in the last section of this review.

### Jaws

The question of the emergence of the gnathostome jaw is closely associated to the acquisition of a new feeding behaviour and to the presence of novel features related to the perception of and response to environmental stimuli. As the jaw is one of the most obvious external morphological differences between cyclostomes and gnathostomes, it was one of the first characteristics to be investigated by evo-devo biologists. A large number of anatomical and gene expression studies (Cohn 2002; Horigome et al. 1999; Kuratani 2005a; Kuratani et al. 2002; McCauley and Bronner-Fraser 2003; Neidert et al. 2001; Ogasawara et al. 2000; Shigetani et al. 2002) have provided insights into the mechanisms of tissue patterning, cell specification and migration and morphogenetic movements underlying the formation of the lamprey head and jaw (reviewed in (Kuratani 2005b; Shigetani et al. 2005)).

In this context, it is probably worth mentioning here the controversy which occurred about the "*Hox* hypothesis" for the acquisition of jaws. Indeed in gnathostomes, the first pharyngeal arch (PA1) does not express any *Hox* genes. When Cohn (2002) reported that *HoxL6* was expressed in *L. fluviatilis* PA1, it was tempting to hypothesise that a withdrawal of *Hox* genes from PA1 could have facilitated the evolution of jaws at the agnathan/gnathostome transition. The subsequent demonstration that another lamprey, *Lethenteron japonicum*, shows no *Hox* expression in PA1 (Takio et al. 2004) has provided evidence that *Hox* expression in PA1 is not a general lamprey feature and is, therefore, unlikely to be related the jawlessness.

As said above, in gnathostomes, the jaw originates from the mandibular arch. Importantly, genes involved in specification of the mandibular arch are expressed with similar patterns in the oral regions of chick and lamprey embryos: Shigetani et al. (2002) have shown that *Fgf8* and *Bmp4* expression in the proximal and distal aspects of the arch epidermis are in register with *Dlx* and *Msx* expression in the proximal and distal ectomesenchyme (that is, neural crest-derived). In this context, the authors were able to confirm the conservation of the signaling cascades between lampreys and gnathostomes by application of protein-soaked beads, a method widely used in chick embryological studies. Fgf8- and Bmp4-soaked beads implanted on one side of early lamprey embryos induced unilateral ectopic expression of *Dlx1/6* and *MsxA*, respectively (Shigetani et al. 2002). However, careful analysis of the exact origin of the cephalic neural crest invading the arch showed that, in lamprey, the forebrain- to hindbrain-derived crest cells contribute more extensively to the upper and lower lips than do those of gnathostomes to the mandibular arch. The authors propose that a positional shift in the interactions between the epidermis and the neural crest-derived cells

(a heterotopic epithelial-mesenchymal shift) could be at the origin of the appearance of jaws, with the mandibular arch differentiating into maxillary and mandibular processes in gnathostomes, whereas it differentiates into the lower lip and the velum (a special oral pumping apparatus) in lampreys.

### Neural crest

The neural crest gives rise (among other derivatives) to the cranio-facial skeleton and has, therefore, been thoroughly investigated in lampreys. Isolation, expression and phylogenetic analysis of lamprey homologues of known neural crest markers has revealed a global conservation of the general mechanisms of neural crest cell specification and migration in craniates (Horigome et al. 1999; Kuratani 2004; McCauley and Bronner-Fraser 2002, 2003, 2004, 2006; Meulemans and Bronner-Fraser 2002; Meulemans et al. 2003; Neidert et al. 2001; Ogasawara et al. 2000; Tomsa and Langeland 1999). However, neural crest migration pathways in the hindbrain are different in lampreys (Horigome et al. 1999), and lamprey crest cells are less restricted in their movements, with cells from a given axial level contributing to multiple branchial arches, leading some authors to propose that migratory constraints may be a gnathostome innovation (McCauley and Bronner-Fraser 2003). In addition, lampreys have fewer neural crest-derived cell types (e.g., no sympathetic chain ganglia), indicating a possibly restricted differentiation potential.

A recent study on the *SoxE* gene represents an important technical advance, as it was the first to report morpholino oligonucleotide injections in lamprey embryos to disrupt *SoxE1* translation. *SoxE* is expressed in the neural crest cells which invade branchial arches in a similar way in both lampreys and gnathostomes (McCauley and Bronner-Fraser 2006). This observation raises the possibility that one or more *SoxE* genes might have an ancestral role in neural crest and pharyngeal arch development. The knock-down of SoxE1 led to profound defects in the pharyngeal arches, similar to effects reported in *Xenopus* (Spokony et al. 2002), which supports the homology of pharyngeal elements between lampreys and gnathostomes (McCauley and Bronner-Fraser 2006). More recently, the same laboratory has made a huge contribution to the field by systematically analysing a set of 50 genes from the "Neural Crest Gene Regulatory Network" (NC-GRN; Sauka-Spengler et al. 2007). They have also pushed further the technical limits in lamprey embryology, as they coupled morpholino-based knock-down strategy with mRNA injections to rescue the induced phenotypes. Their data demonstrate that the basic upstream core of the NC-GRN (including Bmp and Wnt signaling, and *MsxA*, *ZicA*, *Dlx* and *Pax3/7* "lateral plate" genes) was already fixed in the first Craniates, whereas downstream effector genes which function later or downstream in the NC-GRN are more divergent and may underlie species-specific traits (Sauka-Spengler et al. 2007). It is worth noting that the recent advances in hagfish embryology, although much less exhaustive, confirm that a NC-GRN indeed already existed at the dawn of Craniates (Ota et al. 2007).

### Skeletal muscle

The expression of many skeletal, muscle-specific genes is now known in lamprey (reviewed in (Kusakabe and Kuratani 2005). As in amphioxus and gnathostomes (but not ascidians), the paraxial post-otic mesoderm of lampreys is segmented into myotomes. However, the lamprey head mesoderm never develops into cephalic myotomes, and a primarily unsegmented mesoderm may be a pan-craniate feature (Kuratani et al. 1999). In the first transgenesis experiment ever reported in lamprey, eggs were injected with constructs where green fluorescent protein (GFP) expression was driven either by a virus promoter or by the 5' regulatory regions of medaka (a teleost fish) actin gene. Reporter gene expression was recorded for more than a month starting 2 days after injection. Although the expression patterns were highly mosaic and differed among individuals, GFP was expressed predominantly in the striated muscles of lamprey embryos when driven by the 5' upstream regions of the medaka muscle actin. This suggests that a pan-craniate muscle-specific gene regulatory mechanism may have evolved before the cyclostome or gnathostome divergence (Kusakabe et al. 2003). On the other hand, this study has also shown that some of the most rostral post-otic myotomes and the hypobranchial muscle grow rostrally and cover the cranial region, which does not occur in gnathostomes (Kusakabe et al. 2003).

## The lamprey brain: adult features

Great similarities between the lamprey brain and that of gnathostomes were revealed by anatomical, hodological, immunohistochemical and gene expression studies. This high degree of conservation was not necessarily apparent in a first approach, as the lamprey brain morphology looks quite different from that of known gnathostome brains (Fig. 2a,b).

The adult lamprey brain is very small—one of the smallest among craniates, as a proportion of total body mass but within the size range of some teleost fish (reviewed in (Wullimann and Vernier 2006). The large olfactory bulbs are a very conspicuous feature, as well as the well-developed pineal and parapineal organs, which connect to the brain via long stalks. The relative size of these brain regions reflects the importance of the olfaction

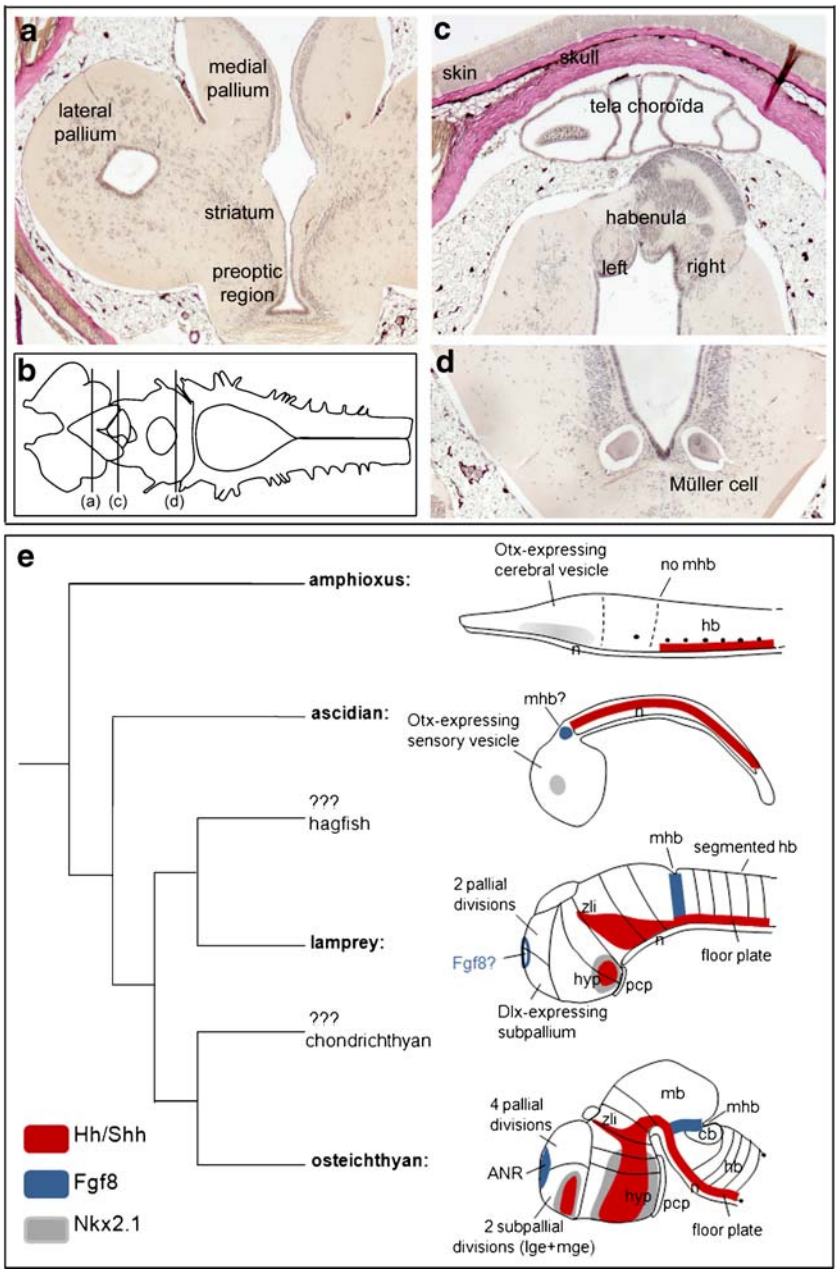


**Fig. 2** Introducing the lamprey brain: **a**–**d** anatomy of the adult lamprey brain. **a, c**, and **d** show transverse sections through the brain of *Lampetra fluviatilis*, after Van Gieson coloration (courtesy of Franck Bourrat); **a** shows the peculiar structure of the telencephalon, **b** illustrates the high degree of asymmetry of the habenula and **c** shows a pair of giant Müller cells in the ventral mesencephalon. Anatomical structures discussed in text are indicated, and approximate section levels are indicated in **b**, where the shape and general structure of the adult brain is viewed from the dorsal side, with anterior to the left. **e** Secondary organisers and forebrain evolution; the expression patterns for Shh (*red*) and Fgf8 (*blue*) are depicted on schematic drawings of the embryonic brain of an amphioxus, an ascidian, a lamprey and a gnathostome. Nkx2.1 (*grey*) expression is also indicated to help the reader understand the paragraph concerning the evolution of the telencephalon. A major innovation present in lampreys but not prochordates is the presence of a neuromeric diencephalon and a telencephalon proper. A major innovation present in gnathostomes but not lampreys in the presence of a large and highly regionalised telencephalon. These events can be correlated to the expression of *Shh* and *Fgf8* in organising centres located in the more anterior parts of the nervous system. *ANR*, anterior neural ridge; *cb*, cerebellum; *hb*, hindbrain; *hyp*, hypothalamus; *mb*, midbrain; *mhb*, mid-hindbrain boundary; *n*, notochord; *pcp*, prechordal plate

and the regulation of the circadian rhythm that are of major importance for these animals. Of note, the habenula of lampreys is prominently asymmetric (Fig. 2c). The lamprey telencephalon is only partly evaginated into two vesicles, and the non-evaginated middle portion is rather large, including parts of both the pallium and the subpallium (Fig. 2a). All of the lamprey axons are unmyelinated, as those from hagfish and cephalochordates, and this is likely

to be an ancestral chordate characteristic (reviewed in (Nieuwenhuys and Nicholson 1998). The majority of the neural cell bodies in the lamprey brain are concentrated around the ventricular walls. This pattern of organisation, characterised by a small degree of migration from the zone where the neurons develop, has appeared several times in evolution, being present in different taxa, and has been referred to as laminar (Butler and Hodos 1996). Lampreys possess three types of giant neuronal cells associated to escape response: ten pairs of Müller neurons (Fig. 2d), one pair of Mauthner cells in the hindbrain (also present in some fish and amphibians) and giant interneurons in the spinal cord. Curiously, lampreys have mixed or dual synapses which have both electrical and chemical modes of transmission (Nieuwenhuys and Nicholson 1998). These animals also possess several circumventricular organs with a neurohemal function (Joly et al. 2007; Tsuneki 1986).

A rudimentary cerebellum, absent from hagfish, has been recognised in the lamprey. However, this region is devoid of Purkinje cells and cerebellar nuclei, as well as components of the rhombic lip-derived cerebellar and pre-cerebellar systems (reviewed in Murakami et al. 2005; Nieuwenhuys and Nicholson 1998). The absence of *Pax6* expression in the rhombic lip of the developing brain has been related to the absence of these latter components (Murakami et al. 2005).

Classical comparative neuroanatomical techniques have started to be applied to the lamprey brain more than a century ago. More recently, immunohistochemical, tract-tracing (HRP, DiI, etc.), electrophysiological and behavioural studies have provided a clearer picture of the organisation and functioning of the lamprey central nervous system(CNS). Notable advancements have been made using the lamprey spinal cord as a model to study the regeneration of nerves after injury, the respiration and the swimming behaviour (e.g. Gravel et al. 2007; Grillner and Wallen 2002; McClellan 1994; reviewed in Rovainen 1996). Notably, the Grillner laboratory has strongly contributed to the understanding and modelling of the neural control system for goal-directed locomotion, including steering and control of body orientation. Indeed, lamprey is one of the few Craniates where the central pattern generator (a neuronal network which is able to exhibit rhythmic activity in the absence of sensory input) is well described at a cellular level, and it has even been recently used to design a controller for a simulated belly-dancing humanoid robot (Grillner et al. 2007; Or 2006).

In the book *The Central Nervous System of Vertebrates*, the chapter dedicated to the description of the lamprey CNS gives a survey of the model, even if much has been found since its publication (Nieuwenhuys and Nicholson 1998). The chapter contains an extensive list of the localisation of neurotransmitters and other neuronal substances in the lamprey brain. More recent data on brain neurochemical distribution, proliferation and connectivity, or data not mentioned in this list, include the work of (Abalo et al. 2005; Auclair et al. 2004; de Arriba Mdel and Pombal 2007; de Miguel et al. 1990; Del Carmen De Andres et al. 2002; Frontini et al. 2003; Gonzalez et al. 1999; Laframboise et al. 2007; Melendez-Ferro et al. 2002a, 2002b, 2003; Menard et al. 2007; Osorio et al. 2006; Perez-Costas et al. 2002, 2004; Pflieger and Dubuc 2004; Pierre-Simons et al. 2002; Pombal et al. 1997a, 1997b, 2001; Robertson et al. 2006, 2007; Root et al. 2005; Vidal Pizarro et al. 2004; Villar-Cheda et al. 2002, 2006; Weigle and Northcutt 1999). Neuronal phenotype distribution and fibre connectivity in the lamprey brain have also been reviewed by Wullimann and Vernier (2006).

Globally, besides some distinctive peculiarities, the adult lamprey brain, therefore, shares many features with the prototypic gnathostome brain. Of note, a prosomeric organisation of the lamprey diencephalon has been recognised (Pombal and Puelles 1999), strengthening the unity of Craniates in this respect, as the Urochordates and Cephalochordates (ascidians and amphioxus) do not show any pseudo-segmental organisation of their anterior nervous system. The developmental origin and evolutionary emergence of such an organisation is discussed in the next section.

## The lamprey brain: insights from evo-devo

### Embryonic development through primary neurulation

The embryonic development of the CNS in lampreys involves the formation of a neural plate from which neural folds elevate and then fuse to form a rod and, later, a tube (Damas 1944). This type of neurulation is classified as "primary neurulation" and is shared with most gnathostomes (Lowery and Sive 2004). In *Lampetra reissneri* (Tahara 1988), the neural groove forms on the fifth day post-fertilisation (dpf) in the middle of the neural plate (stage 17). The neural folds start to contact and fuse in the dorsal midline by stage 20 (6 1/4 dpf). A median region of the dorsal ectoderm gradually detaches itself from the surface to form a cord of solid cells (Nieuwenhuys and Nicholson 1998). Finally, at stage 22 (8 dpf) a longitudinal slit appears in the nerve cord and the neural tube is formed. When hatching (stage 24), the anterior neural tube is already relatively well developed, and the fore-, mid- and hindbrain become discernible due to the formation of the epiphysis and the folding of the cerebral commissure (Tahara 1988).

### Atypical proliferation and neurogenesis patterns

In a recent important analysis of neuronal proliferation and differentiation in the brain of embryonic, larval and adult

lamprey (*Petromyzon marinus*), proliferation has been correlated to lamprey brain morphogenesis (Villar-Cheda et al. 2006). These authors have shown that differences in the thickness and appearance of the ventricular zone (the portion of the neural tube closest to the ventricle), as well as the presence of proliferation discontinuities, correspond to distinct neuroanatomical regions. The presence of conspicuous late-proliferating regions during larval life and metamorphosis is in remarkable contrast to the limited proliferative activity in the adult brain. Analysis of both proliferating cell nuclear antigen and 5'-bromo-2-deoxyuridine markers has revealed that the cell cycle in lamprey is very long, explaining both the slow growth of the brain and the presence of an extensive ventricular zone for many years. In addition, analysis of neuronal differentiation markers suggests that there is little, if any, tangential migration in the lamprey forebrain (Villar-Cheda et al. 2006). Many of these characteristics show a striking contrast to the proliferation patterns in teleost brains, where postembryonic neurogenesis is rapidly restricted to small specialised areas (Wullimann and Knipp 2000; Wullimann and Puelles 1999) or to the complex neurogenesis and tangential migration patterns, which are classically described in the amniote forebrain (reviewed in (Marin and Rubenstein 2001). Some of these differences were interpreted with respect to the very particular life cycle of these animals, which, as described above, consists in an embryonic or prolarval period, a very long larval phase, a metamorphosis event and an adult life.

## A craniate *Bauplan* to build the brain

The comparison of the expression domains of genes involved in brain development in representative species of Cephalochordates, Urochordates and Craniates has led to the idea of a common tripartite organisation of the ancestral chordate brain. In the anterior region of the neural tube of both ascidians and craniates, these three regions are: (1) an *Otx*-expressing domain (which corresponds to the forebrain and midbrain of Craniates), (2) a central region expressing *Pax2/5/8* genes and (3) a more posterior region expressing *Hox* genes (the craniate hindbrain and spinal cord). In amphioxus, *Pax2/5/8* expression is not detected in an intermediate domain between the anterior *Otx*-expressing and the posterior *Hox*-expressing regions (Kozmik et al. 1999; see also Fig. 2e).

Due to the long divergence time between the three chordate taxa, the establishment of unequivocal homologies (a notion that implies a common evolutionary origin) within the neural tube has been a difficult task. Despite the presence of similarities in the organisation of the anterior neural tube between the three main chordate groups, the craniate brain has a very distinctive type of regionalisation not present in cephalochordates and urochordates. This craniate-type brain organisation (the craniate brain *Bauplan*) appears during development under the influence of unique patterning centres, sometimes called secondary organisers (see below).

The study of a number of genes expressed during brain development in lamprey has provided insight into the mechanisms of brain patterning and organisation (neural crest cell specification and migration has been discussed above). The expression patterns of representatives of the *Otx* (Tomsa and Langeland 1999; Ueki et al. 1998), *Emx* (Myojin et al. 2001), *Pax6* (Derobert et al. 2002; Murakami et al. 2001), *Dlx* (Myojin et al. 2001; Neidert et al. 2001), *Fgf8/17* (Shigetani et al. 2002), *Hh* (Osorio et al. 2005; Uchida et al. 2003), *Nkx2.1* (Ogasawara et al. 2001), *Lhx1/5, Lhx2/9* (Osorio et al. 2005), *Pax2/5/8*, *Pax3/7* (McCauley and Bronner-Fraser 2002; Osorio et al. 2005), *Bmp2/4* (McCauley and Bronner-Fraser 2004; Shigetani et al. 2002), *Pax1/9* (Ogasawara et al. 2000), *Hox* (Cohn 2002; Murakami et al. 2004; Takio et al. 2007), *Krox20* and *Eph* (Murakami et al. 2004) families have been particularly important to understand brain development and evolution. Such analysis, and comparison with ascidians and amphioxus, has revealed a high degree of conservation of brain organisation between lampreys and gnathostomes. The presence of neuromeres throughout the neuraxis and of a telencephalon proper with a *Pax6*-expressing pallium and a *Dlx*-expressing subpallium are distinctively shared features of the craniate brain (Murakami et al. 2005).

## Secondary organisers: a craniate novelty?

Secondary organisers are signaling centres which secrete diffusible morphogen molecules with properties to control the growth and patterning of the neuroepithelium (they are called “secondary” to distinguish them from “the” gastrula organiser). In Craniates, these secondary organisers include the roof plate and the floor plate, which secrete Wingless-Int, Bone Morphogenetic Protein and Shh (Sonic Hedgehog), and have crucial roles in dorso-ventral patterning (Wilson and Maden 2005), together with the mid-hindbrain boundary (MHB; Alexandre and Wassef 2003), the *zona limitans intrathalamica* (zli; Lim and Golden 2007) and the anterior neural ridge (ANR; Rubenstein et al. 1998) which secrete *Fgfs* and *Shh* and rather control antero-posterior patterning (Fig. 2e). After years of analysis of these signaling centres in chordate embryos, a picture clearly emerges. A roof plate and a floor plate are shared craniate features. Whereas amphioxus and ascidians have exclusively posterior expression of *Shh* (Shimeld 1999; Takatori et al. 2002), the expression of this morphogen in the forebrain and particularly the zli of craniates is strikingly correlated with the emergence of a neuromeric diencephalon (Osorio et al. 2005). A “MHB-like” region expressing *Fgf8* and

*Pax2/5/8* can be recognized at the posterior edge of the *Otx*-expressing domain in tunicates (Imai et al. 2002) but not in amphioxus (Kozmik et al. 1999). The ANR which secretes *Fgf8* at the rostral tip of the gnathostome telencephalon may be absent in lamprey (fragmentary data in Fig.1h of Shigetani et al. 2002). Future studies dealing with the expression of morphogen molecules in the telencephalon will be of importance to understand the emergence of a telencephalon proper and its evolution among Craniates. It nevertheless appears that the evolution of secondary organising centres was probably of crucial impact for emergence of novelties in the anterior brain of Craniates and for the increasing importance taken by the forebrain during Craniate evolution (sometimes referred to as "encephalisation" of the brain, Fig. 2e).

## A case study: telencephalic GABAergic and cholinergic neurons

Figure 3 gives a schematic comparison of some aspects of forebrain organisation between lamprey and mouse based on the expression patterns of some of the genes mentioned above. The absence in lampreys of a *Hh*- and *Nkx2.1*-expressing region in the developing subpallium has been correlated to the absence of a pallidum (the gnathostome subpallial telencephalic region derived from the medial ganglionic eminence or MGE (Murakami et al. 2005; Osorio et al. 2005), a region which cannot be recognised in adult lampreys (Nieuwenhuys and Nicholson 1998; see also Fig. 2a). This raises important and fundamental questions on the embryonic origin of important neuronal populations, such as GABAergic neurons or cholinergic neurons in the telencephalon. Indeed, in gnathostomes, it now well established that almost all gamma-aminobutyric acid (GABA) interneurons of the pallium (or cortex) and of the striatum originate in the MGE, and the same holds true for cholinergic striatal interneurons (Fig. 3, left panel). These specification and migration processes are highly dependent on the expression of Lhx6 and Lhx7 (Alifragis et al. 2004; Zhao et al. 2003), two LIM-homeodomain transcription factors which are downstream of *Shh* and *Nkx2.1* in the MGE developmental cascade and which were unsuccessfully searched for in lamprey in silico and by various molecular cloning attempts (our unpublished data). On the other hand, there are GABA pallial neurons in lampreys, but they appear very late in development (Melendez-Ferro et al. 2002a), and they may actually prove to be projection neurons (Robertson et al. 2007), a possibility which is spectacularly different from the gnathostome situation where pallial projection neurons are exclusively glutamatergic (Fig. 3). There are also some (scarce) cholinergic cells in the lamprey striatum (Pombal et al. 2001). The completed *Petromyzon* genome will confirm (or not) the absence of *Lhx6/7* in lampreys. In any case, even in the absence of the *Shh* or *Nkx2.1* developmental cascade in their subpallium, lampreys possess GABAergic pallial neurons and cholinergic subpallial cells which are inevitably generated through totally different specification mechanisms from those used in gnathostomes and, therefore, represent a typical case of homoplasy (Fig. 3). The

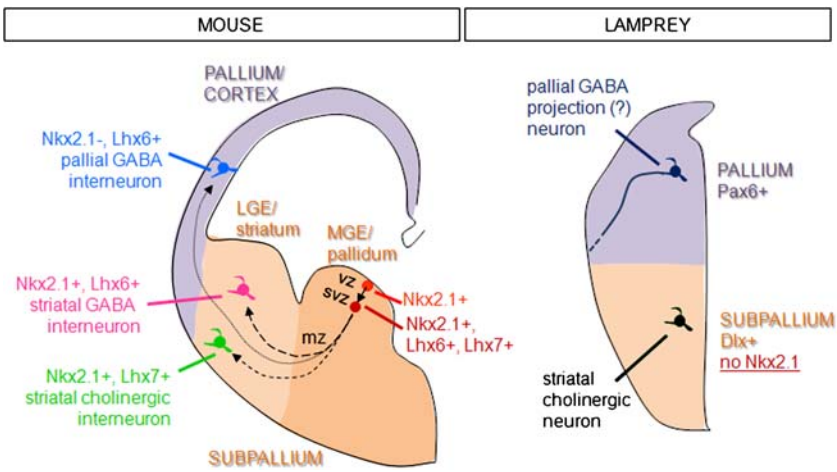


**Fig. 3** Divergent mechanisms to specify forebrain neurons among Craniates: a hemi-section through the developing telencephalon of a mouse (*left*) and a lamprey (*right*) are schematised. Both are composed of a *Pax6*-expressing pallium (dorsal telencephalon, *purple*) and a *Dlx*-expressing subpallium (ventral telencephalon, *orange*). *In mouse*, the subpallium is further subdivided into the *l*ateral and *m*edial *g*anglionic *e*minences (LGE and the MGE). The LGE expresses only *Dlx*, whereas the MGE expresses both *Dlx* and *Nkx2.1*. Several tangential long distance migratory pathways (*arrows*) originating in the *Nkx2.1*-expressing MGE populate the entire telencephalon with GABAergic and cholinergic interneurons, whose migration and specification are dependent on the expression of *Nkx2.1*-downstream transcription factor Lhx6 and/or Lhx7 (*blue*, *pink* and *green* neuronal populations). *In lamprey*, there is only one *Dlx*-expressing subpallial division (thus, mouse LGE-like). This situation raises important questions on the genetic specification of the GABAergic and cholinergic neurons, which must be generated through *Nkx2.1*-independent mechanism in the lamprey telencephalon

knowledge of the patterning scheme in the hagfish telencephalon should bring some clues on the ancestral craniate situation at this level.

Hox code and rhombomeres: not in register

In the hindbrain, the different reticulospinal neurons develop from specific neuromeres both in lampreys and gnathostomes, and lamprey orthologues of *Krox20* and *Eph* are similarly expressed in rhombomeres 3 and 5. The coupling of the rhombomeric organisation of the hindbrain and the rhombomeric-specific distribution of reticulospinal neurons is probably a craniate novelty (Murakami et al. 2005). On the other hand, it is important to note that the hindbrain Hox code is not correlated to the rhombomeric borders in lamprey. While in gnathostomes the motor nuclei of cranial nerves develop in register with the rhombomeric boundaries, it is not so in lamprey. A striking case is that of the transition between the trigeminal (V) and facial (VII) motoneurons. In gnathostomes, this transition occurs at the r3–r4 border; while in lamprey, it occurs instead in the middle of r4, where it coincides with the rostral expression boundary of *Hox3* (Murakami et al. 2004). This shows that the association between the rhombomeres and the development of motoneurons (defined by the Hox code) has only appeared in gnathostomes after the split from the lineage leading to lampreys. These findings can be seen as a challenge to the definition of rhombomeres as seen from gene expression patterns, and raise important questions about the nature of the hindbrain "compartments" in lampreys and gnathostomes.

## Conclusion

Lampreys are odd animals, with a peculiar life cycle and lifestyle, strange head and body anatomy, yet they possess an ever greater number of shared characters with their gnathostome sister group. Throughout this review, we have tried to emphasise the commonalities and divergences recently found between the two groups. It appears that, even though the developmental and molecular mechanisms are not always shared, the lampreys function with many equivalent, but not all, homologous systems as those of jawed vertebrates. Striking examples include the immune system or telencephalic neuronal systems of lampreys, which can be taken as living examples of the spectacular evolutionary diversification among Craniates.

Lampreys are now entering their genomic era, and this will undoubtedly shed light in the next few years on long-standing issues concerning genome duplications and, more generally, gene content in the Craniate ancestor. Although less straightforward, analysis of gene regulatory sequences will also probably help understand the emergence of de novo expression or the changes in expression of some important molecules at the origin of craniate innovations.

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