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Forebrain Architecture and Development in Cyclostomes, with Reference to the Early Morphology and Evolution of the Vertebrate Head

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Lamprey · Hagfish · Cyclostome · Forebrain · Telencephalon

Abstract
The vertebrate head and brain are characterized by highly complex morphological patterns. The forebrain, the most anterior division of the brain, is subdivided into the diencephalon, hypothalamus, and telencephalon from the neuromeric subdivision into prosomeres. Importantly, the telencephalon contains the cerebral cortex, which plays a key role in higher order cognitive functions in humans. To elucidate the evolution of the forebrain regionalization, comparative analyses of the brain development between extant jawed and jawless vertebrates are crucial. Cyclostomes – lampreys and hagfishes – are the only extant jawless vertebrates, and diverged from jawed vertebrates (gnathostomes) over 500 million years ago. Previous developmental studies on the cyclostome brain were conducted mainly in lampreys because hagfish embryos were rarely available. Although still scarce, the recent availability of hagfish embryos has propelled comparative studies of brain development and gene expression. By integrating findings with those of cyclostomes and fossil jawless vertebrates, we can depict the morphology, developmental mechanism, and even the evolutionary path of the brain of the last common ancestor of vertebrates. In this review, we summarize the development of the forebrain in cyclostomes and suggest what evolutionary changes each cyclostome lineage underwent during brain evolution. In addition, together with recent advances in the head morphology in fossil vertebrates revealed by CT scanning technology, we discuss how the evolution of craniofacial morphology and the changes of the developmental mechanism of the forebrain towards crown gnathostomes are causally related.

Introduction

During development, in most jawed vertebrates (or gnathostomes), the anterior end of the neural tube primarily differentiates into three brain vesicles: forebrain, midbrain, and hindbrain. Subsequently, the forebrain and hindbrain are further subdivided into two subregions each, the telencephalon and diencephalon the former, and the metencephalon and myelencephalon the latter, therefore resulting in a five-vesicle brain. This stage in

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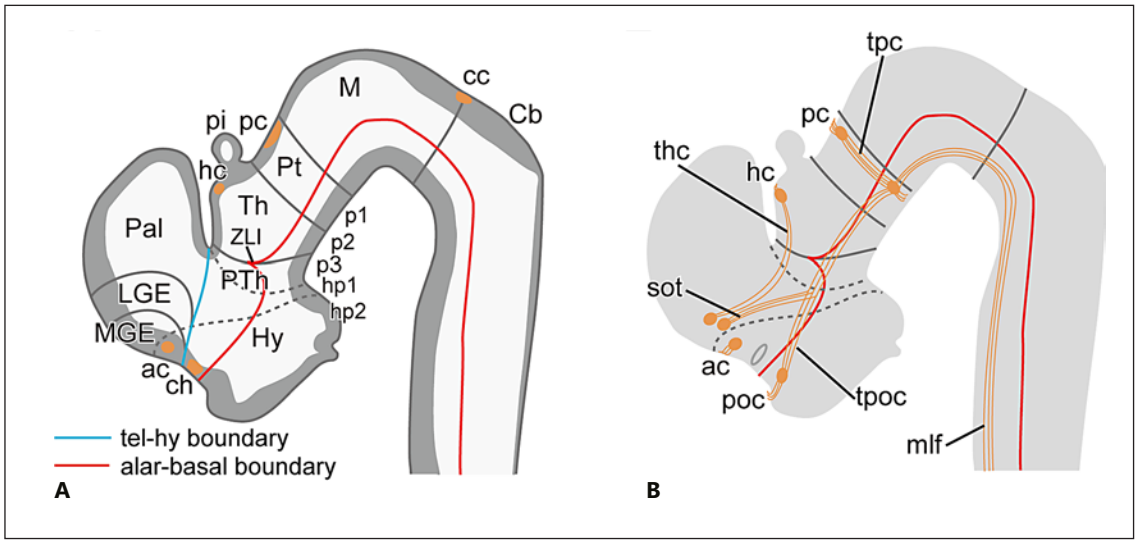

Fig. 1. Brain organization of the jawed vertebrates. **A** Embryonic brain architecture of the cloudy catshark, *Scyliorhinus torazame*, as representative of jawed vertebrates, based on the gene expression patterns and axon bundles reported in Sugahara et al. [2016]. Dashed lines indicate postulated borders of hypothalamo-telencephalic prosomeres [Puelles and Rubenstein, 2003, 2015]. The red line indicates the alar-basal boundary; the blue line indicates the telencephalo-hypothalamic boundary from the prosomeric model. **B** Nerve tracts and commissures in the embryonic brain [Barreiro-Iglesias et al., 2008; Ware et al., 2015; Sugahara et al., 2016]. ac, anterior commissure; Cb, cerebellum; cc, cerebellar commissure; ch, optic chiasma; hc, habenula commissure; hp1 and 2, hypothalamo-telencephalic prosomeres; Hy, hypothalamus; LGE, lateral ganglionic eminence; M, mesencephalon; MGE, medial ganglionic eminence; mlf, medial longitudinal fascicle; p1–3, prosomeres; Pal, pallium; pc, posterior commissure; pi, pineal organ; poc, postoptic commissure; Pt, prethalamus; PTh, pretectum; sot, supraoptic tract; Th, thalamus; thc, tract of the habenular commissure; tpc, tract of the posterior commissure; tpoc, tract of the postoptic commissure; ZLI, zona limitans intrathalamica.

brain morphogenesis is widely conserved among all extant gnathostomes [Ishikawa et al., 2012]. Each primordium is further regionalized by differential, specific gene expression patterns (or genoarchitecture) induced by secreting signals that act as secondary organizers [Kiecker and Lumsden, 2005]. For instance, clear transverse segmental structures, called rhombomeres, appear along the anterior-posterior axis of the hindbrain, each one of them characterized by combinatorial, differential expression patterns of *Hox* genes and other transcription factors. Repulsive interaction between the Eph receptor and the ephrin ligand plays a crucial role for the lineage-dependent neuronal cell restriction of rhombomeres [Cooke et al., 2001]. Such antero-posterior regionalization is also found in the diencephalon, in this case into "prosomeres" [Puelles and Rubenstein, 1993; Rubenstein et al., 1994; Puelles, 1995]. According to this model, the diencephalon can be molecularly subdivided into three regions along the antero-posterior axis: the prosomeres 3, 2, and 1, from anterior to posterior. Each prosomere also consists of a basal and alar region. The alar subdivisions of these regions clearly correspond to the prethalamus (p3), thalamus (p2), and pretectum (p1), while the basal diencephalon mainly consists of the posterior tuberculum [Striedter and Northcutt, 2020] (Fig. 1A). The boundaries of each of these domains serve as scaffolds for axonal growth, leading to a conserved pattern of early axon scaffolds [Barreiro-Iglesias et al., 2008] (Fig. 1B).

The hypothalamus was traditionally classified as a ventral component of the diencephalon. However, it has recently been proposed that the hypothalamus is included in a region called the "secondary prosencephalon" together with the region of the telencephalon rostrodorsal to the rest of the diencephalon [Puelles and Rubenstein, 2003, 2015; Nieuwenhuys and Puelles, 2016]. The developmental basis for this terminology is that the diencephalic region is influenced by the notochord, while the secondary prosencephalon is influenced by the prechordal plate. According to this model, the dorsal part of the secondary prosencephalon is the telencephalon and its ventral part is the hypothalamus, which means that the hypothalamus can be interpreted as the most anterior region of the neural tube and the telencephalon as the dorsal expansion of the alar part of the secondary prosencephalon (Fig. 1A).

According to the current prosomeric model, the secondary prosencephalon is further divided into the hypo-

thalamo-telencephalic prosomere 1 (hp1) and hp2 (Fig. 1A) [Puelles et al., 2012; Puelles and Rubenstein, 2015]. The hypothalamic regions of HP1 and HP2 are called peduncular hypothalamus (PHy) and terminal hypothalamus (THy), respectively. In the telencephalon, HP1 occupies much of the pallium and subpallium, while HP2 includes the preoptic area and anterior commissure.

The telencephalon can be divided into two major regions, the pallium and subpallium. The former differentiates mainly into the olfactory bulb, hippocampus, cerebral cortex, and parts of amygdala, and the latter gives rise to the basal ganglia (striatum and pallidum), parts of amygdala and septum. The direction of this subdivision has been conventionally considered to be "dorso-ventral," yet, according to the prosomeric model, it should be "caudal-rostral." However, to avoid confusion in this review, the conventional terminology about telencephalic subdivision is included along with the terminology from the prosomeric model. The embryonic subpallium is further divided in to lateral and medial ganglionic eminences (LGE, MGE), giving rise to the striatum and pallidum (or globus pallidus), respectively. These subdomains are also known to be sources of migratory neurons. Namely, olfactory bulb interneurons arise from a subregion of the LGE and migrate rostrally [Waclaw et al., 2006], while cortical interneurons arise from the MGE and migrate tangentially to the cortex [Marin and Rubenstein, 2001].

When was the regionalized forebrain established in evolution? Since the forebrain regionalization is also found in cartilaginous fishes (Fig. 1) [Sugahara et al., 2016; Santos-Duran et al., 2018], it would have likely been present in the last common ancestor of crown jawed vertebrates. Amphioxus, a non-vertebrate chordate, has a small vesicle, called the cerebral vesicle, at the anterior end of the neural tube, which does not show apparent morphological regionalization as seen in vertebrates. However, a recent comprehensive gene expression study has revealed that the developing cerebral vesicle of amphioxus has two distinct regions: the rostral hypothalamo-prethalamic primordium (HyPTh), corresponding to the HP2, HP1, and prethalamus of vertebrates, and the caudal Di-Mesencephalic primordium (DiMes), corresponding to the vertebrate thalamus, pretectum, and midbrain [Albuixech-Crespo et al., 2017]. Altogether, the data from amphioxus show that a partial subdivision based on a differential molecular identity of the different brain regions probably predated the origin of the vertebrate forebrain, which would have been established before the split between cephalochordates and other chordates.

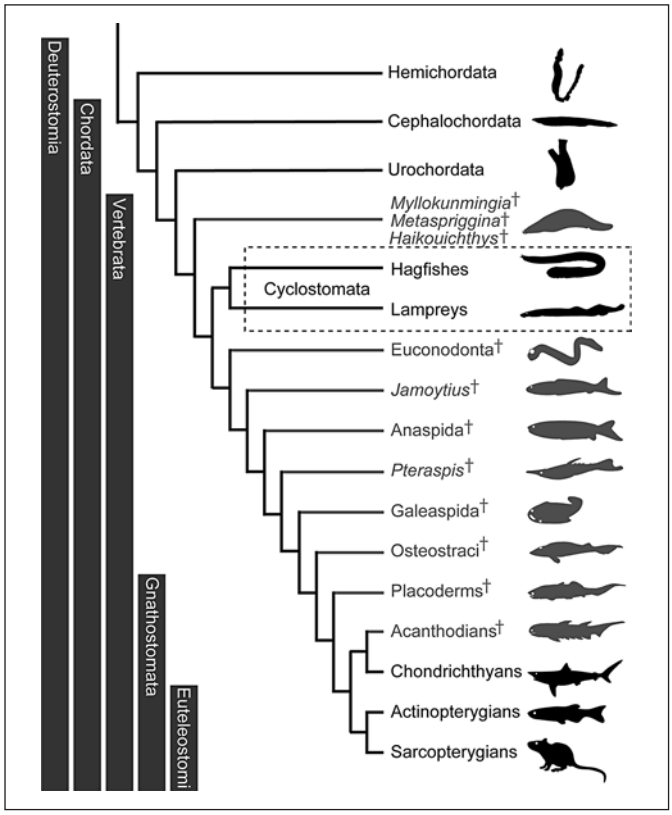

Fig. 2. Phylogeny of the major vertebrate lineages including fossil species. The tree is based on Morris and Caron [2014] for the fossil jawless vertebrates and Zhu et al. [2013] for the jawed vertebrates. Gray lines and "†" indicate extinct lineages. Note that the cyclostomes are a monophyletic group (lamprey and hagfish share a common ancestor).

Cyclostomes and Their Brains

Early vertebrates did not have an articulated jaw, hence they are called agnathans ("without mouth"). Most agnathan species are extinct; lampreys and hagfishes are the only surviving lineages, branching off from the rest of vertebrates more than 500 million years ago [Kuraku and Kuratani, 2006]. Based on molecular phylogenetic analyses they form a monophyletic group, the Cyclostomata (Fig. 2) [Kuraku and Kuratani, 2006; Heimberg et al., 2010]. Both lampreys and hagfish have a conserved program of craniofacial development [Oisi et al., 2013]. Cyclostomes are important model organisms for understanding the early vertebrate evolution since they are the most distantly related group to jawed vertebrates, and comparative analyses between the two are thus essential to infer the condition of the last common ancestor of crown vertebrates [Sugahara, 2021].

The external morphology of the lamprey brain is similar to that of teleost fishes in appearance (Fig. 3). However, lampreys do not have a proper cerebellum, but do have a "cerebellar plate" at the anterior end of the hindbrain which consists of small granule cells but no Purkinje cells [Striedter and Northcutt, 2020] (Fig. 3C). In the lamprey forebrain, an epiphysis (or pineal organ) is well developed, and each cerebral hemisphere is composed of an evaginated portion (laterally expanded) and a non-evaginated (medial) portion [Nieuwenhuys, 1966]. On the other hand, the hagfish brain shows a distinct morphology (Fig. 3). First, the olfactory bulb and cerebral hemisphere are significantly larger than its lamprey counterparts. Second, the epiphysis and cerebellum are missing. Finally, in the telencephalon, the lateral ventricles are rudimental, but there is a highly differentiated organization of the pallium [Conel, 1929]. These morphological oddities made the identification by comparative morphology of the brain regions of the adult hagfish brain very difficult [Conel, 1929].

To depict the ancestral brain regionalization and its developmental program, it is crucial to compare the gnathostome condition with both cyclostome lineages, because it enables us to exclude derived characters that are independently evolving in either the lamprey or hagfish lineages [Sugahara et al., 2017]. For example, a recent study has suggested that the hagfish single-canal inner ear is a derived feature, since early development of the inner ear in lampreys and gnathostomes share the same initial developmental patterning with two pillars [Higuchi et al., 2019]. Another example is that the hagfish lacks all extraocular muscles and innervating nerves (cranial nerves III, IV, and VI) as happens likewise in non-vertebrate chordates. However, since both of these features are evident in lampreys and gnathostomes, it is considered to be a secondary degeneration of the hagfish as a consequence of its deep-sea habitat devoid of light [Locket and Jørgensen, 1998].

Genoarchitecture of the Lamprey Forebrain

Developmental studies of the cyclostome brain have been conducted mainly in lampreys due to some advantages compared to hagfish regarding the obtainment of embryos. Fertilized lamprey eggs are easily obtained by artificial fertilization [Sugahara et al., 2015], and to better identify the different brain regions, embryos can be made transparent with a mix of Benzyl-alcohol and Benzyl-benzoate, what makes each brain region morphologically identifiable in similar ways to zebrafish embryos (Fig. 4A). This technique, combined with in situ hybridization of key markers, permits deciphering the genoarchitecture of the lamprey brain during early development.

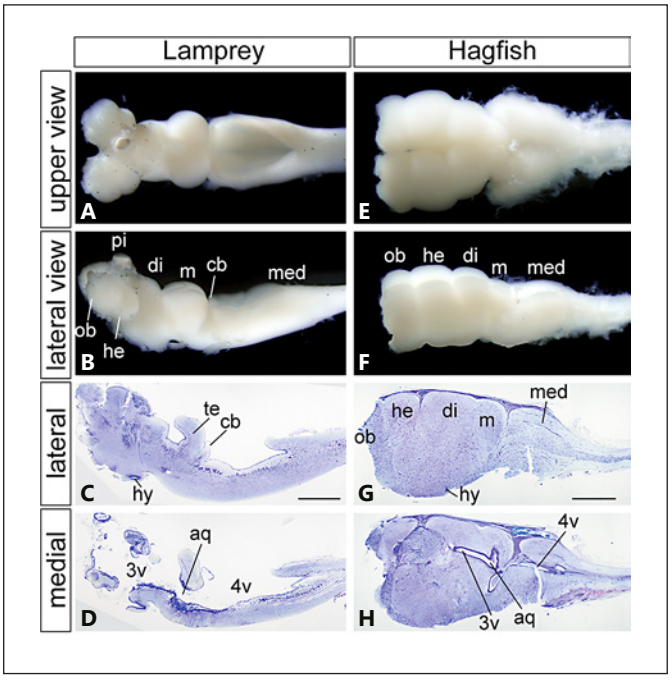

Fig. 3. Adult brain of cyclostomes. **A–D** Brain of Arctic lamprey, *Lethenteron camtschaticum*. **E–H** Brain of the inshore hagfish, *E. burgeri*. **C**, **D**, **G**, **H** Nissl staining of brain sections. Note that the lamprey has a small cerebellar plate caudal to the tectum, whereas the hagfish brain does not possess such a structure in the corresponding region. Brain ventricles are significantly reduced in the hagfish adult brain (**G**, **H**). aq, aqueduct of midbrain; ob, olfactory bulb; di, diencephalon; he, cerebral hemisphere; te, optic tectum; med, medulla oblongata; 3v, third ventricle; 4v, fourth ventricle. See Figure 1 for other abbreviations. Scale bars, 1 mm.

In the early neurula embryo of the lamprey, the *Hedgehog* (*Hh*) gene is expressed in the notochord and the prechordal plate (Fig. 4D), and *Nkx2.2*, a gene encoding for a transcription factor crucial for the formation of the alar-basal boundary, is expressed in the ventral neural tube, which is induced by Shh protein released from the underlying basal and floor plate longitudinal zones, whose *Shh* expression is activated by Shh released from the notochord [Sugahara et al., 2011]. At this stage, *Nkx2.2* expression extends to the anterior end of the neural tube (Fig. 4B). Subsequently, the anterior neural tube bends ventrally to form the cephalic flexure and the dorsal forebrain expands antero-dorsally. Along with these changes, *Hh* expression shows a transversal spike-like expression domain in the diencephalon, forming the *zona limitans intrathalamica* (ZLI; Fig. 4E). The ZLI marks the boundary between the prosomere 2 (thalamus) and 3 (prethalamus), and it is the

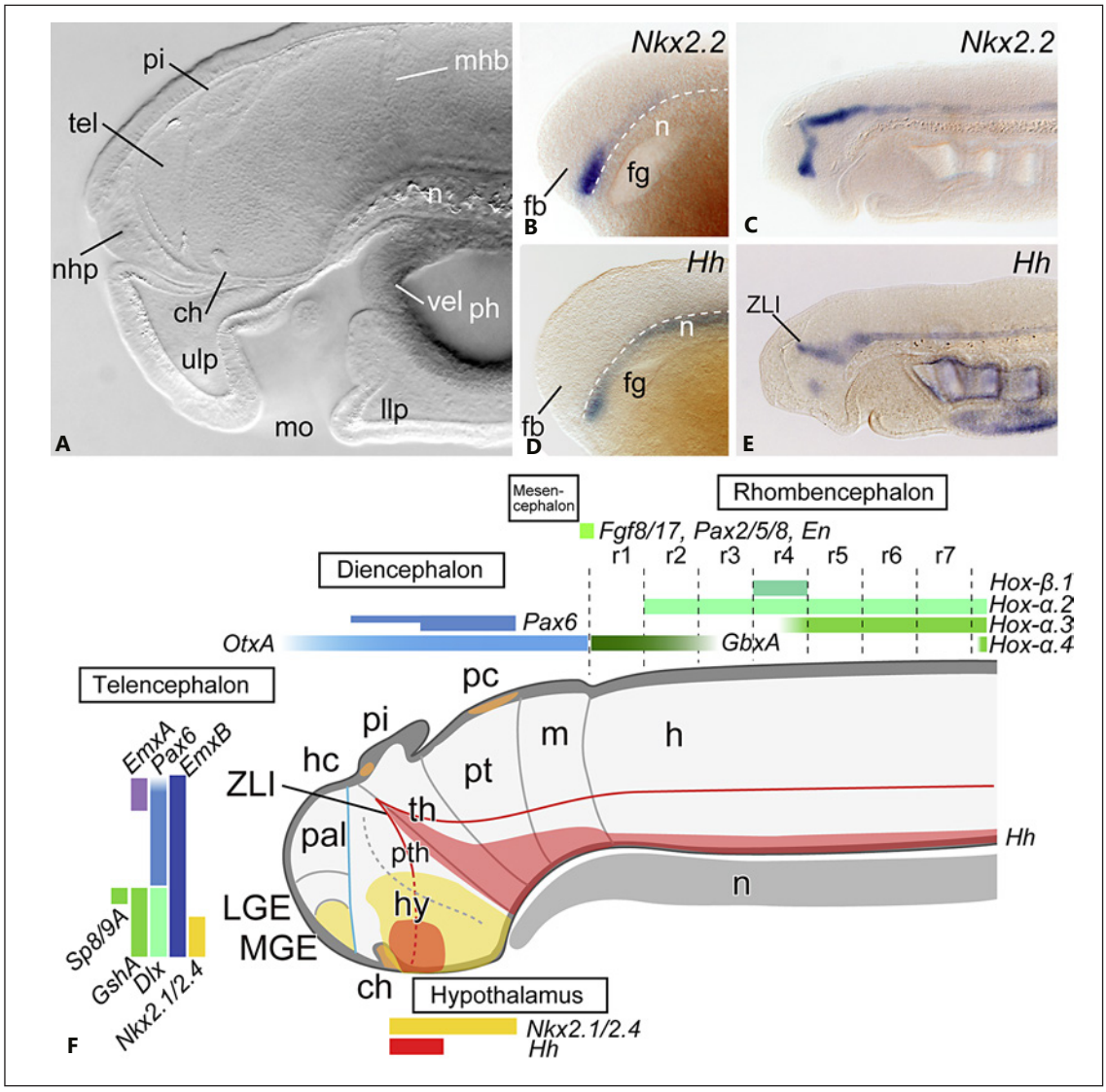

Fig. 4. Brain genoarchitecture of the lamprey *L. camtschaticum* embryo. **A** A DIC microscopy image of the head region of a lamprey larva at stage 27, cleared by BABB (1:2 mixture of Benzyl-alcohol and Benzyl-benzoate). *Nkx2.2* expression in the alar-basal position of the neural tube at stage 20 (**B**) and 26 (**C**). Dashed lines in **B** and **D** indicate the boundary between the neural tube and axial mesoderm. **D**, **E** *HhA* (one of four *Hedgehog* paralogs in lamprey) expression at stage 20 and 26. Note that *Hh* is only expressed at the notochord and prechordal plate in early neurogenesis (**D**). Later, *Hh* is seen in the floor plate, ZLI, and hypothalamus (**E**), while the notochordal *Hh* expression is downregulated [Sugahara et al., 2011]. **F** Regionalization of the lamprey brain revealed by gene expression studies cited in the main text and Takio et al. [2004] for the *Hox* genes in the rhombomeres. The red line indicates the alar-basal boundary; the blue line indicates the telencephalo-hypothalamic boundary from the prosomeric model. The gray dashed line indicates the rostral boundary of the prethalamus deduced from the adult lamprey [Pombal et al., 2011], which has not yet been well defined by gene expression in the embryo. fb, forebrain; fg, foregut; llp, lower lip; mo, mouth opening; n, notochord; nhp, naso-hypophyseal placode; ph, pharynx; tel, telencephalon; ulp, upper lip; vel, velum. See Figure 1 for other abbreviations.

Hh signaling emanating from it that controls this regionalization [Kiecker and Lumsden, 2005; Puelles and Martinez 2013]. In the mammalian diencephalon, Shh signaling is also involved in the arrangement of the thalamic progenitor cells [Vue et al., 2009]. In hemichordates, a sister group of chordates, there exists a similar gene expression pattern reminiscent of the ZLI signaling in the vertebrate diencephalon and was secondarily lost in non-vertebrate chordates [Pani et al., 2012], suggesting that this signaling center dates back to deuterostome ancestry.

Subdivision of the Lamprey Telencephalon

Previous expression analyses of *Pax6* and *Dlx* showed that the pallial-subpallial subdivision as seen in mammals exists in the lamprey embryonic telencephalon [Murakami et al., 2001] (Fig. 4F). In addition, *EmxA* is expressed in a subdomain of the pallium, suggesting that at least a rostro-caudal subdivision (dorso-ventral according to the conventional columnar model) of the pallium exists [Murakami et al., 2001; Myojin et al., 2001]. On the other hand, from recent cytoarchitecture and synaptic connectivity studies of the adult lamprey brain, the lateral portion of the evaginated pallium has been suggested to be homologous to the mammalian cortex [Ocaña et al., 2015; Suryanarayana et al., 2017].

In the gnathostome subpallium, *Gsh2* is crucial for the development of the LGE [Toresson and Campbell, 2001; Yun et al., 2001]. Also, *Isl1* is required for the differentiation of the striatal projection neurons [Wichterle et al., 2001]. Both of these genes are expressed in the lamprey subpallium, suggesting that the LGE is present also in the lamprey telencephalon (Fig. 4) [Sugahara et al., 2011]. Furthermore, the gene for the zinc finger transcription factor *Sp8*, which regulates the generation of olfactory bulb interneurons [Waclaw et al., 2006], was found to be expressed in a distinct region of the lamprey LGE, suggesting that a caudal (dorsal according to the conventional columnar model) subdomain of the LGE (dLGE) might be present in lampreys as in gnathostomes [Sugahara et al., 2011, 2013].

The identity of the MGE is controlled by the transcription factor encoded by *Nkx2.1*, the expression of which is maintained by *Shh* in mammals [Fuccillo et al., 2006]. Traditionally, the MGE has been considered to be absent in the lamprey since *Nkx2.1* and *Hh* gene expressions were hitherto unreported in the lamprey subpallium [Ogasawara et al., 2001; Uchida et al., 2003; Osorio et al., 2005; Sugahara et al., 2011], suggesting a gnathostome origin after the split of cyclostomes [Murakami et al., 2001; Osorio et al., 2005; Sugahara et al., 2011]. This was in disagreement with physiological studies that suggested the presence of a pallidum-like region in the adult lamprey [Stephenson-Jones et al., 2011]. Also, Dlx- and GABA-immunoreactive cells were found in the pallium of the lamprey brain, implying the migration of the MGE-derived GABAergic interneurons from the subpallium to the pallium [Martinez-de-la-Torre et al., 2011; Pombal et al., 2011].

To address the above controversy, we cloned all *Nkx2.1* paralogous genes and observed their expression across a wide range of developmental stages, taking into account the possibility of an heterochronic delay of the MGE development in the lamprey [Richardson et al., 2010; Sugahara et al., 2017]. The result showed that two of the three *Nkx2.1* paralogues were expressed within the lamprey subpallium, just before the respiration stage (Fig. 5) [Sugahara et al., 2016]. In other words, our gene marker expression analysis, together with previous physiological and histological works, demonstrated that an MGE region is present in the lamprey telencephalon, and that it develops relatively late as compared to its gnathostome counterpart [Sugahara et al., 2017]. On the other hand, none of the *Hh* paralogs were expressed in the MGE, suggesting that either the specification of the lamprey MGE is somehow divergent, or an undiscovered *Hh* paralog may be expressed there. Regarding the hypothalamo-telencephalic prosomeres (HP1/2) in the secondary prosencephalon, it has been proposed that they exist in the adult brain [Pombal et al., 2009]. In the future, detailed genetic analyses of the hypothalamus in lamprey embryos will elucidate the presence of the specification mechanism of this regionalization.

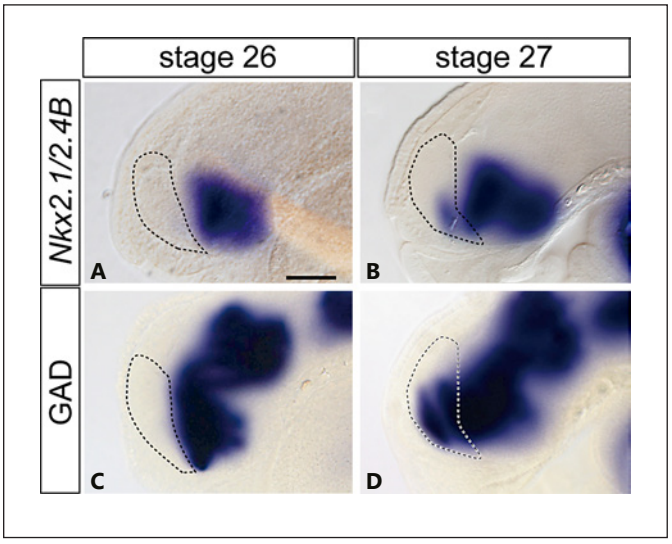

Fig. 5. Late emergence of the MGE in the lamprey telencephalon. Dashed circles are telencephalic territories. At stage 26, *Nkx2.1/2/4B* are not expressed (**A**) but evident at stage 27 within the subpallium (**B**). The timing of *GAD* (glutamate decarboxylase) expression is coincident with the *Nkx2.1/2.4B* expression in the telencephalon (**C**, **D**). Scale bar, 50 μm.

Genoarchitecture of the Hagfish Embryonic Forebrain

Historically, the brain development of the hagfish was first described by von Kupffer [1899; 1900; 1906]. He utilized embryonic specimens of the Pacific hagfish, *Bdellostoma stoutii* (nowadays *Eptatretus stoutii*), originally prepared by Bashford Dean [1899] from Monterey Bay, and tried to identify brain regions by using alcohol-fixed

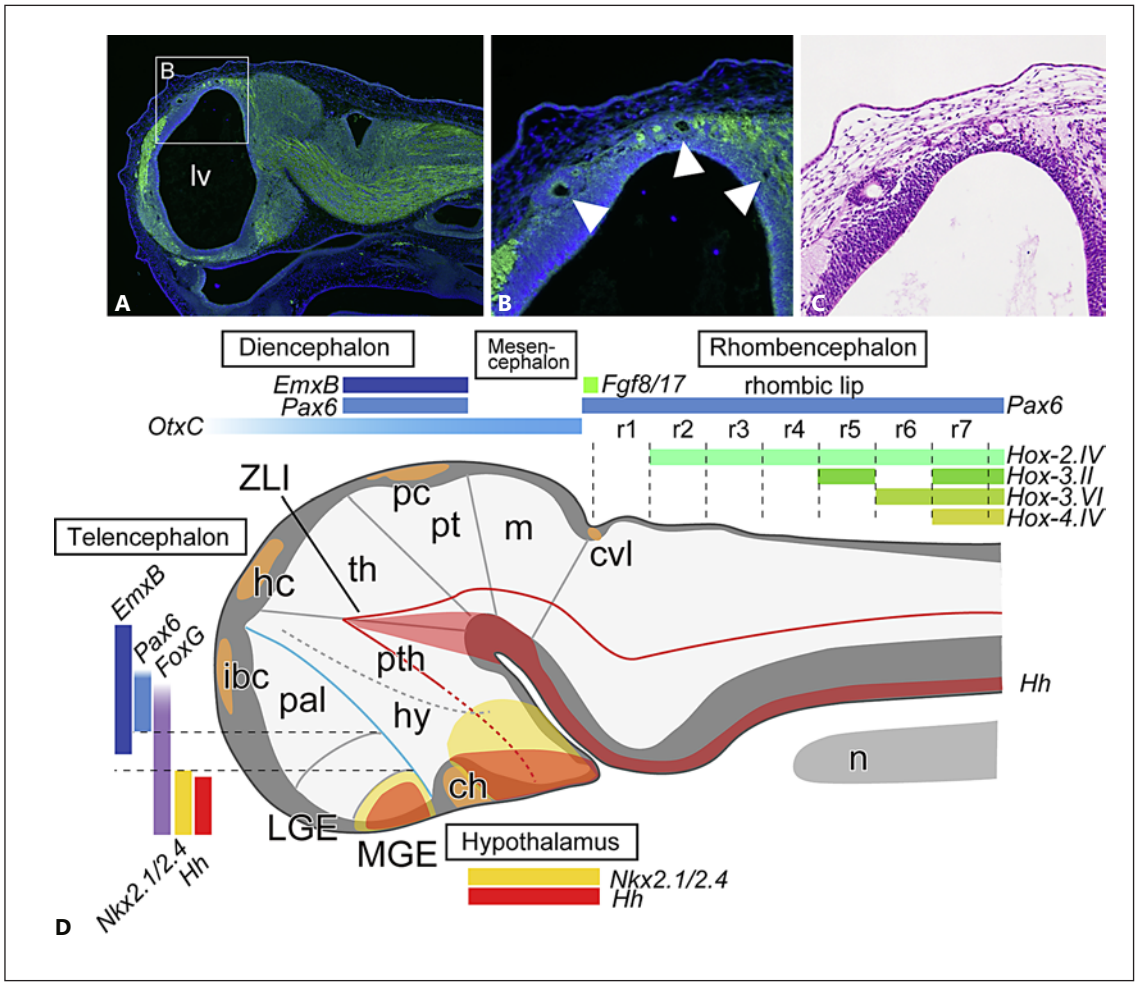

Fig. 6. Brain genoarchitecture of the hagfish *E. burgeri* embryo. **A** Head region of a hagfish stage 53 embryo, immunostained by anti-acetylated tubulin (green) and DAPI (blue). **B** High magnification image of **A**. The arrowheads indicate neuroepithelial cysts, which might have been misidentified as the epiphysis in a previous study [von Kupffer, 1906]. **C** Bright field image of **B** stained with hematoxylin and eosin. **D** Regionalization of the hagfish brain revealed by gene expression patterns [Sugahara et al., 2016; Pascual-Anaya et al., 2018]. The gray dashed line indicates the rostral boundary of the prethalamus deduced from the adult lamprey [Pombal et al., 2011], which has not yet been well defined by gene expression in the hagfish embryo. Note that anterior end of the notochord is secondarily posteriorized in this stage [Oisi et al., 2013]. cvl, commissura vestibulo-lateralis; ibc, interbulbar commissure; lv, lateral ventricle. See Figure 1 for other abbreviations.

embryos. However, since the embryos were fixed while within their hard eggshell, they were actually kept severely deformed, leading to several long-standing misunderstandings. For example, the adenohypophysis was thought to have originated in the endoderm because the primary oropharyngeal membrane was destroyed during fixation and instead the distal part of the oral cavity is closed secondarily [Gorbman, 1983; Oisi et al., 2013]. In the embryonic brain, von Kupffer described an epiphysis (or pineal organ), which is not seen in the adult hagfish [von Kupffer, 1900]. It is now considered that what von Kupffer described was a neural tissue fragment that was detached from the brain during fixation [Sugahara et al., 2016] (Fig. 6A–C).

Since most hagfish species dwell in deep-sea habitats, it has traditionally been extremely difficult to obtain embryos. However, since 2006, our group has continuously obtained a few fertilized eggs of the inshore hagfish, *Eptatretus burgeri* [Ota et al., 2007; Oisi et al., 2015]. This has enabled us to analyze key gene expression patterns in the embryonic brain of the hagfish. For instance, the telencephalic territory of the hagfish embryo is identified as

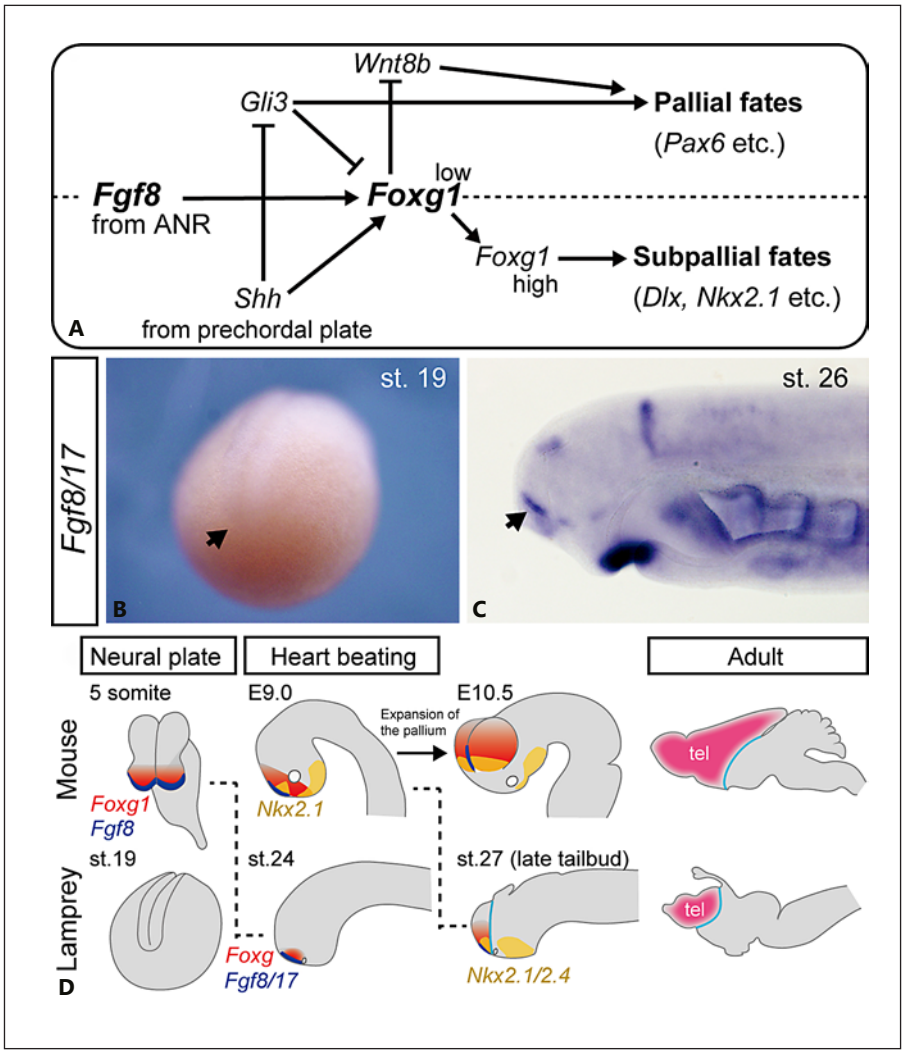

Fig. 7. ANR signaling for forebrain patterning. **A** Regulatory network controlling telencephalic identity in the vertebrate forebrain, based on Danesin et al. [2009]. *Fgf8* to *Foxg1* signaling in the early neurula stage is the key for the telencephalic regionalization. Note that the rostralizing (ventralizing according to the conventional columnar model) factor for the telencephalon is not the notochordal-derived Shh but prechordal Shh [Fuccillo et al., 2006]. This prechordal Shh-mediated axis patterning is conserved in lamprey [Sugahara et al., 2011]. **B** Lamprey embryo at stage 19. *Fgf8/17* could not be observed at the ANB in the neurulation stage (arrow). **C** Lamprey larva at stage 26. From the mid pharyngula stage (after stage 24, not shown), *Fgf8/17* is detectable in the anterior end of the forebrain (arrow). **D** Comparison of telencephalic development between mouse and lamprey. Dashed lines indicate similar telencephalic gene expression patterns between them.

a *Pax6*-, *FoxG*-, and *EmxB*-expressing domain [Tank et al., 2009; Noro et al., 2015; Sugahara et al., 2016]. In gnathostomes, the pretectum (p1) is defined by the presence of the posterior commissure and *Pax6* expression [Watson et al., 2012], features that we found in the hagfish [Sugahara et al., 2016]. In addition, the expression pattern of hagfish *Hh* clearly marked the very characteristic position of the ZLI. Given the regionalizing activity of Shh secreted from ZLI in gnathostomes [Kiecker and Lumsden, 2005; Martinez-Ferre et al., 2013], its presence in hagfish is consistent with the existence of the boundary between the prethalamus (p3) and thalamus (p2; Fig. 6). As for the pineal organ, since the lamprey possesses this organ, there is no doubt that the common ancestor of the extant vertebrates had it. However, as mentioned above, the pineal organ has not been found in the adult hagfish (Fig. 6). So far, it is unknown whether a regionalization of the pineal organ is present during embryogenesis, although the habenula commissure is present in the epithalamus. Studies using appropriate regional molecular markers will be necessary to confirm its presence or secondary absence.

In the subpallium, although the pallidum has not been identified in adult hagfish [Wicht and Northcutt, 1994; Wicht and Nieuwenhuys, 1998], a domain co-expressing *Nkx2.1* and *Hh* was found within the subpallium, corresponding to a part of the MGE and preoptic area at late embryogenetic stages of *E. burgeri* [Sugahara et al., 2016], possibly associated with an LGE adjacent to it (Fig. 6D).

Expression Timing of the Signaling Center and Telencephalic Development

The anterior neural ridge (ANR) or border (ANB) is one of the crucial signaling centers for the forebrain de-

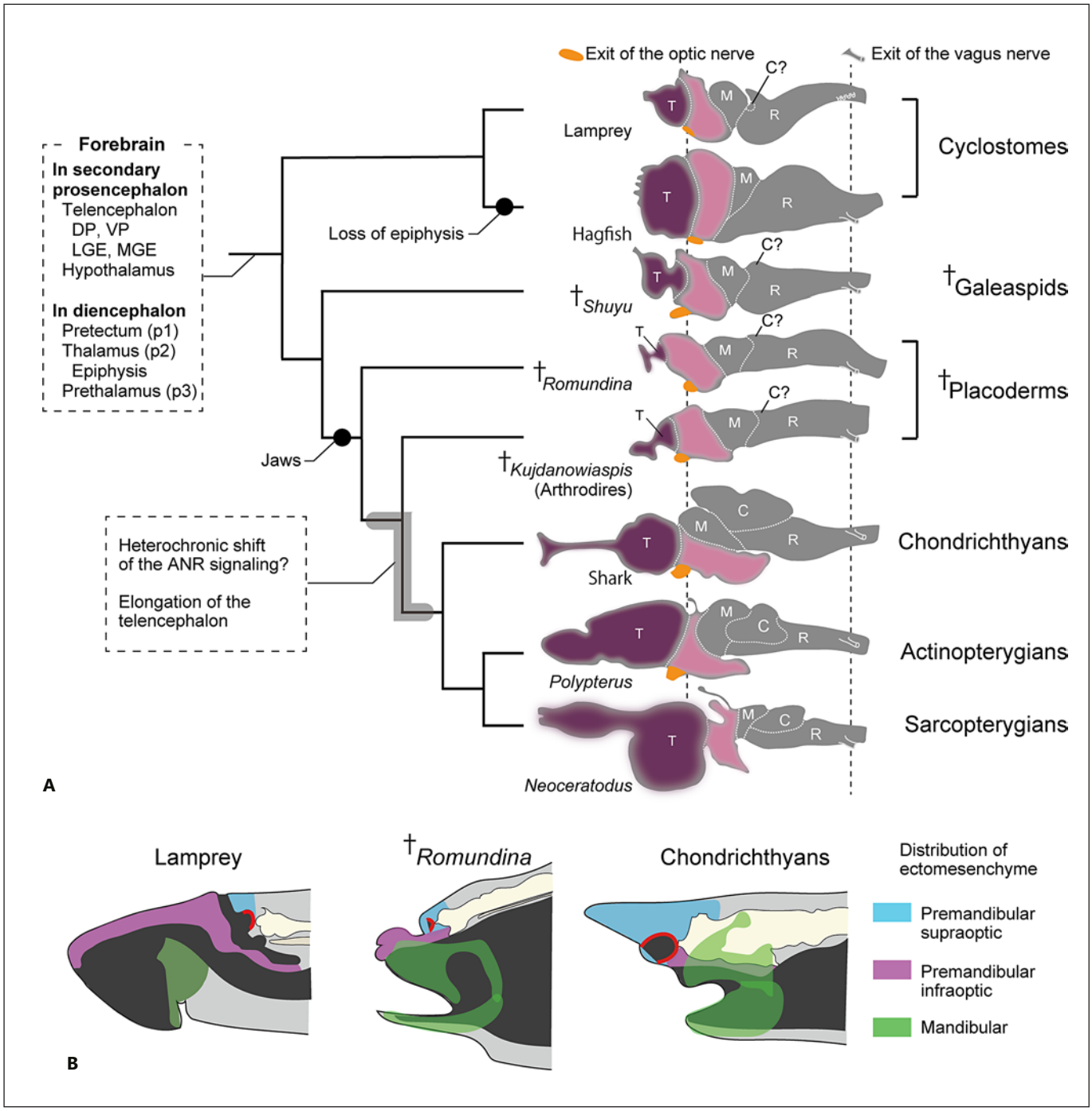

Fig. 8. Evolutionary transition of the forebrain and craniofacial morphology. **A** A phylogenetic tree of vertebrate including fossil species. Brains are aligned on the exits of the optic nerve and vagus nerve. **B** Comparison of the craniofacial morphology and distribution of the ectomesenchyme, modified from Dupret et al. [2014]. Light purple indicates the upper lip or trabeculae region (premandibular infraoptic ectomesenchyme is distributed), light blue indicates the nasal and rostrum region (supraoptic ectomesenchyme), green indicates the lower lip or jaws (mandibular ectomesenchyme), red indicates nasal capsule. Note that although *Romundina* were jawed vertebrates, their brain, craniofacial morphology, and presumptive distribution pattern of the mesenchyme are similar with those of cyclostomes. DP, dorsal pallium; VP, ventral pallium (note that these were named according to the conventional columnar model).

velopment [Echevarria et al., 2003]. In gnathostomes, *Fgf8* is initially expressed at the ANR, in the rostral margin of the neural plate. Subsequently, *Foxg1*, a forkhead box transcription factor induced by *Fgf8*, regulates both the telencephalic cell proliferation and the subsequent neurogenesis [Shimamura and Rubenstein, 1997; Echevarria et al., 2003; Paek et al., 2009]. Also, *Foxg1* directly induces subpallial identity (Fig. 7A) [Danesin et al., 2009; Manuel et al., 2010] as well as the differentiation of pallial projection neurons [Kumamoto et al., 2013]. In tunicates, the sister group of vertebrates, *Foxg* induced by MAPK signaling specifies sensory neurons in the ANB [Liu and Satou, 2019]. Moreover, hemichordates possess similar gene expression patterns in their rostral ectoderm [Pani et al., 2012], suggesting a deep deuterostome ancestry of this genetic program.

As mentioned above, the onset of expression of *Fgf8* and *Foxg1* are relatively early in gnathostome CNS development. For example, *Fgf8* and *Foxg1* expression onset is at 5 somite (open neural plate) stage in mice [Shimamura and Rubenstein, 1997] or 12 somite stage in zebrafish [Thisse et al., 2001; Walshe and Mason, 2003]. Interestingly, lamprey *Fgf8/17* is not expressed during the early neurulation stage [Guerin et al., 2009] (Fig. 7B) but later, and only in the surface ectoderm where the nasal placode is formed. Subsequently, *Fgf8/17* expression is seen in the telencephalon adjacent to the nasal placode at mid pharyngeal stage (stage 24) [Sugahara et al., 2011] (Fig. 7C). Also, the onset of lamprey *Foxg1* expression is significantly late, consistent with the *Fgf8/17* expression [Ermakova et al., 2019]. In the hagfish, the rostral end of the telencephalon remains negative for *Fgf8/17* even after the neural tube closure (see extended data Fig. 3 in Sugahara et al. [2016]).

Regarding telencephalic subdivisions, telencephalic cells in *Foxg1*$^{-/-}$ mice are incompetent to generate the subpallium, suggesting that *Foxg1* regulates directly telencephalic cells to adopt subpallial fates [Manuel et al., 2010]. As mentioned above, the onset of *Nkx2.1* expression is relatively late in lampreys (stage 27; Fig. 5) [Tahara, 1988; Sugahara et al., 2016]. For instance, in mice and zebrafish *Nkx2.1* is expressed as early as stages E8.5 and 24 hpf, respectively, the stages at which the heart beating commences [Kimmel et al., 1995; Shimamura et al., 1995; Thisse et al., 2001; Chen et al., 2010] (Fig. 7D). Also in hagfish, *Nkx2.1* is not detected in the telencephalon even after stage 45 [Dean 1899], when the heart starts to beat, and then it is found at stage 53 [Sugahara et al., 2016]. Altogether, the late expression of ANR signaling factors and the subsequent subpallial regionalization might be shared in cyclostomes.

Relationship between Forebrain and Craniofacial Morphology

What are the changes in the early evolution of the vertebrate brain caused by the above-mentioned differences in the timing of genetic signaling? The morphology of the brain might be related not only to the shape of the cranium that covered it, but also the craniofacial morphology, including the distribution of ectomesenchyme and sensory organs [Kuratani et al., 2001; Kuratani, 2012]. Although the brain is a soft tissue that is difficult to see fossilized, the endocast of the cranium sometimes reflects the brain morphology. Generally, it is difficult to identify the brain morphology in anamniotes because the space between the brain and the internal surface of the skull is often occupied with fat tissues in extant lampreys or teleost fish. However, there are reports of some well-preserved endocasts from early fishes indicating the morphologies of their contained brain [Stensiö, 1927].

Recent progress in synchrotron radiation X-ray tomographic microscopy has also made it possible to reconstruct the brain morphology of early vertebrates without damaging the fossils. For example, a Silurian jawless vertebrate, *Shuyu*, has a brain similar to lampreys, but the hypophyseal duct opens towards the oral cavity, and the nasal sacs are paired like in gnathostomes [Gai et al., 2011]. An early placoderm fish, *Romundina*, is one of the earliest jawed vertebrates, from the Early Devonian period (410–419 mya). This species differs from other jawed vertebrates in that the nostrils are located proximally between the eyes, and a large "upper lip" region is located at the rostral position, where premandibular neural crest cells occupy the mesenchyme, like in cyclostomes [Oisi et al., 2013; Dupret et al., 2014]. This means that, although *Romundina* has an articulated jaw, the proportions of other head morphologies are reminiscent of those of cyclostomes rather than gnathostomes (Fig. 8). Interestingly, synchrotron radiation X-ray tomographic microscopy has revealed that *Romundina*'s telencephalon is extremely short along the longitudinal axis and the hypophysis is located more anteriorly than in other jawed vertebrates [Dupret et al., 2014]. This characteristic proportion is shared with lamprey and hagfish. In other words, the morphology of the head of early jawed vertebrates is closely related to the size and proportion of the forebrain, suggesting that the extension of the forebrain might have been the driving force behind the evolution of the head morphology towards gnathostomes (Fig. 8).

Is there a relationship between the size of the telencephalon and the ANR signaling center? In *Foxg1*$^{-/-}$

mutant mice, the telencephalon is substantially reduced in size [Hanashima et al., 2004]. Temporal inactivation and delayed activation experiments of *Foxg1* in mice suggest that *Foxg1* is a crucial initiator of production of neocortical neurons [Kumamoto et al., 2013; Toma et al., 2014]. It is difficult to elucidate the ancestral state of the timing of the ANR signaling in the common ancestor of vertebrates (early or late) without an appropriate outgroup. However, if we assume the ancestry of cyclostome condition, it is possible that heterochronic changes might have occurred during the early gnathostome evolution that led to the expansion of the forebrain territory. Namely, a shift of the ANR signaling towards early embryonic stages might increase the telencephalic neuron precursors prior to the formation of the skull, and also enable the production of sequential differentiation of the cortical neuron subtypes [Kumamoto et al., 2013; Toma et al., 2014] (Fig. 8). Functional genetic approaches in lamprey thus might provide insights into the developmental transition towards crawn gnathostome.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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F.S. and Y.M. wrote the first draft; all authors then contributed to editing the manuscript.

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