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Involvement of Hedgehog and FGF signalling in the lamprey telencephalon: evolution of regionalization and dorsoventral patterning of the vertebrate forebrain

Fumiaki Sugahara[1,2], Shin-ichi Aota[1], Shigehiro Kuraku[3], Yasunori Murakami[4], Yoko Takio-Ogawa[1], Shigeki Hirano[5] and Shigeru Kuratani[1,*]

SUMMARY

Dorsoventral (DV) specification is a crucial step for the development of the vertebrate telencephalon. Clarifying the origin of this mechanism will lead to a better understanding of vertebrate central nervous system (CNS) evolution. Based on the lamprey, a sister group of the gnathostomes (jawed vertebrates), we identified three lamprey *Hedgehog* (*Hh*) homologues, which are thought to play central signalling roles in telencephalon patterning. However, unlike in gnathostomes, none of these genes, nor *Lhx6/7/8*, a marker for the migrating interneuron subtype, was expressed in the ventral telencephalon, consistent with the reported absence of the medial ganglionic eminence (MGE) in this animal. Homologues of *Gsh2*, *Isl1/2* and *Sp8*, which are involved in the patterning of the lateral ganglionic eminence (LGE) of gnathostomes, were expressed in the lamprey subpallium, as in gnathostomes. Hh signalling is necessary for induction of the subpallium identity in the gnathostome telencephalon. When Hh signalling was inhibited, the ventral identity was disrupted in the lamprey, suggesting that prechordal mesoderm-derived Hh signalling might be involved in the DV patterning of the telencephalon. By blocking fibroblast growth factor (FGF) signalling, the ventral telencephalon was suppressed in the lamprey, as in gnathostomes. We conclude that Hh- and FGF-dependent DV patterning, together with the resultant LGE identity, are likely to have been established in a common ancestor before the divergence of cyclostomes and gnathostomes. Later, gnathostomes would have acquired a novel *Hh* expression domain corresponding to the MGE, leading to the obtainment of cortical interneurons.

KEY WORDS: DV patterning, Telencephalon, Lamprey

INTRODUCTION

The vertebrate telencephalon represents a highly sophisticated portion of the central nervous system (CNS). One component, the neocortex with its multilayered cortical neurons, can perform complicated processing of information, especially in mammals. Although the amphioxus *FoxG1*, considered a telencephalic marker in vertebrates, is expressed in a few cells in the cerebral vesicle (Toresson et al., 1998), detailed neuroanatomy supports the idea that a homologue of the vertebrate telencephalon is lacking in cephalochordates and urochordates (Wicht and Lacalli, 2005). How the telencephalon was obtained in evolution remains elusive, and this question is tightly linked with that of the origin of vertebrates per se. As a sister group of the jawed vertebrates (gnathostomes), lampreys serve as a valuable model system for studying the evolution of the telencephalon. This is because this animal lineage appears to have diverged in the earliest phase of vertebrate evolution and probably possesses ancient developmental features that have been modified or lost in gnathostomes (Kuratani and Ota, 2008; Murakami and Kuratani, 2008).

The vertebrate telencephalon is divided dorsoventrally into the pallium and subpallium. Of these, the subpallium is crucial because it not only differentiates into the basal ganglia but also serves as the source of various types of neurons (Moreno et al., 2009). This structure is further divided into lateral and medial ganglionic eminences (LGE and MGE, respectively). The MGE is specifically known to be the source of cortical interneurons, whereas the LGE gives rise to striatal projection neurons and olfactory bulb interneurons. The MGE produces GABAergic interneurons (Wichterle et al., 2001); curiously, precursors of these interneurons show active migration in gnathostome embryos, migrating along the tangential pathway to populate the cortex to yield most of the interneurons (Marin and Rubenstein, 2001). Although it is unknown whether the shark has an equivalent MGE region in the embryonic brain, migratory neuroblasts have been observed in this animal (Carrera et al., 2008). In the lamprey, the expression patterns of *Pax6*, *Emx* and *Dlx* homologues suggest that the embryonic brain also appears to possess a distinction equivalent to that found between the pallium and subpallium (Murakami et al., 2001) (see Fig. S4 in the supplementary material). Furthermore, based on the expression pattern of the *Emx* homologue, the pallium can be further divided into at least two domains that are specified dorsolaterally (Murakami et al., 2001). In addition, because the *Nkx2.1* (also known as *TTF-1*) expression domain is absent in the subpallium (Ogasawara et al., 2001) and no *Hh* cognates are expressed in the telencephalon (Uchida et al., 2003; Osorio et al.,

[1]Laboratory for Evolutionary Morphology, Center for Developmental Biology (CDB), RIKEN, 2-2-3 Minatojima-minami, Kobe 650-0047, Japan. [2]Graduate School of Science, Kobe University, Kobe 657-8501, Japan. [3]Laboratory for Zoology and Evolutionary Biology, Department of Biology, University of Konstanz, Universitätsstrasse 10, 78464 Konstanz, Germany. [4]Graduate school of Science and Engineering, Ehime University, 2-5, Bunkyo-cho, Matsuyama, 790-8577, Japan. [5]Department of Medical Technology, School of Health Sciences, Faculty of Medicine, Niigata University, Niigata 951-8518, Japan.

*Author for correspondence (saizo@cdb.riken.jp)

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2005), the lampreys were assumed to lack a domain equivalent to the gnathostome MGE (Murakami et al., 2005). These traits pose a few questions regarding forebrain patterning in the lamprey. Does the lamprey really lack the MGE domain together with the *Hh-Nkx2.1* expression domain (i.e. no other Hh genes expressed)? If so, which developmental and neurological functions does the lamprey subpallium possess? Finally, are there any differences between the lamprey and gnathostomes in the developmental mechanisms involved in the dorsoventral (DV) patterning of the telencephalic region?

In the present study, we aimed to isolate all the *Hh* paralogues from the lamprey, as well as those of *Ptc*, the genes encoding the suspected Hh protein receptors, to clarify whether the lamprey embryonic telencephalon exhibits functional *Hh* expression domains. We also characterized the gene expression profile of the forebrain to detect any LGE-associated properties in the lamprey subpallium. Finally, we performed inhibitory experiments for Hh and fibroblast growth factor (FGF) signalling using inhibitors to detect changes in developmental patterning in the lamprey telencephalon. We confirmed that there are no *Hh* expression domains at any of the observed stages, and the expression patterns of *Gsh2*, *Isl1/2* and *Sp8* suggest the presence of an LGE-like property of the lamprey subpallium. We also showed that although the lamprey subpallium does not possess any *Hh* expression domains, Hh signalling functions in the early DV patterning of the telencephalon at specific developmental stages, followed by involvement of FGF signalling. Thus, we have reconstructed an evolutionary scenario for the vertebrate telencephalon in terms of changes introduced to the developmental systems of ancestral animal lineages during evolution.

MATERIALS AND METHODS

Animals

Adult lampreys, *Lethenteron japonicum*, were collected from Miomote River, Niigata and Shiribetsu River, Hokkaido, Japan, during the breeding season (early June). Eggs were fertilized artificially and incubated in 10% Steinberg's solution (Steinberg, 1957) at 16-23°C. Lamprey embryos were staged according to Tahara's staging of *Lethenteron reissneri* (Tahara, 1988). For in situ hybridization, the embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), then dehydrated in a methanol dilution series and stored in 100% methanol at –20°C.

cDNA cloning and sequencing

Total RNA of *L. japonicum* was extracted from whole embryos of stages 25-27 using TRIZOL reagent (Invitrogen). Degenerate reverse transcription polymerase chain reaction (RT-PCR) was performed to amplify fragments of respective genes. For the *LjHh* genes, the degenerate primers 5′-GARGGNTGGGAYGAYGAYGGNCAYCA-3′ (forward) and 5′-ATGC-GGTACAGCAGWCGCGAGTACCARTG-3′ (reverse) were designed based on the amino acid sequences EGWDEDGHH and HWYSRLLYRI, respectively. For *LjIsl1/2* genes, the degenerate primers 5′-TTCAGC-AAGACGGACTTCGTNATG-3′ (forward) and 5′-CGCGAACTCGCT-GAGSGTYTTCCA-3′ (reverse) were designed based on the amino acid sequences FSKTDFVM and WKTLSEFA, respectively. For the *LjGliA*, *LjSp8/9A and LjNkx2.2* genes, we used specific primers designed based on the nucleotide sequence of putative orthologue sequences found in the draft genome obtained by the *Petromyzon marinus* genome project (http://genome.wustl.edu/pub/organism/Other_Vertebrates/Petromyzon_marinus/). The *LjPtcA* gene was obtained using specific primers based on the nucleotide sequence of *P. marinus Patched* (Hammond and Whitfield, 2006). The PCR products were cloned into the pCRII-TOPO vector (Invitrogen). Amplified fragments were sequenced with the 3130 Sequence Analyzer (Applied Biosystems). The 5′ and 3′ ends were amplified with the GeneRacer Kit (Invitrogen). The cDNA sequences identified here have been deposited in GenBank under accession numbers AB583548-AB583554. Cloning of *LjSproutyA* (AB586026) and *LjLhx6/7/8A* (AB498801) will be published elsewhere.

In situ hybridization

Whole-mount in situ hybridization was performed as described previously (Takio et al., 2007) with minor modifications. Hybridization and post-hybridization washes were performed at 70°C to avoid non-specific cross-hybridization among paralogues. Some embryos were embedded in Tissue-Tek compound (Sakura, Japan) and sectioned using a cryostat microtome (HM505E; MICROM, Germany). Section in situ hybridization was performed in a Ventana automated machine (Roche).

Treatment of embryos with chemical inhibitors

Cyclopamine (Calbiochem) was dissolved in ethanol (EtOH) at 20 mM. SU5402 (Calbiochem) was dissolved in DMSO at 10 mM. Embryos were punctured with sharpened forceps and treated with 100 μM cyclopamine at 23°C or with 20 μM SU5402 at 16°C. Treatments were performed in 12-well plates, 50 embryos per well, in 1 ml of 10% Steinberg's solution. No effects were observed by exposure to DMSO or EtOH vehicle alone at the same concentrations used for the experimental treatments. Treated embryos were collected and fixed in 4% paraformaldehyde in PBS for in situ hybridization and sectioning.

RESULTS

Identification of the lamprey Hh homologues

We identified two *Hh* homologues from *L. japonicum*, designated *LjHhB* and *LjHhC*, in addition to *LjHhA* (Uchida et al., 2003). The genome of a closely related species, *P. marinus*, does not reveal any *Hh* homologues other than the orthologues of *LjHhA*, *B* and *C*. Two *Hh* homologues have been identified from another closely related species, *Lampetra fluviatilis* (Kano et al., 2010). Although the lack of sequence detail hinders confirmation of their orthology to any of the *LjHh* genes, *LjHhA* and *LjHhB* probably correspond to *LfHha* and *LjHhb*, respectively.

Our molecular phylogenetic analysis supported the orthology of *LjHhA* to gnathostome *Shh* (see Fig. S1 in the supplementary material). By contrast, *LjHhB* and *LjHhC* appeared to be products of a gene duplication unique to the lamprey (or cyclostome) lineage (see Fig. S1 in the supplementary material). Although not strongly supported, these two genes showed a closer relationship to *desert hedgehog* (*Dhh*) (see Fig. S1 in the supplementary material). Overall, this phylogenetic analysis resulted in many tree topologies supported with similarly high likelihood.

Expression patterns of lamprey *Hh* and related genes

We performed expression analyses of the *Hh* genes *LjHhB* and *LjHhC* together with the previously identified *LjHhA*. *LjHhA* was expressed in the midline mesoderm from stage 18 (neural fold stage) onwards (Fig. 1A,A′). At this stage, *LjPtcA*, encoding the Hh receptor, was expressed only in the anterior boundary between neural and non-neural ectoderm (see Fig. S5E,F in the supplementary material). However, at stage 19, expression of *LjPtcA* appeared in the ventral neural ectoderm adjacent to the Hh-expressing mesoderm (see Fig. S5G,H in the supplementary material). At stage 21, *LjHhA* was expressed in the notochord, the floor plate and particularly strongly in the prechordal plate (Fig. 1B,B′). In a stage 23 embryo, the notochordal expression of *LjHhA* started to disappear in a rostral to caudal direction, while it started to be expressed in the pharyngeal endoderm (Fig. 1C). By stage 24, *LjHhA* transcripts had not been detected in the neural tube rostral to the zona limitans intrathalamica (zli), but they began to be detected in the hypothalamus from stage 26, which was

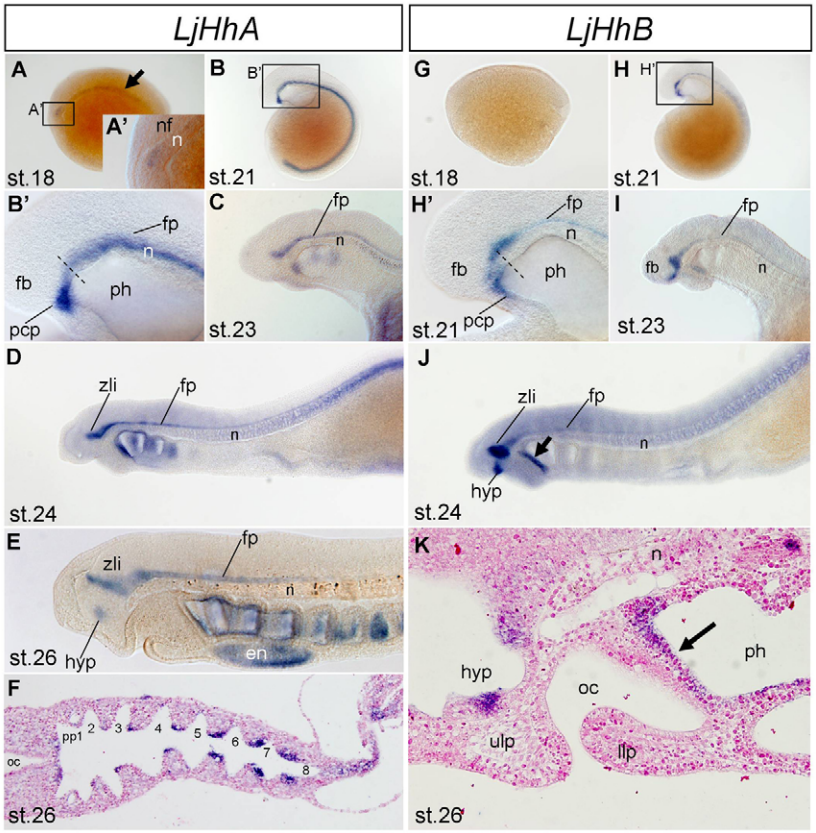

Fig. 1. The expression patterns of the *L. japonicum* Hh genes. (**A-K**) In situ hybridization of the lamprey *Hh* genes, *LjHhA* (A-F) and *LjHhB* (G-K). (A,A′) At stage 18, expression of *LjHhA* was first detected in the midline mesoderm (arrow). Boxed area is enlarged in A′. (B,B′) At stage 21, expression was observed in the notochord, the prechordal plate and the floor plate. Boxed area is enlarged in B′. The dashed line indicates the boundary at the anterior tip of the notochord. We were able to detect the expression of *LjHhA* at stage 18 in the midline and at stage 21 in the prechordal plate in addition to the previously reported expression domains (Osorio et al., 2005; Uchida et al., 2003). (C,D) In later embryonic stages, expression of *LjHhA* was observed in the floor plate, the zona limitans intrathalamica and the pharyngeal endoderm. Notochordal expression of this gene started to disappear in a rostral to caudal direction. (E) At stage 26, *LjHhA* was expressed in the hypothalamus. (F) In situ hybridization was performed on paraffin wax sections because whole-mount in situ hybridization often gives false positives in the pharyngeal endoderm. *LjHhA* was expressed in restricted regions of the pharyngeal endoderm. (G) *LjHhB* expression was not detected in stage 18 embryos. (H,H′) At stage 21, *LjHhB* was expressed in the floor plate and the prechordal plate. Boxed area is enlarged in H′. The dashed line indicates the boundary at the anterior tip of the notochord. (I,J) At later stages, *LjHhB* was expressed in the hypothalamus, the zona limitans intrathalamica and in pharyngeal endoderm adjacent to the stomodeal ectoderm (arrow in J). (K) In situ hybridization on a paraffin wax section. The arrow indicates anterior expression in the pharyngeal endoderm. en, endostyle; fb, forebrain; fp, floor plate; hyp, hypothalamus; llp, lower lip; n, notochord; nf, neural fold; oc, oral cavity; pcp, prechordal plate; ph, pharynx, pp1, 2-8, pharyngeal pouches; ulp, upper lip; zli, zona limitans intrathalamica.

discontinuous from the more caudal expression domain (Fig. 1D,E) (Osorio et al., 2005). Otherwise, transcripts were also distributed in the endostyle (Fig. 1E).

LjHhB was not expressed in the notochord, but it started to be expressed in the floor plate and the prechordal plate at stage 21 (Fig. 1G,H,H′). At stage 23, it was also expressed in the ventral part of the forebrain. Unlike the report on *LfHh* expression by Osorio et al. (Osorio et al., 2005), this expression domain of *LjHhB* was apparently continuous posteriorly with that in the floor plate (Fig. 1I), reminiscent of a gnathostome embryo pattern. Its expression in the pharyngeal endoderm peaked adjacent to the stomodeal ectoderm (Fig. 1J,K). Expression of *LjHhC* could not be detected by in situ hybridization, so its expression level must be extremely low. From these results, *LjHhA* and *LjHhB* exhibited expression patterns similar to those seen in gnathostomes for diencephalic expression but showed slight differences in some minor domains. By observing the expression patterns of *Pax6* and *Dlx* cognates, the lamprey telencephalon is probably regionalized into a pallium and a subpallium (Murakami et al., 2001). Histologically, *LjPax6* and *LjDlxA* expression domains in the stage 26 telencephalon were complementary in a DV fashion (see Fig. S4A,B in the supplementary material) (Kuraku et al., 2010; Murakami et al., 2001).

In the lamprey, the boundary between the telencephalon and the diencephalon can be found in the anterior intraencephalic sulcus (sa in Fig. 2A) (von Kupffer, 1906; Murakami et al., 2001). In the lamprey forebrain before embryonic stage 26, although the *LjHh* genes were expressed in a similar manner to those in gnathostomes, especially in the floor plate and zli, they were not expressed in the ventral telencephalon (Fig. 2A,B). To rule out possible heterochrony specifically associated with the telencephalic upregulation of these genes, we performed expression analyses on sections of a stage 30 ammocoete larva, which also gave no signals (data not shown). In gnathostome embryos, *Nkx2.1* expression is maintained by *Shh* signals to specify the MGE (Machold et al., 2003). *LjNkx2.1*, the lamprey homologue of *Nkx2.1*, is also

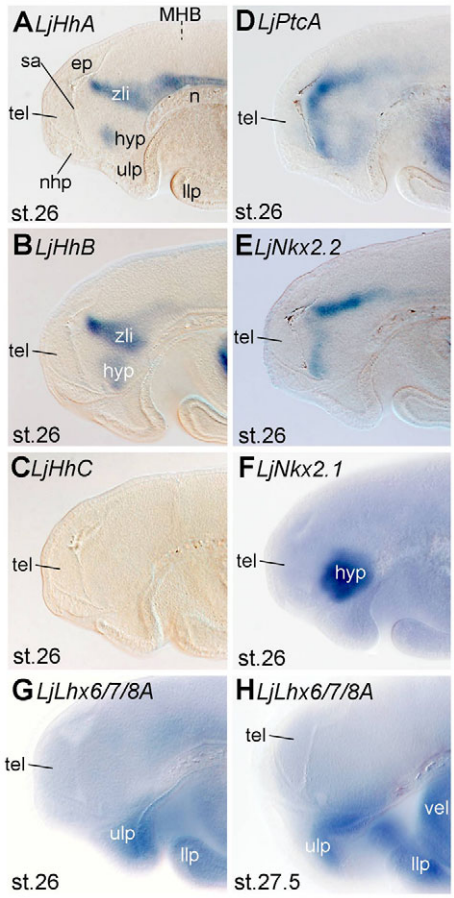

Fig. 2. Comparative expression of medial ganglionic eminence (MGE)-related genes in the lamprey telencephalon. Whole-mount in situ hybridization staining of *LjHhA* (**A**), *LjHhB* (**B**), *LjHhC* (**C**), *LjPtcA* (**D**), *LjNkx2.2* (**E**), *LjNkx2.1* (**F**) and *LjLhx6/7/8A* (**G**,**H**) in stage 26 or stage 27.5 lamprey embryos in lateral view. The boundary between the telencephalon and the diencephalon is shown by the anterior intraencephalic sulcus (sa) (von Kupffer, 1906). Note that the expression pattern of *LjPtcA* is similar to that of *Nkx2.2*. Moreover, none of these genes was detected in the telencephalon. ep, epiphysis; hyp, hypothalamus; llp, lower lip; MHB, midbrain-hindbrain boundary; n, notochord; nhp, nasohypophyseal placode; sa, anterior intraencephalic sulcus; tel, telencephalon; ulp, upper lip; zli, zona limitans intrathalamica.

expressed in the hypothalamus but not in the telencephalon (Murakami et al., 2001; Ogasawara et al., 2001; Uchida et al., 2003) (Fig. 2F). To exclude the possible involvement of unknown *LjHh* paralogues, the expression of *LjPtcA*, the lamprey homologue of *Ptc* and *Nkx2.2*, was studied. In gnathostomes, *Ptc* is both a receptor for Hh signalling and a transcriptional target of the Hh pathway; *Nkx2.2* is induced by Hh signals expressed caudally to the diencephalon and is required for specification of the p3 domain in the spinal cord (Dessaud et al., 2008). For both genes, expression patterns were similar to those in gnathostomes in the diencephalon and more caudal domains but were undetectable in the telencephalon (Fig. 2D,E).

In gnathostomes, LIM-homeodomain protein-encoding genes *Lhx6* and *Lhx7* (also called *Lhx8*) play a role in the differentiation of interneurons derived from the MGE; *Lhx6* contributes to GABAergic interneurons, whereas *Lhx7* is involved in the formation of the striatal cholinergic interneuron (Marin and Rubenstein, 2001). Therefore, we isolated *LjLhx6/7/8A*, a putative homologue of gnathostome *Lhx6/7/8* (see Fig. S3C in the supplementary material). Although *LjLhx6/7/8A* was expressed in the oral mesenchyme as in gnathostomes (Grigoriou et al., 1998), it could not be observed in the telencephalon (Fig. 2G,H). From these results, we conclude that the lamprey telencephalon lacks the *Hh*, *Nkx2.1* and *Lhx6/7/8* expression domains, together with a region corresponding to the gnathostome MGE as suggested by Murakami et al. (Murakami et al., 2001).

Expression patterns of LGE-related genes

Targeted disruption of *Nkx2.1* in the mouse leads to a smaller MGE and a concomitant expansion of the LGE (Sussel et al., 1999). Thus, the entire lamprey subpallium might be equivalent to the LGE because this animal lacks expression domains for *Hh* and *Nkx2.1* (Murakami et al., 2005; Osorio et al., 2005). However, it is unknown whether this region possesses the expected developmental traits as in the LGE.

Gsh2 (*Gsx2* – Mouse Genome Informatics) is known to be necessary for regional specification of the LGE in the mouse (Hebert and Fishell, 2008). The orthology of *LjGshA* to gnathostome *Gsh2* was confirmed (Kuraku et al., 2009). When we observed the embryonic expression of this gene in the lamprey, it was expressed in the subpallium in a pattern similar to that observed for *LjDlxA* (Fig. 3B,C).

In the mouse, a zinc finger transcription factor-encoding gene, *Sp8*, is expressed in the dorsal LGE, which might be a prerequisite for the differentiation of olfactory bulb interneurons (Waclaw et al., 2006). Expression of *Isl1* is also necessary for the differentiation of striatal projection neurons in the LGE (Stenman et al., 2003). Therefore, we isolated *Sp8* and *Isl1* cognates in the lamprey to observe their embryonic expression patterns. We identified *LjSp8/9A* as a member of the *Sp8/9* subfamily, being clustered with the gnathostome *Sp8* (see Fig. S3B in the supplementary material). It was detected slightly dorsally in the subpallium at stage 26 (Fig. 3D). Transcripts of *LjIsl1/2B*, a member of the *Isl1/2* subfamily, were also present in the lamprey subpallium (Fig. 3E; see Fig. S3A in the supplementary material). Thus, the lamprey embryonic subpallium exhibits a similar gene expression profile to that observed in the gnathostome LGE.

Expression of genes involved in the DV patterning of the telencephalon

Hh signalling is crucial for the DV patterning of the neural tube. The signal is induced by the notochord and emanates ventrally from the floor plate, counteracting the dorsally originated factor Gli3 (Lupo et al., 2006). *Shh* restricts the dorsalizing function of *Gli3* and controls the positioning of the DV boundary (Hebert and Fishell, 2008). As with gnathostomes, *Hh* expression patterns in the lamprey CNS are distributed in the diencephalon as well as in the ventral moiety of the more posterior neural tube. Curiously, no transcripts were observed in the telencephalon, unlike in gnathostomes. This suggests that in the lamprey, Hh signalling might not act in the DV patterning of the telencephalon. To test this, we isolated a *Gli3* homologue in the lamprey.

LjGliA clustered with gnathostome *Gli3* (see Fig. S2A in the supplementary material). Its transcripts were detected in the dorsal neural fold (see Fig. S5I-L in the supplementary material), the dorsal neural tube and in the dorsal brain (Fig. 4C,C′) including the telencephalon (Fig. 4C″). Therefore, it seems that the isolated *LjGliA*

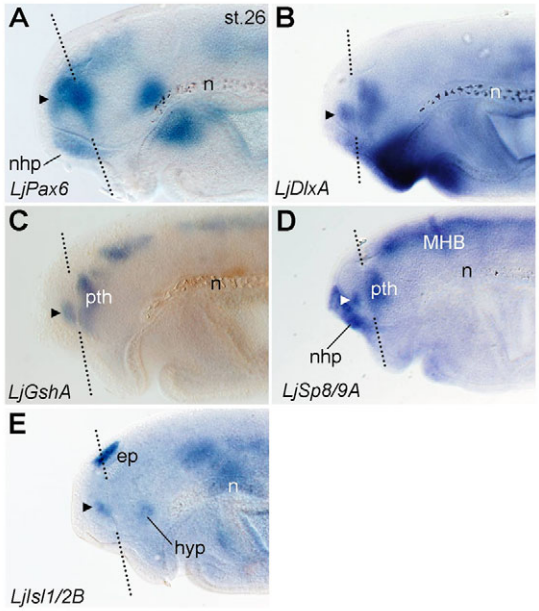

Fig. 3. Comparative expression of lateral ganglionic eminence (LGE)-related genes in the lamprey telencephalon. Whole-mount in situ hybridization staining of stage 26 lamprey embryos in lateral view. In all panels, dotted lines indicate the telencephalic border. (**A**) *LjPax6* was expressed in the dorsal telencephalon (arrowhead). (**B**) *LjDlxA* was expressed in the ventral part in the telencephalon (arrowhead) and shows a complementary expression pattern to *LjPax6*. (**C**) The expression pattern of *LjGshA* in the ventral telencephalon (arrowhead) was similar to that of *LjDlxA*. (**D**) *LjSp8/9A* was expressed in a medial part of the neural tube, in the midbrain-hindbrain boundary, the prethalamus, the nasohypophyseal placode and a dorsal part of the ventral telencephalon (arrowhead). (**E**) *LjIsl1/2B* was expressed in the epiphysis, the hypothalamus and a caudoventral telencephalon (arrowhead). ep, epiphysis; hyp, hypothalamus; n, notochord; MHB, midbrain-hindbrain boundary; nhp, nasohypophyseal placode; pth, prethalamus.

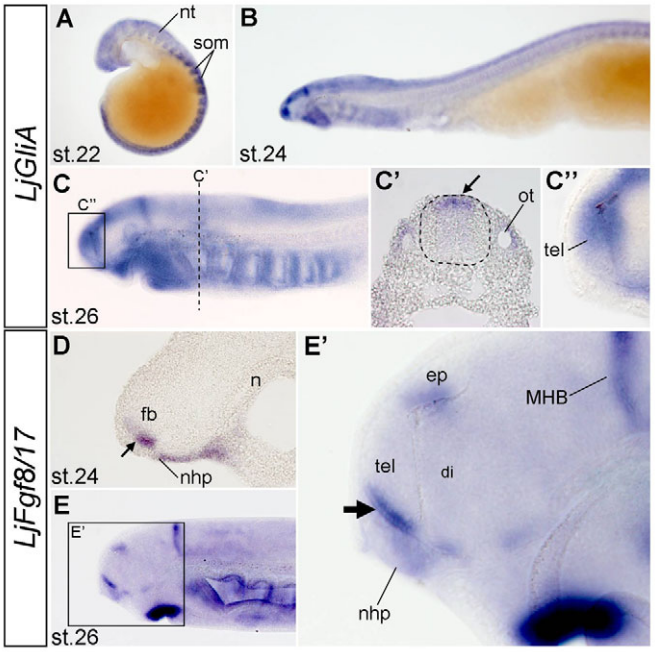

Fig. 4. Expression patterns of the *Gli* and *Fgf8* homologues. In situ hybridization patterns of the lamprey *LjGliA* (**A-C″**) and *LjFgf8/17* (**D-E′**). (A) At stage 22, *LjGliA* was expressed in the neural tube and the somites. (B) At stage 24, *LjGliA* was expressed in the forebrain, the dorsal neural tube, the mouth and the pharyngeal region. (C-C″) At stage 26, *LjGliA* was expressed in the dorsal spinal cord (arrow) and the otic vesicle. Dashed line indicates the level of the transverse cryosection shown in C′. The dashed circle indicates the neural tube. Boxed area is enlarged in C″ and shows *LjGliA* expression in the dorsal telencephalon. (D) At stage 24, *LjFgf8* expression was detectable in the ventral forebrain (arrow). (E,E′) At stage 26, *LjFgf8/17* expression remained in the anterior part of the ventral telencephalon (arrow in E′). Boxed area around the head region is enlarged in E′. di, diencephalon; ep, epiphysis; fb, forebrain; MHB, midbrain-hindbrain boundary; n, notochord; nhp, nasohypophyseal placode; nt, neural tube; ot, otic vesicle; som, somites; tel, telencephalon.

corresponds functionally to the gnathostome *Gli3* that represses Hh signalling. Thus, *LjGliA* is expressed in a gnathostome-like dorsally restricted pattern when the ventral Hh signal is absent. What could be the ventral signal for the lamprey telencephalon?

Blocking Hh signals

In some gnathostomes, Shh involved in DV patterning of the telencephalon can be found in the prechordal mesoderm, and its inductive function appears only in a small window of development. In the chicken, it can be detected before Hamburger-Hamilton (HH) stage 6 (Gunhaga et al., 2000), in the mouse before embryonic day (E) 9 (Fuccillo et al., 2004) and in the zebrafish before the eight-somite stage (Danesin et al., 2009). Curiously, during these stages, *Shh* expression in the telencephalon is not detected in any of these animals, possibly implying that the telencephalic Hh expression is not necessarily involved in the initial DV patterning of the gnathostome telencephalon.

In stage 18 and 19 lamprey embryos, *LjHhA* was already upregulated in the midline mesoderm corresponding to the notochord and prechordal plate (Figs 1A,A′; see Fig. S5A-D in the supplementary material), whereas at these stages, *LjHhA*, *LjNkx2.1* and *LjNkx2.2* could not be detected in the neural ectoderm (see Fig. S5A-D,O-R in the supplementary material). *Hh* expression in the prechordal plate persisted up to stage 21 (Fig. 1B′,H′). To detect whether these expressions are responsible for DV patterning of the telencephalon, we administered cyclopamine, an Hh signal inhibitor (Chen et al., 2002; Taipale et al., 2000), to early lamprey embryos. Because *LjHhA* starts to be expressed from stage 18, we studied stage 17 embryos corresponding to the neural plate stage of the lamprey. As negative controls, we employed stage 20 embryos just after neurulation.

In the embryos treated from stage 17, the ventral part of the telencephalon was smaller, whereas the dorsal half was enlarged (Fig. 6A,B). The morphology of the ventral domain was also altered in the diencephalic region (Fig. 6C,D). In these embryos, expression of putative dorsal specifiers such as *LjPax6* and *LjGliA* expanded ventrally at stage 26, covering almost the entire telencephalon (Fig. 5A,B,E,F). On the other hand, the ventrally upregulated *LjDlxA* and *LjSp8/9A* had lost their transcripts in the experimental embryos (Fig. 5C,D,G,H). However, embryos treated from stage 20 did not show any difference in the developmental patterns from non-treated embryos (Fig. 5I-L). Therefore, we conclude that the prechordal mesoderm-derived Hh signal is responsible for DV patterning of the lamprey telencephalon by stage 20 (before neurulation), and its

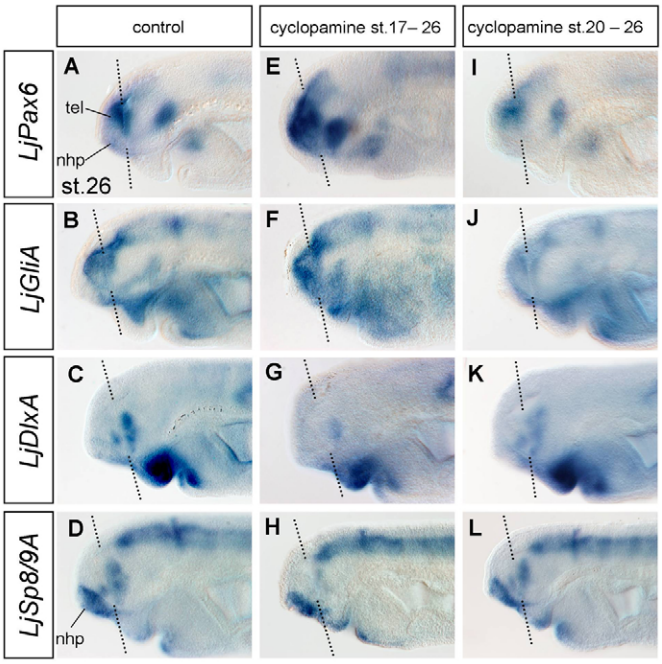

Fig. 5. *Hh* signalling is involved in establishing the dorsoventral axis in the lamprey telencephalon. Control embryos (**A-D**) were treated with ethanol at the same concentration as used for the experimental treatments from stages 17 to 26. Lamprey embryos were treated with 100 μM cyclopamine from stages 17 to 26 (**E-H**) or from stages 20 to 26 (**I-L**). The expression patterns of the telencephalic markers were monitored in stage 26 embryos. Dotted lines indicate the telencephalic border. (E,F) The expression patterns of the dorsal telencephalon markers *LjPax6* and *LjGliA* extended to the ventral side. (G,H) The expression patterns of the ventrally expressed genes *Ljlsl1/2B* and *LjSp8/9A* were greatly reduced. (I-L) Embryos treated with cyclopamine from stages 20 to 26 showed no changes in expression patterns. nhp, nasohypophyseal placode; tel, telencephalon.

inhibition results specifically in the ventral patterning of the telencephalon, possibly via dorsalization of the telencephalon. In the $Shh^{-/-}$ mutant mouse, there is no *Fgf* expression because of the repressive action of Gli3 (Aoto et al., 2002). Curiously, in the cyclopamine-treated lamprey embryos, *LjFgf8/17* and *LjSproutyA*, a putative downstream target of the FGF signalling, were still expressed in the ventral telencephalon, as in controls (Fig. 8).

Blocking FGF signals

Shh signalling in the telencephalon patterning might suppress dorsalization by repressing GLI3 and might be insufficient for subpallial patterning (Rallu et al., 2002). Thus, in the $Shh^{-/-}Gli3^{-/-}$ double knockout (KO) mouse, DV patterning of the telencephalon was restored and expression of subpallial genes was maintained. Therefore, the required inductive signal for the subpallium could be the FGF signal (Gutin et al., 2006).

In the lamprey telencephalon, *LjFgf8/17* were expressed from stage 24, and by stage 26, they were active in the anteroventral telencephalon (Fig. 4E,E′) (Guerin et al., 2009; Uchida et al., 2003). To test whether FGF signalling is responsible for the lamprey subpallium patterning, we applied SU5402, a potent FGF receptor (FGFR) inhibitor (Mohammadi et al., 1997), onto embryos from stage 24 to stage 26. The experimental embryos had lost the expression of *LjSproutyA*, a downstream gene of FGF and a putative homologue of the gnathostome *Sprouty* (Komisarczuk et al., 2008) (Fig. 7E,J), suggesting that SU5402 is also capable of inhibiting lamprey FGF signalling. At stage 26, the expression of *LjPax6* expanded ventrally, whereas that of *LjDlxA*, *LjGshA* and *LjSp8/9A* was lost in embryos treated with SU5402 between stages 24 and 26 (Fig. 7A-D,F-J). Thus, besides the early Hh signalling shown previously, FGF signalling is responsible for DV patterning of the telencephalon in the lamprey.

DISCUSSION

Gene duplications and functional differentiation

Our degenerate RT-PCR amplified two novel *Hh* homologues. In general, the landscape of regulatory gene repertoires in the lamprey has been obscured by insufficient gene sampling and by ambiguous signatures in lamprey sequences when assigning their orthology (Kuraku, 2008). Nonetheless, our analysis supported the orthology of *LjHhA* to gnathostome *Shh* genes (see Fig. S1 in the supplementary material), together with *Gli*, *Ptc*, *Isl1/2* and *Sp8/9* (see Figs S2 and S3 in the supplementary material), for which we also observed gene duplications in early vertebrate evolution. Thus, the last common ancestor of all extant vertebrates had already experienced gene duplications resulting in multiple subtypes with differential expressions (Kuraku et al., 2009). This needs to be confirmed with reinforced data sets after thorough gene sampling in genome-wide resources of hagfishes and lampreys.

MGE evolved as an evolutionary novelty

The ventral telencephalon of the lamprey does not express *LjNkx2.1* or *LjHhA*, leading to the hypothesis that this animal does not possess a mechanism to specify the MGE (Murakami et al., 2001; Osorio et al., 2005). Here, we isolated two more *LjHh* genes, *Nkx2.2* and *Ptc* homologues of the lamprey. Their expression patterns indicated the absence of the *Hh* expression domain in the ventral telencephalon.

In gnathostomes, the MGE gives rise to GABAergic interneurons in a Hh-dependent manner, which later migrate cortically (Marin and Rubenstein, 2001) and play important roles in ensuring the function of the gnathostome cerebral cortex. This migration has been observed in the chicken (Cobos et al., 2001), turtle (Métin et al., 2007), *Xenopus* (Moreno et al., 2008) and shark (Carrera et al., 2008) embryos. Thus, this pattern of telencephalic development is widespread among gnathostomes. In gnathostomes, cholinergic interneurons are also generated in the MGE and migrate to the striatum (Marin and Rubenstein, 2001). However, no migratory interneurons have been identified in the lamprey. *Lhx6* is required for the migratory GABAergic interneurons, whereas *Lhx7/8* regulates development of the migratory striatal cholinergic interneurons (Marin and Rubenstein, 2001). A lack of expression of at least one of the *Lhx6/7/8* homologue genes, *LjLhx6/7/8A*, was observed in the telencephalon (Fig. 2G,H). Although GABA-immunoreactive cells and cholinergic neurons were found in the pallium (Melendez-Ferro et al., 2002; Robertson et al., 2007) and in the striatum (Pombal et al., 2001) in the developing and adult lamprey, respectively, it is unclear whether these cells are migrated interneurons or projection neurons. Based on the expression of *LjLhx6/7/8A*, we speculate that these cells are pallial projection neurons or migrated interneurons, which are generated through a different specification mechanism from that of gnathostomes. Moreover, they lack the pallidum, the MGE derivative identified in the telencephalon (Nieuwenhuys and Nicholson, 1998). Altogether, it appears most likely that MGE and

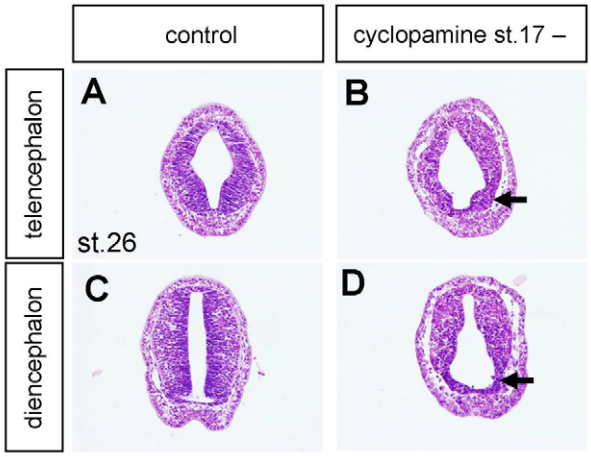

Fig. 6. Morphology of the ventral forebrain was disrupted by inhibiting *Hh* signalling. (**A-D**) Haematoxylin and Eosin-stained sections of the stage 26 lamprey forebrain. At the telencephalic level in the treated embryos, the dorsal part was expanded and the ventral part reduced in size (B; arrow), as in the control (A). At the diencephalic level, the ventral part became thinner (D; arrow), compared with the control (C).

migrating interneurons derived from MGE were acquired secondarily in the gnathostome lineage after divergence from the lamprey lineage (Fig. 9), because the key regulatory genes for MGE development (*Hh* and *Nkx2.1*) and for migrating interneurons (*Lhx6/7/8*) are absent in the lamprey telencephalon.

In mammals, migrating interneurons are also generated in the caudal ganglionic eminence (CGE) of the basal telencephalon (Nery et al., 2002). In the mouse, ~30% of all cortical interneurons are CGE derived (Miyoshi et al., 2010). However, it is unknown whether other gnathostomes possess a CGE. We could not identify a CGE in lampreys in this study.

LGE functions in the lamprey telencephalon

Expression of *Gsh2* in the gnathostome LGE is necessary for its development (Toresson and Campbell, 2001; Yun et al., 2003). Here, the expression of *LjGshA*, the *Gsh2* orthologue in the lamprey, colocalized with the region of the subpallium that also expresses *LjDlxA* (Fig. 3B,C), suggesting that the LGE is present in the lamprey telencephalon.

The gnathostome LGE is also known as the developmental source for striatal projection neurons and olfactory bulb interneurons (Wichterle et al., 2001). Normal expression levels of *Isl1 and Sp8* are required for the differentiation of these neurons, respectively (Stenman et al., 2003; Waclaw et al., 2006). Lamprey cognates of these genes (*LjIsl1/2B*, *LjSp8/9A*) are also expressed in the ventral telencephalon (Fig. 3D,E), and this animal also possesses olfactory bulb interneurons and striatal cholinergic neurons, as found previously (Nieuwenhuys, 1967; Pombal et al., 2001). Although *Isl1* is also expressed in MGE, *Isl1*-expressing cells require *Nkx2.1* and *Lhx6/7* (Fragkouli et al., 2009), and these genes are absent in the lamprey telencephalon. Therefore, it appears that the expression of *LjIslet1/2B* corresponds to that of LGE and not to that of MGE in gnathostomes.

Altogether, the subpallium domain of the lamprey appears to possess properties similar to the gnathostome LGE, which suggests that this domain was established in the last common ancestor of cyclostomes and gnathostomes.

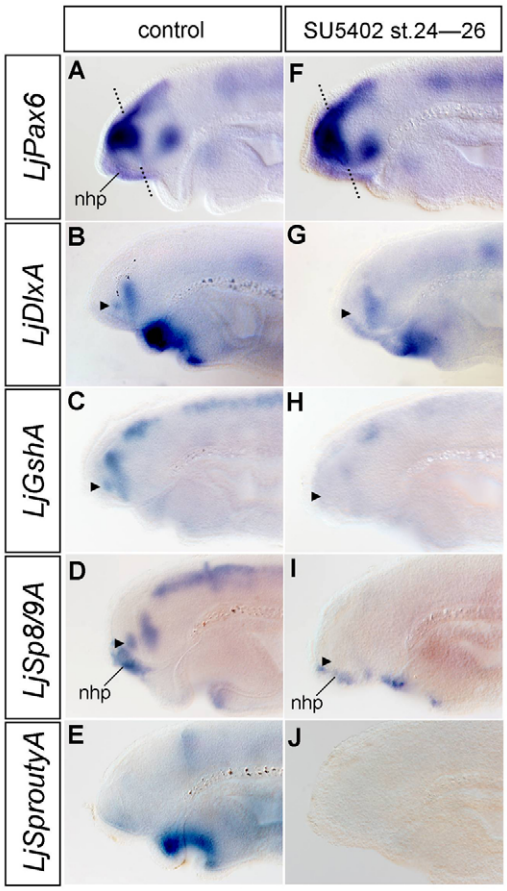

Fig. 7. FGF signalling is involved in establishment of the dorsoventral axis in the lamprey telencephalon. Control lamprey embryos (**A-E**) were treated with DMSO at the same concentration as used for the experimental treatments from stages 24 to 26. Experimental embryos were treated with 20 µM SU5402 (**F-J**). In panels A and F, dotted lines show the telencephalic border. (A,F) The expression pattern of a dorsal marker, *LjPax6*, extended to the ventral part in the treated embryos. (B-D,G-I) The expression of ventral telencephalon markers, *LjDlxA* (arrowheads in B,G), *LjGshA* (arrowheads in C,H) and *LjSp8/9A* (arrowheads in D,I) was greatly reduced. (E,J) *LjSproutyA*, a putative downstream target of FGF signalling, was examined to test whether SU5402 actually inhibited FGF signalling in the lamprey. Expression of *LjSproutyA* was greatly reduced in the SU5402-treated embryos (H), suggesting that the inhibitor is effective in this animal. nhp, nasohypophyseal placode.

DV patterning in the lamprey telencephalon

Inhibiting Hh signalling with cyclopamine from embryonic stage 17 led to a change in the developmental pattern of the ventral telencephalon. This was associated with the ventral expansion of the *LjPax6*/*LjGliA* domain that is normally restricted to the dorsal telencephalon, as well as the loss of *LjDlxA*/*LjSp8/9A* expression normally observed in the ventral telencephalon (Fig. 5). This treatment also led to the anatomical pattern of the ventral forebrain (Fig. 6). These effects were only observed when the treatment was applied from stage 17, and were not observed when cyclopamine was administrated after stage 20 (Fig. 5). Moreover, *LjPtcA* was expressed from stage 19 in the ventral forebrain (see Fig. S5H in the supplementary material), whereas expression of the *Hh* genes was observed only in the mesoderm from stage 18 (Fig. 1A,A′; see Fig. S5A-D in the supplementary material), suggesting that the

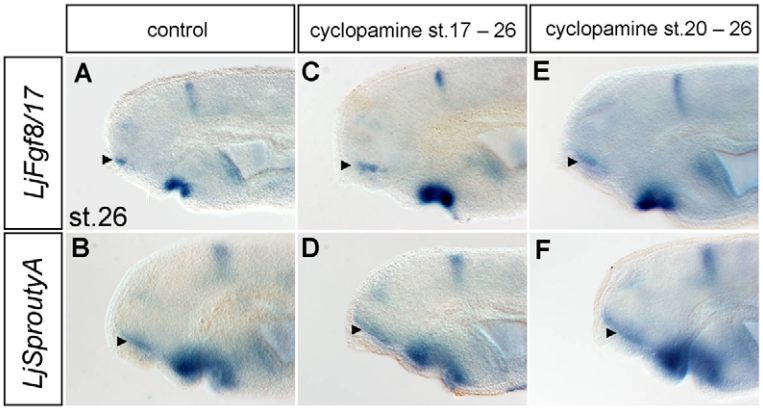

Fig. 8. Inhibition of Hh signalling showed no effect on fibroblast growth factor (FGF) signalling. To investigate whether inhibition of *Hh* signalling affected FGF signalling, we observed the expression patterns of *LjFgf8/17* and *LjSproutyA* in cyclopamine-treated embryos. Control embryos were treated with ethanol at the same concentration used for the experimental treatments from stages 17 to 26 (**A**,**B**). Lamprey embryos were treated with 100 μM cyclopamine from stages 17 to 26 (**C**,**D**) or from stages 20 to 26 (**E**,**F**). (A,C,E) *LjFgf8/17* was expressed in an anterior part of the ventral telencephalon (arrowheads) and no changes were observed in cyclopamine-treated embryos. (B,D,F) *LjSproutyA*, a downstream target of FGF signalling, was also expressed in the ventral telencephalon and no changes were observed in the cyclopamine-treated embryos (arrowheads).

forebrain might receive Hh signalling from the prechordal mesoderm at this stage. Therefore, it appears to be most likely that Hh signalling is functional in DV patterning of the telencephalon at early stages before the completion of neural tube closure in this animal, as in other gnathostomes (Danesin et al., 2009; Fuccillo et al., 2004; Gunhaga et al., 2000).

In gnathostomes, there appear to be two phases in telencephalon patterning in terms of *Shh* expression patterns. The first is the inductive activity derived from the prechordal plate, leading to the DV patterning of the telencephalon before E9 of mouse development. The second depends on expression in the MGE anlage to function in the maintenance of *Nkx2.1* expression, active after E9 (Fuccillo et al., 2006). In lampreys, because there are no *Shh* expression domains in the telencephalon anlage, the second phase of Shh signalling might represent a function that was new to gnathostomes in evolution. In other words, the first phase of action of Shh would be primary and more ancestral than the second function, most probably possessed by the common ancestor of vertebrates. The secondary acquisition of the telencephalon-derived Shh signalling appears to be a key innovation that would have permitted further evolution of the MGE and cortical interneurons specific for gnathostomes (Fig. 9).

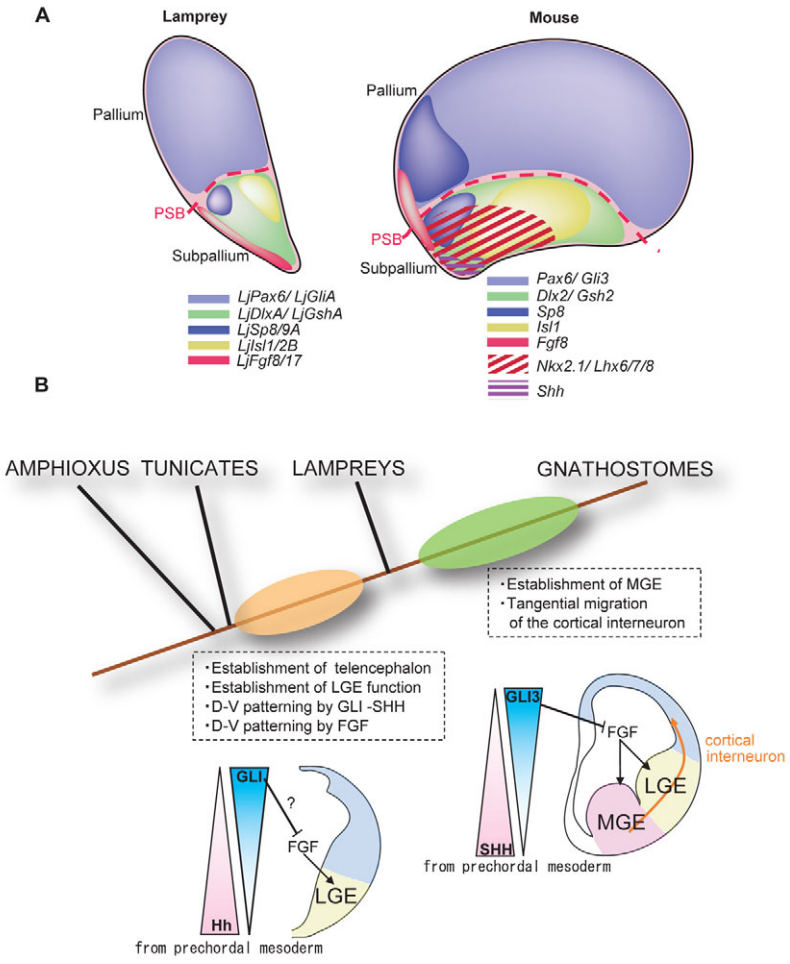

Fig. 9. Diagrams of the developmental plan of the vertebrate telencephalon. (**A**) Comparison of the developmental plans in the lamprey and mouse telencephalon, based on the gene expression patterns described in this study. PSB, pallium-subpallium boundary. (**B**) Our proposed scenario for the evolution of the vertebrate telencephalon, which is absent in the invertebrate deuterostomes, such as amphioxus and tunicates. Gnathostomes and lampreys share the dorsoventral (DV) patterning of the telencephalon (pallium-subpallium), the lateral ganglionic eminence (LGE) and involvement of Hh (from the prechordal mesoderm) and fibroblast growth factor (FGF) in the patterning. We propose that gnathostomes acquired a medial ganglionic eminence (MGE) along with the expression domains of *Shh*, *Nkx2.1* and *Lhx6/7/8A* in the telencephalon and MGE-associated tangentially migrating interneurons after the cyclostome-gnathostome divergence.

Recent studies have demonstrated that not only Hh but also FGF signalling is involved in DV patterning of the telencephalon in gnathostomes. In the *Shh*$^{-/-}$*Gli3*$^{-/-}$ double KO mouse, ventral patterning is restored greatly over that observed in single *Shh* mutants, suggesting that Hh-independent signals are involved in the DV patterning of the telencephalon (Rallu et al., 2002). FGF signalling has important roles in this process (Gutin et al., 2006).

Here, *LjFgf8/17* was expressed from embryonic stage 24 in the ventral forebrain (Fig. 4D) and upregulated in the anterior part of the ventral telencephalon at stage 26 (Fig. 4E,E′). To determine whether FGF signalling is responsible for this DV patterning, we applied an inhibitor against FGF signalling from stages 24 to 26 (Fig. 7). The expression pattern of *LjPax6*, a dorsal marker, expanded ventrally, whereas the ventral telencephalon markers *LjDlxA*, *LjGshA* and *LjSp8/9A* disappeared. Thus, FGF signalling is likely to be responsible for DV patterning of the telencephalon, in addition to early Hh signalling in the lamprey. We also estimated the effective time window of this signalling during lamprey development. Hh signalling during neurulation (stages 18-20) is essential for DV patterning in the forebrain, and FGF signalling is likely to act between stage 24 and 26. Actual changes in DV patterning were observed at stage 26.

Generally, FGF signalling also controls cell proliferation (Mason, 2007). However, the lamprey telencephalon did not seem to be reduced in size by inhibiting FGF signalling (Fig. 7). Furthermore, inhibition experiments of FGF signalling using zebrafish embryos resulted in no significant changes in cell proliferation in the dorsal or ventral telencephalon (Shinya et al., 2001). Therefore, inhibiting FGF signalling might not affect cell proliferation in the lamprey telencephalon at these stages.

Although the *Shh*$^{-/-}$ mutant mouse shows loss of *Fgf* expression, the *Shh*$^{-/-}$*Gli3*$^{-/-}$ double mutant restores *Fgf* expression (Aoto et al., 2002). It is thus conceivable that *Gli3* represses FGF signalling (Gutin et al., 2006). By contrast, FGF signalling did not seem to be repressed in the lamprey by blocking Hh signalling (Fig. 8). It seems likely that these phenomena represent differences in developmental mechanisms for the telencephalon between gnathostomes and lampreys.

An evolutionary scenario for the vertebrate telencephalon

We propose an evolutionary scenario for the vertebrate telencephalon (Fig. 9). This domain is most likely to have been established in the vertebrate ancestor before the dichotomy between the gnathostome and cyclostome lineages. There would have already been a distinction between the pallium and the subpallium. It is likely that the ventral telencephalon of the ancestor would have possessed a function similar to that of the gnathostome LGE. Even in the absence of the Hh expression domain in that region, Hh signals emanating from the prechordal plate and FGF signals from the ventral telencephalon would have functioned in the DV patterning of the telencephalon. After the divergence of the cyclostome and gnathostome lineages, an *Hh-Nkx2.1* expression domain arose in the gnathostome ventral telencephalon de novo, which led to an apomorphic specification of the MGE in this animal lineage independently. Acquisition of an MGE provided migratory neuroblasts for cortical interneurons, leading to the sophisticated function of the gnathostome pallium.

Finally, how could the MGE region have been established in the gnathostome lineage? In this regard, both in gnathostomes and in lampreys, transcripts of *Nkx2.1* and *Hh* colocalize in the hypothalamus in the ventral diencephalic domain, which appears as a serial homologue of the pallidum. This suggests that co-option of a gene network to pattern the hypothalamus into a more rostral domain (the ventral telencephalon) might have generated the MGE in the gnathostomes de novo. To test this hypothesis, we need to perform further comparisons of gene expression profiles between the hypothalamus and the pallidum, and to investigate the regulatory mechanisms of these genes in the ventral forebrain. Further studies on regulatory genes in the lamprey will provide valuable insights into the evolution of the vertebrate forebrain.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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