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A comparative examination of neural circuit and brain patterning between the lamprey and amphioxus reveals the evolutionary origin of the vertebrate visual center

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ABSTRACT

Vertebrates are equipped with so-called camera eyes, which provide them with image-forming vision. Vertebrate image-forming vision evolved independently from that of other animals and is regarded as a key innovation for enhancing predatory ability and ecological success. Evolutionary changes in the neural circuits, particularly the visual center, were central for the acquisition of image-forming vision. However, the evolutionary steps, from protochordates to jaw-less primitive vertebrates and then to jawed vertebrates, remain largely unknown. To bridge this gap, we present the detailed development of retinofugal projections in the lamprey, the neuroarchitecture in amphioxus, and the brain patterning in these animals. Both the lateral eye in larval lamprey and the frontal eye in amphioxus project to a light-detecting visual center in the caudal prosencephalic region marked by *Pax6*, which possibly represents the ancestral state of the chordate visual system. Our results indicate that the visual system of the larval lamprey represents an evolutionarily primitive state, forming a link from protochordates to vertebrates and providing a new perspective of brain evolution based on developmental mechanisms and neural functions.

A major question remaining to be answered in vertebrate evolution is how image-forming vision was established in a complicated eye from a simple, ocellus-like eye. Image-forming vision in vertebrates is thought to have evolved independently from arthropods and cephalopods and enabled their success as active predators (Lacalli, 2001).

Basal chordates, amphioxus, have an ocellus-like 'frontal eye', but this appears to function only in photoreception and not in image-forming vision. Though amphioxus has multiple photoreceptors, not only the frontal eye but also Hesse ocelli, Joseph cells and lamella body and this indicates that multiple photoreceptors are probably appeared in chordate ancestors, it is considered homologous to the vertebrate paired eyes due to its topology, *Pax6* expression and photoreceptor type (Lacalli, 2004). Recent molecular analyses gave further support for homology through the molecular fingerprinting of *Rx*, *Gi* and *c-opsin* in photoreceptor cells and *Mitf* and *Pax2/5/8* in pigment cells (Vopalensky et al., 2012). Lacalli (1996) found the visual center by tracing innervation through electron microscopy and named it "tectum" as a homologous region to the vertebrate mesencephalon. However, the mesencephalon-specific marker gene *Dmbx* is not expressed in the nerve chord of amphioxus, suggesting that amphioxus lacks a mesencephalic region (Takahashi and Holland, 2004). Therefore, there is some disagreement on amphioxus neuroarchitecture and brain patterning, making the evolution of vision obscure.

A key animal for resolving this issue is the lamprey, a basal vertebrate, because it possesses unique "dual visual development". Adults have well-developed camera eyes, which can process well-focused color vision (Gustafsson et al., 2008). The retino-tectal projection of adult lampreys is topographically organized like that of gnathostomes (Jones et al., 2009). In contrast, in the larval stage, the eye is covered by thick, non-transparent skin, and the lens is immature, indicating that it is not an image-forming eye (Villar-Cheda et al., 2008). In this stage, their retina consists at least of opsin immunoreactive receptors and ganglion cells (Melendez-Ferro et al., 2002), and photoreceptors may contact directly ganglion cell dendrites (De Miguel et al., 1989).

As larvae grow, cells in the peripheral part of the retina actively proliferate until the metamorphic stage (Villar-Cheda et al., 2008), while most cells remain neuroblastic (Villar-Cerviño et al., 2006). The retino-tectal projection newly develops in larvae longer than 70–80 mm with topographical manner (De Miguel et al., 1990, Corinde-Petronio et al., 2011). During metamorphosis, neuroblasts differentiate into photoreceptor, bipolar, amacrine and horizontal cells in the peripheral retina, and the eyes become the 'truly functional', 'camera-type' eyes of adults (Villar-Cerviño et al., 2006; Villar-Cheda et al., 2008). These studies indicate that larval lampreys have a unique visual system, possibly indicating the primitive state of the vertebrate visual system, and provide key information on understanding the evolution of the vertebrate visual system. However, the early development of the optic nerve and its projection pattern remain unknown.

Here we focused on the evolution of the vertebrate visual system, especially on its neural circuitry. We performed comparative examinations of the lamprey and amphioxus and, based on the results, propose an evolutionary scenario for the visual system in the chordate lineage.

MATERIALS AND METHODS

Animals

Adult lampreys (*Lethenteron camtschaticum*, synonym *L. japonicum*) were collected from the Shiribeshi-Toshibetsu River, Hokkaido, Japan. Mature eggs were expelled from females and fertilized *in vitro* by sperm. Adults were anesthetized in ethyl 3-aminobenzoate methanesulfonate (MS-222). Embryos were cultured at 16°C. Developmental stages were determined as described by Tahara (1988).

Collection of larvae of *Branchiostoma japonicum* was done as described by Yasui et al. (1998) and of *B. lanceolatum* as described by Fuentes et al. (2007)

Medaka (*Oryzias latipes*) eggs were incubated at 28°C and then used for neurolabeling experiments.

Whole-mount immunostaining

Whole-mount immunostaining of lampreys (*L. camtschaticum*) with anti-acetylated tubulin monoclonal antibody (Sigma, T6793, RRID: AB_477585) was performed according to Kuratani et al. (1997) with some minor modifications. Fixed embryos stored in methanol were washed in TBST containing 5% dimethylsulfoxide (TSTd). The embryos were then blocked with 5% nonfat dry milk in TSTd (TSTM). They were incubated with the primary antibody (diluted 1:1,000 in TSTM) for 2-4 days at room temperature (RT). After washing with TSTd, samples were incubated with secondary antibody (horseradish peroxidase (HRP)-conjugated antibody (Sigma, A2554, RRID: AB_258008)) or fluorescence antibody (Invitrogen, Alexa fluor 555, A21424, RRID: AB_10566287) diluted 1:200 in TSTM. After a final wash in TSTd, embryos treated with HRP-conjugated antibody were incubated with the peroxidase substrate in TBST for 1 hour, reacted in TBST with the same concentration of DAB with 0.01% hydrogen peroxide, and examined through an optical microscope. The embryos treated with fluorescent secondary antibody were dehydrated and clarified in a 1:2

mixture of benzyl alcohol and benzyl benzoate (BABB) and then examined using a confocal laser microscope (LSM 510, Zeiss).

Whole-mount immunostaining of amphioxus with anti-acetylated tubulin (Sigma, T6793, RRID: AB_477585), synaptotagmin (Sigma, S2177, RRID: AB_261464), anti-vesicular acetylcholine transporter (VAChT) (Sigma, V5387, RRID: AB_261875) and anti-serotonin (Sigma, S5545, RRID: AB_477522) antibodies was performed according to Kaji et al. (2001) with minor modifications. The primary and secondary antibodies were added together for the double immunostaining with anti-synaptotagmin and anti-acetylated tubulin. Stained specimens were examined using a confocal laser microscope (LSM510, Zeiss).

Antibody Characterization

Please see Table 1 for a list of all antibodies used.

The acetylated tublin antibody recognized a single band of 50 kDa m.w. on western blots of rat brain (manufacturer's datasheet) and stained a pattern in lampreys and amphioxus that is identical with previous reports (Kuratani et al., 1997, Kaji et al., 2001 respectively).

The synaptotagmin antibody reacts specifically with synaptotagmin, derived from rat brain tissue (65 kDa) and the antibody may be used in immunoblotting of rat brain extract. Staining of synaptotagmin band is specifically inhibited with synaptotagmin peptide (rat, amino acids 1-16 with C-terminally added lysine) (manufacturer's datasheet). Western blots of amphioxus 7days larvae (*B. lanceolatum*) showed a band at 65-70kDa, suggesting the antibody reacts with synaptotagmin in this tissue (Fig. 1).

The VAChT antibody reacts specifically with VAChT (~70 kDa) (manufacturer's datasheet). In immunoblotting VAChT appeared as a doublet band at 67-70 kDa and staining of the VAChT band by immunoblotting is specifically inhibited with the immunizing peptide (VAChT rat, amino acids 512-530 with N-terminally added lysine) (manufacturer's datasheet). On western blots of

amophioxus larvae showed a band at 55-60kDa (Fig. 1). In addition, the cell distribution by immunostaining (see results) is identical with that of *VAChT* in situ hybridization reported previously (Candiani et al., 2012)

The serotonin antiserum specifically stains enterochromaffin cells in formalin-fixed, paraffin-embedded sections of normal human appendix and serotonin-containing carcinoid tumors (manufacturer's datasheet). In the central nervous system, the antiserum reacts with serotonin-containing fibers in perfusion-fixed, free-floating sections of rat brain. Specific staining is inhibited by preincubation of diluted antiserum with 500 μM serotonin or 200 μg/ml serotonin-BSA (manufacturer's datasheet). The staining pattern in and amphioxus that is identical with previous reports (Holland and Holland 1993).

Neurolabeling

To label the neurons, dextran conjugates (tetramethylrhodamine, 3,000 m. w., Invitrogen, D3308; Alexa Fluor 488, 10,000 m. w., Invitrogen, D22910) were injected into the right eyecup or the caudal rhombencephalon of lamprey embryos or larvae (*L. camtschaticum*) and medaka larvae according to the method described by Glover (1995). The one-color triple labeling was performed by the tetramethylrhodamine-dextran conjugates injection to right eyecup, right forebrain surface, and rhombencephalon at the same time. The two-color double labeling was performed by the sequential labeling of Alexa Fluor 488-dextran conjugates to the rhombencephalon and tetramethylrhodamine-dextran to the right eyecup with 30 minuites interval. The injected embryos were incubated at RT for 30 minutes to allow anterograde labeling of neuronal projections with dextran. Embryos were then washed with distilled water and fixed in 4% PFA/PBS. The fixed specimens were dehydrated and clarified with BABB. Labeled neurons were examined using a confocal laser microscope.

Isolation of cDNA clones of lamprey and amphioxus genes

Pax6 lamprey homologs were isolated as described by Murakami et al. (2001).

Pax6 amphioxus homologs were isolated by PCR using adult *B. japonicum* cDNA as a template. Primers for PCR were designed on the *Pax6* sequences of *B. floridae* (AJ223440), which have been cloned previously (Glardon et al., 1998). These primer sequences are F: 5'-ATTTCCCGCCTTCTGCAGGTCTCGAATGG-3' and R: 5'-GCCATATTGCCGGGTACGGAAAAGCTTGG-3'.

We isolated a cloned sequence that was orthologous to *BfPax6* (100% match by amino acid sequence), and it was submitted and assigned the DDBJ/EMBL/GenBank accession number AB915169.

Whole-mount and section *in situ* hybridization

Whole-mount *in situ* hybridization for lamprey larvae (*L. camtschaticum*) was performed according to Ogasawara et al. (2000) with minor modifications. Whole-mount *in situ* hybridization for amphioxus larvae was performed as described previously (Wada et al., 1999).

Double staining by *in situ* hybridization and anti-acetylated tubulin immunostaining was performed following serial treatments. After post-fixation by the NBT/BCIP reaction of *in situ* hybridization, the samples were incubated at RT with 0.1 M glycine-HCl (pH 2.0) for 30 minutes to inactivate alkaline phosphatase. The specimens were then post-fixed with 4% PFA/PBS for 1 hour, washed with PBS and immunostained. The embryos were dehydrated and clarified with BABB and then examined using a confocal microscope. The *in situ* hybridization signals were examined by transmitted light microscopy and the immunostaining signal by specific laser microscopy.

RESULTS

Early development of the lamprey optic nerve

Immunostaining of anti-acetylated tubulin was performed to examine the temporal profile of lamprey optic nerve development (see also Barreiro-Iglesias et al., 2008; Kuratani et al., 1998, 1997). In stage 24 and stage 25 embryos, some neural fibers (for example, fasciculus retroflexus [FR], medial longitudinal fascicle [MLF], supraoptic tract [SOT], tract of the posterior commissure [TPC]. tract of the postoptic commissure [TPOC]) were observed, but there were no optic fibers (Fig. 2A, B). The eyecup (asterisk) and optic fibers (arrow) were first identified during late stage 25 (stage 25.5; 14–15 days post-fertilization; Fig. 2C). The eyecups are located just on the ventral region of the ophthalmicus profundus ganglion (gV_1), and the optic fibers are coursed anteriorly toward the chiasm (Ch). In stage 26, the eyecup and optic fibers were present, although it was difficult to distinguish them from the inner brain fibers (Fig. 2D). In stage 27, the optic nerve was formed of thin fibers, as noted using confocal microscopy (Fig. 2E). In stage 28, the relative position of the eyecup was shifted slightly. It was just ventral to gV_1 at stage 27, but between gV_1 and the trigeminal ganglion ($gV_{2,3}$) at stage 28 (Fig. 2F). The opticnerve is thicker compared with the previous stage. It is also notable that the dorsal region of the mesencephalon (Mes) has relatively low immunoreactivity to anti-acetylated tubulin, indicating that there are only a few fibers in this region.

Neurolabeling of lamprey optic nerve projections

We next examined the projection target of the optic nerve by rhodamine-dextran conjugate injection into lamprey embryos and larvae. The reagent was injected into the right eyecup, and the right optic nerve axons were traced anterogradely (Fig. 3A). The optic fibers could be labeled in embryos older than stage 25.5 (Fig. 3B). This result is consistent with that of the anti-acetylated tubulin immunostaining. As larvae grew, more fibers were labeled (Fig. 3C–F). The tract of

posterior commissure (TPC) was also observed as an artifact (Fig. 3B, C), because some tracer was taken directly by the brain surface. The optic nerve terminated contralaterally in the left dorsal region forebrain at all stages, and this region was rostral to the tectum, which is located just anterior to the midbrain-hindbrain boundary (MHB). Moreover, ipsilateral retinofugal fibers were not observed at any stage.

We triple labeled the optic nerve, the MLF and the TPC to clarify the target position of the optic nerve projection in the brain. The TPC was situated along the dorsocaudal border of the diencephalon, and the nucleus of the MLF in the ventral region of the posterior commissure. The optic nerve projected to the region ventral to the TPC or nucleus of the MLF (Fig. 4A). This region corresponds to the ventral part of the pretectum. Furthermore, we performed two-color double labeling of the optic nerve and the MLF (Fig. 4B) and found optic nerve axons with varicosity (see inset of Fig. 4B) projecting to dendrites of MLF neurons, suggesting that at least a part of the optic fibers directly connects to MLF neurons.

To verify the brain region receiving the optic projection, we compared nerve tract locations with the expression pattern of *Pax6*, a dorsal prosencephalon marker (Murakami et al., 2001). The *Pax6* expression domain covered the TPC and the nucleus of the MLF situated in its ventral region (Fig. 4C). This result is highly consistent with a previous observation (Murakami et al., 2001). Because the region of the optic nerve projection overlapped with the ventral TPC and nucleus of the MLF, the optic nerve likely projects to a *Pax6*-positive prosencephalic region (P1; pretectum) , but not to the mesencephalic region.

For comparison, we examined optic nerve innervation pattern in medaka as a representative species of gnathostomes by two-color double labeling of optic fibers (magenta) and medial longitudinal fascicle (green). (Fig. 4D). In medaka 10 dpf (days post fertilization) larvae, most of the optic fibers (ON) projected to the tectum, and any nMLF-projecting fibers (green) could not be observed in this experiment. These results are consistent with the previous research, which

studied retinotectal pathfinding in medaka (Yoda et al., 2004).

Brain patterning and visual center in amphioxus larvae

We next examined brain patterning in amphioxus. Vopalensky et al. (2012) showed the homology between the amphioxus frontal eye and the vertebrate lateral eye by molecular fingerprintings. In addition, they showed innervation by the serotonergic neuron from the frontal eye to the tegmental neuropile, which they suggested is comparable to the vertebrate hypothalamus, although no clear evidence was provided for this homology. Thus, we traced the position of the visual center and examined its homology with the vertebrate neuroanatomical domain by comparing gene expression in this developmental stage. We performed immunostaining using several neural system-related proteins to determine the position of the visual center in amphioxus larvae.

In four gill slit (4gs) larvae, we found anti-serotonin (5-HT)-immunopositive cells located just on the ventral side of the frontal eye pigment (Fig. 5A). These cells were identified as R2 cells, as described previously (Holland and Holland, 1993; Lacalli, 1996; Vopalensky et al., 2012). Furthermore, there were anti-VAChT-immunoreactive neurons (Fig. 5B) in 4gs larvae. These cholinergic cells are located in the ventral neural nerve chord; thus, they are ventral component (VC) motor neurons (Bone, 1960; Lacalli, 2001; Lacalli and Kelly, 1999; Candiani et al., 2012). Most rostral cells had relatively large cell bodies. Based on position and cell morphology, these large neurons were likely those identified by Lacalli (1996) as giant cells of the primary motor center in the caudal cerebral vesicle. Between these two types of neurons, we found an anti-synaptotagmin (syt) highly-immunoreactive region (Fig. 5C1). Topologically, this region is just rostral to the n2 nerve root (rN2, Fig. 5C2) and thought to correspond to the ‘tectum’(Lacalli, 1996) and its ventral neuropile, containing many synaptic connections. These results suggest that this region may process light information and control movements as a visual center (as at least one of the function of this region), receiving input from rostral sensory neurons and sending output to

caudal motor neurons (Fig. 5E).

We then performed double staining of *Pax6 in situ* hybridization with acetylated-tubulin immunostaining in one-gill slit (1gs) larvae. At this stage, *Pax6* was expressed in the posterior cerebral vesicle (Fig. 5D1, see also Glardon et al., 1998). We found that this region coincides topologically with the rostral region of the n2 nerve root (rN2, Fig.5D2). This indicates that the presumptive visual center in amphioxus larvae is located in the *Pax6* positive region, which may be homologous to the vertebrate prosencephalon as discussed below.

DISCUSSION

The 'primary' optic nerve of lampreys

Lampreys show 'dual (or two-step) development' of the eye and optic nerve. In embryonic or pre-ammocoete larvae (the 'primary' phase), the retina has an ocellus-like form (Melendez-Ferro et al., 2002). In this period, a small number of the optic nerve fibers is formed. In late ammocoete larvae (the 'secondary' phase), new optic fibers are formed again. After metamorphosis, adult lampreys have well-developed camera eyes. Thus, we can term the optic nerve formed in the embryonic period as the 'primary' optic tract and the one in the newly formed in late larvae as the 'secondary' optic tract.

Our experiments showed that the primary optic tract projects into the *Pax6*-positive neural region (Fig. 4). By comparing the expression of *Pax6*, *Otx*, *Dlx1/6*, *Pax2/5/8* and neural tracts marked by anti-acetylated tubulin antibodies, Murakami et al. (2001) indicated that the lamprey prosencephalon can also be identified as a *Pax6* and *Otx*-positive region and the mesencephalon as a *Pax6*-negative and *Otx*-positive region. Therefore, we concluded that the lamprey primary optic tract projects into the prosencephalic region but not the mesencephalic region. In the embryonic period, there are opsin-immunoreactive photoreceptor cells in the retina, which may process light information at this stage (Melendez-Ferro et al., 2002). As we found no other retinofugal fibers, we surmised that this neural tract is the only pathway transferring light information from the retina in early larvae (see also De Miguel et al., 1990). This retino-pretectal projection remains as a retinofugal pathway in adult lampreys (Jones et al. 2009). It is thought to function in escape swimming in response to sudden visual stimuli and in dorsal light response (Ullén et al., 1993, 1997; Deliagina and Fagerstedt, 2000). Furthermore, retino-pretectal projection is conserved among gnathostomes. For example, the light reflex of the rat pupil is controlled by the contralateral pretectum (Trejo and Cicerone,1984). And the retino-pretectal projection is also found in hagfish (Kusunoki and Amemiya, 1983; Wicht and Northcutt, 1990) and blind cave fish (Voneida and

Sligar, 1976), whose retino-tectal projection is mostly degenerate. Although no diencephalic projection was observed in the stage studied in our experiments (Fig. 4D), the adult medaka actually has a number of diencephalic visual centers, such as the pretectum (Deguchi et al., 2005). Also in the zebrafish (Burrill & Easter 1994), the presumptive retio-pretectal projection is observed shortly after the retino-tectal projection (52–54 hpf).

Therefore, the retino-pretectal projection is evolutionarily conserved and probably an ancestral feature of vertebrates. There are fibers of the TPC and nucleus of the MLF in the pretectum region (Fig. 4). The TPC fibers integrate left-right information, and the MLF fibers send the signal into the spinal cord. This neuroarchitecture may represent the ancestral visual system.

Visual center similarity in lampreys and amphioxus

We showed the input/output architecture in the visual system of the amphioxus frontal eye, and that the presumptive visual center in amphioxus is located in the *Pax6* expression domain (Fig. 5). Therefore, this visual center occupies the same prosencephalic region to that of the region receiving the ‘primary’ optic projections in lamprey, though the segmental neural organization in amphioxus is unclear and detailed synaptic connections between row cells of the amphioxus frontal eye remains to be studied. Both regions are *Pax6*-positive, receiving visual input and sending output to the trunk, suggesting that this region functions as an integrative visual center (see also Fig. 6A). Thus, these regions share close morphological/functional similarity. In addition, Lacalli (2002) showed that the overall structure of the anterior cerebral vesicle change little during metamorphosis in amphioxus. This suggests that the visual center does not change during metamorphosis.

Moreover, in the ascidian *Ciona intestinalis*, putative photoreceptor cells project their axon to other neurons in the posterior sensory vesicle (Imai and Meinertzhagen, 2007). Some cholinergic neural cell bodies are located in this region (Yoshida et al., 2004), which is *CiPax6*-positive at the mid-tailbud stage (Mazet et al., 2003). Therefore, the visual center of this species is also located in a

Pax6-positive region.

On the other hand, there are some differences in the visual neuroarchitecture between lampreys and amphioxus. Serotonergic R2 cells in amphioxus are thought to be homologous to the retinal ganglion cells (RGCs) in vertebrates. But there is no 5-HT immunoreactive RGCs in lamprey before metamorphosis (Abalo e al., 2008). There is another 5-HT immuonoreactive cells in the photoreceptor organ in early lampreys, the pineal organ. Although this organ develops from *Pax6*-positive region, the relationship with R2 cells is also unclear. Moreover, the R2 cells projects ipsilateral (Vopalensky et al., 2012), though the retinal projection in early lamprey is contralateral, as is the often case in vertebrates. However, there are other types of neurons corresponding to the vertebrate retinal neurons, R3 and R4 cells, and especially, R4 cells are thought as possible homologues of RGCs (Lacalli 1996). R4 cells are not serotonergic, and have contralateral projections. They locate just caudal to the R2 cells and have backward-projecting axon like R2 cells. Therefore we do not dismiss a possibility that other cell types such as R4 cells also act as sensory neurons in the amphioxus frontal eye visual system, and RGCs are homologous to some of these cells rather than R2 cells.

Evolution of vision in the chordate lineage

Based on our findings, we propose an evolutionary scenario for the visual system in chordates (Fig. 6A). The common ancestor of chordates had an ocellus-like eye(s), and the visual center was in the *Pax6*-positive region, where directional vision was processed. These characters are also conserved in tunicates. Moreover, they can be traced back to more ancestral lineages, because the serotonergic neurons found in the amphioxus frontal eye are thought to be homologous to the serotonergic apical organ neurons found in larval echinoderms (Lacalli et al., 1994). Larval hemichordates also have these serotonergic neurons in the apical organ (Miyamoto et al., 2010; Nakajima et al., 2004; Nielsen and Hay-Schmidt, 2007). Furthermore, recent research

(Marlow et al., 2014) revealed that the origin of the apical organ can be traced back to the common ancestor of cnidarians and bilaterians.

In the common ancestor of vertebrates, the *Pax6*-positive (i.e., prosencephalic) visual center remains one of the main visual center as larval lampreys. Larval lampreys also show some other ancestral states, such as the endostyle (Wright et al. 1980) and the absence of arcualia (Potter and Welsch 1992; Richardson et al. 2010). In addition, the mesencephalic retino-tectal projection was a newly formed 'secondary' optic tract in the mesencephalic region. This projection developed a topographical arrangement that enabled the ancestor to establish image-forming vision (Jones et al., 2009).

This scenario also inspires an idea that the evolution of image-forming vision is associated with the evolution of the mesencephalon. However, the emergence of the mesencephalon remains enigmatic. Figure 6B shows the gene regulatory network establishing the mesencephalic region in a vertebrate neural tube. Pani et al. (2012) proposed that the IsO organizer is conserved in the acorn worm. However, Holland et al. (2013) noted that the arrangement of IsO-related genes is reversed and that the organizer activity might not be directly related to the effects on A/P patterning. The *Otx*/*Gbx* boundary and *Pax6* expression is present in amphioxus (underlined), suggesting the existence of the IsO and conserved A/P patterning in the amphioxus neural tube. As neither *Pax2/5/8* nor *En1/2* is expressed in the neural tube just anterior to the *Otx*/*Gbx* boundary, in addition to a lack of *Dmbx* expression, the amphioxus appears to lack a mesencephalic region. Rather, the expression profile of the patterning gene in the anterior neural tube is strikingly similar to that of the vertebrate prosencephalon. Our observations in this study are consistent with this idea because the amphioxus visual center, located in the posterior cerebral vesicle, is comparable to the prosencephalic 'primary' visual center found in larval lampreys.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: DGS, YM, HW. Acquisition, analysis and interpretation of data: DGS, YM, HE, HW. Drafting of the manuscript: DGS, HW. Obtained funding: DGS. Administrative, technical, and material support: YM, HE. Study supervision: HW.

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RESOURCES CITED

Anti-acetylated tublin antibody (Sigma, T6793, RRID: AB_477585)

Horseradish peroxidase (HRP)-conjugated antibody (Sigma, A2554, RRID: AB_258008))

Alexa fluor 555-conjugated antibody (Invitrogen, A21424, RRID: AB_10566287)

Anti Synaptotagmin antibody (Sigma, S2177, RRID: AB_261464)

Anti-Vesicular acetylcholine transporter (VAChT) antibody (Sigma, V5387, RRID: AB_261875)

Anti-serotonin antibody (Sigma, S5545, RRID: AB_477522)

FIGURE LEGENDS

Figure 1

Western blot analysis of synaptotagmin (syn) and vesicular acetylcholine transporter (VAChT) antibodies. Lane 1 and 2, stained with Syn. Lane 3 and 4, stained with VAChT. Lane 1 and 3, tissue extract from the mixture of amphioxus (*B. lanceolatum*) 7days larvae. Lane 2 and 4, tissue extract from the mixture of mouse cortex.

Figure 2

Whole-mount immunostaining with anti-acetylated tublin antibody in *L. camtschaticum* embryos and early larvae. Asterisks indicate the eyecup and arrows the optic nerve. **A–D:** Optical microphotographs of specimens stained by DAB in the craniofacial region. **A:** Stage 24. Axonal tracts, including the fasciculus retroflexus, medial longitudinal fascicle, supraoptic tract and the tract of the postoptic commissure. **B:** Stage 25. The tract of the posterior commissure is newly formed. **C:** Stage 25.5. The eyecup (asterisk) and optic nerve (arrow) have appeared. The eyecup region is magnified in the inset. **D:** Stage 26. The eyecup and optic nerve are still distinct. The magnified eyecup region is shown in the inset. **E, F:** Confocal microphotographs of specimens marked by fluorescent secondary antibodies in the head region. **E:** Stage 27. The optic nerve extends to the optic chiasm. The dorsal region of the mesencephalon was less immunoreactive. **F:** Stage 28. The relative position of the eyecup has changed slightly. Abbreviations: Ch, chiasm; FR, fasciculus retroflexus; Mes, mesencephalon; MLF, medial longitudinal fascicle; SOT, supraoptic tract; TPC, tract of the posterior commissure; TPOC, tract of the postoptic commissure. Scale bars = 100 μm.

Figure 3

Neurolabeling of lamprey (*L. camtschaticum*) optic nerve fibers. Asterisks indicate the left eyecup.

A: Overview of the labeled specimen at stage 27. Dextran was injected into the right eyecup (arrowhead), and it travelled through the chiasm, terminating in the left side of the brain. **B–F:** Neurolabeling in serial stages showing target regions. Arrows indicate optic fibers. **B:** Confocal microphotographs of the optic nerve projection region at stage 25.5. Some optic fibers are labeled (arrow). The tract of the posterior commissure is also labeled. **C:** Stage 26. More optic fibers are labeled than at stage 25.5. The tract of posterior commissure is also labeled. **D–F:** Stages 28–30. The number of optic nerves increases but terminates in the same region at all stages (dorsal region of the left eye). Abbreviations: Ch, chiasm; MHB, midbrain-hindbrain boundary; TPC, tract of the posterior commissure. Scale bars = 100 μm.

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Figure 4

Analysis of the optic nerve projection region in *L. camtschaticum* embryos and early larvae. **A:** Triple labeling of optic fibers, medial longitudinal fascicle and the tract of the posterior commissure in stage 27. **B:** Two-color double labeling of optic fibers (magenta) and medial longitudinal fascicle (green). The optic nerve projects to the dendrites of neurons of the nucleus of the medial longitudinal fascicle neurons. A magnified picture of optic fibers with varicosities (arrowheads) is shown in the inset. This picture was reconstructed from raw data before making the projection picture (B1). **C:** Double staining of anti-acetylated tubulin antibody immunostaining and *Pax6 in situ* hybridization. (C1) shows anti-acetylated tubulin, (C2) shows *Pax6* expression by transmitted light and (C3) shows the merged microphotographs. The TPC is located in the caudal-most *Pax6*-positive region (arrow), and the nucleus of the medial longitudinal fascicle is located in its ventral region. **D:** Two-color double labeling of optic fibers (magenta) and medial longitudinal fascicle (green) in medaka. (D1) shows dorsal view of the brain region at 10dpf (days post fertilization). (D2) shows magnified left tectal region. Abbreviations: MHB, midbrain-hindbrain boundary; (n)MLF, (nucleus of) medial longitudinal fascicle; TPC, tract of the posterior commissure; tel,

telencephalon; ON, optic nerve fibers. Scale bars = 100 μm in (A), (C1), (D1), and (D2), 50 μm in (B1).

Figure 5

Neuroarchitecture and brain patterning of amphioxus larvae. **A–C:** Immunohistochemistry in *B. lanceolatum* four-gill slit (4gs) larvae. **A:** Immunostaining with anti-serotonin (5-HT) antibody. There are immunoreactive R2 photoreceptor cells just ventral to the frontal eye pigment observed by transmitted light (TR). **B:** Immunostaining with anti-VAChT antibody. Motor neurons in the ventral neural tube were immunoreactive (arrows), and most rostral cells were thought to be giant cells. **C:** Double staining with anti-synaptotagmin (syt, magenta) and anti-acetylated tubulin (ac-tub, green). (C1) shows synaptotagmin immunoreactivity and (C2) shows the merged microphotographs. The presumptive visual center (arrows) is relatively highly anti-synaptotagmin-immunoreactive, and this region is located just rostral to the root of the n2 nerve. **D:** Double staining with anti-acetylated tubulin antibody immunostaining and *Pax6 in situ* hybridization in *B. japonicum* one-gill slit (1gs) larvae. (D1) shows *Pax6* expression and (D2) shows the merged microphotographs. The caudal part of the cerebral vesicle is *Pax6*-positive (arrows), and this region corresponds to the presumptive visual center, located just rostral to the root of the n2 nerve. The arrow indicates the second nerve root. **E:** Schematic illustration of the neuroarchitecture and brain patterning of amphioxus larvae. Abbreviations: FEP, frontal eye pigment; GCs: giant cells; (r)N2, (root of) n2 nerve; NP, neuropore; POP, preoral pit; R2Cs, row 2 cells. Scale bars = 50 μm.

Figure 6

Schematic illustration of the evolution of vertebrate image-forming vision. **A:** Hypothetical evolutionary scenario. The common ancestor of chordates had an ocellus-like eye(s), and the visual center was in the *Pax6*-positive region, which processes directional vision. In the common ancestor

of vertebrates, the *Pax6*-positive (i.e. prosencephalic; Pros) visual center remained the main visual center, since larval lampreys had the same type of visual system as protochordates. The mesencephalic (Mes) retino-tectal projection was newly formed as a 'secondary' optic tract. **B:** The gene regulatory network that establishes the mesencephalic region in the vertebrate neural tube. Genes with conserved expression in chordates are underlined (the *Gbx* gene is lost in tunicates and is dashed-underlined). Genes with conserved expression in tunicates and vertebrates are in bold.

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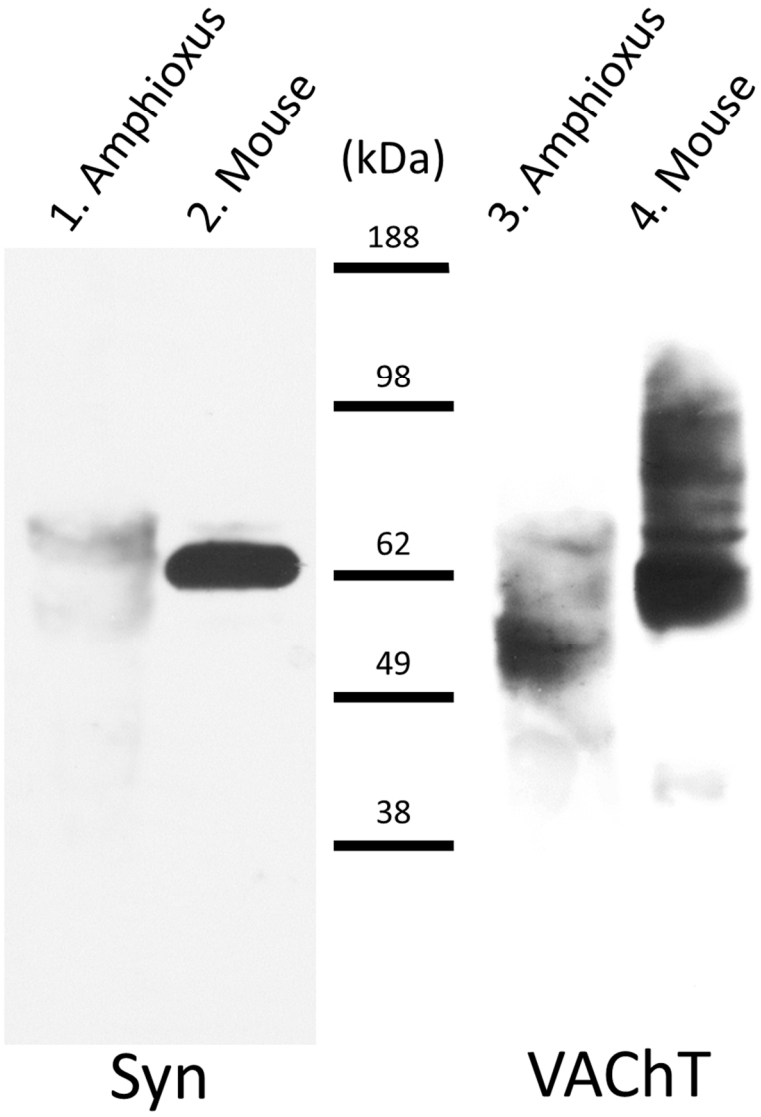
http://www.textcheck.com/certificate/9kOkMh

Table 1. Table of Primary Antibodies Used

Antigen	**Description of Immunogen**	**Source, Host Species, Cat. #, Clone or Lot#, RRID**	**Concentration Used**
Acetylated tublin	α 3 isoform of *Strongylocentrotus purpuratus* acetylated tublin, within four residues of Lys-40 of when this amino acid is acetylated	Sigma, mouse monoclonal, Cat# T6793, RRID:AB_477585	0.1ug/ul
synaptotagmin	KLH-conjugated, synthetic peptide corresponding to corresponding to the N-terminal of synaptotagmin I (SytI) of rat origin (amino acids 1-16 with C-terminally added lysine)	Sigma, rabbit polyclonal, Cat# S2177, RRID:AB_261464	0.1ug/ul
Vesicular acetylcholine transporter (VAChT)	KLH-conjugated, synthetic peptide corresponding to C-terminal of the cloned rat VAChT (amino acids 512-530 with N-terminally added lysine)	Sigma, rabbit polyclonal, Cat# V5387 RRID:AB_261875	0.1ug/ul
serotonin (5-HT)	Rabbit serotonin creatinine sulfate complex conjugated to BSA	Sigma, rabbit polyclonal, Cat# S5545, RRID:AB_477522	0.1ug/ul



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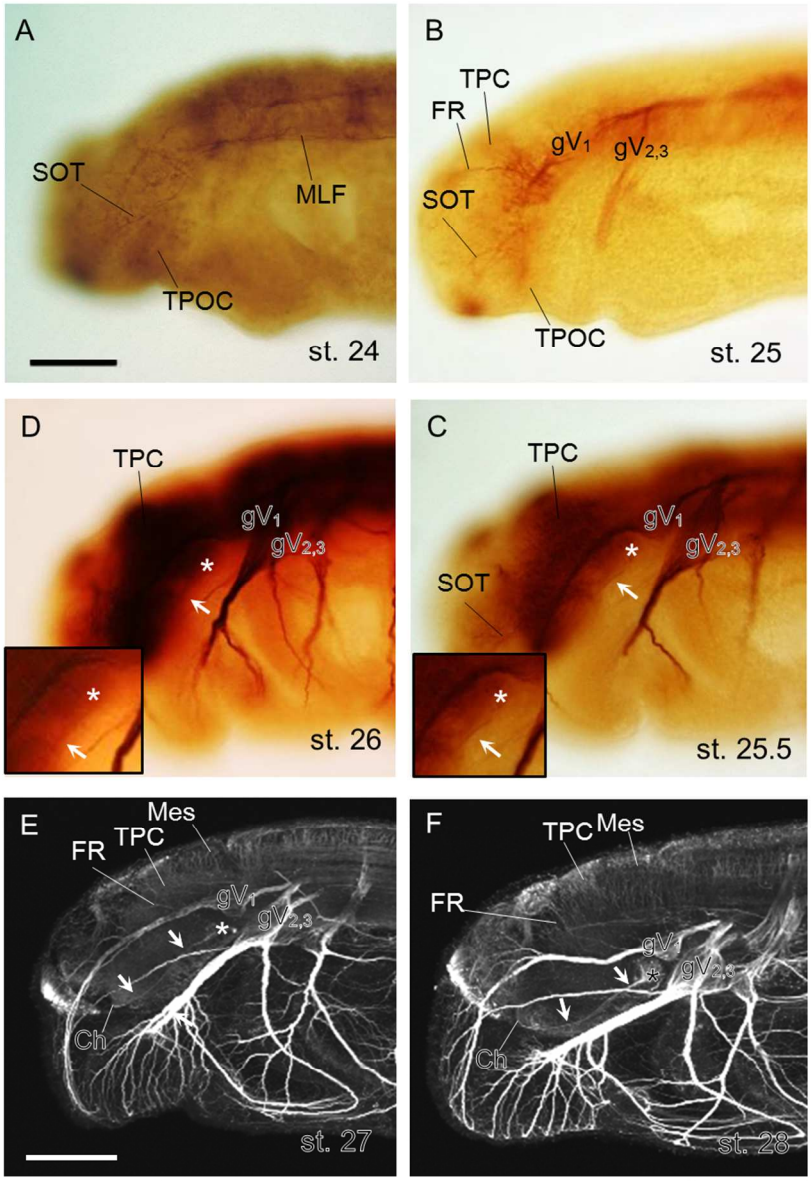

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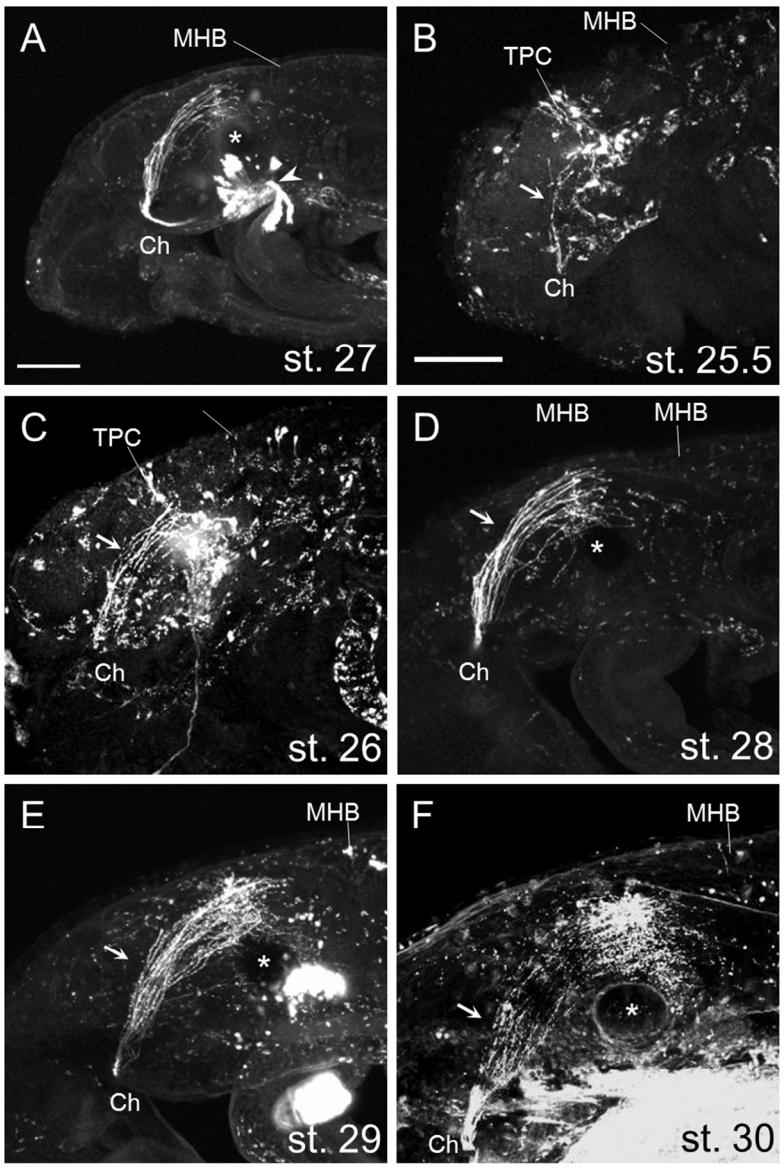

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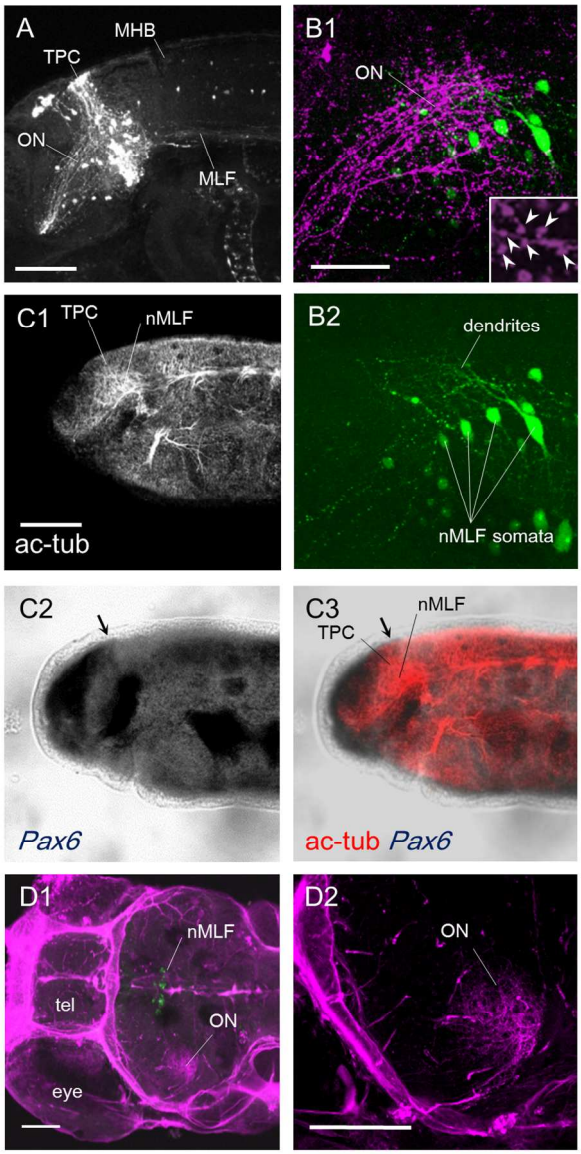

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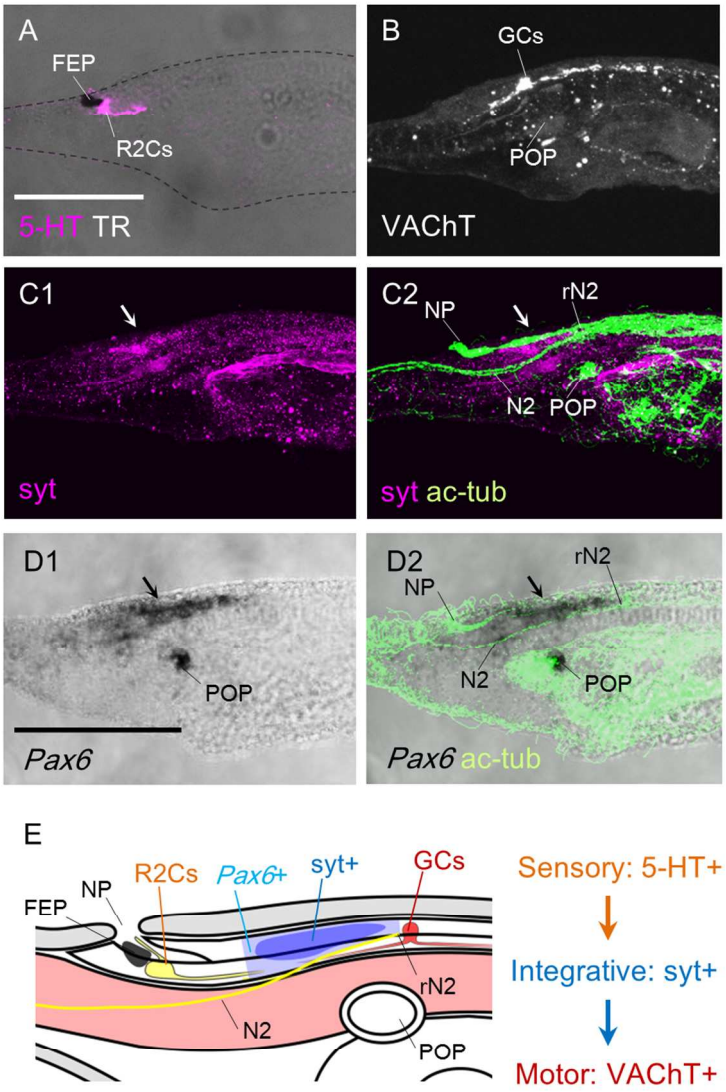

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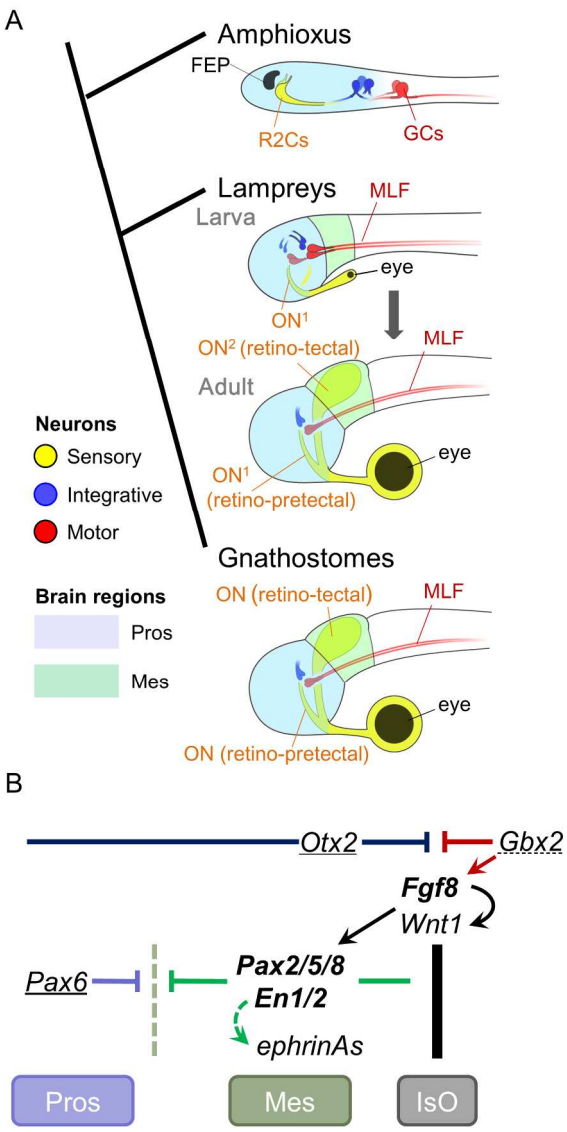

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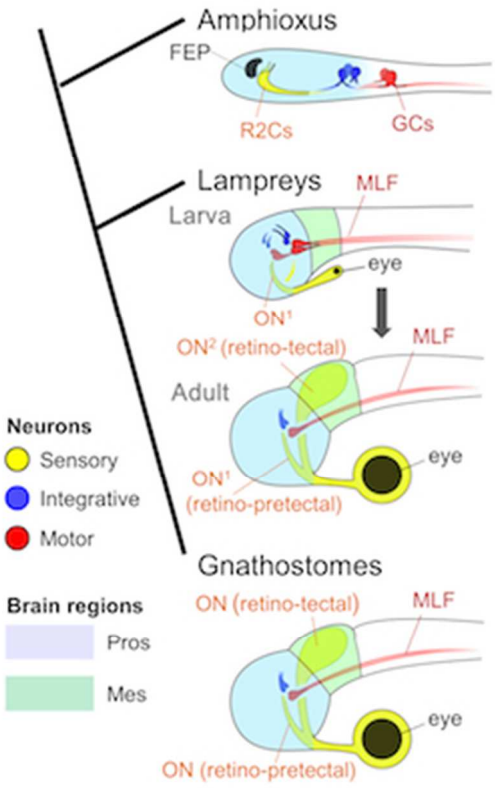

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105x141mm (72 x 72 DPI)

GRAPHICAL ABSTRACT

The common ancestor of chordates had an ocellus-like eye(s), and the visual center was in the *Pax6*-positive region, which processes directional vision. In the common ancestor of vertebrates, the *Pax6*-positive (prosencephalic) visual center remained the main visual center, since larval lampreys also have their visual center in the prosencephalon.