

Introduction

The question of the evolutionary origins of the vertebrate characteristics is of critical importance to the study of the origins of vertebrate cognitive abilities (especially human ones) and has been hotly debated in the literature for over a century (Huxley, 1895). The telencephalon in vertebrates is formed as an evagination of the prosencephalon – an outpouching anterior to the diencephalon in the developing embryo. To study the problem of the origin of the vertebrate telencephalon, evolutionary neuroanatomists have examined the central nervous systems of basally-branching extant vertebrates (*cyclostomata*, including hagfish and lampreys) and chordates (tunicates and cephalochordates). The telencephalon has been thought to be a synapomorphy of vertebrates, as lampreys, belonging to a vertebrate outgroup, have a relatively small telencephalon (Retaux and Kano, 2010), and because amphioxi, a group of invertebrate chordate, was thought to lack it altogether (Wicht and Lacalli, 2005; Holland and Short, 2008).

Amphioxus, commonly called ‘lancelets’, belonging to *Cephalochordata*, has become an important organism to study with regards to chordate evolution due to its place in modern phylogenetic trees of metazoans as a basally branching chordate (Figure 1). While it was initially believed that the tunicates split off the line to vertebrates before amphioxus, due to tunicates’ derived and simplified development into sessile organisms, it was later shown via advancing genetic analyses that amphioxus, and not tunicates, was the more basally branching chordate (Putnam et al., 2008). This reorganization aids the importance of finding vertebrate homologs in amphioxus biology, as such homologies would now form the basis of a strong argument for finding such a feature in the proto-chordate ancestor.

Importantly, the long-standing claim that there is no telencephalic homolog to be found in the nervous system of amphioxus specifically has recently been called into question (Benito-Gutierrez et al., 2021). While earlier claims that amphioxus lacked a telencephalon relied on gross morphological observations, such as the relatively small size of the swelling at the amphioxus anterior neural tube during development, and patterns of gene expression in embryonic and larval stages (Shimeld and Holland, 2005; Wicht and Lacalli, 2005, Holland and

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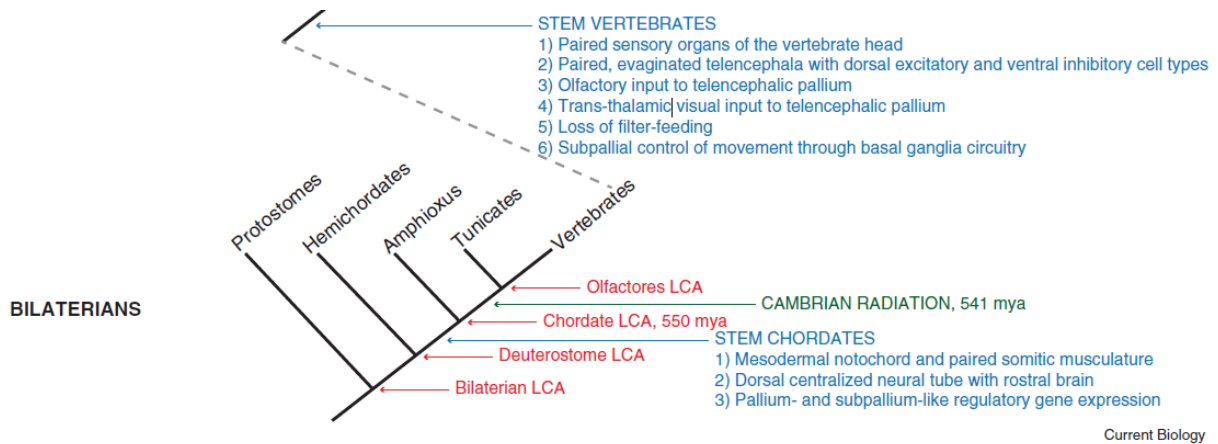


Figure 1: Phylogenetic tree showing Bilaterian evolution on the line leading to vertebrates. *Amphioxus* remains the best species to study to learn about stem chordates. Figure from Briscoe and Ragsdale, 2019.

Short, 2008), these recent proposals marshal new evidence gathered in adult amphioxi, making claims of homology at the levels of cell type and gene expression pattern to the vertebrate telencephalon. However, research as recent as 2017 mapped the most anterior regions of the developing amphioxus embryo to diencephalic vertebrate areas, rather than telencephalic areas (Albuixech-Crespo et al., 2017). These claims of telencephalon homology, initially posted on biorxiv (Benito-Gutierrez, et al., 2018), while persuasive, are highly novel, run against the existing literature, and have yet to be widely accepted or cited.

In this review, I critically consider the recent claims of a telencephalon homolog in amphioxus made by Benito-Gutierrez et al. I first parse their evidence and line of argument in terms of claims of homology, specifically, within the levels of homology framework. I weigh the argument made in those papers against earlier models of amphioxus central nervous system organization and how it may correspond to vertebrate organization. I consider the evolutionary implications of accepting the telencephalon as a chordate, rather than vertebrate, innovation. I conclude by arguing that while Benito-Gutierrez et al. marshal convincing evidence that the patterns of gene expression and cell populations in the amphioxus PAD represent strong evidence for homology by descent with vertebrates for those attributes, we cannot thus far conclude that the PAD and the vertebrate telencephalon are homologous as structures. With regards to future approaches, I additionally describe of a series of experiments that would serve to further elucidate the nature of this problem.

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What constitutes a telencephalon?

While the outpouching of the telencephalon in the development of most vertebrates is unmistakable, the definition of what constitutes telencephalon once we look at basal chordates such as amphioxus does come down to how we would like to define telencephalon. Does it constitute a series of gene expression patterns that we find across vertebrates that govern telencephalon development? Or is it instead a functional requirement of multimodal sensory integration and a structural requirement of pallial and sub-pallial division? Are homologies with vertebrate telencephalon a requisite, or could a convergently evolved structure, if it were to be found to otherwise meet given criteria, be called a telencephalon? In a Current Biology Review, written shortly after the first posting of evidence for an amphioxus telencephalon-like region by Benito-Gutierrez et al, Briscoe and Ragsdale discuss telencephalon evolution, and lay out, both explicitly and implicitly, key telencephalon features as they have been described across vertebrates, even while telencephalon anatomy does vary significantly across vertebrates. These include (brutally summarized here-) acting as a “center for multimodal sensory integration, and... to control a rich repertoire of behaviors,” comprising two areas – a dorsal pallium and a ventral subpallium, itself composed mainly of GABAergic neurons, which eventually form a striatum and pallidum, for which circuitry is conserved across known vertebrates (Briscoe and Ragsdale, 2019). Additional telencephalon features at the gene expression level that give rise to specific cell type organization and appear conserved across vertebrates (while best studied in the developing mouse brain) are expression patterns of essential genes such as FGFs, Shh, BMPs, FoxG1, Pax6, and Lhx2, (Hebert and Fishell, 2009).

The criteria for telencephalon should be obviously broader than that of neocortex and broader than those for the pallium, but not so broad as to include many clades of metazoans across Bilateria as having a telencephalon. The goal of categorization here should be to draw lines that help elucidate the origins of structures that explain the unique cognitive abilities of humans. However, while the definition of telencephalon is somewhat arbitrary, claims of homology between amphioxus brain areas as specific and argumentative, and hold weight in terms of the question of the origins of vertebrate and chordate brain architecture.

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Review of homology claims for the anterior amphioxus nervous system from 2000-2010

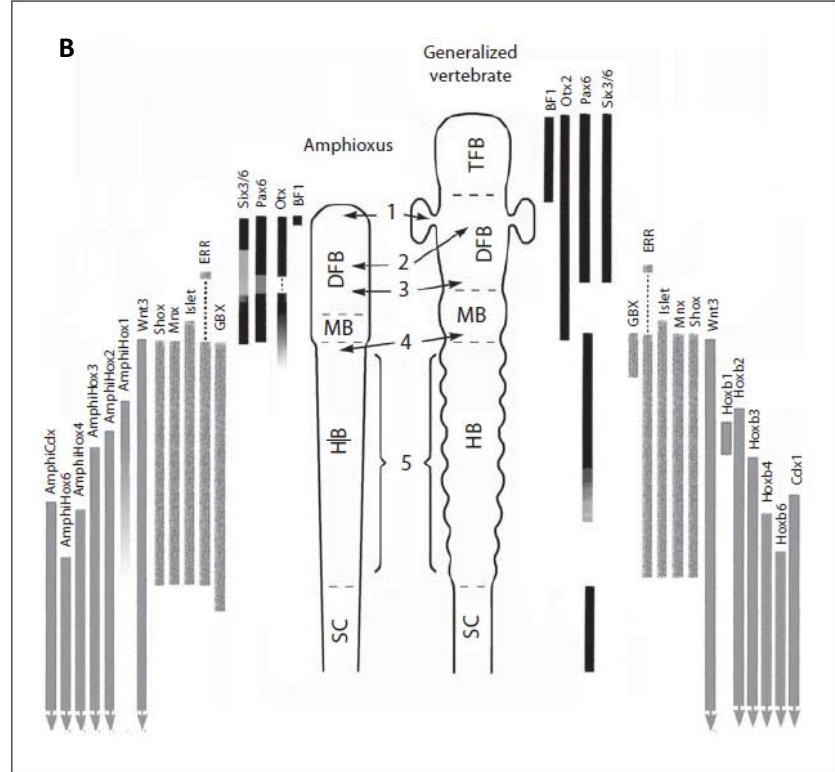
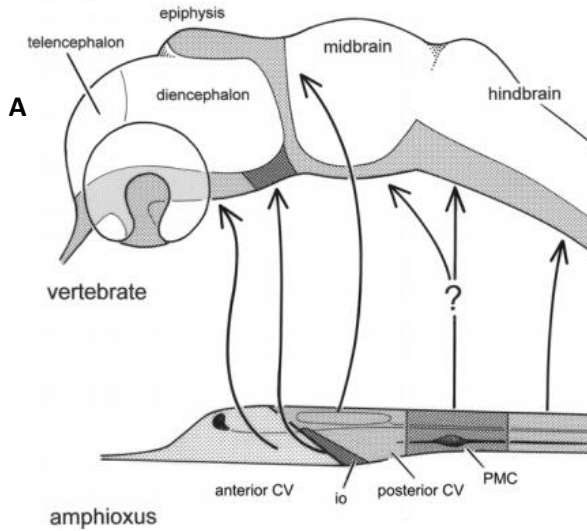


Figure 2: Homology claims, 2000-2010. A: Mapping structures from anterior amphioxus central nervous system to ventral structures in the developing vertebrate. Figure from Wicht and Lacalli, 2005. B: Mapping anterior-posterior gene expression patterns from Amphioxus to vertebrate CNS. DFB, Diencephalic forebrain. MB, midbrain, HB, hindbrain, SC, spinal cord. Figure from Holland, 2008.

By the early 21st century, it became clear that areas surrounding the central ventricle of the amphioxus embryo were comparable to brain structures in the developing vertebrate. In a 2005 review by Wicht and Lacalli, proposed homologues primarily mapped to only ventral structures. Particularly, a proposal of homology between the anterior cerebral vesicle and the ventral diencephalon was made – notably not the telencephalon (Figure 2A). The authors claim that because of a homology between the lamellar body and the pineal gland, the entire cerebral vesicle may be thought of as a “primitive counterpart of the diencephalon” (Wicht and Lacalli, 2005). These ventral diencephalon regions, supported by “microanatomy” analysis, include “the pre-optic area to the hypothalamus and infundibulum, the floor of the midbrain, roughly equivalent of the tegmentum, and the anterior end of the reticulospinal system.” The authors do point out other possibilities for homologies – including the potential for an olfactory bulb in later development and adult stage organisms. They also note the lack of data on these later stages. Gene patterning in amphioxus versus vertebrate embryos indicated homologies in greater detail than solely anatomical analysis – patterns of expression were already being used

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to suggest homology between cerebral vesicle in amphioxus and diencephalon in vertebrates: specifically, *Pax6*, *Six3*, and *BF-1* (or *FoxG1*), as well as *Otx* (Figure 2B). Even with this evidence of homology at the level of gene pattern expression, researchers at this time were hesitant to conclude full structural homology – Shimeld and Holland warn of this in their 2008 review of amphioxus developmental genetics, writing, “The chief problems with this approach are that a given gene may be used at more than one time and placed in the development of the same embryo, and there are unequivocal examples of dissociation between genotype and phenotype (Tautz 1998)” (Shimeld and Holland, 2005).

Recent evidence for detailed mapping of anterior amphioxus nerve cord to vertebrate diencephalon

In the decade following, more powerful genetic toolkits made possible more detailed maps of gene expression patterns in amphioxus. Such an application in 2017 to the developing amphioxus revealed an unexpectedly complex set of gene expression patterns, termed by the authors of the study to be a “genoarchitectonic model”, revealing novel anterior-posterior and dorsoventral patterning. This approach yielded novel homology claims, but as before, concluded that no structure in the developing amphioxus corresponded to telencephalon (Albuixech-Crespo et al., 2017). The results of this study, namely that fore and midbrain structures in vertebrates mapped to the same area in amphioxus, were used by the authors to question the prosencephalic model.

Specifically, the authors in this study proposed a new model of molecularly regionalized areas in amphioxus at the 7-somite mid-neural phase of development on the basis of overlapping morphogenic signals, including *Hox*, *Gbx*, *Fezf*, *Lhx*, *Wnt*, *Otp*, and *Nkx* (Figure 3). Importantly for our purposes, the authors divide the rostral neural tube, which they call the rostral archencephalic portion, into two territories – the more posterior di-mesencephalic primordium (DiMes) (Figure 3B') and the more anterior hypothalamo-prethalamal primordium (HyPTh) (Figure 3B'). Within the HyPTh, the authors further divided anterior nerve cord into Rostral, Interm, and Caudal-HyPTh. From these divisions, the authors propose homologies between the

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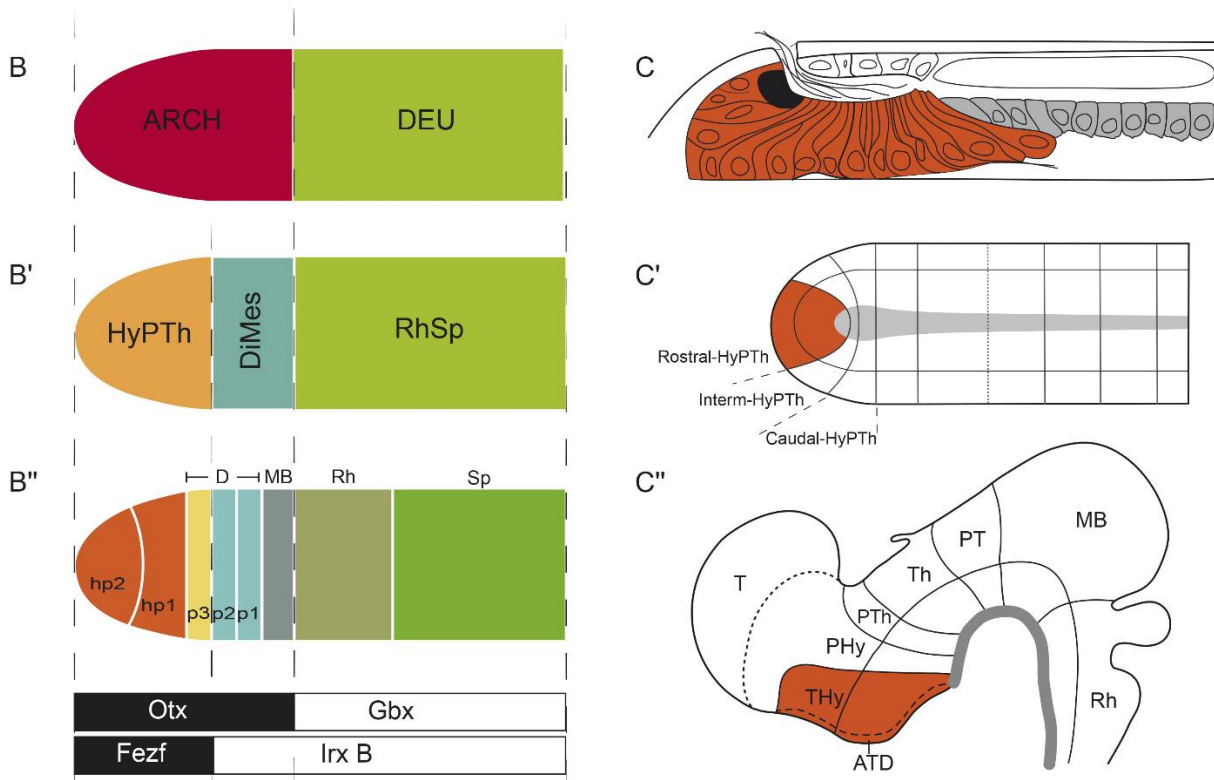


Figure 3: "Genoarchitectonic model of the developing CNS at the amphioxus 7-somite stage." B-B'': Comparison between amphioxus (B') and vertebrate (B'') model subdivisions. C, C': Amphioxus neural plate, with rostral HyPTh in orange and floor plate domain in grey. C'': comparison with vertebrate neural tube and suggested homology. Figure and most of description from Albuixech-Crespo et al., 2017.

non-telencephalic part of the secondary prosencephalon – specifically the terminal and peduncular hypothalamic prosomeres in the vertebrate diencephalon (Figure 3C'').

Claims and evidence for telencephalic homolog in amphioxus

New methods for obtaining serialized slices of amphioxus brain, alongside careful comparative study of adult versus larval amphioxus were novel hallmarks of the recent study that presented evidence for a telencephalic homolog in amphioxus, dubbed the *Pars anterodorsalis* (PAD). This study exhibited evidence of homology at the level of gene expression patterns discussing overlapping expression in the adult amphioxus of *FoxG1*, *Emx*, *Lhx2/9*, *Pax4/6* and *Nkx2.1*, as well as *Shh*. Additionally, the authors made a claim of homology at the level of cell types, producing evidence in the adult amphioxus of populations of dorsal populations of glutamatergic and dopaminergic neurons. Key to their argument in putting forward a claim that

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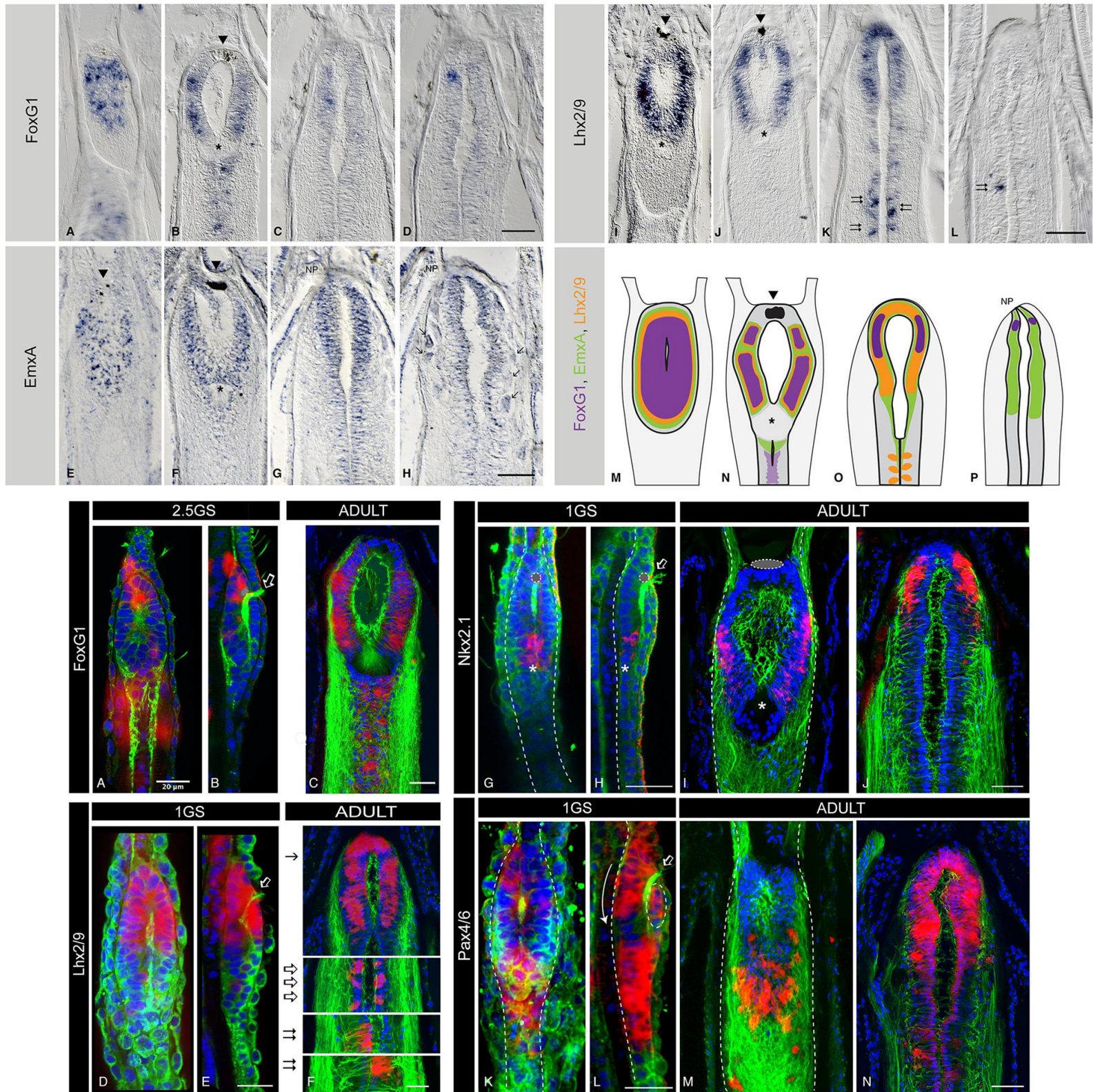


Figure 4: Gene expression data for FoxG1, EmxA, Lhx2/9, Nkx2.1, and Pax4/6 genes in adult amphioxus brain (top) and adult vs. larval brain (black, bottom). Images from left to right for top panel proceed ventrally to dorsally as coronal sections. Top of image is anterior. For top panel: all images are centered on cerebral vesicle (cv). Scale bars are 50 microns. For bottom image: shows post-metamorphic expansion of gene domains for telencephalic genes in vertebrates. First two images in series show dorsal and lateral view of embryo. Pink is gene expression, green is neuropil, and blue is nuclear staining. Adult scale bars are 50 microns and embryo scale bars are 20 microns. GS = gill slit stage. Figures and most of captions from Benito-Gutierrez et al., 2021.

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studies had not collected gene expression data in the *adult* amphioxus, and as the lancelet develops slowly and even after metamorphosis, much of amphioxus development had been skipped over (Benito-Gutierrez et al., 2021).

The authors suggested homology to vertebrate telencephalon patterns of gene expression by showing remarkably similar patterns of overlapping telencephalon organization genes in the adult amphioxus brain (Figure 4, top). This detailed imaging and patterning analysis lies in strong contrast to earlier work (shown in previous figures), which was significantly less precise, and in fact asserted markedly different patterns of expression of similar genes in the larva – for example, BF-1 expression in larval amphioxus, corresponding to *FoxG1*, is represented by a singular square next to the anterior neural tube in Figure 2B. Images from this latest study show that this gene is expressed widely in the anterior amphioxus brain. Holland and Short cite BF-1 expression as evidence for homology between anterior amphioxus brain and diencephalon, but adult expression appears more like telencephalic expression in vertebrates, according to Figure 2B (Holland and Short, 2008). Additional data in Figure 4-Bottom Panel-A-C back up this difference, where we can see gene expression has changed significantly from the larva to the adult. The same is true for other essential vertebrate telencephalic patterning genes shown in the figure that were cited in earlier literature as lacking in amphioxus brain, a result which was used as evidence for the claim of lack of homology.

Additional novel evidence provided in the Benito-Gutierrez paper includes evidence for cell type homologies in terms of neurotransmitter. Given the continuously developing nature of the amphioxus elucidated here, the authors examined the extent to which neurons differentiated into populations through amphioxus' continuing development. Making reference to excitatory, glutamatergic populations of neurons originating in the mouse telencephalon, and shown to be controlled by the same genes found here in the amphioxus, the authors proceeded to demonstrate the localization of similar populations in the adult lancelet (data not shown here – Benito-Gutierrez). Importantly, they found a dorsal population of these neurons. Additionally, the authors located populations of GABAergic cells slightly ventral to the excitatory population

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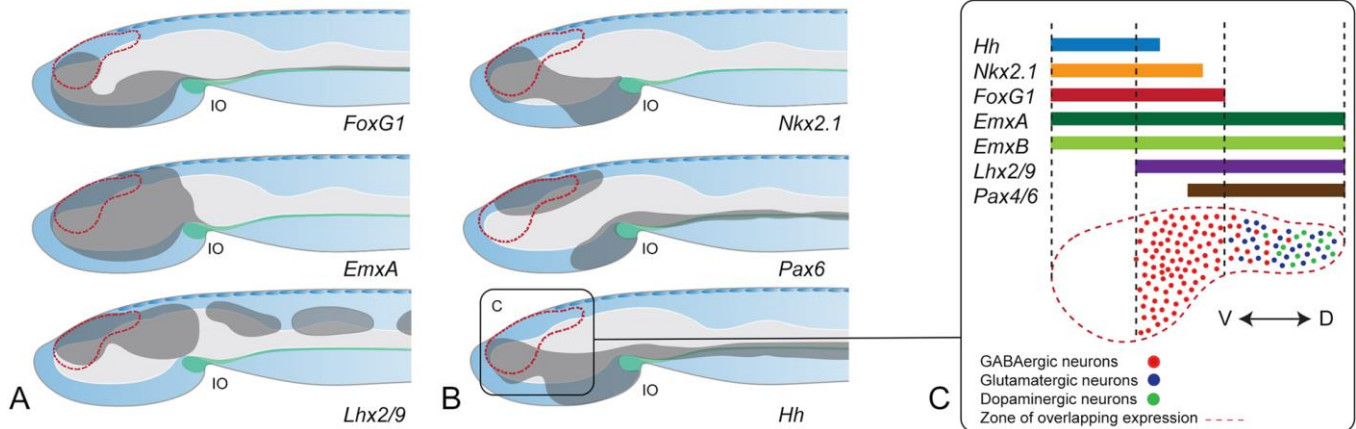


Figure 5: Expression patterns in the proposed telencephalic region of the adult amphioxus brain. A, B - summary of key gene expression patterns. C: view of PAD and dorsal ventral patterning of gene expression domains and cell type. Figure and most of caption from Benito-Gutierrez et al., 2021.

in the PAD. These groupings align with the vertebrate telencephalon cell type organization (Figure 1, stem vertebrate text).

In concluding this section, the claims of homology in this paper on the level of gene expression and cell types are strong between vertebrate characters and amphioxus. This paper succeeds in countering other, earlier claims regarding gene expression in amphioxus embryo that was assumed to apply as well to adults (such as from Wicht and Lacalli, 2005 and Retaux and Kano, 2010).

Critiquing these claims

While these homology claims are persuasive at their respective levels, homology at one level does not imply homology at another (Dugas-Ford and Ragsdale, 2015). Additionally, certain details in the analysis of the paper featured in the preceding section deserve more careful critical attention. I proceed in this section to carefully critique aspects of the telencephalon homolog argument.

In defending the conserved nature (through Chordata instead of just Vertebrata) of the gene expression patterns to which the earlier authors drew our attention, they note, “Prior to this work, the expression of *FoxG1*, *Hh*, and *Emx* was believed to be missing from the anterior amphioxus brain, contrasting with several reports of these genes being present... in nonchordate invertebrates such as insects, annelids, and enteropneusts” (Benito-Gutierrez et

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al., 2021). If these genes are conserved throughout Bilateria, they then either must exist in amphioxus or have been lost. While they should get credit for solving this puzzle in locating the missing genes, this note is a strike against their claims, with respect to the question of how the telencephalon should be defined. Given that these genes are so widely conserved, the burden is now higher for these authors to show how cephalochordate patterning is distinctly like vertebrate patterning in a fashion that is different from protostome patterning.

A second important note is that this paper makes claims regarding the effects of these patterns of gene expression in amphioxus, without having proved them. While the expression of these genes shows remarkable similarity to vertebrates, the conclusion that the structures will then be homologous is not supported. Indeed, this is the note that Shimeld and Holland made, as discussed earlier in this report (Shimeld and Holland, 2005). What is required to back up the claims of functional effects of gene patterning is a repetition of experiments done in mice to prove that the “vertebrate telencephalic genes” which are discussed here are indeed critical to telencephalon development: knockouts. Such a procedure, perhaps paired with a behavioral analysis of lancelet differences across experimental and control animals would then elucidate the question of other, more functional levels of homology.

As discussed earlier, these homology levels may be more important in defining telencephalon. While the PAD area may share localized gene expression patterns with the vertebrate telencephalon, can it be shown to be a sensory integration and/or decision making area in amphioxus? Knockout experiments could provide key context and help answer these questions. In the paper as it is currently, the authors could stand to be more precise with claims of homology. For example, in their abstract, they note: “One way to identify homologous brain parts between distantly related animal groups is to focus on the combinatorial expression of conserved regionalization genes that specify brain regions” (Benito-Gutierrez et al., 2021). This directly conflates levels of homology – one may only identify homologous regionalization genes in this way, without proving the effects of these genes on those brain regions. Additionally, a key underlying homology defining telencephalon across vertebrates is homology at the level of neural circuits (Briscoe and Ragsdale, 2019). This could be further explored by functional circuit

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mapping using electrophysiological methods in the amphioxus PAD area to bolster claims of homology to vertebrate telencephalon.

A final criticism involves expanding the library of homologous genes. The genes studied here are not comprehensive in terms of shaping vertebrate telencephalon development. For example, Wnt and SFRP-1 signaling have been shown to be essential to telencephalon development in vertebrates (Retaux and Kano, 2010; Harrison-Uy and Pleasure, 2012).

In conclusion, at the gene pattern expression and cell type homology levels, the PAD in amphioxus is homologous to the vertebrate telencephalon. However, this is not a strong enough homology claim to conclude that amphioxus does, indeed have a telencephalon.

Further, more conclusive functional studies are necessary to conclude structure level homology. Under the current definition, and given current data, amphioxus does not have a telencephalon.

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