Is There a Prechordal Region and an Acroterminal Domain in Amphioxus?

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Abstract

This essay re-examines the singular case of the supposedly unique rostrally elongated notochord described classically in amphioxus. We start from our previous observations in hpf 21 larvae [Albuiexch-Crespo et al.: PLoS Biol. 2017;15(4):e2001573] indicating that the brain vesicle has rostrally a rather standard hypothalamic molecular configuration. This correlates with the notochord across a possible rostromedian acroterminal hypothalamic domain. The notochord shows some molecular differences that specifically characterize its pre-acroterminal extension beyond its normal rostral end under the mamillary region. We explored an alternative interpretation that the putative extension of this notochord actually represents a variant form of the prechordal plate in amphioxus, some of whose cells would adopt the notochordal typology, but would lack notochordal patterning properties, and might have some (but not all) prechordal ones instead. We survey in detail the classic

and recent literature on gastrulation, prechordal plate, and notochord formation in amphioxus, compare the observed patterns with those of some other vertebrates of interest, and re-examine the literature on differential gene expression patterns in this rostralmost area of the head. We noted that previous literature failed to identify the amphioxus prechordal primordia at appropriate stages. Under this interpretation, a consistent picture can be drawn for cephalochordates, tunicates, and vertebrates. Moreover, there is little evidence for an intrinsic capacity of the early notochord to grow rostralwards (it normally elongates caudalwards). Altogether, we conclude that the hypothesis of a prechordal nature of the elongated amphioxus notochord is consistent with the evidence presented.

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Introduction

Members of the phylum Chordata are characterized by sharing structural components of the body plan, such as pharyngeal gill slits, segmental muscles, a dorsal nerve cord, and the notochord (chordamesoderm) [Brusca and Brusca, 1990; Lacalli, 2012; Di Gregorio, 2020]. Chordate

animals can be divided into three subphyla: cephalochordates (amphioxus or lancelets), urochordates (tunicates), and vertebrates (whose most early branching lineage relative to the human one is represented by the Agnatha, that is, hagfishes and lampreys). The discussion about which of these chordate subphyla should be considered most basal began with early morphological studies that compared the complexity of amphioxus with the relative simplicity of tunicates [Corbo et al., 1997; Passamanek and Di Gregorio, 2005]; these works identified tunicates as probable basal chordates and the cephalochordate amphioxus as the closest living relative of vertebrates. However, more recent phylogenetic studies unequivocally defined cephalochordates as the first-branching chordate subphylum despite their greater structural complexity compared to tunicates [Delsuc et al., 2006; Putnam et al., 2008]. Members of the subphylum tunicates are considered to have fast-evolving genomes with a simpler anatomical structure than cephalochordates, which is possibly due to secondary reduction of fundamental developmental mechanisms. In contrast, both cephalochordates and vertebrates are considered to have slowly evolving genomes and the evidence suggests that the cephalochordate amphioxus experienced relatively few evolutionary changes in the last 500 million years [Holland and Holland, 1989; Holland, 2015].

Genome sequencing projects also have confirmed Ohno's [1970] hypothesis that two rounds of complete genome duplication occurred during the early evolution of vertebrates, while a non-duplicated single genomic set is found in the cephalochordate and urochordate lineages [Holland and García-Fernández, 1996; Putnam et al., 2008; Holland, 2010]. The single genes present in the genome of non-vertebrate chordates thus resulted in four copies (paralogue genes) secondary to double genomic duplications correlated with the evolutionary emergence of jawed vertebrates, although the total number of genes was variously reduced ultimately by gene losses [e.g., in Meis genes; Irimia et al., 2011; Sanchez-Guardado et al., 2011]. Tunicates in fact suffered a significant loss of genes involved in developmental patterning, a feature possibly related to their structural simplification. Despite the important evolutionary distances between the extant cephalochordates and tunicates with extant vertebrates, with strong differences in the number of developmental genes, the basic features of the chordate body plan are remarkably conserved [Passamanek and Di Gregorio, 2005; Holland, 2010].

Amphioxus, as the extant species of the first-branching chordate subphylum, is in a key phylogenetic position for the study of the origin and early evolution of the cen-

tral nervous system (CNS) of vertebrates. The larva of amphioxus was described in detail in the late 19th century by the zoologists Kowalevsky [1877] and Hatschek [1881]. Additional morphological and developmental details were provided later by Lwoff [1894], Willey [1894], and Conklin [1932], among other authors. Willey and Conklin essentially followed Hatschek's concepts, while Lwoff adopted some alternative viewpoints and compared with several vertebrates. However, it was nearly a century later, when molecular studies using *in situ* hybridization probes started, that the comparative study of the brain primordium of cephalochordate larvae again captured the attention of researchers. This organism came to be considered a crucial model to understand the development and evolution of vertebrates [Holland et al., 1992, 1995].

There is a long tradition of comparative studies on the amphioxus brain, as well as of tentative homologies of its parts with those observed in vertebrates. Stieda [1873] hypothesized that the entire brain of vertebrates was homologous to the cerebral vesicle of cephalochordates. Minot [1897], considering contemporaneous research on cephalic neuromeres, speculated that the vertebrate brain was "originally" segmented and suggested some cerebral homologies with the brain vesicle of amphioxus and tunicates (see also Johnston [1910] in this regard). Towards the end of the 20th century, key molecular morphological studies were initiated that shed new light on the fundamental structural organization or general construction plan (Bauplan) of the CNS of vertebrates. Accruing gene expression patterns were interpreted in terms of causal mechanisms leading to dorsoventral (DV) and antero-posterior (AP) patterning of the neural tube, with gradual regionalization along these dimensions into intercrossed patterns of fundamental transverse and longitudinal morphogenetic units which were conserved among all studied vertebrates [Nieuwenhuys and Puelles, 2016]. Puelles and Rubenstein [1993, 2003, 2015] and colleagues developed the so-called prosomeric brain model of vertebrates, which redefines the AP and DV brain dimensions in terms of causal molecular mechanisms; this model was updated several times. Using this evolving model, the invariant topological position of conserved large or smaller brain subdivisions of vertebrates was characterized by particular combinatorial profiles of gene expression in correspondence with specific anatomical landmarks [as studied across lampreys, hagfishes, sharks, teleosts, amphibians, reptiles, birds, and mammals; Puelles and Rubenstein, 1993, 2003, 2015; Hauptmann et al., 2002; Brox et al., 2004; Ferran et al., 2007, 2008, 2009; Pombal et al.,

Fig. 1. **a** Schematic representation of the bipartite initial division of the amphioxus brain (with gene correlations). **b** Schematic representation of the three major AP subdivisions observed in the amphioxus brain at the 21 hpf stage and the correlative distribution of their key gene markers. **c** Schematic dorsal view of the late neural plate of amphioxus and its primordial Bauplan. The HyPTh domain is regionalized into rostral, intermediate, and caudal do-

main. The DiMes and the five rostralmost domains of the RhSp region are represented as well. ARCH, archencephalic prototagma; AT, acroterminal domain; DEU, deuteroencephalic prototagma; DiMes, diencephalo-mesencephalic primordialium; HyPTh, hypothalamo-prethalamic primordialium; RhSp, rhombospinal domain; S1–6, somites 1–6. Modified from Albuixech-Crespo et al. [2017].

2009; Rodríguez-Moldes, 2009; Moreno et al., 2010; Merchan et al., 2011; Morona et al., 2011, 2017; Puelles et al., 2012a, b; Puelles, 2013; Pose-Méndez et al., 2016; Sugahara et al., 2016]. Morphological topologic mapping of gene expression within a structural model or Bauplan established the novel genoarchitectonic approach to comparative neuroanatomy [Puelles and Ferran, 2012] and laid the foundation for systematic search of a possible conserved set of interrelated brain partitions in vertebrates or chordates, with invariant molecularly defined boundaries [Puelles and Ferran, 2012; Ferran, 2017; Puelles, 2018]. The Bauplan construct serves to assess homology versus analogy hypotheses, or variations (key innovations or losses) among the phyla sharing the Bauplan [Puelles and Medina, 2002; Puelles and Ferran, 2012; Ferran, 2017; Puelles, 2021; this issue]. The fundamental question was next put forward: how far do cephalochordates or tunicates reproduce in their simpler brains molecularly definable conformational features corresponding to the brain Bauplan of vertebrates?

With the beginning of gene expression studies at the end of the twentieth century, it was suggested again that

the CNS of vertebrates is homologous to the entire CNS of amphioxus [Holland et al., 1992; Lacalli et al., 1994; Lacalli, 1996]. However, only recently were details of potential topologic and molecular regional correspondence of the brain Bauplan of amphioxus versus that of vertebrates examined in terms of specific rostrocaudal neuromeric or DV longitudinal divisions and gene patterns [Albuixech-Crespo et al., 2017; Ferran and Puelles, 2019]. According to this genoarchitectonic analysis, the brain vesicle of the amphioxus neurula reveals at its rostral end three molecularly distinct neuromeric units forming an AP series along the rostrocaudal dimension. Their distinct genoarchitectonic profiles are consistent with one-to-one homology with the vertebrate bineuromeric rostral hypothalamic forebrain [prosomeres hp2, hp1; Puelles et al., 2012a; Moreno et al., 2017] plus the caudally adjacent prethalamic diencephalic neuromere (p3). These three rostral neuromeric units configure what Albuixech-Crespo et al. [2017] called the hypothalamo-prethalamic primordialium (HyPTh; Fig. 1).

These molecularly recognizable rostral AP units of amphioxus (apparently homologous in position and molecu-

lar profile to corresponding units described in vertebrates) also showed characteristic DV molecular partitions interpretable in terms of floor, basal, and alar/roof plates (Fig. 1). In contrast, in larval amphioxus most of the diencephalo-mesencephalic territory of vertebrates (thalamus, pretectum, and midbrain/forebrain regions) was represented by a very reduced and molecularly undifferentiated territory, identified by Albuixech-Crespo et al. [2017] as a di-mesencephalic (DiMes) primordium. This hardly specified part of the brain vesicle was linked experimentally by these authors to the absence of local secondary organizers in amphioxus, namely both the mid-diencephalic (zona limitans) and mes-rhombencephalic (isthmus) organizers [Albuixech-Crespo et al., 2017; reviewed in Puelles, 2017]. In addition, a simplified rhombospinal primordium (RhSp) was likewise identified genoarchitecturally. A conserved DV pattern implicit in the general presence of floor, basal, and alar/roof longitudinal zones was confirmed through HyPTh, DiMes, and RhSp. The partially conserved brain organization pattern observed in the study by Albuixech-Crespo et al. [2017], also considering the presence of a cyclopic median pigmented eye spot at the front of the HyPTh, and the absence of evaginated telencephalic vesicles and cerebellum, supports a similar yet significantly simpler cephalochordate brain Bauplan compared with that of vertebrates. The DV dimension seems established in a vertebrate-like way, though with scarce if any development of important alar derivatives (eyes, telencephalon, thalamus, midbrain tectum, cerebellum) whereas the AP dimension seems partially abnormal (compared to vertebrates), essentially due to the small and non-differentially specified DiMes component. Interestingly, the HyPTh primordium is the forebrain part that most resembles the vertebrate counterparts, suggesting a similar patterning mode. In vertebrates, this territory normally receives ventralizing patterning signals from the rostral tip of the notochord, rostralizing signals from the prechordal plate, and dorsalizing signals from the septo-commissural roof plate [Puelles et al., 2012a; Ferran et al., 2015; Puelles, 2017; Diaz and Puelles, 2020].

The peculiarly long notochord of amphioxus supports the whole brain vesicle in a manner uncharacteristic for vertebrates, and moreover extends beyond the brain to the tip of the head. Albuixech-Crespo et al. [2017] made the interesting collateral finding that its rostral part actually has a subtly distinct molecular profile. These differential features of the notochordal extension, irrespective of the apparent chordal typology of its differentiated adult cells and its close topographic relationship with the rostral terminal (acroterminal) end of the HyPTh pri-

mordium possibly imply a non-chordal nature of the molecularly distinct rostral portion. It was conjectured that it might be homologous alternatively with the transient migrating prechordal cell population of vertebrates that derives from the early endodermal prechordal plate and participates in rostral hypothalamic patterning [Albuixech-Crespo et al., 2017; Ferran and Puelles, 2019].

The notochord has attracted attention since the start of experimental neuroembryology in the late nineteenth century for its apparent selective role in inducing vertically the floor plate of the neural tube. This initiates crucial ventralizing patterning effects that antagonize roof plate-derived dorsalizing signals to produce the general DV division of the whole neural tube wall into floor, basal, alar, and roof parts [His, 1893, 1904; Johnston, 1910; Kingsbury, 1920, 1922; Shimamura et al., 1995; Puelles, 2018]. The ventralization and dorsalization processes are thus important mechanisms for the development of the brain Bauplan and are crucially involved in the establishment of the neural axis.

In surprising contrast with the long-assumed singular rostral elongation of the amphioxus notochord, in vertebrates this axial mesodermal structure is known during embryonic stages to gastrulate through the node (or dorsal lip of the blastopore) following the pioneering head endoderm (the evolutionary emergence of this process in fact separates invertebrates from chordates). In vertebrates, the earliest chordal cells originate at the node when the latter occupies its rostralmost position and produce the rostral tip of the notochord, later found under the mamillary pouch. The subsequently invaginated chordamesoderm elongates caudalwards jointly with the whole brain and embryonic body, as is indicated by clonal analysis [brain, notochord and somites are composed of caudally elongated clones; Lawson and Pedersen, 1992]. Kopsch [1950] published fate-mapping data on shark embryo gastrulae which he had collected since 1897. He showed conclusively that the notochord originates from the node (at the dorsal lip of the blastopore), but *after* the rostromedian head or anterior endoderm has finished its gastrulation. It thereafter elongates strictly caudalwards as axial mesoderm, in concert with body and brain elongation. These and other modern examples of experimental results show that the notochord of vertebrates only grows caudalwards. Surprisingly, there is no evidence so far indicating that the singular amphioxus notochord grows rostralwards in an active (proliferative) way. Contrarily, there is some evidence of proliferation occurring at the prechordal plate of amphioxus [Holland and Holland, 2006]. It is therefore of interest to carefully

re-examine the hypothesis of Albuixech-Crespo et al. [2017] that amphioxus may have a variant sort of prechordal plate derivative (that imitates the notochord in solidity and cell typology) that elongates and stands for the classic “rostral part of the notochord.” Should this hypothesis be corroborated or made credible by substantial evidence, the specification mechanism leading to a variant chordal phenotype of such prechordal cells would need to be explained.

Our aim in this review is to present existing evidence with regard to this notion, and eventually re-evaluate the evolutionary scenario relative to the prechordal region in the context of cephalochordates, tunicates, and vertebrates.

Notochordal and Prechordal Cells in Cephalochordates

One of the common “new” features of chordates (cephalochordates, urochordates, and vertebrates) is the presence of a notochord [Yasuo and Satoh, 1994]. The notochord (or chordamesoderm) was described for the first time by von Baer [1828] in the chick as a dorsal strand or chorda dorsalis. Early studies of the extinct Middle Cambrian fossil *Pikaia gracilens*, from the Burgess Shale of British Columbia [Morris and Caron, 2012; Mallatt and Holland, 2013] recently traced the evolutionary origin of the notochord to some 500 million years ago. Furthermore, Lauri et al. [2014] proposed that a structure called axocord, described in the annelid *Platynereis dumerilii*, is also present in bilaterian ancestors, and might give rise to the chordate notochord by vacuolization and hardening. Signals diffused from the notochord are involved in the patterning of adjacent tissues such as the midline blood vessels, the endodermal gut, the mesodermal somites, and the ectodermal neural tube [Cleaver and Krieg, 2001; Hogan and Bautch, 2004; Holland et al., 2004; Stemple, 2005]. In cephalochordates and ascidians, the notochord represents an axial support system essential for locomotion, due to the absence of an ossified skeleton [Satoh, 2003; Holland et al., 2004]. In ascidians, the notochord is observed only during larval stages, and is composed of some 40 cells placed in a single row along the tail; however, in the appendicularian tunicate *Oikopleura* the notochord is preserved in the adult animal and is composed of some 120–160 cells [Annona et al., 2015; Almazán et al., 2019; Di Gregorio, 2020]. Importantly, in ascidians the notochord does not extend rostralwards beyond the cerebral vesicle, thus showing the typical topological po-

sition of vertebrates [e.g., Dufour et al., 2006; Kugler et al., 2008; Veeman, 2018].

In contrast, the notochord from the extant basal chordate amphioxus reaches the anterior end of the body, clearly extending beyond the rostral end of the cerebral vesicle [Willey, 1894, p. 124]. This anomalous variation has never been explained with regard to its developmental mechanism. We owe, though, a relevant comment to Willey [1894], who stated that “...the ‘rostral end of the notochord’ presents a *retarded growth*,” which he qualified as a “*secondary phenomenon*.” This pattern was apparently understood by other authors as meaning “recently evolved” [i.e., non-ancestral; Holland et al., 1995], implying its lack of strict homology when compared with the standard rostral tip of the notochord in vertebrates. Indeed, the fossil records of the ancestral cephalochordate *Pikaia* suggest the presence of a shorter notochord that does not reach the anterior tip of the body [Morris, 1993]. We will nevertheless defend below a literal interpretation of Willey’s opinion, namely that in amphioxus larvae *a normal vertebrate-like notochord is formed*, and, *secondarily*, a rostrally protruding axial organ emerges *out of a different origin*, possibly the prechordal plate. This obviously implies a different homology for the notochordal extension.

In any case, modern molecular studies have largely assumed that the entire notochord of cephalochordates displays a common developmental program [Holland et al., 1995]. This contrasts with the recent discovery of molecular differences between the rostral and caudal parts of the notochord in larval amphioxus [Albuixech-Crespo et al., 2017; Ferran and Puelles, 2019]. The distinct “rostral notochordal domain,” which lies topographically along the midline of what we identify as the hypothalamic acroterminal domain (Fig. 2a, b, 3) [see concept in Puelles et al., 2012a; Puelles and Rubenstein, 2015; Puelles, 2018] and projects rostrally beyond it, was tentatively interpreted by Albuixech-Crespo et al. [2017] as a possible prechordal derivative. This was based on its topologic position (Fig. 2a, b), molecular profile, and some associated prechordal-like patterning effects (but not all standard prechordal effects), even though its cells do resemble notochordal cells topologically, at least by superficial observation.

Molecular Evidence

Albuixech-Crespo et al. [2017] examined whether the amphioxus cerebral vesicle satisfies the updated prosomeric model, a molecularly based model of brain region-

Fig. 3. Identification by *Dlk1* expression of the whole rostromedian acroterminal forebrain domain in an E13.5 mouse embryo. Note the extension from the locus where the anterior commissure develops (visible at E15.5) down to the front of the mamillary pouch. Ah, adenohypophysis; LT, lamina terminalis; Mb, mamillary body; MB, midbrain; Nh, neurohypophysis; OC, optic chiasm; p1–3, prosomeres 1–3; PT, pretectum; PTh, prethalamus; TH, thalamus.

alization previously tested in diverse vertebrates (this model was modified from earlier less precise versions by Puelles et al. [2012a], Puelles and Rubenstein [2015], and Puelles [2018]). The analysis of amphioxus was achieved by mapping some 50 relevant gene markers having characteristic brain expression patterns in vertebrates, at a particular larval stage of amphioxus (midneurula; 21 hpf). It was found that the “rostral part of the notochord” lying next to the median acroterminal hypothalamus does not express the marker genes *Shh* and *IrxB* (present in the notochord proper), and also differs from the rest of the notochord in maintaining stronger *Brachyury* expression and expressing differentially *Six3/6* (Fig. 2c, d). However, the “rostral notochord” does not express the gene *Gsc*, held to be a marker of prechordal derivatives in vertebrates. This gene is characteristically absent in the notochord of vertebrates and is involved mainly in the splitting of the rostral forebrain [De Robertis et al., 1992; Filosa et al., 1997; Lopez-Sanchez et al., 2005]. The independent molecular profile and developmental trajectory of the prechordal cell population compared with the notochord in vertebrates is also confirmed by the zebrafish *flh* mutant (*not*) in which prechordal mesendoderm is developed while the notochord is absent [Talbot et al., 1995]. The same conclusion can be obtained from the study of the zebrafish gene *oep* (one-eyed pinhead), which is involved in the formation of the prechordal region but not of the notochord [Schier et al., 1997]. *Oep* mutant em-

bryos are characterized by the absence or severe decrease of *Gooseoid* expression.

In vertebrates, *FoxA2* (*Hnf3β*) mRNA is detected along the entire length of the notochord as well as in tissue derived from the prechordal plate, and in the neural floor plate above the notochord [Sasaki and Hogan, 1993]. Both amphioxus orthologs of the *FoxA2* genes (*AmHNF-3-1* and *AmHNF-3-2*), which resulted from an amphioxus-specific duplication, are expressed in the rostral and caudal parts of the notochord, but only one of them appears expressed in the *Branchiostoma floridae* neural floor plate at the 10-somite stage [see Fig. 6a–c in Shimeld, 1997]. Both the Japanese *B. belcheri* and the European *B. lanceolatum* also express *FoxA2-1* throughout the length of the notochord [Shimeld, 1997; Terazawa and Satoh, 1997; Venkatesh et al., 1999; Albuixech-Crespo et al., 2017]. In the tunicate ascidian *Ciona intestinalis* the ortholog *FoxA-a* gene is expressed in the notochord and also at the correlative neural tube floor plate, but no data are available about possible prechordal plate expression [Corbo et al., 1997; Di Gregorio et al., 2001].

Brachyury, *Chordin*, *Noggin*, and *Not* (*Flh*) genes are expressed in the notochord but are absent in prechordal cells of vertebrates [Talbot et al., 1995; Dale et al., 1999; Lopez-Sanchez et al., 2005]. In vertebrates, the *Brachyury* gene is maintained only in the notochord after gastrulation [Herrmann, 1995; Holland et al., 1995]. Amphioxus *Brachyury* is also duplicated in amphioxus [*AmBra1* and *AmBra2*; Holland et al., 1995; Terazawa and Satoh, 1997]. At neurula stages of *B. floridae*, *AmBra1* and *AmBra2* expression was detected first at the “rostral part of the notochord” and later also in the caudal part [Holland et al., 1995]. Similar data are available for *B. belcheri* neurula embryos, whose in situ signal is more intense at the “rostral notochord” compared with the caudal notochord [see Fig. 3j, k in Terazawa and Satoh, 1997]. The notochord of ascidians, which is vertebrate-like in extent and position, only exists during the larval stage and expresses the *Brachyury* gene *As-T* during early/late gastrula and neurula stages [Yasuo and Satoh, 1994].

The single amphioxus *Hedgehog* gene in amphioxus is the ortholog of the vertebrate *Hedgehog* gene family. Following Shimeld’s [1999] study, in *B. floridae* the expression of *Hedgehog* gene appears in the caudal notochord as well as in the related floor plate of the neural plate at early neurula and midneurula stages, and the published illustrations show that the signal is absent at the rostral notochord [see Fig. 4c–g in Shimeld, 1999]. This result was corroborated by Albuixech-Crespo et al. [2017] in *B. lanceolatum* between stages 18 hpf and 21 hpf. Loss of

function of the *Shh* signaling pathway in the prechordal plate results in cyclopia in zebrafish, mice, and humans [Chiang et al., 1996; Roessler et al., 1996; Roessler and Muenke, 2001; Dubourg et al., 2004; Krauss, 2007].

The Prechordal Hypothesis

Under this notion we refer to Albuixech-Crespo et al.'s [2017] hypothesis that the molecularly differential rostral part of the amphioxus notochord may be a prechordal derivative. In order to explore this idea, we need to compare the prechordal formations of vertebrates with possibly equivalent prechordal elements in amphioxus. This task apparently was never addressed before.

The prechordal material of vertebrates is a biphasic formation. Its earliest cells originate with the rest of the invaginated rostral endoderm at the inception of the early node [Imoh, 1985; Nieuwkoop, 1985 (his Fig. 2, 3); Delarue et al., 1992; Bally-Cuif et al., 1995; Gilbert, 2003]. They form the prechordal plate of the anterior head visceral endoderm, which characteristically contacts directly (adheres to) the prospective tuberal part of the rostral hypothalamus [Wood and Timmermanns, 1988; Wargha and Kimmel, 1990; Schulte-Merker et al., 1994], even before the notochord starts to form out of the node (see a comparable amphioxus stage amphioxus in Fig. 4a of Willey [1894], after Hastchek [1881]). Once the prechordal plate is in place, the notochordal cells start to ingress from their proliferating nodal source, first forming what is going to be the notochordal tip under the prospective mamillary pouch [Imoh, 1985; Nieuwkoop, 1985] (see a comparable amphioxus stage in Fig. 4b of Willey [1894], Fig. 5a, b in Conklin [1932], von Kupffer [1894]; see also Lwoff [1894], Aasar [1931], Hill and Florian [1931], Adelmann [1932], De Lange [1936], Kopsch [1950]). Various authors have suggested that some mesodermal cells incorporate secondarily into the endodermal prechordal plate, implying a mixed endo-mesoderm or meso-endoderm [Adelmann, 1922, 1926; Seifert et al., 1993; Foley et al., 1997; Pera and Kessel, 1997; Nieuwkoop, 1999; Rowan et al., 1999]. However, in our opinion this point does not seem to be clearly established with appropriate labeling of mesodermal or endodermal elements. In a second phase subsequent to closure of the neural tube, the adhering prechordal plate separates from the hypothalamic ectoderm and becomes transiently a part of Seessel's pouch of the pharyngeal endoderm (see for example von Kupffer's [1894] midsagittal illustrations for the lamprey; Fig. 5c in the chick in Puelles et al.

[1987a]). However, some of the originally epithelial endodermal prechordal plate cells do instead make an epithelio-mesenchymal transition, and become in situ free prechordal cells that migrate thereafter dorsalwards in front of the rostromedian acroterminal domain of the hypothalamus, reaching septo-preoptic roof plate levels [Schulte-Merker et al., 1994; Thisse et al., 1994; Bally-Cuif et al., 1995]. Modern studies have revealed several selective gene markers typical of the early (plate-like) and late (freely migrating) prechordal plate cells [*Gooseoid*, *Crescent*, *Hex*, *Bmp7*, *Nodal*, *Shh*, and *FoxA2*; Pera and Kessel, 1997; Dale et al., 1999; Lopez-Sanchez et al., 2005].

Along this biphasic interaction of prechordal plate cells with the hypothalamus various inductive effects take place. The early epithelial prechordal plate produces SHH and possibly other signals acting upon the basal acroterminal ectoderm, possibly reinforcing local ventralization and establishing the prospective median differentiation of the median eminence and the neurohypophysis. Other early effects act upon the mamillary body [Garcia-Calero et al., 2008]. Effects of the second phase apparently involve the suprachiasmatic hypothalamic midline (suprachiasmatic nuclei), the optic chiasma, and the preoptic terminal lamina, leading eventually to the bilateral separation of the optic and telencephalic fields, as well as of the olfactory placodes, by inhibition of these fates at the midline [Mangold, 1931; Adelmann, 1936; Wallis and Muenke, 2000; Roessler and Muenke, 2001; Krauss, 2007; Puelles, 2017; Diaz and Puelles, 2020]. Failure of these effects leads to the holoprosencephalic syndrome, which may involve in its maximal grade absence of the mamillary bodies and full dorsalization of the forebrain [Garcia-Calero et al., 2008]. On the other hand, amphioxus is known not to have bilateral telencephalic vesicles and optic vesicles, but has instead a single median pigmented patch held to be an eye homolog, as well as a median preinfundibular region (a possible neurohypophysis homolog?) at the alar and basal acroterminal domains of its hypothalamus, respectively, rather than anything resembling a notochord-induced floor plate.

Is There a Prechordal Plate in Cephalochordates?

In vertebrates the rostral tip of the notochord normally lies close to the mamillary pouch, at least at embryonic stages [Puelles et al., 2012a; Puelles and Rubenstein, 2015]; at later stages the tip of the notochord normally retracts caudalwards, apparently due to its lack of further growth while the forebrain expands and bends consider-

ably. In contrast, adult amphioxus singularly seem to have a notochord extending well beyond the brain vesicle into the anterior end of the head. Surprisingly, there is no evidence of an ectopic floor plate being induced where this longer notochord contacts the acroterminal part of the brain vesicle [prospective hypothalamus; Albuixech-Crespo et al., 2017]. Tunicates and cephalochordates both have a single (unevaginuated) median pigmented eye spot instead of bilateral eyes [Lacalli et al., 1994; Lacalli, 1996; Ferran and Puelles, 2019]. Such a structure is never formed epichordally in vertebrates. This combination of contradictory data plus molecular differences at the rostral notochord led Albuixech-Crespo et al. [2017] to suggest that the demonstrably molecularly distinct rostral part of the “notochord” is maybe not really notochordal in nature and function and may possibly be a variant evolved prechordal derivative that has adopted a notochord-like cellular configuration. Contrary to this idea, the cyclopic and unevaginuated aspect of the amphioxus eye primordium (the pigmented spot) might be explained as due to the absence of normal prechordal cells. The hypothesis of a fundamental prechordal nature of the rostral notochordal segment of amphioxus nevertheless may be saved under the assumption that the postulated modified prechordal cells may also produce variant signaling that lacks the normal capacity to split the eye field. On the other hand, it seems difficult to ascribe to an ectopic notochordal effect the induction of such recognizable acroterminal structures as the median pigmented eye spot and the underlying preinfundibular and infundibular cells; this would imply a gain in function on the side of the notochord which would need to be taken jointly with the loss of the standard notochordal floor plate-inducing function [Lacalli et al., 1994; Lacalli and Kelly, 2000, 2002]. The notochord clearly never contacts the acroterminal hypothalamic domain in vertebrates and does not induce any acroterminal specializations when explanted ectopically [e.g., Yamada et al., 1991].

In amphioxus and lamprey larvae, respectively, Lwoff [1894] and von Kupffer [1894] both illustrated an early endodermal prechordal plate forming a zone of direct contact (adhesion) between the rostradorsal endoderm and the rostromedian part of the ectodermal cerebral vesicle (the prospective tuberal hypothalamus; Fig. 4a, 5a, b). This attachment imitates what happens after endoderm gastrulation in invertebrates [Gilbert, 2003], and is perfectly distinct from the mesodermal notochordal tip (topologically in a different embryonic leaflet), which is illustrated in both species as ending close to the theoretical prospective mamillary area of the hypothalamus at early

postgastrulation stages (Fig. 4b, c, e, 5a). The same arrangement of rostromedian endoderm and ectoderm relative to notochordal mesoderm was observed in a stage HH10 chick embryo by Puelles et al. [1987a] (Fig. 5c; see similar images in Adelmann [1922] and Seifert et al. [1993]). At later stages the prechordal endodermal plate

Fig. 4. Series of drawings extracted from Willey [1894] after Hatschek [1881] (a, b) and Conklin [1932] (c–f), representing various amphioxus larval stages across early, intermediate, and late phases of gastrulation. a–d are from midsagittal sections, whereas e, f represent horizontal sections (rostral to the top). All drawings in a–d have a common AP axis and imply our interpretation that larval elongation occurs by caudalward growth at the lips of the blastopore (green arrows), simultaneously with the advance of gastrulation. a At an early gastrula stage only the prospective endoderm has gastrulated, occluding the cavity of the blastula and initiating the archenteron (widely open caudally oriented concavity), while the prospective notochordal cells (blue; blue arrows) still lie in the epiblast (at the proliferating dorsal lip of the blastopore), caudally to the prospective neural ectoderm. The presumptive prechordal plate (pink; black arrow) represents the dorsomedian part of the invaginated endoderm that singularly contacts the neuroectoderm. b At a late stage of gastrulation the notochordal material (blue; blue arrows) has invaginated through the dorsal lip of the blastopore, and now occupies the caudally elongated midline of the embryo behind the prechordal plate (pink; black arrow) and under the neural ectoderm, forming transiently the apparent roof of the archenteron; note that no part of the notochord would move rostralward, as the classic concept of “cephalic process” falsely assumes; rather, the rostralmost notochordal cells are the first to invaginate (close to the image in a), being followed by more caudal ones as the blastopore proliferates and extends caudalwards. c At the 16 hpf larval stage the whole embryo has elongated more caudalwards (green arrows), practically closing the blastopore. Note the endodermal prechordal plate (pink; black arrow) and the notochord (blue; blue arrows) maintain their previous relative median positions. d At the 21 hpf larval stage the situation is similar, but now the novel tissue interpreted by us as the “prechordal process” (dark pink) appears in front of the tip of the notochord (blue; blue arrows), exactly intercalated between the endodermal prechordal plate (pink; black arrow) and the neural ectoderm; this process was understood classically as a secondary rostral prolongation of the notochord. We argue in the text that that interpretation would be inconsistent with the molecular patterning observed in the neighboring hypothalamus already at this specific stage [Albuixech-Crespo et al., 2017]. e A horizontal section at 16 hpf stage (like c) shows the distinct boundary observed classically between the median notochord (N; blue; blue arrows) and the rostromedian endodermal prechordal plate (pink; black arrow). f A similar horizontal section at 18 hpf larval stage already starts to show the novel “prechordal process” outgrowth (dark pink; compare with d) specifically at the median part of the prechordal plate and in front of the notochordal tip, which does not change its primary position underlying the floor plate of the neural primordium. A, archenteron; N, notochord.

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loses its contact with the tuberal hypothalamus and is transformed into Seessel's pharyngeal pouch (amphioxus in Fig. 4d; incipient in the chick in Fig. 5c), in temporal coincidence with the parallel formation of Rathke's pouch

[Sánchez-Arrones et al., 2015] (Fig. 5c). Correlative with this separation process, in vertebrates a number of prechordal cells dissociate from the endodermal plate and disperse dorsalwards as freely migrating cells along the

midline of the head, in front of the acroterminal hypothalamic domain [Seifert et al., 1993; their Fig. 6]. These cells may be tracked in the chick using the *gooseoid* marker up to roof plate levels, where they finally disappear [e.g., Bally-Cuif et al., 1995; Lopez-Sanchez et al., 2005; their Fig. 7]. Unfortunately, *gooseoid* is not expressed in the median head region in amphioxus.

Construing on classic earlier embryologic studies on amphioxus by Kowalewsky [1877] and Hatschek [1881], Lwoff [1894] significantly first noticed that the process of gastrulation (with formation and closure of the blastopore) essentially involves active caudalwards proliferative growth that occurs maximally at the dorsal blastopore lip. Neither the embryo nor the notochord elongates rostralwards, as is often assumed in the literature, but caudalwards (see evidence in amphioxus after tracking proliferating cells using EDU-pulse chases [Andrews et al., 2021; Gattoni et al., 2021]; this applies as well to the neural primordium). Lwoff concluded that initial steps of gastrulation, when the endoderm rapidly invaginates (forming the primary archenteron and occluding the blastocoel by contacting the ectoderm), occur much in the same way in amphioxus as in the lamprey, shark, teleost, and axolotl larvae he also studied. A small and strictly endodermal archenteron thus forms initially (A; Fig. 4a), resembling topologically what occurs in the sea urchin and tunicates [e.g.; Gilbert, 2003; his Fig. 8.15 and 8.42]; this initial archenteron is fated to become the definitive rostral gut, including ventrally the prospective mouth. The dorsomedian part of this early archenteron contacts the prospective neural ectoderm, rather than head ectoderm, forming the prechordal plate (pink and black arrow; Fig. 4a). A later gastrulation phase leads to dorsomedian invagination through the dorsal blastopore lip of the notochordal material and adjacent parts of the mesoderm (blue and blue arrow; Fig. 4a, b). Lwoff [1894] concluded from cell typology aspects and mitotic density that this notochordal material came out of the caudal “ectoderm” (we now identify this as *ectoblast*) via the caudally growing dorsal blastopore lip. He thus negated an origin of the notochord out of the endodermal archenteron roof, a notion supported instead by Hatschek [1881], Willey [1894], and Conklin [1932]. Lwoff [1894] thought that, as the embryo elongates caudalwards relative to the precociously formed prechordal plate (green arrows in Fig. 4a–c), the dorsal blastopore vehiculates inwards no more endodermal tissue (whose caudal parts nevertheless continue to ingress through the lateral blastopore lips) but transfers inwards instead notochordal mesoderm, which thus comes to occupy the caudally elongating roof of the archenteron be-

hind the prechordal plate (Fig. 4b, c). The lateral walls and floor of the rostral gut forming caudal to the prechordal plate invaginate through the lateral lips of the blastopore and constitute first a dorsally unclosed endodermal canal, whose lips touch the transient mesodermal components at the archenteron roof (Fig. 5d, e). These lips subsequently grow medialwards under the bilateral primordia of the somites and of the median notochord, thus closing the endodermal canal (Fig. 5f, g; in some vertebrate species, this closure of the endodermal gut apparently occurs primarily, before the notochord is formed, or independently from it [Gilbert, 2003], thus indicating an heterochronic aspect of amphioxus gastrulation). Though fre-

Fig. 5. Postgastrulation stages of the early prechordal plate and notochord in relation with the neural ectoderm, compared with surrounding endoderm and mesoderm (in different species). **a** Drawing extracted from Conklin [1932] of a midsagittal section through a 16 hpf amphioxus larva. Note the endodermal prechordal plate (pink; black arrow) contacting with rostral neural ectoderm rostrally to the initial tip of the gastrulated notochord (blue; blue arrows; somites are also partly visible); the transverse line D marks the section plane of **d–g**. **b** Drawing extracted from von Kupffer [1894] of a similar midsagittal section through a 6-day lamprey larva; prechordal plate (pink; black arrow) and notochord (blue; blue arrows) show the same relationship to neural ectoderm. **c** Midsagittal section through a chick embryo stage HH10 reacted for acetylcholinesterase [extracted from Puelles et al., 1987a] showing analogous relationships at a slightly more advanced stage in which the primordial prechordal plate endoderm (black arrow) starts to separate from the neural ectoderm (the presumptive basal hypothalamus), transforming into a part of Seessel’s pouch (S), but clearly in front of the rostral tip of the notochord (n; blue arrows); note incipient formation of Rathke’s pouch (R). **d–g** Drawings extracted from Lwoff [1894] showing cross-sections through non-identified successive larval stages of amphioxus (only **g** was characterized as a stage with 7 somites). These images illustrate how the endodermal archenteron roof is initially widely open dorsally (D), thus allowing the notochord (N; blue) and bilaterally adjacent somitic material (m.f.) to provide a dorsal closure of the archenteron cavity. At stages illustrated in **e** and **f** the increasingly distinct free lips of the endodermal canal grow medialwards under the mesodermal tissues (N; m.f.), which gradually are thus separated from the archenteron roof. At the stage of 7 somites (**g**) the endodermal tube is nearly closed and the notochord (N) has largely segregated from it, similarly as the fully segregated somites (m). Note also the gradual closure of the neural tube (N). These images demonstrate that the notochord does not “invade” the endoderm and rather remains always independent from it. Species in which this sequence is not observed probably regulate endoderm canal closure heterochronically, so that it closes relatively earlier, in which case the notochord never appears to close the archenteron dorsally. A, archenteron; D, diencephalon; M, midbrain; N, notochord; R, Rathke’s pouch; S, Seessel’s pouch; SP, secondary prosencephalon. Small black arrows: inter prosomeric boundaries.

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quently invoked in the literature, there is no strong evidence of “penetration” or fusion of mesodermal tissue into the underlying endoderm. Both tissues remain distinct, though in contact, even in amphioxus. As a result, the initially unpaired dorsomedian rostral endoderm, which forms the prechordal plate adhered to neuroectoderm, occupies from the beginning of gastrulation the rostral midline of the head under the brain vesicle primordium, in front of the rostral tip of the notochord (Fig. 4c, e), both in amphioxus and in vertebrates.

All the early larval data published on gastrulation stages in amphioxus (see for example the present Fig. 4a–c, which encompass larvae ranging from early gastrulation

up to 16 hpf) are consistent with the normal formation of a primary endodermal prechordal plate at the rostradorsal end of the archenteron roof, where the endoderm directly contacts the rostral neural ectoderm; this characteristic epithelial locus is seen at 16 hpf to lie immediately in front of the rostral end of the notochord and the first somites (Fig. 4e).

As can be observed particularly well in the drawings of Hatschek [1881], the site of the epithelial endodermal prechordal plate later starts to show a local median outgrowth that accumulates at the interface of the prechordal plate with the neural ectoderm, separating them, and later extends rostralwards; this phenomenon starts at the

stage of 18 hpf; this novel cell population apparently originating from the prechordal plate, comparable in principle with the secondarily epithelio-mesenchymal-transformed cells of vertebrates, transforms rapidly into what is known as the elongating “rostral part” of the notochord. In order to emphasize its apparent prechordal origin, and for clarity, we suggest renaming it instead the “prechordal process” (dark pink; Fig. 4d, f). It is difficult to assess from the available classic drawings whether the prechordal process is initially more connected to the prechordal plate than to the tip of the notochord. We would expect the former to be true under the assumption that the notochord cannot grow rostralwards, whereas the prechordal process of amphioxus has been noted to show signs of proliferation [Holland and Holland, 2006]. At which point in time these prechordal process cells adopt a notochordal morphology is not clear. This is a point that probably needs to be addressed with differential markers.

This re-examination of relevant literature leads us to conclude that amphioxus initially forms a perfectly normal prechordal plate, which represents a rostradorsal median specialization of the rostral head endoderm whose cell material invaginates early on through the dorsal lip of the blastopore, preceding any notochordal material. A standard notochord invaginates next through the dorsal blastopore lip, but only after the prechordal endoderm has occupied its definitive place at the rostral end of the archenteron. This causes the rostral tip of the notochord to lie just behind – and in a different layer than – the prechordal plate, as is normally found in all vertebrates (Fig. 5a–c). The initial notochord of amphioxus thus shows a normal length and position under the whole forebrain, but unrelated to the rostral acroterminal domain (like that of tunicates). It accordingly underlies directly the subsequently differentiated floor plate ending rostrally at the hypothalamic floor homolog [Albuxech-Crespo et al., 2017]. The notochord and adjacent somitic primordia form transiently in amphioxus the archenteron roof caudally to the prechordal plate. However, the initially dorsally unclosed endodermal canal soon closes by median fusion of its transiently free lips, which grow actively under the mesodermal elements, thus separating fully the retropharyngeal gut cavity from the notochordal primordium and the adjacent somitic elements (Fig. 5d–g).

This being so, it is remarkable that the first sign at stage 18 hpf of the molecularly distinct “rostral notochord” (named here instead “prechordal process”) appears clearly associated topographically to the pre-existent median prechordal plate. Against the alternative interpretation

that it emerges as a rostral outgrowth of the notochordal tip weighs the absence of any data indicating a proliferative potential of the hypothalamic rostral end of the notochord in any species, whereas the prechordal process has been shown to have strong intrinsic proliferative capacity, present also at the prechordal plate [Holland and Holland, 2006; their Fig. 1g, h]. This property would explain the subsequent elongation of the prechordal process into the tip of the developing amphioxus head. This growth imitates in a variant form the dorsalward migration of second-phase free prechordal cells along the acroterminal neural midline, typical of vertebrates (Fig. 2a) [Seifert et al., 1993; Schulte-Merker et al., 1994; Thisse et al., 1994; Bally-Cuif et al., 1995; Lopez-Sanchez et al., 2005], which later disappear or become undistinguishable. These dispersed rather than compacted prechordal cells induce along their dorsalward pathway the bilateral separation of the eyes and the telencephalic primordia (possibly also the nasal placodes, which also remain single and median in cyclopic disorders, but curiously not so the adenohypophyseal placode) [Mangold, 1931; Adelman, 1936; Wallis and Muenke, 2000; Roessler and Muenke, 2001; Krauss, 2007; Puelles, 2017; Diaz and Puelles, 2020]. The apparent histologic similarity of the prechordal process cells with the notochordal cells is an accompanying singularity likewise only known to have emerged in amphioxus.

Brain Patterning Evidence

According to the analysis of Albuxech-Crespo et al. [2017], the developing cerebral vesicle of amphioxus appears regionalized dorsoventrally into standard floor, basal, alar/roof longitudinal zones, whose rostralmost portions surprisingly approach orthogonally the rostral part of the “notochord” (Fig. 1c, 2b; i.e., being longitudinal zones, they would be expected to be parallel to the notochord). Moreover, the larger rostral part of the cerebral vesicle was characterized molecularly as having three AP prosomere-like units (Fig. 2b): a topologically and molecularly comparable rostral hypothalamic domain is devoid of telencephalic and ocular evaginated appendages, but is subdivided into two prosomere-like units, distinguished molecularly as possible homologs of the hp2 and hp1 prosomeres described in vertebrates (Fig. 2a, b) [Puelles et al., 2012a; Puelles and Rubenstein, 2015; Puelles, 2018]; this is followed by a third prosomere-like unit that might represent the prethalamus unit of the diencephalon of vertebrates (p3; Fig. 2a, b); like the hypothal-

amus this third unit expresses a *Fezf-like* gene (present likewise in the prethalamus of vertebrates). The observed tripartite rostral forebrain region was therefore named hypothalamo-prethalamic primordium (HyPTh; Fig. 1). Behind the possible prethalamus there appears a small, but molecularly distinct, caudal forebrain bridge area expressing *Otx*, *Pax4/6*, and *IrxB*, but not *Fezf* or *Gbx*. This was tentatively identified according to position and by exclusion of a separate hindbrain primordium homolog candidate, as a largely undeveloped DiMes primordium (Fig. 1b, c, 2b). The next identifiable brain portion was clearly a partly undeveloped hindbrain-spinal primordium that expresses *Gbx* and *Hox* genes but lacks the full neuromeric characteristics of the vertebrate hindbrain (RhSp; Fig. 1b, c, 2b). The boundary separating DiMes from RhSp was aberrant, lacking the molecular characteristics of the isthmus organizer.

The amphioxus HyPTh seems quite correctly patterned in both AP and DV dimensions, irrespective of the lack of evaginated components. The prethalamus prosomeric unit was so far ascribed in the prosomeric model to the anterior part of the diencephalon of vertebrates. This unexpected pattern of amphioxus raised the possibility that the prethalamus may actually represent a dependence of the hypothalamic proneuromere instead, as is suggested, for example, by the shared gene markers *Fezf* and *Dlx*. The amphioxus HyPTh displays in its rostralmost end part that contacts the rostral “notochord” or “prechordal process” a histologically singular median acroterminal domain representing the lineal rostralmost end of the neural tube postulated in the prosomeric model (it expresses singularly *Dlk1*; Fig. 3). Some acroterminal loci of amphioxus already are conventionally thought to be homologous to corresponding alar or basal acroterminal structures in vertebrates (Fig. 2a, b). Specifically, the alar acroterminal subdomain has a median pigmented spot with various other associated median cells that are collectively interpreted as representing a cyclopic eye homolog (non-evaginated and obviously unsplit; Lacalli, 1996). Similarly, in the basal acroterminal subdomain there is a rostromedian non-evaginated preinfundibular domain which possibly compares with the infundibulum/hypophysis/median eminence complex of vertebrates [Lacalli et al., 1994; Lacalli, 1996]. Importantly, the amphioxus hypothalamus also has a distinct floor plate (Fig. 1c) [Lacalli et al., 1994; Lacalli and Kelly, 2002] which is consistent in its topological position in the model with that of vertebrates [restricted to the mamillary and retromamillary hypothalamic floor in the updated prosomeric model; Puelles et al., 2012a; Puelles and Rubenstein, 2015]. Importantly,

the amphioxus rostralmost floor plate is epichordal relative to the tip of the caudal notochord but does not relate “epichordally” to the rostral part of the “notochord” or prechordal process (Fig. 2b).

The observed molecular distinction in amphioxus HyPTh of standard hypothalamic alar and basal plates parallel to this floor domain (and orthogonal to the rostral part of the “notochord”) implies that a standard DV patterning process has occurred throughout the hypothalamus, comparable to that reported in vertebrates. According to the experiments in chick embryos of Garcia-Calero et al. [2008], this DV patterning of the hypothalamus may be tentatively attributed to ventralizing floor patterning effects in concert with antagonistic roof plate signals (in this case coming from the anterior neural ridge at neural plate stages). The amphioxus hypophysial and median eminence primordia (the infundibular/preinfundibular complex) are consistent in position with the standard basal acroterminal specialization of the hypothalamic tuberal region, which was adhered to transiently by the prechordal plate, and also gives rise to the evaginated hypophysis in vertebrates. Although the topology of the “rostral notochord” or prechordal process of amphioxus relative to hypothalamic structure is consistent with that of the prechordal plate in vertebrates, a characteristic prechordal function is nevertheless missing, namely the splitting of the eye field. The cited results of Albuixech-Crespo et al. [2017] thus indicate that the amphioxus rostral cerebral vesicle is divided anteroposteriorly into 2–3 prosomeric units (largely hypothalamic in nature) plus a recognizable acroterminal domain at its median front. These neuromeric units are molecularly recognizable as equivalent to those present at the same relative positions and with similar molecular profiles in vertebrates, particularly as regards their standard DV subdivision into floor, basal, and alar/roof plates (Fig. 1c, 2b) [Albuixech-Crespo et al., 2017].

It is therefore surprising in retrospect that amphioxus, with its anomalous notochord, does not show a correspondingly anomalous hypothalamic DV pattern, and shows instead a standard floor plate and alar-basal boundary [see descriptions of amphioxus brain floor plate by Lacalli et al., 1994; Lacalli, 1996; Lacalli and Kelly, 2000, 2002; Wicht and Lacalli, 2005].

Epichordal versus Prechordal Brain

Due to the peculiarly long notochord, classic authors considered that all the neural tube of amphioxus was exceptionally epichordal when compared to the rostral fore-

brain of vertebrates, thought to be in part prechordal. Even in the early formulations of the prosomeric model [Puelles and Rubenstein, 1993; Rubenstein et al., 1994] it was not realized that gene markers selective of both the notochord and the floor plate reached rostrally the mamillary hypothalamus; discovery of this aspect led to the postulate of the present updated version of the prosomeric model [Puelles et al., 2012a; used accordingly by Albuixech-Crespo et al., 2017] in which the whole forebrain is presented as intrinsically epichordal relative to the notochordal tip.

At this point the acroterminal domain was realized to be topologically transversal (i.e., extending from ventral to dorsal), rather than extending the forebrain axis into the telencephalon, as was previously assumed in the columnar model. Interestingly, early comparative work on the notochord primordium had made it clear that the vertebrate notochord has its rostralmost tip under the hypothalamic mamillary area [Dohrn, 1883; Lwoff, 1894; von Kupffer, 1894, 1906; Sterzi, 1907; Johnston, 1909, 1910]. Modern studies have also corroborated this conclusion [Jurand 1974; Morriss-Kay and Tuckett, 1987; Sausedo and Schoenwolf, 1994; Sulik et al., 1994; Barteczko and Jacob, 2002; Puelles et al., 2012a, b; Puelles and Rubenstein, 2015]. The notochord proper is thus strictly coextensive with the neural floor plate, this being a causal relationship, since floor plate specification and differentiation is selectively induced by notochordal morphogens [Marti et al., 1995; Placzek, 1995; Ericson et al., 1996; Harland and Gerhart, 1997; Dodd et al., 1998; Placzek et al., 2000; Stemple, 2005; Sanchez-Arrones et al., 2009, 2012].

Consequently, the developing notochord of vertebrates never appears associated to the infundibular (neurohypophysis), tuberal, chiasmatic, preoptic (lamina terminalis), or septal (anterior commissure) median forebrain regions, which collectively represent the rostromedian acroterminal domain influenced inductively in a temporal sequence first by the basal plate-attached prechordal plate, and later by dispersed migratory prechordal cells related to the alar portion. Typically, no part of the acroterminal domain is floor plate-like in molecular terms (Fig. 3) [Puelles et al., 2012a; Puelles and Rubenstein, 2015]. The updated prosomeric brain model thus holds presently that the entire brain including the hypothalamo-eye-telencephalon complex is epichordal, but it redefines the axis concept and all the brain longitudinal zones (floor, basal, alar, roof) so that the notions of brain axis and rostral end of the brain essentially only refer to the extent of the neural tube found as far rostrally as the embryonic tip of the notochord and the related floor plate. The neural tube is closed rostrally primarily

by the acroterminal domain, which lies under the rostral neuropore [Puelles et al., 2012a; Puelles, 2018]. Accordingly, the acroterminal midline (Fig. 3) is not regarded any longer in this model as extending the axial dimension of the tube, compared to its real end at the mamillary landmark reference, but represents the ventrodorsally patterned terminal wall of the neural tube (the brain's end), derived primarily from a median domain of the rostral neural plate which does not receive notochordal signals other than those coming from the mamillary area [Puelles, 2018]. In contrast with the anterior neural ridge (and refuting His' [1893] original speculation), the acroterminal domain has nothing to do with the closure of the rostral neuropore. The latter is limited to the closure of the roof plate [the rim of the neural plate; Puelles, 2018], which ends rostrally at the septal site of the anterior commissure (as was advanced by von Kupffer [1894, 1906] and demonstrated later experimentally by Puelles et al. [1987b] and Cobos et al. [2001]).

The "prechordal" material, formed first by a static structure forming the median endodermal prechordal plate adhering to prospective tuberal hypothalamus, is a median component of cephalic endoderm which initiates endodermal gastrulation (Fig. 4a). Once this initial prechordal plate dissociates from the basal hypothalamus, the freely migrating median prechordal cells derived from it [Seifert et al., 1993] disperse dorsalwards in front of the acroterminal domain. The latter sequentially receives signals from the prechordal material, becomes itself a source of rostralizing morphogens, and thus comes to represent the hitherto largely unrecognized origin of AP neural patterning signals [Ferran et al., 2015; Puelles, 2017; Diaz and Puelles, 2020].

Conclusion

The foregoing analysis leads us to think that the molecularly distinct rostral part of the amphioxus "notochord," here renamed "prechordal process," does not participate in ectopic notochord-like induction of a floor plate at the acroterminal HyPTh and seems to participate instead in standard AP patterning of the hypothalamo-prethalamo region. It thus acts prechordal-like, as it were, although this effect appears incomplete in its efficiency as regards splitting of rostromedian alar forebrain primordia into paired appendages (eyes and telencephalic vesicles). The missing evagination of eye and telencephalic vesicles is a process that has not been related directly to the prechordal plate or any other agency so far.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Author Contributions

J.L.F. and L.P. analyzed the literature. J.L.F., M.I., and L.P. wrote and revised the manuscript.

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