The evolutionary history of placodes: a molecular genetic investigation of the larvacean urochordate Oikopleura dioica

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Summary

The evolutionary origin of vertebrate placodes remains controversial because divergent morphologies in urochordates, cephalochordates and vertebrates make it difficult to recognize organs that are clearly homologous to placode-derived features, including the olfactory organ, adenohypophysis, lens, inner ear, lateral line and cranial ganglia. The larvacean urochordate Oikopleura dioica possesses organs that morphologically resemble the vertebrate olfactory organ and adenohypophysis. We tested the hypothesis that orthologs of these vertebrate placodes exist in a larvacean urochordate by analyzing the developmental expression of larvacean homologs of the placode-marking gene families Eya, Pitx and Six. We conclude that extant chordates inherited olfactory and adenohypophyseal placodes from their last common ancestor, but additional independent proliferation and perhaps loss of placode types probably occurred among the three subphyla of Chordata.

Key words: Appendicularia, Larvacean, Tunicate, Chordate evolution

Introduction

A popular evolutionary scenario describes the rise of vertebrates from filter feeding ancestors to aggressive raptors, and attributes this change largely to the innovation of two embryonic tissues: neurogenic placodes and neural crest (Gans and Northcutt, 1983; Northcutt and Gans, 1983). Placodes contribute to a number of paired sensory organs, including the olfactory organ, otic vesicles of the inner ear, lenses of the eyes, cranial sensory ganglia such as those involved in gustation, and the electro- and mechanosensory lateral line organs; these organs would have facilitated the evolution of an active, predatory life-style.

Because no placode-like structures or their derivatives have been recognized without controversy in non-vertebrate chordates, biologists generally assume that various placodes rapidly arose together within the early vertebrates. Consistent with this hypothesis, placodes broadly share several characteristics: they originate from an arc of ectoderm around the anterior neural plate, form from thickened epithelia, generate some overlapping cell types (e.g. sensory neurons) and most produce migratory cells by delamination (Baker and Bronner-Fraser, 1997). In addition, placodes express paralogs of a number of gene families (Streit, 2004). Paralogs of eyes absent (eya) are expressed in the lens and adenohypophyseal placodes and all of the neurogenic placodes (David et al., 2001; Xu et al., 1997). Pitx genes mark the most anterior placodes, the olfactory and adenohypophyseal, as well as the stomodeum (Gage and Camper, 1997; Lanctot et al., 1997). Like Eya genes, Six family paralogs are expressed in all placodes (Baker and Bronner-Fraser, 2001; Kawakami et al., 2000; Oliver et al., 1995; Schlosser and Ahrens, 2004). Significantly, Six genes are also important in the developing forebrain, including: the olfactory bulbs and neurohypophysis (posterior pituitary), which become physically and functionally connected to the olfactory and adenohypophyseal organs (e.g. Ghanbari et al., 2001; Jean et al., 1999). Despite examples of shared gene expression and the prevailing opinion that placodes develop from a common primordium (Streit, 2004), Begbie and Graham (Begbie and Graham, 2001) have reasoned that the diversity of placode-derived organs, and the diversity of ways in which they are induced to form indicate that placodes should not be considered closely related embryonic structures and might not all share a common evolutionary origin limited to Vertebrata.

Gans and Northcutt (Gans and Northcutt, 1983) argued that, while the chordate common ancestor probably had a neural crest/placode precursor involved in sensory tissue development, this precursor was part of a diffuse ectodermal nerve plexus like that seen in some modern deuterostomes, including echinoderms and hemichordates, and did not form focal condensations characteristic of vertebrate placodes. Evidence in favor of an ancient, eochordate origin for placodes, however, has come from analysis of ascidian and cephalochordate molecular embryology (Baker and Bronner-Fraser, 1997; Holland and Holland, 2001; Manni et al., 2004b; Mazet et al., 2005; Shimeld and Holland, 2000). For example, ascidians have been proposed to possess an otic placode homolog: the paired, thickened, ectodermal primordia of the exhalent siphon (atrium) in Ciona embryos express Pax2/5/8, an ascidian ortholog of the vertebrate otic placode-marking genes Pax2 and Pax8 (Wada et al., 1998), and the adult atrium wall contains ‘cupular’ cells that are morphologically similar to hair cells of the vertebrate inner ear and lateral line (Bone and Ryan, 1978). Cupular cells, unlike vertebrate hair cells, however, are primary sensory cells (sending axons to the
central nervous system (CNS) rather than being separately innervated), and the role of ascidian Pax2/5/8 in the atrium might instead be interpreted as a non-placodal requirement in outer gill embryogenesis as has been proposed for amphioxus Pax2/5/8 (Kozmik et al., 1999). Many ascidians are found to have rows of ‘coronal’ organs in the incumbent, oral siphon; Burighel et al. and Manni et al. (Burighel et al., 2003; Manni et al., 2004a) propose that these are a lateral line homolog, but they are located inside the equivalent of the stomodeum, which does not contain lateral line receptors in vertebrates.

Cephalochordates and ascidians may have a homolog of a non-neurogenic placode, the pituitary. In amphioxus embryos, the primordium of Hatschek’s pit expresses the adenohypophyseal marker pitx (Boorman and Shimeld, 2002; Yasui et al., 2000), and adult pit cells are immunoreactive for several vertebrate pituitary hormones (Nozaki and Gorbman, 1992). Because the ascidian neurohypophyseal duct, which connects the cerebral ganglion to the oral cavity, is neurogenic and produces migratory cells, it is a candidate homolog for both neural crest and for the combined olfactory and adenohypophyseal placodes (Burighel et al., 2003; Manni et al., 2004a). Although the neurohypophyseal duct resembles Rathke’s pouch in topography and in the expression of pitx in the associated ciliated funnel (Boorman and Shimeld, 2002; Christiaen et al., 2002), the structure is unlike, unlike the vertebrate olfactory placodes. Ruppert (Ruppert, 1990), who observed phagocytic rather than secretory cells in the ascidian duct walls, proposed that the duct regulates blood volume, and is unrelated to the adenohypophysis.

Developmental morphologists proposed that the ‘ventral organ’ and the ‘ciliary funnel’ in the larvacean urochordate Oikopleura dioica, are homologs of the olfactory organ and the pituitary (Bollner et al., 1986; Holmberg, 1982; Olsson, 1969). The dilated cilia of the sensory cells of the ventral organ protrude into the environment from a slit-like pocket of ectoderm (Bollner et al., 1986). These cells are primary sensory cells like vertebrate olfactory receptor cells, and their axons integrate into the rostral-most CNS (Bollner et al., 1986). To reflect these structural similarities to the vertebrate olfactory epithelium, they were called olfactory or chemosensory cells by various authors, although their function is unknown (Bollner et al., 1986; Lohmann, 1933).

The connection of the larvacean ciliary funnel between the brain and the roof of the anterior pharynx topographically resembles the developing vertebrate pituitary when Rathke’s pouch invaginates in the roof of the stomodeum and contacts the primordial hypothalamus. In addition, dorsal funnel cells appear to secrete a substance periodically into the trunk haemocoel (Holmberg, 1982). Because of these characteristics, Olsson (Olsson, 1969) and Holmberg (Holmberg, 1982) argued that the larvacean ciliary funnel is homologous to both the adeno- and neurohypophyses. This hypothesis mirrors the proposed pituitary homology of Hatschek’s pit in cephalochordates and the neurohypophyseal duct or neural gland complex in ascidians (Gorbman, 1995; Olsson, 1990).

At least three other ciliated sensory cell types exist around the mouth and in the lateral trunk in Oikopleura (Olsson et al., 1990). Although they have not been suggested to be homologous to vertebrate placode derivatives, these mechanoreceptors are organized into paired sensory organs, and like the ventral organ, they form thickened areas in otherwise monolayer epithelia.

Several questions remain unanswered regarding the origin of placodes. Do non-vertebrate chordates have placodes? If so, are they orthologous to specific vertebrate placodes? Are they composites of vertebrate placodes? Or did vertebrate and non-vertebrate placodes independently evolve from a common ancestral placode precursor? Given the diversity of morphologies among chordate taxa – which challenges the clear assignment of placode homologies – do different lineages of non-vertebrate chordates (i.e. cephalochordates, ascidians, thaliaceans, and larvaceans) have different or overlapping sets of placode homologs? To infer the nature of developmental mechanisms in the last common ancestor of extant chordates and to reassess the role the origin of placodes might have played in the vertebrate transition, we must try to answer these questions.

We present the isolation and developmental expression patterns of eya, pitx and three Six genes from Oikopleura dioica in the context of the origin of placodes. In this study, we show that homologs of genes important for vertebrate olfactory and pituitary placodes are expressed in the primordia of the larvacean ventral organ and ciliary funnel. We show that these larvacean organs conform to morphological criteria that characterize vertebrate placodes, and report that the primordia of paired larvacean peripheral mechanoreceptor organs express placode-marking genes. Finally, we discuss the relationship of these organs to ascidian mechanoreceptors that have been proposed to be homologs of otic or lateral line hair cells.

Materials and methods

Animal husbandry

O. dioica were collected in the Pacific Ocean near Charleston, Oregon (Oregon Institute of Marine Biology) and Vancouver Island, BC (Bamfield Marine Station). Animals were cultured in natural seawater in 2 or 4 liter jars, and fed algal concentrates (‘Coral and Clam Diet’, Reed Mariculture). Synchronously fertilized embryo clutches were cultured at 12°C or 14°C in tissue culture coated Petri dishes.

Cloning and sequence analysis

Gene fragments were obtained by degenerate PCR amplification from genomic or cDNA templates using primers based on alignment of homologous genes. Primer sequences can be provided on request.

cDNA sequences were obtained by RACE PCR (SMART RACE cDNA Amplification Kit, Clontech), and by amplification from first-strand synthesis cDNA pools. Poly-A mRNA was purified (Micro-FastTrack 2.0 mRNA isolation kit, Invitrogen) from ~1600 hatchlings ranging from just hatching to tailfirst stage, just before house building.

A genomic fosmid library was constructed using the CopyControl Fosmid Library Production Kit (EpiCentre) and DNA from about 50 ripe O. dioica males. Fosmids containing eya, pitx, six1/2, six3/6a and six3/6b were isolated by screening the arrayed library with gene-specific PCR primers. Fosmids were fully sequenced by the Joint Genome Institute, Walnut Creek, California. GenBank accession numbers for Oikopleura genes are: eya (DQ011272, fosmid), (DQ011273, cDNA); pitx (DQ011274, fosmid), (DQ011275, DQ011276, DQ011277, DQ011278, cDNAs); six1/2 (DQ011279, fosmid), (DQ011280, cDNA); six3/6a (DQ011281, fosmid), (DQ011282, cDNA); six3/6b (DQ011283, fosmid), (DQ011284, cDNA).

Sequences were aligned using ClustalW software (http://sf01.bic.nus.edu.sg/clustalw/). Unrooted neighbor-joining phylogenies were calculated using PAUP 4.0b software. GenBank
Accession Numbers for protein sequences used in Fig. 3B can be provided on request.

In situ hybridization
Embryos for in situ analysis were treated as in Bassham and Postlethwait (Bassham and Postlethwait, 2000), or sometimes the probe hybridization time was shortened from overnight to 2.5 to 4 hours.

Scanning electron microscopy
Animals were fixed 2 hours at 4°C in 2% glutaraldehyde, 0.1 M cacodylate and 0.27 M NaCl (pH 7.3), rinsed in 0.1 M cacodylate buffer, postfixed for 1 hour at 4°C in 2% osmium tetroxide, 0.1 M cacodylate buffer, rinsed in distilled water, dehydrated through an ethanol series, and dried in a Quorum Technologies E3100 Critical Point Drying Apparatus. Sputter-coated samples were imaged on a JEOL JSM-6400FV scanning electron microscope.

Light microscopy
Animals were anaesthetized with 0.02% MESAB and photographed on a Leica DMLB compound microscope with a SPOT RT Color digital camera (Diagnostic Instruments). Because high magnification and DIC microscopy greatly limit depth of field, two or more focal planes of some images have been digitally merged using Adobe Photoshop software to clearly illustrate three-dimensional relationships of organs and gene expression domains: Fig. 1B-D; Fig. 5C,D inset; Fig. 6A.

Results
Investigating morphology of candidate placodes in Oikopleura
The organization of the sensory organs and CNS of the larvacean Oikopleura dioica has been described in some detail, providing the framework for hypotheses of homologies to vertebrate anatomy (e.g. Olsson, 1986; Cañestro et al., 2005). Its CNS consists of an enlargement, the ‘anterior brain’, containing fewer than 70 cells (Georges et al., 1988; Martini, 1909) (Fig. 1A-C), connected by an axon bundle to the ‘caudal ganglion’ at the base of the muscular tail, and a tubular spinal cord made up of ependymal glial cells, axons and periodic clusters of motor neuron cell bodies along the length of the tail (Bone, 1998; Cañestro et al., 2005; Lohmann, 1933). Paired nerves emanate laterally from the brain (Fig. 1C) (Olsson et al., 1990): the first pair (the rostral nerves, or n1) connect with ventral organ axons (Bollner et al., 1986); the second pair (n2, Fig. 1C) innervate two kinds of ciliated mechanosensory cells in the mouth (the upper lip cells and a circumoral ring); and the third pair (n3, Fig. 1C) may regulate ciliary beat in the spiral or gill opening (Olsson et al., 1990). A final noteworthy nerve pair connects large, mechanosensory ciliated cells in the posterior trunk to the caudal ganglion (Bone and Ryan, 1979; Holmberg, 1986).

The larvacean ventral organ develops from an ectodermal thickening close to the mouth (Fig. 1C,D). Martini (Martini, 1909) first recognized the nerve tracks that connect the ventral organ to the brain via ganglion-like swellings (bulbs), and referred to the organ as both an olfactory organ (‘Geruchsorgan’) and a taste bud (‘Geschmacksknospe’). Approximately 30 ciliated cells make up the organ (Fig. 1C) (Bollner et al., 1986). Axons of these cells do not appear to reach into the brain, but contact brain processes with which they are bundled by the bulb cells (T. Bollner, personal communication).

![Diagram of Oikopleura morphology](https://example.com/diagram)

**Fig. 1.** Olfactory- and pituitary-like organs in Oikopleura. (A) Schematized adult morphology, lateral right view. (B,C) Lateral right (B) and dorsal (C) views of adult anterior trunk showing arrangement of the anterior brain (br), paired rostral nerves (rn), ciliary funnel (cf). (C) Cell bodies (green dots) line the rostral nerve. Rows of clustered lip receptor (lr) sensory cilia protrude from the lower lip. (D) Right lateral view of a juvenile, showing the rostral nerve in cross section, the ventral organ (vo) and cilia (ci). Thickened epithelia in the mouth are made up of rows of ciliated sensory cells in the lower lip and stomodeal roof (pink dots). (E) Developing hatching (slightly ventral, right lateral view) with thick, immature walls of the funnel connected to the pharynx roof, the rostral nerve thickening (rn), the primordium of the ventral organ (vo) and the sensory bristle cilium (sb) of one of two upper lip cells. (F) Tailshift stage hatching (slightly dorsal, right lateral view) showing the ciliary funnel in cross-section near its base in the pharynx roof and the right rostral nerve in longitudinal section. Insets in E and F schematize the funnel (blue) and rostral nerves (green). bg, buccal gland; br, anterior brain; cf, ciliary funnel; cg, caudal ganglion; ci, sensory cilia; e, endostyle; go, gonad; gu, gut; li, lips; lr, lip receptors; mo, mouth; mu, muscle; n2 and n3, second and third paired nerves; nc, trunk nerve cord; no, notochord; ph, pharynx; rn, rostral nerve; sb, sensory bristle; sc, spinal cord; sl, statolith; sv, sensory vesicle; tf, tail fin; vo, ventral organ. Scale bars: 10 μm.
communication), and are therefore primary sensory cells, such as vertebrate olfactory receptor cells.

Four cells line and ensheath each of the paired rostral nerves (Fig. 1C,F) (Bollner et al., 1986); these cells form synapses with processes from the brain and ventral organ (Bollner et al., 1986). Thinking the paired cell clusters were outgrowths of the brain, Bollner et al. (Bollner et al., 1986) called them the "rostral brain bulbs" and tentatively suggested their homology to the vertebrate olfactory bulbs. Delsman (Delsman, 1912), by contrast, described the development of these bulbs from the rostral ectoderm, not from the brain: he observed that a transverse band of epithelial ectoderm overlying the mouth thickens and protrudes inward and ultimately separates from the epidermis. Delsman’s almost century-old descriptive diagrams are here confirmed and recreated with DIC microscopy (Fig. 2). This cell behavior is similar to the delamination of placode cells in vertebrates (Baker and Bronner-Fraser, 1997).

The ciliary funnel is a cone that slopes rostrally and ventrally from the right side of the brain (Fig. 1B), similar to the right-handed infundibular extension and Hatschek’s pit in amphioxus (Gorbman et al., 1999). The base of the funnel forms an opening into the right, dorsal wall of the pharynx (Fig. 1A-C,E,F). Holmberg (Holmberg, 1982) described three distinct funnel segments. In the pharyngeal wall, a ring of cells guards the funnel opening against particles with a screen of static cilia. In the ventral part of the funnel, the walls are one-cell thick and bear long cilia that constantly beat towards the dorsal end of the funnel. The dorsal funnel is unciliated, and several brain cells extend processes that contribute to the dorsal funnel wall. At this blind, dorsal end, the ‘tip’ cells appear to secrete a substance into the haemocoel (Holmberg, 1982).

Larvaceans have at least three kinds of ciliated mechanosensory organs: (1) the Langerhans receptors, which are bilaterally paired in the posterior trunk; (2) two upper lip clusters of cilia which we here report to bear a stiff, bristle-like cillum previously unrecognized because the cilia are absent in adults (Fig. 1E); and (3) lying around the circumference of the mouth, a band of ciliated mechanosensory cells which each protrude clusters of cilia from a groove formed by overlapping epithelial cells (Olsson et al., 1990).

Cloning and characterization of molecular sequences

Using degenerate PCR primers for the Eya, Pitx and Six gene families, we cloned five genes from Oikopleura. Gene structures were predicted by aligning cDNA with genomic sequences obtained by sequencing our fosmid genomic clones (Fig. 3A). Several lines of evidence, including gene phylogeny and analysis of diagnostic protein motifs (Fig. 3B; see Fig. S1 in the supplementary material), test the contention that each of these larvacean genes is an ortholog to paralogy groups of vertebrate Eya, Pitx and Six genes.

Eya transcription factors contain a unique ‘Eya-domain’ that interacts with other proteins, including Six gene products (Hanson, 2001; Li et al., 2003; Ohto et al., 1999; Pignoni et al., 1997). The Oikopleura Eya-domain is 65.7% to 71.2% identical to the four human EYA proteins and 71.7% identical to Ciona Eya. A catalytic motif critical for Eya phosphatase activity that releases vertebrate Six1 from inhibition (Li et al., 2003) is identical in larvacean, vertebrate and protostome Eyas. A gene phylogeny of Eya amino acid sequences is consistent with the presence of a single Eya gene in the last common ancestor of urochordates and vertebrates, followed by duplications in the vertebrate lineage (Fig. 3B).

Vertebrate Pitx genes display several isoforms resulting from alternative splicing and alternative promoters, and these isoforms differ in tissue and time of expression, and in function (Cox et al., 2002; Essner et al., 2000; Gage and Camper, 1997; Schweickert et al., 2000; Tremblay et al., 2000). Oikopleura pitx RACE products also displayed two distinct 5’ UTRs and translation start sites; if these distinct 5’ ends are transcribed from separate promoters, as in Ciona pitx (Christiaen et al., 2005), then Oikopleura pitx promoter 1 is approximately 3.4 kb upstream of promoter 2. To assess transcript diversity, PCR primers in each 5’ UTR were paired with a primer in the common 3’ UTR and products were amplified from embryonic cDNA pools. We observed at least six splice forms, four of which are schematized in Fig. 3A. Long transcripts from promoter 1 are similar to vertebrate Pitx2 isoforms a and b and to Ciona pitxa/b (Christiaen et al., 2002; Cox et al., 2002; Essner et al., 2000). Vertebrate Pitx2 isoforms differentially affect the development of left-right asymmetry (Liu et al., 2001; Schweickert et al., 2000); a thorough analysis of Oikopleura pitx isoforms in the context of larvacean anatomical asymmetries will be published separately. The Oikopleura transcript from Promoter 2 is similar to vertebrate and Ciona isoform c, except that the C terminus is truncated by alternative splicing. This is the first report of naturally occurring Pitx isoforms with truncated C-termini.

In transcripts from promoter 1, the short 5’ UTR was preceded by a sequence that did not match genomic flanking sequence but that is identical to a trans-spliced leader present in many Oikopleura transcripts (Ganot et al., 2004). Trans-splicing of pitx transcripts might be associated with enhancement of translation, as in other organisms (Maroney et al., 1995; Zeiner et al., 2003). Ciona pitx transcripts also have a trans-spliced leader (Christiaen et al., 2002).
Pitx genes belong to the Aristalless-related subfamily within the Paired-class homeobox gene superfamily. In addition to the DNA-binding homeodomain, Aristalless-related proteins share a protein interaction domain called the OAR domain (Furukawa et al., 1997; Meijlink et al., 1999). We detected no OAR domain in Oikopleura Pitx by alignment with other proteins. Despite this difference from even the reported ascidian pitx cDNA sequences (Boorman and Shimeld, 2002; Christiaen et al., 2002), gene phylogeny (Fig. 3B), GenBank BLAST results and protein alignment indicate this Oikopleura gene is otherwise an indisputable Pitx ortholog.

Drosophila and vertebrate Six genes fall into three clades: one clade includes the three genes Sine Oculis, Six1 and Six2; the second clade includes Drosophila Six3, Optix, Six3 and Six6; and the third includes Drosophila Six4, Six4 and Six5 (Seo et al., 1999). Six proteins have a Six domain required for protein-protein interactions and a homeodomain (Pignoni et al., 1997). We cloned three larvacean Six genes in this study. An unrooted gene phylogeny of protein sequences restricted to the Six domains and homeodomains is consistent with one Oikopleura gene orthologous to the Six1 + Six2 clade, and two Oikopleura genes orthologous to the Six3 + Six6 clade (Fig. 3B). Oikopleura six3/6a and six3/6b share an intron position in the Six domain (Fig. 3A) that is not shared with vertebrate Six3 and Six6 or with a Ciona six3/6 gene (ci0100153410). The larvacean six3/6 genes, therefore, are co-orthologs of ascidian six3/6, and are named six3/6a and six3/6b to reflect an independent duplication in the larvacean lineage. This finding is consistent with the expansion of several larvacean homeobox gene families, including the Six genes (Edvardsen et al., 2005).

Drosophila and vertebrate Six genes are clustered in both fly and vertebrate genomes, and the common ancestor of these lineages may have had a single cluster containing one of each of the three classes of Six genes (Gallardo et al., 1999; Kawakami et al., 2000). Although Six1/2 and Six3/6 were probably adjacent genes in the ancestral state, the genomic regions flanking each Oikopleura Six gene

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**Fig. 3. Oikopleura orthologs of vertebrate placode-marking genes. (A) Gene structures for five larvacean genes (red, Eya domain; black, homeodomain; blue, Six domain; green, intron unique to larvacean Six3/6 duplicates). (B) Topologies of neighbor-joining gene phylogenies of aligned protein sequences are consistent with assignment of Oikopleura genes to Eya, Pitx, Six1/2 and Six3/6 orthologies.**
on our completely sequenced fosmids contain segments with strong BLAST similarities only to non-Six-related genes (not shown), implying that the larvacean Sixes are not tightly clustered. If this larvacean gene arrangement represents the breaking of an ancestral Six gene cluster in the larvacean lineage, it would parallel the fragmentation of the *Oikopleura* Hox cluster (Seo et al., 2004).

**pitx and six3/6a expression and the ANR**

Having isolated *Oikopleura* homologs of vertebrate ‘placode genes’, we tested the hypothesis that *Oikopleura* possesses homologs of the most anterior vertebrate placodes by investigating whether the expression of Eya, Pitx and Six genes may mark a pre-placodal domain at the anterior margin of the embryonic CNS.

In vertebrate embryos, the primordia of the stomodeum and of the olfactory and adenohypophysial placodes lie in adjacent parts of the anterior neural ridge (ANR), an arc of ectoderm surrounding the rostral margin of the neural plate (Couly and Le Douarin, 1985; Kawamura and Kikuyama, 1992). Genes important for stomodeal and placode development, including Pitx, Eya and Six family genes, are expressed in the ANR (e.g. Baker and Bronner-Fraser, 2001; Schlosser and Ahrens, 2004; Streit, 2004). Similar to their vertebrate counterparts, larvacean Eya, Six and Pitx genes are expressed early in overlapping patterns at the anterior margin of the developing CNS: in rostral ectoderm, in the presumptive mouth and in the rostral-most pharynx (Fig. 4).

Because *Oikopleura* embryos undergo neurulation when they have few cells and limited anatomical landmarks, we began our analysis when the presumptive CNS has just completed internalization (Cañestro et al., 2005). At incipient-tailbud stage, the morphological division between the trunk and tail first becomes apparent, and ectodermal cells begin to transition from a rounded, cleavage stage morphology to an epithelial morphology. The anterior-most CNS is marked by homologs of *pax6* and *otx* at this and later stages (S. Bassham, PhD Thesis, University of Oregon, 2002) (Cañestro et al., 2005). A rectangular field of ectodermal cells just rostral to the CNS strongly expresses *six3/6a* (Fig. 4A,C). Two cells probably in the presumptive rostral pharynx, adjacent to the anterior CNS, express *pitx* (Fig. 4B,C). We did not detect *eya* expression above background in incipient-tailbuds, and *six1/2* expression was broad in the ectoderm (not shown).

By mid-tailbud stage, the *six3/6a* ectodermal domain, consisting of six cells, organizes into a three-sided box open on the ventral side (Fig. 4D). At this stage, transverse ranks of cells in the superficial ectoderm adjacent to the pharyngeal cells also express *pitx* (Fig. 4E; purple, pink arrowheads). The *pitx* and *six3/6a* domains overlap (pink arrowhead), although *pitx* signal is strongest in cells (purple arrowhead) that lie dorsal to the *six3/6a* domain, as revealed by one-color double in situ hybridization with probes for both genes (Fig. 4F,G). A few ectodermal cells also express *eya*, apparently overlapping the stronger *pitx* ectodermal domain (Fig. 4H,I).

As with the expression of vertebrate *Pitx1*, *Pitx2* and *Six3* genes (Oliver et al., 1995; Schweickert et al., 2000), *Oikopleura* *pitx* and *six3/6a* expression begins early in overlapping domains at the rostral border of the CNS, far preceding the terminal differentiation of cell types such as sensory neurons. The region marked by these larvacean genes is probably homologous to the vertebrate ANR and rostral pre-placodal region, although, unlike the vertebrate ANR, these

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**Fig. 4.** Orthologs of ANR-marking genes are expressed anterior to the CNS. All panels are left lateral views, all insets are frontal. (A) In incipient-tailbud stage, the stomodeal primordium expresses *six3/6a*. (B) The anterior pharynx primordium and the tail muscle precursors express *pitx* at incipient-tailbud stage. (C) Schematic overlay of *pitx* and *six3/6a* in which the anterior *pitx* domain lies just interior to the ectodermal *six3/6a* expression. (D) In mid-tailbud stage the *six3/6a* ectodermal expression (blue arrowhead) changes shape (see inset). (E) Ectodermal cells begin to express *pitx* by mid-tailbud stage in weak (purple arrowhead) and strong (purple double arrowheads) domains. (F) Double in situ reveals that only the ventral rank of ectodermal cells weakly expressing *pitx* overlaps *six3/6a* expression, while the dorsal *pitx*-expressing cells do not. (G) Schematic summarizing overlapping (checkered) and non-overlapping *pitx* and *six3/6a* domains. (H) A horizontal row of ectodermal cells expresses *eya* in mid-tailbud embryos. (I) Schematic shows inferred overlapping (checkered) and non-overlapping *eya* and *pitx* domains. ec, ectoderm; en, endoderm; gi, endodermal gill pouch primordium; ph, pharynx primordium. Black arrowhead indicates separation between trunk and tail. Scale bars: 20 μm.
**EyA, Pitx and Six gene expression in the ventral organ**

The hypothesis that the larvacean ventral organ is homologous to the vertebrate olfactory epithelium (Bollner et al., 1986) predicts that *Oikopleura* orthologs of vertebrate genes important for olfactory placode development should also be expressed in the developing larvacean ventral organ. To test this prediction, we analyzed the expression of larvacean eya, pitx and six genes in *Oikopleura* embryos and hatchlings.

In early larvacean hatchlings, when organogenesis is still ongoing in the trunk, eya, pitx and six1/2 genes appear to be co-expressed in ectodermal cells concentric with cells immediately surrounding the presumptive mouth (Fig. 5A-D). For eya and pitx, this expression is a continuation of rostral ectodermal expression from mid-tailbud stages (discussed above). Ventrally, paired patches of six1/2 expression meet at the midline (Fig. 5B). Similar patterns are observed for both eya and pitx (not shown and Fig. 5C). The ventral organ, which forms in this region, develops from ectodermal cells that sink into a groove perpendicular to the body axis (Bollner et al., 1986), and although the ventral organ appears unpaired in adults, this early gene expression pattern together with bilateral innervation reveals that the organ originates as a paired structure. In lateral view, six1/2 expression is just rostral to the thickened ectodermal epithelium at the anterior border of the underlying endostyle; if these six1/2-expressing cells are presumptive ciliated receptor cells, then this represents a stage before their involution (Fig. 5B).

Pitx expression around the presumptive mouth in hatchlings includes cells in the ring domain shared with six1/2 and eya, and also cells immediately adjacent to the mouth (Fig. 5C,D), but excludes at least the upper lip cells, which in late hatchling stages carry sensory bristles. six1/2 is also expressed in cells immediately adjacent to the mouth (Fig. 5B), in the region where the bristle-bearing cells and other ciliated mechanoreceptors will form (discussed below).

In late hatchling stages, the cells lining the rostral nerves express both pitx (Fig. 5E,F) and six3/6a (Fig. 5G).

**pitx, six3/6a and six3/6b expression in the ciliary funnel**

The hypothesis that the larvacean funnel is homologous to both the adeno- and neurohypophyses of vertebrates (Holmberg, 1982) predicts that orthologs of genes expressed in the vertebrate adenohypophyseal placode should be expressed in part of the developing ciliary funnel. Pitx should be expressed...
in tissue homologous to the vertebrate stomodeum or foregut endoderm, both of which express Pitx genes (e.g. Lanctot et al., 1997; Pommerreit et al., 2001) and contact the adenohypophyseal placode in amphibians (Kawamura and Kikuyama, 1992) and hagfish (Gorbman, 1983). We tested these predictions by analyzing the expression of Oikopleura pitx and Six genes in larvacean embryos and hatchlings.

Duplicate larvacean homologs of six3/6 are expressed in the rostral pharynx. In young hatchlings, the expression domain of six3/6b is nested within the broader six3/6a expression domain (Fig. 6A-D). While six3/6a is expressed in the entire roof of the anterior pharynx, in the rostral brain, in the esophagus and in posterior ectodermal domains, six3/6b appears only in a trio of cells on the right-hand side (Fig. 6B,D); these cells may give rise to the three or four cells that make up the most ventral part of the adult ciliary funnel (Holmberg, 1982) and, later, the ventral part of the morphologically distinct funnel expresses six3/6b (Fig. 6F). Six3/6a expression in later stages marks the entire ciliary funnel from its base to the brain (not shown) and is therefore presumably in both pharynx-derived and CNS-derived parts of the funnel.

Oikopleura pitx is expressed in the stomodeum and pharyngeal endoderm. Pitx expression expands in hatching stages from the stomodeum into the rostral pharynx, but does not reach into the endodermal gill pouches (Fig. 5D, inset). The ciliary funnel expresses pitx at the level of the pharynx, but not in the more dorsal parts (Fig. 6G,H). In addition to the ventral funnel, cells in two domains in the brain express pitx (Fig. 5D,E); the rostral of these appears to be the origin of paired nerve n2 that innervates a circumoral sensory organ (discussed below). The dorsal pitx domain in the brain is posterior to the ciliary funnel tip and it seems unlikely that it is in cells that form a connection with the funnel wall that were described by Holmberg (Holmberg, 1982).

The expression patterns of larvacean pitx and six3/6 genes are consistent with the orthology of the ciliary funnel and the vertebrate pituitary rather than other placode-derived organs, particularly because vertebrate Pitx genes are expressed in only the most anterior placodes (pituitary, olfactory and lens) (Baker and Bronner-Fraser, 2001). Pitx is expressed in the ventral-most part of the funnel, while six3/6 paralogs are expressed in both the ventral and more dorsal parts of the funnel. As vertebrate Pitx genes are expressed in the adenohypophysis while Six3 and Six6 are expressed in both the adenohypophyseal placode and the hypothalamic primordium (e.g. Ghanbari et al., 2001), the Pitx-expressing and non-Pitx-expressing domains of the larvacean funnel might correspond to adeno- and neurohypophysial components, respectively.

**eya and six3/6a in the Langerhans and circumoral ciliated mechanoreceptors**

Oikopleura has other paired peripheral mechanoreceptor organs, the Langerhans and oral sensory organs. The two Langerhans organs include a receptor cell bearing a long rigid cilium, supporting cells, and a neuron in the caudal ganglion that sends processes to each of the Langerhans organs and towards the anterior brain (Holmberg, 1986). In the rostral part of the anterior brain, two neurons innervate respectively right and left sides of the oral sensory complex; e.g. with a single branching axon, the left neuron innervates both the left upper lip cell and the left side of the circumoral ring of ciliated cells (Olsson et al., 1990).

Are the larvacean sensory cells homologous to either ascidian cupular or coronal receptor cell types? Are they homologous to vertebrate hair cells? Hypotheses that the Langerhans organ is homologous to the inner ear neuroepithelium and that the larvacean oral sensory system is homologous to the lateral line neuromasts predict that larvacean homologs of Eya and Six genes important for otic and lateral line placode development should be expressed in the primordia of these larvacean peripheral mechanosensory organs. We found larvacean eya and six3/6a expression in lateral paired patches in the trunk of tailbud embryos and early hatchlings where the Langerhans organs develop (Fig. 7A-F), and eya and six1/2 expression in the oral region in late hatchlings where the sensory cells of the lips develop (Fig. 7H,I).
Development

In tailbud stages, paired lateral ectodermal patches at the junction of the trunk and tail express *eya* in the region where the Langerhans mechanoreceptors will develop (Fig. 7A,B). Expression narrows to a pair of cells at the posterior, ventral trunk in early hatchlings (Fig. 7D). The pharyngeal endoderm of the proximal gill pouches also expresses *eya* (Fig. 4A); similarly, murine *Eya1* is expressed in the pharyngeal pouch endoderm (Xu et al., 1997).

In vertebrates, the lateral line placode expresses *Six1* and the otic placode expresses *Six1* and *Six4*, but neither placode is known to express *Six3* or *Six6* (Baker and Bronner-Fraser, 2001; Streit, 2004). Unexpectedly, *Oikopleura six3/6a* (rather than *six1/2*) is expressed like *eya* in paired ectodermal patches. In young hatchlings, *six3/6a* is expressed strongly in two lateral trunk cells at the base of the tail in the region where the Langerhans cells develop (Fig. 7E); this expression originates in tailbud stage embryos when unilateral *six3/6a* expression in a single right side cell (Fig. 7C) precedes the later paired expression (Fig. 7E). The early, right side expression of *six3/6a* is narrower than that of *eya*, which is bilaterally symmetrical at tailbud stages and extends to more than one cell on each side.

Cells immediately surrounding the presumptive mouth express *six3/6a* and *pitx* in tailbud stages (Fig. 4A-G); they express *six1/2* in early hatchlings (Fig. 4B), and they express both *six1/2* and *eya* in late hatchings (Fig. 7H). In addition, the lower lip and the stomodeal roof, presumably including the precursors of the ciliated cells, express *pitx* in hatchling stages (Fig. 4B,E; Fig. 5C,D,F; Fig. 6H). Interestingly, the two bristle-bearing cells of the upper lip (Fig. 6H) in these stages appear not to express *pitx*, but *pitx* is expressed in a prominent cell bulging from the left rostral brain (Fig. 5E); this brain expression is almost certainly one of the paired cells described by Olsson et al. (Olsson et al., 1990) that conspicuously bulge from the left rostral brain and originate nerve n2 to both the upper lip and ciliated cells of the mouth.

Although neither the larvacean Langerhans nor the circumanal mechanoreceptor organs have been proposed on morphological grounds to be homologous to vertebrate placode-derived sensory organs, the embryonic primordia of these tissues appear to express orthologs of vertebrate placode-marking genes. These larvacean sensory systems could have independently evolved from a placode precursor in the common ancestor of chordates and may have no counterpart in vertebrates. Alternatively, the Langerhans organ primordia may be orthologous to both the ascidian atrial primordia and the vertebrate otic placodes.

**Discussion**

The chordate anterior neural ridge

In developing larvaceans, cells adjacent to the anterior CNS express *pitx* and *six3/6a*, resembling expression of orthologous genes in the vertebrate anterior neural ridge (ANR). In gnathostome embryos, the primordia of the adenohypophyseal andolfactory placodes are initially part of the ANR; the primordia are contiguous with each other and with the hypothalamus primordium (Coulby and Le Douarin, 1985; Kawamura and Kikuyama, 1992). The stomodeum primordium lies superficial to the placodal arc in the ANR (Kawamura and Kikuyama, 1992). Vertebrate Pitx genes are early markers of the ANR, and continue to be expressed in the stomodeum and the adenohypophyseal and olfactory placodes as these separate from the neural tube and become distinct (Dutta et al., 2005; Lancot et al., 1997; Schweickert et al., 2001). Vertebrate *Six3* also marks the ANR and anterior neural plate, and continues to be expressed in ANR derivatives and in the forebrain after neurulation (Oliver et al., 1995). In larvacean tailbud embryos,
the stomodeal region expresses pitx and six3/6a, and putative pituitary and olfactory placode homologs continue to express these genes at later stages. *Oikopleura* pitx expression is also comparable with ascidian and amphioxus Pitx genes, which are expressed anterior to the CNS, in the stomodeum, and in the ciliated funnel (ascidian) and Hatschek’s pit (amphioxus), strengthening the homology of all chordate mouths and adenohipophysial-like organs (Boorman and Shimeld, 2002; Christiaen et al., 2002; Yasui et al., 2000).

In vertebrates, evidence suggests that specific placodes individualize from a pan-placodal field by a series of inductive fate restrictions (Streit, 2004), and, at least in zebrafish, placode precursor cells move to condense from an initially broad field (Whitlock and Westerfield, 2000). By contrast, larvacean placode specification probably occurs when the embryo consists of fewer than 200 cells (before incipient taillbud stage). In concert with reduced cell number and mosaic development, the gradual cascade of placode specification could have become secondarily simplified in the urochordate lineage, leaving no broad pan-placodal field marked by simultaneous expression of Eya, Pitx and Six genes, but rather the expression of at least some of these markers first appears when placode subtypes are already spatially separated and distinguished by unique, although overlapping, sets of markers.

**The ventral organ and the vertebrate olfactory organ**

Eya and Six proteins (excluding Six3 and Six6) interact synergistically, both in vertebrates and in flies, to activate transcription of downstream genes (Ohno et al., 1999). *Oikopleura* six1/2 and eya expression domains overlap in a ring of ectodermal cells centered on the presumptive mouth (Fig. 4). The overlap of six1/2 and eya would be predicted if their protein products interact in the same way as their homologs do in vertebrates and flies.

Larvacean pitx expression also overlaps eya and six1/2 in a ring of ectodermal cells around the mouth (Fig. 3Ci; Fig. 4C,D). Vertebrate Pitx1 and Pitx2 genes are expressed early in the stomodeum, this gene expression persists in the olfactory and adenohipophysial placodes as these invaginate, and Pitx1 may directly regulate vertebrate olfactory receptor genes (Hoppe et al., 2003). The location and timing of larvacean six1/2, eya and pitx expression in the rostral ectoderm are appropriate to mark two cell groups (the ciliary cells of the ventral organ and the ensheathing ‘rostral brain bulb’ cells) that form by a placode-like process. Consistent with Delsman’s (Delsman, 1912) and our observations that the ensheathing cells along the rostral nerves delaminate from the ectoderm, initially ectodermal epithelial cells express pitx and then the rostral brain bulb cells express the gene (Fig. 6C-F). The rostral nerves also express six3/6a at the stage when the separation of the bulb cells from the rostral epidermis is visible (Fig. 6G), consistent with the expression of vertebrate Six3 genes in the olfactory placode (e.g., Ghanbari et al., 2001).

We propose, based on the expression of eya, six1/2, six3/6a and pitx, that the rostral bulbs represent part of the ventral organ placode, and that this placode is the ortholog of the vertebrate olfactory placode. In vertebrates, the olfactory placode produces not only ciliary sensory cells, but also several other cell types, some of which are ultimately located at a distance from the olfactory epithelium; these types include supporting, basal and ensheathing cells (Farberman, 1992). Bollner et al. (Bollner et al., 1986) sorted the four ensheathing cells of the larvacean rostral bulbs into three classes, one of which had mesaxon-like structures similar to those typical of vertebrate olfactory ensheathing cells (OECs), which bundle the unmyelinated axons of the receptor cells. Unlike the Schwann cells of other sensory nerves, OECs originate in the placode itself rather than from neural crest, and, like the larvacean rostral bulb cells, they embrace bundles of axons, rather than enveloping axons one at a time (Farberman, 2000). However, the bulb cells are also like vertebrate terminal nerve cells, some of which appear to originate in the olfactory placode (Wirsig-Wiechmann, 2001). Terminal nerve cells exert a neuromodulatory influence on the olfactory neurons; similarly, the larvacean bulb cells exhibit immunoreactivity for GABA, an inhibitory neurotransmitter (Bollner et al., 1991).

There is no structure morphologically comparable with the ventral organ described in cephalochordates or ascidians. In cephalochordates, although the rostral epidermis that has been suggested to be olfactory is marked by *AmphiPax-6* in post-gastrula *Branchiostoma floridae* (Gladson et al., 1997), the corresponding tissue does not appear also to express pitx (Boorman and Shimeld, 2002). Furthermore, individual ciliated sensory cells identical to those that form in the Pax6 domain are also scattered throughout the trunk epidermis and are not discretely clustered (Holland and Yu, 2002; Northcutt, 1996), making them unlike placode-derived olfactory organs. Burighel et al. (Burighel et al., 1998) have proposed that the ascidian neural gland complex is both an olfactory and pituitary homolog (Burighel et al., 1998). The idea that a single organ served for both olfaction and hormone regulation in a chordate ancestor and that this organ split into separate olfactory and pituitary organs in vertebrates (Gorbman, 1995) is weakened by the existence of separate probable olfactory and adenohipophysial organs in *Oikopleura*, and by the finding of Whitlock et al. (Whitlock et al., 2003) that cells of the terminal nerve that produce GnRH (otherwise a hormone of the pituitary) are products of neural crest rather than the olfactory placode, as was originally thought. Mazet et al. (Mazet et al., 2005) recently proposed that the ascidian palps might be an olfactory organ homolog, based on expression of eya and neuronal marker COE; it seems likely, on the basis of gene expression and topography, that the *Oikopleura* ventral organ placode is homologous to the ectoderm of the ascidian palps, although, unlike the larvacean ventral organ, no ciliated sensory cells have been identified on the palps. Other olfactory markers in non-vertebrate chordates should be analyzed to resolve these apparent differences among chordates.

**The ciliary funnel and the vertebrate pituitary**

Like early developmental stages of the vertebrate adenohipophysial, the larvacean ciliary funnel forms a pouch that connects the pharyngeal roof with the brain. Much of the larvacean funnel, however, is an outgrowth of the brain (Delsman, 1912). The neural origin and secretory behavior of the ciliary funnel prompted Holmberg (Holmberg, 1982) to propose that this larvacean organ represents the combined homolog of the adenohipophysial and neurohypophysial. Fate-mapping in chick (Takor and Pearse, 1975) and amphibians (Kawamura and Kikuyama, 1992) shows that immediately adjacent regions give rise to the hypothalamus and the adenohipphysial, though they separate after neural tube
Development proximal to the brain and because vertebrate downregulated in the olfactory organ, Boorman and Shimeld pitx the systems for collecting olfactory information, including sex synchronizing sexual maturation and reproductive behavior, chemicals from the environment. Because of the importance of would be in constant communication with waterborne buccal cavity, where, in an aquatic organism, these organs Rathke’s pouch, Hatschek’s pit and the ciliated funnel in the pharyngeal opening, ultimate acts to regulate transcription of Pitx genes. Vertebrate Pitx1 is an early marker of the stomodeum and the foregut endoderm, and its expression persists in the olfactory and adenohypophyseal epithelia into late development (Lanctot et al., 1997). In the pituitary, Pitx1 ultimately acts to regulate transcription of hormone genes, and Pitx2 is important for proper morphogenesis of Rathke’s pouch (Drouin et al., 1998; Suh et al., 2002). Larvacean pitx is also expressed in the mouth and rostral foregut, where it overlaps with the expression of six3/6a and six3/6b. pitx, however, does not appear to be expressed in dorsal parts of the funnel. This is similar to ascidian development, where only the ciliated funnel in the pharyngeal wall expresses pitx but the ciliated duct that connects to the brain does not (Boorman and Shimeld, 2002).

Arguments that the origin of the adenohypophysis predated the origin of the vertebrates and was originally chemosensory (Gorbman, 1995; Manni et al., 1999; Nozaki and Gorbman, 1992; Olsson, 1990) are based in part on the location of Rathke’s pouch, Hatschek’s pit and the ciliated funnel in the buccal cavity, where, in an aquatic organism, these organs would be in constant communication with waterborne chemicals from the environment. Because of the importance of synchronizing sexual maturation and reproductive behavior, the systems for collecting olfactory information, including sex pheromones, and for eliciting maturation and behavioral changes could have been anciently linked (Muske, 1993). Because ascidian pitx is not expressed in the part of the duct proximal to the brain and because vertebrate Pitx is eventually downregulated in the olfactory organ, Boorman and Shimeld (Boorman and Shimeld, 2002) tentatively proposed that the ascidian duct could be homologous to the olfactory component of a combined adenohypophyseal/olfactory organ; this hypothesis, similar to that of Burighel et al. (Burighel et al., 1998), implies a conflation of organs in ascidians that is at odds with the separate olfactory and adenohypophyseal homologies that we propose in Oikopleura dioica and Mazet et al. (Mazet et al., 2005) propose for ascidians.

In any case, both ascidian and larvacean urochordates apparently differ from vertebrates, which continually use Pitx in adenohypophyseal development through adult pituitary function. Functional tests are needed to determine if the ability of Pitx to regulate transcription of hormone genes is conserved in urochordates.

**Larvacean mechanosensory organs and placode-marking genes**

In the ascidian Ciona, the walls of the exhalant atrial siphon contain hair cell-like cupular organs that Bone and Ryan (Bone and Ryan, 1978) have proposed are homologous to the acoustico-lateralis (otic + lateral line) system. Wada et al. (Wada et al., 1998) proposed that the atrial primordia are, more specifically, orthologs of vertebrate otic placodes. Larvaceans also have peripheral ciliated mechanoreceptors. In the posterior larvacean trunk, the paired ciliated Langerhans receptors elicit an escape response when stimulated (Bone and Ryan, 1979). Several characteristics make these organs plausible homologs of vertebrate otic placode derivatives. The larvacean Langerhans cells are unusual for non-vertebrate sensory cells in that they are secondary receptors, meaning they do not themselves send an axon to the CNS but instead are separately innervated (Bone and Ryan, 1979). In this respect, they are more like hair cells than are cupular cells, which are primary receptors (Bone and Ryan, 1978). Vertebrate Eya1 and Six1 are expressed in the otic placode where they are crucial for the development of the sensory epithelium (Zheng et al., 2003). Paired patches of larvacean nya expression appear in tailbud stage at the junction between the trunk and the tail (Fig. 7A,B) where the Langerhans receptors will develop. The paired larvacean nya expression pattern is adjacent to the caudal ganglion, the larvacean homolog of the vertebrate hindbrain (Cañestro et al., 2005), where the axons that innervate the receptors originate (Holmberg, 1986). This topographic relationship is similar to vertebrate otic development in which a combination of signals from the nearby hindbrain and mesendoderm may induce otic placode formation (e.g. Liu et al., 2003) and the otic neuron axons project to the hindbrain.

Oikopleura has linearly arranged ciliated mechanoreceptors that ring the mouth (Fig. 7I); similar to the vertebrate lateral line organs, they are secondary receptors and lie in a groove formed by overlying epithelial cells (Olsson et al., 1990). Touching these cilia causes a reversal of the feeding current driven by the spiral ganglion, the response predicted to reject particles too big for the fixed-gape mouth (Galt and Mackie, 1971; Lohmann, 1933). Eya1 is important for survival of hair cells in the developing ear and lateral line placodes of zebrasfish (Kozlowski et al., 2005), and Six1 is expressed in lateral line hair cells in amphians and fish (Bessarab et al., 2004; Pandur and Moody, 2000). The developing larvacean stomodeum expresses both nya and six1, suggesting that, as in vertebrates, these genes could play a role in the development of ciliated mechanosensory cells.
Superficial structural parallels between urochordate and vertebrate ciliated mechanosensory systems suggest that the common chordate ancestor already had lateral line and otic placodes. The variety of morphologies among mechanoreceptor cells and organs in larvaceans, ascidians and vertebrates, however, might indicate that, rather than necessarily being specific otic and lateral line orthologs, these organs could have evolved by the independent proliferation in chordate lineages of a mechanosensory placode precursor. Although Eya1 is expressed in vertebrate otic placodes, Six3 and Six6 are not. The unpredicted expression of a larvacean six3/6a in the developing Langerhans receptor could represent a larvacean co-option of this homeobox gene into otic development, or it could be evidence that the Langerhans organs are paralogs, rather than orthologs of the otic placodes. The fact that Ciona six1/2 is expressed in the atrial primordia (Mazet et al., 2005) is evidence in favor of the first of these hypotheses.

The projection of axons from the larvacean circumoral sensory cells into the rostral brain and the projection of axons from the ascidian coronal organ into the cerebral ganglion (Burighel et al., 2003) are not predicted for a lateral line ortholog, whose nerves should enter the hindbrain as in vertebrates (Gompel et al., 2001). The presence of ciliated, secondary mechanoreceptors in the oral ectoderm is common to larvaceans, ascidians and cephalochordates (Lacalli and Hou, 1999), however, and a common ancestral organ cannot be excluded, despite differing cell morphologies. Vertebrate Eya1 and Six1 expression is persistent in the stomodeum (Schlosser and Ahrens, 2004), suggesting that the stomodeum of the vertebrate/urochordate common ancestor expressed Eya and Six1/2 and a role for these genes in oral mechanosensory organ development may have been retained in filter-feeding non-vertebrate chordates. Expression analysis of Eya and Six1/2 in coronal organ-bearing ascidians and in amphioxus, and functional analysis of the genes is needed to test this hypothesis.

Conclusions
In summary, we found that developmental expression of larvacean eya, pitx and six genes corresponds with predictions stemming from morphology-based homology assignments between larvacean and vertebrate placode-derived organs, making a strong case not only for placodes, but for specific placodes as plesiomorphies, or primitive character states, of the phylum Chordata. We propose that separate olfactory and pituitary organs were already present in the common ancestor of modern chordates (Fig. 8).

In addition, mechanosensory organs orthologous to the otic placode, represented in urochordates by the larvacean Langerhans and ascidian atrial placodes, might also be ancestral to all modern chordates. Finally, a fourth, oral mechanosensory placode may be ancestral in the chordates, but lost or modified in the vertebrates. We caution, however, that there could have been an independent proliferation and parallel evolution of mechanosensory placodes in the different chordate lineages, and that, although these placodes retain ancestral gene expression of placode markers, they might be paralogous organs. Likewise, the presence of both orthologous and paralogous placodes in vertebrates with respect to urochordates implies independent proliferation and diversification of some placodes in the vertebrates, but this is not incompatible with a common placodal primordium in early vertebrate development. Differences in morphology and complement of sensory organs among larvaceans and ascidians highlight the need for broad sampling among chordate clades before drawing generalizations about the inferred chordate ancestor.

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Supplementary material
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