The ascidian mouth opening is derived from the anterior neuropore: Reassessing the mouth/neural tube relationship in chordate evolution

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ABSTRACT

The relative positions of the brain and mouth are of central importance for models of chordate evolution. The dorsal hollow neural tube and the mouth have often been thought of as developmentally distinct structures that may have followed independent evolutionary paths. In most chordates however, including vertebrates and ascidians, the mouth primordia have been shown to fate to the anterior neural boundary. In ascidians such as *Ciona* there is a particularly intimate relationship between brain and mouth development, with a thin canal connecting the neural tube lumen to the mouth primordium at larval stages. This so-called neurohypophyseal canal was previously thought to be a secondary connection that formed relatively late, after the independent formation of the mouth primordium and the neural tube. Here we show that the *Ciona* neurohypophyseal canal is present from the end of neurulation and represents the anteriormost neural tube, and that the future mouth opening is actually derived from the anterior neuropore. The mouth thus forms at the anterior midline transition between neural tube and surface ectoderm. In the vertebrate *Xenopus*, we find that although the mouth primordium is not topologically continuous with the neural tube lumen, it nonetheless forms at this same transition point. This close association between the mouth primordium and the anterior neural tube in both ascidians and amphibians suggests that the evolution of these two structures may be more closely linked than previously appreciated.

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Introduction

Recent phylogenies have shown that the closest relatives of the vertebrates are not the cephalochordates (amphioxus) but the tunicates (ascidians, larvaceans, and salps) (Delsuc et al., 2006). Ascidians have a small, simple swimming larval stage with a stereotypical chordate body plan (Fig. 1A), but then metamorphose into sessile filter-feeding adults (Fig. 1B). The ascidian mouth is derived from a dorsal, placode-like structure just anterior to the neural tube, variously known as the oral siphon primordium (OSP), stomodeum or the primordial pharynx (Katz, 1983; Manni et al., 2005; Satoh, 1994). The primordial pharynx is not an apt name, however, as the OSP is an ectodermal and not an endodermal structure. The OSP expresses a suite of genes, including *Pitx* and *Six3/6*, that are characteristic of all chordate mouth primordia (Boorman and Shimeld, 2002; Christiaen et al., 2002, 2007). The early morphogenesis of the OSP has not been extensively characterized.

The neurohypophyseal duct

Several generations of ascidian researchers have been perplexed by a thin canal connecting the OSP and the adjacent neural tube lumen at larval stages (Fig. 1C). This Neurohypophyseal Duct (ND) or canal

was first described by Willey in 1893 (Willey, 1893), and its existence was confirmed by Katz in 1983 using transmission electron microscopy (Katz, 1983). Willey described closure of the anterior neuropore followed by the independent invagination of the OSP from dorsoanterior ectoderm, with the ND forming as a thin canal protruding from the sensory vesicle (anterior neural tube) to contact and perforate the OSP (Willey, 1893) (Fig. 1D). Willey described this as being a secondary neuropore, but only in the sense of being a connection between the neural tube lumen and the outside, and not in relation to the original site of neural tube closure. Manni et al. used TEM to examine the morphogenesis of the ND at post-hatching stages and argue in favor of Willey's model, but their earliest timepoint was too late to address the initial formation of either the ND or the OSP (Manni et al., 2005).

The ascidian neural plate

The neural plate can be separately defined by morphology, gene expression, inductive mechanism and cell fate. In ascidians, these separate criteria give rise to overlapping but not perfectly congruent definitions of the neural plate. Cell morphology (Nicol and Meinertz-hagen, 1988a,b), the expression of the panneural marker *Etr1* (Yagi and Makabe, 2001), and a common requirement for FGF signaling (Bertrand et al., 2003; Hudson and Lemaire, 2001) all define a similar patch of cells on the dorsal midline. This contiguous group of cells, which includes the descendants of blastomeres a6.5, a8.25, A7.4 and

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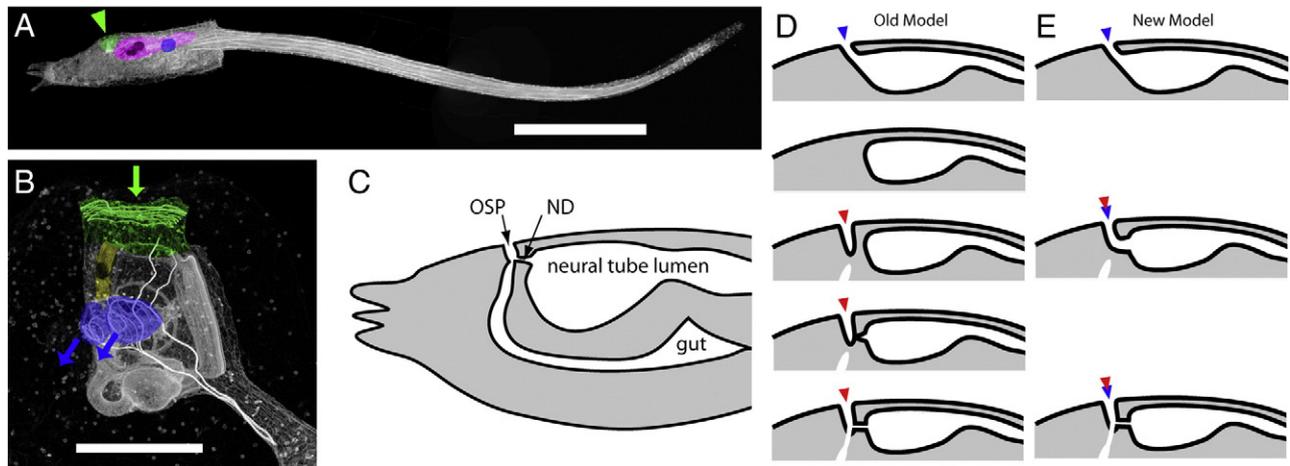
Fig. 1. Ascidian mouth formation. (A) Confocal image (maximum intensity projection) of phallacidin-stained *Ciona savignyi* swimming tadpole larva. Anterior is to the left; dorsal is to the top. The brain, composed of the sensory vesicle, neck region and visceral ganglion, is highlighted in magenta. The oral siphon primordium is highlighted in green, with the presumptive mouth opening indicated with a green arrowhead. The left atrial siphon primordium is highlighted in blue. Scale bar: 200 µM. (B) Confocal image (maximum intensity projection) of phallacidin-stained *C. savignyi* juvenile (post-metamorphosis) showing the openings of the oral siphon (highlighted in green) and the two atrial siphons (highlighted in blue). The forming ciliated duct is highlighted in yellow. Water flow through the oral and atrial siphons is indicated with arrows. Scale bar: 200 µM. (C) Schematic view of the swimming larval stage *Ciona* neural tube lumen, gut lumen, oral siphon primordium (OSP) and neurohypophyseal duct (ND). (D) The classical model of OSP and ND morphogenesis. In this model, the anterior neuropore (blue arrowhead) closes and the neural tube becomes detached from the overlying epidermis. The OSP (red arrowhead) then forms as an independent epidermal invagination. The ND forms as a small evagination of the neural tube that reaches out to contact and then fuse with the OSP. E) Our revised model of OSP and ND morphogenesis. After neurulation, the anterior neural tube remains connected to the surface ectoderm via the anterior neuropore. OSP morphogenesis is centered on the neuropore and preserves its topology. The ND is thus present from the end of neurulation rather than being a secondary connection between neural tube and OSP.

A8.15, is widely referred to as the neural plate. In terms of cell fate, however, this field of cells gives rise not just to the definitive neural tube, but also to the adhesive palps and the OSP (Nishida, 1987).

Imaging anterior neural morphogenesis

To better visualize the morphogenesis of the neural plate into the neural tube, palps and OSP, we have engineered a line of *Ciona savignyi* stably expressing the fast-folding fluorescent protein Venus (Nagai et al., 2002) under the control of the *Etr1* promoter. The transgene faithfully recapitulates the expression pattern of *Etr1* in the neural plate and its derivatives. There is also expression at later stages in epidermal sensory neurons, but this PNS expression is easily distinguishable from the neural plate-derived expression.

Using the *Etr1* transgene together with probes for cellular morphology, we find that Wiley's historical description of neural tube closure and OSP formation is inaccurate in several key respects. In particular, we find that the anterior neuropore remains evident at all stages after neurulation, and itself becomes the OSP (compare Fig. 1E with Fig. 1D). The ND thus represents the anteriormost neural tube lumen and does not involve the formation of a secondary connection between neural tube and OSP.

While chordate mouths generally fate to near the anterior neural boundary, this is the first known example of a mouth opening being directly derived from the neuropore. This result suggests an unexpectedly close interplay between the development and evolution of the mouth and neural tube. To compare our ascidian results with a vertebrate, we fate mapped the *Xenopus* mouth at the time of neural tube closure. While we find no evidence of topological continuity between the *Xenopus* neural tube lumen and mouth primordium, *Xenopus* is nonetheless strikingly similar to *Ciona* in that the mouth primordium is derived not just from the anterior neural border but from the precise topological transition on the anterior dorsal midline between deep neural tube and surface ectoderm.

Materials and methods

An *Etr1:Venus* stable transgenic line was generated using the *I-Sce1* method essentially as described (Deschet et al., 2003), using 2847 bp of *Etr1* upstream sequence amplified by primers atgggagtcgtcaggatacg and tctggataaagcaatacatacgagtg, then Gateway cloned into p1.72BSSPE/ISCE1/RfA-Venus (gift of J.S. Joly). The transgenic line was propagated by standard methods at the UCSB Marine Lab.

Ascidian labeling reagents included rabbit polyclonal antiGFP (1:500–1:1000), Alexa 564 antirabbit (1:1000), Bodipy FL phallacidin (1 U/100 µL) and Draq5 (1:1000). Embryos were fixed in 2% paraformaldehyde in seawater for 1–2 h, washed with PBS + 0.2% Triton-X100 (PBSTr), and blocked for 30 min in PBSTr + 5% heat-inactivated goat serum. Primary and secondary antibody incubations were performed overnight at 4 °C in PBSTr + 5% goat serum. After each incubation, the embryos were washed five times for at least 20 min per wash in PBSTr. Draq5 and phallacidin were added to the second wash after the secondary antibody, and incubated for 45 min at room temperature. Fixed and stained embryos were dehydrated in isopropanol, mounted in Murray Clear and imaged on an Olympus Fluoview 1000 with a 40× 1.3NA oil immersion objective.

Live *Ciona* embryos were imaged with a 40× 1.05NA water immersion objective or a 20× 0.75NA dry objective on an Olympus Fluoview 1000. Dechorionated embryos were mounted in seawater using double-sided sticky tape spacers between slide and coverslip and sealed with VALAP.

Xenopus timelapses used incident light from a fiber optic light source on a Leica DMRB compound microscope with an automated focus drive. Focus stacks were combined into extended depth of field reconstructions using CombineZP software (http://www.hadleyweb.pwp.blueyonder.co.uk/).

For *Xenopus* fate mapping, DiI in aqueous solution was introduced by pneumatic microinjection just under the epidermis and imaged on a Leica stereomicroscope with epifluorescence optics. *Xenopus* laminin staining used polyclonal antilaminin (Sigma) at 1:150 and was bleached, cleared and imaged with a 10× 0.4NA objective on an Olympus Fluoview 1000.

Both *Ciona* and *Xenopus* images were contrast adjusted and visualized using ImageJ and Imaris (Bitplane AG). In particular, the 'Oblique Slicer' feature of Imaris was used to reslice confocal volumes along arbitrary planes.

Results

Ciona Etr1:Venus marks the neural plate and its derivatives

To characterize the *Etr1:Venus* transgene, we first examined its expression pattern during gastrulation, when individual blastomeres in the forming neural plate can be easily identified by morphology. At stage 11 [early gastrula, Hotta's staging series (Hotta et al., 2007)], expression is seen in eight a-line blastomeres (left and right pairs for a8.17, a8.18, a8.18 and a8.20) and 12 A-line blastomeres (A9.13, A9.14, A9.15, A9.16, A9.29 and A9.30) (Fig. 2A). By stage 12 (mid gastrula), the a-line cells have all divided once, and *Etr1:Venus* is expressed in all 16 daughter cells (Fig. 2B). By stage 13+ (late gastrula/neural plate), weak expression can also be seen in a9.50 and a9.49 (Fig. 2C). A summary diagram of these expression patterns is shown in Fig. 2D. The only discrepancy between these results and

previously published in-situ hybridization patterns (Yagi and Makabe, 2001) is that the *Etr1:Venus* transgene is not expressed in a9.52 and a9.51. Our results fit with the fate map in that these blastomeres, daughter cells of a8.26, are thought to give rise only to epidermis [Fig. 2E, (Nishida, 1987)]. The transgene is expressed in all neural precursors except for a small number of b-line cells that contribute to dorsal spinal cord and visceral ganglion, which also do not express *Etr1* by in-situ hybridization. There is also weak expression in several cells that will give rise to posterior tail muscle. Overall, however, the transgene is a faithful marker of the broadly-defined neural plate. 3D renderings of the stage 12 and 13+ confocal volumes are shown in Supplemental Movie 1.

Figs. 2F–K shows dorsal views of *Etr1:Venus* expression from stage 14 (early neurula) to stage 18 (initial tailbud II). The expression domain remains contiguous over this period as the neural folds are elevated and seal up along the midline in a posterior to anterior
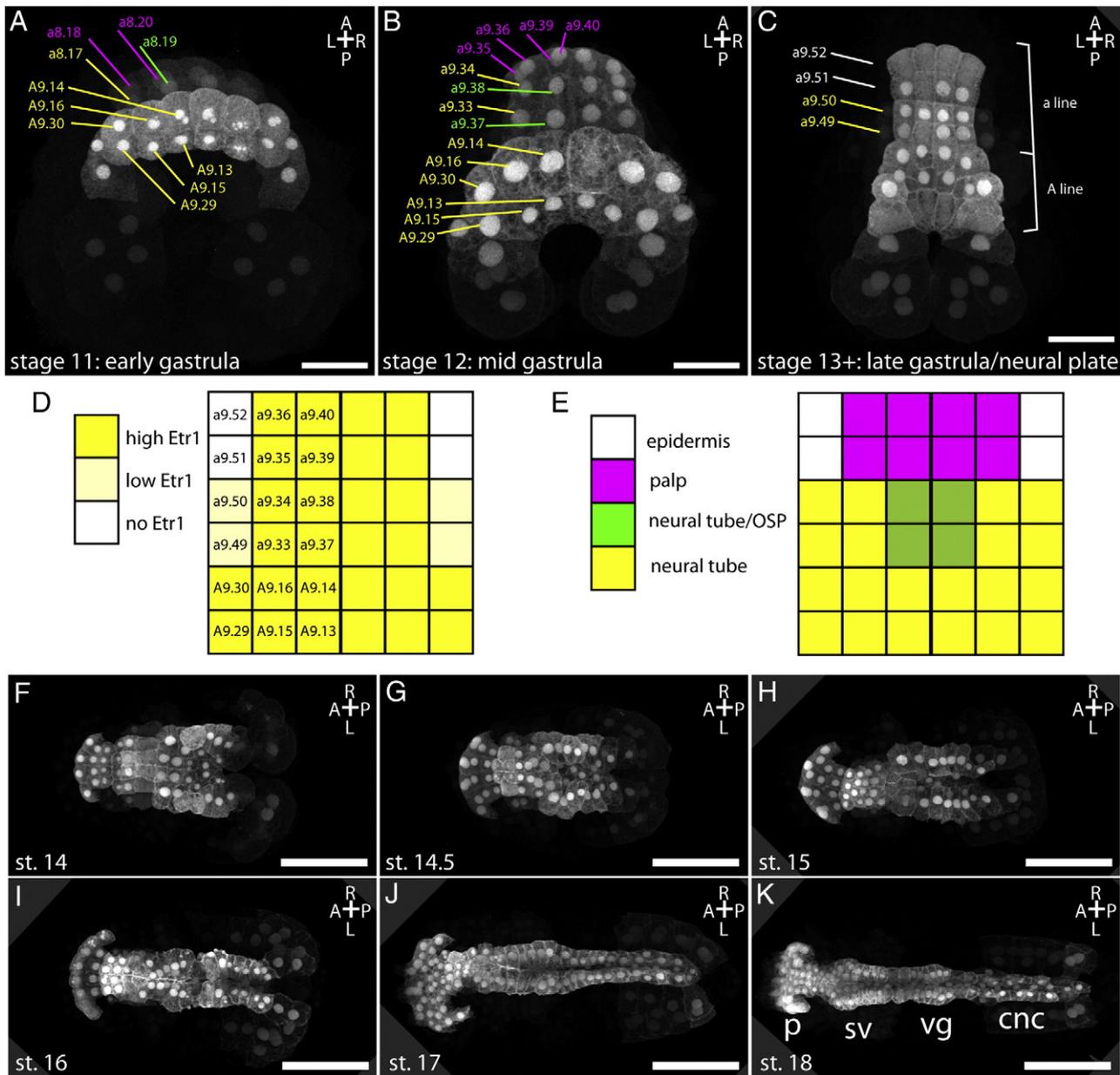
Fig. 2. Early expression of *Etr1:Venus*. (A–C) Maximum intensity projections of confocal stacks through fixed, cleared embryos at the indicated stages. Dorsal view with anterior to the top. *Etr1:Venus* expressing blastomeres are labeled using the system of Conklin (1905). Only the left side blastomeres are labeled. Label colors indicate the fate of each blastomere according to the fate map of Nishida (1987). Scale bar: 20 μm. (D) Summary cartoon of *Etr1* expression in the open neural plate. (E) Summary cartoon of neural plate fates according to Nishida (1987). (F–K) Maximum intensity projections of confocal stacks through fixed, cleared embryos at the indicated stages. Dorsal view with anterior to the left. In (K), the palps (p), sensory vesicle (sv), visceral ganglion (vg) and caudal nerve cord (cnc) are indicated. Scale bar: 40 μm.

progression. At the end of this period, the distinct morphology of the incipient palps, sensory vesicle, visceral ganglion and caudal nerve cord can be clearly identified (Fig. 2K). In *Halocynthia roretzi*, scattered expression in putative epidermal sensory neurons has been reported at early tailbud stages, but such staining is not apparent at these stages with the *Etr1:Venus* transgene, nor with any of the early tailbud stage *Ciona Etr1* in-situ in the ANISEED database (http://aniseed-ibdm.univ-mrs.fr/). It is also important to note that *Etr1* is expressed in all of the presumptive palp cells, and not just in the small subset of those cells that will form the six palp sensory neurons. Neural plate-derived *Etr1* staining in the palps is thus distinct from the much later and more transient expression seen in differentiating epidermal sensory neurons.

Although tangential to the goals of this study, this transgene also helps to resolve a controversy between two ascidian fate maps. One fate map, derived by labeling individual blastomeres in *H. roretzi* by HRP injection, shows the anteriormost neural plate cells forming palps (Nishida, 1987), whereas another, derived by serially sectioning many *Ciona* embryos, has the anteriormost neural plate forming neurohypophysis (Nicol and Meinertzhagen, 1988b). The *Etr1:Venus* transgene suggests that the anteriormost neural plate cells form palps, not neurohypophysis. In support of this, DiI labeling in *C. intestinalis* shows that the anterior neural plate precursor a6.5 contributes to palps as well as OSP and anterior sensory vesicle (Deschet and Smith, 2004). This indicates that the *Halocynthia* neural fate map generally appears to be valid for *Ciona*, and that later details of the *Ciona* neural lineages may need to be revisited.

Imaging neuropore closure and OSP formation

To better relate the *Etr1:Venus* expression pattern to tissue- and cell-level morphology, we imaged the transgene together with phallacidin to label the actin cytoskeleton and Draq5 to label cell nuclei.

The anterior neuropore is still open at Hotta stage 18.5 (large blue arrowhead in Fig. 3A) and there is no tissue separation at the anterior between the deep *Etr1:Venus* positive cells of the neural tube and the *Etr1:Venus* positive cells on the surface ectoderm that give rise to the palps and part of the OSP.

The neuropore then closes but does not lose its tubular topology as revealed by the concentration of filamentous actin on its lumenal surfaces at Hotta stage 24 (Fig. 3B; follow between the small blue arrowhead in the lumen of the sensory vesicle to the large blue arrowhead at the neuropore). At this stage, the superficial *Etr1:Venus* positive cells have segregated into two domains: an anteriormost domain which is already beginning to differentiate into the columnar palps (indicated by the red asterisk), and a slightly more caudal domain which remains continuous with the anteroventral neural tube.

By Hotta stage 25, the palps are further elongated and the remaining superficial *Etr1:Venus* positive cells have largely been internalized while preserving the topology and topography of the neural tube lumen, which continues to exit the body at the precise boundary of *Etr1:Venus* expression (Fig. 3C). The anteriormost *Etr1: Venus* positive cells that are contiguous with the definitive neural tube
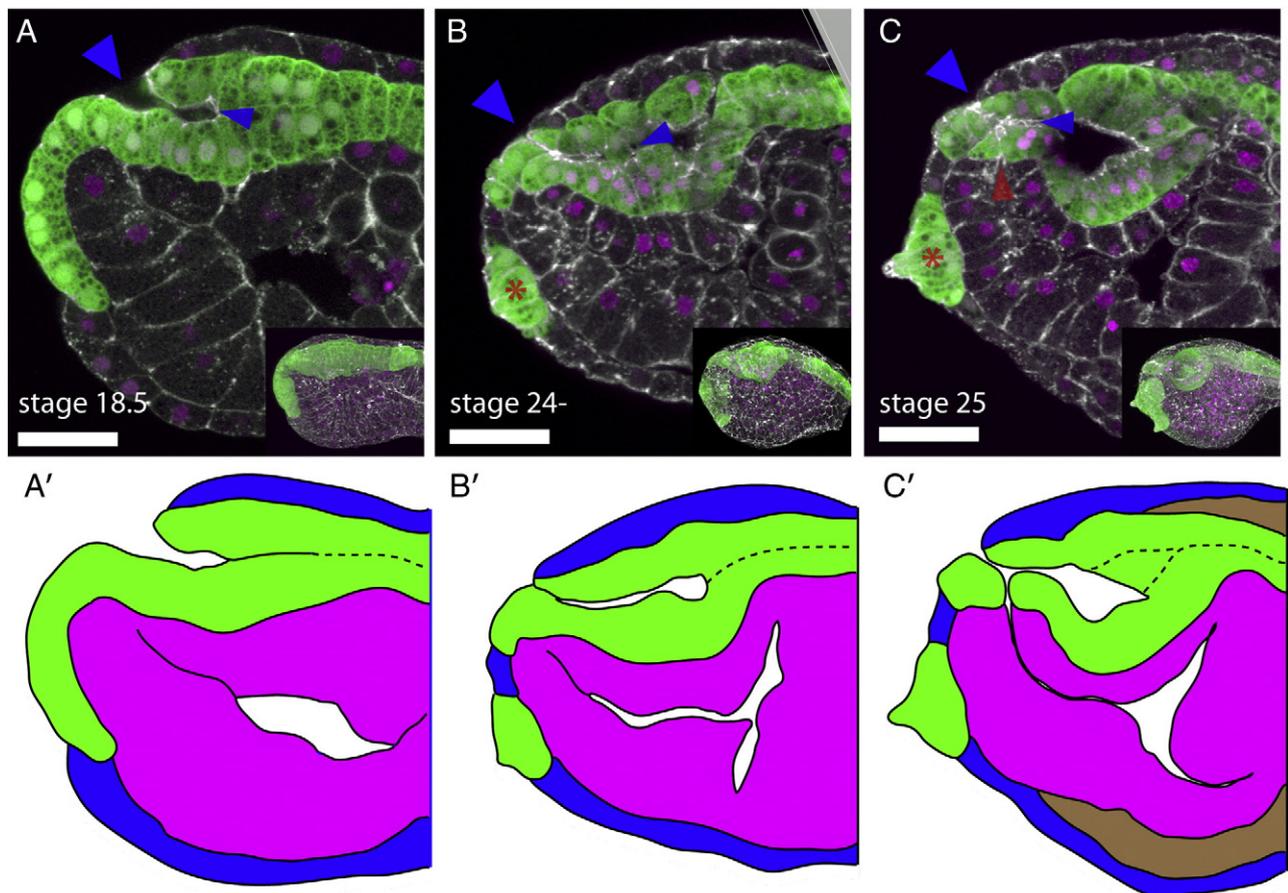
Fig. 3. Confocal imaging of *Ciona* oral siphon primordium development. (A–C) Confocal images of fixed and cleared *Ciona savignyi* stained for *Etr1:Venus* (green), actin (phallacidin; white) and nuclei (Draq5; magenta). Scale bar: 20 μm. Lateral view with anterior to the left and dorsal to the top. Main image shows a thin section near the dorsoventral midline. Inset (bottom right corner) shows a projection of the entire 3D stack. Large blue arrowhead marks the neuropore/stomodeum. Small blue arrowhead marks the inside of the neural tube lumen. Red arrowhead marks the forming junction between gut lumen and oral siphon primordium. Red asterisk marks adhesive palp. Stages: (A) Hotta stage 18.5 (early tailbud); (B) Hotta stage 24; (C) Hotta stage 25. (A′–C′) Cartoon views of A–C showing neural plate derivatives (green), endoderm (magenta), epidermis (blue) and mesenchyme (brown).

now form a beak-like protrusion from the dorsoanterior sensory vesicle, and a new connection can be seen centered in this protrusion connecting the neural tube lumen with the forming lumen of the gut endoderm (red arrowhead in Fig. 3C).

In Fig. 4 we show several oblique slices derived from reslicing a confocal volume of a stage 26 embryo along several anatomically important planes. Fig. 4A shows a lateral (sagittal) view, with the neuropore marked with a red arrowhead on the schematic version in Fig. 4A′. A distinct, well-formed three-way junction (small red asterisk) can be seen connecting the neural tube lumen (large red asterisk), the gut lumen, and the outside of the embryo. Fig. 4B shows a dorsal view slightly superior to the three-way junction. In this view, a rosette of Etr1:Venus expressing cells is seen just anterior to but contiguous with the sensory vesicle. Heavy phalloidin staining at the center of the rosette surrounds the narrow neuropore lumen. Rosette morphology just anterior to the sensory vesicle indicates that this
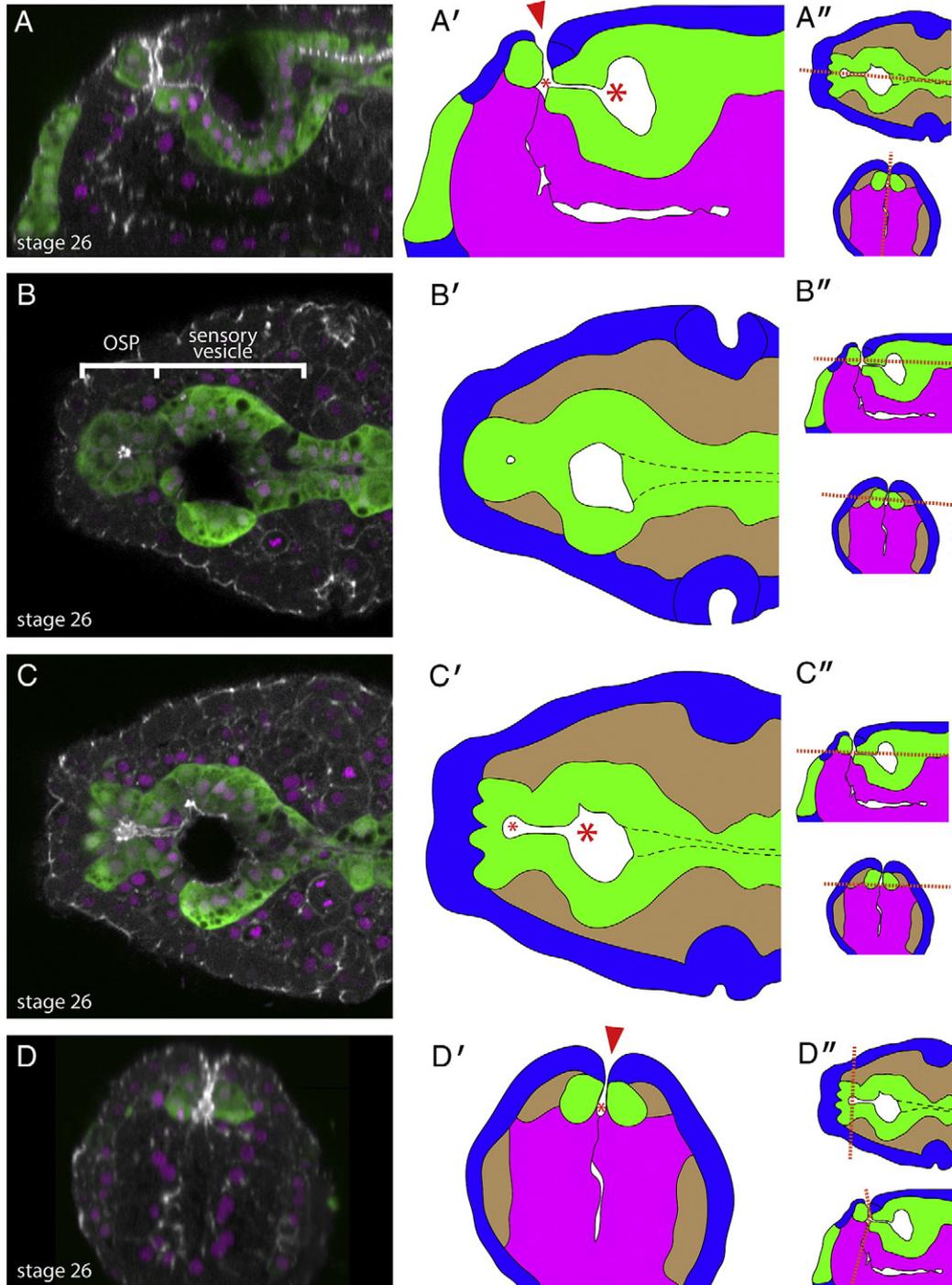
Fig. 4. Morphology of the ascidian oral siphon primordium. (A–D) Oblique sections derived from a single confocal volume of a Hotta stage 26 *Ciona savignyi* embryo stained for *Etr1: Venus* (green), actin (phallacidin; white) and nuclei (Draq5; magenta). The confocal stack was acquired with the optical axis roughly parallel to the dorsoventral axis, so the dorsal views are higher resolution than the lateral and cross-section views. (A) Lateral view through the dorsoventral midline (same view as in Fig. 3.). (B) Dorsal view through the center of the neural tube. Anterior is to the left. (C) Dorsal view, slightly deeper than (A) to show the neurohypophyseal duct. (D) Cross-sectional view through the oral siphon primordium (composite view of two sections to better follow the lumen as shown in Fig 4D″). (A′–D′) Cartoon views of (A–D) showing *Etr1:Venus* positive neural derivatives (green), epidermis (blue), trunk mesenchyme (brown) and endoderm (magenta). The small red asterisk is at the center of the OSP where it forms a three-way junction, the large red asterisk is in the sensory vesicle lumen, and the red arrowhead marks where the neuropore/OSP opens to the embryonic surface.

structure is also the OSP. Fig. 4C shows a similar dorsal view at a slightly deeper dorsoventral location, where concentrated actin staining defines the ND as a thin canal connecting the OSP/neuropore lumen (small red asterisk) with the sensory vesicle lumen (large red asterisk). Fig. 4D is a cross-section at the level of the OSP that shows the connection to the gut lumen.

At stage 26 the palps are extremely elongated, and Etr1:Venus expression can now be seen laterally in several forming pairs of epidermal sensory neurons (Fig. 5A). At the topmost level of the OSP, only the anterior half of the rosette is Etr1 positive (Fig. 5B). Fig. 5C again shows the top layer of the OSP being bisected by Etr1:Venus expression, but in a live embryo in which the OSP has an unmistakable rosette morphology by DIC. The split expression of Etr1 in the top layer of the OSP is presumably because neurulation movements have brought Etr1 negative epidermal cells to the midline posterior to the anterior neuropore. This suggests that the OSP is a hybrid structure incorporating not just neural plate cells, but also a small number of epidermal cells.

The neuropore could be easily distinguished at all stages, both as the posterior edge of the Etr1:Venus expressing cells that remained on the surface, and also by actin morphology. The anteriormost ventral neural tube remains epithelially contiguous with the surface ecto-derm anterior to the neuropore at all stages. At no stage was there any invagination of dorsoanterior epidermis independent of the neuro-pore. There does appear to be a small invagination as the OSP adopts its placode-like morphology, as seen by the internalization of the superficial Etr1 positive OSP cells (Figs. 4B, 4C, 5A), but this invagination is centered on the neuropore and preserves its topology. We conclude that the historical model is incorrect and that instead of being an independent invagination of surface ectoderm, the OSP develops from the anterior neuropore. The neurohypophyseal duct is not a novel topological connection that forms after hatching but the remnant of neural tube lumen anterior to the sensory vesicle.

Live imaging of Etr1:Venus

We also confirmed key aspects of anterior palp/stomodeal/neural morphogenesis by timelapse imaging in live embryos. Figs. 6A–E show a dorsal view of Etr1:Venus expression over 3 h following neural tube closure. The presumptive palp and stomodeal territories of Etr1 expression are initially contiguous but then pinch apart to form two distinct domains (green arrowheads). This appears to be driven by the stomodeal domain rounding up to adopt a more placodal morphology.

See Supplemental Movie 2 for the full image sequence. Figs. 6F–I shows a lateral view of another embryo that starts roughly where the previous timelapse finishes. The palp and stomodeal domains of Etr1 expression are initially adjacent to one another, but gradually move apart (red and blue arrowheads). This separation appears to be driven by both the columnarization of the palps and the invagination of the OSP. See Supplemental Movie 3 for the full image sequence. After hatching, the palps and stomodeum are further separated by the elongation of the trunk (not shown). It is clear from these timelapses that although the palps and OSP end up at a considerable distance from one another, they originate from an initially contiguous, Etr1-expressing neural plate.

A new model for ascidian OSP morphogenesis

We summarize these findings in Fig. 7. Before neurulation, Etr1 expression marks the broadly-defined neural plate on the dorsal midline (Fig. 7A). After neurulation, the posterior two thirds of the neural plate has rolled inwards to form the neural tube, leaving Etr1 expressing cells on the surface anterior to the neuropore (Fig. 7B). These superficial Etr1 positive cells pinch off into two domains: an anterior domain that will form the palps and a posterior domain that will contribute to the OSP (Fig. 7C). These two domains are then pulled apart as the palp precursors become columnar and the more posterior Etr1 positive superficial cells begin to roll into the neuropore (Fig. 7D). The definitive OSP is formed through a relatively subtle invagination that internalizes the superficial Etr1 positive non-palp cells and a small number of epidermal cells (Fig. 7E). This invagination is centered on the neuropore, and preserves its topological connection to the neural tube lumen. At this point, an incipient connection can be seen with the gut lumen. There are no clear-cut tissue boundaries between the cells forming the sensory vesicle, ND and OSP, and it is not clear precisely where the boundary is between cells that were internalized during neurulation and cells internalized during OSP morphogenesis. It is clear, however, that the OSP is not an independent epidermal invagination but rather develops from the anterior neuropore and is topologically continuous with the neural tube lumen from its earliest stages.

Early fate map of the Xenopus mouth

This topological relationship between neural tube closure and mouth formation was sufficiently unexpected that we wished to
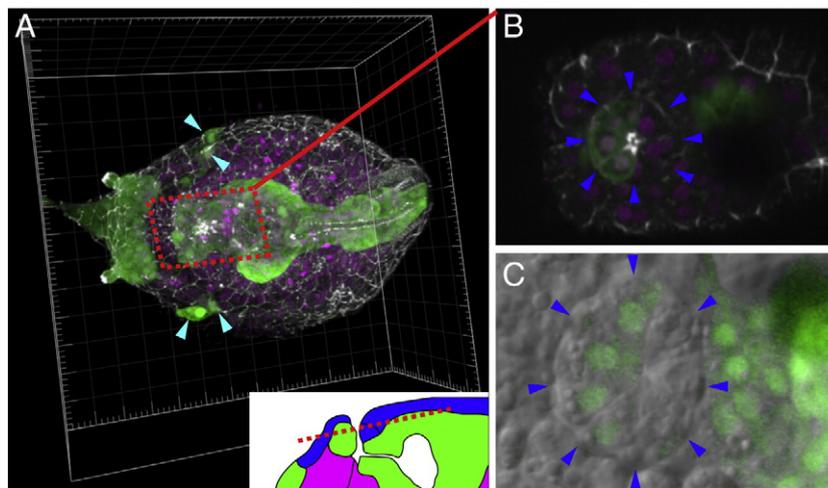
Fig. 5. Etr1 expression bisects the top layer of the OSP. (A) Volume rendering of stage 26 embryo. Dorsal view with anterior to the left. Etr1:Venus expression in forming epidermal sensory neurons is indicated with cyan arrowheads. The dashed red box and insert indicate the position of the oblique section in (B). (B) Oblique section tangential to the top layer of the OSP. The OSP is highlighted with blue arrowheads and Etr1 is only expressed in the anterior half of the rosette. (C) A comparable section from a living, late-tail stage embryo. Etr1: Venus fluorescence (green) is overlayed on a DIC image of the OSP rosette (highlighted with blue arrowheads).
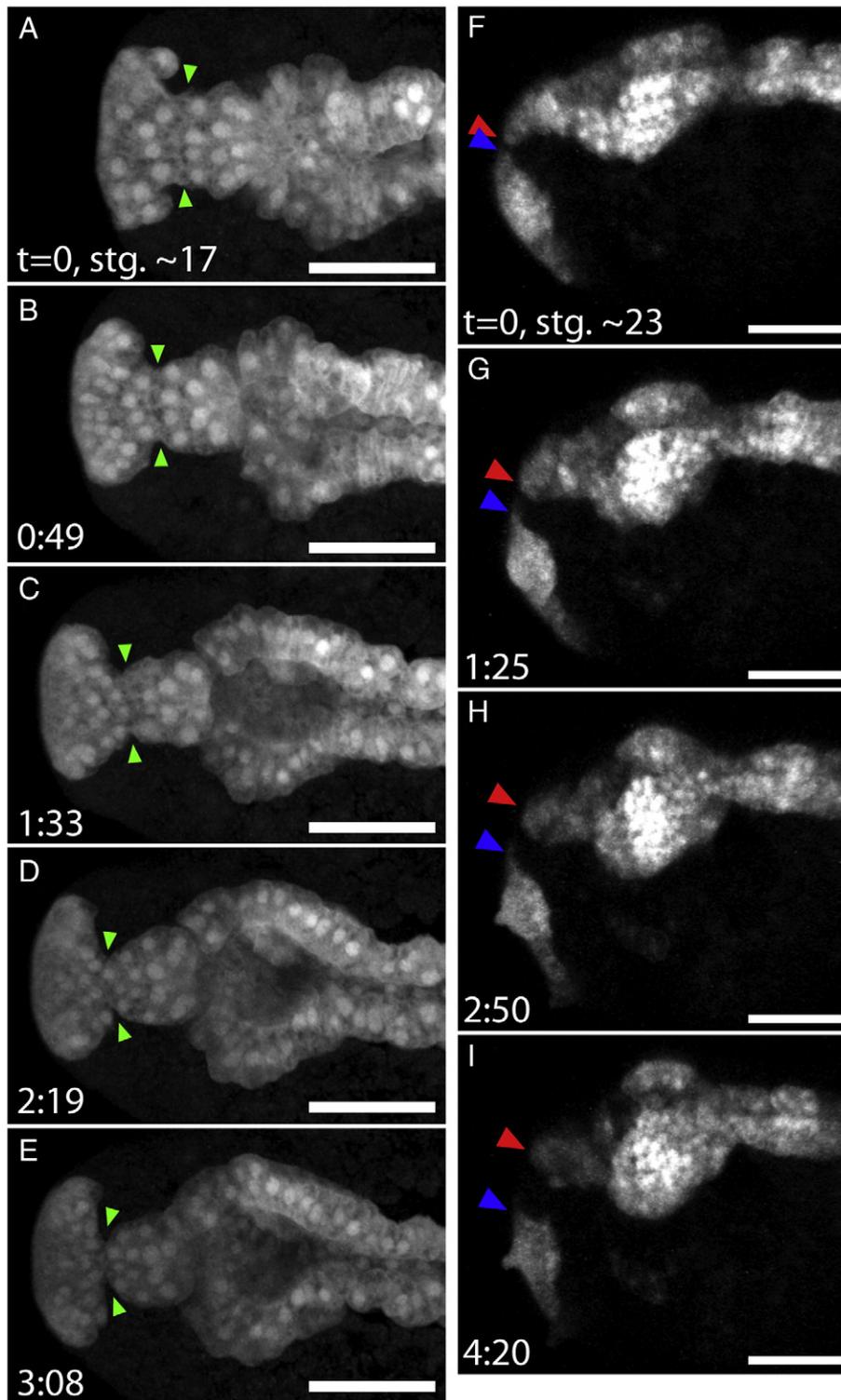
Fig. 6. Live imaging of *Etr1:Venus*. Confocal timelapses of *Etr1:Venus* in *C. savignyi*. Images shown are 3D volume renderings at the specified timepoints. Scale bars: 40 μm. (A–E) Dorsal view, anterior to the left. Green arrowheads indicate the site of separation between palp and OSP. Starts at approximately Hotta stage 17. (F–I) A different embryo. Lateral view, anterior to the left. Blue arrowhead indicates the posterior margin of the palps. Red arrowhead indicates the anterior edge of the OSP. Starts at approximately Hotta stage 23.

test whether a similar relationship might exist in vertebrate embryos. Adenohypophyseal precursors, which may represent a subset of the mouth field, have been mapped to the anterior neural boundary in several vertebrates (Couly and Le Douarin, 1985; Eagleson et al., 1995; Kawamura and Kikuyama, 1992; Kawamura et al., 2002), but the anterior neural boundary is a poorly defined region that has not been precisely related to the morphology of neural tube closure. In *Xenopus*, the primary mouth is thought to

form from a Stomodeal-Hypophyseal Anlage (SHA) apparent after neurulation between the neural tube and the cement gland (Nieuwkoop and Faber, 1994). Only recently, however, has the *Xenopus* mouth primordium actually been fate-mapped. Dickinson and Sive show that the mouth primordium is just dorsal to the cement gland at stage 24 (Dickinson and Sive, 2006) but this is approximately 5 h later than neural tube closure at stage 19/20 and the neuropore is no longer obvious.

Sagittal Section View   **Dorsal Surface View**

A

B

C

D

E
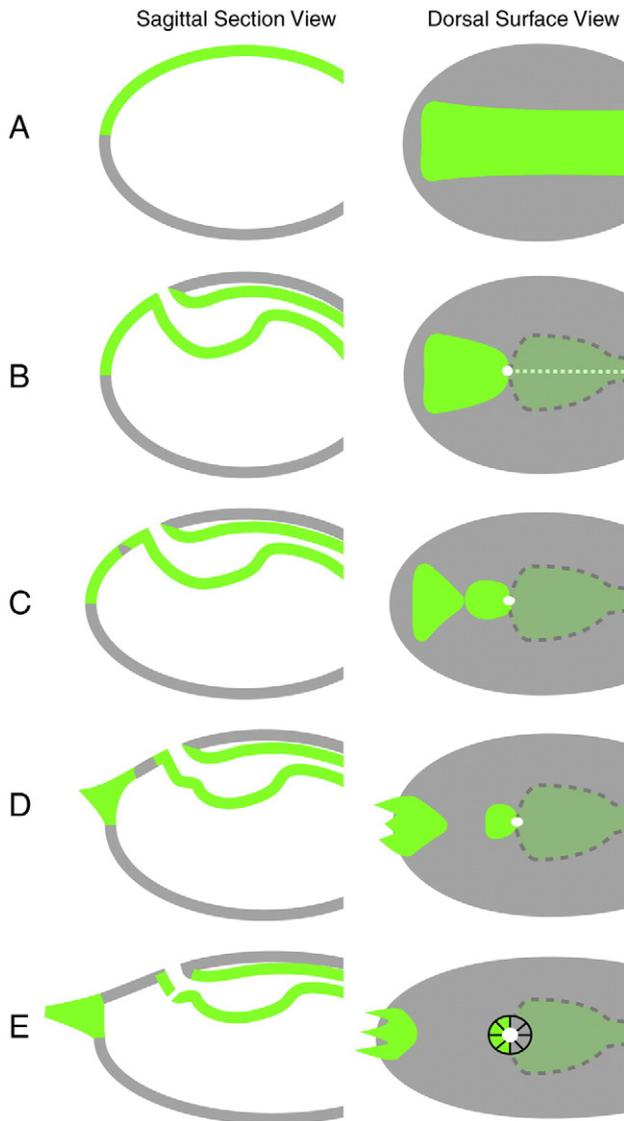
Fig. 7. Summary of *Ciona* neural/OSP/palp morphogenesis. Cartoon sketches (not to scale) of key stages in anterior neural plate morphogenesis, with the *Etr1*-expressing neural plate and its derivatives in green and epidermis in gray. (A) Open neural plate. (B) After neurulation, the posterior two thirds of the neural plate has formed the neural tube, and the neuropore is evident as the posterior boundary of superficial *Etr1* expression. (C) The superficial field of *Etr1* expressing cells pinches into two domains: an anterior domain that will form the palps and a posterior domain that will contribute to the OSP. (D) The palp and OSP domains are pulled apart as the palps become columnar and OSP morphogenesis begins. Non-*Etr1* expressing cells move in from more lateral positions to fill the resulting gap. (E) The definitive OSP is formed by a small invagination that is centered on the neuropore and preserves the topological connection between the neuropore and the sensory vesicle lumen. This invagination internalizes the modest number of *Etr1* expressing non-palp cells on the embryonic surface just anterior to the neuropore, as well as approximately 4 non-*Etr1* expressing epidermal cells just posterior to the neuropore. The OSP appears to be a hybrid structure that includes *Etr1* expressing cells from the anteriormost neural tube, *Etr1* expressing superficial cells from just in front of the neuropore, and a few epidermal cells from just behind the neuropore. A connection forms between the base of the OSP and the forming gut lumen.

As a starting point, we captured 3D timelapse images of *Xenopus* neurulation with the embryo oriented to give an anterior view. Extended-depth-of-field images were then reconstructed from the 3D stacks. (Figs. 8A–H). These recordings show that the anterior neuropore is quite close to the site of presumptive stomodeal invagination. It is not, however, perfectly congruent, with the neuropore typically closing approximately 5 cell diameters more dorsal [compare just-closed neuropore (blue arrowhead) with the dorsal margin of the cement gland (white arrowhead) in Fig. 8E]. It is

also clear from these recordings that the inverted Y-shape of the hatching gland forms independently of neural tube closure and does not reflect a prominent backwards-folding anterior neural lip as proposed by Drysdale and Elinson (1991).

Xenopus embryos are opaque, so these timelapse images were only able to capture the behavior of the most superficial cells. We also performed fate mapping experiments with the lineage tracer DiI to unambiguously locate the mouth precursors at the time of neural tube closure. We labeled two small, adjacent locations on the dorsoventral midline at stage 19: either just anterior to the neuropore (Fig. 8I), or else just posterior to the cement gland (Fig. 8J). Clones labeled just anterior of the neuropore only contributed to the anterior brain (Fig. 8I′), whereas clones labeled at the boundary of the cement gland contributed to cement gland, anterior brain and presumptive mouth primordium (Fig. 8J′).

To determine the location of these labeling sites with respect to the topology and topography of the forming neural tube, we performed whole mount imaging of embryos labeled with an antibody against laminin to visualize tissue boundaries, and Draq5 to counterstain for nuclei. The dorsal boundary of the presumptive cement gland can be recognized in cross-section by the prominent inflection point it forms with the bulge of the neural tube (Hausen and Riebesell, 1991). At stage 18/19, this boundary corresponds precisely with a small wedge-shaped protrusion from the dorsoanterior neural tube (white arrowhead in Fig. 8K). This represents the site of topological transition between the cement gland ectoderm on the embryonic surface and the subsurface, tubular topology of the forming neural tube. It is important to note that, unlike later when the presumptive SHA fills the gap between the neural tube and the cement gland (Fig. 8L), at the time of neural tube closure the anterior neural tube and cement gland are directly juxtaposed and there is no such gap (Fig. 8K). Thus, although the *Xenopus* mouth primordium is not topologically continuous with the neural tube lumen as in *Ciona*, it has its embryonic origins, as in *Ciona*, at the anterior transition between neural tube and surface ectoderm.

Discussion

We have shown here that *Ciona* embryos use the anterior neuropore to form their mouth opening. This is a novel mechanism for mouth formation, and a significant revision to the ascidian body plan. This mouth opening/neuropore relationship was only evident through the three-dimensional imaging of a neural plate-specific transgene together with probes for cell morphology. It is important to note that although there are compelling reasons to consider the OSP and palps as part of the neural plate, our observation that the OSP opening is derived from the neuropore opening is in no way contingent on this definition. It is also important to note that the slight invagination at the neuropore during OSP morphogenesis indicates that the neuropore is a dynamic structure. The continuity of the neural tube lumen to the outside of the embryo is preserved, but the cells forming the neuropore change as these cells are internalized into the OSP. It will be interesting in the future to determine the precise boundary between cells internalized during neurulation and cells internalized during OSP morphogenesis, and to determine when the topological connections between OSP, neural tube and gut become open to the passage of fluids.

Comparing the mouth/neural tube relationship between divergent taxa

Although the *Ciona* mouth primordium differs from vertebrates in being topologically continuous with the neural tube lumen, the mouth primordia in both *Ciona* and *Xenopus* are derived from remarkably similar locations with respect to neural tube morphogenesis. The mechanics of folding an epithelial sheet into a chordate neural tube imply that there must be at least a transient stage where the
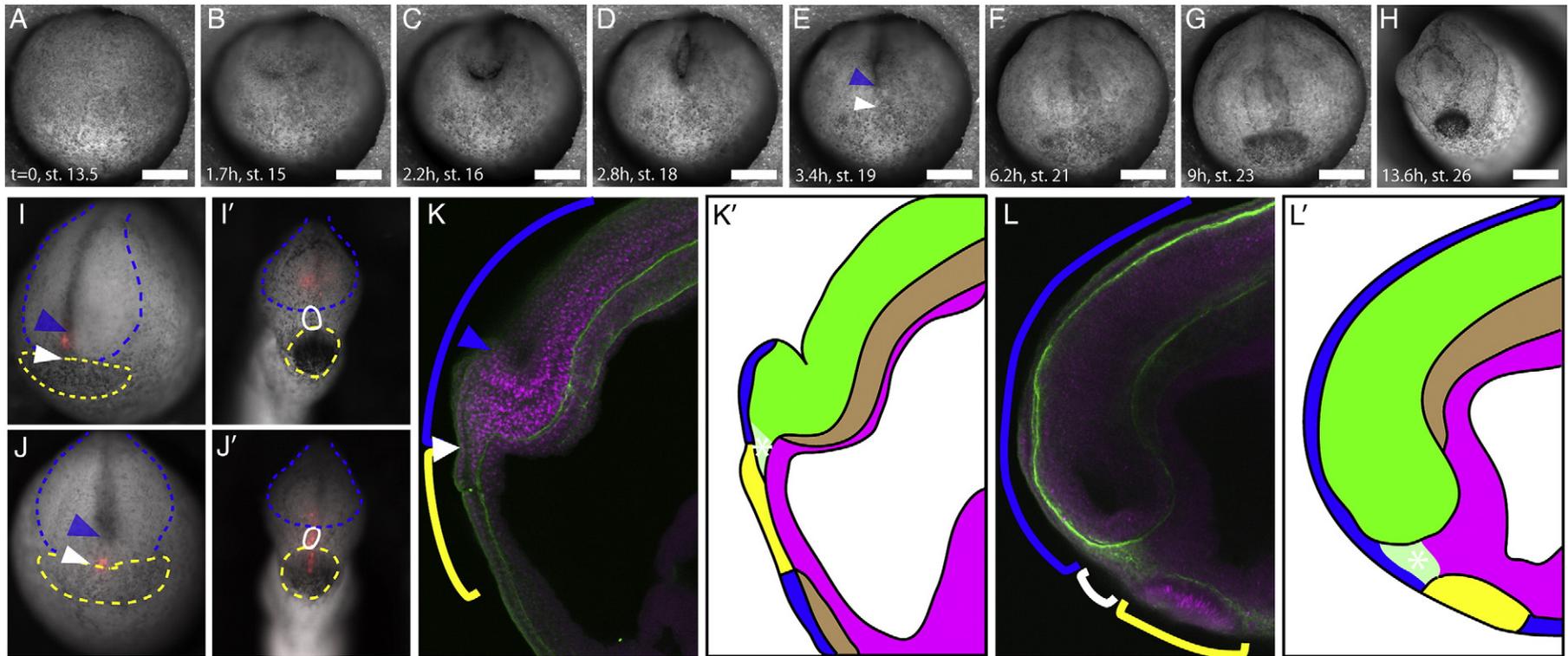
Fig. 8. Early fate mapping of the *Xenopus* mouth. (A–H) Selected timepoints from a timelapse of *Xenopus* neural tube closure. The embryo is oriented in a modeling clay well with anterior facing the camera and dorsal to the top. Time and approximate Nieuwkoop and Faber stages are indicated. Scale bar: 300 μM. In (E), the blue arrowhead indicates the anterior neuropore and the white arrowhead the dorsal border of the forming cement gland. (I–L) Small groups of cells were labeled with DiI as the neuropore was closing, either (I) just ventral to the neuropore (neuropore indicated with blue arrowhead) or (J) just dorsal to the cement gland (boundary indicated with white arrowhead). The neural tube is outlined with a blue dashed line and the cement gland with a yellow dashed line. (I′, J′) The same embryos imaged again at stage 28. The white circle indicates where the mouth will form. (K, L) Wholemount confocal imaging of fixed, cleared *Xenopus* embryos stained for laminin (green) and nuclei (magenta). (K) Stage 18/19. (L) Stage 27/28. The blue arrowhead marks the anterior neuropore. The white arrowhead marks the dorsal boundary of the cement gland. The yellow bracket marks the cement gland. The blue bracket marks the neural tube. The white bracket marks the site of mouth formation. (K′, L′) Schematic cartoons of K and L showing the neural tube (bright green), mouth primordium (pale green, also marked with a white asterisk), epidermis (blue), endoderm (magenta) and mesoderm (brown).

anteriormost cells of the forming neural tube remain epithelially contiguous with the surface midline cells just in front of the neural tube. This anterior transition zone between the deep neural tube and the surface ectoderm may coincide with the site of neuropore closure, as in *Ciona*, or may be anterior to it, as in *Xenopus*. In both cases, however, we find that it is this transition zone that is the precise site of mouth formation (schematized for a generic chordate in Figs. 9A, A′, B, B′). While ascidian and vertebrate mouths have been widely recognized as being derived from the anterior neural boundary, this is the first demonstration of them being derived specifically from the anterior midline transition between neurectoderm and ectoderm, as opposed to the surface ectoderm anterior to the neural tube more generally.

Although the chordate mouth is frequently described as being a ventral feature, it is clear that the vertebrate and ascidian mouths are actually dorsoanterior in their embryonic origin (Christiaen et al., 2007; Couly and Le Douarin, 1985; Dickinson and Sive, 2006). In vertebrates, the mouth is moved ventrally by cephalic flexure and the massive growth of the brain, whereas in ascidians it remains dorsal (Christiaen et al., 2007; Dickinson and Sive, 2007). As discussed by Nielsen (1999), this indicates that the dorsoventral inversion hypothesis (Arendt and Nubler-Jung, 1994; De Robertis and Sasai, 1996; Garstang, 1894; Saint-Hilaire, 1822) does not necessarily imply a radical relocalization of the chordate mouth from dorsal to ventral. Many protostome mouths pass through the CNS, suggesting that the palps and cement gland, which are likely homologous (Sive and Bradley, 1996), may represent the remnants of a ventral, pre-inversion neurogenic region anterior to the ancestral ventral mouth opening. In this view, dorsoventral inversion would not require any relocation of the chordate mouth other than a small anterior shift with respect to the molecular map (Arendt et al., 2008; Arendt and Nubler-Jung, 1999) conserved between protostome and deuterostome brains.

Although the cephalochordates (amphioxus) are basal within the chordates, the cephalochordate mouth is unusual among the chordates in that it originates on the left flank, possibly from a modified gill slit, and then migrates ventrally (Whittaker, 1997). This is likely a derived feature to accommodate the peculiar rostral extension of the notochord in cephalochordates (Ruppert, 2005). With respect to mouth development, amphioxus is thus not a good model for the ancestral chordate.

In the hemichordates, which are potentially the least derived of the non-chordate deuterostomes (Gerhart et al., 2005), there is a ventral mouth and both dorsal and ventral nerve cords (Nomaksteinsky et al., 2009). A small section of the dorsal nerve cord passing through the collar is internalized by movements resembling chordate neurulation, though opinions are mixed as to whether this is a true homology (Gerhart et al., 2005; Nomaksteinsky et al., 2009). Interestingly, although *Pitx* is not expressed in the ventral mouth, it is expressed in the proboscis pore immediately anterior to the collar nerve cord (Lowe et al., 2006). It is currently unclear, however, whether the chordate CNS is homologous to the hemichordate dorsal nerve cord, ventral nerve cord, or both; it is equally unclear whether the chordate mouth is homologous to the hemichordate mouth, proboscis pore, or neither. It is thus difficult to meaningfully compare the relative positions of mouth and brain between hemichordates and chordates.

In hagfish and lampreys, the most basal vertebrates, there is a single midline nasal opening just dorsal to the mouth that develops from a so-called nasohypophyseal placode. The mouth and nasal aperture develop from a common domain of *Pitx* expression (Uchida et al., 2003). It would be valuable to determine the site of neuropore closure with respect to the developing cyclostome oral/nasal apparatus.

Alternate models to account for the juxtaposition of anterior neural tube with the mouth primordium

With the anterior neuropore at the mouth and the posterior neuropore at the blastopore, neurulation bears a surprising resemblance to gastrulation through a slit blastopore. This resemblance has typically been used largely as a helpful simile to explain the Amphistomy model (Arendt and Nubler-Jung, 1997), but it is also reminiscent of Delsman's generally discounted theory that the neural tube was derived from an ancestral gut (Delsman, 1922). The idea that neurulation might represent a duplication or recapitulation of gut formation is only an extreme interpretation, however, of the increasingly plausible idea that the hollow dorsal neural tube and the mouth are connected in their development and evolution.

Although the mouth develops from the anterior neural boundary in Olfactores, it is unclear whether that is the ancestral condition or whether neurulation first evolved posterior to the mouth. In the latter scenario, it is possible that the neural tube first evolved independent of the mouth, with the mouth eventually acting as a structural barrier to the anterior expansion of the brain. The ascidian mouth developing from the actual neuropore might then be a consequence of the simplification and miniaturization of the ascidian tadpole larvae.

Alternatively, however, ascidian mouth formation may reflect the ancestral state, suggesting that the chordate CNS might have evolved its tubular topology as an adjunct structure to the mouth. For a putative chordate ancestor with a ventral mouth and a ventrally-centralized but non-tubular nervous system, a small groove or pouch continuous with the posterior aspect of the mouth could have acted as a sensory structure (Fig. 9C). This would then have expanded over time to encompass the whole neurogenic region posterior to the mouth, with broader neural functions coming to dominate the original sensory function. This model would imply a heterochronic
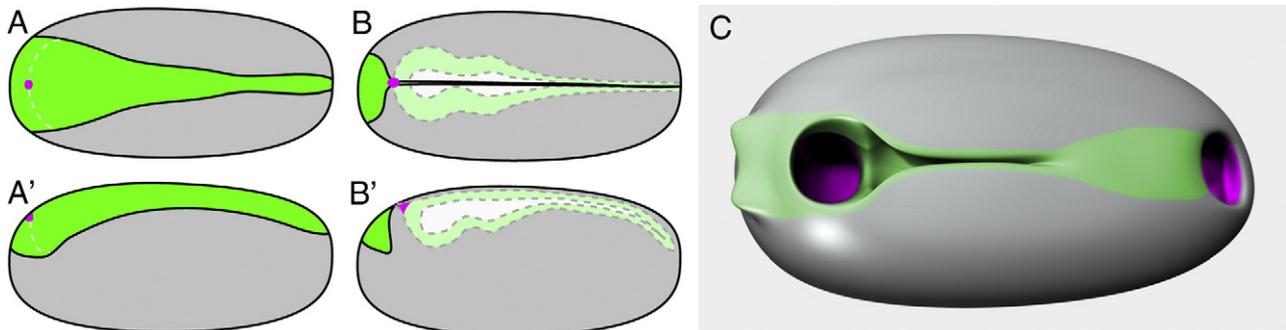
Fig. 9. Model of the mouth/neural tube relationship for a generic chordate and chordate ancestor. (A, B) Schematic representation based on our observations in *Ciona* and *Xenopus* of a generic chordate both before (A, A′) and after (B, B′) neurulation. The neural plate is marked in green and the presumptive site of mouth formation is shown in magenta. Anterior is to the left. (A, B) Dorsal views. (A′,B′) Lateral views. Note that the mouth primordium arises at the precise topological transition between the definitive neural tube (internalized by neurulation) and the anterior adhesive organ (cement gland/palps), which remains on the surface. (C) Schematic view of the ventral side of a hypothetical pre-inversion chordate ancestor. The mouth (left) and anus (right) are shown in magenta. The ventral neurogenic region (green) is partly folded to form an accessory pocket of the mouth.

shift between our proposed ancestor, where the movements of neurulation would have first appeared at a stage where the mouth had already formed, versus modern chordates where neurulation precedes mouth formation. The idea that the tubular CNS may have originated as a sensory fold or pocket of the mouth is speculative, but is compelling in that it suggests not just a series of developmentally plausible intermediates but also the selective pressures connecting them.

Implications for the evolution of the pituitary

Although we have emphasized here the relevance to mouth and CNS evolution, our revised model of the early morphogenesis of the ascidian OSP and ND also has potential implications for the evolution of the pituitary. At larval stages, the ND connects the OSP and the neural tube lumen, whereas at adult stages, a ciliated funnel and duct connect the wall of the mouth opening to a poorly understood organ called the neural gland (Ruppert, 1990). The ND has typically been assumed to give rise to the ciliated duct, but this has never been conclusively tested by timelapse imaging or fate mapping. The origin of the neural gland is also unclear. It has been suggested to arise from the ND itself (Manni et al., 2005), or else from a poorly defined primordium posterior to the sensory vesicle (Joly et al., 2007), but there is little data to support either case.

Both the ND and the neural gland have been widely discussed as potential homologs to the vertebrate pituitary, but the details remain extremely contentious (Boorman and Shimeld, 2002; Gorbman, 1995; Joly et al., 2007; Manni et al., 2005). These discussions typically assume that the ND represents a secondary connection between the stomodeum and the anterior neural tube, much like how Rathke's pouch contacts the forming infundibulum. Here, however, we have shown that the ND is not a secondary connection but rather seems to represent the anteriormost neural tube lumen itself. It is thus derived from a similar anatomical location to the neurohypophysis, but has a much different topology. The ND remains a fascinating structure, but the argument for pituitary homology based on comparative embryology is now less straightforward. It is important not to overinterpret the expression patterns of genes such as *Pitx* and *Six3* that are expressed in both mouth and pituitary precursors. We concur with Joly et al. (2007) that it is premature to assign any vertebrate homology to the ascidian ND. Future progress on this subject will require proper fate mapping of the ciliated duct/neural gland complex.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.04.028.

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