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De novo neurogenesis in a budding chordate: Co-option of larval anteroposterior patterning genes in a transitory neurogenic organ

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ABSTRACT

During metamorphosis of solitary ascidians, part of the larval tubular nervous system is recruited to form the adult central nervous system (CNS) through neural stem-like cells called ependymal cells. The anteroposterior (AP) gene expression patterning of the larval CNS regionalize the distribution of the ependymal cells, which contains the positional information of the neurons of the adult nervous system.

In colonial ascidians, the CNS of asexually developed zooids has the same morphology of the one of the post-metamorphic zooids. However, its development follows a completely different organogenesis that lacks embryogenesis, a larval phase and metamorphosis.

In order to describe neurogenesis during asexual development (blastogenesis), we followed the expression of six CNS AP patterning genes conserved in chordates and five neural-related genes to determine neural cell identity in *Botryllus schlosseri*.

We observed that a neurogenesis occurs *de novo* on each blastogenic cycle starting from a neurogenic transitory structure, the dorsal tube. The dorsal tube partially co-opts the AP patterning of the larval CNS markers, and potentially combine the neurogenesis role and provider of positional clues for neuron patterning. This study shows how a larval developmental module is reused in a direct asexual development in order to generate the same structures.

1. Introduction

During the development of most bilaterian, the central nervous system (CNS) becomes regionalized molecularly along its dorso-ventral and anteroposterior (AP) axes. Such partitioning is defined by a patterned expression of transcription factors (TFs). Dorso-ventral regionalization has been suggested to have evolved independently (Martín-Durán et al., 2018). The AP order of gene expression is conserved even in animals with no centralized nervous system (Pani et al., 2012) and such patterning can be important for the axis of entire organisms (Holland et al., 2013).

In ascidians (Tunicata), the larval CNS is formed like in other chordates by the rolling up of a neural plate into a dorsal hollow tube, which becomes organized along the AP axis into four regions: the sensory vesicle (SV), the neck, the visceral ganglion (VG), and the tail nerve cord (Hudson, 2016). While the homology of these structures to corresponding regions in chordate CNS regionalization is still debated (Hudson, 2016; Wada et al., 1998; Ikuta and Saiga, 2007; Dufour et al., 2006), the expression of some TF genes characterizing the AP axis are conserved (Holland et al., 2013). For instance, along the AP axis, *Otx* is expressed anteriorly (Hamada et al., 2011; Moret et al., 2005), and *Pax2/5/8* and *Gli* more posteriorly (Imai et al., 2009), while several *Hox* genes are expressed even more posterior, in the VG (Locascio et al., 1999) and in the nerve cord (Ikuta et al., 2004). This order of gene expression reflects the patterns of expression of orthologous genes not only of vertebrate, but also of the cephalochordate and the hemichordate nervous systems (Holland et al., 2013).

During their biphasic life history ascidians undergo a severe remodeling of their nervous system. The swimming tadpole-like larva with the typical chordate body plan metamorphoses into a sessile filter feeding zooid (Cloney, 1982). Throughout this process it loses the larval sensory organs and tail used in locomotion. The post-metamorphic zooid substitutes the larval CNS by the adult neural complex. The neural complex (Mackie and Burighel, 2005) consists of a cerebral ganglion, which innervates the body wall (Tiozzo et al., 2008; Burighel, 1997; Osugi et al., 2017) and the neural gland (NG) (Lane, 1972; Deyts

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et al., 2006), which opens anteriorly into the branchial chamber through a ciliated funnel-shaped duct (CF) and continues posteriorly forming the dorsal strand, or dorsal organ (DO) (Terakado, 2009).

The adult ganglion is derived from cell progenitors of the transitory larval CNS. In *Ciona* only one third of the larval nervous system are neurons and the rest are glia-like cells, classified as ependymal cells (Nicol and Meinertzhagen, 1991). Most larval neurons disappear during metamorphosis, whereas the ependymal cells of the larval brain differentiate into the neurons of the adult ganglion (Horie et al., 2011). Interestingly, cell tracing experiments showed that the patterning of the larval CNS provides the positional information along the AP axis of the adult CNS neurons (Dufour et al., 2006; Horie et al., 2011; Sasakura and Hozumi, 2018; Hozumi et al., 2015). This is an example where a transitional structure, the larval CNS, serves as a scaffold to build the adult definitive structure, the adult CNS (Minelli, 2003).

In colonial species of ascidians, such as *Botryllus schlosseri*, the post-metamorphic zooid (oozooid), derived from a larva, begins a cyclic asexual budding process named blastogenesis that leads to the development of other adult zooids (blastozooids) (Manni et al., 2014). During blastogenesis all organs, including the nervous system, are created anew from pre-existing epithelia, without passing through a larval stage (Suppl. Mat. 1). Regardless their different ontogenetic origin, the overall anatomy of the CNS in oozooids and blastozooids is identical. During blastogenic neurogenesis, the neural complex originates from an epithelial thickening that evaginates from the dorsal inner vesicle of the young bud forming a tubular structure, the dorsal tube, which elongates anteriorly along the AP axes (Manni et al., 2014; Burighel et al., 1998). The dorsal tube gives rise to the neural gland and by delamination of migratory cells to the cerebral ganglion (Manni et al., 2014; Burighel et al., 1998).

To explore whether the larval AP patterning process has been co-opted during blastogenetic CNS development in *Botryllus schlosseri*, we studied the expression of six genes with highly conserved roles among chordate CNS AP patterning. To further extend comparisons with non ascidian chordates CNS patterning we included *Botryllus* orthologues of *IrxB and Pou3*, where presence or expression in ascidians is less documented (Suppl. Mat. 3) (Imai, 2004; Candiani et al., 2002) Five additional genes were chosen to determine neural fate of cells originating in the dorsal tube . The selected genes have been described in Table 1. Briefly, *Otx, Gli, IrxB, Pou3, Hox3 and Pax 3/7* have been reported to play a role during the AP patterning in many chordates (Holland et al., 2013) including in some ascidian (Wada and Satoh, 2001), whereas *Zic-r.a, Ebf, Etr, Notch* and *Pou4* have been shown to be involved in neural fate determination and neurogenesis in ascidians and other chordates .

We followed the expression dynamics of these 11 genes during blastogenetic development and observed that the dorsal tube, partially co-opt the anteroposterior patterning of the larval CNS markers, potentially providing positional clues for the establishment of the entire neural complex.

2. Materials and methods

2.1. Animal husbandry

Botryllus schlosseri colonies were raised on 50 × 70 × 1 mm glass slides as described previously (Langenbacher et al., 2015). A *Botryllus* colony consists of three coexisting generations that arise asexually: the adult filter feeding zooids, their buds, called primary buds, and their buds (secondary buds or budlets). Budding (blastogenesis) was staged according to Lauzon et al. (2002). First, a secondary bud appears as a thickening of the peribranchial epithelia and overlying epidermis of the previous generation (stages A1-A2). Second, the peribranchial evagination closes off and forms a vesicle, whereas the overlying epidermis maintains a connection to the parental zooid giving the impression of a double vesicle (stages B1-B2). Third, organogenesis begins as the inner vesicle folds into three distinct chambers: the central branchial chamber, and two lateral peribranchial chambers (stages C1-C2). While the adult zooid undergoes programmed cell death, and the primary bud takes over his place to become the next generation of zooid (stage D) and the secondary bud becomes the primary (A).

2.2. Gene identification and phylogeny

RNA sequences were retrieved by tblastn from the *Botryllus schlosseri* transcriptome database http://octopus.obs-lfr.fr/public/botryllus/blast_botryllus.php, full length sequences of the tunicate proteins are retrieved from Aniseed (https://www.aniseed.cnrs.fr/), and others from NCBI (Suppl. Material 2). Alignments were generated using MAFFT, and sequences trimmed by the TrimAl Gappyout method. Maximum likelihood trees were compiled using PhyML (Guindon et al., 2018) (Suppl. Material 3–11).

2.3. Fluorescent in situ hybridization (FISH)

Antisense mRNA probes were designed within the coding region of each gene (Supp. Material 12) FISH was carried out as previously described in Ricci et al. (2016a) with the following modifications: 1% Dextran sulfate was added to the Hybridization buffer and the revelation solution. The anti-Digoxigenin Antibody (HRP) (Roche, #11207733910) was pre-adsorbed for 1 h in hybridization solution with a mix of fixed colonies at different stages. When the tunic was exhibiting a very strong background the animals were manually removed from the tunic after rehydration, post fixed in 4% PFA for 1 h and transferred into washing baskets in 24-well plates. DIG-probe detection was performed with bench-made FITC-Tyramide by 3 h incubation. For double FISH, the hybridization of DIG labeled and Fluorescein labeled probes was performed at the same time, fluorescein probes were detected with Cy3-Tyramide.

2.4. Imaging

Confocal images were acquired using a Leica SP8 (40x/1.1 Water WD 0.6 HCX PL APO CS2) and processed with ImageJ (Rueden et al., 2017) and Inkscape.

3. Results

3.1. Early expression of neural patterning genes during blastogenesis

At the onset of blastogenesis the secondary bud forms a double vesicle, the inner vesicle starts to fold giving rise to various structures including the dorsal tube (Manni et al., 2014)(Materials and methods). The dorsal tube begins as a dorsal thickening of the epithelium of the inner vesicle (Burighel et al., 1998). In this region *Otx* and *IrxB* were expressed starting from the double vesicle stage (stage B2, Fig. 1 A, B). This expression was maintained when the primordium of the dorsal tube started to evaginate (stage C1, Fig. 1C). At this stage the first AP pattern was detected (Fig. 1 D-D′′): *Pou3* was expressed anteriorly in few cells in the forming dorsal tube (Fig. 1D), moving posteriorly *Pou3* was co-expressed with the posterior marker *Hox3* (Fig. 1D’) and more posteriorly few cells expressed only *Hox3* (Fig. 1D′′). The area of the evaginating dorsal tube co-expressed also the neural makers *Zic-r.a* and *Ebf* (Fig. 1E-G). While the domain of expression of *Zic-r.a* was broader (Fig. 1F), *Ebf* was expressed only in a set of cells at the posterior side of the *Zic-r.a* domain (Fig. 1G).

3.2. Dynamic of expression during the elongation of the dorsal tube

In the secondary bud, during stages C2 the forming dorsal tube extends anteriorly as a blind tube (Fig. 2A) to meet the inner vesicle

Table 1
List of genes analyzed in this study divided in AP patterning genes and neural markers.

	Gene / aliases	Expression in Tunicata		Expression in chordates and hemichordates NS
		Larval	Post-metamorphic	
AP patterning genes	*Otx*	*Halocynthia roretzi,* anterior CNS (Wada et al., 1998),	*Ciona* endostyle, pharynx, heart (Sasaki et al., 2003; Yoshida et al., 2017)	(Holland et al., 2013; Albuixech-Crespo et al., 2017)
		Ciona , SV & ant. ectoderm (Hudson and Lemaire, 2001),	*Oikopleura dioica anterior* endostyle (Cañestro et al., 2008),	
		Oikopleura dioica, OtxB anterior NS (Cañestro et al., 2005),	*Herdmania curvata* pharynx, CF (Hinman and Degnan, 2000),	
	Gli (zinc finger TF)	*Ciona intestinalis* neck (Imai et al., 2009; Islam et al., 2010),	*Ciona* endostyle, pharynx and heart (Ogasawara and Satoh, 1998),	(Holland et al., 2013)
	IrxB (homeobox TF)	*Ciona robusta:*Irx posterior sensory vesicle and visceral ganglion (https://www.aniseed.cnrs.fr/) (Holland et al., 2013)	NA	(Holland et al., 2013; Albuixech-Crespo et al., 2017; Rodríguez-Seguel et al., 2009)
	Pou3 (Pou class III homeobox TF)	(Albuixech-Crespo et al., 2017; Spaniol et al., 1996)		
	Hox3 (homeobox TF)	*Ciona* anterior region of the visceral ganglion (Locascio et al., 1999),	*Ciona intestinalis* posterior part of the CG (Nakayama et al., 2016),	(Holland et al., 2013; Albuixech-Crespo et al., 2017)
	Pax3/7 (paired box TF)	*Ciona* 3 patches in brain, post. neural tube (Mazet et al., 2005),	NA	(Albuixech-Crespo et al., 2017; Matsunaga et al., 2001; Miyamoto and Wada, 2013)
Neural/neural tube marker	*Zic-r.a* (zinc finger TF) Macho-1	*Ciona* in CNS with 2 gaps (Gostling and Shimeld, 2003), *Halocynthia roretzi,* not in CNS (Aruga, 2004),	NA	(Gostling and Shimeld, 2003; Aruga, 2004)
	Ebf (HLH TF) COE, Unc-3	*Ciona* CNS (Islam et al., 2010),	NA	(Albuixech-Crespo et al., 2017; Dubois and Vincent, 2001)
	Etr (ELAV-like family member) Celf/CUGBP	*Halocynthia roretzi* CNS (Akanuma et al., 2002; Yagi et al., 2001),	NA	(Knecht et al., 1993)
	Notch (transmembrane receptor)	*Halocynthia roretzi* preferentially in CNS (Akanuma et al., 2002; Hori et al., 1997)	NA	(Holland et al., 2001; Louvi and Artavanis-tsakonas, 2006)
	Pou4 (Pou class IV homeobox TF)	*Ciona* PNS and posterior sensory vesicle (Candiani et al., 2005),	*Ciona* endostyle (Mazet et al., 2005),	(Candiani et al., 2006; He et al., 1989)

The genus *Ciona*, refers to either *Ciona robusta* or *Ciona intestinalis* (Brunetti et al., 2015), if the reference dates before 2015 and no indication of the type A or B was given. NS: nervous system; SV: sensory vesicle; CF: ciliated funnel.

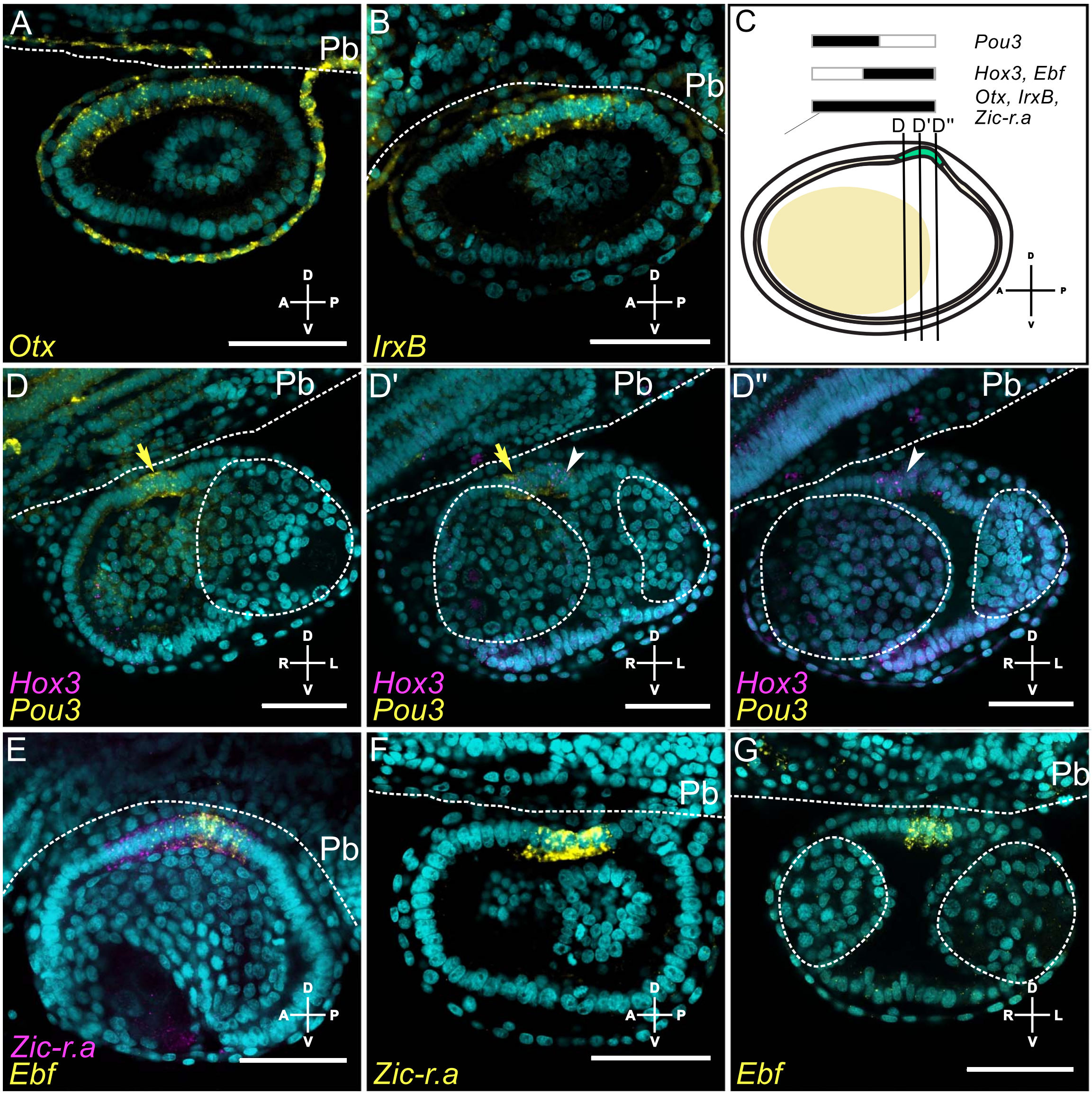

Fig. 1. AP patterning and neurogenic markers are expressed early in the *Botryllus schlosseri* secondary bud stage B2. Confocal sections of budlets (A, B, D-G), dorsal is upwards. (C) Schematic drawing of *Botryllus schlosseri* budlet in stage C1 shows the first event of dorsal evagination of the inner vesicle (green) and the formation of peribranchial chambers that will give rise to the atrial chamber (ochre), with an expansion of mRNA expression projected on top. (D-D") yellow arrow pointing to *Pou3*, with arrowhead to *Hox3* expression. Genes (yellow or magenta) are indicated in the lower left corner. Dashed lines indicate the border with the primary bud (Pb) and dashed circles outline the gonads. Scale bar is 50 µm. Nuclei are stained with Hoechst (cyan).

and fuse to the future branchial chamber at stage D (Fig. 2B-C). In the meantime, cells detach from the walls of the dorsal tube to the overlying mesenchyme (Manni et al., 2014; Burighel et al., 1998). During the elongation of the dorsal tube *Otx* transcripts were expressed at the anterior pole with a gradual increase in expression to the ventral side, over the dorsal epithelia of the future branchial basket and atrial chambers, as well as in other more posterior regions (Fig. 2D). During dorsal tube elongation, *IrxB* was expressed around the dorsal tube and in scattered cells of the tube (Fig. 2E). The expression of *Gli* was first detected along the entire dorsal tube (Fig. 2F) and then became restricted in the median region of the dorsal tube after the anterior fusion of the tube (Fig. 2G). During dorsal tube elongation *Pou3* expression shifted posteriorly and overlaps with *Hox3* expression (Fig. 2H). During anterior fusion of the dorsal tube *Hox3* was detected in the posterior end of the tube, and in mesenchymal cells around the dorsal tube (Fig. 2I). *Pax3/7* patterns *Ciona* CNS in three regions anterior and posterior part of the SV, the neck and the caudal nerve cord (Mazet et al., 2003). In the *Botryllus* C2 secondary bud, *Pax3/7* was expressed in the anterior dorsal side of the elongating dorsal tube (Fig. 2J), and in the epidermis that overlies the tube. After the fusion of the tube (stage D), *Pax3/7* transcripts were no longer localized in the epidermis, but in mesenchymal cells overlying the dorsal tube (Fig. 2K). The detection of the pan-neuronal marker ELAV related gene *Etr* was observed from stage C2 in scattered cells of the dorsal tube and in the mesenchymal cells around the tube (Fig. 2J). Some *Etr*+ cells in the tube and in the mesenchyme also co-expressed *Pax3/7* (Fig. 2J). The larval neural tube marker *Zic-r.a* retained expression along the dorsal tube during elongation (Fig. 2L) and after fusion. In stage C2 the neural marker *Ebf* was expressed in single cells along the entire dorsal tube and the overlying mesenchyme (Fig. 2L, M), some of these cells co-expressed the second Pou-domain containing transcription factor *Pou4* (Fig. 2M), a TF necessary for terminal differentiation of specific sensory neurons in different metazoan phyla (Candiani et al., 2005). *Notch* (Fig. 2N) was expressed in scattered cells all along the tube during its elongation and fusion.

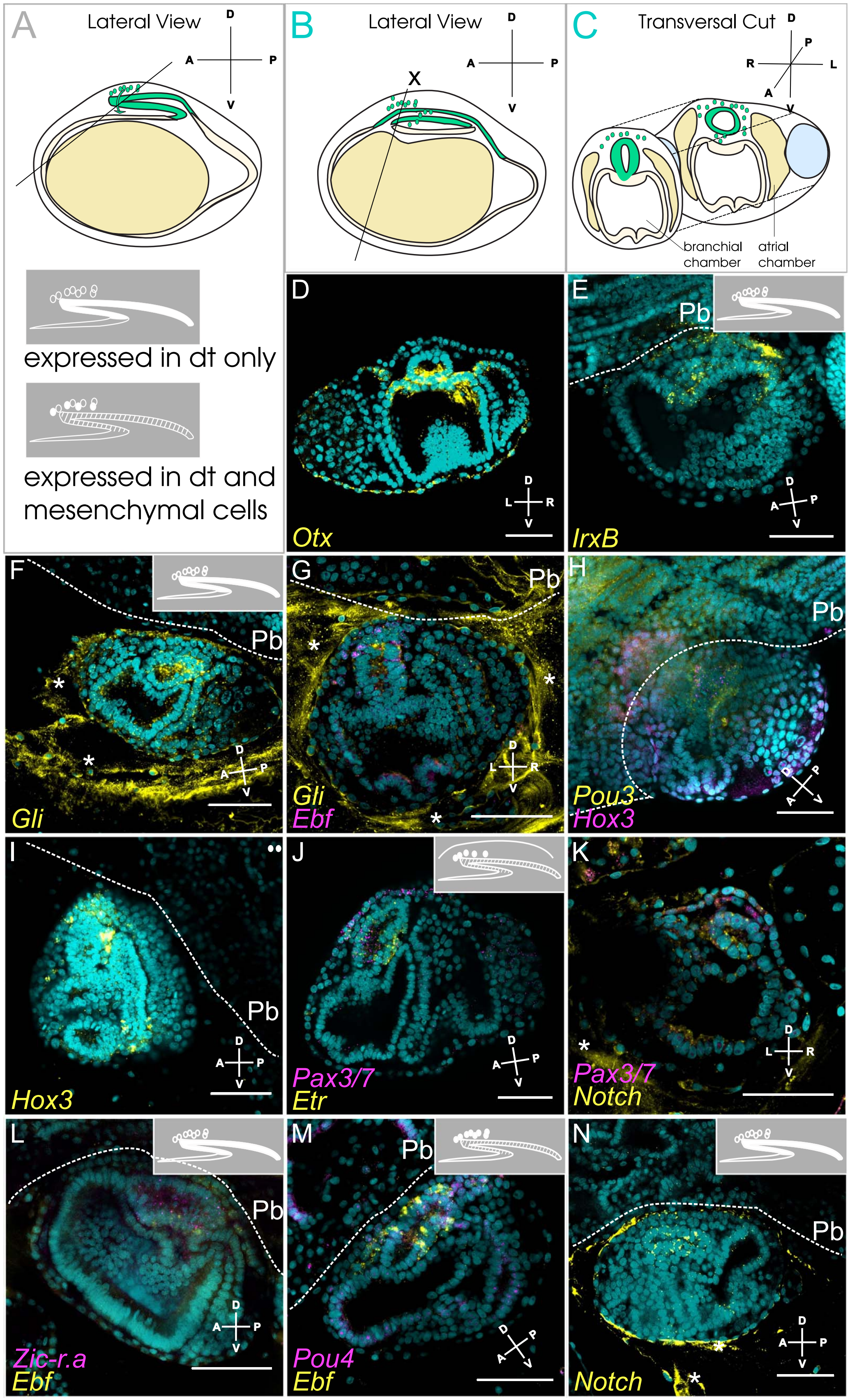

Fig. 2. Dynamic anteroposterior marker genes are expressed coupled with neurogenic genes in *Botryllus schlosseri* during dorsal tube elongation and fusion. (A) Schematic drawing of *Botryllus schlosseri* budlet in stage C2 shows dorsal evagination, elongation, and tubularization of the inner vesicle. Overlying mesenchymal cells (green) move ventrally (green arrow), while the peribranchial chambers form (ochre). States of expression (close-up in grey) are represented in the upper right corner of corresponding figures. (B-C) Schematic drawing of the budlet in stage D shows the dorsal tube (green) attaching anteriorly to the branchial chamber (light ochre); presumptive gonads (blue) are located laterally (B) and transversally (C). (E, F, H, J, L, M, N) Confocal sections of budlets in stage C2 and stage D (D, G, I, K). Genes (yellow or magenta) are indicated in the lower left corner in. The color border indicates colony stages (A-C). Dashed lines indicate the border with the primary bud (Pb). Asterisk indicates unspecific staining of the probe in the tunic. The scale bar is 50 µm. Nuclei are stained with Hoechst (cyan).

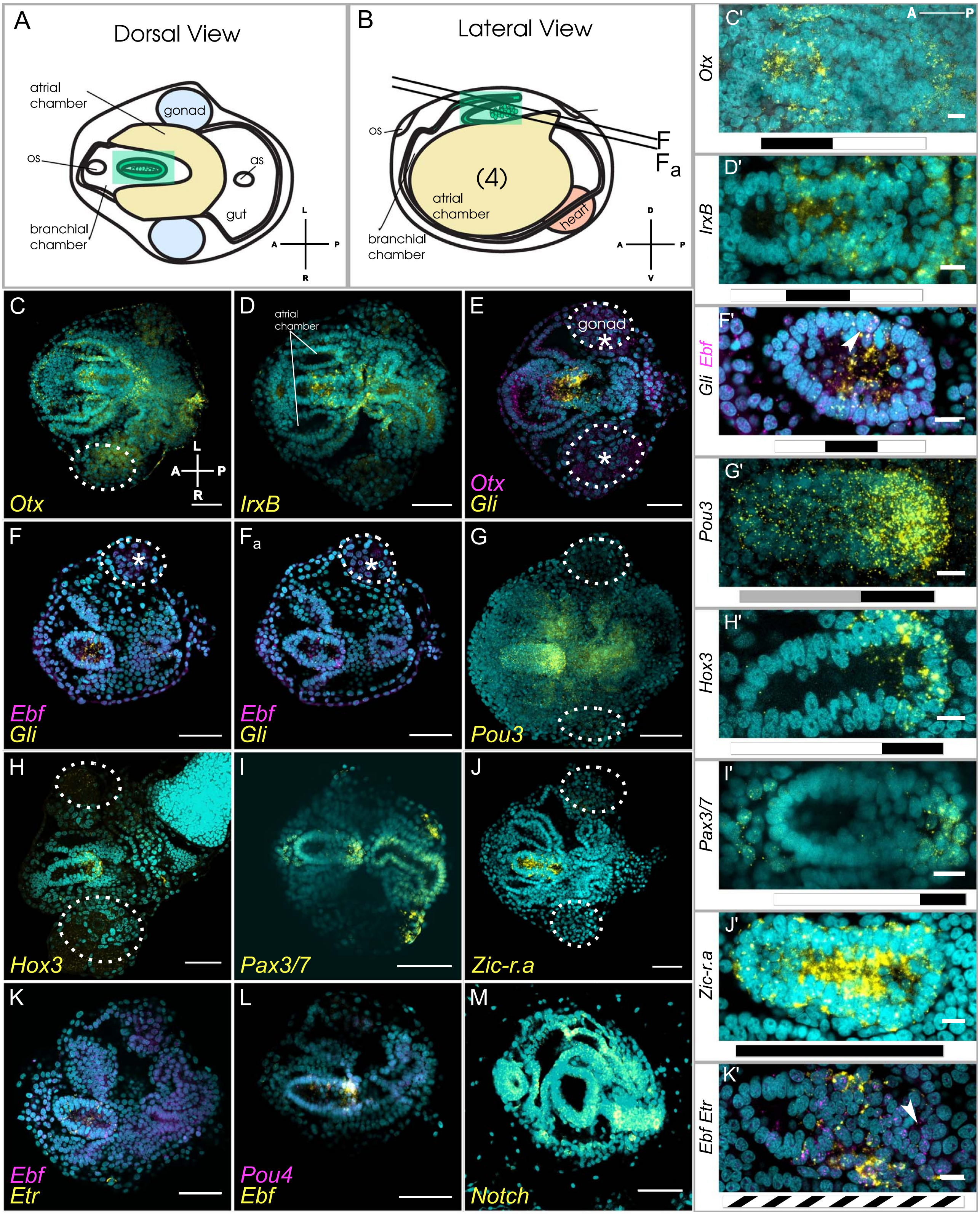

Fig. 3. AP neurogenic genes during ganglion development in the *Botryllus schlosseri* primary bud. (A-B) Schematic drawing of *Botryllus schlosseri* bud in stage A1, the dorsal tube is detached posteriorly and the cerebral ganglion is shown as a cluster of cells ventral to the tube (green), and the forming peribranchial chambers are fused dorsally (ochre). The green box in (A) represents a close-up view of the dorsal tube shown in the pictures on the right column. Oral siphon (os) and atrial siphon (as) remain closed. (B) Cuts trough dorsal tube indicate where confocal section of F and Fa were taken. (C-F′, H,J-M) Confocal images of the dorsal side in stage A1 buds (G, I). Genes are indicated in the lower left corner of the pictures in yellow or magenta. (C′, D′,F′′,G′-K′) Close-up view of the dorsal tube, black bar indicates the expansion of expression, dashed bar indicates scattered expression, grey relative low expression. (F′) Arrow indicates co-expression of *Ebf* and *Gli* (K′) *Ebf* expression. Asterisks indicate nonspecific staining of the probe trapping in the tunic and gonad, dashed circles outline the gonads. Scale bar is 50 μm and 10 μm in right column. Nuclei are stained with Hoechst (cyan).

3.3. AP patterning of the dorsal tube during the formation of cerebral ganglion

At stage A1 of the colony, the CNS of the primary bud is further developed than the secondary bud at stage C mentioned above (Fig. 3A-B). The posterior side of the dorsal tube detaches (Manni et al., 2014), and a delamination of cells from the tube migrate ventrally forming the cerebral ganglion (Manni et al., 2014, 2001; Burighel et al., 1998). At this stage *Otx* expression was confined to the anterior pole of the tube, as well as to the dorsal epithelia of the branchial basket and peribranchial chambers (Fig. 3 C, C′). *IrxB* expression was restrained to a central region of the dorsal tube (Fig. 3D, D′). *Gli* maintained its domain of expression in the middle of the dorsal tube posterior to the *Otx* expression domain and showing only a small overlap (Fig. 3E). Only a scattered part of the *Gli* expressing cells co-expressed the neural marker *Ebf* (Fig. 3F, F_a, F′). *Pou3* expression split in two domains: low expression in anterior cells and strong expression on cells located on the posterior third of the dorsal tube (Fig. 3G, G′).

The patterning markers *Hox3* (Fig. 3H, H′) and *Pax3/7* (Fig. 3I, I′) were both expressed in the posterior portion of the dorsal tube. *Pax3/7* was no longer expressed in the anterior tube, but showed an additional expression in mesenchymal cells located in the dorsal and anterior region of the dorsal tube. *Zic-r.a* (Fig. 3J, J′) was expressed over the entire length of the dorsal tube. The neural markers *Ebf*, *Etr* (Fig. 3K, K′), and *Pou4* (Fig. 3L) were expressed in scattered cells along the dorsal tube. Only part of the *Ebf*+ cells co-expressed *Etr* (Fig. 3K′). *Notch* (Fig. 3M) was expressed over the entire length of the dorsal tube.

3.4. Expression of CNS patterning genes and neural markers in the adult neural complex

The mature neural complex of a *Botryllus schlosseri* blastozooid is composed of four structures: the ciliated funnel (CF), which is in continuity with the neural gland (NG), the dorsal organ (DO), and the cerebral ganglion (CG) (Manni et al., 2014) (Fig. 4A).

The CNS patterning genes that were still expressed in the fully developed neural complex include: *IrxB*, which was expressed in scattered cells in the NG and CG (Fig. 4B); *Pou3*, which was expressed in NG, CG and DO (Fig. 4C); *Hox3*, expressed in the posterior region of the NG, the DO, and in a patch of cells in the postero-ventral side of the CG (Fig. 4D); and *Pax3/7* that was expressed at the anterior pole of the NG and the antero-dorsal region of the CG (Fig. 4E). *Gli* and *Otx* were not detected in the adult neural complex. *Zic-r.a* was expressed throughout the CF, NG and DO but not in the CG (Fig. 4F, F′, F′′). *Ebf* was expressed in scattered cells of both CG and NG (Fig. 4G, H). *Etr* was expressed in the entire CG and in scattered cells of the NG (Fig. 5H). Expression of the transmembrane protein *Notch* was restricted to the NG region (Fig. 4I), whereas *Pou4* was expressed in the CG and NG (Fig. 4J).

4. Discussion

4.1. Dorsal tube formation and neurulation share similar AP patterning

Our data highlight comparable AP expression patterns between the stereotypical chordate neurulation (Holland et al., 2013; Wada et al., 1998; Albuixech-Crespo et al., 2017) (Fig. 5), and the formation of the blastogenetic dorsal tube in *Botryllus schlosseri*. As described before the dorsal tube is a developmental structure that generates the adult neural complex during asexual development (Manni et al., 2014). Here we show a common tripartite molecular regionalization during chordate CNS tubular formation, independent of its developmental origin, whereas *Otx* is expressed anteriorly, *IrxB* and *Gli* in a central region, and *Hox3* and *Pax3/7* at the posterior end of the tube. The regionalized expression of these genes is transient and begins in the young bud, at stage B2, in a region defined by the presence of TFs associated to ectodermal tissue fates (Ricci et al., 2016a). Within this epithelial domain of the internal vesicle of the young bud, the dorsal tube arises as a thickening, and displays a bipartite expression of the transcription factors *Pou3* and *Hox3*. While not reported in ascidian neural patterning, *Pou3* has been shown to regionalize the nervous system during development of several taxa (Wollesen et al., 2014; Alvarez-Bolado et al., 1995). In early stages of larval development of ascidians, the CNS exhibits an early bipartition reflected by an absence of a gap between *Otx* and *Hox* gene expression (Ikuta and Saiga, 2007), which is followed by a the later insertion of a *Pax2/5/8a+/Otx-/Hox-* domain that establishes the tripartite partitioning of the larval brain (Wada and Satoh, 2001). Concordantly, in *Botryllus* blastogenesis, we find the formation of a middle region during the progression of the dorsal tube elongation, which corresponds to a region of expression of *IrxB* and *Gli* between the *Otx* and *Hox3* domains at stage D. While present in the *Botryllus schlosseri* genome, in this study we were not able to amplify *Pax2/5/8a*, a marker of the neck region in *Ciona*; hence we used the zinc finger TF *Gli*, a downstream target of *Pax2/5/8a* (Imai et al., 2009). In addition, we analyzed *IrxB,* which has not been clearly described in solitary ascidians, but in vertebrates is expressed posterior to the *zona limitans intrathalamica*, the border between the thalamus and prethalamus (Rodríguez-Seguel et al., 2009; Sena et al., 2016). Both *Gli* and *IrxB* were expressed in the central gap region between *Otx* and *Hox* in stage A1 bud. Interestingly, before expression became restricted to the mid region prior the dorsal tube differentiation, the expression of both TFs started broader during dorsal tube elongation in the early tubulogenesis. Such extended AP domain might reflect a generally broader expression of both, *Gli and IrxB,* observed in non-ascidian chordates (Fig. 5C) (Holland et al., 2013).

The tripartite regionalization of the dorsal tube reflects largely the divisions of the larval ascidian brain. In contrast, genetic markers of the nerve cord in the larval tail were not expressed during dorsal tube blastogenetic development. For instance, posterior *Hox5* (Gionti et al., 1998) was detected in the peribranchial chamber only, but not in the dorsal tube (Suppl. Material 13).

The AP patterning genes analyzed here are not exclusively expressed during larval CNS development, but are also involved in the regionalization of other tunicate tissues with AP extension, such as the endostyle (Cañestro et al., 2008). They are also known to be expressed in post-metamorphic structures, such as the heart and pharynx (Yoshida et al., 2017; Hinman and Degnan, 2000; Ogasawara et al., 1996). The three AP tripartite molecular domains observed here may be responsible for the morphogenetic boundaries of the neural gland complex, *i.e.* CF/NG/DO (Fig. 3. A). Hence, an anterior *Otx*+ domain delimits the territory of the CF, a median *Gli*+/*IrxB*+ delimits the median NG, and a posterior *Pax3/7*+/*Hox3*+ domain defines the posterior DO respectively (Fig. 5). We hypothesize further that the three domains may also be important for generating different neuronal cell types that delaminate and eventually reside in the CG.

4.2. Dorsal tube as center of neurogenesis during blastogenetic development

Histological and ultrastructural description of asexual neurogenesis defines the dorsal tube also as a cellular source for cerebral ganglion development. In particular Burighel et al. (1998) described the delamination and migration of cells from the dorsal tubular epithelium which migrate, cluster and differentiate into the neurons of the cerebral ganglion. In addition, the patterning observed in the dorsal tube occurs in tandem with the expression of neural and neurogenic markers such as *Etr*, *Zic-r.a*, *Ebf* and *Pou* genes. Thus expression dynamics suggests a progressive neural fate determination from a population of cells in the dorsal tube epithelia, and in cells delaminating from it, which corresponds to morphological observations present in literature (Burighel et al., 1998; Manni et al., 1999). It has previously been

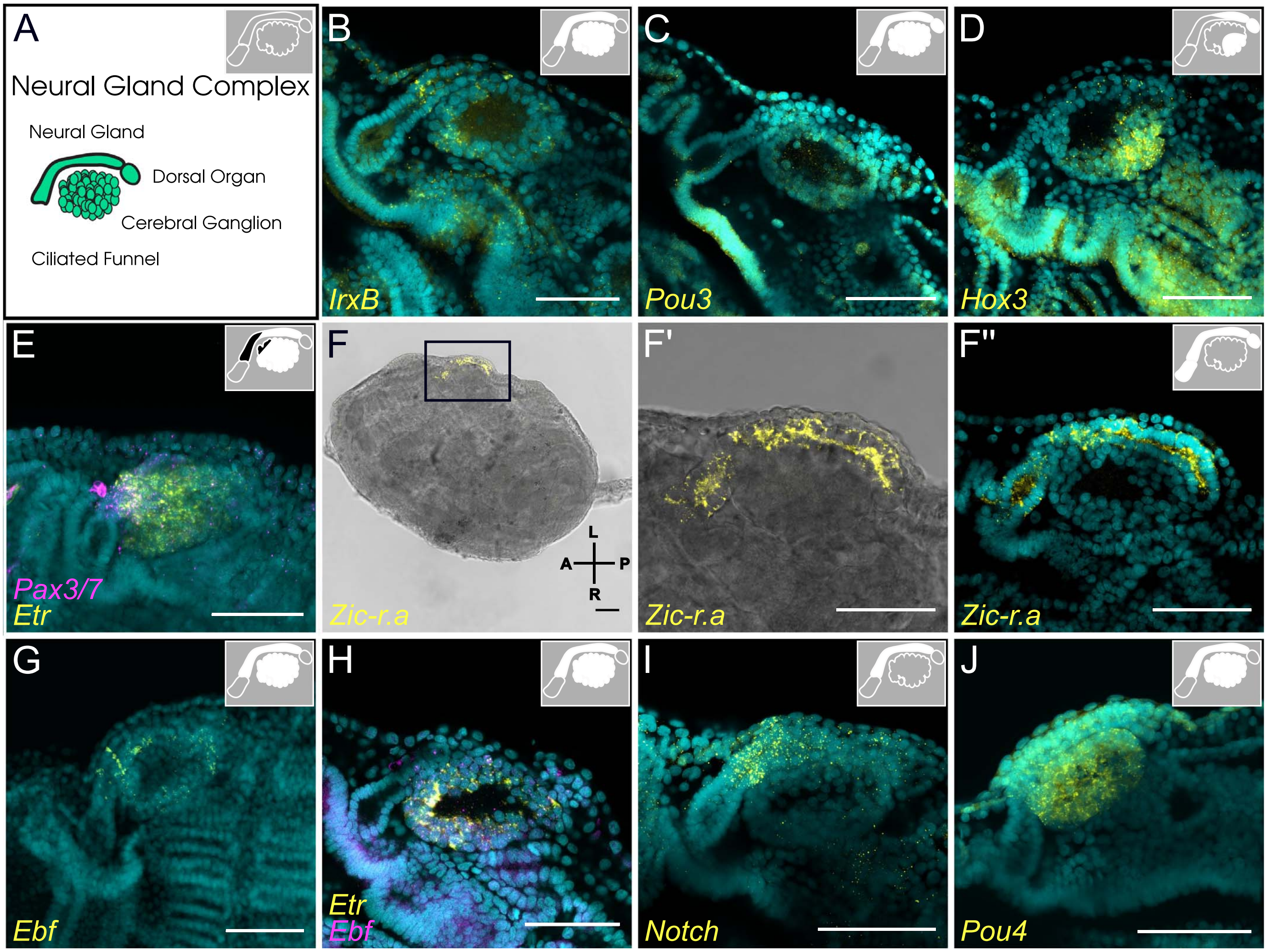

Fig. 4. AP patterning and neurogenic markers in the neural complex of *Botryllus schlosseri*. (A) Schematic drawing of *Botryllus schlosseri* neural complex. (B, C,E,G,I,J) Confocal projection images of stacks of the neural complex and (D, F, F′, F′′, H) confocal sections. (F) Bright field images of a zooid prior to siphon opening, close-up in (F′). Genes (yellow or magenta) are indicated in the lower left corner. Scale bar is 50 µm. Nuclei are stained with Hoechst (cyan).

suggested that during larval development and metamorphosis in *Botryllus schlosseri*, most of the adult neural complex originated from the neurohypophyseal duct in the larval CNS (Manni et al., 1999), which followed a similar morphogenetic mechanism to that described during blastogenesis (Manni and Burighel, 2006). On the other hand, in *Ciona* metamorphosis, the post-metamorphic ganglion was formed from ependymal cells of the CNS, and the position of cells along the AP axis reflects their eventual positioning in the adult cerebral ganglion, suggesting that it was the patterning of the larval nervous system that provide the positional information to pattern the adult cerebral ganglion (Dufour et al., 2006; Horie et al., 2011; Sasakura and Hozumi, 2018; Hozumi et al., 2015). While the patterning of the larval CNS is conserved in *Botryllus schlosseri* (Suppl. Material 13) we did not detect any patterning genes in the neurohypophyseal duct. It is then intriguing to speculate that, in a non-embryonic context, the patterning of the dorsal tube may provide the spatial cues for neuronal guidance and development in the cerebral ganglion.

4.3. Dorsal tube: neural and non-neural nature

During dorsal tube differentiation, not all the *Ebf*+ mesenchymal cells express the neural differentiation markers *Etr* (Yagi et al., 2001) or *Pou4*. In *Ciona*, *Ebf* is not only responsible for the decision of cholinergic motor neuron over ependymal cells fate in the larval CNS (Kratsios et al., 2011) but is also important for muscle formation (Razy-Krajka et al., 2014). Interestingly, we also detected myogenic TFs in the *Botryllus* dorsal tube and delaminating cells (data not shown). Therefore, we argue that the dorsal tube and delaminating mesenchymal cells are not exclusively committed to neural fates.

Even though the ascidian specific transcription factor *Zic-r.a* is a maternal factor that determines muscular fate primary (Nishida and Sawada, 2001; Sawada et al., 2005) its zygotic expression in the anterior part of the sensory vesicle, in the neck, the ganglion, and along the nerve cord suggests a secondary role in neural development (Satou et al., 2002; Satou and Imai, 2018). In *Botryllus* asexual development *Zic-r.a* represents a pan-dorsal tube marker, from the onset of dorsal tube formation until its final differentiation into the CF, NG and DO. Without clear live tracking of the dorsal tube derived epithelial mesenchymal cells it is not possible to follow the dynamics of any delaminating cells. Nevertheless, these patterns of expression suggest that the dorsal tube is not exclusively a source of neural cells but may contribute to other cell types. While the role of the dorsal tube has not been functionally tested, this structure shows complex dynamics of cell commitment, cell differentiation, and cell migration. In this context, the presence of *Notch*+ cells, could play an inhibitory role on their differentiation (Nye et al., 1994; Tanigaki and Honjo, 2010). Unfortunately, chemically blocking Notch pathway resulted into a lethal phenotype (data not shown), so any effect on cell type specification could not be addressed.

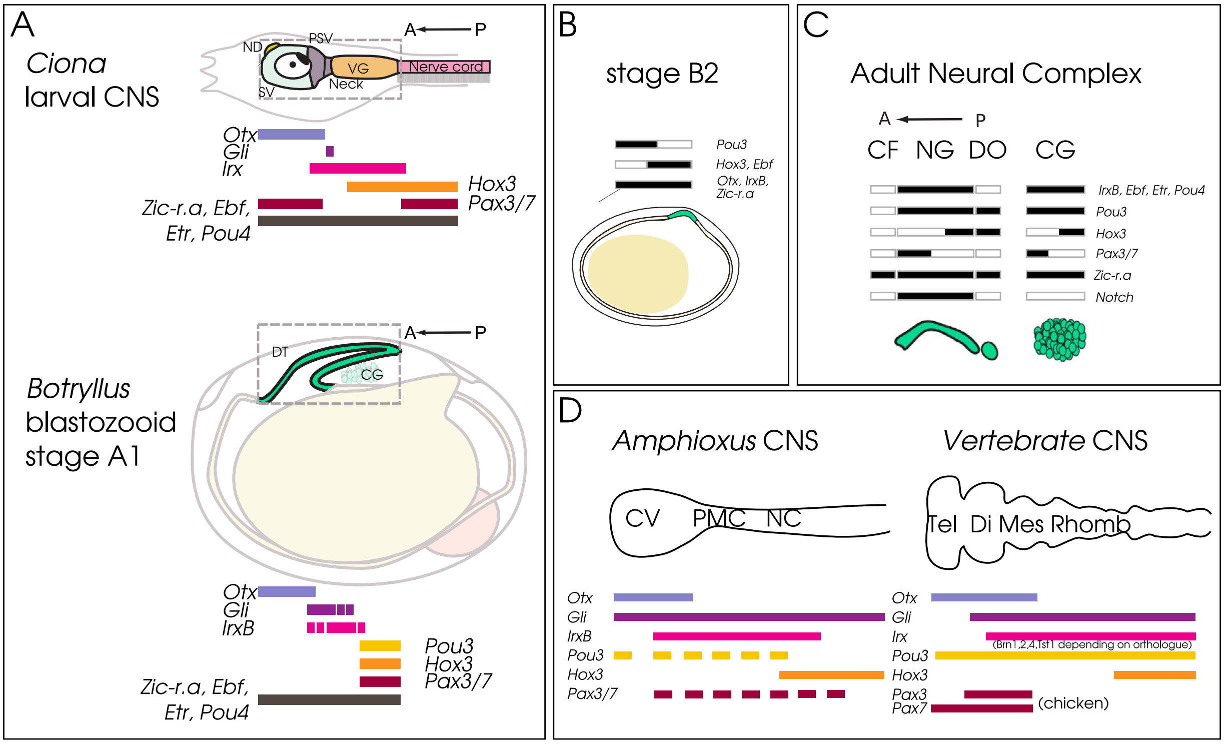

Fig. 5. AP patterning genes during larval CNS development and asexual dorsal tube development. (A) AP patterning domains of expression in the larval CNS of *Ciona* and the dorsal tube in a stage A1 bud of *Botryllus schlosseri*. (B) Stage B2 (C) adult neural complex prior to siphon opening in *Botryllus schlosseri*. (D) Chordate AP patterning genes in Amphioxus or a generic vertebrate (rat or chicken). Gene expression domains based on Alvarez-Blado et al. (1995), Hudson and Lemaire (2001), Matsunaga et al. (2001), Mazet et al. (2003), Imai et al. (2009), Holland et al. (2013) and Albuxech-Crespo et al. (2017). ND, neurohypophyseal duct; SV, sensory vesicle; PSV, posterior sensory vesicle; VG, visceral ganglion; DT, dorsal tube; CG, cerebral ganglion; CF, ciliated funnel, NG, neural gland, DO, dorsal organ, CG, cerebral ganglion, CV, cerebral vesicle, PMC, Primary motor center, NC, Nerve chord, Tel, Di, Mes, Rhomb, Tel-,Di-,Mes-,Rhombenchephalon.

4.4. Neural complex: neural and non-neural nature

Despite its name, the neural gland and associated structures, including the CF and the DO, have been considered the non-neuronal part of the neural complex (Mackie and Burighel, 2005). Some authors support the homology of the ascidian neural gland complex with the vertebrate pituitary or adenohypophysis (Burighel et al., 1998; Pestarino, 1985), whereas others dismiss this homology and infer an osmoregulatory function (Deyts et al., 2006) (reviewed in Burighel and Cloney, 1997) (Burighel, 1997). When analyzed histologically, the NG is a homogeneous structure with no obvious differences along the AP axis (Deyts et al., 2006; Lane, 1971) and composed of mainly phagocytic cells, without evidence of neuronal cell types (Ruppert, 1990). Our observations of a differential expression of *Pax3/7* and *Hox3* along the AP axis of the NG in *Botryllus* indicates a molecular maintenance of territory identity after the separation of the CF, NG, and DO. In addition, a continuous expression of the neural marker *Etr* and *Pou4* both in the differentiated ganglion as well as in cells of the NG suggests a perpetual differentiation of neurons in this region.

5. Conclusion

During non-embryonic developments such as *Botryllus* blastogenesis, different morphogenetic and ontogenetic events lead to similar structures, often co-opting molecular regulatory pathways from embryogenesis (Ricci et al., 2016b; Tiozzo et al., 2005; Tiozzo and De Tomaso, 2009). The observation of a transitory tubular structure that leads to the development of an adult CNS during asexual development, allows us to draw a raw map of gene expression patterns that show a remarkable similarity to AP patterning of larval CNS development. This is the first study that describes a molecular patterning event during a non-embryonic development of an ascidian. The development of two tubular neurogenic structures, one during embryogenesis and metamorphosis, and another during blastogenesis, strongly suggests co-option of a patterning between two different ontogenesis. In this case, the partial co-option of the dorsal tube patterning could represent a provisional scaffolding in blastogenesis, just like the larval CNS functions as a provisional scaffold for the adult CNS during the sexual development.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.10.009.

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