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Nervous system development in cephalopods: How egg yolk-richness modifies the topology of the mediolateral patterning system

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ABSTRACT

Cephalopods possess the most complex centralized nervous system among molluscs and the molecular determinants of its development have only begun to be explored. To better understand how evolved their brain and body axes, we studied *Sepia officinalis* embryos and investigated the expression patterns of neural regionalization genes involved in the mediolateral patterning of the neuroectoderm in model species. *SoxB1* expression reveals that the embryonic neuroectoderm is made of several distinct territories that constitute a large part of the animal pole disc. Concentric *nkx2.1*, *pax6*/*gsx*, and *pax3/7*/*msx*/*pax2/5/8* positive domains subdivide this neuroectoderm. Looking from dorsal to ventral sides, the sequence of these expressions is reminiscent of the mediolateral subdivision in model species, which provides good evidence for "mediolateral patterning" conservation in cephalopods. A specific feature of cephalopod development, however, includes an unconventional orientation to this mediolateral sequence: median markers (like *nkx2.1*) are unexpectedly expressed at the periphery of the cuttlefish embryo and lateral markers (like *Pax3/7*) are expressed centrally. As the egg is rich with yolk, the lips of the blastopore (that classically organizes the neural midline) remain unclosed at the lateral side of the animal pole until late stages of organogenesis, therefore reversing the whole embryo topology. These findings confirm – by means of molecular tools – the location of both ventral and dorsal poles in cephalopod embryos.

1. Introduction

The high diversity of nervous systems (NS) within Bilateria, notably with regard to their architecture and degree of condensation, raises the question of the origin of nervous centralization. A strong argument in favour of a unique origin is the high level of similar molecular signatures that have been found in the developing NS of model species as different as *Mus musculus* (Chordate-Vertebrate), *Drosophila melanogaster* (Ecdysozoa-Arthropoda-Insect) and *Platynereis dumerilii* (Lophotrochozoa-Annelida). Along the Antero-Posterior (AP) axis of these organisms, a similar *otx-pax2/5/8-Hox* gene expression sequence can be described all along their regionalized nervous axis (neural plate and tube in vertebrates, ganglionic chain in insects and annelids) with the cerebral structures (the brain, the cerebellum or the cerebral ganglia, respectively) on the anterior side (see Urbach (2007) and Holland et al. (2013)). Along the transversal dorso-ventral axis (from the median line of the neurogenic territory to more lateral parts), molecular data also reveals a similar mediolateral sequence of developmental gene expression. First, similar *nkx2-gsx-msx* (respectively *vnd-ind-msh* orthologs in *Drosophila)* positive neurogenic domains have been described in vertebrates and insects (Arendt and Nübler-Jung, 1994, 1999). It has also been shown that in vertebrates, insects and annelid, the similar staggered expression of medial (*foxA*, *nkx2.2*) and lateral (*pax6, pax3/7, gsx, msx, dll*) groups of genes control the mediolateral patterning of neurogenic regions centered along the neural midline (Wheeler et al., 2005; Denes et al., 2007). The most convincing point is that these molecular regions determine the differentiation of conserved neuron types: serotonergic neurons and motoneurons are found mostly in median regions, sensory neurons in lateral regions, and interneurons in-between (Denes et al., 2007; Arendt et al., 2008). Taken all together, these molecular similarities support the homology hypothesis between the dorsal central nervous system (CNS) of vertebrates and the ventral CNS of non-vertebrates and therefore revive the old hypothesis of a "body axis inversion" during

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vertebrate evolution (Dohrn, 1875; De Robertis and Sasai, 1996).

Beyond these strong similarities, noticeable differences can also be found. For instance, the hedgehog ortholog (*hh*) is involved in establishing the dorso-ventral (DV) neural axis in vertebrates (Patten and Placzek, 2002), but does not seem to play this role in insects and annelids (Tashiro et al., 1993; Dray et al., 2010). Orthologs of *gsx/ind* are involved in the intermediate neuroectoderm specification in insects and vertebrates, but not in annelids (Kulakova et al., 2008; Hui et al., 2009). In vertebrates and annelids, *dbx* and *pax6* seem to play a role in neural specification but not in insects (Mizutani et al., 2006; Kammermeier et al., 2001). These few examples underline that the occurrence of divergence or convergence events during the course of evolution cannot be simply set aside. Because of the large diversity of NS architectures, confirming the hypothesis of a single origin of nervous centralization (Moroz, 2009; Holland et al., 2013) requires non-model species to be taken into account in molecular studies.

To do such investigations, cephalopods are particularly interesting organisms because they possess a large and highly centralized NS (called a "brain"; Boycott, 1961; Young, 1971) but also a large number of innovations that may have largely affected the molecular program of their body plan, body axes development, and NS regionalization. As part of the mollusc family, their CNS is not made of a linear chain of ganglia (like in insects) or a unique neural anlage (like in annelids or Chordates) but of a secondary interconnection of ganglia and cords emerging first in distinct neurogenic territories (Jacob, 1984; Shigeno et al., 2001; Yamamoto et al., 2003; Shigeno et al., 2015). In addition, in cephalopods, an important growth and fusion of several pairs of these ganglia (namely the cerebral, pedal, and visceral) leads to a large brain surrounded by two optic lobes (Marquis, 1989; Budelmann, 1995; Shigeno et al., 2001; Yamamoto et al., 2003; Buresi et al., 2012). Cephalopod development is also unique among molluscs for several reasons. First, they possess yolk-rich telolecithal eggs, in which a discoidal cleavage leads to a flat and circular animal pole. Second, the organogenesis is completely direct and results in the hatching of a juvenile without passing through any larval stage (trochophore or veliger as in most molluscs) or any metamorphosis step (Boletzky, 2003). As a result, the evolution of cephalopod development has long been a puzzling question, mostly investigated by anatomical comparisons between species (embryos, larvae, or adults), by the research of archetypes based on fossil records or analogies, or more recently, by comparisons of developmental gene expression patterns within molluscs and model species (e.g. Brooks, 1880; Naef, 1928; Lee et al., 2003; Shigeno et al., 2008, 2010; Buresi et al., 2012; Wollesen et al., 2015a).

In molluscs, investigations into developmental genes and transcription factors involved in NS development are quite recent and dispersed over several species. *Hox* gene patterns of expression have been essentially investigated in larvae of gastropods (*Patella vulgata*, Nederbragt et al., 2002; *Haliotis asinina*, Hinman et al., 2003; O'Brien and Degnan, 2003; *Gibbula varia*, Samadi and Steiner, 2010) but also during the mid- and late organogenesis of two cephalopod species (*Euprymna scolopes*, Lee et al., 2003; *Sepia officinalis*, Forecata et al., 2014). These studies have revealed that comparisons with model species are complex and that very few homologies can be clearly assessed with such distant species. *pax6* has been studied for its role in photoreceptor development (Tomarev et al., 1997; Hartmann et al., 2003; Yoshida et al., 2014), and has been shown to be expressed in non-overlapping territories with the *hh* ortholog like in vertebrates (Navet et al., 2009). Other transcription factors like engrailed (Baratte et al., 2007), Dll (*Nautilus pompilius*, *Idiosepius paradoxus*, Shigeno et al., 2008; Shigeno et al., 2015), and other developmental genes like *apterous* (Farfán et al., 2009), *otx* (*S. officinalis*, Buresi et al., 2012), or *gsx* (Wollesen et al., 2015b) have been studied. For now, no global molecular interpretation about NS development or regionalization has been realized in one cephalopod species. However, a recent article on *pax2/5/8* expression in several mollusc groups, including cephalopods with *Idiosepius notoides*, suggests that the *otx-pax2/5/8-Hox* gene expression sequence of model species resembles the one of cephalopods but not the one of several other molluscs (Wollesen et al., 2015a).

A similar search for a molecular footprint is conducted in our present work, but on the mediolateral axis. This study explores the expression of some orthologous "mediolateral genes" in the developing neuroectoderm of *Sepia officinalis* (the *gsx*, *msx*, *pax* and *nk* gene families) during the first steps of organogenesis. At this stage, the animal pole is a disc whose ventral pole is acknowledged to be at the periphery and dorsal pole at the center (Naef, 1928; Boletzky, 2003; Lee et al., 2003; Shigeno et al., 2008, 2010; Wollesen et al., 2015a; Wanninger and Wollesen, 2015; Sumner-Rooney et al., 2015, Fig. 1). The gene expression patterns we observed are compared with that in other molluscs. Along the DV axis, the sequence of their expression patterns is compared with that of metazoan model species. The global similarity we describe suggests that the mediolateral footprint has been conserved (or recruited) along the DV axis in cephalopods. The completely reversed order of this sequence also reveals that this footprint has been impacted by the specificity of cephalopod development: because of the large amount of yolk, the gastrulation line of the embryo (on its ventral side) is not closed during the first steps of organogenesis, so what should be the medial region of the embryo NS is pushed to the embryo's periphery and what should be the nervous lateral regions become *de facto* central parts of the animal pole disc. We discuss these topological consequences and focus on what they can teach us about the evolution of NS development among molluscs.

2. Results

2.1. Main features of Sepia embryogenesis and organogenesis

Cephalopod eggs are telolecithal. A meroblastic and non-spiral cleavage leads to a discoidal animal pole (discoblastula) lying on the large yolk syncytium (Boletzky, 1988). As organogenesis takes place, the animal pole grows and reaches the juvenile morphology at hatching, whereas the yolk reserve is metabolized and decreases in size (Fig. 1A). In *S. officinalis*, gastrulation corresponds to an internal splitting-off of blastomeres at the edge of the proliferating animal disc ectoderm (Fig. 1B; Boletzky et al., 2006), forming first a ring-shape, and then a full endo-mesoderm layer (Fig. 1C). At this stage, an extra-embryonic ectoderm also starts growing from the gastrulation lip, covering and integrating the large uncleaved yolk syncytium into a nutritive yolk organ (Fig. 1C) (Boletzky, 1988; Boletzky et al., 2006). Before organogenesis (from fertilization to stage 12), it is almost impossible to preserve a cephalopod egg's integrity while removing the chorion, because the yolk syncytium is both fragile and highly adherent to the chorion. Therefore these stages remain inaccessible to immunostaining or hybridization studies for now. Once organogenesis begins and first organs appear (early organogenesis from stages 13–19), presumptive territories are concentrically arranged within the animal disc: future arms at the ventral periphery, future mantle at the dorsal center (Fig. 1D). From mid-organogenesis (stages 20–23), the animal pole protrudes above the yolk mass and stretches out in a DV outgrowth: peripheral parts of the animal disc (close to the former gastrulation lip, fGL) become the future ventral organs of the animal (arm crown, future head) whereas central territories acquire their future dorsal location (the mantle, containing the

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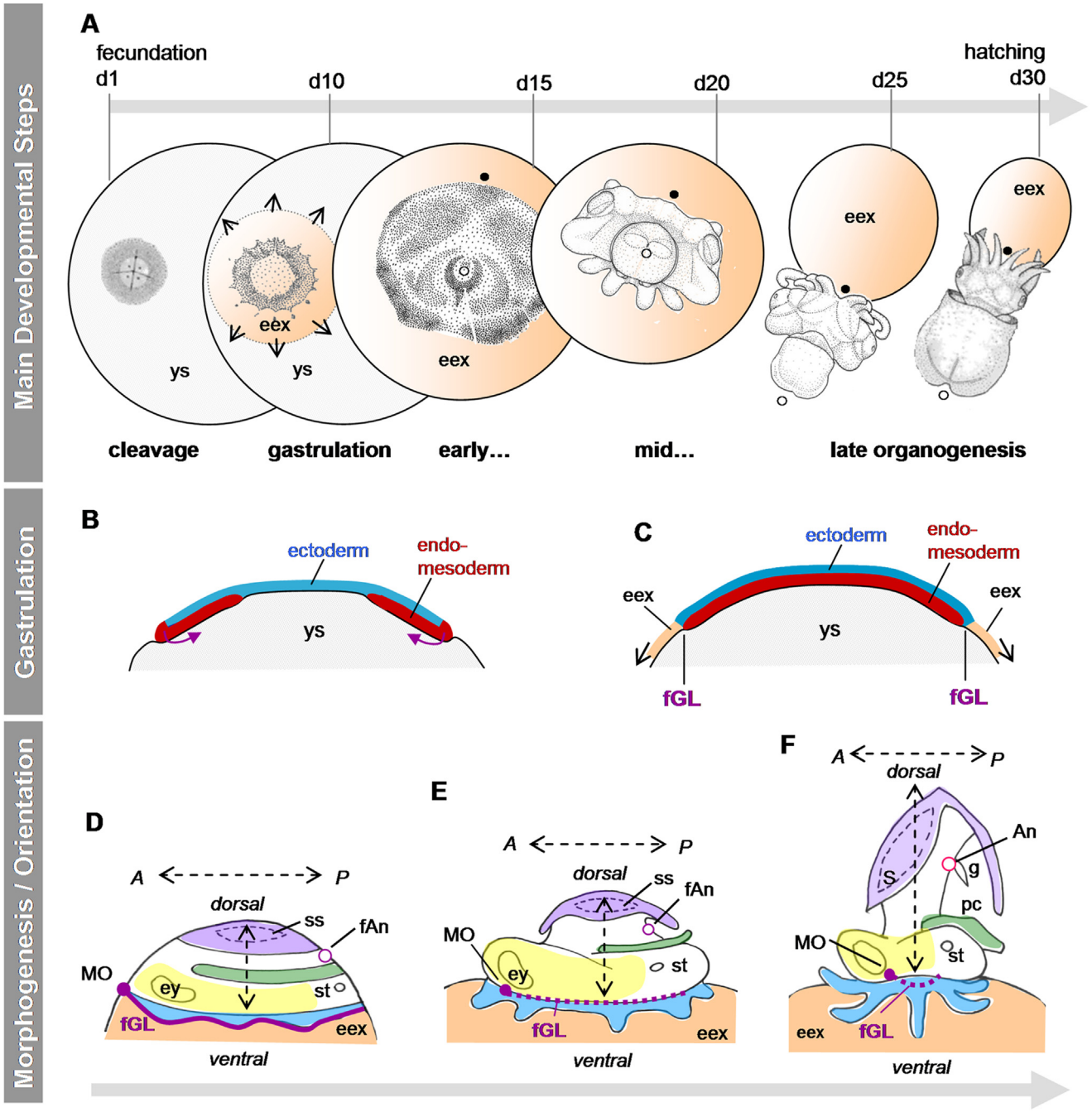
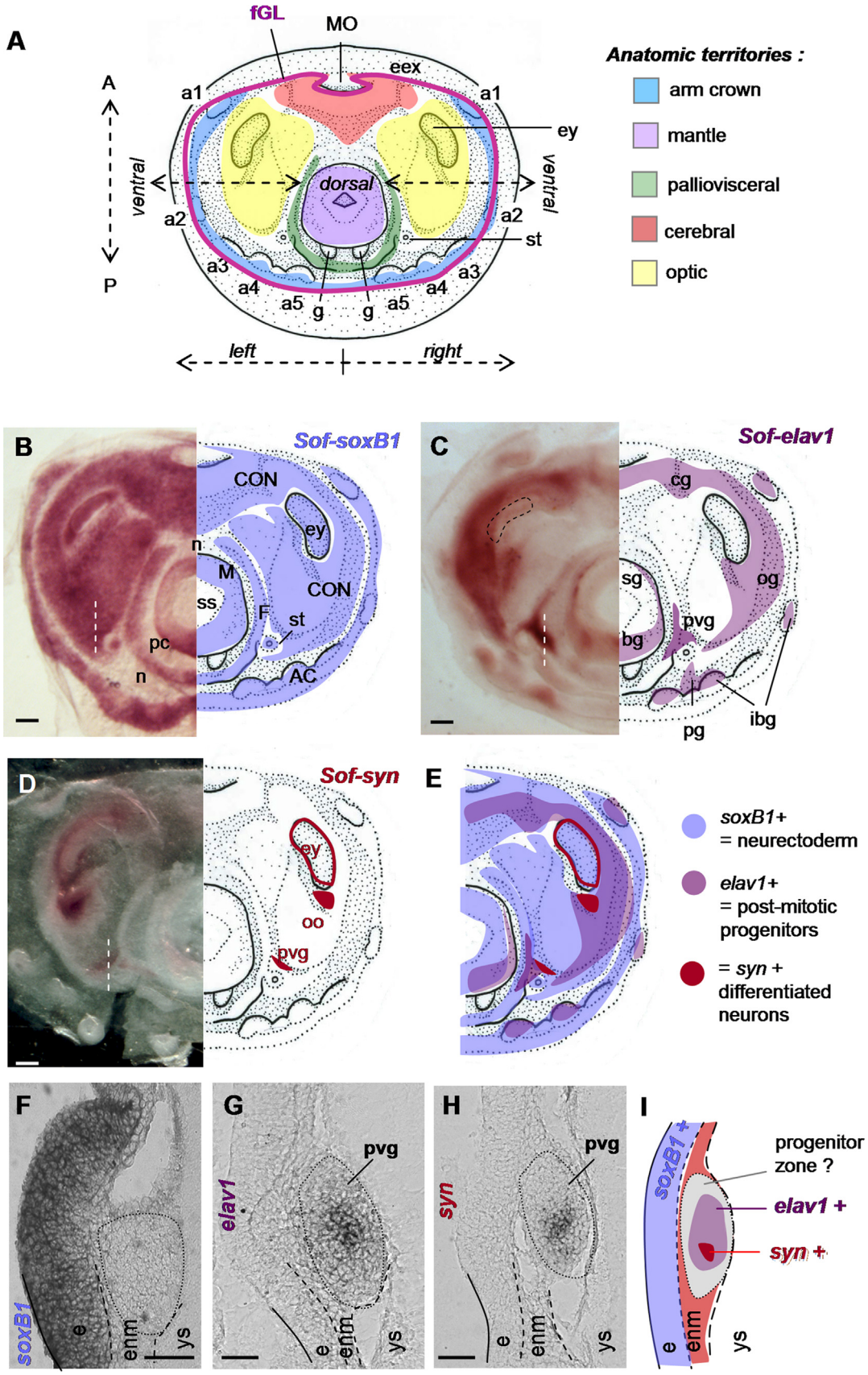


Fig. 1. Main features of *Sepia* embryogenesis and organogenesis. A – Main steps of the embryonic development of *Sepia officinalis*: first cleavage events (d1), gastrulation, and epiboly of the extra-embryonic ectoderm (eex) over the yolk syncytium (arrows, d10), first visible organs at the disc-shaped animal pole (early organogenesis, d15), mid-organogenesis when the animal disc stretches out (apical view, d20), and growth until hatching (late organogenesis, anterior view at d25 and d30). A black circle marks the mouth location in all drawings, and an empty circle marks the tip of the mantle in all drawings (not at the same scale). B, C – schematic representations of a cross-sectioned disco-blastula showing the establishment of germ layers at gastrulation stages (d10-d14; Boletzky et al., 2006). The peripheral blastocones migrate under the ectoderm plate (see arrows) and form a disc-shaped endo-mesoderm layer. After gastrulation (C), an extra-embryonic ectoderm (eex) starts growing from the gastrulation lip, covering the large uncleaved yolk syncytium (ys). D, E, F – schematic representations of *S. officinalis* animal pole (lateral left views) showing the main morphological changes of the animal pole between early (D), mid- (E), and late (F) organogenesis. Identical anatomical main territories are colored similarly: the mantle in purple, the palliovisceral area in green, the optic area in yellow and the arm crown in blue. In E and F, the mantle territory and the pallial cavity are represented in cross sections. An: anus; e: ectoderm; eex: 'extra-embryonic' ectoderm over the yolk syncytium; ey: eye; fAn: future anus; fGL: former gastrulation lip; g: gill; MO: mouth; pc: pallial cavity; s: shell; ss: shell sac; st: statocyst; ys: yolk syncytium. Orientation: A is for anterior, P is for posterior.

viscera and surrounding the pallial cavity) (Fig. 1E). The mouth (anterior) is eventually surrounded by the ventral arm crown, while the anus (posterior) that opens in the pallial cavity becomes more dorsal. At the end of organogenesis (from stage 24 to hatching), the animal pole reaches the juvenile shape (Fig. 1F). Throughout organogenesis, the arm crown narrows around the mouth so that the former gastrulation lip reduces into a small circle at the junction with the yolk sac, close to the mouth (Fig. 1E and F).

2.2. Early organogenesis and early neurogenesis

Early organogenesis corresponds to stages where a two-



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dimensional layout of organs starts emerging within the discoidal animal pole (stages 13–19). At the periphery of the early animal disc, the ten-arm primordia form a large "arm crown" territory (blue in Fig. 2A), shaped like a horseshoe, externally delimited by the fGL and only interrupted by the mouth opening (Fig. 2A). At the center of the animal disc is the mantle territory (purple in Fig. 2A). The center of the mantle invaginates early and forms the shell sac that will later secrete the internal calcareous shell. Around the mantle territory is the palliovisceral territory, also shaped like a horseshoe (green in Fig. 2A). Between the mantle and the mouth is the "cerebral territory" (red in Fig. 2A) in which the buccal and cerebral ganglia will later develop. Finally, territories between the palliovisceral territory and the arm crown will be part of the future head and are here called "optic territories" (yellow in Fig. 2(A) since they bear both eye placodes and will form the future optic lobes.

Using the neuroectodermic marker *soxB1* and the neural differentiation markers *elav* and *synaptotagmin*, we determined the spatial extent of the *S. officinalis* neuroectoderm. SoxB1 proteins belong to the SOX family of transcription factors widely known for their role in neural determination and specification in numerous phylogenetic lineages (Miyagi et al., 2009). Elav is an RNA-binding protein specific for proneural cells and post-mitotic differentiating neurons (Soller and White, 2004; Buresi et al., 2013, 2014). Synaptotagmin is a transmembrane protein required for synaptic vesicle trafficking, thus its detection indicates the existence of functional neurons (Poskanzer et al., 2003). During early organogenesis, *Sof-soxB1* is expressed in a large proportion of the embryonic ectoderm, divided into eight discrete territories: the whole arm crown, the mantle, the palliovisceral horseshoe, both eyes, both statocysts and a large surface including all cerebral and optical territories, called the "cerebro-optic neuroectoderm" (CON) (Fig. 2B and F). The *Sof-soxB1* negative ectoderm corresponds to the invaginating shell sac; the future pallial cavity between the palliovisceral horseshoe and the mantle, a few surfaces that will form the neck and the mouth (Fig. 2B). Under the neuroectoderm, some cell clutches of future ganglia show early differentiating neurons with *Sof-elav1* gene expression (Fig. 2C, G, and I): in each separate arm primordium (corresponding to the future intrabrachial ganglia), in the most peripheral part of the CON (future optic and cerebral ganglia), in the mantle territory (future stellate and branchial ganglia), and both palliovisceral ganglia which show the strongest staining (as already described in Buresi et al. (2013)). Finally, inside these clutches, a few neurons are differentiated and functional as shown by *Sof-synaptotagmin* gene expression: in the eyes, the olfactory organs and the palliovisceral ganglia (Fig. 2D, H, and I).

2.3. Expression of neural regionalization genes during early neurogenesis

To determine whether a neural mediolateral pattern is present in cephalopods and similar to that of vertebrate, insect or annelid model species, we analyzed the expression patterns of some of the neural regionalization orthologues during early neurogenesis stages in *S. officinalis* (stages 16–18). In addition to already published fragments of *Sof-pax6* (Navet et al., 2009) and *Sof-pax3/7* (Buresi et al., 2014), we cloned and sequenced fragments of putative *nkx2.1*, *pax2/5/8*, *msx* and *gsx* orthologues found in an embryonic EST library (Bassaglia et al., 2012). Orthologies were assigned by neighbor-joining analyses and phylogenetic constructions place all *S. officinalis* sequences within clades of Lophotrochozoa orthologues (Fig. S1; accession numbers: *Sof-nkx2.1*: KP867643; *Sof-pax2/5/8*: KP867644; *Sof-msx*: KP867645; *Sof-gsx*: KP867646).

In vertebrates, insects and annelids, the *nkx2.2* (*vnd*) gene specifies medial neurons (Briscoe et al., 1999; Wheeler et al., 2005; Denes et al., 2007) and is complemented by the median expression of its sister gene *nkx2.1* in the developing brain (Zaffran et al., 2000). In early neurogenesis of *Sepia*, only the *nkx2.1* gene was found. *Sof-nkx2.1* is expressed at the ventral and anterior periphery of the CON both in the cerebral and in the optic territories (Fig. 3A) and within the ectodermic layer (Fig. 3A1 and A2). No expression was found in the arms (Fig. 3A3), mantle, or palliovisceral territory (Fig. 3A).

In vertebrates and annelids, a broad *pax6+* progenitor domain laterally abuts the *nkx2.2+* domain (Ericson et al., 1997; Denes et al., 2007). Similarly, in the head, *nkx2.1* and *pax6* show complementary territories that specify the anterior and median part of the developing forebrain (Tessmar Raible et al., 2007). This aspect of early neural patterning seems to be conserved in *S. officinalis* where *Sof-pax6* presents a large ectodermic and mesodermic expression in the optic area (Fig. 3B and B1) and a more restricted expression in the cerebral area, at the ecto-mesodermic limit (Fig. 3B and B2). In the arms, *Sof-pax6* is expressed in the whole ectoderm (Fig. 3B and B3). However, *Sof-nkx2.1+* and *Sof-pax6+* territories are not strictly complementary in *S. officinalis*, since they clearly overlap next to the eyes at the periphery of the optic area (Fig. 3A and B).

Another feature of vertebrate and annelid neural patterning is the activity of *pax3* and *pax7* (*pax3/7* in non-vertebrates) in progenitor domains laterally overlapping *pax6* expression (Ericson et al., 1996; Goulding et al., 1993; Denes et al., 2007). We found *Sof-pax3/7* expressed at early stages in multiple domains within the animal pole (Fig. 3C). *Sof-pax3/7* is expressed in a central *Sof-pax6* positive region of the optic area (Fig. 3C1), in the ectoderm of the funnel and mantle (Fig. 3C2) and, for each arm, in the dorsal ectodermic surface (the ventral half that will develop suckers remained unstained) (Fig. 3C3).

Next, in the vertebrate neural plate, the domain of *pax3* and *pax7* laterally overlap with that of the *msx* genes, *msx1,* and *msx2* (Ramos and Robert, 2005). In early organogenesis of *S. officinalis*, *Sof-msx* is expressed in the developing branchial (Fig. 4A and A1) and stellate ganglia (Fig. 4A2) within the mantle area but not in the *Sof-pax3/7* positive mantle ectoderm (Fig. 3C2). *Gsx* is a parahox gene expressed in the developing cerebral ganglia of gastropod larvae (Samadi and Steiner, 2010). In vertebrates, *gsx* is expressed in a region overlapping that of *pax6* but in annelids, *gsx* is located in the *nkx2+* domain (Denes et al., 2007). In *S. officinalis*, *sof-gsx* shows discrete expression in the mesodermic tissue of

Fig. 2. Neuroectoderm development during early organogenesis of *Sepia officinalis*. A – main presumptive anatomical territories in the animal pole at stage 17. B – *Sof-soxB1* expression at stage 16 in eight distinct neuroectodermic territories: eyes, mantle, palliovisceral, statocysts, arm crown, and cerebro-optic (schematic drawing on right). C – *Sof-elav1* expression at stage 17 in the cerebro-optic neuroectoderm (CON) (future cerebral and optic ganglia), in the future stellate, branchial, palliovisceral, pedal and intrabrachial ganglia (schematic drawing on right). D – *Sof-syn* expression at stage 18 in the eye vesicle (border), the olfactory organ, and the palliovisceral ganglia (schematic drawing on right). E – schematic drawing with superimposed *Sof-soxB1*, *Sof-elav1*, and *Sof-syn* expression patterns in early organogenesis of *S. officinalis*. F – thin section through the CON of a stage 17 embryo hybridized against *Sof-soxB1*. G – thin section through the palliovisceral ganglion of a stage 17 embryo hybridized against *Sof-elav1*. H – thin section through the palliovisceral ganglion of a stage 18 embryo hybridized against *Sof-syn*. I – schematic drawing summarizing *Sof-soxB1*, *Sof-elav1*, and *Sof-syn* expressions at the palliovisceral ganglion level in early organogenesis of *S. officinalis*. a1, a2, a3, a4, a5: arms; A: anterior; AC: arm crown; bg; branchial ganglion; cg: cerebral ganglion; CON: cerebro-optic neuroectoderm; enm: endo-mesoderm; ey: eyes; F: funnel; fGL: former gastrulation lip; g: gills; ibg: intrabrachial ganglion; M: mantle; MO: mouth; og: optic ganglion; oo: olfactory organ; P: posterior; pg: pedal ganglion; pvg: palliovisceral ganglion; sg: stellate ganglion; st: statocysts; ys: yolk sac. Whole embryos are oriented with anterior up (A) and posterior down (P). Sections are oriented with anterior up. Scale bar is 200 μm.

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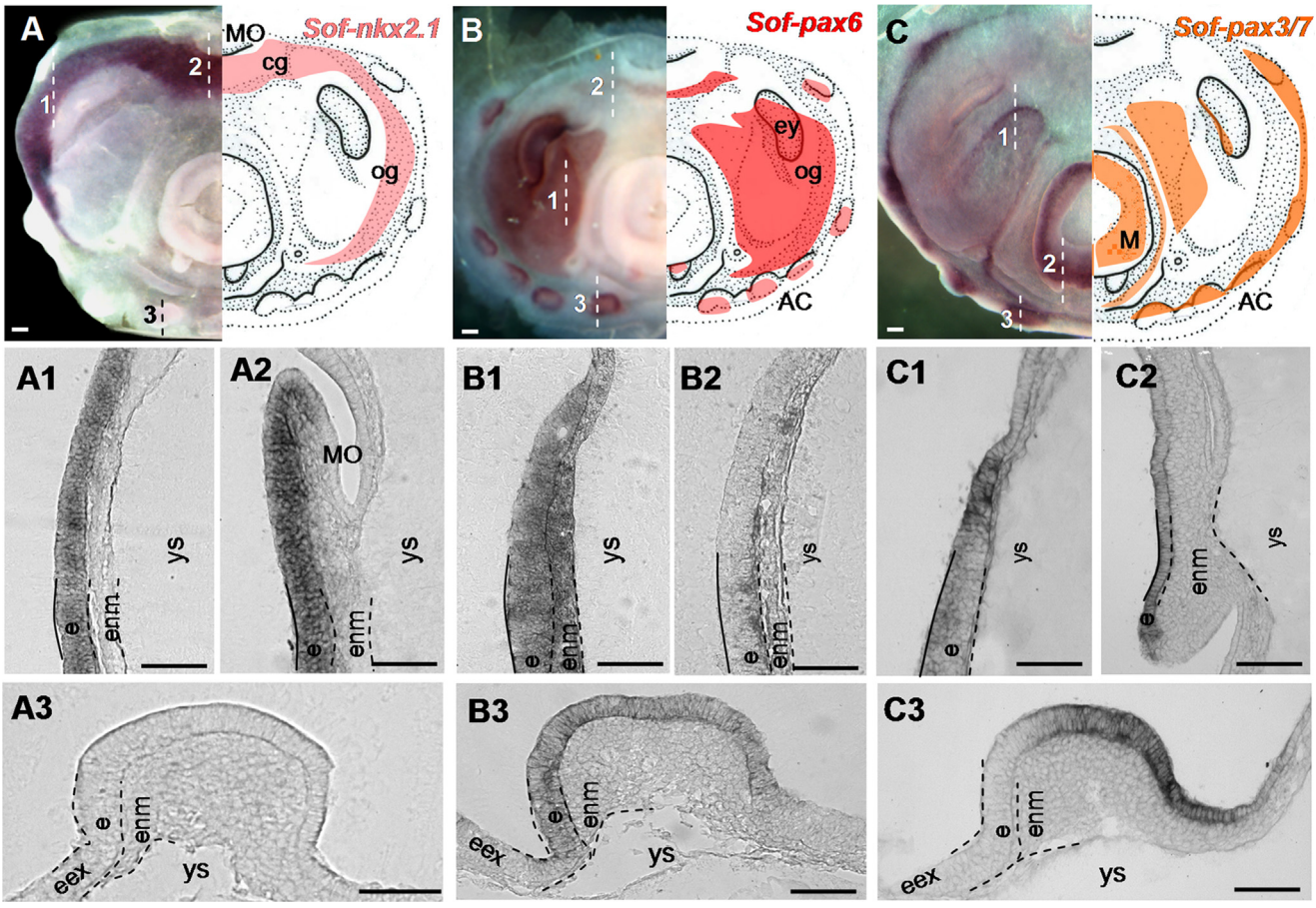

Fig. 3. Expressions of *Sof-nkx2.1*, *Sof-pax6*, and *Sof-pax3/7* during early organogenesis of *S. officinalis* (see Fig. 2 for anatomic territories and orientation). A – *Sof-nkx2.1* expression at stage 17 at the periphery of the animal pole (schematic drawing on right); A1: thin section at the lateral level (ectodermic expression); A2: thin section at the mouth level (ectodermic expression); A3: thin section at the arm 5 level (no expression). B – *Sof-pax6* expression at stage 17 in arms, optic area, and cerebral area (schematic drawing on right); B1: thin section at the optic level (ectodermic and endo-mesodermic expression); B2: thin section at the cerebral level (expression at the ecto-mesodermic boundary); B3: thin section at the arm 5 level (ectodermic expression). C – *Sof-pax3/7* expression at stage 17 in arms, optic area, and mantle (schematic drawing on right); C1: thin section at the optic level (ectodermic expression); C2: thin section at the mantle level (ectodermic expression); C3: thin section at the arm 5 level (ectodermic expression in the half most anterior territory). AC: arm crown; cg: cerebral ganglion; e: ectoderm; enm: endo-mesoderm; eex: extra-embryonic ectoderm; ey: eye; M: mantle; MO: mouth; og: optic ganglion; ys: yolk syncytium. Whole embryos are oriented with anterior up (mouth) and posterior down. Sections are oriented with anterior up (or right for arm sections). Scale bar is 200 μm.

cerebral (Fig. 4B, B1, and B3) and posterior optic area (olfactory region and palliovisceral region) (Fig. 4B2) that both express *Sof-pax6* (Fig. 3B1 and B2).

In vertebrates and annelids, *pax2/5/8* is a gene whose lateral expression overlaps *pax3/7* and *pax6* domains (Burrill et al., 1997). In *Haliotis asinina* (Gastropoda), *pax2/5/8* is expressed in the statocysts and in the mantle area (O'Brien and Degnan, 2003). The *Sepia pax2/5/8* gene is also expressed in the mantle ectoderm (Fig. 4C and C1), funnel tube ectoderm, ectoderm over the eye vesicle (Fig. 4C3), and in most of the posterior mesodermic tissues in the arms (Fig. 4C2). Therefore, *Sof-pax2/5/8* expression overlaps *Sof-pax3/7* only in the mantle ectoderm, and overlaps *Sof-pax6* only in the eye.

2.4. Patterns of regionalization genes during mid and late organogenesis

In model species, regionalization genes of the mediolateral system determine specific functional regions and specific neuron subtype differentiation; *nkx2* genes mostly specify motoneurons, *pax6* genes are expressed in catecholaminergic areas, and *pax3/7* is mostly involved in sensory neuron differentiation (Denes et al., 2007). To determine whether such a relationship is conserved in cephalopods, we investigated the expression patterns of *Sof-nkx2.1*, *Sof-pax6* and *Sof-pax3/7* during mid- (stages 20–23) and late (stage 24 to hatching) organogenesis.

During the mid-organogenesis phase, most ganglia are well circumscribed but still not connected (Buresi et al., 2013; Fig. 5A). Later, in late organogenesis phase, some of the ganglia fuse and form the brain; both cerebral ganglia form the supra-esophageal brain mass (future vertical lobes involved in memorization and learning), both pedal ganglia form the most anterior part of a sub-esophageal brain mass (ASM), and both palliovisceral ganglia form the median and posterior parts of the same mass (MSM, PSM) (Fig. 5E). During mid and late organogenesis, *Sof-nkx2.1* is expressed in the cerebral ganglia in the most anterior part of the optic lobes, and in some ectodermic tissues of the head (Fig. 5B, B1, and F). *Sof-pax6* remains expressed in the cerebral and peripheral tissues of the optic area (Fig. 5C, C1, and G). *Sof-pax3/7* is expressed in the ectodermic layers of the mantle, funnel, head and arms (Fig. 5D, D1, and H).

3. Discussion

The objective of this study was to evaluate whether a mediolateral pattern could be found in cephalopods, like in model species in Chordates, insects and other Lophotrochozoa such as annelids (Denes et al., 2007). We looked at the earliest stage we could study in *Sepia officinalis*

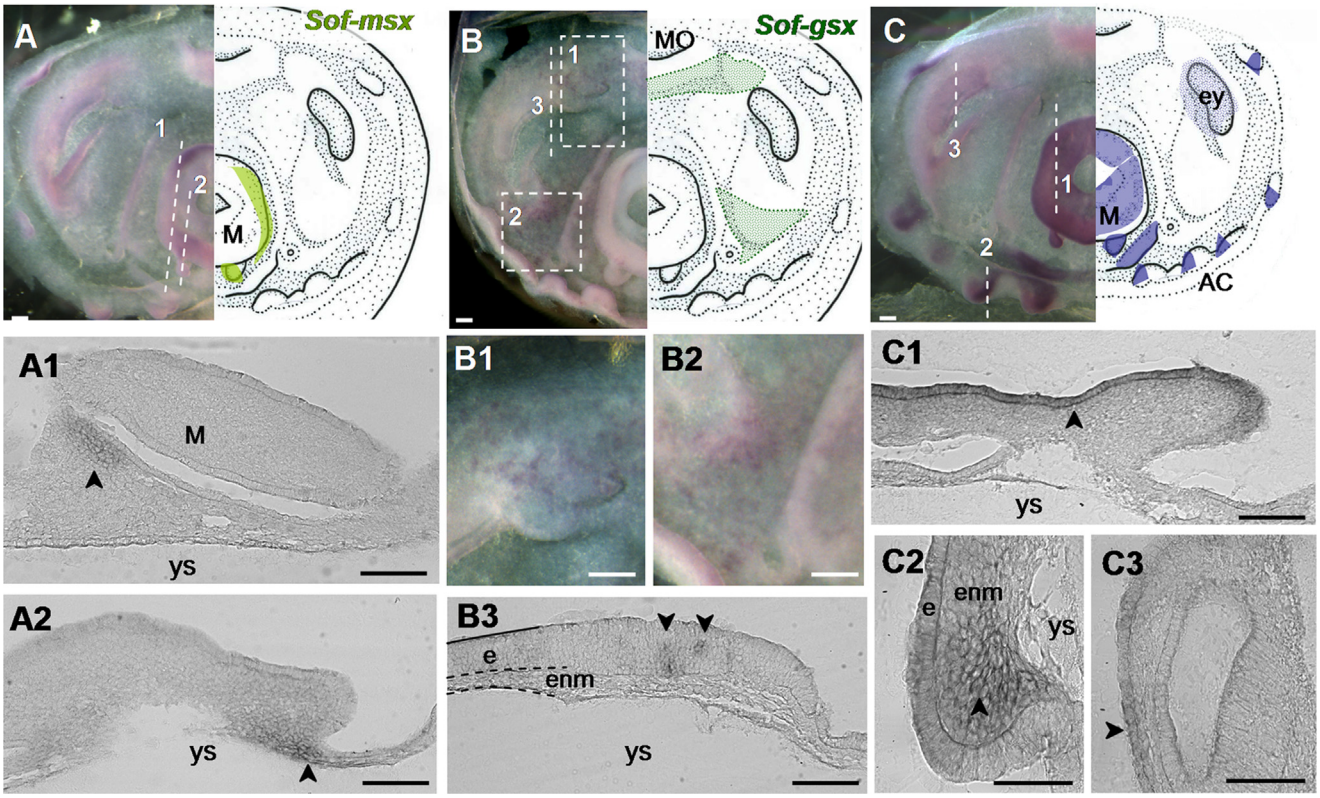

Fig. 4. Expressions of *Sof-gsx*, *Sof-msx* and *Sof-pax2/5/8* during early organogenesis of *S. officinalis* (see Fig. 2 for anatomic territories and orientation). A – *Sof-msx* expression at stage 18. Staining in sections is only detectable in the mantle area, coloration in eyes and mouth is likely background; A1: thin section at the gill level; A2: thin section at the mantle level (endo-mesodermic expression). B – *Sof-gsx* expression at stage 18 in optic and cerebral areas (schematic drawing on right); B1: detail of the cerebral expression; B2: detail of the optic expression; B3: thin section at the optic ganglion territory level (discrete ectodermic expression). C – *Sof-pax2/5/8* expression at stage 18 in arms, eyes, mantle and funnel (schematic drawing on right). The pink color in eyes and around the mouth is background: no staining is visible through sections; C1: thin section at the mantle level (ectodermic expression); C2: thin section at the arm 5 level (mesodermic expression in the half most median part); C3: thin section at the right eye level (ectodermic expression). AC: arm crown; e: ectoderm; enm: endo-mesoderm; ey: eye; M: mantle; MO: mouth; ys: yolk syncytium. Whole embryos are oriented with anterior up (mouth) and posterior down. Sections are oriented with anterior up or right (for arm pictures). Scale bar is 200 μm.

embryos (i.e. in early organogenesis from stage 15) to describe the neuroectodermic anlage and investigated how neural regionalization genes were expressed. The neuroectoderm in *S. officinalis* embryos corresponds to a very large proportion of the animal pole ectodermic surface, as shown by the very few ectoderm surfaces that did not express *Sof-soxB1* (Fig. 2B), a marker of early neuroectodermic determination in many Metazoa (Miyagi et al., 2009). The neuroectoderm is much larger than what we expected from earlier histological studies that described nervous placodes in other cephalopod embryos (Marquis, 1989; Shigeno et al., 2001; Yamamoto et al., 2003). However, since the adult epidermis houses many isolated sensory cells (Baratte and Bonnaud, 2009), it makes sense that a large part of the embryonic ectoderm possesses neurogenic capacities and expresses SoxB1. *Sof-elav1* is a marker of differentiating neurons. Its expression in *S. officinalis* revealed that the developing nervous structures comprise large surfaces of this neuroectoderm (Fig. 2C), which is congruent with recent finding in *Idiosepius* showing that the elements of the future CNS in cephalopods are organized as concentric cords (Shigeno et al., 2015). Finally, differentiation of neurons may appear early in some ganglia (*Sof-syn* expression, Fig. 2D, and I), which is consistent with earlier studies in *S. officinalis* that detected neurons in early stages of organogenesis (Baratte and Bonnaud, 2009; Buresi et al., 2013; 2014). These data provide the molecular confirmation that the NS of cephalopods does not emerge from a unique anlage but from multiple and non-contiguous territories like in previously studied molluscs (Bullock and Horridge, 1965; Jacob, 1984; Budelmann, 1995; Shigeno et al., 2015).

With the noticeable exception of the arm crown, where gene expression patterns are distributed in each arm bud (see below), the expression patterns concern large areas of the animal pole and are not restricted to one organ (Fig. 6A); the cerebral and optic areas of the CON (for *Sof-nkx2.1*, *Sof-pax6*, *Sof-gsx* and *Sof-pax3/7* genes) and the palliovisceral and mantle territories (for *Sof-msx*, *Sof-pax3/7* and *Sof-pax2/5/8* genes). The expression of NS regionalization genes inside the neuroectoderm appears to be clearly concentrically arranged, paralleling the concentric anatomical territories (Fig. 6A). The *Sof-nkx2.1* gene expression pattern is peripheral to the arm crown (ventral and anterior) (Fig. 3A). In other Lophotrochozoa, *nkx2.1* also shows an anterior but no ventral expression (*Leptochiton asellus* larvae, Vöcking et al., 2015; *Terebratalia transversa* Brachiopoda, Santagata et al., 2012). *Sof-pax6* expression has a medial position, occupying most of the CON region (including both optic and cerebral areas, Fig. 3B). This is consistent with expression in other cephalopods (Navet et al., 2009; Hartmann et al., 2003) and in chiton larvae (Vöcking et al., 2015). The *Sof-pax3/7* and *Sof-pax2/5/8* genes are expressed in central parts of the early embryo (dorsal pole) the palliovisceral and mantle territories (Figs. 3C and 4C). In early steps of organogenesis, *pax2/5/8* is expressed in the mantle territories of cephalopods (in *Sepia* and in *I. notoides*, Wollesen et al., 2015a), chitons and bivalvia (Wollesen et al., 2015a), but also in the funnel of cephalopods (present study, Wollesen et al., 2015a). *Gsx* gene expression patterns in *Sepia officinalis* also show some consistencies with other molluscs where it has been studied: it is expressed in cerebral and visceral areas (cephalopod *I. notoides* and scaphopod *Antalis entalis*: Wollesen et al., 2015b; gastropod *Gibbula varia*, Samadi and Steiner, 2010). Here in *S. officinalis*, for the first time in molluscs, all these genes have been studied at the same stages, in the same species, which allows comparing their different pattern

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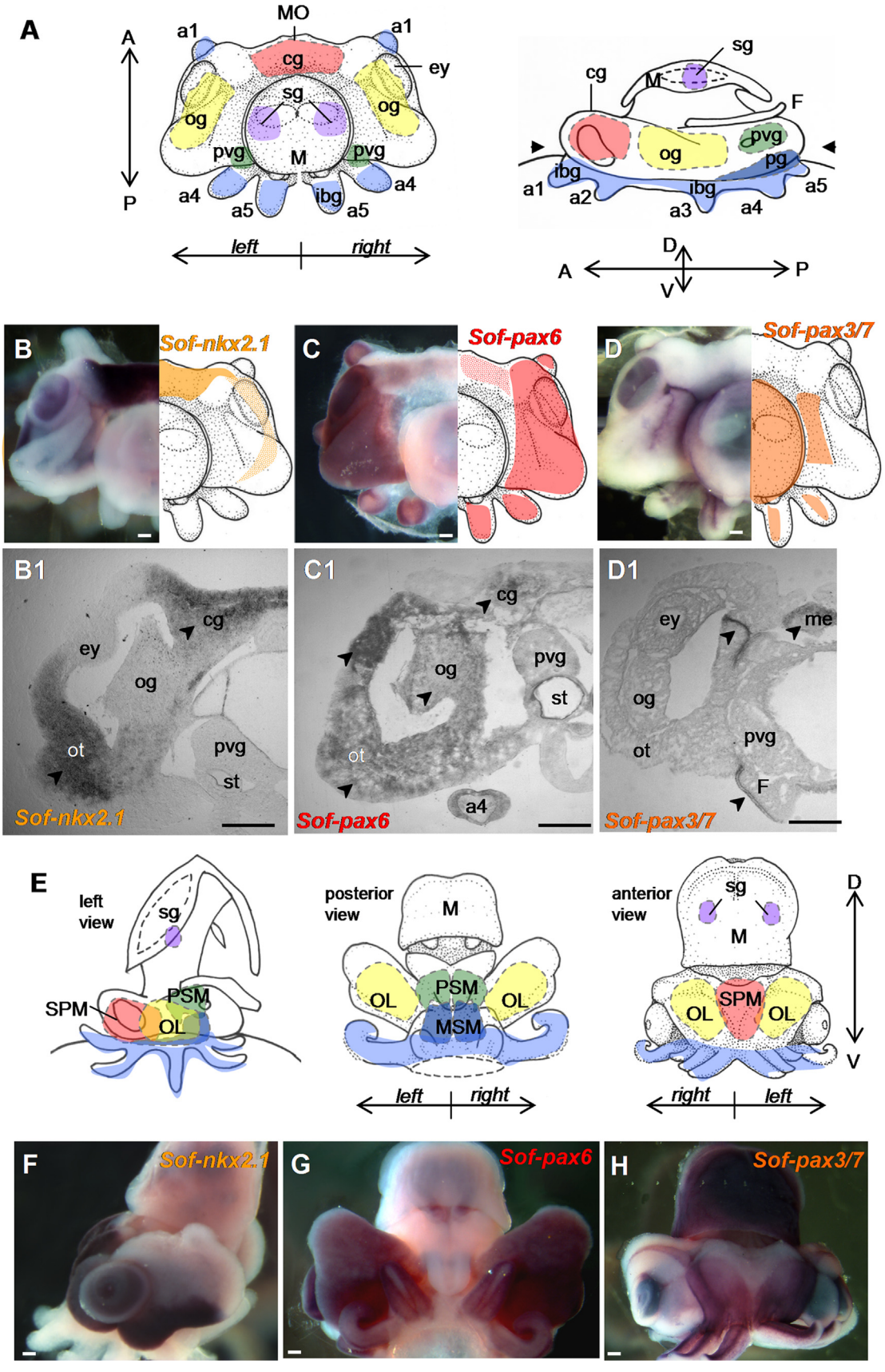

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of expressions.

3.1. A molecular mediolateral architecture in S. officinalis?

The whole scheme of gene expression patterns is clearly complex (see the summary in Fig. 6A). However, despite the flat and concentric organization of early *S. officinalis* embryos, it remains possible to distinguish a ventral-to-dorsal axis: the ventral pole is located at the periphery and the dorsal pole is central. As compared to model species, this axis could also correspond to the medio-lateral axis (delineated by two dashed arrows in Fig. 6A, for the left and right sides). This axis begins at the arms (ventral), runs across the large CON region, through the funnel structures, and then ends in the mantle nervous region. Along this axis, on both sides of the line of bilateral symmetry, a sequence of gene expression can be found that interestingly parallels the sequence order found in the annelid nervous trunk (Fig. 6B) and the vertebrate neural plate (Fig. 6B); namely from periphery to center: *nkx2*, *pax6*, *gsx*, *msx* and other *pax* genes. Some topological similarities can even be found; *Sof-pax2/5/8* and *Sof-pax6* expression patterns do not overlap like in vertebrates, where mutual coordinated repression between *pax6* and *pax2,* or *pax5* and *pax8* regulate the development of interneurons (Bel-Vialar et al., 2007; Burrill et al., 1997). In vertebrates and annelids, *pax3* and *pax7* expressions largely overlap with *pax6* expression (Pattyn et al., 2003; Denes et al., 2007) and we observed the same for *Sof-pax3/7* and *Sof-pax6*. Some differences were noticed as well. For instance, *Sof-nkx2.1* and *Sof-pax6* positive regions overlap in *S. officinalis*, which is similar to insects (Kammermeier et al., 2001), but is not the case in vertebrates or annelids (Ericson et al., 1997; Denes et al., 2007).

The overall similarity suggests that, within Lophotrochozoa, the mediolateral system found in model species is not only present in annelids (Denes et al., 2007) but also at least in cephalopods. This demonstrates that mediolateral organization may also concern non-metamerised animals and animals with a NS made of multiple gangliogenic regions (Jacob, 1984). A first explanation could be the recruitment of the same set of genes for neural regionalization in cephalopods. However a more tempting possibility could be that the mediolateral system has been inherited from an ancestral Urbilateria scheme (see Fig. 6B) (Tosches and Arendt, 2013). If so, this implies that the mediolateral has closely accompanied the emergence of the specific NS development of cephalopods and should be also detectable in other molluscan classes, which has not been done yet. For instance, in cephalopods, the huge development of the optic function may explain the large expression area of the *Sof-pax6* gene in the CON territory (Figs. 3B and 6A).

3.2. A specific molecular signature in arms

Another important observation is the peculiar molecular signature of the arm crown. First, whereas the arm crown corresponds to the most peripheral circle of the animal pole (ventral side), *Sof-nkx2.1* is not expressed in arms but remains restricted to a less peripheral part (Fig. 3A). If a mediolateral organization seems to exist in cephalopods, the arm crown is definitely not included. Second, most of the regionalization genes we studied show an iterated expression in the arm crown, following the same pattern in each arm regardless of its location within the embryo. No difference in gene expression patterns was found between arm 1 (the most anterior arm), arm 4 (the future tentacle), or arm 5 (the most posterior arm): *Sof-pax6* is expressed in the whole arm ectoderm (Fig. 3B3), *Sof-pax3/7* is expressed in the dorsal half of the arm ectoderm (Fig. 3C3) and *Sof-pax2/5/8* is expressed in the posterior half of the arm mesoderm (Fig. 4C2) (consistent with expression in *I. notoides*, Wollesen et al., 2015a). Arms are acknowledged to be homologous to the molluscan foot (Brooks, 1880; Naef, 1928; Raven, 1966; Boletzky, 1988; Shigeno et al., 2008) and the comparisons of gene expression patterns with the foot of other molluscs show some consistencies with our own results in *S. officinalis*. In the chiton *Leptochiton asellus,* the ventral region of the foot shows no *nk2.1* expression. *Pax2/5/8* is also found in the developing foot of the *Haliotis asinina* (gastropod, O'Brien and Degnan, 2003), in the lateral borders of the foot area in the chiton *Acanthochitona crinita* (Wollesen et al., 2015a), and lacking in the bivalve *Nucula tumidula* (Wollesen et al., 2015a). The evolution of the foot among molluscs and more specifically the origin of arms in cephalopods (head or foot origin) are long-held questions (Boletzky, 2003; Shigeno et al., 2008; Shigeno et al., 2010). The gene expression patterns we obtained here can hardly elucidate these questions, however the occurrence of a posterior *pax2/5/8* expression in each arms (even in the most anterior arms) could at least suggest a posterior or pedal origin of the arm crown rather than an anterior or head origin.

3.3. A conserved mediolateral patterning but modified by the egg yolk-richness

If the global mediolateral sequence seems conserved in *S. officinalis*, its polarity is surprisingly reversed. Actually, if we first consider the line of symmetry of the embryo as the midline of the whole neuroectoderm and the periphery of the embryo as its lateral side, all the mediolateral sequences show a mirrored order as compared to model species (see Fig. 6B): median markers (like *nkx2.1*) are unexpectedly expressed at the periphery of the embryo and lateral markers (like *Pax3/7*) are expressed at its central side. In fact, this reversion can be explained by the specificities of the cephalopod gastrulation due to the yolk-richness of their eggs.

During the development of insects, annelids, and vertebrates, it is acknowledged that the neural midline originally emerges from the median fusion of the blastopore lips after gastrulation (*nkx2* genes are expressed close to the gastrulation edge) (Arendt and Nübler-Jung, 1997) and that across bilaterians the mediolateral

Fig. 5. Expressions of *Sof-nkx2.1*, *Sof-pax6* and *Sof-pax3/7* during mid and late organogenesis of *S. officinalis.* A – Main nervous territories (ganglia) at mid-organogenesis in *S. officinalis* (stage 20–23): apical (i.e. dorsal) view of the animal pole (left drawing) and left lateral view (right drawing). Arrowheads point out the level of thin sections in b', c' and d'. B – *Sof-nkx2.1* expression at stage 21 at the cerebral level and in the cerebral tissues above the arm crown (schematic drawing on right); B1: thin section at the cerebral, optic and palliovisceral level with staining in the optic and cerebral tissues (arrowheads). C – *Sof-pax6* expression at stage 20 at the optic and cerebral level (schematic drawing on right); C1: thin section showing expressions in left eye, optic ganglion, cerebral and optic tissues (arrowheads). D – *Sof-pax3/7* expression at stage 21 at the mantle level (schematic drawing on right); D1: thin section at the cerebral, optic and palliovisceral level showing staining in the epidermis of the funnel, the mantle and head (arrowheads). E- Main nervous territories (brain lobes and peripheral ganglia) during late organogenesis in *S. officinalis* (stage 24 to hatching): left lateral, posterior and ventral views. The supra-esophageal mass (SPM), is derived from both cerebral ganglia, the subesophageal mass is made from the former pair of pedal ganglia (anterior sub-esophageal mass, ASM) and of palliovisceral ganglia (median and posterior subesophageal mass, MSM and PSM). F – left lateral view of a stage 24 embryo hybridized against *Sof-nkx2.1* showing staining of the anterior cerebral tissue. G – posterior view of a stage 24 embryo hybridized against *Sof-pax6* showing staining of the cerebral and optic tissue. H – anterior view of a stage 24 embryo hybridized against *Sof-pax3/7* showing staining of the mantle and head epidermis. a1, a2, a3, a4, a5: arms; A: anterior; cg: cerebral ganglion; D: dorsal; ey: eye; F: funnel; ibg: intra-brachial ganglion; M: mantle; me: mantle edge; MO: mouth; MSM: median sub-esophageal mass; og: optic ganglion; OL: optic lobe; ot: optic tissue; P: posterior; pg: pedal ganglion; PSM: posterior sub-esophageal mass; pvg: palliovisceral ganglion; sg: stellate ganglion; SPM: supra-esophageal mass; V: ventral. Scale bar is 200 μm.

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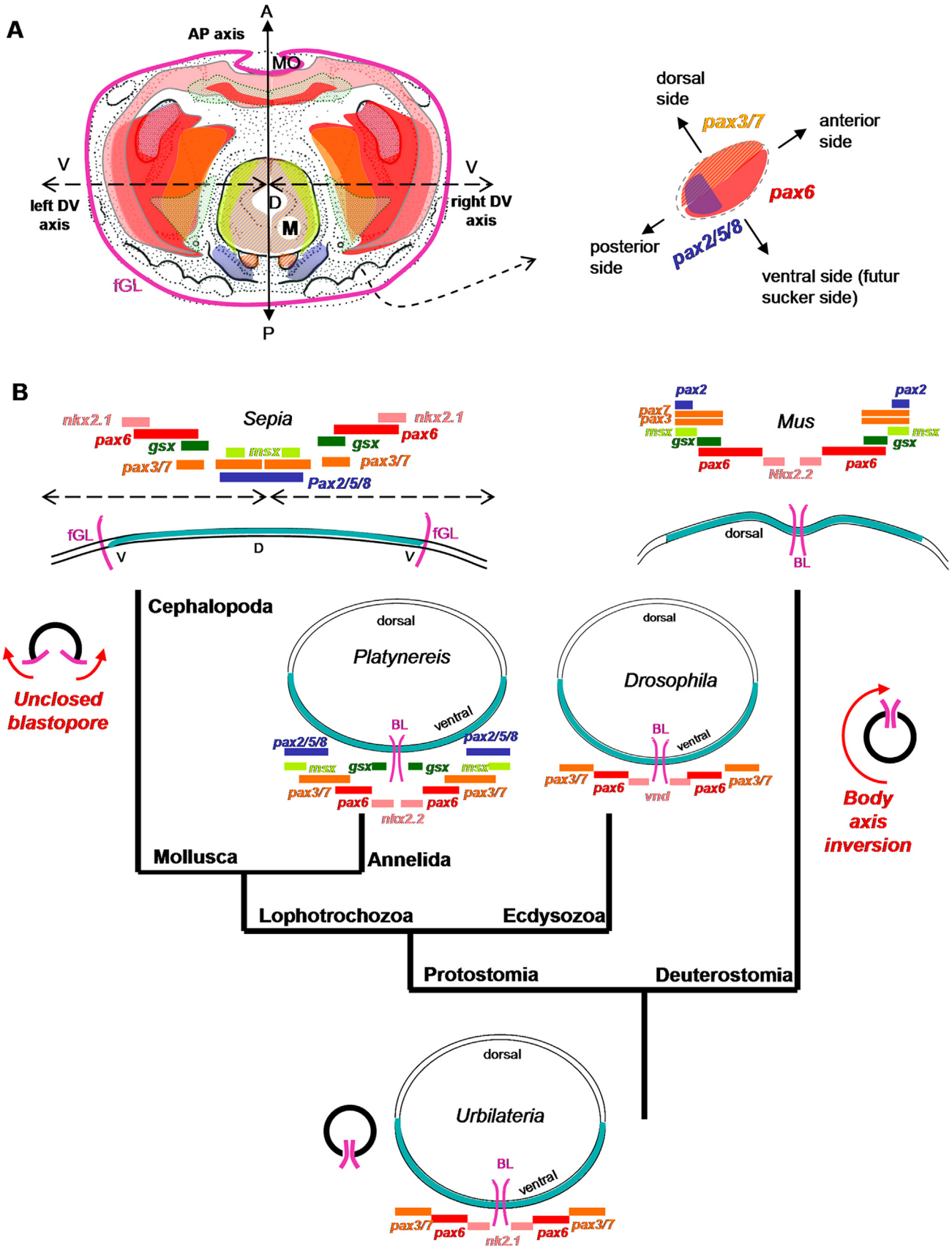

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system is active alongside this line of fusion (blastoporal patterning hypothesis) (Tosches and Arendt, 2013). In molluscs with a trochophore stage, the blastopore fusion line corresponds to the midline of the ventral surface and is the region where the foot develops (Raven, 1966). In cephalopods, it appears that the large amount of yolk prevents the fusion of the blastopore lips (at the site of the intra-embryonic internalization of mesoderm) and leaves them "wide open" in the early stages of organogenesis (see the fGL pink circle on Fig. 1). One evidence for this is that the arm crown is split into two lateral halves (the two peripheral rows of arms). The blastopore lips are closed only when the arm crown later constricts around the thin stalk of the outer yolk sac, next to the mouth (see Fig. 1D–F). A similar rearrangement of gastrulation has been observed in birds and other amniotes with yolk-rich eggs (Arendt and Nübler-Jung, 1999). In these species, the blastopore divides into two functional parts; one as the site of mesoderm internalization (the intra-embryonic blastopore) and another as the site of ectodermal epiboly, called an extra-embryonic blastopore (similar to the extra-embryonic ectoderm covering the yolk in *S. officinalis*) (Arendt and Nübler-Jung, 1999).

Because of the large amount of yolk in cephalopod early embryos, the fGL stays therefore unclosed at the animal pole periphery, i.e. at its ventral pole (see Fig. 1), so the putative neural midline will not be found at the bilateral symmetry line but at its periphery. With this in mind, there is no more apparent reversion: median markers for nervous system regionalization, such as *nkx2.1*, are expressed at the periphery (consistently next to the gastrulation line) and lateral markers, such as *pax3/7* and *pax2/5/8*, are expressed in more central embryonic territories (consistently far from the gastrulation line). These results also confirm – by means of molecular tools – that the ventral pole in the embryological orientation of cephalopods is rightly located at the arm side, as commonly acknowledged in all former studies (Naef, 1928; Boletzky, 2003; Lee et al., 2003; Shigeno et al., 2008, 2010; Wollesen et al., 2015a).

3.4. Mediolateral architecture and neural subtype's determination

As development proceeds, the expressions of nervous regionalization genes remain unchanged at the histological level (Fig. 5). As the animal pole acquires its definitive shape, peripheral expression patterns become ventral (*Sof-nkx2.1* and *Sof-pax6* are located ventrally in the head, Fig. 5B, C, F, and G), while central expression patterns become more dorsal (*Sof-pax3/7* is located dorsally in the head and in the mantle Fig. 5D and H). In vertebrates, insects and annelids, these gene expressions delineate a bilateral sequence of molecular regions centered along the neural midline that specifies different subsets of neurons. In vertebrates as well as in annelids and insects, *nkx2.1+* regions are associated with a medial contractile-motor region (serotonergic motor and interneurons) (Briscoe et al., 1999; Schmidt and Jordan, 2000; Pattyn et al., 2003; Denes et al., 2007). In *S. officinalis*, the *Sof-nkx2.1+* regions also correspond to the most ventral part of the head containing the motor region of the arms (the brain lobes issued from the pedal ganglia: ASM and MSM, Fig. 5B and F). These parts of the brain correspond to the lobes where the somatic motoneuron marker *mnx1* is expressed in *S. officinalis* (Nomaksteinsky et al., 2013). In vertebrates and annelids, the *pax6* progenitor domain is associated with cholinergic somatic neurons (Ericson et al., 1997; Denes et al., 2007). In *S. officinalis*, *Sof-pax6* is expressed in cerebral and optic regions, which is consistent with *pax6* visual functions in most Metazoa (Navet et al., 2009), but also corresponds to the most cholinergic-rich regions of the cephalopod CNS; the optic lobes (derived from the optic region) and the supra-esophageal mass of the brain (issued from the cerebral area) (Florey and Winesdorfer, 1968; Messenger, 1996). In vertebrates, *pax3* and *pax7* are expressed at the neural edge of the early neural plate preceding neural tube closure (Basch et al., 2006; Otto et al., 2006), where they dorsalize cells that will form sensory neurons and interneurons (Goulding et al., 1991). In annelids, the *pax3/7+* region is associated with a lateral sensory-integrative region (sensory and interneurons) (Denes et al., 2007) and this is also the case in *S. officinalis* (Buresi et al., 2014). Beyond the apparent conservation of the mediolateral molecular architecture, a similar mechanism of neuronal subtype determination seems to have been conserved- or recruited- in the evolution of the mollusc's and cephalopod's NS.

4. Conclusions

The idea of a yolk mass deforming the embryonic territories and splitting the arm territory into two halves parts is a very old idea (Brooks, 1880). More than 100 years later, we see that this developmental specificity has real consequences on molecular signatures: markers of the neural midline (supposed to be organized close to the blastopore line) have become lateral, and markers of the lateral neural territories have become central in the discoidal and flattened topology of the cuttlefish early embryo. Going beyond this specificity, our results show good evidence for a global conservation of the mediolateral system of model species in cephalopods. Surprisingly, this architecture does not concern specific ganglionic territories (except for each arm bud), but involves large parts of the neuroectoderm within the whole animal pole (Fig. 6).

It has been recently showed that the anterior-posterior sequence of *otx-pax2/5/8-Hox* genes can be found in cephalopods (in *I. notoides*, Wollesen et al., 2015a). In *S. officinalis*, *otx* expression in mid- and late organogenesis is restricted to subesophageal masses of the brain (anterior) (Buresi et al., 2012) and *Sof-pax2/5/8* in some more posterior masses of the brain (interbasal lobe and ASM, data not shown). In the mollusc family, the occurrence of *pax2/5/8* expression in the nervous system of a gastropod (*Gibbula varia*) (O'Brien and Degnan, 2003) but not in any stage in the developing nervous

Fig. 6. Overview of the expression patterns during early organogenesis in *S. officinalis* (A) and illustration of a conservative evolutionary scenario (B). A – Overview of the expression patterns of mediolateral nervous regionalization genes in early organogenesis embryos. On the left: the drawing sum up the different expression patterns from Figs. 3 and 4, using the same color codes. In the mantle and gills, overlapping expressions are figured by hatched colors. In the cerebro-optic neuroectoderm (CON), expressions are figured by transparent colored surfaces. On the right: detail of the expressions in arm buds (apical view). The expression patterns are similar regardless the location of the arm. *Sof-pax3/7* is expressed in the ectoderm of the dorsal side (overlapping *Sof-Pax6*), *Sof-pax6* is expressed in all the ectoderm and *Sof-pax2/5/8* is expressed in the mesoderm of the posterior half side. B – Comparison of mediolateral gene expression patterns in model species and in *Sepia officinalis* (sequence of gene expression along the Dorso-Ventral (DV) axes, dashed double arrows in A). The global similarity of mediolateral expression patterns in *Mus musculus*, *Platynereis dumerilii* and *Drosophila melanogaster* support the idea of an ancestral mediolateral scheme inherited from the Urbilateria ancestor (gene expression data from Denes et al. (2007); Arendt et al. (2008)). The mediolateral gene expression patterns are arranged into two symmetrical domains at both side of the neural midline, which corresponds to the ventral line of the blastopore lip (BL) fusion (Tosches and Arendt, 2013). Similar ventral patterning in annelids and *Drosophila* could be inherited from the ancestral orientation while, in vertebrates, the dorsal position of the neuroectoderm would be due to a global "body axis inversion" (Arendt and Nübler-Jung, 1994). In *S. officinalis*, the sequence is similar but the orientation appears inverted. We propose that the yolk-richness of the egg prevents the ventral blastopore lips (fGL) from being fused, which maintains the neural midline, and then the median markers *nk2.1*, at the periphery of the animal pole (hypothesis of "unclosed blastopore"). The branch lengths of the phylogenetic tree have no evolutionary meaning. BL: blastopore lip; fGL: former gastrulation lips (pink line); M: mantle; MO: mouth.

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system of polyplacophoran *Acanthochitona crinita* or of the basal bivalve *Nucula tumidula* remind that all is not clear-cut and that multiple events of functional loss or functional recruitment certainly may have complicated the whole evolutionary history.

To evaluate whether the mediolateral system in cephalopods is a conserved or a recruitment feature, similar studies are now required in other mollusc species. The expressions we obtained are often consistent with some results in other gastropods, chitons, and bivalves (O'Brien and Degnan, 2003; Samadi and Steiner, 2010; Wollesen et al., 2015a, 2015b). However, each time an intellectual difficulty appears: we compare expressions found in a cephalopod embryo to expressions in larvae (trochophore or veliger) without knowing if this comparison between different stages of development is homologically relevant or not. Therefore, we believe that multiplying the number of gene expression comparisons between different molluscs species and stages should allow great progress to uncover this issue in molluscs.

5. Material and methods

5.1. Animal care and staging of embryos

Fertilized egg batches of *Sepia officinalis* were collected in the English Channel from the marine stations at Luc-sur-Mer (Université de Caen Basse-Normandie) or at Roscoff (Sorbonne Universités, UPMC) between April and September and kept at 18 °C in oxygenated artificial sea water (Instant ocean) in the laboratory. Specimens were sampled daily to obtain a complete collection of different stages over the approximately 30 days of development. For embryos of stage 15 and older, the darkly pigmented egg capsule and the chorion were removed by dissection in seawater. Embryos were anesthetized in 4 °C sea water for 10 min and then fixed in 3.7% paraformaldehyde (PFA) in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) overnight at room temperature. After viewing with a stereo dissecting microscope to determine stages of development according to Lemaire (1970), embryos were washed three times for 5 min each in PBS, dehydrated in baths of increasing glycerol concentration and then stored at −20 °C in 30% glycerol in PBS. Before stage 15, the chorion is closely attached to the animal pole and cannot be removed without releasing the yolk pressure or damaging the embryo. For now, this technical limitation prevents any in situ hybridization for these early stages. All animal procedures were in compliance with the guidelines of the European Union (directive 86/609) and the French law (decree 87/848) regulating animal experimentation. All efforts were made to minimize animal suffering and to reduce the number of animals used.

5.2. Isolation of Sepia gene fragments

Some of the genes in this study have been previously described: *Sof-pax6* (Navet et al., 2009), *Sof-elav1* (Buresi et al., 2013), *Sof-syn* (Nomaksteinsky et al., 2013) and *pax3/7* (Buresi et al., 2014). For *Sof-soxB1*, a GenBank sequence (KC545795.1) was used to design specific primers for PCR (Forward: 5′-tcactcgacattgttagacac-3′; Reverse: 5′-attgcgcgcaaacgtttcgct-3′). Finally, for *Sof-pax2/5/8*, *Sof-msx*, *Sof-gsx* and *Sof-nkx2.1,* fragments of mRNA were identified in an embryonic EST library of *S. officinalis* (Bassaglia et al., 2012). The orthology of each of these sequences was determined using phylogenetic analyses (Mega5.2 software, Fig. S1) and specific primers were designed for PCR amplification (*Sof-pax2/5/8*: Forward: 5′-acctaaccacagcgtaccgt-3′; Reverse: 5′-gaccatgtttgcctgggaga-3′; *Sof-msx*: Forward: 5′-agcgaaaattcaggacgaaa-3′; Reverse: 5′-tggtagatggcgctgttgta-3′; *Sof-gsx*: Forward: 5′-ccagagcagcaaaagaatcc-3′; Reverse: 5′-cttgacttgacggtgaatcg-3′; *Sof-nkx2.1*: Forward: 5′-gtctctcagcccgaagcat-3′; Reverse: 5′-cagttctggacaaaattcgt-3′). Gene fragments were amplified by PCR under the following conditions: 95 °C for 5 min + (95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min) for 35 cycles + 72 °C for 10 min. As templates, total RNA of *S. officinalis* was extracted from a pool of embryos from stages 15–23 using Tri Reagent (MRC, Cincinnati, OH, USA) and RNA purification kit (Qiagen, Valencia, CA, USA). Then mRNAs were isolated with the gDNA Eliminator column and converted into cDNA by Omniscript reverse-transcriptase (Qiagen). PCR products were cloned into TOPO vectors (Invitrogen, Carlsbad, CA, USA) and sequenced by GATC Biotech (Konstanz, Germany).

5.3. Whole-mount in situ hybridization

RNA probes were obtained with the digoxigenin (DIG) RNA labeling mix kit from Roche (Mannheim, Germany). According to the sense of PCR product insertion into the vector, sense and antisense probe were obtained with T3 and T7 polymerase (Roche). *In situ* hybridizations were performed, using at least three embryos at each stage 15–30 (hatchlings). Embryos were treated in PTW (PBS plus 0.10% Tween20) with proteinase K (10 μg/ml, 20–45 min, depending on the stage of the embryos). They were then fixed again for 1 h in 3.7% PFA in PTW. A prehybridization step was done in hybridization solution (HS) (50% formamide, 5X standard saline citrate, 0.5% sodium dodecyl sulfate, 1% Tween20) with 33 μg/ml heparin and 400 μg/ml tRNA, during 6 h at 65 °C. Embryos were next incubated overnight at 65 °C in HS with the riboprobes, 25 μg/ml heparin and 100 μg/ml tRNA. Excess probe was eliminated by four rinses (30 min each, 55 °C) in HS and by progressive impregnation in standard 2X saline citrate (30 mM trisodium citrate, 0.3 M NaCl). Embryos were then bathed in MABT (100 mM Maleic acid, 150 mM NaCl, 1% Tween20, pH 7.5). Saturation was accomplished in blocking solution (MABT, 4% Blocking powder (Roche), 15% fetal bovine serum) for one hour, at room temperature, followed by incubation overnight at 4 °C with anti-digoxigenin antibodies (Roche) coupled to alkaline phosphatase (AP) and diluted at 1:2000 in blocking solution (MABT, 2.4% Blocking powder, 20% fetal bovine serum). AP activity was revealed using 370 μg/ml NBT (nitro-blue tetrazolium chloride) and 185 μg/ml BCIP (5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt) (Roche). The reaction was stopped by washing in PTW solution. Embryos were then fixed again in 3.7% PFA in PBS for 24 h. To visualize internal staining, some *S. officinalis* embryos were incubated in 15% sucrose, 7.5% gelatin in a 0.12 M phosphate buffer (pH 7.2) for 24 h before being frozen in isopentane at −80 °C, and finally cut in 20 μm cryostat sections. Specimens used for negative controls (DIG-labeled sense probe and anti-digoxigenin antibody conjugated to AP) did not show any staining.

5.4. Microscopy and image processing

Embryos labeled by in situ hybridizations were observed with a Leica M16 2F binocular stereomicroscope and a Leica DMLB compound microscope. All images were adjusted for contrast and brightness using Adobe Photoshop 8 (Adobe, San Jose, CA, USA).

Author contributions

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but were not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Acquisition of data: Buresi,

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Andouche, Navet; Analysis and interpretation of data: Buresi, Bonnaud, Baratte; Drafting of the manuscript: Buresi, Baratte; Critical revision of the manuscript for important intellectual content: Andouche, Baratte, Bonnaud.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2016.04.027.

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