Patterning of brain precursors in ascidian embryos

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ABSTRACT

In terms of their embryonic origins, the anterior and posterior parts of the ascidian central nervous system (CNS) are associated with distinct germ layers. The anterior part of the sensory vesicle, or brain, originates from ectoderm lineages following a neuro-epidermal binary fate decision. In contrast, a large part of the remaining posterior CNS is generated following neuro-mesodermal binary fate decisions. Here, we address the mechanisms that pattern the anterior brain precursors along the medial-lateral axis (future ventral-dorsal) at neural plate stages. Our functional studies show that Nodal signals are required for induction of lateral genes, including Delta-like, Snail, Msxb and Trp. Delta-like/Notch signalling induces intermediate (Gsx) over medial (Meis) gene expression in intermediate cells, whereas the combinatorial action of Snail and Msxb prevents the expression of Gsx in lateral cells. We conclude that despite the distinct embryonic lineage origins within the larval CNS, the mechanisms that pattern neural precursors are remarkably similar.

KEY WORDS: Ascidian, Ciona, Brain, Sensory vesicle, Neural patterning

INTRODUCTION

The chordate super-phylum is characterised by a well patterned dorsal tubular central nervous system (CNS) (Satoh et al., 2014). Ascidians belong to the urochordates, or tunicates, a phylum of invertebrate chordates closely related to vertebrates (Delsuc et al., 2001). Ascidian embryos develop with very few cells and a fixed cell lineage, features enabling the step-by-step analysis of developmental cell fate choices with a single-cell level of precision (Hudson, 2016).

Founder cell lineages of the ascidian embryo are established at the 8-cell stage, when the embryo divides along the animal-vegetal axis to produce two pairs of animal cells (the a- and b-lineages) and two pairs of vegetal cells (the A- and B-lineages). The CNS arises from the a-, b- and A-lineages (Nicol and Meinertzhagen, 1988a,b; Nishida, 1987). The anterior-most part of the sensory vesicle, including the pigmented cells, has an a-lineage origin and thus shares a common origin with anterior epidermis. The dorsal-most cells of the remaining CNS arise from the b-lineage, with the rest of the CNS arising from the A-lineage cells, which share a common lineage origin with mesoderm (notochord). At mid-gastrula stages, A- and a-lineage CNS precursors are arranged in a neural plate that consists of six rows of cells along the anterior-posterior (A-P) axis, such that row I is the most posterior and row VI the most anterior (Fig. 1A). The anterior-most two rows (I-II) of cells are A-lineage, and the anterior four rows (III-VI) of cells are a-lineage. Cells are aligned in columns along the medial-lateral axis, with column 1 the medial-most pair of columns and column 3 the lateral-most, although the A-lineage has an additional fourth column. The b-lineage cells are positioned lateral to this grid-like array. Of the four rows of a-lineage cells, only rows III and IV will actually contribute to the CNS, generating the anterior part of the sensory vesicle, the ascidian ‘brain’, and contributing to the oral siphon primordium (Christiaen et al., 2007; Cole and Meinertzhagen, 2004; Nishida, 1987; Taniguchi and Nishida, 2004; Veeman et al., 2010). Rows V and VI will form a specialised region of anterior epidermis, including a placode-like territory and the palps (Abitua et al., 2015; Nishida, 1987).

Patterning of the A-lineage-derived neural plate involves combinatorial inputs of FGF/ERK, Nodal and two temporally separable Delta/Notch signals (Hudson and Yasuo, 2005; Hudson et al., 2007; Imai et al., 2006; Mita and Fujiwara, 2007). Each cell, present on both sides of the bilaterally symmetrical embryo, receives a unique combination of these three signalling pathways, which determine the eight distinct cell types (Hudson et al., 2007). Like the A-lineage-derived neural plate, differential FGF/ERK signalling also patterns the a-lineage-derived neural plate along its anterior-posterior axis. Specifically, FGF/ERK signalling is required to promote row III over row IV cell identities (Haupax et al., 2014; Racioppi et al., 2014). Similarly, as in the A-lineage neural plate, Nodal signalling is implicated in specification of the lateral part of the a-lineage neural plate, as lateral gene expression is lost in the a-lineage cells when Nodal signalling is inhibited (Hudson and Yasuo, 2005; Imai et al., 2006; Ohtsuka et al., 2014). In this study, we investigate in detail the mechanisms responsible for patterning of the a-lineage row III brain precursors of Ciona embryos.

RESULTS AND DISCUSSION

Nodal is required for medial-lateral patterning of the a-lineage-derived neural plate

In order to investigate patterning of the ascidian brain precursors, we used a set of three genes, Trp, Gsx and Meis, which label row III cells in columns 3 (lateral), 2 (intermediate) and 1 (medial), respectively, at neurula stages. The expression of Trp and Meis was analysed at the neurula stage (~8.25 h of development at 18°C), when all of the 6-row neural plate cells have divided along the A-P axis (Fig. 1A). Trp is expressed in column 3, with stronger expression in the posterior cell, a10.97, whereas Meis is expressed in column 1, with stronger expression in the posterior cell a10.73 (Fig. 1A, Fig. 2A). Gsx expression was analysed in slightly earlier neurula stage embryos (7.5 h of development at 18°C), when it is expressed in both row IIIa and row IIIp (a10.66 and a10.65...
respectively), because at 8+ hours of development, Gsx expression also commences in column 1.

We first investigated the role of Nodal during medial-lateral patterning of the a-lineage-derived neural plate. From the 32-cell stage, Nodal is expressed in cells that contact the lateral-most a-lineage neural precursors (Fig. 1B). To inhibit Nodal activity, we treated embryos with a pharmacological inhibitor of TGFβ type I receptors ALK4, ALK5 and ALK6 (SB431542), or inhibited Nodal mRNA translation by injection of anti-sense morpholino oligonucleotides (Nodal-MO) (Fig. 2A). These treatments resulted in loss of Trp expression from column 3. Gsx expression in column 2 was also strongly reduced following Nodal signal inhibition. However, in many embryos, while expression of Gsx was lost from column 2, we observed its ectopic expression in column 3 (Fig. 2A). Thus, Nodal is required both to promote Gsx expression in column 2 as well as inhibit its expression in column 3. In Nodal-inhibited embryos, Meis was ectopically expressed in column 2 of most embryos (88% of Nodal-MO; 96% of SB431542-treated) and in column 3 in a proportion of embryos (18% of Nodal-MO; 27% of SB431542-treated). Overexpression of Nodal had the opposite effect to inhibition of Nodal (Fig. 2A). We overexpressed Nodal using the upstream regulatory sequences of FOG (pFOG>Nodal) to drive expression of Nodal throughout the animal hemisphere from the 16-cell stage of development (Hudson et al., 2015; Pasini et al., 2006; Rothbächer et al., 2007). This led to ectopic expression of Trp throughout the row III daughters and loss of both Gsx and Meis expression (Fig. 2A). Thus, Nodal promotes column 3 identity and represses column 1 and 2 identity. Taken together, we conclude that Nodal signals are required for the correct specification of both columns 2 and 3 and to repress medial column gene expression in lateral cells.

**Delta/Notch specifies column 2 over column 1 fates**

One of the transcriptional targets of Nodal signals, Delta-like (previously Delta2), is expressed in b-lineage neural precursors as well as a vegetal A-lineage cell at the 64-cell stage (Fig. 1B). At the early gastrula stage, Delta-like is expressed in the lateral A-lineage neural precursors and b-line cells and later, at neural plate stage, it is expressed in the lateral borders of the neural plate (Fig. 1B). Thus, from the 64-cell stage, cells expressing Delta-like are in contact with lateral a-lineage precursors. Notch receptor transcripts are present ubiquitously during early cleavage stages, with expression detected from the late gastrula stage in the developing nervous system (Imai et al., 2004). Consistent with a role for Notch signalling during patterning of the a-lineage-derived neural plate, Hesb, a transcriptional target of Delta-like/Notch signals, is expressed in both column 2 and 3 of row III (Hudson et al., 2007). To inhibit Delta-like/Notch signalling, we treated embryos from the 76-cell stage with DAPT, an inhibitor of γ-secretase, an enzyme required for Notch receptor processing. Alternatively, we injected mRNA encoding a dominant negative form of Suppressor of Hairless, a transcription factor known to
mediate Notch signalling. Either of these treatments resulted in a strong reduction in \(Gsx\) expression and concomitantly, ectopic expression of \(Meis\) in column 2 (Fig. 2B). Overexpression of \(Delta-like\), by electroporation of \(pFOG>Delta-like\), had the opposite effect: expression of \(Meis\) was lost and ectopic expression of \(Gsx\) was observed in column 1 (Fig. 2B). These data indicate that \(Delta-like/Notch\) signals promote column 2 fates at the expense of column 1 fates in the a-lineage neural plate.

### Fig. 2. Nodal and Notch pattern the a-lineage CNS precursors.

(A,B) Marker analysed is indicated to the left, embryo treatment indicated above the columns. All embryos are at neurula stage in dorsal view. Red arrowheads or brackets indicate ectopic expression. Some embryos are stained with DAPI to confirm cell identification. The graphs show the percentage of embryos in each category of expression following the key below. The blue/red bars for \(Gsx\) expression in A indicate that at least one column 2 and one column 3 cell exhibited detectable \(Gsx\) expression (i.e. we did not distinguish strong or weak levels of expression for this category). \(n=\)total number of embryos analysed.

Snail and Msxb repress \(Gsx\) in column 3

So far we have shown that Nodal signals are required for the correct specification of the column 2 and 3 cells and to repress medial gene expression in the lateral neural plate, whereas Notch signalling specifies column 2 over column 1 cell identity. Based on \(Hesb\) expression, column 3 cells also respond to \(Delta-like/Notch\) signalling, yet they do not express \(Gsx\). We hypothesised that a factor, induced by Nodal in column 3 cells, acts to repress \(Gsx\)
expression in response to Notch signals. Snail, which encodes a transcription factor that can act as a repressor (Nieto, 2002) would be a good candidate for the repression of Gsx transcription in column 3. Indeed, Snail has been shown to mediate Nodal-dependent repression of medial genes in the A-lineage-derived neural plate (Hudson et al., 2015; Imai et al., 2006). Furthermore, Snail is expressed downstream of Nodal in the row III/column 3 precursor at the 6-row neural plate stage (Fig. 1B; Fig. S1). In order to address the role of Snail, we knocked it down using Snail-MO or overexpressed it throughout the neural plate using the ETR promoter (pETR>Snail) (Fig. 3A) (Hudson et al., 2015). Overexpression of Snail resulted in downregulation of both Meis and Gsx (Fig. 3A). Knockdown of Snail resulted in a downregulation of Trp, but only a very occasional ectopic expression of Gsx in column 3 (Fig. 3A). However, we saw strong ectopic expression of Gsx in column 3 of embryos injected with Snail-MO when analysed at the 6-row neural plate stage (Fig. 3B). This suggests that Snail represses Gsx in column 3 at the 6-row neural plate stage, but that other factors act, during later neurula stages, to repress Gsx in column 3. One candidate is Msxb, which is expressed a little later than Snail in a9.49 (row III/column 3) (Fig. 1B). Msxb expression in a-lineage column 3 is also downstream of Nodal (Fig. S1), as has been shown previously for b-lineage Msxb expression (Roure et al., 2014). Using Msxb-MOs, we found that while knockdown of Msxb alone had no effect on Gsx expression, combined inhibition of both Msxb and Snail resulted in strong ectopic expression of Gsx in column 3 at the neurula stage (Fig. 3C;
Fig. S2A). Thus, Snail and Msxb both act downstream of Nodal to repress Gsx expression in the column 3 cells.

### Conclusion

Our data are consistent with the model shown in Fig. 4A. Medial-lateral patterning of the a-lineage neural plate, much like medial-lateral patterning of the A-lineage neural plate, depends upon patterning mechanisms initiated by Nodal signals. Nodal is required for correct specification of columns 2 and 3 and to prevent ectopic expression of medial genes in the lateral neural plate. Nodal induces expression of Delta-like, and Notch signals are required to specify column 2 over column 1 fates. In column 3, Nodal-dependent expression of Snail and Msxb is required to repress Gsx expression in column 3. We conclude that despite the distinct lineage origins of the anterior and posterior nervous system, these cells are subsequently patterned by very similar mechanisms (Fig. 4B).

Patterning across the medial-lateral (future ventral-dorsal) axis of the neural plate in ascidians involves distinct signalling molecules compared with vertebrates (Dessaud et al., 2008; Hudson et al., 2007, 2011; Le Dréau and Marti, 2012; Urbach and Technau, 2008). Nonetheless, for many genes, the order of transcription factor gene expression along this axis appears to be well conserved (e.g. dorsal Snail and Mxx, intermediate Gsx, ventral FoxAa) (Corbo et al., 1997). Indeed, for some genes, their relative order of dorsal-ventral expression may be traceable to the bilaterian ancestor (Buresi et al., 2016; Cornell and Von Ohlen, 2000; Denes et al., 2007; Urbach and Technau, 2008; Winterbottom et al., 2010).

**Fig. 4. Model for patterning of the a-lineage derived brain precursors in Ciona.**

(A) A gene regulatory network constructed using Biotapestry (Longabaugh et al., 2005). Genetic interactions may be direct or indirect. Nodal signals from lateral b-lineage cells induce Msxb, Snail, Delta-like and Trp in the lateral column (col. 3). Delta-like/Notch induces Gsx and represses Meis in col. 2. Col. 1 receives neither Nodal nor Notch signals and expresses Msxs. In col. 3 Msxb and Snail prevent col. 3 cells expressing Gsx in response to Notch signalling. Snail repression of Meis in column 3 is based on overexpression data (Fig. 3A). However, simultaneous inhibition of Snail, Msxb and Notch did not result in ectopic expression of Meis in column 3 of the majority of embryos (Fig. S2). This suggests that other factor(s) (Gene X?, in red) prevent Meis expression in column 3 of Notch-inhibited embryos.

(B) a- and A-lineage neural plates are patterned by very similar mechanisms. Nodal is required for the entire lateral domain where it induces Snail expression. Snail (together with Msxb in a-line) represses medial gene expression in lateral cells. Delta-like is induced by Nodal, and Notch signalling promotes columns 2 over column 1 gene expression, as well as inducing column 4 gene expression (A-line only).

**Embryological experiments**

Adult Ciona intestinalis were purchased from the Station Biologique de Roscoff (France) or from Stazione Zoologica Anton Dohrn (Italy). Blastomere names, lineage and the fate maps were described previously (Conklin, 1905; Nishida, 1987). Ascidian embryo culture and microinjection have been described (Sardet et al., 2011). All microinjections were carried out in unfertilised eggs. The electroporation constructs pFOG>Nodal, pFOG>Delta-like and pETR>Snail have been previously described (Hudson et al., 2007, 2015; Pasini et al., 2006).

**In situ hybridisation**

Gene markers used for in situ hybridisation have been described (Aniello et al., 1999; Hudson and Lemaire, 2001; Imai et al., 2004) (http://ghost.zool.kyoto-u.ac.jp) and named according to recent guidelines (Stolfi et al., 2015). The Ciona TRP (L-dopachrome tautomerase) used corresponds to the
GenBank entry reported previously (Hudson et al., 2003). In situ hybridisation was carried out and photographed as described (Hudson and Yasuo, 2006; Hudson et al., 2013, 2016; Wada et al., 1995).

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Competing interests
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C.H., H.Y., A.S.: acquisition analysis and interpretation of data. C.H.: drafting the article. All authors revised the article.

Supplementary information
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References


