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RESEARCH REPORT

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., 2006; Satoh et al., 2014). Ascidian embryos develop with very few numbers of 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 posterior-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 (Haupaix 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

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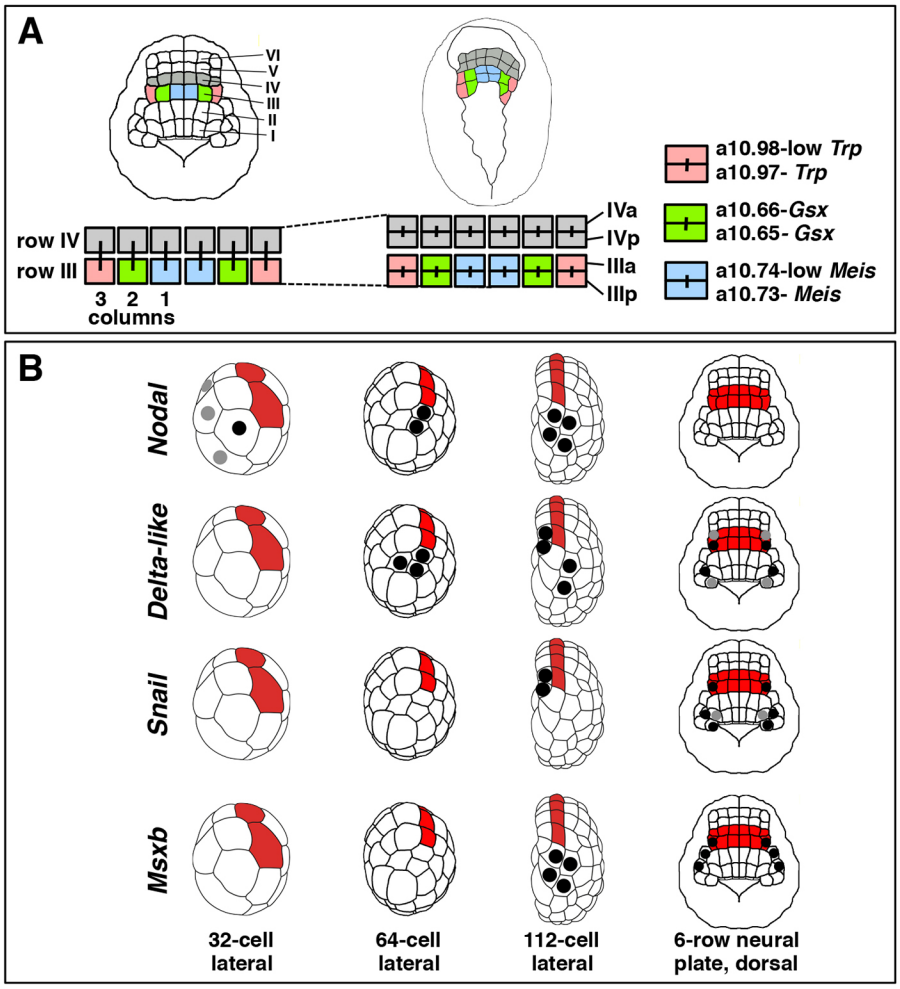

Fig. 1. Expression patterns of genes analysed in this study. (A) Schematic drawings of 6-row neural plate stage and mid-neurula stage highlighting the different columns of row III. *Trp*, *Gsx* and *Meis* are expressed within distinct columns of row III. *Trp* and *Gsx* expression begins at the 6-row neural plate stage, whereas *Meis* expression is first detected at the neurula stage. (B) Sequential activation of *Nodal*, *Delta-like*, *Snail* and *Msxb* during the 32-cell stage to 6-row neural plate stage, based on data in Fig. S1 and Hudson and Yasuo (2005); Hudson et al. (2007); Imai et al. (2009); Roure et al. (2014). The stage and orientation of the embryo in each drawing is indicated below each column. Animal pole is to the right for the 32-, 64- and 112-cell stage embryo drawings. Gene expression is indicated by black dots, with weaker expression represented by grey dots. The a-lineage neural plate cells that generate the CNS are coloured in red.

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

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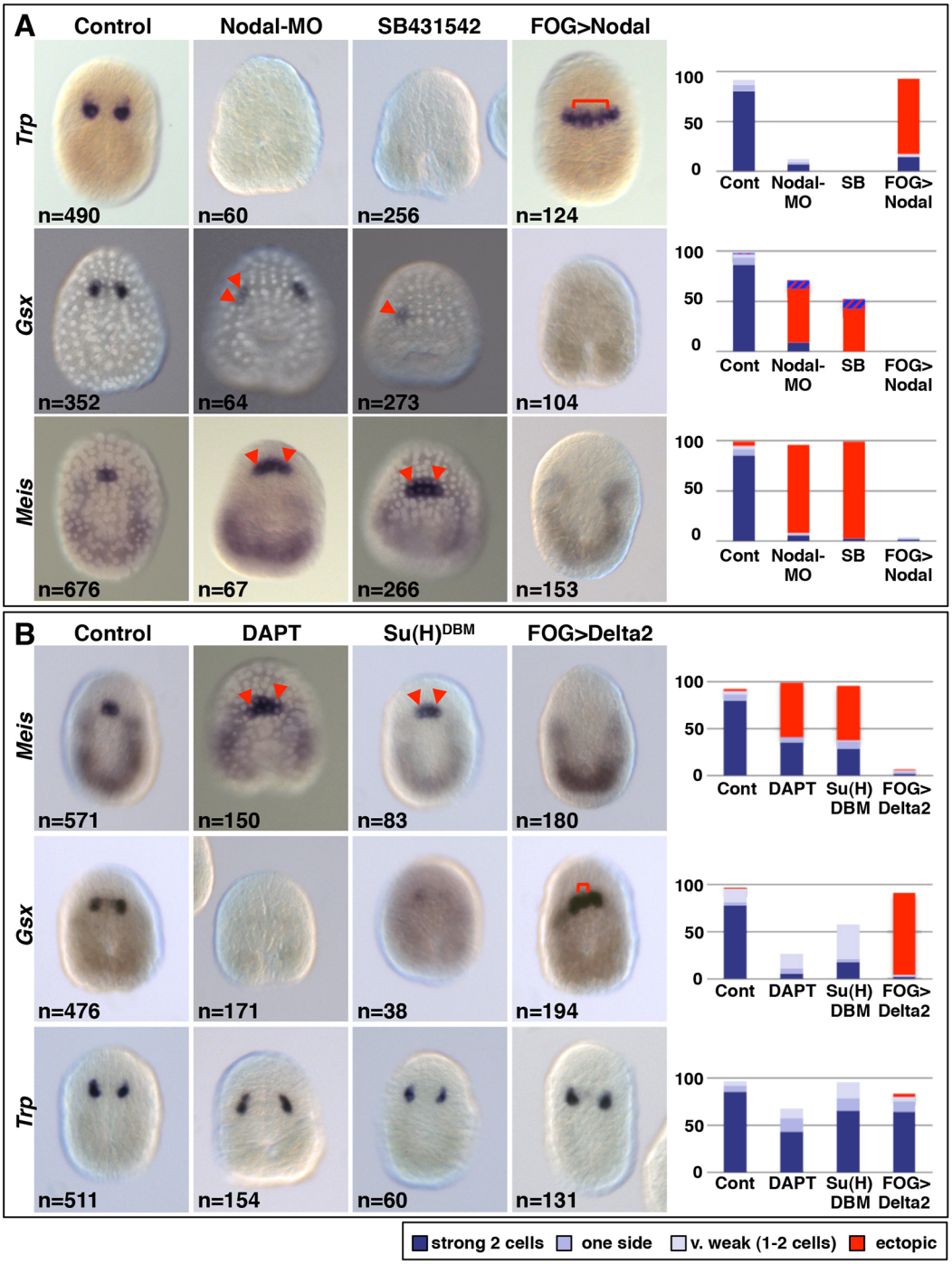

Fig. 2. Nodal and Notch pattern the a-lineage CNS precursors. (A,B) Marker analysed is indicated to the left, embryo treatment indicated above the columns. All embryos are at neurula stage in dorsal view. Red arrowheads or brackets indicate ectopic expression. Some embryos are stained with DAPI to confirm 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.

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.

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*

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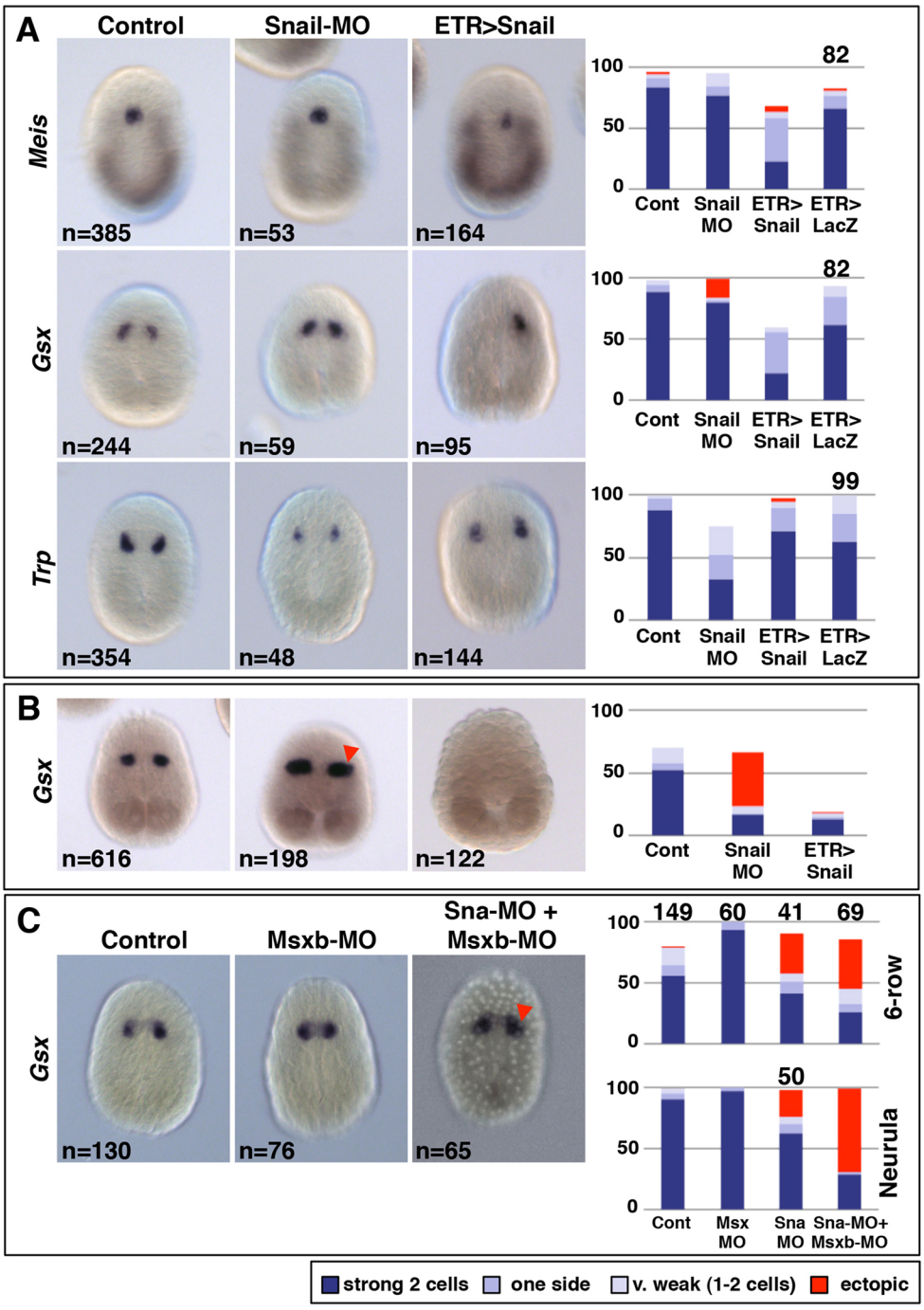

Fig. 3. *Snail* and *Msxb* repress *Gsx* expression in column 3. (A-C) Marker analysed is indicated to the left, embryo treatment indicated above the columns. Embryos were analysed at neurula stage (A,C) or 6-row neural plate stage (B and C, top graph) and shown in dorsal view. Red arrowheads indicate ectopic expression. Some embryos were stained with DAPI to confirm cell identification. The graphs show the percentage of embryos in each category of expression. *n*=total number of embryos analysed. Numbers above bars in graphs indicate the number of embryos analysed.

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;

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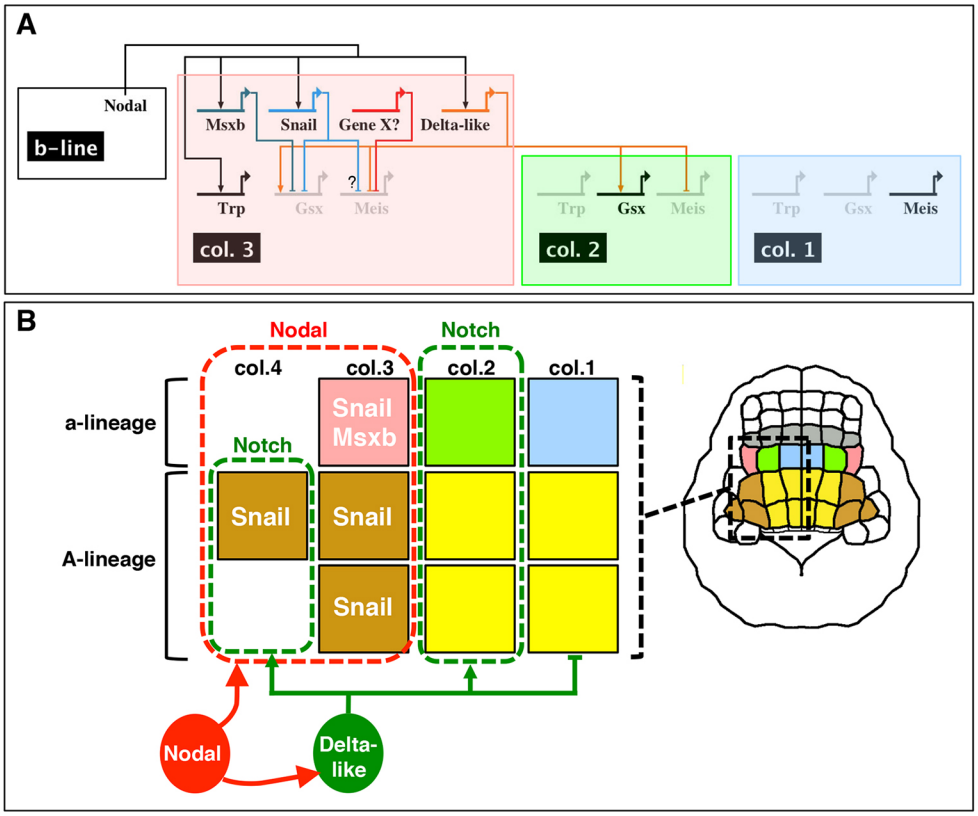

Fig. 4. Model for patterning of the a-line-derived brain precursors in *Ciona*. (A) A gene regulatory network constructed using Biotapestry (Longabaugh et al., 2005). Genetic interactions may be direct or indirect. Nodal signals from lateral b-line cells induce *Msxb*, *Snail*, *Delta-like* and *Trp* 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 *Meis*. 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 pattered 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 column 2 over column 1 gene expression, as well as inducing column 4 gene expression (A-line only).

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 Martí, 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 *Msx*, 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).

MATERIALS AND METHODS

Overexpression and knockdown tools

Morpholinos for *Snail* (MO1), *Nodal* and *Msxb* and *Ciona* Su(H)^{DBM} are described previously (Hudson and Yasuo, 2005, 2006; Hudson et al., 2015; Imai et al., 2006; Roure and Darras, 2016). SB431542 (Tocris) and DAPT (Calbiochem) treatments have been described previously (Hudson and Yasuo, 2005, 2006). SB431542 was added to embryos at the 16- or 32-cell stage and DAPT at the 76-cell stage. Although previously DAPT gave consistent results (Hudson et al., 2007), recent lots purchased did not give consistent phenotypes among different batches of embryos. We therefore treated batches of embryos and analysed them at the 6-row stage for *Ebf* (previously *COE*) expression (which should be lost) and *Foxb* (previously *FoxB*) expression (which should be ectopically expressed in column 2) (Hudson et al., 2007). Only batches of embryos that gave the expected result were processed further. Su(H)^{DBM} on the other hand, gave consistent results in all experiments. The electroporation constructs *pFOG>Nodal*, *pFOG<Delta-like* and *pETR>Snail* have been previously described (Hudson et al., 2007, 2015; Pasini et al., 2006).

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 protocol was based on Christiaen et al. (2009). All data were pooled from at least two independent experiments (i.e. on different batches of embryos). For data shown in Fig. S2, embryos were first injected with Snail-MO or Snail+Msxb-MO. Uninjected, or MO-injected embryos were then split into two groups and one group injected with *Ciona* Su(H)^{DBM} RNA. After fertilisation and culturing to neurula stages, the uninjected and MO-injected embryos were further divided into two groups for *Meis* and *Gsx* analysis.

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

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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
The authors declare no competing or financial interests.

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Author contributions
C.H., H.Y., A.S., R.E.: conception and design of the project. C.H., H.Y., R.E., A.P., C.S., A.S.: acquisition analysis and interpretation of data. C.H.: drafting the article. All authors revised the article.

Supplementary information
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