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RESEARCH ARTICLE

A gene regulatory network for apical organ neurogenesis and its spatial control in sea star embryos

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ABSTRACT

How neural stem cells generate the correct number and type of differentiated neurons in appropriate places remains an important question. Although nervous systems are diverse across phyla, in many taxa the larva forms an anterior concentration of serotonergic neurons, or apical organ. The sea star embryo initially has a pan-neurogenic ectoderm, but the genetic mechanism that directs a subset of these cells to generate serotonergic neurons in a particular location is unresolved. We show that neurogenesis in sea star larvae begins with *soxc*-expressing multipotent progenitors. These give rise to restricted progenitors that express *lhx2/9*. *soxc*- and *lhx2/9*-expressing cells can undergo both asymmetric divisions, allowing for progression towards a particular neural fate, and symmetric proliferative divisions. We show that nested concentric domains of gene expression along the anterior-posterior (AP) axis, which are observed in a great diversity of metazoans, control neurogenesis in the sea star larva by promoting particular division modes and progression towards becoming a neuron. This work explains how spatial patterning in the ectoderm controls progression of neurogenesis in addition to providing spatial cues for neuron location. Modification to the sizes of these AP territories provides a simple mechanism to explain the diversity of neuron number among apical organs.

KEY WORDS: Sea star, *Patiria*, Neurogenesis, GRN

INTRODUCTION

Knowledge of how stem cells form distinct, differentiated neural cell types in the correct place, time and numbers is important for understanding how nervous systems develop, function and repair. The specification of neural cell fate involves a complicated combination of temporal identity factors and positional information that directs the changing competence of the progenitor states (Kohwi and Doe, 2013). Together, these types of information instruct multipotent neural progenitors to first become restricted progenitors of a particular type, and later to execute a particular terminal differentiation program (Guillemot, 2007).

Much of the work towards understanding neurogenesis has utilized either vertebrates or invertebrate protostomes, namely *Drosophila melanogaster* and *Caenorhabditis elegans*, as model systems. Vertebrates, however, are exceptional among metazoa owing to their highly complex nervous systems that contain astonishingly large numbers of neurons and neuronal cell types. The genetic programs that control neurogenesis are therefore extraordinarily difficult to comprehensively study and understand in vertebrate systems. For this reason, many lines of research have been dedicated to understanding neurogenesis in invertebrate models. However, there are some fundamental differences in neurogenesis between invertebrate models and commonly used vertebrate models. For example, in *Drosophila* neurogenesis proceeds as an invariant chain of asymmetric divisions, whereas the radial glia of vertebrates show more flexibility in their mode of division, allowing for more proliferative cell divisions and, ultimately, more neurons (Price et al., 2011). These differences make it unclear which features of the neurogenesis genetic programs are common to bilateria, and which might be tied to the increase in the number and diversity of neurons in vertebrates. Knowledge of the gene regulatory networks (GRNs) that control neurogenesis in a broader sampling of invertebrate taxa, especially invertebrate deuterostomes, which are closely related to vertebrates, is needed to understand basal mechanisms of neurogenesis and how complexity arose in vertebrates.

The larvae of echinoderms offer such a deuterostome invertebrate model. These larvae, which are bilaterally symmetrical, have neurons associated with ciliary bands that transverse the ectoderm, as well as an anterior localization of serotonergic neurons called the apical organ (Bisgrove and Burke, 1986; Chee and Byrne, 1999; Nakajima et al., 2004b). Many marine larval forms, including those of other deuterostomes, such as the tornaria larvae of hemichordates (Nakajima et al., 2004a), and also protostomes such as mollusks (Kempf et al., 1997) and annelids (Marlow et al., 2014), form apical organs. These apical organs exhibit diversity in their size and morphology. Cnidarian embryos lack an anterior concentration of serotonergic neurons, but have a molecularly homologous territory with sensory function, suggesting a common origin of metazoan apical organs and a potentially ancient gene regulatory process (Sinigaglia et al., 2013). Because apical organs are found in such diverse organisms they offer a potentially homologous metazoan neural structure that is simple enough to dissect at the GRN level. While apical organ territory patterning is relatively well studied and conserved (Range, 2014), the process of neurogenesis that directs an ectodermally derived progenitor cell to progress to a differentiated serotonergic neuron in this territory remains poorly understood in any of these taxa, although recent work has shown a role for *soxc* in this process (Garner et al., 2015; Wei et al., 2016).

Previously, we began to characterize neurogenesis in the bipinnaria larvae of the sea star (Yankura et al., 2013). We also found that the ectoderm of this organism is initially broadly neurogenic, as it expresses the extremely well-conserved neural stem cell transcription factor *soxb1* (Miyagi et al., 2009) throughout

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this territory in early development (Yankura et al., 2013). *soxc*-expressing (*soxc*$^+$) cells are then partitioned from this ectoderm through Delta-Notch signaling (Yankura et al., 2013). Knockdown of Soxc leads to the loss of all neural cell types, both ciliary band and serotonergic, and potentially also other cell types. Therefore, we predicted that these *soxc*$^+$ cells are likely to be multipotent progenitors (Yankura et al., 2013). Intriguingly, although *soxc*$^+$ cells are found throughout the ectoderm, differentiated neurons only form in the ciliary bands and apical organ. Therefore, we sought here to understand how anterior-posterior (AP) patterning could direct pan-ectodermal *soxc*$^+$ cells to progress to more restricted progenitors, and finally to differentiated neurons, within a particular ectodermal territory. We focus specifically on the formation of the neurons in the apical pole domain (APD), as these most likely represent homologous structures and are clearly defined by serotonin immunoreactivity.

We first confirm that *soxc*$^+$ cells are indeed a proliferating population of multipotent progenitors. These progenitors give rise to more restricted progenitors, which become serotonergic neurons, and are further defined by the expression of the LIM homeodomain factor *lhx2/9*. This only occurs within the retinal homeobox *rx* regulatory environment. These restricted progenitors differentiate into serotonergic neurons within the APD, which is the anteriormost region of the ectoderm. This territory is defined by the expression of the forkhead factor *foxq2*. Therefore, surprisingly, we show that the role of AP-restricted patterning genes, such as *foxq2* and *rx*, is to regulate the progression of multipotent progenitors, first to restricted progenitors and finally to postmitotic differentiated neurons, such that each step occurs in a discrete spatial domain. Patterning of the AP domains also regulates the size of the neural proliferation zone and the ultimate number of neurons in the apical organ. This presents a possible conserved basal role for these AP-restricted patterns in controlling the progression of neurogenesis.

RESULTS

soxc$^+$ multipotent progenitors produce *lhx2/9*$^+$ restricted progenitors in the anterior ectoderm

The serotonergic neurons of the apical organ are found in two bilateral clusters within the anterior dorsal ectoderm of *Patiria miniata* larvae (Fig. 1A,B) and other sea star species at this stage (Chee and Byrne, 1999; Nakajima et al., 2004b). *soxc*$^+$ cells, which are required for the formation of all neural cell types, are found scattered throughout the ectoderm at 48 hours post-fertilization (h) (Yankura et al., 2013), although the differentiated neurons of the apical organ are restricted to the dorsal regions of the APD. In the present study, we specifically demonstrate that some of these *soxc*$^+$ cells comprise the cell lineage that will give rise to serotonergic neurons. We predict that other *soxc*$^+$ cells will give rise to other

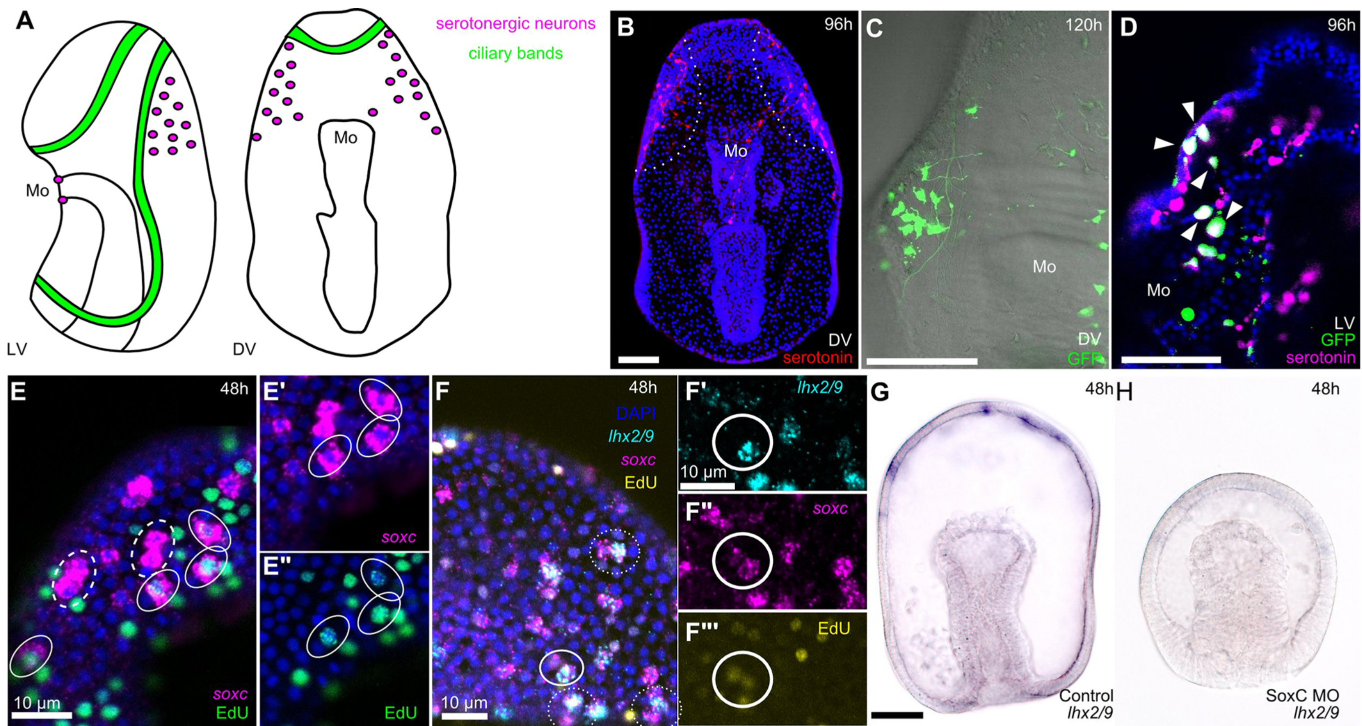

Fig. 1. *soxc*$^+$ cells are multipotent progenitors and generate *lhx2/9*$^+$ restricted progenitors in the anterior ectoderm. (A) Schematic depicting the positions of serotonergic (magenta) and ciliary band (green) neurons in sea star larvae. (B) Dorsal view (DV) of a 96 h sea star larva. Apical organ neurons are stained by rabbit anti-serotonin immunofluorescence (red) and appear in two clusters in the anterior ectoderm. Nuclei are stained with DAPI (blue in B,D,E,F). Mo indicates the location of the mouth. Dotted lines demarcate the two lateral clusters of neural bodies. (C) A 120 h larva injected with *soxc*-GFP recombinant BAC construct showing a labeled population of cells of the correct position and morphology to be serotonergic neurons when compared with B. (D) Lateral view (LV) of a 96 h larva injected with *soxc*-GFP recombinant BAC construct and stained by immunofluorescence for GFP and serotonin. Colocalization (arrowheads) demonstrates that some formerly *soxc*$^+$ cells eventually go on to become serotonergic neurons. (E-E″) FISH (magenta) combined with EdU labeling (green) at 48 h results in colabeling of *soxc*$^+$ cells that are actively proliferating in the anterior ectoderm (circled). Pairs of *soxc*$^+$ cells are indicated by dashed ovals. (F-F‴) Double FISH plus EdU labeling reveals that *soxc* expression (magenta) frequently occurs in pairs of cells, and co-expression with *lhx2/9* (cyan) occurs in one of the two *soxc*$^+$ cells, as indicated by both solid and dashed circles. Solid circle in F indicates the region enlarged in F′-F‴, showing that asymmetric pairs are the result of recent cell division, as demonstrated by light EdU label (yellow) persisting in the pair of cells after a brief labeling period plus 30 min chase (F‴). (G) *lhx2/9* is normally expressed in spots in the anterior ectoderm of 48 h embryos. (H) Knockdown of Soxc by MO results in loss of *lhx2/9* expression. Scale bars: 50 µm, except where labeled otherwise.

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types of neurons, including those of the ciliary band. To help demonstrate that some *soxc*$^+$ cells are indeed the lineage that leads to serotonergic neurons we developed a GFP knock-in BAC for *Pmsoxc*, in which *gfp* was inserted in place of the single *soxc* coding exon. When injected into embryos, the BAC becomes stably incorporated into a clonal patch of cells that express *gfp* under the regulatory control of the *soxc* locus. As GFP protein is relatively stable, it can be used to track cells that had once expressed *soxc*, even when the *soxc* transcripts are no longer being expressed (Fig. S1). Some larvae injected with this construct eventually give rise to cells with GFP label in what appear to be serotonergic neurons based on their location and morphology (Fig. 1B compared with Fig. 1C). We demonstrate, using immunofluorescence against GFP and serotonin, that the mosaic expression of GFP driven by this BAC construct indeed overlaps with clusters of serotonergic neurons at larval stages (Fig. 1D). This demonstrates that at least some *soxc*$^+$ cells eventually take on a serotonergic neuron fate.

Given the role of *soxc* orthologs in regulating proliferation during the development of neural precursors in vertebrates (Bergsland et al., 2011; Wang et al., 2013), we tested whether these *soxc*$^+$ cells are multipotent progenitors in the sea star. On closer inspection, we show that *soxc* is often expressed in a pair of adjacent cells, which appear to be the products of recent division (Fig. 1E, dashed ovals). To determine whether these cells were dividing, we labeled proliferating cells by bathing embryos in a short pulse of EdU to identify those in S phase. Labeling occurred in the solitary cells that are about to divide (circled), but not in the pairs of cells (dashed oval), which have already undergone cell division and therefore did not take up EdU during the short pulse. This showed that 19±2% of *soxc*$^+$ cells on the dorsal face of the embryo, as identified by fluorescent *in situ* hybridization (FISH), are indeed undergoing cell division (*n*=6 embryos) (Fig. 1E-E″). This is consistent with a model in which *soxc*$^+$ cells represent a population of multipotent progenitors.

We next turned our attention to *lhx2/9*, which is expressed in individual cells within the anterior dorsal ectoderm at 48 h (Fig. S2A,B). We had previously studied the expression of orthologs of transcription factors known to pattern the anterior of vertebrate embryos, and noted that *lhx2/9* is expressed in scattered ectodermal cells (Yankura et al., 2010). We examined whether these spots overlap with the *soxc*$^+$ cells described above. Intriguingly, by 96 h, *lhx2/9* is expressed in two bilateral clusters in the anterior dorsal ectoderm (Fig. S2C,D) which is reminiscent of the pattern of serotonergic neurons in 96 h larvae (Fig. 1B) (Chee and Byrne, 1999; Nakajima et al., 2004b). We speculated that *lhx2/9* could be important in apical organ development and examined its expression and function more rigorously. First, we show that *lhx2/9* is frequently expressed in one of the two paired *soxc*$^+$ cells (Fig. 1F). We find that 45±3% of *soxc*$^+$ pairs on the dorsal side express *lhx2/9* in one of the two cells (*n*=20 embryos). This co-expression occurs only in the dorsal anterior ectoderm where *lhx2/9* is expressed, and hence pairs of *soxc*$^+$ cells are completely *lhx2/9*$^-$ in the more posterior ectoderm and also on the ventral side of the embryo (Fig. S2E). There are also some *soxc*$^+$ pairs in the dorsal anterior ectoderm that do not express *lhx2/9*, but it is unclear whether they are still in an earlier phase of neurogenesis or if they give rise to other ectodermal cell types. Because these pairs are reminiscent of recent cell division, we performed an EdU pulse-chase to label recently divided cells, which appear as pairs of adjacent cells marked by faint EdU incorporation. We were able to label some *soxc*$^+$ *lhx2/9*$^+$ pairs with EdU in this way (Fig. 1F-F‴), which further confirms that *lhx2/9*$^+$ cells are the progeny of *soxc*$^+$ cells and that *soxc*$^+$ cells can undergo asymmetric cell division – that is, one daughter cell maintains a stem cell state whereas the other, which expresses *lhx2*/9, may now be restricted to serotonergic versus ciliary band neural fate. Finally, we show that Soxc function is needed for the formation of the *lhx2/9*$^+$ cells in the anterior ectoderm (Fig. 1G,H), as *lhx2/9* expression is entirely absent when Soxc is knocked down using a specific morpholino antisense oligonucleotide (MO). Thus, both ciliary band and apical organ neurons originate from an ectoderm that has broad neurogenic potential, as indicated by the broad territory of *soxc* expression, but subsequent expression of *lhx2/9* may mark restriction towards apical organ neuron fate.

Pmlhx2/9$^+$ restricted progenitors produce the serotonergic neurons of the apical organ in the APD

We next aimed to determine whether *lhx2/9* is needed for the formation of apical organ neurons. We previously showed that ectodermal *elav* is a marker of postmitotic neurons and is expressed by both the ciliary band neurons and anterior dorsal ganglia (Yankura et al., 2013). *elav* is also expressed within migratory mesenchyme, and thus it is likely that the single sea star *elav* ortholog takes on the roles of the multiple paralogs of this gene in vertebrates (Yankura et al., 2013). We can, however, readily distinguish between the mesenchymal cells and ectodermal cells because the mesenchyme does not embed itself within the ectoderm (Fig. S1F). Additionally, we had shown that correct Soxc function is needed for the ectodermal expression of *elav* in gastrula stage larvae (Yankura et al., 2013) (Fig. S3). When Lhx2/9 is knocked down, *elav* is no longer expressed in the APD of 48 h embryos [Fig. 2A,B; control embryos have 6.2±1.5 *elav*$^+$ cells (range 4-8) whereas Lhx2 morphants have 0.4±0.7 (range 0-1) *elav*$^+$ cells], although we note that mesodermal *elav* expression appears unaffected. We had previously speculated that the *elav*$^+$ cells in the APD at this stage will contribute to the apical organ and that later expression of *elav* represents the ciliary band neurons that differentiate later in development (Yankura et al., 2013). In support of this, we now show that *elav* expression is specifically lost from the apical organ of 120 h Lhx2/9 morphants (Fig. 2C,D; and in 96 h larvae, Fig. S3). By this stage, the neurons are normally located more posteriorly, as seen in Fig. 1B. Other domains of *elav* expression, namely the mesodermal bulb of 48 h embryos and ciliary band neurons of 120 h larvae, appear unaffected by the loss of Lhx2/9, demonstrating a very specific role for Lhx2/9 (Fig. 2A-D). We had previously shown that all *elav* expression is lost in Soxc morphants (Yankura et al., 2013), and thus *lhx2/9*$^+$ cells have a more restricted potential than *soxc*$^+$ cells, which is why we refer to these *lhx2/9* expressing cells as restricted progenitors. It is not yet known whether any non-neuronal cell types are affected by *lhx2/9* knockdown, although Lhx2/9 morphant larvae have largely normal morphology, indicating that most cell types and tissues are likely to be specified properly. As predicted by these results, Lhx2/9 morphants do not produce serotonergic neurons, whereas these are present in control MO-injected larvae (Fig. 2E,F).

We further investigated the relationship between *lhx2/9* and the formation of these serotonergic neurons. We show that *lhx2/9* colocalizes with *elav*$^+$ cells in the APD at 48 h (Fig. 2G). We also see that, in the APD, *lhx2/9*$^+$ cells undergo asymmetric divisions whereby one cell expresses *elav* and the other does not (Fig. 2H-H″). We find that 22±4% of *lhx2/9*$^+$ cells are also *elav*$^+$ (*n*=6 embryos). *elav* expression in this territory marks postmitotic neurons that will differentiate rather than produce additional neural precursors. Therefore, we hypothesize that the other cell in the pair,

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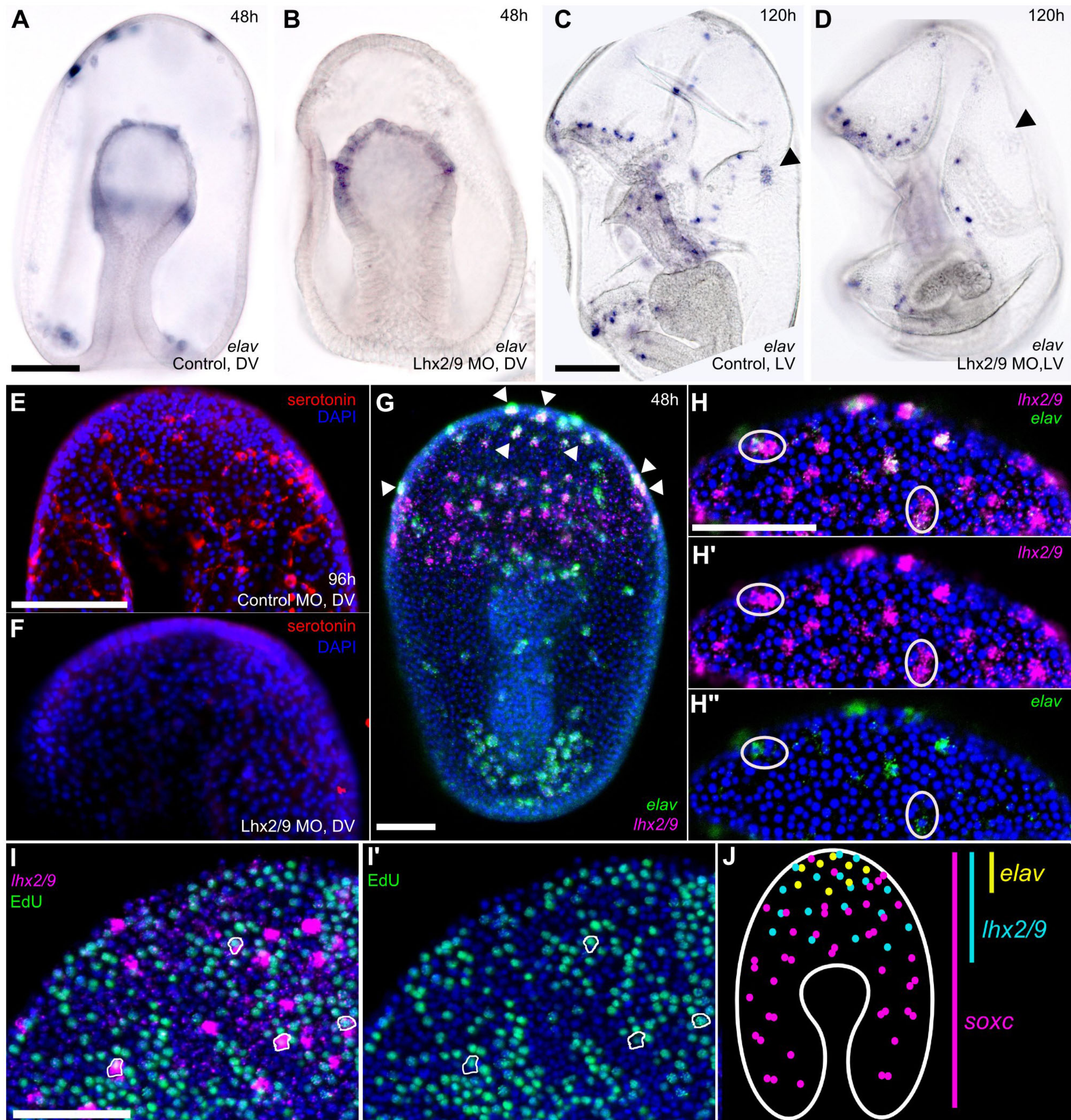

Fig. 2. *Pmlhx2/9*⁺ restricted progenitors give rise to the serotonergic neurons of the apical pole domain. (A) In 48 h embryos, *elav* is normally expressed in the apical pole domain (APD) and also in the mesodermal bulb. (B) Expression of *elav* is lost only from the APD when Lhx2/9 is perturbed by a MO. (C,D) Lhx2/9 is specifically needed for serotonergic neuron development. At 120 h, larvae have two neuron populations marked by *elav* expression (C). Only the apical organ *elav* expression (arrowhead) is lost in Lhx2/9 morphant embryos (D). (E,F) Additionally, Lhx2/9 morphants do not exhibit serotonin staining, indicating a lack of this neuron population (E versus F; which depict only the anterior dorsal region of 96 h larvae). (G) Double FISH reveals *lhx2/9* expression (magenta) in the anterior ectoderm, and co-expression with *elav* (green) in the APD (arrowheads); mesenchymal *elav* expression is also noted in the posterior embryo. (H-H″) APD view of double FISH for *lhx2/9* (magenta) and *elav* (green) at 48 h. *lhx2/9* expression frequently occurs in pairs of cells. Asymmetric divisions generate pairs in which one cell expresses *elav*, denoting a transition to postmitotic neuron state (circled). (I,I′) FISH (magenta) combined with EdU labeling (green) at 48 h results in colabeling of *lhx2/9*⁺ cells that are actively proliferating in the anterior ectoderm (outlined). (J) A schematic depicting *soxc*, *lhx2/9* and *elav* expression in the dorsal ectoderm of a gastrula stage embryo. Scale bars: 50 µm.

which does not express *elav*, will continue to proliferate. We also see pairs of *lhx2/9*⁺ cells in which neither cell expresses *elav* more posterior to the APD, which might represent cells dedicated to proliferation at that time (Fig. 2G). There are instances of *lhx2/9*⁺ pairs in the APD that do not express *elav*, either because they are newly generated by *soxc*⁺ cells or because they are proliferative, but such pairs are much less plentiful in this region. In any case, this suggests that serotonergic neurons are specified continuously for some as yet undefined time as opposed to being produced simultaneously.

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To test this hypothesis, we next determined whether these *lhx2/9*$^{+}$ cells are postmitotic cells that are likely to be undergoing differentiation or proliferative cells that could serve as restricted progenitors. We again combined EdU labeling with FISH to determine whether some *lhx2/9*$^{+}$ cells are dividing. We find that 34±3% of *lhx2/9*$^{+}$ cells are EdU positive (*n*=5 embryos; Fig. 2I,I′), which supports the idea that these cells are progenitors. Collectively, these data indicate that *lhx2/9* is crucial for the development of serotonergic neurons because *lhx2/9*$^{+}$ cells are restricted progenitors, at least some of which take on serotonergic neuron fate. This establishes a pathway of neurogenesis from multipotent progenitor to differentiated serotonergic neuron.

Neurogenesis depends on the regulatory state established spatially along the AP axis

A striking observation is that the progression appears to proceed according to AP position. We observe *soxc*$^{+}$ multipotent progenitors throughout the ectoderm at 48 h, but only those in the anterior half of the ectoderm progress to *lhx2/9*$^{+}$ restricted progenitors (Fig. S2E). Likewise, only *lhx2/9*$^{+}$ cells in the APD at 48 h differentiate into serotonergic neurons and therefore become *elav*$^{+}$ (Fig. 2G). This is summarized in Fig. 2J. We investigated whether neurogenesis might be tied to a GRN for AP patterning, particularly because AP patterning domains tend to be well conserved across metazoans, although CNS morphologies are not (Lowe et al., 2003; Marlow et al., 2014; Yankura et al., 2010). We especially focused on the roles of the transcription factors *foxq2*, *rx* and *six3* in patterning distinct AP neurogenic domains. These were previously shown to be expressed in nested concentric domains along the AP axis within the ectoderm (Yankura et al., 2010). The domain of *six3* expression abuts *wnt8* expression posteriorly, while *foxq2* expression defines the most anterior ectoderm. *rx* is expressed in the anterior half of the ectoderm, and is expressed more broadly than *foxq2*, but more anteriorly than *six3*. For simplicity, we will refer to these nested AP territories as 1-4, where 1 indicates the most anterior ectoderm and 4 the most posterior (Fig. 3A).

We sought to determine the role that each of these genes has in establishing these domains. Our goal is to understand how these territories in turn may regulate neurogenesis. We first examined the role of *foxq2* in establishing domain 1. Foxq2 is required for the expression of *lhx2/9* (Fig. 3B,C) and *rx* (Fig. 3D,E) in domains 1 and 2, as well as of several markers of differentiated APD specific to domain 1, including *elav* and *α2-tubulin* (Fig. 3F-I, Fig. S4). Foxq2 therefore has a role in directing the differentiation of appropriate cell type in this anteriormost ectoderm and in establishing the regulatory state of domain 2. We note that Foxq2 morphant embryos are shorter overall along the AP axis, consistent with a crucial role of this factor in establishing an anterior pole. By contrast, our results show that the endomesoderm appears to be specified normally, which provides confidence that development is not simply delayed overall. Rx, in turn, functions to repress *foxq2* from only within domain 2, although they are co-expressed in domain 1 (Fig. 3J,K). This suggests that Rx might require additional factors not present in domain 1 for repression of *foxq2*. We previously demonstrated that *six3* abuts the domain of *wnt8* expression, and one of its functions is to maintain this boundary, delineating domains 3 and 4 (Yankura et al., 2013). Wnt signaling, meanwhile, restricts the expression of *six3*, *lhx2/9* and *foxq2* to their respective domains (Fig. S4C-H). Domain 1 appears unaffected in Six3 morphants, as the *foxq2* domain of expression is maintained, if not expanded (Fig. 3L,M). These morphants have, however, lost the distinct regulatory domains between *wnt8* and *foxq2* (Fig. 4A,B), as we show that *wnt8* expression now abuts or even overlaps with *foxq2* in Six3 knockdowns. Therefore, a function of both Six3 and Rx is to maintain a distinct domain 2, which is *rx* positive but negative for *foxq2* and *wnt8* (Fig. 3A).

Having established, in part, the epistatic relationships that describe the initial GRN that delineates these domains, we used this knowledge to experimentally manipulate the regulatory state to determine how these territories might govern neurogenesis. We show that Six3 is not required for the correct expression of *soxc* (Fig. S4I,J). This is consistent with the broad expression of *soxc* throughout the ectoderm, i.e. *soxc*$^{+}$ cells are not limited to a particular AP domain. This result also corroborates our previous finding that the ciliary band neurons develop normally in Six3 morphants in spite of the altered morphology of these embryos (Yankura et al., 2013).

We next examined the expression of *lhx2/9* in Six3 and Rx morphants. In both of these perturbations there is no longer a molecularly distinct domain 2. In Six3 morphants, in which *wnt8* expands into the anterior ectoderm such that it now abuts the expression domain of *foxq2* (Fig. 4A,B), *lhx2/9* is still expressed, but the number of *lhx2/9*$^{+}$ cells is reduced (Fig. 4C,D). In later development, the *lhx2/9*$^{+}$ cells and serotonergic neurons, although still present, are also much reduced in number (Fig. 4E,F, Fig. S4K,L). Interestingly, these cells are positioned in a single central cluster, rather than the typical bilateral clusters observed in control larvae. This indicates that Six3 normally functions to maintain an *lhx2/9*$^{+}$ progenitor population. Whereas the more posterior *lhx2/9*$^{+}$ cells do not normally express *elav* and instead can continue to proliferate in control embryos (22±4% of *lhx2/9*$^{+}$ cells are also *elav*$^{+}$, *n*=6 embryos), all *lhx2/9*$^{+}$ cells now co-express *elav* in Six3 morphants (Fig. 4C,D). This is presumably because all *lhx2/9*$^{+}$ cells are now within domain 1, as the distinct domain 2 is lost. *elav* is only expressed within domain 1 in normal embryos at this stage (Fig. 4G), suggesting that *foxq*2 could be important for serotonergic neuron cell cycle exit and differentiation.

To test this further, we examined the expression of *lhx2/9* in Rx morphants, where *foxq2* expression expands posteriorly (Fig. 4H,I). This again places all *lhx2/9*$^{+}$ cells into the *foxq2* territory, but now the domain of *foxq2* is extended. We show that, as predicted, all of these cells also express *elav* rather than maintaining a progenitor state (Fig. 4J,K). Therefore, all *lhx2/9*$^{+}$ cells become *elav*$^{+}$ in Six3 and Rx morphants because they are within domain 1. This provides further evidence that *foxq2* promotes exit from proliferation and the differentiation of restricted progenitors. As a result, the proliferating reserve of *lhx2/9*$^{+}$ cells is lost in these AP domain perturbations. These results indicate, therefore, that domain 2 serves as a neural proliferation zone.

DISCUSSION

A key question in developmental biology is how neural stem cells progress to their correct differentiated fate, and how this is coordinated in space and time. We show here that the sea star larva uses a simple and elegant patterning system to direct a subset of broadly localized *soxc*$^{+}$ proliferative cells to give rise to *lhx2/9*$^{+}$ restricted progenitors. At least some of these in turn will exit their proliferative state to form serotonergic neurons in the *foxq2*$^{+}$ APD. Combined, our data indicate that *soxc*$^{+}$ cells are multipotent progenitors in sea stars and that spatial domains along the AP axis establish regulatory states that control neural progression.

Both here, and previously (Yankura et al., 2013), we show that *soxc*$^{+}$ cells are the initial precursors required for the eventual production of neurons in the sea star larva. This role of Soxc in

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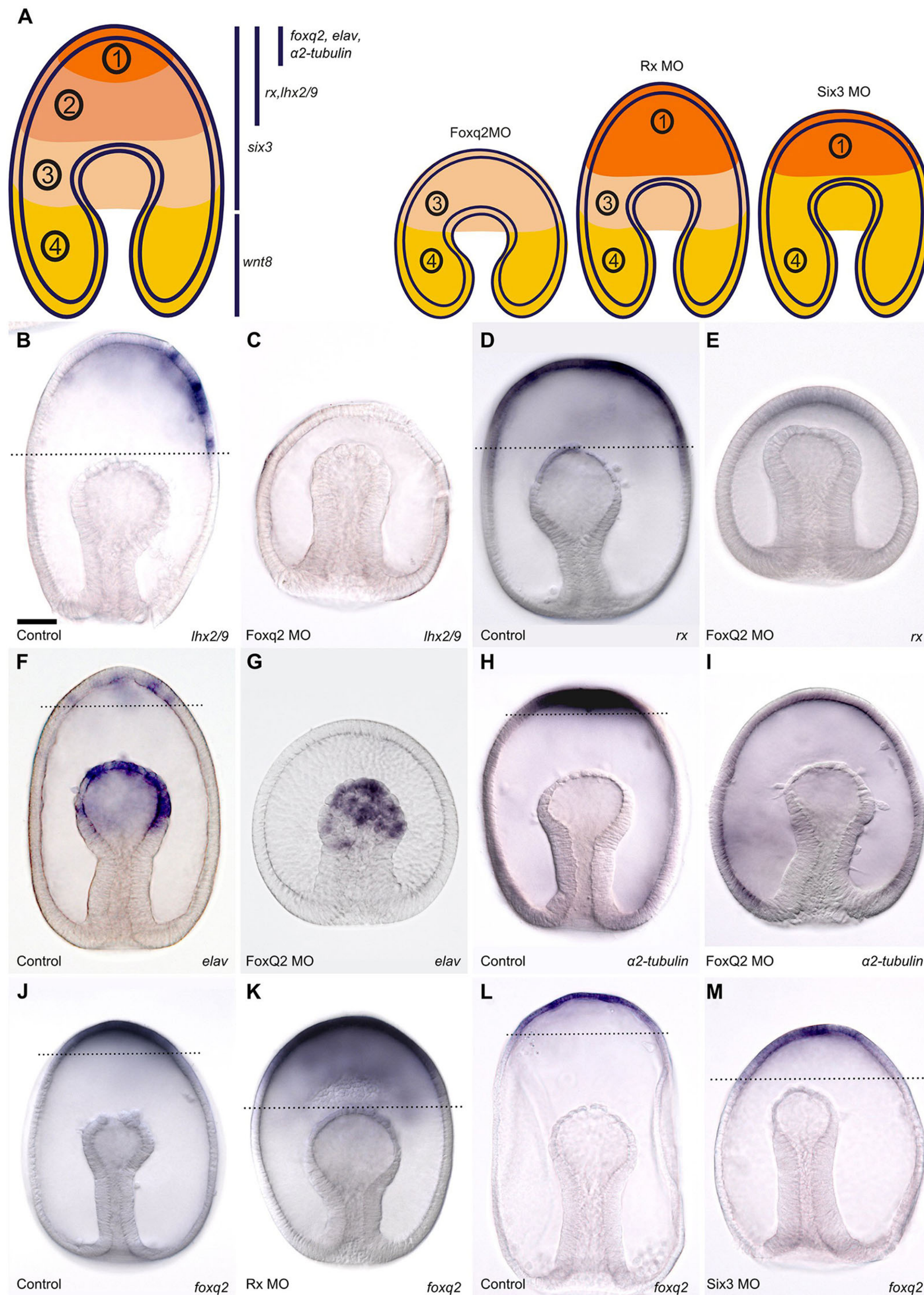

Fig. 3. Establishment of neurogenic regions by AP domains of regulatory genes. (A) Schematics depicting AP regulatory gene expression domains and alterations to these domains that occur upon knockdown of the indicated transcription factors. Schematics are based upon data from Fig. 3B-M and Fig. 4 and our previous work (Yankura et al., 2010, 2013). (B,C) Normal expression of *lhx2/9* in the anterior dorsal ectoderm is lost upon perturbation of Foxq2. (D,E) *rx* expression throughout domain 2 (D) is also lost in Foxq2 morphants (E). (F-I) The APD (domain 1) expresses differentiation genes such as *elav* and *α2-tubulin*, which also require Foxq2 function. Mesodermal bulb expression of *elav* is not dependent upon Foxq2 (F,G). (J) *foxq2* expression is normally restricted to the APD. (K) Rx function is required to repress *foxq2* expression within domain 2. Rx morphants exhibit expanded *foxq2* expression. (L,M) *foxq2* expression is not dependent upon Six3 function, but Six3 may somewhat restrict *foxq2* to domain 1. Dotted line delineates delineates posterior limit of staining. Scale bars: 50 µm.

maintaining cell proliferation is most likely ancient, as its expression has recently been observed in regions of high cell division in ctenophores (Schnitzler et al., 2014). Recent work also indicates that *soxc*-expressing cells contribute to the serotonergic neurons in sea urchins (Garner et al., 2015). Additionally, in the vertebrate forebrain, *Sox11*, a *soxc* ortholog, facilitates the transition from multipotent progenitor to differentiating postmitotic neuron by turning on genes such as *Lhx2* (Bergsland et al., 2011). Sox11 is

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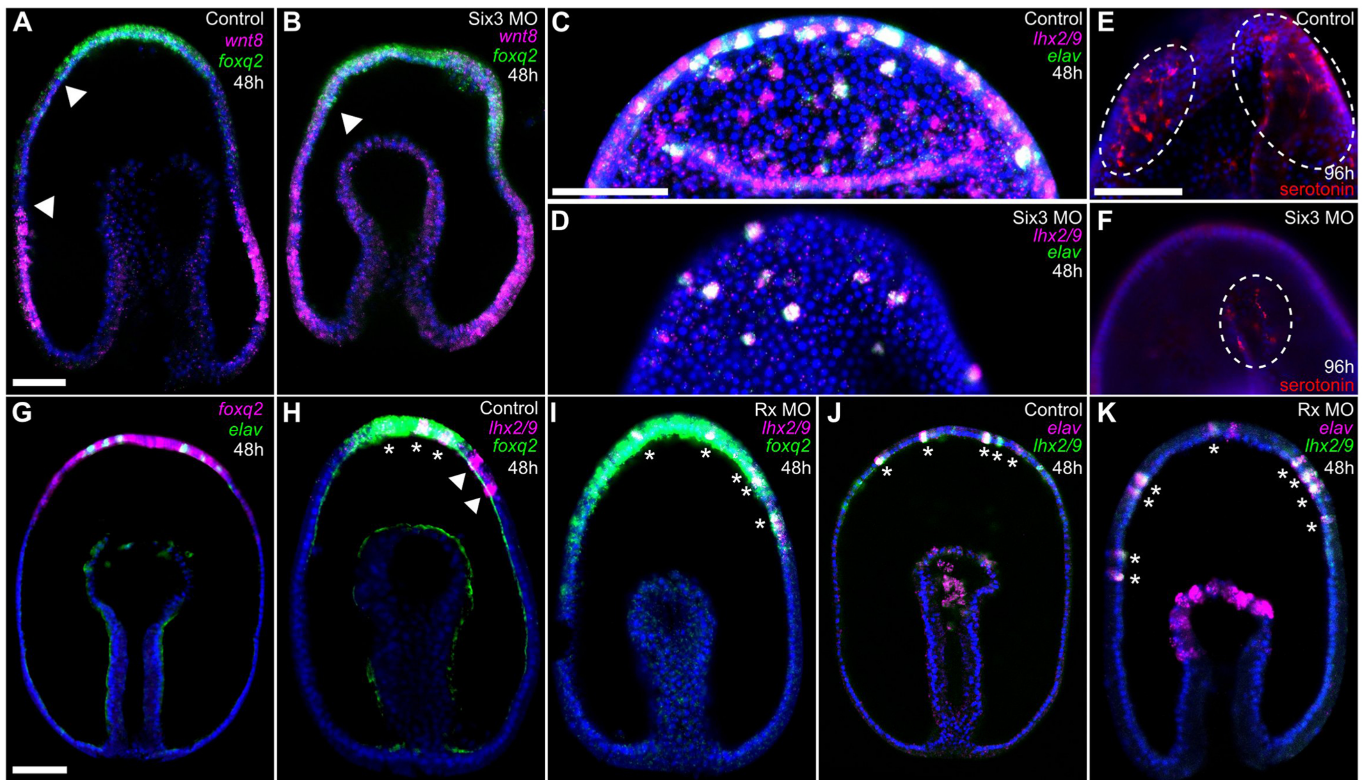

Fig. 4. Alteration of AP domains affects the quantity and location of neurons produced. (A) Two-color FISH of *wnt8* (magenta) and *foxq2* (green). *wnt8* is restricted to domain 4 and *foxq2* to domain 1, with domains 2 and 3 in between, which express neither gene. Arrowheads indicate the boundaries of these gene expression domains. (B) Six3 morphants exhibit expansion of *wnt8* and *foxq2*, such that domain 1 and 4 abut, and the molecularly distinct domains 2 and 3 are lost. (C) In the anterior ectoderm, *lhx2/9* (magenta) and *elav* (green) are normally co-expressed in the APD (domain 1), and pairs of cells only expressing *lhx2/9* are present more posteriorly, in domain 2. (D) All *lhx2/9*$^+$ cells also express *elav* in Six3 morphants. (E,F) Anterior dorsal views of 96 h larvae. (E) Control larvae have two large clusters of serotonergic neurons (dashed ovals), as indicated by serotonin immunostaining (red). (F) Six3 morphants produce fewer serotonergic neurons by comparison, which are in a single central cluster (dashed oval). (G) In control embryos, all ectodermal *elav* expression (green) is contained within domain 1, indicated by *foxq2* expression (magenta). (H) *lhx2/9* expression (magenta) occurs in both domains 1 and 2, and therefore some *lhx2/9*$^+$ cells (arrowheads) are outside of the *foxq2* expression domain (green). (I) In Rx morphants, *foxq2* expression is expanded and all *lhx2/9* expression is within the *foxq2* domain. (J) *lhx2/9* (green) and *elav* (magenta) co-expression normally occurs in domain 1, with additional cells expressing only *lhx2/9* in domain 2. (K) In Rx morphants, all *lhx2/9*$^+$ cells co-express *elav*. Asterisks (H-K) indicate coexpression of genes indicated at top right. Scale bars: 50 µm.

also needed for the proper proliferation of neural progenitor cells (Wang et al., 2013). Combined, these data suggest that this early neurogenic step is likely to be conserved not only among deuterostomes, but also potentially across metazoa. We had also previously correlated the presence of neurons in both the ciliary bands and the apical organ with *elav* expression (Yankura et al., 2013). Until now, we have had little knowledge of the transition state that occurs between these multipotent *soxc*$^+$ cells and the resulting unique neural populations. Here, we demonstrate in sea stars that *lhx2/9*$^+$ cells are the subset of multipotent progenitor progeny that are restricted to becoming the serotonergic neurons of the apical organ, as opposed to ciliary band neurons. *lhx2/9* promotes both the proliferation of restricted progenitors and their eventual differentiation into serotonergic neurons.

An important finding from our present work is that the progression of neurogenesis is dependent on position along the AP axis. A great body of work demonstrates similarities between the AP patterning of gene expression domains within the invertebrate ectoderm and the vertebrate CNS and in particular the forebrain (Range, 2014). The purpose of such domains in the larvae of marine invertebrates has been unclear, as they do not appear to use these domains to produce a complex CNS with discrete territories and cell types, as vertebrates do. Here, we explain for the first time that these expression domains define territories devoted to particular steps of neurogenesis during the development of an anterior sensory structure in sea star larvae. The entirety of this process is depicted in our model shown in Fig. 5. Activating and repressive interactions between Wnt signaling, *six3*, *rx* and *foxq2* result in nested gene expression of the aforementioned genes. Although multipotent progenitors, marked by expression of *soxc*, are present throughout the four resulting ectodermal domains, they are only able to progress to a restricted progenitor state, indicated by the expression of *lhx2/9*, in domains 1 and 2. We demonstrate that Foxq2 provides cues needed for neural precursors to differentiate into serotonergic neurons in domain 1. Domain 2 is a designated neural proliferation zone, as restricted progenitors are produced and continue to divide here, rather than differentiating. It is not yet clear whether these cells migrate to domain 1 to differentiate at a later point, or if the regulatory state of this territory changes over time. The shape, size and location of the apical organ change considerably between 48 h and 96 h, indicating that much remains unknown about how the development of serotonergic neurons occurs in this organ. We find that Rx is important for the maintenance of a restricted progenitor population adjacent to the APD, as it represses *foxq2*. Additionally, we find that the role of

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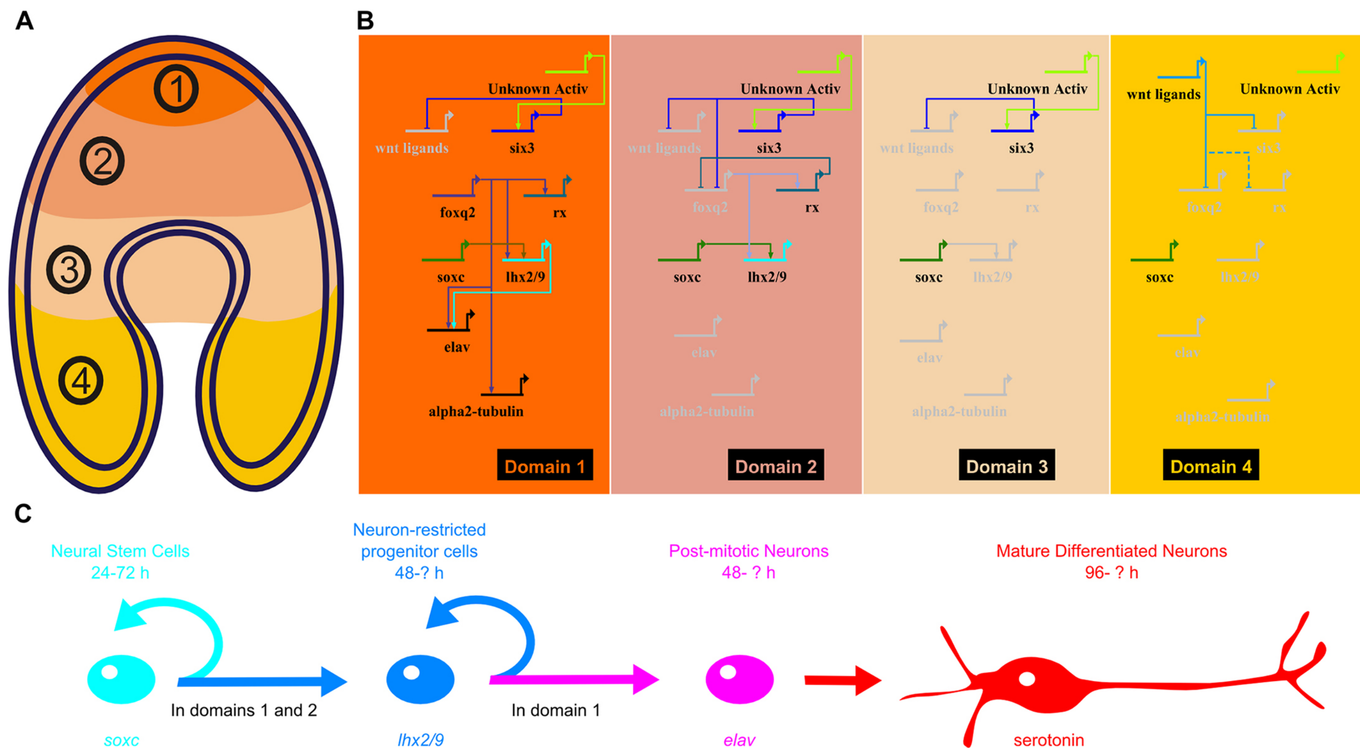

Fig. 5. A GRN for apical organ neurogenesis. (A) Schematic of AP regulatory domains in the sea star 48 h embryo. (B) GRN activity is specific to each domain, resulting in different steps of neurogenesis occurring in specific regions. It is important to note that, at this time, any of these regulatory inputs could be direct or indirect. Direct cis regulatory analyses of genes in this network has not yet been performed. (C) Schematic of neurogenesis. First, restricted progenitors are produced from multipotent progenitors through asymmetric divisions. Next, postmitotic neurons arise from asymmetric divisions of restricted progenitors. Finally, postmitotic neurons mature into differentiated serotonergic neurons.

Six3 is to maintain domain 2 by preventing both Wnt8 and Foxq2 activity from encroaching into this region. The presence and extent of these domains is crucial to ensuring that the apical organ attains a proper size by determining the number of restricted progenitors produced.

Interestingly, Foxq2 is able to control regulatory events in domain 2, even though its expression is limited to domain 1. We find that Foxq2 morphants do not express *lhx2/9* at all (Fig. 3C), rather than only missing these cells in the most anterior region of the ectoderm. The same is true for *rx* expression (Fig. 3E). There are several possible explanations for this phenomenon. First, Foxq2 might be able to exert an effect on domain 2 through diffusible molecules such as the Wnt agonist Dkk3 (Fig. S4A,B). Alternatively, this function might result from Foxq2 protein lingering in domain 2 from earlier time points, as this gene is known to progressively restrict its expression domain in other echinoderms (Yaguchi et al., 2008). However, if this is the case, then the progression of *lhx2/9*$^{+}$ cells to serotonergic neurons must either require additional cues in domain 1, or be prevented in domain 2 by some unknown mechanism; otherwise, we should observe *elav*$^{+}$ cells in domain 2. Further exploration into the regulatory states of each domain and the wiring between regulatory genes expressed in these domains will enhance our understanding of neurogenesis in the sea star and other organisms.

It had previously been suggested that *six3* must function at the top of the GRN for apical organ development in the sea urchin (Wei et al., 2009), as knockdown of this gene resulted in loss of a differentiated APD, including the loss of serotonergic neurons and apical tuft cilia. Six3 MO sea star larvae, by comparison, develop serotonergic neurons, although they are fewer in number. We think that this discrepancy is the result of different network wiring between *six3* and *foxq2*. Whereas Wei and colleagues found evidence for Six3 positively regulating *foxq2* (Wei et al., 2009), we see that, in sea stars, Six3 is a likely repressor of *foxq2* (Fig. 3L,M). During mammalian telencephalon development Six3 promotes the proliferation of neural precursors by keeping these cells in an immature state and by prolonging their amplification period (Appolloni et al., 2008). As in sea star larvae, in vertebrates the loss of Six3 does not alter the early specification of the anterior neural region; however, it does ultimately lead to truncation of the forebrain, presumably due to expansion of Wnt signaling into this region (Lagutin et al., 2003). Thus, Six3 has a highly conserved role in maintaining patterning that is tightly coupled to the regulation of neural precursor proliferation.

Finally, we anticipate that evolutionary changes to the relative sizes of these domains might provide a mechanism for altering the ratio of proliferation to differentiation. There is a great diversity in neuron number among larval forms; even among echinoderms, there are potential differences in the size of both the neural proliferative zone and the resulting apical organ. The 96 h sea star apical organ is composed of 30-50 serotonergic neurons located in two broad dorsal ganglia, whereas sea urchins of a comparable larval stage have only eight serotonergic neurons and these are restricted to a small apical pole territory (Byrne et al., 2007). We have previously compared gene expression along the AP axis of the sea star and sea urchin and suggested that although the sea urchin utilizes the same suite of regulatory genes and similar nested expression domains, the domains are extremely compacted in sea urchins compared with sea stars (Yankura et al., 2010). Study of the sea urchin reveals at least two concentric domains of gene expression in the anteriormost ectoderm: an outer ring of *six3* and

an inner ring of *foxq2* (Wei et al., 2009). It is unclear whether there is a distinct ring of *rx* expression in the sea urchin ectoderm. If so, domains 2 and 3 are still much smaller in sea urchins. Meanwhile, the wide domain of *six3* expression in the sea star allows a broader region of ectoderm to be permissive to the proliferation of neuronal precursors. We find that when the functions of Six3 are blocked in the sea star, this region of proliferating restricted progenitors is lost, resulting in fewer serotonergic neurons and a restriction of these neurons to a small region in the anterior of the embryo. This phenotype resembles the normal pattern of serotonergic neurons in the sea urchin (Bisgrove and Burke, 1986; Wei et al., 2009), suggesting that changes in *six3* expression and function might have contributed to differing neural proliferative zone size and apical organ morphologies among echinoderms.

MATERIALS AND METHODS

Embryo culture and microinjections

Patiria miniata embryos were cultured and injected as described previously (Cheatle Jarvela and Hinman, 2014). MOs were designed by GeneTools. Sequences are provided in the supplementary Materials and Methods. Images depicting MO-injected embryos are representative of phenotypes observed in at least three experiments, each containing 30-50 morphant embryos. Control embryos are siblings of corresponding gene knockdown embryos and were injected with 400 µM standard control MO construct (GeneTools). MO-injected embryos are collected at an equivalent developmental stage relative to control-injected embryos rather than at the same number of hours post-fertilization to correct for developmental delays. Hours post-fertilization listed in figure panels indicate the age of an equivalently staged control. Stage is determined by morphological features such as gastrulation, mouth formation and the overall shape of the embryo or larva.

BAC construct development and injections

The *P. miniata* BAC library was screened with a *soxc*-specific RNA probe. Five possible *soxc*-containing BACs were selected and sequenced. Two were found to have a 150 kb insert with ~75 kb of sequence upstream and downstream of the *soxc* transcript start, and standard protocols were used to recombineer a *gfp* sequence into the *soxc* single exon in each. Sequencing confirmed successful integration (Yu et al., 2000). Constructs were prepared and injected as described previously (Hinman et al., 2007).

Whole-mount *in situ* hybridization (WMISH) and fluorescent *in situ* hybridization (FISH)

WMISH was performed as previously described (Hinman et al., 2003; Yankura et al., 2010) using digoxigenin- or dinitrophenol-labeled antisense RNA probes. FISH embryos were mounted in Slowfade media (Life Technologies) and imaged by confocal microscopy with an LSM-510 Meta DuoScan inverted confocal microscope (Zeiss).

Immunofluorescence

Embryos were fixed as described for WMISH and stained with rabbit anti-serotonin (Sigma, S5545; 1:250 dilution) and, in some cases, also anti-GFP (Rockland, 600-4010215; 1:1000 dilution). Further detail is provided in the supplementary Materials and Methods.

EdU labeling

EdU labeling was performed using the Click-It Plus EdU 488 and 647 Imaging Kits (Life Technologies), with some modifications to the manufacturer's instructions, as described in the supplementary Materials and Methods. To capture cells in S phase, embryos were fixed immediately after 15 min EdU incubation. For the pulse-chase experiment, embryos were also treated with EdU for 15min, EdU was subsequently flushed out with artificial sea water, and embryos were incubated for an additional 30 min prior to fixation.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.M.C.J., K.A.Y. and V.F.H. devised the study, performed the experiments and analyzed the results. A.M.C.J. and V.F.H. wrote the manuscript.

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.134999.supplemental

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Supporting Information:

Supporting Materials and Methods:

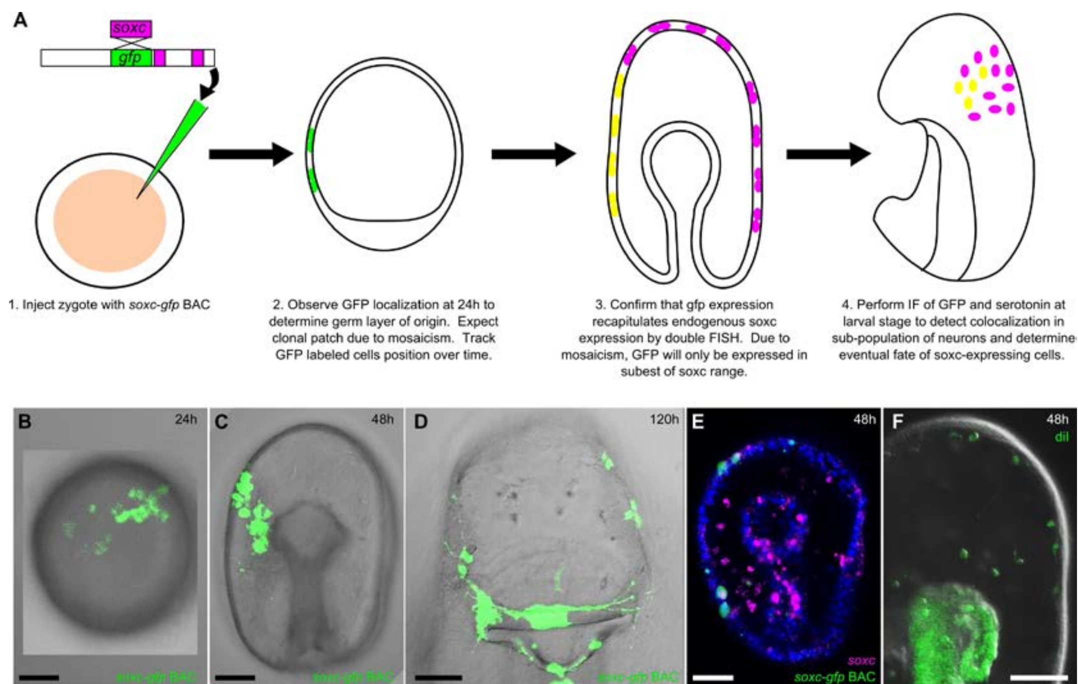
Morpholino Sequences:

*Pm*Lhx2/9: 5' CCGGTTGCAAAGTGAAATACATTCA 3'
*Pm*Rx: 5' GCACAGCTCCAACCCAGATAGCATC 3'
*Pm*FoxQ2_MASO2: 5' TAAACGATGTTCTCTGCAAGACGGT 3'
*Pm*Lhx2/9_MASO2: 5' AGTGTCCGAGCAAAACAAGAGTTGA 3'
*Pm*Soxc_MASO2: 5' TCTGAAGATACGTGGATGAAACTGA 3'
*Pm*Rx_MASO2: 5' TGGTCGCACGGTCCAGATACCAATT 3'
Other MASO sequences (i.e., *Pm*Foxq2, *Pm*Six3 1 and 2, *Pm*Soxc) can be found in our previous publication (1).

Immunofluorescence: Embryos were fixed in 4% paraformaldehyde/MOPS (100mM MOPS, 2mM $MgSO_4$, 1mM EGTA, 80mM NaCl, 0.1% Triton x-100) at room temperature for 90 minutes. They were subsequently stored in 70% EtOH at -20°C until use. Rehydration was accomplished by stepping embryos into MAB/0.1% Triton x-100 (100mM maelic acid, 150mM NaCl), followed by four washes in MAB/0.1% Triton x-100, and blocked in 2% BSA/MAB/0.1% Triton x-100. Embryos were next incubated with 1:250 rabbit anti-serotonin (Sigma), and in some cases also mouse anti-GFP (Pierce), diluted in 2% BSA block, overnight at 4°C. Antibodies were removed by washing four times with MAB/0.1% Triton x-100, and the embryos were next incubated with 1:1000 anti-rabbit alexa-fluor 568 or 1:1000 anti-mouse alexa-fluor 488 as appropriate, diluted in 2% BSA block. Finally, embryos were rinsed twice, incubated with 1:10,000 DAPI for 20 minutes, and rinsed four more times in MAB/0.1% Triton x-100. Imaging was performed as described for FISH.

EdU Labeling: Briefly, EdU was added to embryo culture at a final concentration of 10 µM and incubated for 15 minutes at 15°C. For pulse-chase experiment, EdU was subsequently flushed out with artificial sea water, and embryos were incubated an additional 30 minutes. Embryos were fixed and processed for FISH as previously described. After completion of the FISH protocol, embryos were washed three times in PBS, permeabilized in 0.5% Triton-x100/PBS, and then washed three times in 3% BSA/PBS. Embryos were incubated for 30 minutes in Click-It cocktail (prepared according to manufacturer's instructions, purchased from Thermo Fisher). After three additional PBS washes, nuclei were stained with DAPI, and embryos were imaged by confocal microscopy as described above.

Supplemental Figures:



Supplemental Figure 1: Soxc-GFP knock-in BAC recapitulates endogenous ectodermal *soxc* expression. A. Schematic depicting GFP BAC injection and subsequent expression. B. BAC-directed GFP expression in a 24h embryo. C. BAC-directed GFP expression in a 48h embryo. D. BAC-directed GFP expression in a sub-population of mature neurons in the apical organ and mouth. E. *gfp* transcripts (green) resulting from BAC expression overlap in clonal patches with endogenous *soxc* expression (magenta) as determined by double FISH. This indicates that the BAC construct faithfully directs *gfp* expression in a pattern that mimics *soxc*'s expression. F. A lineage of mesodermal cells was labeled with diI by injection at the 8-cell stage. At 48h, some of these cells ingress into the blastocoel, but do not embed themselves within the ectoderm. This indicates that *elav*+ cells found in the ectoderm are of ectodermal, rather than mesodermal origin. Scale bars indicate 50 μm.

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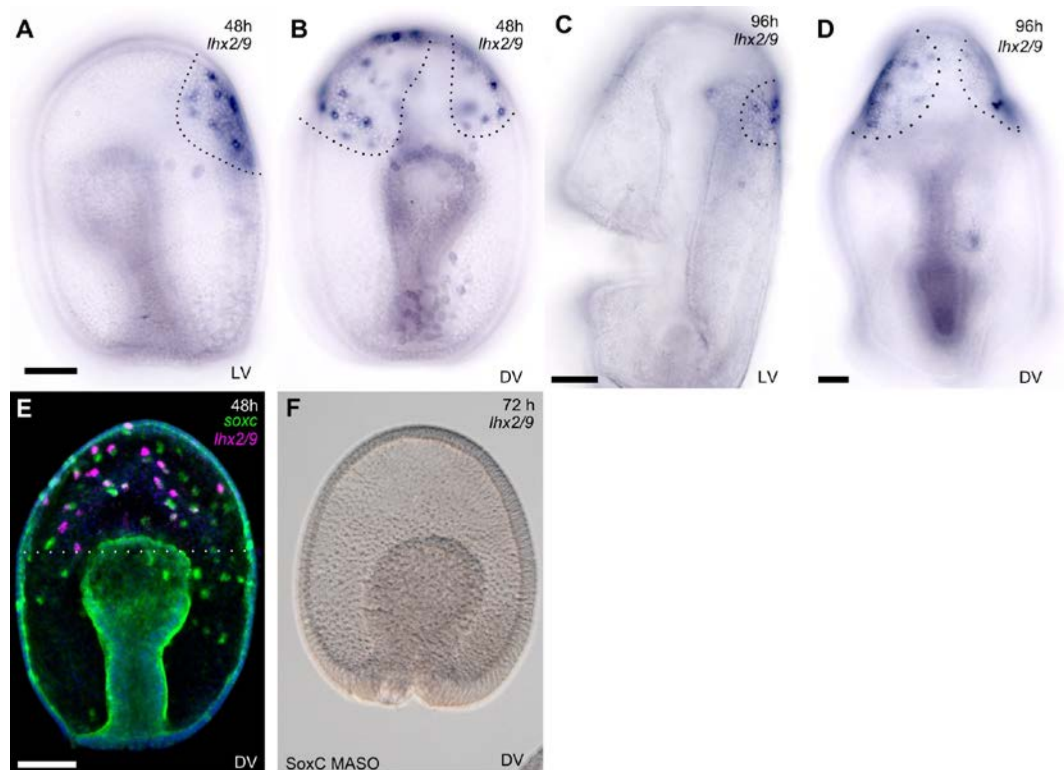

Supplemental Figure 2: *Lhx2/9* expression is specific to the presumptive apical organ. A. Lateral view (LV) of a 48 h embryo. The dorsal face is on the right. *Lhx2/9* expression occurs in spots in the anterior dorsal ectoderm, outlined by dashes. B. Dorsal view of a 48 h embryo reveals lhx2/9 expression in two anterior ectoderm clusters. C. A lateral view of a 96 h larvae, with the dorsal face positioned to the right. *Lhx2/9* expression is localized to the dorsal ectoderm. D. A dorsal view of a 96 h larvae. *Lhx2/9* is expressed in two clusters in the anterior ectoderm. E. *Soxc* and *lhx2/9* are co-expressed in the anterior ectoderm, but pairs of *soxc+* cells without *lhx2/9* are also present more posteriorly (below the dotted line). F. 72 h SoxC morphant embryo still does not express *lhx2/9* in spite of additional time to develop. SoxC morphants do not achieve a normal 72 h phenotype, most likely because early ectodermal progenitors require this gene product. Scale bars indicate 50 µm.

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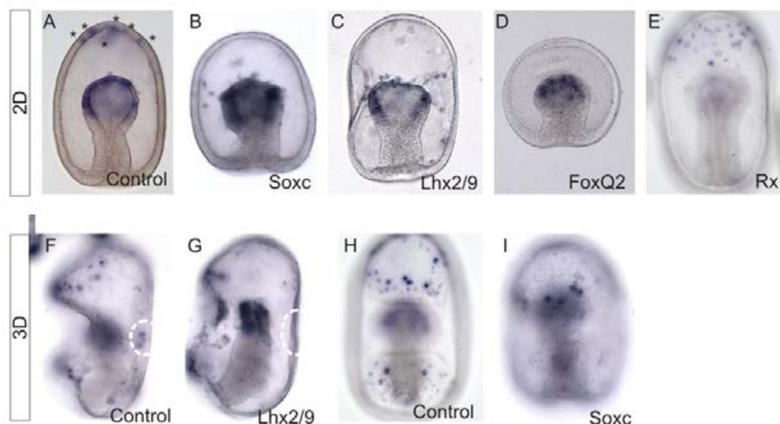

Supplemental Figure 3: A-G Confirmation of morpholino phenotypes with use of second independent MASO sequence. All staining is for the expression of Elav. Second morpholinos to *soxc*, *lhx2/9*, *foxq2*, and *rx* were injected into embryos at 600 micro molar and expression of *elav* was analyzed to confirm knock-down phenotypes match those described in the main text and figures. In all cases, phenotypes correspond to previous results; i.e. loss of apical ectodermal *elav* expression in Soxc, Lhx2/9 and FoxQ2 morphants, and expansion of *elav* cells in Rx morphants. H-I Older stage soxc morphants (3d) injected with lower doses of MASO (400 micromolar) are able to develop to larval stage, but still show a very reduced number of *elav*+ neurons. Stars in A show location of *elav* positive cells in control 48h embryo. Dashed circles in F and G show location of dorsal ganglion in this stage, which has no elav+ cells in Lhx2/9 morphants.

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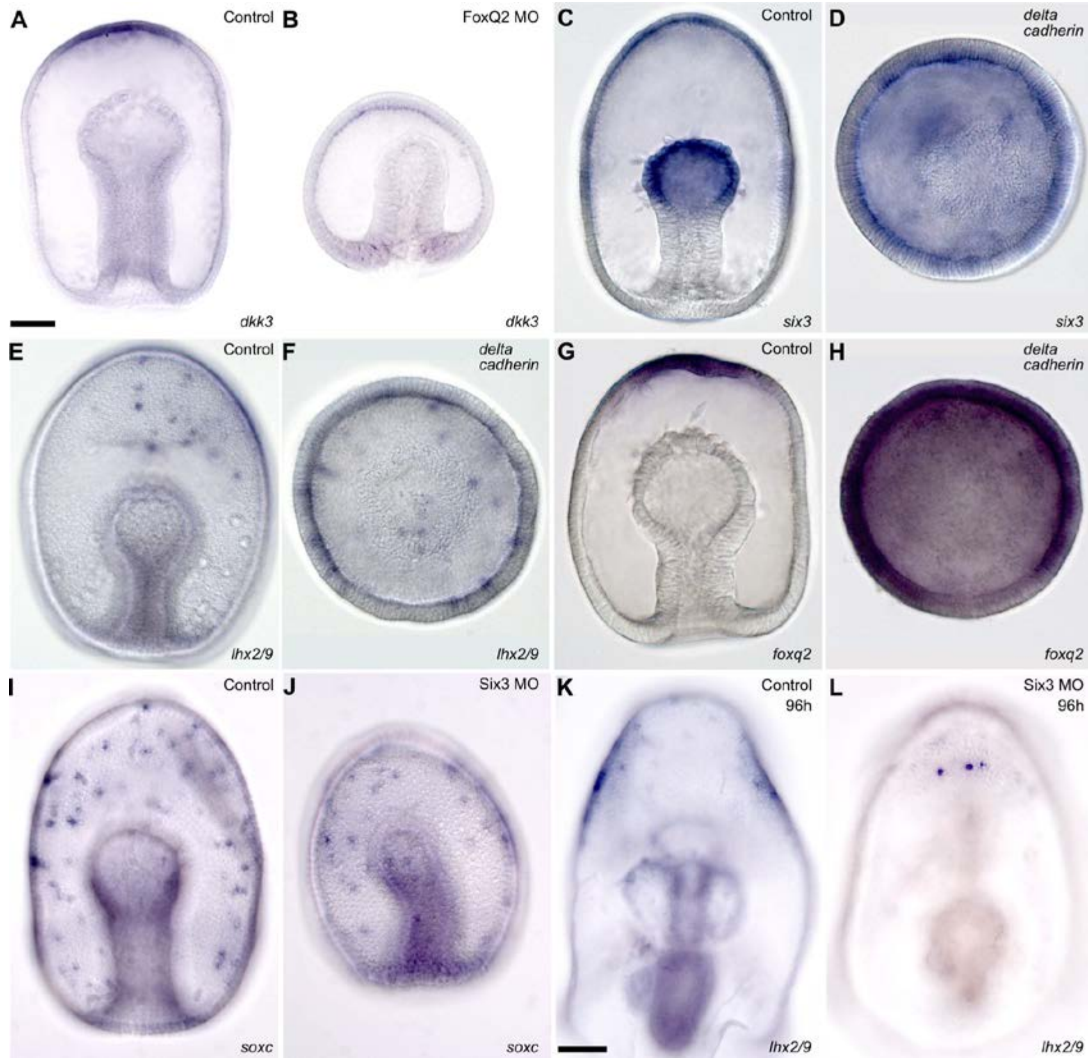

Supplemental Figure 4: Roles of AP patterning genes in establishing unique neurogenic domains. All embryos are 48 h unless otherwise indicated. A. *dkk3* is expressed in domain 1, the apical pole domain. B. *dkk3* expression is lost when Foxq2 is knocked-down. C-H. Wnt signaling limits expression of AP domains to their proper boundaries. Canonical wnt signaling is perturbed by the introduction of *delta-cadherin* mRNA. C. *six3* is expressed in the mesodermal bulb, and also in domains 1-3 of the ectoderm. D. *Delta-cadherin* injected embryos ubiquitously express *six3*. E. *lhx2/9* is normally expressed in domains 1-2, but expands throughout the ectoderm in *delta-cadherin* embryos (F). G. *foxq2* expression is limited to domain 1 in control embryos. H. *Delta-cadherin* embryos exhibit ubiquitous *foxq2* expression. I. *soxc* is expressed in pairs of cells throughout the ectoderm. J. Six3 morphants express *soxc* normally. K. *lhx2/9* is expressed in bilateral clusters in the anterior dorsal ectoderm at 96 h. L. 96 h Six3 morphants produce notably reduced numbers of *lhx2/9*-expressing cells. Scale bars indicate 50 µm.

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