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RESEARCH ARTICLE

Neurogenic gene regulatory pathways in the sea urchin embryo

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ABSTRACT

During embryogenesis the sea urchin early pluteus larva differentiates 40-50 neurons marked by expression of the pan-neural marker synaptotagmin B (SynB) that are distributed along the ciliary band, in the apical plate and pharyngeal endoderm, and 4-6 serotonergic neurons that are confined to the apical plate. Development of all neurons has been shown to depend on the function of *Six3*. Using a combination of molecular screens and tests of gene function by morpholino-mediated knockdown, we identified *SoxC* and *Brn1/2/4*, which function sequentially in the neurogenic regulatory pathway and are also required for the differentiation of all neurons. Misexpression of *Brn1/2/4* at low dose caused an increase in the number of serotonin-expressing cells and at higher dose converted most of the embryo to a neurogenic epithelial sphere expressing the Hnf6 ciliary band marker. A third factor, Z167, was shown to work downstream of the Six3 and SoxC core factors and to define a branch specific for the differentiation of serotonergic neurons. These results provide a framework for building a gene regulatory network for neurogenesis in the sea urchin embryo.

KEY WORDS: Sea urchin, *Strongylocentrotus purpuratus*, Neurogenesis, Embryogenesis, SoxC, Brn1/2/4, Six3

INTRODUCTION

At the end of embryogenesis the nervous system of the sea urchin early pluteus larva includes an apical ganglion in the apical plate at the anterior (animal) pole, a set of 40-50 peripheral neurons, most of which differentiate in, or adjacent to, the ciliary band, and a few neurons that differentiate in the pharyngeal endoderm (Yaguchi et al., 2006; Wei et al., 2009; Range et al., 2013; Burke et al., 2014) (Fig. S1). The apical organ develops within a distinct regulatory domain termed the animal pole domain (APD) (Angerer et al., 2011), which encompasses 10-15% of the blastula surrounding the anterior pole. The patterns of gene expression in the APD become increasingly complex during later larval development and, in addition to the apical ganglion, it generates the apical tuft of cilia and several different groups of cells identified by expression of specific genes with neurogenic functions in other organisms (Burke et al., 2014); the first of these to appear are 2-6 neurons at the posterior margin within the APD, which express serotonin and tryptophan hydroxylase (Tph), an enzyme in the serotonin biosynthesis pathway. The peripheral neurons send projections along the ciliary band to the apical organ and out into the dorsal ectoderm (Burke et al., 2014); the apical ganglion is thought to have sensory and integrating functions, while the ciliary band neurons are thought to have sensory function, and together they are proposed to coordinate the ciliary beat. All of these differentiated neurons can be identified by expression of the pan-neuronal marker synaptotagmin B (SynB) (Burke et al., 2006).

The initial positioning of the neurons in the sea urchin embryo ectoderm is achieved in part through downregulation of neurogenic potential by separate signaling mechanisms operating along the maternally established animal-vegetal (future anterior-posterior) axis and the embryonic dorsal-ventral axis (Angerer et al., 2011). When all known early cell-cell signaling in the embryo is blocked by inhibiting the inductive cascade normally initiated at the posterior (vegetal) pole, a strong underlying maternally driven neurogenic bias is revealed: virtually the whole embryo activates a developmental program characteristic of the APD that generates the apical organ and ciliary tuft, and serotonergic and non-serotonergic neurons differentiate throughout the hyperciliated embryoid (Yaguchi et al., 2006; Wei et al., 2009; Range et al., 2013). This is the severely ‘animalized’ embryo of classical experimental embryology (Hörstadius, 1973). In normal embryos a cascade of multiple Wnt signaling events, initiated at the posterior pole at fertilization and operating, at least in part, through the FoxQ2 transcription factor, progressively restricts the posterior margin of the APD toward the anterior pole (Range et al., 2013; Yaguchi et al., 2008). By the blastula stage, activation of Wnt antagonists within the APD opposes this posterior signaling and defines the final APD domain. Comparison of functional and expression data from ambulacrarians, invertebrate chordates and vertebrates strongly suggests that this Wnt network may be a mechanism that is shared by deuterostomes for positioning the anterior neuroectoderm (Range, 2014). Along the ventral-dorsal axis, differentiation of non-serotonergic neurons in the lateral ectoderm is restricted to a region close to, or within, the ciliary band by a combination of Nodal signaling on the ventral side and the functions of BMP2/4 and the atypical BMP ligand ADMP on the future dorsal side of the embryo (Yaguchi et al., 2010; Lapraz et al., 2009, 2015). Surprisingly, relatively late in embryogenesis, two additional neurons that are thought to function in feeding differentiate within the pharyngeal endoderm (Wei et al., 2011).

Although we have some understanding of the mechanisms that position neurogenic territories in the sea urchin embryo, little information is available in this or other lower deuterostome embryos on the gene regulatory networks (GRNs) that direct the differentiation of different types of neurons. To begin such an analysis, in a previous study we used the leverage provided by the difference in the phenotype and gene expression of ‘animalized’ versus normal embryos to search for potential regulatory genes that might function in neurogenesis. This effort identified a large number of orthologs of vertebrate genes that are expressed in the APD and function in neurogenesis in other systems, especially in the vertebrate forebrain (Wei et al., 2009). One of these, *Six3*, was found to be required for the differentiation of all neurons in the embryo (Wei et al., 2009), and global misexpression of Six3 was shown to drive embryos to the expanded APD phenotype. *Six3* expression begins during cleavage stages, broadly and

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encompassing the neurogenic regions of the embryo. The fact that its expression is not restricted to individual neural precursors suggests that *Six3* is involved in establishing neuroectoderm territories but is not directly involved in initiating the terminal differentiation of neurons. In this work we have identified three factors that function downstream of *Six3* in the pathways to serotonergic and SynB-positive neurons using RNA-Seq-based screening and analysis by multicolor *in situ* hybridization: *SoxC* and *Brn1/2/4* function in the specification of all neurons, whereas *Z167* defines a branch point leading to the differentiation of serotonergic neurons.

RESULTS

SoxC function is required downstream of *Six3* for development of all neurons

SoxC is an early, zygotically activated gene that is expressed in scattered individual cells in the lateral ectoderm [Fig. 1A,B (red) and G-I (green)] and in the APD (future apical plate), as well as in a group of contiguous cells at the posterior end (vegetal plate; Fig. 1A, arrowheads) at the blastula stage and foregut at the gastrula stage (Fig. 1B, arrowheads) (Poustka et al., 2007). In embryos developed from eggs injected with a *Six3* morpholino (morphants), *SoxC* mRNA levels were greatly reduced, as shown here by *in situ* hybridization (Fig. 1C) and consistent with previous microarray and qPCR experiments (Wei et al., 2009). Simultaneous hybridization with probes for *Hnf6* (also known as *onecut*), which is an APD/ciliary band marker, and *SoxC* transcripts showed that *SoxC*-expressing cells lie adjacent to, or within, the ciliary band and APD (Fig. 1G-I′, arrowheads), as expected for neural precursors (Burke et al., 2014). Further supporting this identity, cells in both the apical plate and lateral ectoderm that express *SoxC* also express *Delta* (Fig. 1D,E), which is characteristic of neural precursors in embryos (Vässin et al., 1987; Yang et al., 2009). Furthermore, when Delta-Notch signaling is blocked by treatment with DAPT, which is a gamma secretase inhibitor of Notch signaling, *SoxC*-expressing cells increase in number and form small clusters (Fig. 1F), reflecting the loss of lateral inhibition that is also characteristic of neural precursors (Formosa-Jordan et al., 2013). The number and positions of *SoxC*-expressing cells varied among embryos at the same time of development (Fig. 1G,H,I), suggesting expression is asynchronous and/or that *SoxC* is expressed transiently.

To determine whether it is required for the differentiation of some or all neurons, we knocked down SoxC by morpholino injection and stained with antibodies specific for serotonergic neurons in the APD and for all neurons as recognized by the pan-neural marker *SynB*. As shown in Fig. 2, signals for both neural markers were greatly reduced and, in many cases, completely eliminated, with minimal effect on other structures of the embryo (Fig. 2A versus B and C). The same phenotype was observed with two different morpholinos, one of which was tested and shown to prevent accumulation of SoxC protein (Fig. S2A,B). This suggested that SoxC could be an immediate regulator of neural terminal differentiation. However, in double *in situ* hybridization experiments for *SoxC* and *SynB* transcripts, the two were rarely found colocalized (Fig. 2D-F). Similarly, in embryos treated with DAPT, both *SoxC*-positive and *SynB*-positive cells formed clusters, but doubly labeled clusters were very rare (Fig. 2G-I). This suggests that SoxC has an early transient function in neurogenesis and is not an immediate regulator of terminal differentiation genes, such as *SynB*.

In vertebrates, the *SoxC* family member *Sox11* functions in neural progenitors or neuroblasts (Hong and Saint-Jeannt, 2005; Reiprich and Wegner, 2015), suggesting that *SoxC* might play a similar role in sea urchin embryos. To test this possibility, we determined whether some *SoxC*-expressing cells are still dividing. We labeled embryos with anti-phospho-Histone H3 (H3p) antibody, which specifically labels chromatin between prophase and anaphase (Hendzel et al., 1997). In embryos analyzed at late gastrula and early prism stages, the metaphase nuclei of a few *SoxC*-expressing cells were labeled by the antibody (Fig. 3A-D, arrowheads). The frequency of doubly labeled cells was low, consistent with the low rate of cell division at this stage, the small number of *SoxC*-expressing cells in any individual embryo and the short duration of metaphase of dividing cells. We conclude that at least some *SoxC*-expressing cells are still cycling, consistent with SoxC

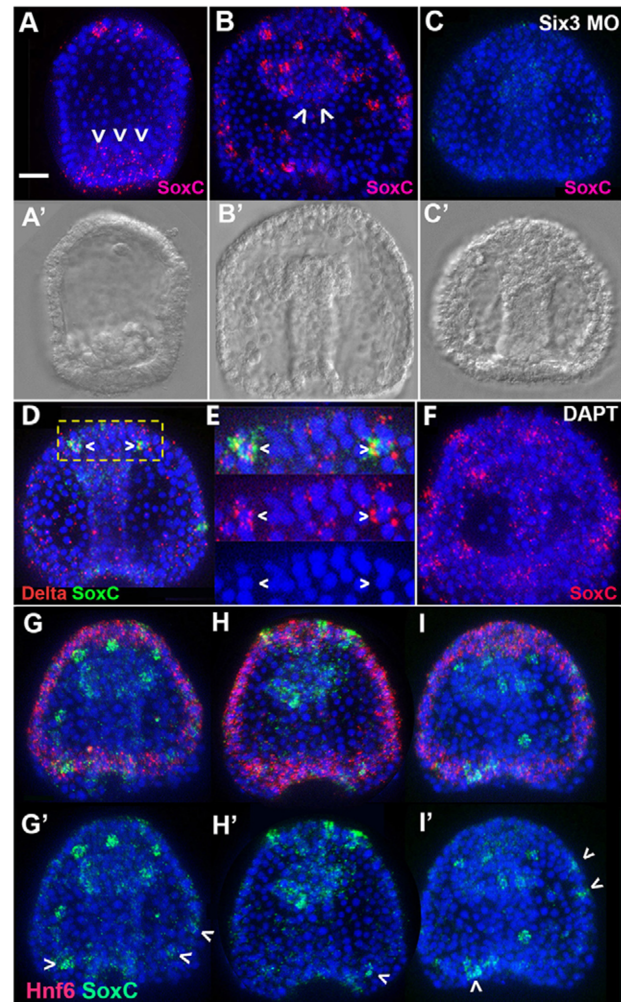

Fig. 1. *SoxC* expression in control sea urchin embryos and embryos treated with DAPT or *Six3* morpholino. (A,B) *In situ* hybridization for *SoxC* (red) shows signal in individual cells of the anterior end (apical plate) and posterior end (vegetal plate; arrowheads) of a 28 h embryo (A) and in lateral ectoderm and foregut (arrowheads) of a 48 h embryo (B). (C) *SoxC* expression in all regions is greatly reduced in *Six3* morphants. (A′,B′,C′) The corresponding DIC images. (D,E) *SoxC* (green) transcripts are detected in some cells expressing *Delta* (red, arrowheads). Doubly labeled cells are shown at higher magnification in E. (F) *SoxC*-expressing cells (red) form small clusters in DAPT-treated embryos. (G-I) Three embryos illustrating that the locations and number of *SoxC*-expressing cells (green) vary among embryos. Magenta signal is the ciliated band/APD marker *Hnf6*. Arrowheads indicate *SoxC*-expressing cells in the ciliated band. (G′,H′,I′) The corresponding green channels. All images are ventral (oral face) views with the animal pole at top. All embryos except A are at the 2-day late gastrula stage. Nuclei are labeled with DAPI (blue). Scale bar: 20 µm.

Development (2016) 143, 298-305 doi:10.1242/dev.125989

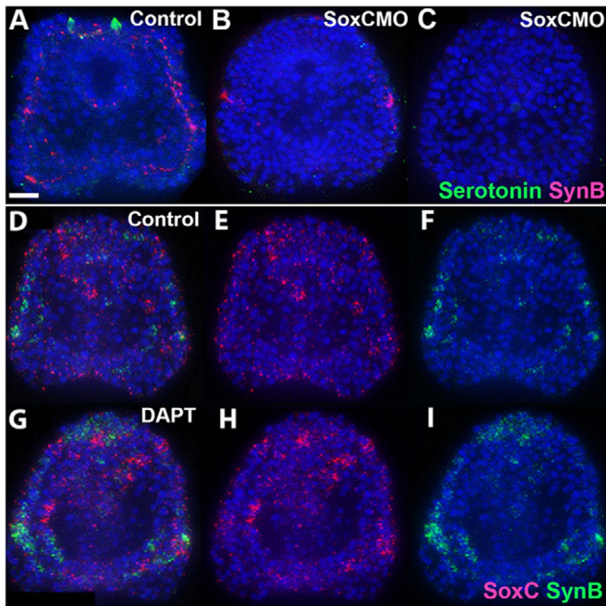

Fig. 2. SoxC is required for the differentiation of all neurons. (A-C) Immunostaining for serotonin (green) and the pan-neural marker SynB (red) is greatly reduced when SoxC is knocked down by morpholino injection (A, control; B and C, two morphants). (D-F) *SoxC* (red) and *SynB* (green) transcripts are expressed in different cells (D, overlap of two channels; E and F, individual channels). (G-I) *SoxC*-expressing and *SynB*-expressing cells both form clusters in embryos treated with DAPT, but labeling for the two markers is mutually exclusive. Nuclei are labeled with DAPI (blue). All embryos are at 68-70 h and shown with the anterior pole at the top. Scale bar: 20 µm.

operating early in the neurogenesis pathway. In a few cases, nuclei of two adjacent cells were labeled by H3p (Fig. 3E-N, arrowheads), only one of which was *SoxC* positive, raising the possibility that *SoxC* might continue to be expressed in one daughter of an asymmetric division. Although it is not possible from these data to define the fates of these two cells, we suggest it is likely that the *SoxC*-positive cell has retained its precursor identity.

Identifying genes that function downstream of *SoxC*

The above observations suggested that regulators of terminal differentiation genes function downstream of *SoxC*. To search for such factors we carried out an RNA-Seq screening experiment to identify genes that depend on SoxC for expression. We tested RNA from 30 h *SoxC* morpholino-injected embryos because, at this stage, many neural genes are likely to be active, but mRNAs characteristic of differentiated *SynB*-positive neurons have not yet appeared (Burke et al., 2006). Among mRNAs affected we identified a few that encode transcription factors, the abundance of which decreased significantly in the absence of SoxC. We pursued *Z167* and *Brn1/2/4*, which were suppressed ∼8-fold and ∼4-fold, respectively, as candidates for intermediate functions in the neural development pathway.

Z167 is encoded by one of 377 zinc finger transcription factor genes that have been annotated in the sea urchin genome (Materna et al., 2006); a specific vertebrate homolog cannot be determined because of the high degree of sequence conservation among zinc finger proteins. Brn1/2/4 is related to three Class III POU domain transcription factors expressed in vertebrate embryos that have demonstrated roles in neural specification (Ryan and Rosenfeld, 1997; Wegner et al., 1993). During mouse embryogenesis, Brn1 (Pou3f3), Brn2 (Pou3f2) and Brn4 (Pou3f4) are expressed in all levels of the CNS and their expression is more tissue-restricted in adults (Wegner et al., 1993). Deletion of *Brn2* is lethal in mouse but not until as late as embryonic day 10. No general defects in neurogenesis were identified, but an essential role for Brn2 has been demonstrated relatively late in the differentiation of specific neural lineages of the endocrine hypothalamus and anterior pituitary (Nakai et al., 1995; Schonemann et al., 1995). Brn2 is highly overexpressed in melanoma, in which it behaves as a pro-proliferative transcription factor (Vance and Goding, 2004). It is required for melanocyte development and lack of Brn2 expression in migrating melanoblasts has led to the suggestion that it is required early and transiently (Goding, 2008).

Z167 links SoxC function to the differentiation of serotonergic neurons in the APD

Consistent with the large reduction in *Z167* expression observed in the screen, in control embryos *Z167* is expressed only in individual cells in the APD (Fig. 4A, red), and transcripts are essentially undetectable in *SoxC* knockdowns (Fig. 4B). As is observed for cells expressing *SoxC* or *SynB*, *Z167*-expressing cells increase in number and form small clusters when embryos are treated with DAPT (Fig. 4C, green). These observations suggested that the

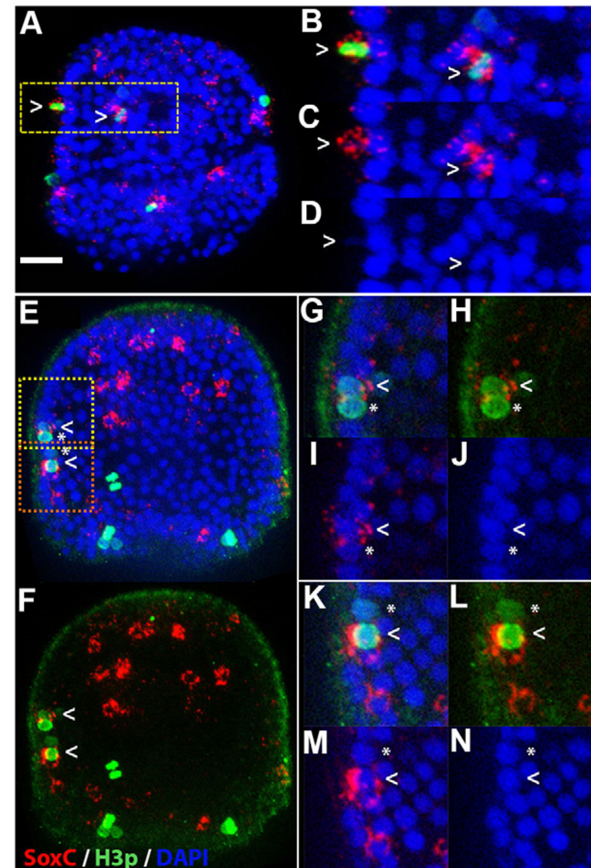

Fig. 3. Some dividing cells express *SoxC*. Embryos were immunostained with the H3p antibody, which labels cells from prophase to anaphase, and hybridized with a probe for *SoxC* mRNA. (A) Two *SoxC*-expressing cells (red) are H3p positive (green, arrowheads). (B) Higher magnification image of H3p-positive cells outlined by the yellow box in A. (C) *SoxC* (red) and DAPI (blue). (D) DAPI only. (E) Two adjacent cells: one *SoxC* positive (arrowhead) and the other *SoxC* negative (asterisk). (F) The *SoxC* and H3p channels of E. (G-J) Higher magnification images of the region in E outlined in yellow. (K-N) Higher magnification images of the region in E outlined in brown. All embryos are at 66 h and are shown with anterior poles at the top. Scale bar: 20 µm.

Development (2016) 143, 298-305 doi:10.1242/dev.125989

Z167-positive cells might be developing serotonergic neurons. As an initial test of whether Z167 links SoxC function to the expression of neural terminal differentiation genes, we performed a triply labeled *in situ* hybridization for *SoxC*, *Z167* and *Tph* [a marker specific for serotonergic neurons (Yaguchi and Katow, 2003)]. Analysis of a representative embryo is shown in Fig. 4D-I. Shown in these optical sections are five *SoxC*-positive cells (Fig. 4H, blue), three *Tph*-positive cells (Fig. 4E, green) and three *Z167*-expressing cells (Fig. 4G, red). All three *Tph*-positive and all three *Z167*-positive cells are in the APD, whereas only two of the five *SoxC*-positive cells are located there. When the image is separated into more ventral (Fig. 4F) and dorsal (Fig. 4I) planes, the ventral image shows two *Tph*-positive cells and one *Z167*-expressing cell (arrowhead). In the dorsal image, two of the three *Z167*-positive cells also express *SoxC*, while the third also expresses *Tph* (arrowhead; the signal from this cell is also shown in the ventral section). Therefore, some cells express both *SoxC* and *Z167*,

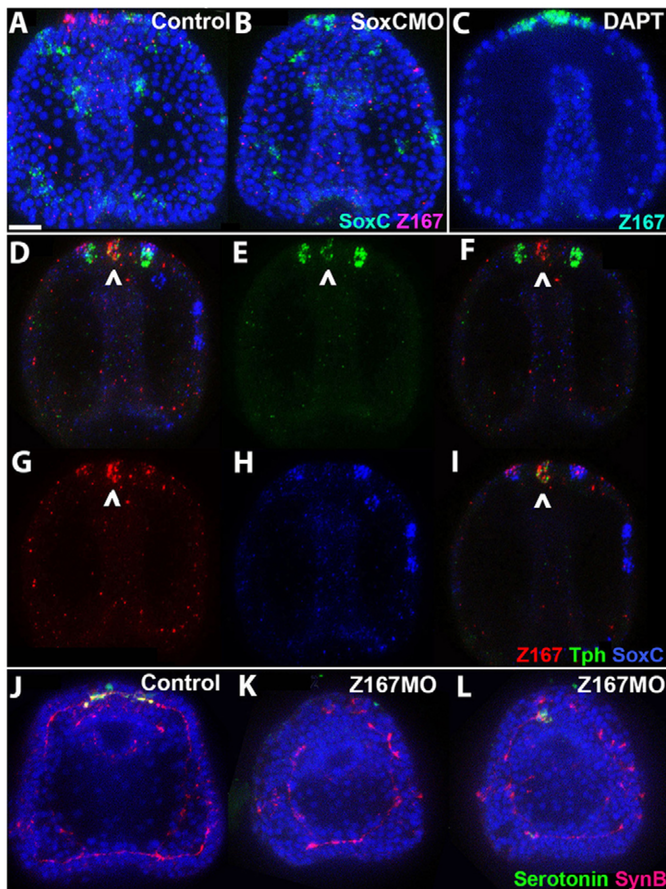

Fig. 4. Z167 is required for differentiation of serotonergic neurons in the APD. (A,B) Doubly labeled *in situ* hybridization for *Z167* (red) and *SoxC* (green), showing that *Z167* is expressed in individual cells at the anterior pole of a control embryo (A), whereas its expression is greatly reduced in *SoxC* morphants (B). (C) *Z167*-expressing cells (green) form clusters in embryos treated with DAPT. (D-I) Triple *in situ* hybridization for *SoxC* (blue), *Z167* (red) and *Tph* (green), showing that some *Z167*-expressing cells also express *SoxC*, whereas others also express *Tph*, a marker for serotonergic neurons. (D) The stack of multiple optical sections further analyzed in E-I. (E) *Tph*, (G) *Z167*, (H) *SoxC*. The combined signals are shown in more ventral (F) and more dorsal (I) optical sections. (J-L) Immunostaining for serotonin (green) and SynB (red), showing that serotonin expression is reduced in *Z167* morphants. (J) Control; (K,L) two morphants. Nuclei in A-C and J-L are labeled with DAPI (blue). Embryos in A-I are at 46 h and in J-L at 70 h. All embryos are shown with anterior poles at the top. Scale bar: 20 µm.

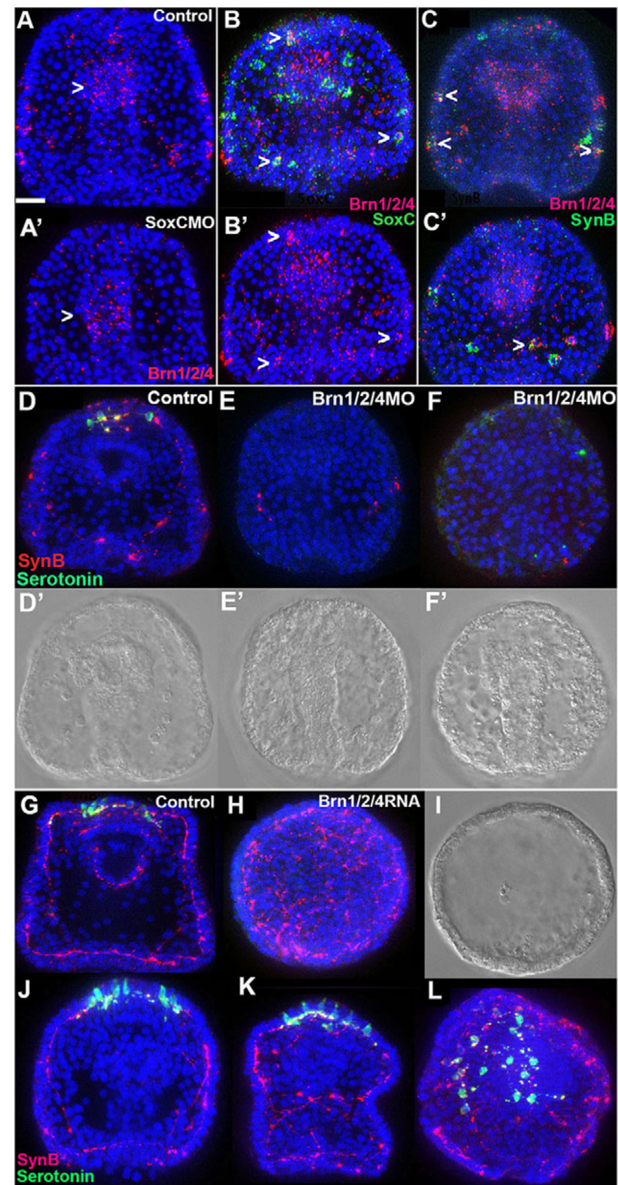

Fig. 5. Brn1/2/4 is required downstream of SoxC for differentiation of all neurons. (A,A′) *Brn1/2/4* (red) is expressed in individual cells in ectoderm and in a group of contiguous cells in the foregut (arrowhead) of a control embryo (A); the number of individual labeled cells is greatly reduced in ectoderm, whereas expression in contiguous endoderm cells (arrowhead) is not detectably affected in *SoxC* morphants (A′). (B,B′) *Brn1/2/4* and *SoxC* doubly labeled *in situ* hybridization of an embryo, showing a few *Brn1/2/4*-expressing cells (red) that also express *SoxC* (green, arrowheads). (B′) Red channel only. (C,C′) Doubly labeled *in situ* hybridization for *Brn1/2/4* and *SynB* in two different embryos, showing that some *Brn1/2/4*-expressing cells (red) also express *SynB* (green, arrowheads). (D-F) *Brn1/2/4* morpholino greatly reduced the number of neurons (green, serotonin; red, SynB). (D) Control; (E,F) two morphants. (D′-F′) Corresponding DIC images. (G-I) Immunostaining for serotonin (green) and SynB (red) in a 3-day control embryo (G) and a *Brn1/2/4* mRNA-injected (H) embryo (higher dose). The latter embryo is an epithelial sphere that lacks serotonin staining but has expanded SynB signal. (I) DIC image of embryo in H. (J-L) Three different injected embryos showing that, at the lower *Brn1/2/4* mRNA dose, the serotonergic cell number is increased. At ∼1.0 µg/µl *Brn1/2/4* mRNA, most embryos resembled that shown in H; at 0.5 µg/µl, more embryos resembled those in J-L. Embryos in A-C are at 46 h and in D-L at 72 h. All embryos are shown with the anterior poles at the top, except H,I,L; L is an anterior view of the APD. Nuclei are labeled with DAPI (blue). Scale bar: 20 µm.

Development (2016) 143, 298-305 doi:10.1242/dev.125989

whereas others express both *Z167* and *Tph*. These observations are consistent with a dynamic transition in the regulatory state of serotonergic neurons: SoxC→Z167→Tph/serotonin.

To determine whether Z167 activity links SoxC function to the differentiation of serotonergic neurons, we characterized a morpholino knockdown. As shown in Fig. 4, *Z167* morphants have greatly reduced immunostaining for serotonin in the anterior pole but, as expected, staining throughout embryos for the pan-neural marker SynB was little affected (Fig. 4J versus K and L). Interestingly, knockdown of Z167 also resulted in loss of *Z167* transcripts (Fig. S2C versus D), suggesting that, once activated, it is locked in a positive autoregulatory loop. Although *Z167* expression was reduced in *Z167* morphants, *SoxC* expression was not reduced (Fig. S2D), indicating that Z167 does not reciprocally activate *SoxC*.

Brn1/2/4 function is required downstream of SoxC for all neurons

Since SoxC is required for the development of all neurons, whereas Z167 is required only for serotonergic neurons, other factors must relay SoxC function in other neural precursors. Of the genes affected by SoxC knockdown, *Brn1/2/4* was selected for further study because, like *SoxC*, it is expressed in scattered individual cells in the ectoderm, including the APD, and throughout the foregut region (Cole and Arnone, 2009) (Fig. 5A, red). *Brn1/2/4* is also expressed maternally, and transcripts gradually decrease in abundance until they are undetectable at the blastula stage (Fig. S3). It is likely that these early transcripts are uniformly distributed in the embryo since they have not been detected by *in situ* hybridization (Cole and Arnone, 2009; Z.W., unpublished observations).

The dependence of *Brn1/2/4* on SoxC function was confirmed by morpholino-mediated knockdown and *in situ* hybridization analysis: in *SoxC* morphants, labeling of individual ectoderm cells for *Brn1/2/4* transcripts was dramatically reduced, whereas expression in the foregut was not detectably affected (Fig. 5A,A′, arrowhead). To examine the possibility that Brn1/2/4 links SoxC function to the expression of terminal differentiation genes in neurons, we carried out double *in situ* hybridizations of *Brn1/2/4* and *SoxC* or *SynB*. In embryos probed for *SoxC* and *Brn1/2/4*, some *Brn1/2/4*-expressing cells also expressed *SoxC* (Fig. 5B,B′, arrowheads); as was the case for *Z167*, only a few cells were doubly labeled. Similarly, some *Brn1/2/4*-positive cells also expressed *SynB* (Fig. 5C,C′, arrowheads). These results are consistent with Brn1/2/4 linking SoxC function to terminal differentiation of neurons throughout the ectoderm.

To determine whether *Brn1/2/4* is required for neurogenesis, we carried out morpholino-mediated knockdown. At a morpholino dose that minimized other developmental effects, the number of neurons was markedly reduced (Fig. 5D,D′ versus E,E′ and F,F′) for both serotonergic and other *SynB*-positive neurons. At this dose, many embryos gastrulated and appeared relatively normal with respect to major structures; some were noticeably smaller, with fewer nuclei (Fig. S2). In *Brn1/2/4* morphants, *SoxC* expression was not detectably affected (Fig. S2E,F), indicating that Brn1/2/4 functions downstream of SoxC, rather than in a parallel, cross-regulatory mode.

To determine whether Brn1/2/4 is sufficient to drive neural fate in the embryo, we also tested the effects of gain-of-function by misexpressing synthetic *Brn1/2/4* mRNA. At a low dose, the number of serotonergic neurons approximately doubled (Fig. 5G versus J-L, green), whereas endomesoderm development was variably reduced. At a higher dose, the resulting embryoids formed epithelial spheres with *SynB* signal expanded to encompass the whole embryo and no detectable staining for serotonin (Fig. 5G versus H and I, red). Further analysis of the high-dose phenotype with probes for territory-specific proteins showed that most nuclei contained Hnf6 protein (Fig. S4A-A″ versus B-B″), which in the normal embryo is expressed only in ciliary band and the APD. The embryos appeared to be radialized around the anterior-posterior (animal-vegetal) axis, since oral ectoderm-specific GSC was not detectable (Fig. S4C-C″ versus D-D″) and Spec1, which is confined to the aboral ectoderm of control embryos, accumulated in all cells (Fig. S4A versus A′). Differentiation of the endomesoderm, as indicated by the Endo1 marker for mid- and hindgut, was greatly suppressed (Fig. S4C′ versus D′); only a few cells continued to express Endo1, marking the location of the vegetal pole. The APD territory was at least partially retained, as evidenced by a contiguous patch of expression of *Nk2.1*, which is a driver of a separate regulatory pathway that constructs the apical tuft of cilia within the APD (Dunn et al., 2007) (Fig. S4E versus F and G). We conclude that Brn1/2/4 is required for the development of serotonergic and all *SynB*-expressing neurons and can directly or indirectly drive the production of excess neurons at low dose, although high-level global misexpression produces additional gain-of-function effects.

If Brn1/2/4 functions in a ‘single-file’ regulatory pathway leading to neural differentiation, then it might be sufficient to rescue neural differentiation in the absence of SoxC. We tested this possibility by co-injecting *SoxC* morpholino and synthetic *Brn1/2/4* mRNA. *SoxC* morpholino (Fig. 6B) and *Brn1/2/4* RNA (Fig. 6C) individually showed their expected phenotypes. When the two were co-injected, the embryoid phenotype resembled that produced by *Brn1/2/4* RNA misexpression alone, but without any detectable SynB or serotonin (Fig. 6D). This result suggests that other factors working in parallel with Brn1/2/4 are required downstream of SoxC.

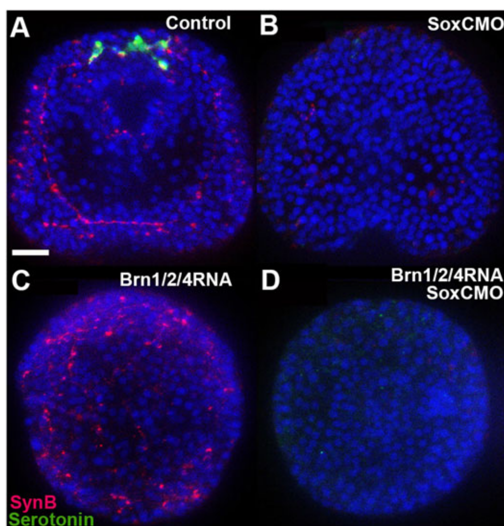

Fig. 6. The Brn1/2/4 overexpression neural phenotype requires SoxC function. Embryos were injected with *SoxC* morpholino or *Brn1/2/4* synthetic mRNA alone or in combination and immunostained for serotonin (green) or SynB (red). (A) A control embryo. (B) A *SoxC* morphant that lacks all neurons. (C) An embryo overexpressing *Brn1/2/4* mRNA (higher dose) shows expanded labeling for SynB and lacks serotonergic neurons. (D) Injection of *Brn1/2/4* mRNA does not rescue SynB-expressing neurons in the *SoxC* morphant. Nuclei are labeled with DAPI (blue). All embryos are at 72 h. Scale bar: 20 µm.

DISCUSSION

In this work we have used molecular screens and gene knockdown approaches to define a temporal and functional sequence of gene

Development (2016) 143, 298-305 doi:10.1242/dev.125989

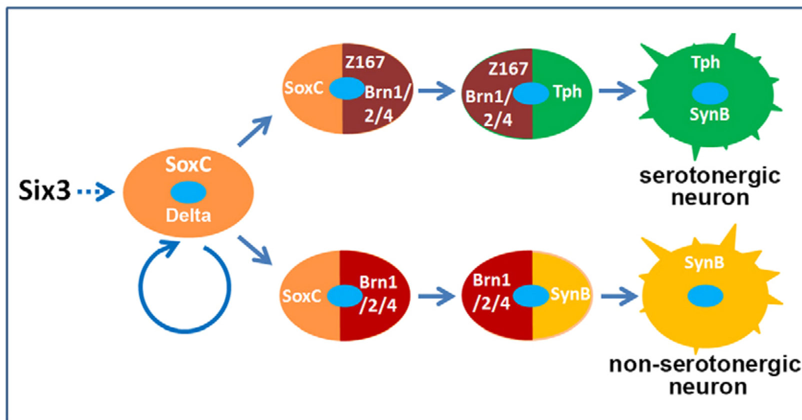

Fig. 7. Model for the neurogenic pathways defined in this work. *SoxC* was previously identified as being dependent on *Six3*. *SoxC* expression is activated in isolated neural precursors by unidentified factors and cell signaling mechanisms (dotted arrow). Cells expressing *SoxC* are dividing (circular arrow) and at least some express Delta and are subject to lateral inhibition. SoxC is required for the development of neurons expressing serotonin and Tph (upper pathway) and also for all other neurons expressing SynB (lower pathway). SoxC is required for expression of *Brn1/2/4* in both pathways; SoxC is transiently expressed and some cells are observed to contain both mRNAs. *Brn1/2/4* also is transiently expressed, and in later embryos some Brn1/2/4-expressing cells also express the differentiated marker SynB. *Z167* is expressed only in developing serotonergic neurons (upper pathway) and is essential for their development. Its position in the hierarchy relative to Brn1/2/4 remains to be defined, but Z167 is likely to function in parallel or downstream.

activities required for the development of all neurons and a branch in that pathway that is specific for serotonergic neurons in the APD. Our entry point to this pathway was the observation that the transcription factor Six3 is required for the expression of many candidate neurogenic genes. From this set, SoxC was selected as a likely positive regulator of neurogenesis because it is downregulated in the absence of Six3 function and is expressed in all neurogenic territories, including isolated individual cells in the lateral ectoderm, which is characteristic of presumptive neurons. Morpholino-mediated knockdown of SoxC function confirmed its requirement for the differentiation of all neurons in the embryo (Fig. 2). In vertebrates, expression of SoxC proteins (Sox 4, 11 and 12) marks the transition from neuroepithelial cells to dividing neuroblasts (Reiprich and Wegner, 2015). A similar function is suggested for the single SoxC representative in sea urchin embryos by the observation that the number of *SoxC*-expressing cells increases when Notch signaling is inhibited by DAPT treatment (Fig. 2) and our finding that at least some SoxC-positive cells are still dividing (Fig. 3). Although overexpression of Sox4, 11 or 12 in chicken and mouse has been shown to have an inductive effect on pan-neuronal gene expression and differentiation (Reiprich and Wegner, 2015), in our hands overexpression of *SoxC* mRNA in sea urchin embryos did not produce a detectable phenotype, suggesting that SoxC is necessary, but not sufficient, to specify neurons (Garner et al., 2016; Z.W., unpublished observations). In addition, the fact that, like *Six3*, *SoxC* is clearly expressed in cells that never adopt neural fate (e.g. the high-level expression in early endomesoderm; Fig. 1), suggests that it has additional functions in sea urchin embryos and is not sufficient to specify neuronal fate.

The fact that *SoxC* expression appears to be transient and that individual cells did not co-express differentiated neuronal markers implied that another regulatory layer connects SoxC function to the expression of genes involved in terminal differentiation, such as *SynB*. In addition, in the absence of lineage tracing, it prevented a firm conclusion as to whether SoxC function in neurogenesis is cell-autonomous and whether *SoxC*-expressing cells include actual neural precursors. Therefore, we carried out an additional molecular screen to identify genes that might link SoxC function to differentiating neurons. This effort identified *Brn1/2/4* as a potential downstream effector of SoxC action, based on its similar pattern of expression and its association with neural development in other embryos (Ryan and Rosenfeld, 1997; Wegner et al., 1993). *Brn1/2/4* expression was shown to depend on SoxC and, in turn, was shown to be required for the differentiation of serotonergic neurons in the APD and non-serotonergic neurons in the ectoderm and foregut (Fig. 5). The distribution of *Brn1/2/4*-expressing cells in the ectoderm varied among embryos at the same time of development, suggesting that it also is expressed asynchronously and in individual neural precursors. *Brn1/2/4* thus provided a link between *SoxC* and *SynB*, since some *Brn1/2/4*-expressing cells were shown to express *SoxC*, whereas others expressed *SynB* (Fig. 5).

In vertebrates, Brn2 expression, in combination with that of two other transcription factors, can convert fibroblasts into neural cells (Pang et al., 2011; Pfisterer et al., 2011). Interestingly, we observed that misexpression of *Brn1/2/4* in sea urchin embryos at low dose increased the number of serotonergic neurons, whereas at higher dose it converted the whole embryo to a novel phenotype (Fig. 5 and Fig. S4). Almost all cells in these embryos accumulated the APD/ciliary band transcription factor Hnf6, with SynB-positive fibers distributed throughout the embryo. Anterior-posterior polarity was retained but differentiation of endomesoderm derivatives at the vegetal pole was dramatically reduced. By contrast, the APD at least partially differentiated, as shown by expression of the Nk2.1 transcription factor, which functions in production of the apical tuft of cilia through a different regulatory pathway (Dunn et al., 2007). This phenotype suggests the interesting possibility that the two phases of *Brn1/2/4* expression (Wei et al., 2006; Materna et al., 2010) (Fig. S3) might have different but related functions: the early, uniform expression, which is amplified and prolonged by *Brn1/2/4* uniform misexpression, might promote or insulate a common precursor to ciliary band cells and neurons, whereas later Brn1/2/4 might function in a much more limited set of neural precursors.

The SoxC knockdown screen also identified Z167 as a transcription factor that is specifically expressed in, and required for, development of serotonergic neurons in the APD. Although another zinc finger protein, Zfhx/Z81 (Yaguchi et al., 2012), has been shown to be required for the development of serotonergic neurons, and several other transcription factors have been shown to be expressed within them (Yaguchi et al., 2011), Z167 is the first factor to be shown to functionally link to the shared core regulatory pathway of Six3→SoxC→Brn1/2/4 that is required for all neurons.

Among the outstanding questions for the future is the identity of mechanisms by which the expression of genes in the neurogenic regulatory pathways becomes restricted to individual neural precursors. Neurons in all three major regions – APD, ciliary band and foregut – have no known origins in cell lineage. Although the APD arises from the an_1 octet of blastomeres of the 32-cell embryo (Hörstadius, 1973), the arrangement of cell types within it is only determined after dorsal-ventral polarity is established. For example, the serotonergic neurons discussed in this work normally differentiate

Development (2016) 143, 298-305 doi:10.1242/dev.125989

at the dorsal edge of the APD, but can develop throughout the APD under a variety of experimental treatments that radialize the embryo. The distribution of other molecular markers further indicates a complex and evolving fine-scale patterning of different cell types. The ciliary band is defined by persistence of the neurogenic bias, driven at least in part by maternal and early zygotic SoxB1 (Barsi et al., 2015). Recent work by these authors shows that the ciliary band comprises four separate gene regulatory territories, the dorsal and ventral (oral and aboral) borders of which are defined by the combined functions of a set of at least ten transcriptional repressors. Neurons appear to be specified within the ciliary band progressively and asynchronously, and except for the involvement of Delta-Notch signaling, nothing is known about the local mechanisms that position them. Finally, the specification of the pair of individual pharyngeal neurons poses an intriguing regulatory phenomenon: they arise within endoderm after it is specified via an extensively characterized GRN (http://sugp.caltech.edu/endomes/) through a neurogenic pathway that includes *Six3* and *Nkx3-2* (Wei et al., 2011), which we now show also includes *SoxC* and *Brn1/2/4*. Interestingly, all of these factors are initially more broadly expressed in endomesoderm, implying that additional regulatory interactions among cells are required for specifying the neurons.

The data presented here allow us to outline a model for gene regulatory pathways leading to the differentiation of SynB-positive and serotonergic neurons in the sea urchin embryo (Fig. 7). The most upstream gene, *Six3*, is activated in early cleavage, probably by maternal factors among those that are sufficient to initiate the APD developmental program throughout the embryo (Yaguchi et al., 2006; Wei et al., 2009; Range et al., 2013). Although Six3 is necessary for differentiation of all neurons in the embryo, its function is neither specific nor sufficient for their production. Instead, it is crucial for development of the APD per se: embryos lacking Six3 fail to produce neurons, the apical ciliary tuft and the cuboidal epithelium characteristic of the APD, whereas global misexpression converts the embryo to an expanded APD (Wei et al., 2009). Downstream of Six3, SoxC and Delta are activated by unknown mechanisms in isolated individual cells that have properties of neural precursors. These are capable of proliferation and probably undergo asymmetric division, with one daughter cell activating *Brn1/2/4* and/or *Z167* expression. *SoxC* expression is transient, and it is required for all neurogenesis, but its overexpression is not sufficient to overproduce neurons, indicating that it must operate in parallel with other factors at this point. As *SoxC* expression declines, *Brn1/2/4* and *Z167* mRNAs begin to accumulate; their expression also is transient and leads to the activation of differentiated gene products, including SynB, Tph and serotonin.

The SoxC→Brn1/2/4→SynB pathway is clearly incomplete. Homologs of a number of genes implicated in neurogenesis in other systems have been identified that also depend on Six3 for expression (Wei et al., 2009) and the requirement for several has been demonstrated by functional assays, including Zfhx for serotonergic neurons (Yaguchi et al., 2012) and Nkx3.2 for pharyngeal neurons (Wei et al., 2009). Here, we have provided a scaffold pathway for integrating other genes into a GRN for neurogenesis in sea urchin embryos.

MATERIALS AND METHODS

Embryo culture

Adult sea urchins (*Strongylocentrotus purpuratus*, Stimpson 1857) were obtained from Monterey Abalone (Goleta, CA, USA) and Patrick Leahy (Point Loma, CA, USA) and maintained in seawater at 10°C. Embryos were cultured in artificial seawater (ASW) at 15°C. In some experiments, embryos were cultured in 5 µM *N*-[(3,5-difluorophenylacetyl)-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester (DAPT) beginning at 4 h after fertilization.

Microinjection of morpholino antisense oligonucleotides (MOs) and synthetic mRNAs

Eggs were prepared as described previously (Wei et al., 2009). Approximately 2 pl of solution containing 25% glycerol and morpholino (Gene Tools) were injected. The knockdown phenotypes were confirmed in each case by two different morpholinos with non-overlapping or partially overlapping sequences. Morpholino sequences and concentrations were as follows (5′-3′): *SoxCMO1*, TTTTGAGGAACCATCTTGAAGTCAG (0.6 mM); *SoxCMO2*, CTTGAAGTCAGCATTCACTTTCGTG (1.2 mM); *Brn1/2/4MO1*, TACTCCCTCGCCCTGATCTGAATTG (0.3 mM); *Brn1/2/4MO2*, GATCTGAATTGCAAAACAACCAGGG (0.6 mM); *Z167MO1*, GAAAGCGCCTCTCCTGTCATTCTGT (0.3 mM); *Z167MO2*, CTGTCAGTACTGCCTCTAGATCATG (1.2 mM). All data shown were derived using *SoxCMO1*, *Z167MO1* and *Brn1/2/4MO2*. Embryos showed a steep dose-response to both *Brn1/2/4* morpholinos, and careful calibration of the dosage was required to separate loss of neurons from effects on other tissues (see main text). Both morpholinos gave the same phenotype, although the dose response sometimes differed. For SoxC, an antibody was available and the morpholinos were shown to efficiently eliminate the protein signal (Fig. S2). For all microinjection experiments, at least three repeats were performed and for each experiment 50-100 embryos were analyzed. *Brn1/2/4* mRNA was synthesized using the mMESSAGE mMACHINE Kit (Ambion), according to the manufacturer's protocol. The injection solution was 0.5 µg/µl or 1.0 µg/µl *Brn1/2/4* mRNA.

Whole-mount *in situ* hybridization

Embryos were fixed, hybridized and stained as described previously (Minokawa et al., 2004), except that each RNA *in situ* hybridization probe was purified with a Qiagen QiaQuik PCR column after adding EDTA to 10 mM. *SoxC*, *Delta*, *Z167*, *Brn1/2/4*, *Tph* and *SynB* (*Syt1*) probes were labeled with digoxigenin, FITC or DNP and detected with Cy3-TSA, FITC-TSA or Cy5-TSA (PerkinElmer). Two-color or three-color fluorescent *in situ* hybridization was carried out as described previously (Yaguchi et al., 2008; Sethi et al., 2014). The concentration of probes was 0.1 ng/µl.

Immunohistochemistry

Embryos were fixed in 2% formaldehyde for 10 min at room temperature. They were incubated overnight at 4°C with primary antibodies using the following dilutions: serotonin, 1:1000 (S5545, Sigma); synaptotagmin B, 1:2000 (Garner et al., 2016); phospho-Histone H3 (H3p), 1:4000 (06-570, Upstate Cell Signaling Solutions); SoxC, 1:500 (gift of Robert Burke). Bound primary antibodies were detected by incubation with Alexa Fluor-coupled secondary antibodies (A11034, Invitrogen) for 1 h and nuclei were stained with DAPI. Embryos were observed using a Zeiss Axiovert 200 M microscope. Optical sections were obtained with an Apotome unit (Zeiss) and stacked images were prepared using Adobe Photoshop.

RNA-Seq screening

RNA from 800-1000 glycerol-injected control or *SoxC* morpholino-injected embryos was purified using Nucleospin columns (Macherey-Nagel). Further RNA-Seq processing and sequencing were carried out by Beckman Coulter Genomics service. PERL programming was used for data analysis and exploration. The RNA-Seq data are available at GEO under accession number GSE76067.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Z.W., L.M.A. and R.C.A. planned experiments and analyzed the data. Z.W. performed experiments. Z.W. and R.C.A. prepared the manuscript.

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Supplementary information

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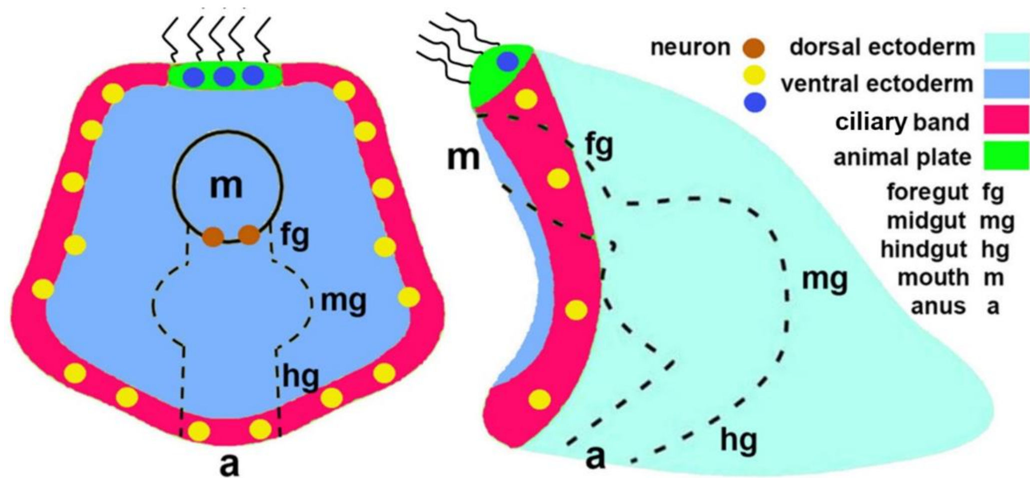

Supplementary Figure S1. Diagram of neurogenic regions of a 3-day pluteus larva discussed in this work. Left, a view from the ventral (oral) side, with the APD at top. Right, a lateral view. SynB neurons (yellow) differentiate in or adjacent to the APD (green) and ciliary band (magenta), while serotonergic neurons (dark blue) are restricted to the apical plate. Two additional neurons differentiated in the pharyngeal endoderm (brown).

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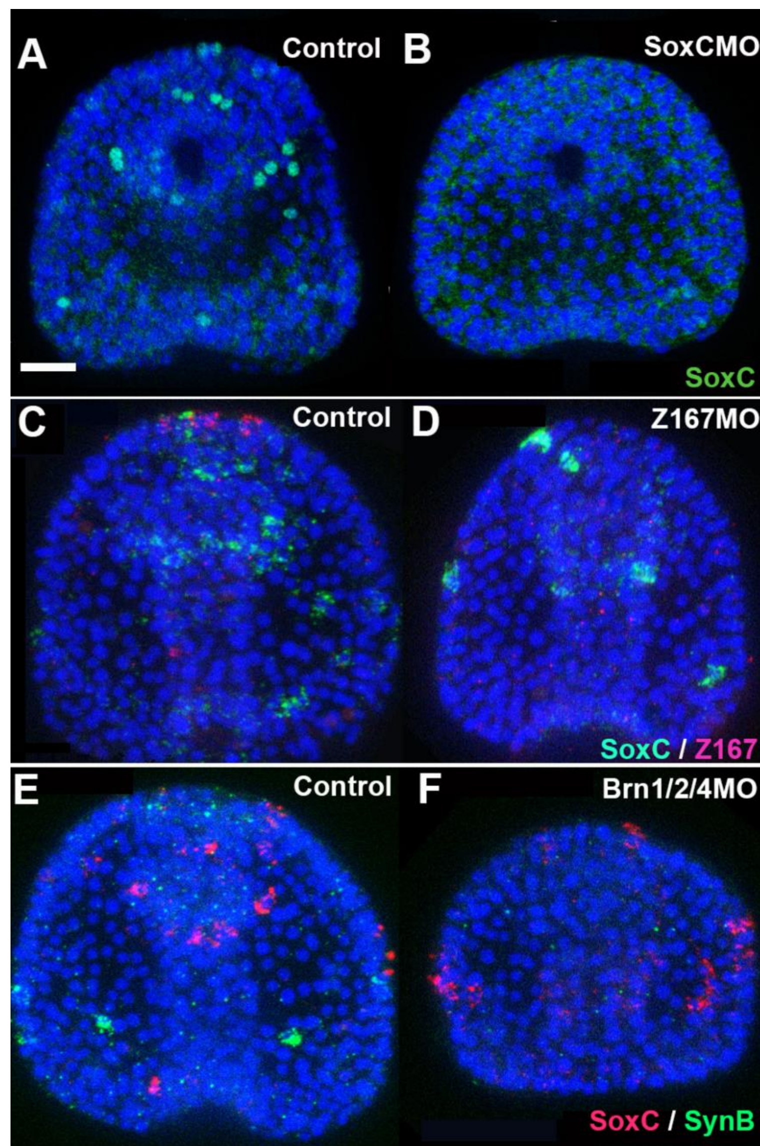

Supplementary Figure S2. ***SoxC* morpholino knockdown prevented accumulation of SoxC protein and *Z167* or *Brn1/2/4* knockdown did not reduce *SoxC* expression.** (A and B) SoxC antibody immunostained (green) control (A) and *SoxC* morpholino-injected (B) embryos. SoxC protein signal was eliminated in the morphant. Embryos were at 52 hours. SoxC antibody courtesy of Dr. Robert Burke. (C and D) Control embryo (C) and *Z167* morphant hybridized with probes for *SoxC* (green) and *Z167* (red) transcripts. Z167 knockdown does not affect SoxC expression, but *Z167* transcripts are down regulated, indicating its probable negative autoregulation. Control (E) and Brn1/2/4 knockdown embryos (F) hybridized in situ for SoxC (red) and SynB (green). Brn1/2/4 knockdown does not affect expression of *SoxC*, but prevents formation of *SynB*-positive neurons. Embryos were at 46 hours and are shown in lateral view with the APD at top. Nuclei were stained with DAPI (blue). The white bar in A represents 20 micrometers.

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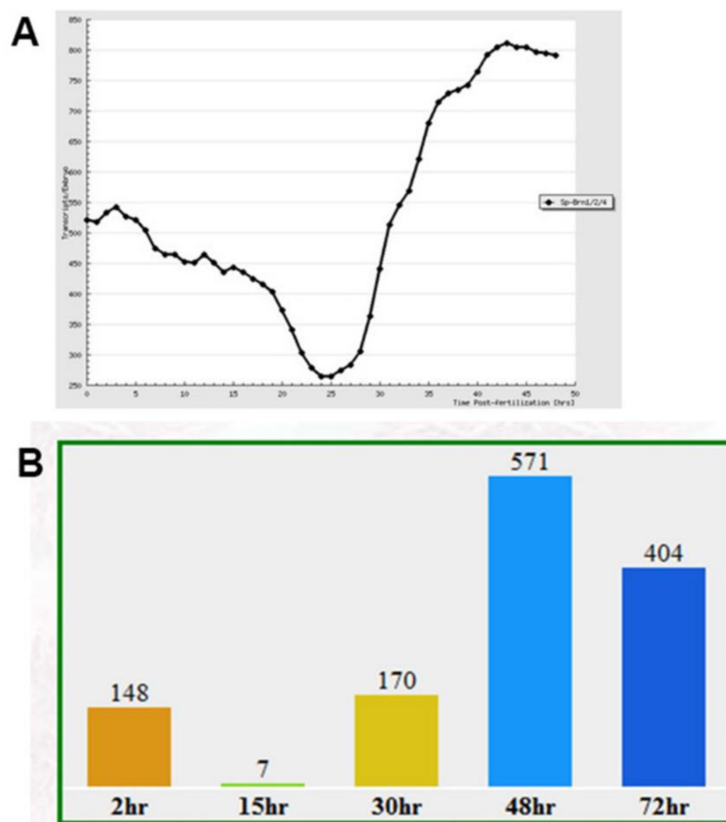

Supplementary Figure S3. SoxC mRNA accumulates in two phases. (A) Temporal expression pattern from a high-resolution transcription analysis (Materna et al., 2010). (B) Temporal expression pattern from a microarray experiment (Wei et al., 2006).

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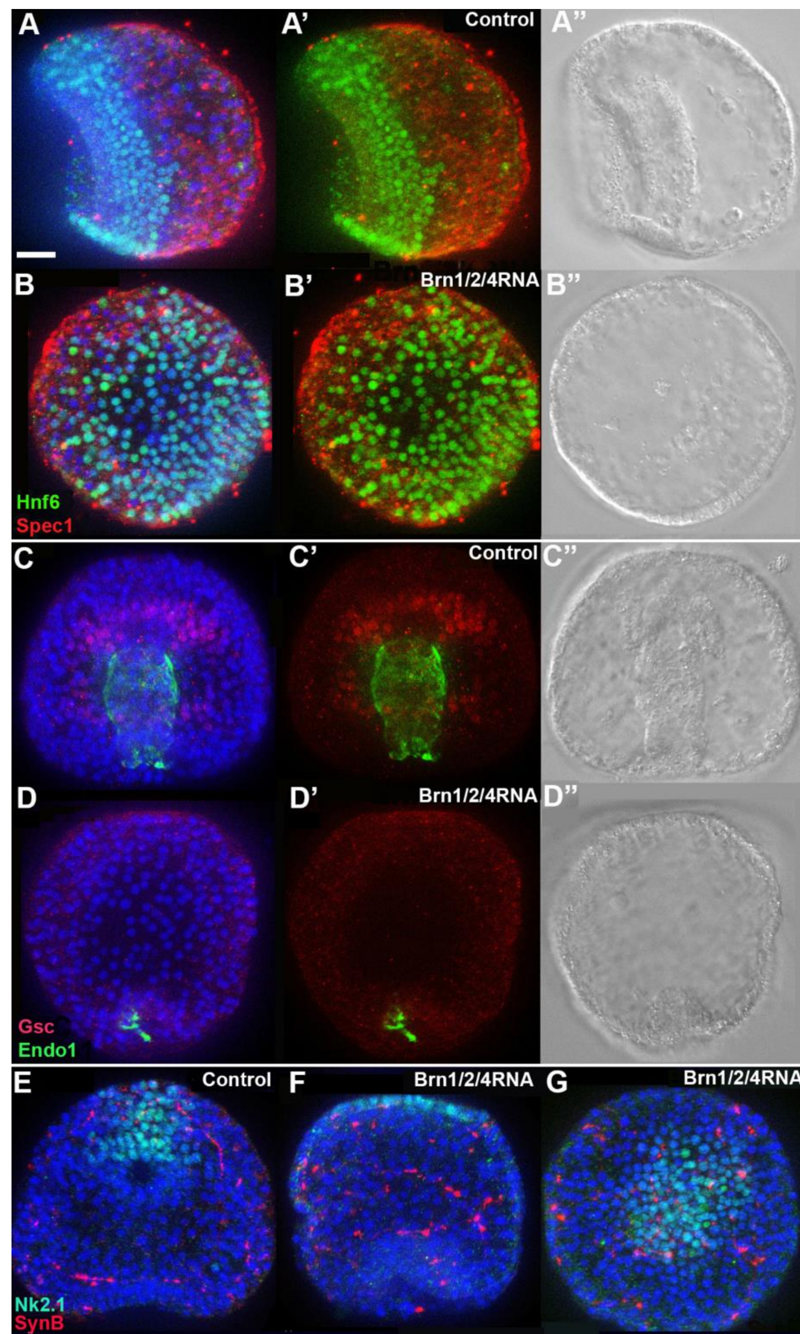

Supplementary Figure S4. Further analysis of the *Brn1/2/4* mis/overexpression phenotype. (A and B) Control (A) and Brn1/2/4-overexpressing embryos (B) immunostained for Hnf6 (cillary band and APD, green) and Spec1 (aboral ectoderm, red). (C and D) Control (C) and Brn1/2/4-overexpressing (D) embryos immunostained for GSC (oral ectoderm, red) and Endo1 (midgut and hindgut, green). (A"-D") are the corresponding DIC images and DAPI fluorescence (blue) is shown in (A-D). (E, F and G), Control (E) and Brn1/2/4-overexpressing (F and G) embryos immunostained for SynB (red) and Nk2.1 (green), a transcription factor operating in a gene regulatory pathway separate from that for serotonergic neurons, which leads to production of the ciliated apical tuft (Dunn et al., 2007). SynB staining encompasses most of the ectoderm and Nk2.1 staining is radialized, lacking dorsal-ventral polarity. Nuclei are stained with DAPI (blue). E and F are lateral views and G is toward the APD. The white bar in A represents 20 micrometers.

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