Gene regulatory networks underlying the compartmentalization of the Ciona central nervous system

Kaoru S. Imai\textsuperscript{1,2}, Alberto Stolfi\textsuperscript{2}, Michael Levine\textsuperscript{2} and Yutaka Satou\textsuperscript{1,*}

The tripartite organization of the central nervous system (CNS) may be an ancient character of the bilaterians. However, the elaboration of the more complex vertebrate brain depends on the midbrain-hindbrain boundary (MHB) organizer, which is absent in invertebrates such as Drosophila. The Fgf8 signaling molecule expressed in the MHB organizer plays a key role in delineating separate mesencephalon and metencephalon compartments in the vertebrate CNS. Here, we present evidence that an Fgf8 ortholog establishes sequential patterns of regulatory gene expression in the developing posterior sensory vesicle, and the interleaved ‘neck’ region located between the sensory vesicle and visceral ganglion of the simple chordate Ciona intestinalis. The detailed characterization of gene networks in the developing CNS led to new insights into the mechanisms by which Fgf8/17/18 patterns the chordate organizer. The precise positioning of this Fgf signaling activity depends on an unusual AND/OR network motif that regulates Snail, which encodes a threshold repressor of Fgf8 expression. Nodal is sufficient to activate low levels of the Snail repressor within the neural plate, while the combination of Nodal and Neurogenin produces high levels of Snail in neighboring domains of the CNS. The loss of Fgf8 patterning activity results in the transformation of hindbrain structures into an expanded mesencephalon in both ascidians and vertebrates, suggesting that the primitive MHB-like activity predates the vertebrate CNS.

\textbf{KEY WORDS:} Ciona intestinalis, Gene regulatory network, Fgf8

\section*{INTRODUCTION}

The expression profiles of neural genes in both protostomes and deuterostomes suggest that the tripartite structure of the CNS might be evolutionarily ancient (Wada et al., 1998; Lowe et al., 2003; Reichert, 2005). The vertebrate CNS has elaborated on this basic tripartite structure to produce complexity and novelty. The anterior neural tube develops into the brain, whereas posterior regions form the spinal cord. The brain becomes regionalized into forebrain, midbrain and hindbrain, which are further subdivided at later stages. The MHB organizer is one of the mechanisms used for the elaboration of the complex vertebrate brain (Liu and Joyner, 2001; Wurst and Bally-Cuif, 2001; Rhinn et al., 2006). A number of transcription factors and signaling molecules are expressed in the MHB region, and these molecules constitute a complex genetic network that establishes and maintains features of the organizer. Fgf8 and Wnt1 are the key factors in this network controlling the development of the midbrain and hindbrain.

The ascidian tadpole represents the closest living relative of the vertebrates and possesses a simplified CNS derived from the dorsal neural tube. The ascidian CNS consists of a centralized sensory vesicle (SV), visceral ganglion (VG) and nerve cord composed of \textasciitilde260 cells (Meinertzhagen et al., 2004). There is a morphologically distinguishable domain called the ‘neck’ between the SV and VG (Nicol and Meinertzhagen, 1988). Although the SV corresponds to the forebrain, it is unclear whether the neck and VG correspond to the hindbrain and/or spinal ganglia of vertebrates (Wada et al., 1998; Dufour et al., 2006). It is conceivable that a distinct midbrain counterpart is absent in the ascidian larva (Takahashi and Holland, 2004). Therefore, the MHB organizer has been regarded as a novel property of the vertebrate CNS. The vertebrate MHB organizer secretes Wnt1 and Fgf8, which are important for the MHB organizing activity. Although the Ciona genome does not contain Wnt1 (Hino et al., 2003), an ortholog of Fgf8 exhibits localized expression during the development of the Ciona CNS at the late gastrula stage (Imai et al., 2004; Hudson and Yasuo, 2005; Imai et al., 2006) and at the middle tailbud stage (Imai et al., 2002), thereby raising the possibility that an MHB organizer-like structure operates in ascidians.

To determine the extent to which the compartmentalization of the ascidian and vertebrate CNS are controlled by conserved and non-conserved gene circuits, we have examined the expression and function of a comprehensive set of neural regulatory genes. These studies permitted the reconstitution of a provisional gene regulatory network for the development of the ascidian CNS based on systematic gene disruption assays. The resultant network can explain the causalities of the gene expression profiles seen in the CNS and provide insights into the evolutionary origin of the vertebrate CNS.

\section*{MATERIALS AND METHODS}

\textbf{Ascidian embryos}

\textit{C. intestinalis} adults were obtained from the Maizuru Fisheries Research Station of Kyoto University (Kyoto, Japan) and from Half Moon Bay harbor (CA, USA). We used late gastrula embryos just after the neural plate formation and late gastrula embryos just prior to the next division in the present study, and early and mid-tailbud embryos, which correspond to E55 and E75-E80 (Cole and Meinertzhagen, 2004).

\textbf{Expression profiles of regulatory genes}

Most cDNA clones were obtained from our EST collection (Satoh et al., 2002). The detailed procedure for whole-mount in situ hybridization has been described (Satoh and Satoh, 1997). Cell identities were determined by DAPI staining of nuclei.

\textbf{Gene knockdowns}

MO oligonucleotides were purchased from Gene Tools: BMP2/4, AAGTGCATCCGATGCGCCACCAT; Cdx, TTGTGCGTTTCTCCTCA - CAATGGTTGC; Delta-like, GAAGTAATATAAGCTTGATGCTCAT;
Fig. 1. Development of the A- and b-line central nervous system in the Ciona embryo. (A) Schematic representations of A- and b-line neuronal cell lineages of the bilaterally symmetrical embryos at the late gastrula stage. Cell names are shown in the lower panel. (B,C) A schematic representation of the CNS cells at the tailbud stage from the (B) dorsal and (C) lateral views, and their lineages. Arrows indicate cell lineages and the cells with the same colors are derived from the single cells at the late gastrula stage (enclosed by pink lines). Cells enclosed by thick black lines are post-mitotic cells destined to become motoneurons. At the early tailbud stage, three pairs of presumptive motoneurons are post-mitotic. Until the mid-tailbud stage, two pairs of post-mitotic presumptive motoneurons are differentiated from the remaining two pairs of the visceral ganglion (VG) cells after cell divisions, as shown in the upper part of B. (D) The central nervous system of a tailbud embryo developed from an egg electroporated with Fgf8/17/18&>RFP/AchTP>GFP. (E) A late tailbud embryo developed from an egg electroporated with Fgf8/17/18&>RFP and AchTP>GFP. Arrowheads indicate motoneurons. (F) The central nervous system of a tailbud embryo developed from an egg electroporated with Fgf8/17/18&>RFP / FoxB&>RFP. RFP marks the A9.30-descendants and GFP marks neurons. LNC, the lateral rows of the nerve cord; DNC, the dorsal row of the nerve cord; VNC, the ventral row of the nerve cord.

Embryos were electroporated as described (Imai et al., 2000). Blast searches of the MO sequences against specificities of the MOs, we injected second MOs (data not shown), indicating that there were no generalized disruptions. For further confirmation of specificities of the MOs, we injected second MOs against Fgf8/17/18 (CATATTGTGATATCCAGAGGA) and Snail (TATTGCAGTAAGATTTAT), and MOs against Otx (AGT-GTGAGATTCTAAGTGATGCATCTG) and Neurogenin (ATCGGTGGCA-GAATAATCCACATAC) of Ciona savignyi, a closely related species, into Ciona savignyi eggs. We carried out in situ hybridization of Otx and Pax2/5/8/A for Fgf8/17/18 morphant embryos, of Fgf8/17/18 for Snail morphant embryos, of Snail for Neurogenin morphant C. savignyi embryos and of Cyp26, FoxB and FoxI for Otx morphant C. savignyi embryos (see Fig. S1 in the supplementary material). They recapitulated the original phenotypes. All of these observations support specificities of the MOs used in the present study. Whole-mount in situ hybridization was used for determining genes expressed in the downstream of genes that were knocked down.

Electroporation

4.8 kb and 4.2 kb of flanking 5' sequences of Fgf8/17/18 and FoxB respectively were PCR-amplified from genomic DNA. AchTP promoter was obtained as described previously (Yoshida et al., 2004). All were cloned into pCESA vector upstream of unc-76-tagged GFP or mCherry reporter gene (Dynes and Ngaí, 1998). The detailed procedure for electroporation has been described previously (Corbo et al., 1997).

RESULTS

The ascidian brain has distinct domains with different properties

According to previous descriptions (Nicol and Meinertzhagen, 1988), the posterior region of the sensory vesicle (PSV) and VG are derived from the posterior neural plate (Fig. 1A). The short region...
between the PSV and VG is called the ‘neck’. Both the PSV and neck are derived from a single pair of neuronal progenitor cells in gastrulating embryos, A9.16, whereas the VG is derived from two neighboring blastomeres, A9.30 and A9.29 (Fig. 1B). The latter cell also contributes to the caudal nerve cord, which consists of non-neuronal ependymal cells. The ventral and dorsal rows of the neural tube are derived from A9.13/A9.14/A9.15 and b9.37/b9.38, respectively (Fig. 1C).

It is thought that the PSV and VG produce the core synaptic activity for the swimming behavior of the tadpole, because the VG includes five pairs of motoneurons that innervate the tail muscles and are modulated by cholinergic neurons located in the PSV (Cole and Meinertzhagen, 2004). To confirm this idea, we traced the A9.30 lineage with an RFP fusion gene containing the Fgf8/17/18 enhancer (Fig. 1D-F) throughout development, as Fgf8/17/18 expression is restricted to A9.30 at the late gastrula stage (Imai et al., 2004). Co-electroporation of this fusion gene with a GFP fusion gene containing the enhancer of the acetylcholine transporter gene (AchTP) identifies two pairs of early post-mitotic cholinergic motoneurons derived from A9.30 (yellow cells, Fig. 1D). The anterior A9.30 lineage forms non-neuronal ependymal cells (red cells, Fig. 1E), as well as two additional pairs of motoneurons in the VG of older embryos (anterior two yellow cells, Fig. 1E). The posterior-most pair of motoneurons (A10.57) arises from the A9.29 lineage (posterior green cells, Fig. 1D,E). Embryos electroporated with a GFP fusion gene containing the \textit{FoxB} enhancer permitted visualization of the anterior lineage arising from A9.16, which forms the PSV. As seen for endogenous \textit{FoxB} transcripts, GFP is strongly expressed in the anterior A9.16 lineage that forms the PSV, but not in the posterior A9.16 lineage that forms the neck region (Fig. 1F). The neck consists of quiescent undifferentiated precursors that form the neurons homologous to cranial motoneurons of vertebrates after metamorphosis (Dufour et al., 2006). These molecular data confirmed the idea that the PSV, neck and VG have distinctive properties (Cole and Meinertzhagen, 2004).

The regulatory states of cells in the developing CNS

An earlier study identified a comprehensive list of regulatory genes expressed in the developing nervous system (Imai et al., 2004). The recent advances in imaging technology and the knowledge of the cell lineages of the CNS (Cole and Meinertzhagen, 2004) allowed us to elaborate on this description at single cell resolution by in situ hybridization (summarized in Fig. 2A-C; see also in situ hybridization).
hybridizations of control embryos in Fig. S1 in the supplementary material). The resultant diagrams of the expression profiles of individual cells define the regulatory states of the cells from the late gastrula to the mid-tailbud stage.

The hierarchical clustering of the expression profiles confirmed and refined the morphological differences of the brain regions from a molecular viewpoint (Fig. 2D). Two pairs of the PSV cells (A11.63 and A11.64 in the early tailbud embryo) are in the same regulatory state, based on selective expression of Otx, FoxB and En. The neck cells (A11.61 and A11.62) selectively express Pax2/5/8-A, Hox1 and Gli. The VG and caudal nerve cord cells express different combinations of regulatory genes. Anterior and posterior motoneurons in the VG express Hox1/En/Neurogenin and Lhx3/Hnf6/Neurogenin, respectively. Posterior portions of the neural tube express Cdx, Snail and Pax6. The dorsal and ventral rows of the neural tube also express different sets of regulatory genes.

Provisional gene regulatory networks in the developing CNS

On the basis of the expression profile data, we systematically perturbed the functions of regulatory genes expressed in the CNS with specific morpholino oligonucleotides (MOs) in order to determine the molecular basis for the compartmentalization of the Ciona brain. We succeeded in disrupting the activities of a total of 25 regulatory genes (Tables 1 and 2). In situ hybridization assays were used to monitor the effects of the different MO-induced mutants on the expression of all of the regulatory genes expressed at the stages examined (see Fig. S1 and Table S1 in the supplementary material).

This information, along with earlier results (Imai et al., 2002; Lemaire et al., 2002; Hudson and Yasuo, 2005; Moret et al., 2005; Imai et al., 2006; Hudson et al., 2007; Ikuta and Saiga, 2007), permits the elucidation of gene networks at single cell resolution, where the interconnections among the regulatory genes controlling the regionalization of the CNS (Fig. 3A). The simplicity of the system also contributes to specification of the dorsal nerve cord cells by activating Pax3/7 (see Fig. S3 in the supplementary material).

For example, Fgf9/16/20 expression in the PSV and neck progenitors is controlled by Emc and Pax3/7 (Fig. 3B). In the neck region, Pax2/5/8-A also contributes to the expression of Fgf9/16/20 (Fig. 3C). Neurogenin was found to be a crucial determinant of the motoneurons in the VG (Fig. 3D). Cdx is required for the development of ependymal cells in posterior regions of the neural tube (Fig. 2E), whereas Lmx contributes to specification of the dorsal nerve cord cells by activating Pax3/7 (see Fig. S3 in the supplementary material).
and Otx.

This Fgf signal acts on the neighboring A9.16 cell to define the neck, which forms most of the VG (A11.117 through A11.120) at later stages. Expression of Otx and FoxB is normally restricted to the anterior-most regions of the CNS, including the presumptive PSV (Fig. 4A; see Fig. S1Q in the supplementary material; see also Fig. 2B). There is a posterior expansion of both expression patterns in morphant embryos (Fig. 4D). There is also a loss of En expression in the neck, thereby restricting their activities to the PSV. En normally displays periodic expression in the PSV and VG (Fig. 4C), but expression extends into the neck of morphant embryos (Fig. 4D). There is also a loss of Pax2/5/8-A expression (Fig. 4F), which is normally restricted to a tight stripe of expressing cells in the neck (Fig. 4E). Gli, Fgf9/16/20 and Arix, which are normally expressed in the neck, are lost in morphant embryos, because these genes are under the control of Pax2/5/8-A (see Fig. S1A,O,S in the supplementary material). Finally, Hox1 expression is normally restricted to the neck and VG (Fig. 4G), but expression is lost in the neck of morphant embryos (Fig. 4H). Altogether, the network analysis suggests that the neck is not formed in Fgf8/17/18 morphants, but is transformed into an expanded PSV (Fig. 4I,J).

Fgf8/17/18 is first expressed at the 64-cell stage in A7.6, which abuts A7.8 (a progenitor of A9.29-A9.32, the VG and caudal nerve cord lineage cells), but this expression disappears before the late gastrula stage. Although this early expression is also suppressed in the Fgf8/17/18 morphant, this early Fgf8/17/18 expression is not likely to be required for the CNS regionalization, because Pax2/5/8-A is not expressed in embryos treated with a MEK inhibitor, U0126, from the late gastrula (Fig. 5). The same Fgf gene is again expressed in the VG at the middle tailbud stage (Imai et al., 2002), but this expression is later than Pax2/5/8-A expression. Therefore, the expression in A9.30 at the late gastrula stage is most likely to be responsible for the regionalization of the CNS.

Localized Fgf8/17/18 delineates PSV and neck regions of the Ciona CNS

The reconstituted networks reveal a central role of Fgf8/17/18 in generating regional patterns of gene expression. Fgf8/17/18 is expressed at the late gastrula stage in A9.30 (see Fig. 5C), which forms most of the VG (A11.117 through A11.120) at later stages. This Fgf signal acts on the neighboring A9.16 cell to define the neck region of the definitive tadpole CNS, which forms between the PSV and VG. Otx and FoxB expression is normally restricted to the anterior-most regions of the CNS, including the presumptive PSV (Fig. 4A; see Fig. S1Q in the supplementary material; see also Fig. 2B). There is a posterior expansion of both expression patterns in morphant embryos injected with an Fgf8/17/18 MO (Fig. 4B; see Fig. S1Q in the supplementary material). Thus, Fgf8/17/18 inhibits Otx and FoxB expression in the neck, thereby restricting their activities to the PSV. En normally displays periodic expression in the PSV and VG (Fig. 4C), but expression extends into the neck of morphant embryos (Fig. 4D). There is also a loss of Pax2/5/8-A expression (Fig. 4F), which is normally restricted to a tight stripe of expressing cells in the neck (Fig. 4E). Gli, Fgf9/16/20 and Arix, which are normally expressed in the neck, are lost in morphant embryos, because these genes are under the control of Pax2/5/8-A (see Fig. S1A,O,S in the supplementary material). Finally, Hox1 expression is normally restricted to the neck and VG (Fig. 4G), but expression is lost in the neck of morphant embryos (Fig. 4H). Altogether, the network analysis suggests that the neck is not formed in Fgf8/17/18 morphants, but is transformed into an expanded PSV (Fig. 4I,J).

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This phenotype of Fgf8/17/18 morphants is evocative of the transformation of the metencephalon into an expanded mesencephalon seen in ace (Fgf8a) mutants in the zebrafish CNS where there is expanded expression of Otx2 (mesencephalon) and a loss of Pax8 (metencephalon) (Jaszai et al., 2003). Similar transformations are also seen in conditional knockout mice of Fgf8 (Chi et al., 2003). Thus, in the vertebrate CNS, Fgf8 is expressed in the MHB region and required for proper specification of the midbrain and anterior hindbrain. We might therefore be able to regard the PSV and neck as counterparts of the vertebrate midbrain and anterior hindbrain, although this has been a debatable issue (Wada et al., 1998; Takahashi and Holland, 2004; Canestro et al., 2005; Canestro et al., 2005; Dufour et al., 2006). Regardless of their exact evolutionary counterparts and the timing of the signaling, we propose that the recruitment of Fgf8 signaling might have been a crucial event for the compartmentalization of the chordate brain. It is less likely that the same signaling system was independently acquired for similar uses by tunicates and vertebrates.

Despite the apparent similarities in the patterning of the ascidian PSV and neck with the compartmentalization of the vertebrate midbrain and anterior hindbrain, we note a number of differences in these processes. First, Ciona Fgf8/17/18 acts much earlier – during late gastrulation – than it does in vertebrate embryos. Fgf8 might act

Table 1. Summary of knockdown experiments with morpholino oligonucleotides for genes expressed at the late gastrula stage

<table>
<thead>
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<th>Number of genes</th>
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<tr>
<td>A Genes analyzed in detail</td>
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<tr>
<td>Genes whose downstream genes were found</td>
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<tr>
<td>Emc, FoxB, Delta-like, EphrinA-b, Gli, Hnf6, Lmx, Mnx, Neurogenin, Otx, Pax2/5/8-A, Pax3/7, Pax6, Sox8B1</td>
</tr>
<tr>
<td>14</td>
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<tr>
<td>Genes whose downstream genes were not found</td>
</tr>
<tr>
<td>En, Hedgehog2, Irx-C, Lhx3, Nk6, Tbx2/3</td>
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<tr>
<td>6</td>
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<tr>
<td>B Genes whose functions in the later development were hardly examined, because of their expression in the early embryo</td>
</tr>
<tr>
<td>EphrinA-d, efts/poindset2, Fgf9/16/20, FoxA-A, macho-1</td>
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<tr>
<td>5</td>
</tr>
<tr>
<td>C Genes not analyzed</td>
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<tr>
<td>Genes for which no good morpholino oligonucleotides were obtained</td>
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<tr>
<td>COE, E(spl)/hairy-b, Mytf, ZF-C2H2-33</td>
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Table 2. Summary of knockdown experiments with morpholino oligonucleotides for genes expressed in the brain, boundary and visceral ganglion at the early tailbud stage

<table>
<thead>
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DEVELOPMENT
early in the ancestral chordates, as seen in the Ciona embryos, or the timing of Fg8 signaling might have shifted to earlier stages during the retrograde evolution of tunicates. Second, Wnt1 is absent in the Ciona genome (Hino et al., 2003), and, hence, it is not involved in the regionalization of the Ciona CNS. It is possible that the ancestral chordate had a regionalization mechanism directed by Fgf8 and Wnt1, but that Wnt1 was subsequently lost by ascidians. However, in amphioxus, Wnt1 is not expressed around the boundary of the putative midbrain and hindbrain, and therefore the ancestral chordate might have relied solely on an Fgf8-dependent mechanism for regionalization of the CNS.

Localized expression of Fgf8/17/18 depends on the Snail repressor
In vertebrate embryos, localized expression of Fgf8 in the MHB is directed by the interaction of Otx and Gbx. However, restricted expression of Fgf8/17/18 in the A9.30 lineage of ascidian embryos does not depend on Otx and Gbx. MO-induced suppression of Otx gene activity did not affect Fgf8/17/18 expression (see Fig. S1N in the supplementary material). Moreover, Gbx is not present in the Ciona genome (Dehal et al., 2002; Wada et al., 2003). In contrast to vertebrates, Fgf8/17/18 is positively regulated by Nodal signals emanating from the b-line neural cells (blue cells in Fig. 1) (Imai et al., 2006). The key component of Fgf8/17/18 regulation is the differential expression of the Snail repressor in the A9.30 and A9.32 neural progenitors. Snail expression is explicitly stronger in A9.32 than A9.30 (Fig. 6A). This augmented expression depends on Neurogenin, which is expressed only in A9.32. MO-induced suppression of Neurogenin resulted in reduced levels of Snail expression in A9.32, similar to the levels normally seen in A9.30 (white arrowhead, Fig. 6C). There is a similar de-repression of Fgf8/17/18 expression upon MO-mediated disruption of Snail activity (Fig. 6C).

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**Fig. 4. Fgf8/17/18 delineates PSV and neck regions.**
(A-H) Expression of (A,B) Otx, (C,D) En, (E,F) Pax2/5/8-A and (G,H) Hox1 in A,C,E,G control embryos and (B,D,F,H) experimental embryos developed from eggs injected with Fgf8/17/18 MO. Cell identities were determined by DAPI staining of nuclei (inserts in A-H). Cells from the posterior end of the SV (A11.63) to the middle part of the VG (A11.118 or its descendants) are enclosed by red and light-blue lines, which show the expression or lack of expression of the indicated genes, respectively. Broken white lines indicate the boundaries of the PSV/neck and the neck/VG. Note that A11.119 is about to divide, or has recently divided, into two daughter cells in A,B,E,F. For simplicity, they are enclosed by single lines. The embryos shown in G and H are at a slightly later stage, when Hox1 gene expression is more prominent. (I,J) Schematic representations of the brain regionalization mechanism by Fgf8/17/18 in (I) the normal embryo and (J) Fgf8/17/18-morphant embryo. Note that Fgf8/17/18 is not expressed in the VG at the tailbud stage but is expressed in the progenitor cells (A9.30) in the neural plate.

**Fig. 5. The Fgf signaling between the late gastrula stage and the neurula stage is required for Pax2/5/8-A expression.**
(A-D) Expression of Fgf8/17/18 from the 44-cell stage to the neurula stage. Expression in A7.6 is shown by black arrowheads and expression in the A9.30 is shown by white arrowheads. (E-H) Expression of Pax2/5/8-A following U0126 treatment at different time points. Numbers indicate the total number of embryos analyzed. The developmental time point when embryos were placed in sea water containing U0126 is shown in the top. Embryos treated with U0126 from the late gastrula stage when Fgf8/17/18 expression in A9.30 begins do not express Pax2/5/8-A, whereas embryos treated with U0126 from the neurula stage express Pax2/5/8-A.
expression in A9.30. These low levels fail to block Fgf8/17/18 expression, but might restrict the levels of expression. The enhanced expression of Snail seen in A9.32 arises from a feedback loop: Nodal induces Neurogenin and the two regulators work together to activate Snail (Fig. 6D). These augmented levels of Snail result in the complete repression of Fgf8/17/18 in the A9.32 lineage of the CNS.

The localized expression of Neurogenin is achieved by two signaling molecules. Nodal is expressed in the b-line cells (blue cells in Fig. 1 and Fig. 6D), which are juxtaposed to A9.32 but not A9.30. Similarly, Delta-like is expressed in the b-line cells under the control of Nodal signaling (Hudson and Yasuo, 2005; Imai et al., 2006), and the Delta-Notch signaling contributes to activation of Neurogenin (see Fig. S1Ad in the supplementary material). Thus, these two inputs induce localized expression of Neurogenin in A9.32 cooperatively or by a simple cascade mechanism.

It has been proposed that the ancestral chordates had the Otx/Gbx gene circuit but lacked an apparent MHB organizer, as the Otx and Gbx expression patterns abut in amphioxus CNS, although other components of the MHB organizer such as En, Pax2/5/8 and Wnt1 are not expressed at this boundary (Castro et al., 2006). If so, the recruitment of Fgf8 signaling as an MHB organizer might have evolved after the divergence of the cephalochordate and tunicate/vertebrate lineages. There are two possible scenarios for the advent of Fgf8 in the compartmentalization of the vertebrate CNS. First, the Otx/Gbx gene circuit might have been used to regulate Fgf8 in the ancestral chordate, and the switch in Fgf8 regulation to the Nodal/Neurogenin/Snail gene circuit might reflect the streamlined cell lineages in the early Ciona embryo and the need for the precise regulation of Fgf8 at single-cell resolution. Alternatively, Fgf8 regulation by the Nodal/Neurogenin/Snail gene circuit might be ancient, and in vertebrates the upstream regulatory mechanism was integrated into a pre-existing Otx/Gbx gene circuit. In this regard, we note that orthologs of Fgf8 are regulated by Snail during gastrulation of the Drosophila embryo (e.g. Stathopoulos et al., 2004).

**Hox1 expression is controlled by Fgf8/17/18 through retinoic acid signaling**

As discussed earlier, Fgf8/17/18 signaling inhibits Otx expression in the posterior A9.16 lineage, thereby restricting its expression to the anterior lineage, the future PSV. Otx either directly or indirectly activates the forkhead regulatory gene FoxB, which represses Hox1 expression in the PSV. It is well known that Hox genes are regulated by retinoic acid (RA) signaling in chordates (Holland and Holland, 1996; Maden, 2002). It has previously been shown that RA enhances Hox1 expression in the Ciona embryo (Nagatomo and Fujiwara, 2003). RA is synthesized by Raldh2 (retinaldehyde dehydrogenase 2), which is expressed in the most anterior muscle cells at the tailbud stage (Fig. 7A) (Nagatomo and Fujiwara, 2003). Indeed, knockdown of Raldh2 eliminates Hox1 expression (Fig. 7B,C). Endogenously synthesized RA is therefore responsible for Hox1 expression. Interestingly, Cyp26 is expressed in the presumptive PSV region of the Ciona CNS, and this expression is lost in morphant embryos injected with either Otx or FoxB MOs (see Fig. S1F in the supplementary material). Cyp26 encodes an enzyme responsible for degrading RA. These observations raise the
Development 136 (2)

The possibility that FoxB indirectly represses Hox1 expression in the presumptive PSV by activating Cyp26, which in turn inhibits RA signaling (Fig. 7D). Previous studies suggested a possible connection between Fgf signaling and Hox expression (Irving and Mason, 2000; Shimizu et al., 2006; Skromne et al., 2007) and between Cyp26 and Hox expression in the vertebrate CNS (Hernandez et al., 2007). We propose that this connection might reflect a direct regulatory connection between the MHB organizer and RA signaling.

**DISCUSSION**

The comprehensive analysis of CNS regulatory genes led to the elucidation of a provisional gene network in the early Ciona tadpole. These networks provide a number of key insights into the compartmentalization of the chordate CNS. First, a localized Fgf8 signaling center was probably used by the last shared ancestor of ascidians and vertebrates to delineate two regions of the chordate brain (mesencephalon and metencephalon). Second, Fgf8 signaling in Ciona leads to restricted expression of Otx and FoxB in the PSV, as well as restricted expression of Pax2/5/8-A in the neck. Otx and FoxB might inhibit Hox1 expression in the forebrain via Cyp26, whereas Pax2/5/8-A might coordinate the expression of the regulatory genes required for the differentiation of metencephalon motoneurons, such as Phox2a/Aris (e.g. Engle, 2006). Finally, although the regulatory genes responsible for the compartmentalization of the vertebrate CNS (e.g. Otx, Pax2, Neurogenin, etc.) exhibit comparable patterns of expression in the Ciona CNS, there are both conserved and distinctive features of the underlying mechanism. Localized Fgf8 signaling is used to deploy these expression patterns in both systems, even though different regulatory mechanisms are used to restrict Fgf8.

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