Regulatory gene expressions in the ascidian ventral sensory vesicle: evolutionary relationships with the vertebrate hypothalamus

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Abstract

In extant chordates, the overall patterning along the anteroposterior and dorsoventral axes of the neural tube is remarkably conserved. It has thus been proposed that four domains corresponding to the vertebrate presumptive forebrain, midbrain–hindbrain transition, hindbrain, and spinal cord were already present in the common chordate ancestor. To obtain insights on the evolution of the patterning of the anterior neural tube, we performed a study aimed at characterizing the expression of regulatory genes in the sensory vesicle of Ciona intestinalis, the anteriormost part of the central nervous system (CNS) related to the vertebrate forebrain, at tailbud stages. Selected genes encoded primarily for homologues of transcription factors involved in vertebrate forebrain patterning. Seven of these genes were expressed in the ventral sensory vesicle. A prominent feature of these ascidian genes is their restricted and complementary domains of expression at tailbud stages. These patterning markers thus refine the map of the developing sensory vesicle. Furthermore, they allow us to propose that a large part of the ventral and lateral sensory vesicle consists in a patterning domain corresponding to the vertebrate presumptive hypothalamus.

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Keywords: Hypothalamus; Ascidian ventral sensory vesicle; Regulatory gene expressions

Introduction

In the vertebrate central nervous system (CNS), regionalization is a developmental process that precedes the establishment of highly diversified neural structures and is characterized by dynamic expression patterns of regulatory genes. Such genes specifically expressed in domains of the anterior neural plate or ventral forebrain encode transcription factors belonging to several families with structurally different DNA binding domains: homeodomain, zinc finger domain, winged helix domain, bHLH-PAS domain. Spatial and temporal combinations of these factors account for the definition of specific anatomical units in the developing brain. Furthermore, abnormal expression of several transcription factors alters early patterning of the ventral forebrain (Six3, Kobayashi et al., 1998, 2002; Lagutin et al., 2003) or the morphogenesis and differentiation of ventral forebrain structures (Otp, Acampora et al., 1999; Nkx2.1, Kimura et al., 1996; Gsh1, Li et al., 1996; Sim1, Michaud et al., 1998; Wang and Lufkin, 2000; FoxB1, Wehr et al., 1997).

Comparing the regionalization of the neural plate and tube between nonvertebrate chordates and vertebrates is a powerful means to gain information on the evolutionary origins of brain patterning. This approach has already been successfully applied to the vertebrate phylum. Whereas early embryogenesis (blastula and gastrula stages) and late CNS morphogenesis are quite divergent between distant vertebrate groups, regionalization of the neural tube, as viewed from the prosomeric model, displays a puzzling conservation in vertebrates (Bachy et al., 2001; Fidgor and Stern, 1993; Milan and Puelles, 2000; Pombal and Puelles, 1999; Puelles, 2001; Puelles and Rubenstein, 1993). Furthermore, lineage studies have shown that the topological relationships between
presumptive territories of the main brain divisions are already established in the neural plate and are well conserved in vertebrates (Cobos et al., 2001; Inoue et al., 2000; Woo and Fraser, 1995). As a starting point for comparison between distant chordates, a simple model of vertebrate forebrain regionalization will be considered. The forebrain, which arises from anterior neuroectoderm, is composed of the telencephalic areas, the eyes, and the diencephalon. This latter region consists of two main domains separated by the zona limitans intrathalamica. The rostral diencephalon encompasses ventrally the hypothalamus, dorsally the anterodorsal diencephalon, and caudally the ventral thalamus. The posterior diencephalon encompasses the dorsal thalamus and the pretectum.

The vertebrate neural tube plan may have evolved through the elaboration and expansion of a simpler neural tube already present in the last common chordate ancestor. In this respect, gross anteroposterior and dorsoventral regionalization observed in vertebrates is conserved in the nervous system of cephalochordates and urochordates, the closest living relatives to vertebrates (Wada and Satoh, 2001). Although the CNS of the urochordate Ciona intestinalis comprises less than a hundred neurons (Nicol and Meinertzhagen, 1991), it has been divided into four anteroposterior domains of patterning marker expression, homologous to regions present in vertebrates (for review, see Lemaire et al., 2002; Wada and Sato, 2001). Furthermore, the regionalization of ascidian and vertebrate neural tubes along the dorsoventral axis is also comparable (Corbo et al., 1997; for review, see Lemaire et al., 2002; Wada and Sato, 2001).

The sensory vesicle represents the most complex part of the ascidian nervous system. Most of this cerebral structure derives from the anterior Otx-positive domain of the neural plate and may correspond to the vertebrate prosencephalon. In Ciona, the major part of this territory originates from the so-called a8.17, a8.19, and a8.25 blastomeres, which themselves derive from the a4.2 blastomere (Hudson and Lemaire, 2001; Nishida, 1987). Interestingly, it has been shown recently that anterior neural fate, marked by Otx expression, is specified through FGF-dependent mechanisms also crucial for vertebrate neural induction (Bertrand et al., 2003; Sheng et al., 2003). Beyond these early steps of development, the later patterning of the anterior CNS in Ciona remains largely unknown.

The expression patterns of genes homologous to vertebrate ventral forebrain markers were studied in C. intestinalis embryos to gain insight into the evolution of ascidian sensory vesicle and vertebrate brain patterning. Homologues of seven genes expressed in vertebrate anterior neural plate, hypothalamus, or other regions of the diencephalon, namely, Ci-Otp, Ci-Meis, Ci-Hif, Ci-Nx2.1, Ci-Six3/6, Ci-FoxB, and Ci-FoxHa, were found to be expressed in the ventral sensory vesicle in C. intestinalis, with strikingly regionalized patterns. Based on restricted expression patterns of these genes in the ventral and lateral parts of the ascidian sensory vesicle, we propose that a patterning domain encompassing the ventral sensory vesicle corresponds to the vertebrate hypothalamic territory. This refined map should allow more detailed developmental studies of patterning mechanisms in the sensory vesicle of C. intestinalis. Moreover, comparisons between urochordates, cephalochordates, and vertebrates provide a basis for understanding the origins of vertebrate forebrain regionalization.

Materials and methods

Adult C. intestinalis were purchased at the Station de Biologie Marine de Roscoff (France) and maintained in artificial sea water at 15°C under constant illumination. Eggs and sperm were collected from dissected gonads and used in cross fertilizations. Fertilized eggs were dechorionated (according to Mita-Miyazawa et al., 1985) and raised at 13–18°C on 1% agarose-coated dishes in artificial sea water supplemented with 50 μg/ml gentamycin (Hudson and Lemaire, 2001).

In situ hybridizations (ISHs) were carried out using digoxigenin-labeled or fluorescein-labeled cRNA probes detected by the alkaline phosphatase chromogenic reaction. For single ISH with digoxigenin-labeled probes, NBT/BCIP staining (Roche) was performed as described in Christiaen et al. (2002). Double ISHs were performed using a mix of fluorescein- and digoxigenin-labeled probes. The probes were successively revealed using NBT/BCIP or Fast-Red staining (Roche), respectively. Samples were mounted with 50% glycerol in PBS.

Genes analyzed here are listed in Table 1. Antisense RNA probes were synthesized from bacterial clones picked from the C. intestinalis gene collection or isolated by specific RT-PCR in the laboratory (Table 1). Ci-Nx2.1 antisense probe was synthesized from a cDNA clone kindly provided by Dr. Ristoratore (Table 1). The identity of the clones was systematically checked by sequencing.

Interpretation of the gene expression patterns at tailbud stages with respect to the reciprocal spatial relationships

<p>| Table 1 |</p>
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<td><strong>Gene name</strong></td>
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*a* Original cDNA isolated in the lab.

*b* Clones obtained from the C. intestinalis gene collection.
and their lineage origin was strongly refined based on three lines of arguments. Close analysis of the morphology of the neural tube under Nomarsky microscope (Leica, Wetzlar, Germany) allows us to define a few invariant morphological landmarks. For instance, at mid-tailbud stages, whereas pigmentation of the otolith is not detectable, the apical side of ventromedial cells located in an intermediate anteroposterior level in the sensory vesicle protrudes in the lumen of the neural tube (black arrowhead in Fig. 2L, for example). Furthermore, the size, shape, and spatial arrangement of cells in the ventral sensory vesicle were analyzed precisely using a confocal analysis of early, mid, and late tailbud embryos stained with Alexa488-phalloidin (Molecular probes). This analysis allowed us to clearly identify these small and densely packed ventromedial cells, as well as four large median cells posterior to them and aligned along the ventral midline of posterior sensory vesicle (Figs. 1A and B). According to Cole and Meinertzhagen (2004), these latter structures were assigned to A+/A10.27–28 cells, whereas more anterior ventral cells were assigned to a/g8.19 and a/g8.17 derivatives. In addition, the relative relationship of Ci-Hif, Ci-Meis, Ci-Nkx2.1, and Ci-FoxHa expression territories at midtailbud stage was confirmed by comparing each one to the restricted expression domain of a transgenic construction in the ventral sensory vesicle by double labeling and confocal analysis on electroporated embryos (for example, refer to Fig. 1C). This construction contains a fragment of the 5′ flanking region of the C. intestinalis Tyrosine Hydroxylase gene upstream from a green fluorescent protein (Moret et al., unpublished results). Images were captured with a Leica DMRXA2 microscope equipped with a TCS SP2 confocal scanning system.

Results

We performed in situ hybridization (ISH) experiments at different gastrula, neurula, and tailbud stages with C. intestinalis genes chosen for expression of their vertebrate or cephalochordate homologues in the ventral forebrain. Expressions of seven genes were detected in the ventral sensory vesicle. Only these genes considered relevant for a comparative analysis were described in this report (Table 1). Additional genes did not display specific expression in the ventral sensory vesicle although their vertebrate homologues are involved in the development of the ventral forebrain. Among these latter genes, the Ciona homologues of vertebrate Sim (Fan et al., 1996; JGI name: ci0100133264), ARNT1 and ARNT2 (Michaud et al., 2000; ci0100143114), as well as Fez-like (Hashimoto et al., 2000; ci0100154186) were not expressed in the sensory vesicle. In contrast, ubiquitous expression of the Ciona Tubby homologue (Guan et al., 1998; ci0100131237) was detected in the central nervous system. Therefore, these genes will not be further described in this part. The phylogenetic relationships between the genes analyzed here and their vertebrate counterparts have been established in previous studies (Satou et al., 2003; Wada et al., 2003; Yagi et al., 2003). Their sequences can be found in the JGI C. intestinalis genome assembly (http://genome.jgi-psf.org/ciona4/ciona4.home.html) and in the C. intestinalis cDNA resource (http://ghost.zool.kyoto-u.ac.jp/index1.html).

Expression of Ci-Otp, Ci-Meis, Ci-Nkx2.1, and Ci-Hif in restricted and complementary areas of the ventral and lateral sensory vesicle

Orthopedia (Otp), a member of the family of Paired class homeodomain transcription factors, is encountered in protostomes, as well as in deuterostomes (Di Bernardo et al., 1999; Lowe et al., 2003; Nederbragt et al., 2002; Simeone et al., 1994). Otp expression in mammalian embryos is restricted to discrete areas of hindbrain, spinal cord, and hypothalamus. Moreover, Otp is essential for the differentiation of several neuroendocrine hypothalamic nuclei (Acampora et al., 1999; Wang and Lufkin, 2000).

The C. intestinalis genome contains a single Orthopedia orthologue (Wada et al., 2003). Its transcripts were detected in a few ventromedial cells in the anterior neuroectoderm at tailbud stages (Figs. 2A–C). In late tailbud embryos and

![Fig. 1. Examples of results obtained with confocal microscopy, improving the spatial resolution of our interpretation. (A and B) Trunk of midtailbud embryos stained with Alexa488-phalloidin and observed in confocal microscopy. Lateral view (A), dorsal view (B). White arrowhead, cell cluster protruding in the lumen of the neural tube. Asterisk indicates A+/A10.27–28 cells. (C) Fluorescence double labeling revealing coexpression of Ci-Meis (in red) and a transgenic lacZ reporter controlled by the Ci-TH promoter (see Materials and methods) (in green). On electroporated embryos, Fast-Red staining (Roche diagnostics) following an ISH against Ci-Meis and a subsequent Alexa488 immunolabeling against LacZ were performed and analyzed in confocal microscopy.](image-url)
larvae, its expression was clearly excluded from the stomodaeum and persisted in the anterior ventral sensory vesicle (Fig. 2D).

In vertebrates, the Tale class homeodomain transcription factors Meis1 and Meis2 are expressed in various neural domains including the ventral forebrain, the telencephalon, as well as the midbrain, the hindbrain, and the spinal cord, whereas Meis3 expression is restricted to the posterior neural tube (Biemar et al., 2001; Cecconi et al., 1997; Maeda et al., 2001; Salzberg et al., 1999; Toresson et al., 2000). Surprisingly, at tailbud stages, the single homologue of vertebrate Meis1/2/3, Ci-Meis (Wada et al., 2003), was specifically expressed in a small ventromedial cell cluster of the sensory vesicle. The latter was slightly protruding in the lumen of the neural tube and localized at the level of the differentiating otolith with respect to the anteroposterior axis (Figs. 2E–H).

In vertebrates, early expression of Nkx2.1 (NKX2.1, T/EBP, TTF1) delineates the hypothalamus primordium. Its expression also encompasses a dorsal diverticule corresponding to the subpallium in all gnathostome groups (Gonzalez et al., 2002; Lazzaro et al., 1991; Nakamura et al., 2001; Ogasawara et al., 2001; Per a and Kessel, 1998). Interestingly, the amphioxus homeobox gene Nkx2.1 is also expressed in the ventral cerebral vesicle (Venkatesh et al., 1999). In previous studies on Ci-tifl, the C. intestinalis homologue of vertebrate Nkx2.1, focused on its endodermal expression (Ristoratore et al., 1999). In our study, Ci-Nkx2.1 transcripts were detected at mid- and late tailbud stages and in the posterior sensory vesicle in two ventrolateral bands (Figs. 2I and J), lateral and posterior to the median Ci-Meis-positive domain.

Several bHLH and PAS domain-containing transcription factors are expressed in the ventral forebrain in vertebrates. Hypoxia inducible factor α (Hif-1α) is a vertebrate bHLH-PAS transcription factor widely expressed in the vertebrate brain (Jain et al., 1998). However, specific expression was reported in the developing hypothalamus (Etchevers, 2003). We show here that at all tailbud stages examined, the neural Ci-Hif expression was restricted to a small group of cells located in the anterior and ventromedian neural tube between the Ci-Otp- and Ci-Meis-positive territories (Figs. 2K–N).

Ci-Otp, Ci-Meis, and Ci-Nkx2.1 are homologous to specific vertebrate hypothalamic markers and their expression territories are adjacent in Ciona ventral sensory vesicle and their expression territories are adjacent in Ciona ventral sensory vesicle suggesting a evolutionary relationship between the region-alization of the vertebrate hypothalamus and Ciona sensory vesicle. We therefore investigated the spatial relationships between the expression patterns of the above Ciona genes and the homologues of vertebrate markers expressed in a large anterior neural domain including the hypothalamus.

Dynamic expression of Ci-Six3/6 encompasses a broad domain in the rostral neuroectoderm

Vertebrates Six3 and Six6 are members of the Six-class homeobox genes. The expression patterns of these para-
logues are highly similar and well conserved in vertebrates (Bovolenta et al., 1998; Jean et al., 1999; Loosli et al., 1998; Lopez-Rios et al., 1999; Oliver et al., 1995; Zhou et al., 2000). *Six3* is expressed in an anterior neural domain encompassing the presumptive eyes, adenohypophysis, olfactory placode, and anterior forebrain (including the presumptive hypothalamus). It is also expressed in the developing hypothalamus at later stages, as well as in the eyes and the telencephalon.

*Ci-Six3/6* is a pro-orthologue of vertebrates *Six3* and *Six6* (Wada et al., 2003). In *Ciona*, high levels of *Ci-Six3/6* transcripts were detected in the anterior neuroectoderm at the gastrula and neurula stages (Figs. 3A–C). This expression domain encompassed the presumptive anterodorsal sensory vesicle and the anterior neural boundary (ANB) at early tailbud stage (Figs. 3D and E). Expression became progressively restricted anteriorly to the sensory vesicle and was excluded from the stomodaeeum in the hatching larva (Fig. 3F). At this stage, *Ci-Six3/6* was also weakly expressed in the vicinity of the ocellus and in the posterior sensory vesicle.

Therefore, at tailbud stage, the *Ci-Six3/6* expression domain overlapped with that of no other above-mentioned marker except *Ci-Otp*. However, the earliest expression of *Ci-Six3/6* was detected in the neural plate at late gastrula stage and neurula stages in the neural plate fourth row of cells and particularly in a/a9.34 and a/a9.38 blastomeres (Figs. 3A and B; Nicol and Meinertzhagen, 1998). According to Nishida (1987), these are daughter cells of a/a8.17 and a/a8.19, which give rise to the anterior lateral and medial parts of the sensory vesicle. According to Cole and Meinertzhagen (2004), a/a9.33 and a/a9.37, the posterior derivatives of a/a8.17 and a/a8.19, constitute the rostral part of the lateral walls of the posterior sensory vesicle, which includes *Ci-Nkx2.1*-expressing cells at tailbud stage. It is thus likely that *Ci-Hif* and *Ci-Meis* are expressed in the progeny of *Ci-Six3/6*-positive a/a9.34 or a/a9.38 cells. In contrast, the *Ci-Nkx2.1* expression domain is probably not included in the progeny of *Ci-Six3/6*-expressing cells.

### Biphasic *Ci-FoxB* expression in progenitors of the median posterior sensory vesicle and dorsolateral sensory vesicle

In vertebrates, the winged helix transcription factor *FoxB1* is expressed in a large domain of the neural plate, posterior to the *Six3* expression domain. At later stages, *FoxB1* expres-
sion becomes restricted to two lateral stripes in the diencephalon (posterior to the zona limitans intrathalamica) and midbrain (Ang et al., 1993; Gamse and Sive, 2001; Grinblat et al., 1998; Mazet and Shimeld, 2002). In the zebrafish gastrula, it has been shown that the anterior-most part of the FoxB1-positive territory corresponds to the hypothalamic primordium (Varga et al., 1999). In addition, FoxB1/Fkh5 and FoxB2/Fkh4 expressions persist in the posterior hypothalamus in mammalian embryos. Moreover, the development of this ventral structure is affected in posterior hypothalamus in mammalian embryos. Moreover, Grinblat et al., 1998; Mazet and Shimeld, 2002). In the zebrafish gastrula, it has been shown that the anteriormost and midbrain (Ang et al., 1993; Gamse and Sive, 2001; Ci-FoxB is required for the specification of the vertebrate hypothalamus, we examined the expression patterns of several vertebrate CNS patterning genes (Wada et al., 1998). It was also proposed to be a domain encompassing the expression domains of Ci-Otp and Ci-HIF and is in the vicinity of Ci-Meis- and Ci-Nkx2.1-positive territories.

In conclusion, CiFoxHa displays a broad expression in the ventral part of the sensory vesicle that precedes the expression onset of Ci-Otp, Ci-Hif, Ci-Meis, and Ci-Nkx2.1. Thus, this situation is compatible with a role of Ci-FoxHa in the patterning of the ventral part of the sensory vesicle.

Discussion

When comparing development and patterning of the neural tube between chordates, many similarities can be observed, which were likely inherited from their common ancestor. This ancestral neural tube may have been subdivided into at least four gross anteroposterior domains delineated by the early expression pattern of Otx, Pax2/5/8, and Hox genes (Wada et al., 1998). It was also proposed to be regionalized along its dorsoventral axis as revealed by the expression of HNF3ß, Pax3/7, and Snail (Corbo et al., 1997). A detailed analysis of the Otx-positive domain, at the origin of the most complex part of the nervous system in chordates, however, has so far been lacking. The expression patterns of homologues of several vertebrate CNS patterning genes within the Otx-positive domain have previously been reported in the presumptive ascidian sensory vesicle (Giuliano et al., 1998; Glardon et al., 1997; Imai et al., 2002; Ma et al., 1996), but little attention has been paid to the ventral part of this structure. This work thus focuses on expression patterns of C. intestinalis homologues of regulatory genes expressed in the vertebral ventral forebrain.

Relevance of the set of markers used in a comparative analysis

Comparative studies of nervous system patterning upon gene expression data require that these latter fulfil several
criteria. First, expression pattern similarities are more informative if the genes provide positional cues to developing neural structures. Genetic and functional data have demonstrated the role of Six3, Nkx-2.1, and Otp homologues during early neural patterning in protostomes and vertebrates (Acampora et al., 1999; Kimura et al., 1996; Kobayashi et al., 2002; Lagutin et al., 2003; Wang and Lufkin, 2000). Previous comparative studies on FoxB homologues carried out in vertebrates and cephalochordates suggested that the position of their neural expression domain has been globally conserved during chordate evolution (Mazet and Shimeld, 2002).

Second, gene duplication events, which occurred in the vertebrate lineage, can obscure expression pattern similarities because they could have led to so-called sub- or neo-functionalization of the newly formed paralogues. Only one Otp gene and one FoxH gene have been identified in vertebrates (Chen et al., 1997; Simeone et al., 1994). Two Nkx2.1 paralogues were encountered in fishes but their combined expression domains recapitulate that of the single Nkx2.1 gene found in other vertebrate groups (Rohr et al., 2001). Several semi-orthologous genes to Six3/6, FoxB, and Meis exist in vertebrates, but the expression patterns of paralogues are globally similar.

Therefore, Six3/6, Nkx2.1, Otp, FoxB, and Meis markers seem particularly informative for the detailed comparative analysis of neural patterning between chordates.

Neural patterning genes exhibit conserved expression domains in the vertebrate forebrain and ascidian sensory vesicle

In this study, homologues of regulatory genes were selected for their restricted expression in the forebrain. Ci-Six3/6, Ci-Otp, Ci-NKX2.1, Ci-Meis, Ci-FoxB, and Ci-FoxHa expressions are expressed in Ciona anterior neural tube (i.e., the region delineated by Otx expression). Among these, the expression of Nkx2.1 and FoxB has already been reported in the cerebral vesicle of cephalochordates. The vertebrate forebrain, the cephalochordate cerebral vesicle, and the urochordate sensory vesicle essentially arise from the Otx-positive part of the neural tube, which suggests that the patterning of the neural tube by Otx is an ancestral feature among chordates. Our results extend this conclusion and strongly suggest that these neural patterning genes were already acting in the anterior Otx-positive domain in the chordate ancestor.

In vertebrates, the bHLH transcription factors Sim1, Sim2, and ARNT2 are required for the differentiation of hypothalamic neuroendocrine cell types of the paraventricular and supraoptic nuclei (Hashimoto et al., 2000; Michaud et al., 2000). No expression of their Ciona homologues was detected in the sensory vesicle at tailbud stages. Thus, these genes may not share a similar role in Ciona embryos. In addition, expression of the vertebrate homeodomain transcription factor Gsh1 was reported in the hypothalamus (Li et al., 1996). Expression of its Ciona homologue Ci-Gsx was observed in the dorsal and posterior parts of the sensory vesicle. These expression domains are unlikely to correspond to that of vertebrate Gsh1 in the hypothalamus but rather in the posterior diencephalon, midbrain, or rhombencephalon (Hudson and Lemaire, 2001; Imai et al., 2004). Furthermore, homologues of several vertebrate forebrain marker genes, like Vax and Anf, are apparently missing from the C. intestinalis genome (Wada et al., 2003). This suggests that the hierarchy of transcription factors involved in the development of the forebrain is only partially conserved between urochordates and vertebrates. Further studies are necessary to understand in which extent the acquisition of the role of these genes in vertebrates or their loss in urochordate may explain the differences in the complexity and the cell type diversity in the forebrain and the sensory vesicle.

Early establishment of fine-grained expression patterns of regulatory genes suggests a simple logic of cell type specification and differentiation

The regulatory genes studied here display highly restricted and often unique expression domains in the sensory vesicle at tailbud stages. Furthermore, although Ci-Otp and Ci-Hif are expressed within Ci-Six3/6 and Ci-FoxHa expression territories, respectively, the regulatory genes studied here are expressed in adjacent and complementary domains that define a fine-grained pattern encompassing most of the ventral and lateral sensory vesicle (Figs. 4A and C). These markers provide refined, often stably localized and useful landmarks in the ventral sensory vesicle. Despite the fact that the cell number in ascidian sensory vesicle is reduced, this structure appears to be highly regionalized. Interestingly, the expressions of Ci-Otp, Ci-Hif, Ci-Meis, Ci-Nkx2.1, and Ci-FoxB remain relatively unchanged during all tailbud stages. This situation is in contrast with the idea of a broad and highly dynamic expression of ascidian patterning genes, as previously reported for Pax6 (Glardon et al., 1997).

In this work, we found that fine details of neural patterning are established in the early tailbud stages and early during embryogenesis. The short developmental time course of Ciona embryo and the simplicity of its nervous system may imply that cell-fate specification is relatively straightforward, bypassing the fine-tuning steps necessary in more complex embryos. Indeed, the fate of some major tissues (like notochord and neuroectoderm) is known to be committed very early, even before gastrulation (Bertrand et al., 2003; for review, see Satoh, 2003). The situation observed at tailbud stages in Ciona could thus be more comparable, to some extent, to late embryonic or adult stages in vertebrates where Otp and Nkx2.1 expression territories are more specific of differentiated hypothalamic nuclei (Acampora et al., 1999; Nakamura et al., 2001).

Thus, the patterning logic of the ascidian ventral sensory vesicle could be governed by a simple coupling between a
regulatory gene and a small cell population, rather than by the specificity of spatial combinations provided by overlapping expressions of several regulatory genes. This could be linked to the observed diversity of locally differentiated cell types arising in the ventral sensory vesicle of the larva (Meinertzhagen and Okamura, 2001; Sakurai et al., 2004).

**Conserved anteroposterior marker topology in chordate anterior dorsal neural tube**

Detailed observation of the gene expression patterns studied here reveals numerous similarities to the expression profiles of homologous vertebrate genes. In vertebrate embryos, the *Six3*-expressing territory is anterior to that of *FoxB1* in the neural plate and the neural tube. Whereas both genes display broad expression in the neural plate, *Six3* later becomes restricted to the anteriormost part of the neural tube and *FoxB1* to lateral bands slightly away from *Six3*-expressing cells.

The overall anteroposterior topology and temporal dynamics of *Ci-Six3/6* and *Ci-FoxB* expressions at gastrula, neurula, and tailbud stages are strikingly similar to that of vertebrates. This observation suggests that common ancestral anteroposterior patterning mechanisms are deployed...
during development of the vertebrate forebrain and ascidian sensory vesicle.

The posterior limit of *Six3* expression in the vertebrate neural plate has indeed been proposed to correlate with the position of the future zona limitans intrathalamicus (zli), boundary between the anterior forebrain (also called secondary prosencephalon) and the posterior diencephalon (Kobayashi et al., 2002). At later stages, this boundary is still marked by the anterior limit of the dorsolateral bands of expression of *FoxB* genes (Kaestner et al., 1996). Thus, our results provide clues to the definition of urochordate patterning domains corresponding to the vertebrate anterior forebrain and posterior diencephalon, respectively. The presence of a homologue of the vertebrate zli in ascidian remains highly speculative at present. How such a border has been established in vertebrates is an exciting subject of investigation.

**Identification of a ventral patterning domain in the presumptive sensory vesicle of *Ciona* corresponding to the vertebrate hypothalamus primordium**

At tailbud stages, the ventral localization of *Nkx2.1*, *Six3/6*, *Otp*, and *Meis*-positive territories, with regard to the more lateral expression of *Gsx* (Hudson and Lemaire, 2001) and *FoxB*, seems to be conserved between *Ciona* and vertebrates. Furthermore, ventral expression of *Nkx2.1* and more lateral expression of *FoxB* were also reported in amphioxus, supporting the ancestry of this large dorsoventral arrangement (Mazet and Shimeld, 2002; Venkatesh et al., 1999). Therefore, we propose to define a patterning domain within the ventral sensory vesicle. This domain encompasses the expression territories of *Ci-Otp*, *Ci-Hif*, *Ci-Meis*, and *Ci-Nkx2.1*. There are indeed striking correspondences between this domain and the presumptive hypothalamus in vertebrates. In vertebrates, *Otp*, *Meis1/2*, and *Nkx2.1* have overlapping expression domains in the presumptive hypothalamus. In contrast, *Ci-Otp*, *Ci-Hif*, *Ci-Meis*, and *Ci-Nkx2.1* are expressed in consecutive anteroposterior territories of the ventral sensory vesicle. However, lineage data suggest that both median (*Ci-Otp*, *Ci-Hif*, and *Ci-Meis* positive) and lateral (Ciona *Nkx2.1* positive) neural cells are derived from the a/a8.19 and a/a8.17 pairs of blastomeres (Fig. 4B; Cole and Meinertzhagen, 2004; Nishida, 1987). Thus, these data strongly suggest that the ascidian anterovenal domain delineated by the expression of *Ci-Otp*, *Ci-Hif*, *Ci-Meis*, and *Ci-Nkx2.1* and the vertebrate hypothalamus derive from early embryonic fields that are evolutionary related (Fig. 4D). An anterovenal domain patterned by *Otp*, *Meis*, and *Nkx2.1* could have been present in the neural tube of the common ancestor of extant chordates.

In early neural plate, *Ci-FoxB* expression could be assigned to A/A10.27–28 blastomeres, which lay medially and posteriorly to the progenitors of the above-mentioned domain. Subsequently, these cells are incorporated anteriorly in the posterior sensory vesicle, presumably between the *Ci-Nkx2.1*-positive lateral bands (Figs. 4B and C; Cole and Meinertzhagen, 2004). In zebrafish, progenitors of the hypothalamus expressing *FoxB1/Fkh5* have been shown to shift from posterior to anterior along the ventral midline of the neural plate at gastrula stages (Varga et al., 1999). This similarity may lead to the hypothesis that similar patterning aspects may be shared by the vertebrate hypothalamus primordium and the gastrula *Ci-FoxB*-positive cells and thus that the latter could be included in the ventral patterning domain of *Ciona*. However, since early vertebrate *FoxB* expression also extends to diencephalic and mesencephalic domains, this hypothesis remains speculative as long as no other homologues of hypothalamic markers are found in these ascidian median cells.

**Vertebrate innovation may account for some differences observed in topological relationships between markers of the anterior nervous system**

Beyond overall topological conservation, our study also points to meaningful differences between vertebrate and *Ciona* anterior patterning. In vertebrates, the dorsal telencephalon and ventral presumptive hypothalamus share *Nkx2.1* and *Meis1/2* expression. In *Ciona*, neither *Ci-Nkx2.1* nor *Ci-Meis* transcripts were detected in the anterodorsal part of the sensory vesicle at tailbud stages. Similarly, no dorsal expression of *Nkx2.1* has been reported in the cerebral vesicle of amphioxus and in the telencephalon of lamprey (Ogasawara et al., 2001). Thus, *Nkx2.1* and *Meis* gene expression may have been co-opted in the dorsal neural tube (presumptive telencephalon) in the course of vertebrate evolution. This is consistent with the absence of a telencephalic-like territory in the sensory vesicle of *Ciona*, as suggested previously (Oda and Saiga, 2001).

Two other differences between ascidian and vertebrate neural patterning are worth noting. At tailbud stages, an overlapping expression of *Ci-Otp* and *Ci-Six3/6* was detected in the anteriormost part of the ventral anterior sensory vesicle in a manner reminiscent of the vertebrate situation where they overlap in the anterior hypothalamus (Bovolenta et al., 1998; Kobayashi et al., 1998; Simeone et al., 1994). In contrast, whereas the vertebrate *Nkx2.1*-expression territory overlaps with *Otp*, *Meis1/2*, and *Six3* expression in the neural tube and derives from the *Six3*-positive domain observed in the neural plate, the *Ci-Nkx2.1*-expressing domain is posterior to *Ci-Otp*, *Ci-Six3/6*, and *Ci-Meis* domains. Furthermore, according to lineage studies, *Ci-Nkx2.1*-positive cells derive from *Ci-Six3/6*-negative progenitors localized in the neural plate posterior to the *Ci-Six3/6+* ones. In addition, the *Ci-Nkx2.1*-expressing territory extends caudally to the anterior end of the *Ci-FoxB* domain at mid-tailbud stage, whereas vertebrate *Nkx2.1* transcripts are anterior to the dorsolateral bands expressing *FoxB1* in the neural tube.
We suggest that these differences are related to differences previously reported between cephalochordates and vertebrates. In amphioxus, the frontal eye, an unpaired organ located on the midline, occupies the anterior tip of the neural tube and \( Nkx2.1 \) is expressed in a ventral domain, posterior to this eye (Venkatesh et al., 1999). In vertebrates, separation of the eye fields occurs after the specification of the hypothalamic primordium in the anterior and ventral region of the neural tube by the prechordal plate (for review, see Wilson and Houart, 2004). Interestingly, in fish, the eye field is split by the forward movement of ventral hypothalamic precursors arising from the diencephalon (Varga et al., 1999). The caudal localization of the \( Nkx2.1 \) territory with respect to the frontal eye in amphioxus or with respect to the \( Ci-Six3/6 \) domain in \textit{Ciona} may be viewed as a conserved and presumably ancestral feature of neural tube patterning shared by chordo-plates. The specification of the \( Nkx2.1/Meis \) domain may have shifted rostrally in the vertebrate lineage. This evolutionary event could have been part of general patterning modifications leading to the ventral expansion of the FoxB-expression domain, and the separation of the eye field in two bilateral eyes.

In \textit{C. intestinalis}, the homologue of the vertebrate pituitary homeobox gene (\textit{Pitx}) is expressed in the anterior neural boundary (ANB) (Boorman and Shimeld, 2002; Christiaen et al., 2002). In addition, our study shows that the ascidian hypothalamic-like patterning domain is just posterior to the \textit{Pitx}-expressing ANB. This situation is similar to the early topological association between the presumptive \textit{Pitx}-expressing cells of the ANB and the hypothalamic primordium in the vertebrate neural plate (for review, see Rubenstein et al., 1998). We thus propose that this feature was already present in the chordate common ancestor. However, no contact between the \textit{Ci-Pitx} and \textit{Ci-Nkx2.1} expression domains exists in \textit{Ciona} embryos. This is in contrast with the vertebrate situation where, at least during hypophysis development, an association occurs between stomodeal \textit{Pitx}-expression territory and \( Nkx2.1 \)-positive hypothalamic primordium. \( Nkx2.1 \) expression in the presumptive hypothalamus has been shown to be required for normal morphogenesis and differentiation of the hypophysis involving complex cross-talks with the hypothalamus (Dasen and Rosenfeld, 1999; Kimura et al., 1996). This suggests that the putative evolutionary rostralward shift of \( Nkx2.1 \) expression in the vertebrate lineage could have been a crucial event related to the emergence of a new developmental context underlying the hypothalamo-hypophysal axis differentiation.

In conclusion, this study emphasizes the possibility that the ventral sensory vesicle, patterned by the expression of \textit{Ci-Otp}, \textit{Ci-Meis}, and \textit{Ci-Nkx2.1}, originates from an ancestral topology of neural patterning markers, which was also the evolutionary basis for the patterning of the vertebrate hypothalamic primordium. Beyond the conservation of the topography of neural patterning genes, the evolution of the coupling between patterning territories and cell type differentiation needs to be addressed. A case in point comes from the presence of coronet cells in the ventral sensory vesicle of ascidian larvae (Katz, 1983). These cells have been proposed to be homologous to the coronet cells observed in an expansion of the ventral wall of the hypothalamus called the \textit{saccus vasculosus}, which is present in some bony and cartilaginous fishes. The differentiation of coronet cells in the \textit{Ciona} larva from precursors present in the ventral sensory vesicle is under investigation (Moret et al., submitted for publication). Such a coupling would be in favor of an ancient association between the \( Nkx2.1 \)- or \( Meis \)-positive patterning domain and the coronet cells, and thus could provide clues to the presence of a protohypothalamus in the chordate common ancestor.

The mechanisms controlling early steps of the vertebrate forebrain patterning have received much attention recently (for review, see Wilson and Houart, 2004). This study, together with previous reports, gives us the opportunity to use ascidians as an alternative model to investigate the ontogenetic and phylogenetic origin of vertebrate forebrain.

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