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Dynamic change in the expression of developmental genes in the ascidian central nervous system: Revisit to the tripartite model and the origin of the midbrain–hindbrain boundary region

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Abstract

Comparative studies on expression patterns of developmental genes along the anterior–posterior axis of the embryonic central nervous system (CNS) between vertebrates and ascidians led to the notion of "tripartite organization," a common ground plan of the CNS, consisting of the anterior, central and posterior regions expressing *Otx*, *Pax2/5/8* and *Hox* genes, respectively. In ascidians, however, descriptions and interpretations about expression of the developmental genes regarded as region specific have become not necessarily consistent. To address this issue, we examined detailed expression of key developmental genes for the ascidian CNS, including *Otx*, *Pax2/5/8a*, *En*, *Fgf8/17/18*, *Dmbx*, *Lhx3* and *Hox* genes, in the CNS around the junction of the trunk and tail of three different tailbud-stage embryos of *Ciona intestinalis*, employing double-fluorescence *in situ* hybridization, followed by staining with DAPI to precisely locate expressing cells for each gene. Based on these observations, we have constructed detailed gene expression maps of the region at the tailbud stages. Our analysis shows that expression of several genes regarded as markers for specific domains in the ascidian CNS changes dynamically within a relatively short period. This motivates us to revisit to the tripartite ground plan and the origin of the midbrain–hindbrain boundary (MHB) region.

Keywords: CNS; Gene expression; MHB; Ascidian

Introduction

Ascidians belong to the subphylum Urochordata, a branch in the phylum Chordata. Ascidian eggs develop into tadpole larvae, which share the prototypical morphogenesis and the body plan of chordates. A chordate character of particular interest in the ascidian morphogenesis is the central nervous system (CNS), which is formed from the rolling of the neural plate into a neural tube (Satoh, 1994) in a manner resembling that seen in vertebrate neurogenesis. The structure of the ascidian neural tube is surprisingly simple. It is composed of about 330 cells, among which only about 100 are neuronal cells, and forms without extensive cell migration or cell death (Cole and Meinertzhagen, 2004; Nicol and Meinertzhagen, 1991). Thus, the ascidian CNS has served as a simple model for the study of the nervous system development in chordates (Lemaire et al., 2002; Meinertzhagen and Okamura, 2001; Okamura et al., 1993). The small cell number and almost invariant cleavage pattern of the ascidian development enabled identification of individual cells and tracing the cell lineages and their fate in the ascidian CNS. In *Ciona intestinalis*, the detailed cell lineages for 226 cells in the CNS have been described (Cole and Meinertzhagen, 2004). In *Halocynthia roretzi*, the fates of the precursor cells for the anterior larval CNS, six in the animal hemisphere (a-line) and four in the vegetal hemisphere (A-line) have been well documented (Taniguchi and Nishida, 2004). On the other hand, draft genome sequencing of *C. intestinalis* was completed (Dehal et al., 2002) and the broad gene expression profiles of transcription factors and signaling molecules are available (Imai et al., 2004). To link this information to the understanding of mechanisms of formation and patterning of the ascidian CNS, the detailed analysis of the expression pattern of key developmental genes with respect to the cell lineage has become urgent task.

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As for the evolutional history of patterning of the CNS, comparative analysis of gene expression pattern and developmental anatomy among chordates has yielded some striking findings. Above all, the discovery of the tripartite pattern shared by vertebrates and ascidians has come under the spotlight. In vertebrate embryos, the expression of *Otx* and *Gbx* in the neuroectoderm during gastrulation marks the future fore/midbrain and hindbrain (Lumsden and Krumlauf, 1996), respectively. The midbrain–hindbrain boundary (MHB) organizer emerges at the boundary between them during neurulation. The MHB organizer patterns the midbrain and hindbrain primordia and is characterized by the expression of a highly conserved set of genes, including *En*, *Fgf8*, *Wnt1* and genes of the *Pax2/5/8* subfamily (Raible and Brand, 2004; Rhinn and Brand, 2001). The region of hindbrain and spinal cord, the rhombospinal region, is then characterized by *Hox* gene expression. Surprisingly, embryos of the ascidian *H. roretzi* also exhibit the anterior *Otx* and posterior *Hox* expression with an intervening gap, in which *Pax2/5/8* is expressed (Wada et al., 1998). This finding led to the proposal that the ancestral chordate neural tube had a tripartite structure along the anterior–posterior axis, and the ascidian CNS includes three regions homologous to the vertebrate fore/midbrain, MHB and the hindbrain/spinal cord (Wada et al., 1998). For the existence of MHB in ascidians, further support came from expression of *Fgf8/17/18* in the region just posterior to the *Pax2/5/8a* domain of the early tailbud stage embryo of *C. intestinalis* in a manner reminiscent of *Fgf8* expressed and functioning in the vertebrate MHB organizer (Imai et al., 2002). Additionally, since a similar tripartite gene expression pattern has been reported for *Drosophila*, it has been suggested that the origin of this ground plan may go back earlier than chordates (Hirth et al., 2003). However, in the larvacean *Oikopleura* (an appendicularian tunicate), although *Otx* and *Hox1* are expressed in the comparative domain, *Pax2/5/8* is expressed at the anterior tip of the CNS, while *En* is expressed posterior to the anterior limit of *Hox1* expression (Canestro et al., 2005). Furthermore, in amphioxus, there is no distinct zone of initial neural expression of *AmphiWnt1*, *AmphiEn* and *AmphiPax2/5/8* between the anterior *Otx* and posterior *Hox* expression domains (Kozmik et al., 1999). These data argue against the presence of the MHB homologue in larvaceans and amphioxus and have raised another issue: whether the pattern in larvacean and amphioxus is secondarily derived or whether *Drosophila*, ascidian and vertebrate show convergent evolution. Making things worse in ascidians, descriptions about the expression of developmental genes regarded as specific for each domain have turned out to be hard to interpret consistently. This controversy is largely due to the lack of detailed expression map of the developmental marker genes for the CNS of protochordates. Especially, temporal shift of the positional relationship of their expression domains has never been investigated with direct comparison using multi-color labeling method.

To address these issues, we investigated by two-color fluorescence whole-mount *in situ* hybridization the expression of key developmental genes for the ascidian CNS, including *Otx*, *Pax2/5/8a*, *Hox*, *En*, *Fgf8/17/18*, *Dmbx* (marking the midbrain in vertebrates), *Lhx3* (marking the motor neuron cell lineage in *H. roretzi*) and *SoxB1* (ascidian homologue of *Sox1*, *Sox2*, and *Sox3*, generally involved in neural development), in the CNS at the junction between the trunk and tail of three different tailbud-stage embryos of *C. intestinalis*. In order to precisely understand the position of the expression domain, we counted the number of the cells that express each gene. Furthermore, we re-examined the expression pattern of *Hox* genes in the CNS of *Ciona* at the cellular level. Based on these observations, we present detailed expression maps for the developmental genes in this region at the three tailbud stages of *C. intestinalis*. Our analysis shows that the expression of several genes regarded as markers for specific domains in the ascidian CNS changes dynamically within the tailbud stage. We also present data suggesting that the CNS of *Ciona* exhibits dipartite pattern at early tailbud stage. These observations motivate us to revisit to the tripartite ground plan of the CNS and the origin of the MHB region.

Materials and methods

Ascidians

C. intestinalis were cultivated at the Maizuru Fisheries Research Station of Kyoto University or at the International Coastal Research Center the University of Tokyo. Eggs and sperm were obtained surgically from the gonoducts. After insemination, eggs were dechorionated and raised in filtered seawater at 18 °C. Embryos at appropriate stages were collected and fixed for whole-mount *in situ* hybridization (WISH).

WISH probes

Antisence digoxigenin and/or fluorescein-labeled RNA probes for *Ci-Otx*, *Ci-En*, *Ci-SoxB1*, *Ci-Lhx3* were synthesized by using EST clones as templates, which were from *C. intestinalis* Gene Collection release 1 (http://ghost.zool.kyoto-u.ac.jp/indexr1.html). DNA fragments for probe synthesis of the following genes were obtained through RT-PCR using total RNA prepared from tailbud-stage embryos: *Ci-Pax2/5/8a* (1155 bp; forward primer, TGGGGATCAGCAATGGCGG; reverse primer, CATTTAGTGGCAGTGCGACA), *Ci-Fgf8/17,18* (1759 bp; forward primer, TTGCGAGTATAAATAGTAAATCC; reverse primer, AAGCAATATATAGGCAATCGAAC), *Ci-Dmbx* (832 bp; forward primer, TTCGTGCAATGTCAGTGTTCA; reverse primer, TAGACAGTGTGCAGCGCATT), *Ci-Hox1* (1239 bp; forward primer, CCTATACCAACCACATCAC; reverse primer, ACCGAATCCAAATACTTAGAC), *Ci-Hox3* (2390 bp; forward primer, CACCGTAACGTCACAGATTG; reverse primer, GCGGTGAGCTGCATTAAGTT), *Ci-Hox5* (1103 bp; forward primer, TGAGGTGTTTGTGACGATATAA; reverse primer, CATGGTCCACTCCGTTTTAAT), *Ci-Hox10* (1412 bp; forward primer, CGAAGGAAAGCGACAAACAC; reverse primer, TGCAGCATTCACACGTCACA), *Ci-vAChTP* (2304 bp; forward primer, CTTTATTGTTCATCATGGACGTT; reverse primer, GAAGCGAAATCACAGCATAAC), and *Ci-vGAT* (1808 bp; forward primer, GGGACCAACAAGTGAAGAGA; reverse primer, TCGTGTCGTCGTTTGTTTT). The DNA fragments were cloned into the pBluescript KS vector (Stratagene).

Fluorescence WISH

After rehydration by stepwise transfer into 50%, 30% ethanol in PBST (PBS containing 0.1% Tween 20), and into PBST for 10 min twice, the specimens were treated with 2 μg/ml proteinase K in PBST at 37 °C for 30 min and digestion was stopped by washing with PBST for 5 min twice. The embryos were post-fixed with 4% paraformaldehyde in PBST for 1 h, followed by washing in PBST for 5 min twice. The specimens were incubated in prehybridization buffer (50% formamide, 5× SSC, 100 μg/ml tRNA, 50 μg/ml heparin, 1% SDS) at 42 °C for 1 h. The prehybridization buffer was replaced by hybridization buffer containing