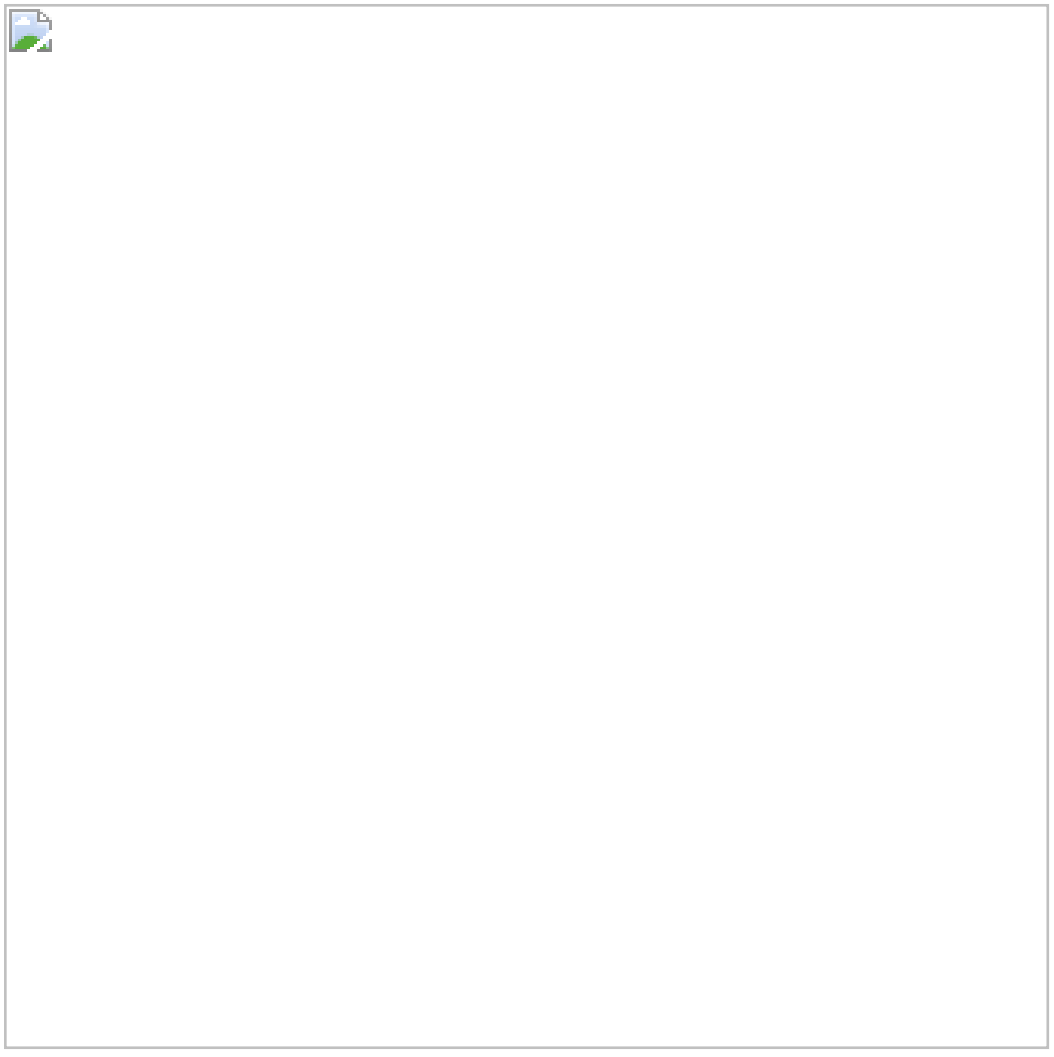

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Source: Zoological Science, 19(8) : 885-889

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.19.885

ZOOLOGICAL SCIENCE **19**: 885–889 (2002)

Neural Marker Genes Differently Expressed in Subsets of Embryonic Neural Cells of the Ascidian *Halocynthia roretzi*

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ABSTRACT—Ascidians are lower chordates that possess a possible prototype of the vertebrate nervous system. The central and peripheral nervous systems of ascidian larvae are composed of only a few hundred cells (Nicol and Meinertzhagen, 1991). To investigate how these ascidian nervous systems develop, dissection at the molecular level using subset-specific markers is essential. Here we describe four new genes zygotically expressed in subsets of the ascidian neural cells. The spatial expression domains of these genes overlap in some parts but not in other parts of the nervous systems. Our results suggest that there are functionally different regions in the nervous systems owing to the gene expression differences. Further analyses of these genes will enable us to determine the molecular neuro-developmental characteristics of various clusters of neural cells.

Key words: ascidian, neural marker genes, calnexin, cellubrevin, doublecortin

INTRODUCTION

Ascidians belong to subphylum Urochordata, the sister group to vertebrates and cephalochordates, and their larvae have many characteristics of a prototype vertebrate (Satoh, 1994). In particular, the central nervous system (CNS) of the ascidian larvae is formed in a typical vertebrate fashion with dorsal neural tube formation. At the molecular level, the antero-posterior axis of the ascidian neural tube has comparable gene expression patterns to the vertebrate CNS (Wada *et al*., 1998). Precise analyses of the ascidian nervous system are expected to provide insights into the ancestral condition of the chordate nervous system, and, on the other hand, into complicated vertebrate systems in miniature as a model chordate nervous system. Ascidian possesses a small genome (Simmen *et al*., 1998) and develop with simple, invariant embryonic cleavage patterns, which facilitate the analysis of gene expression patterns during neurogenesis (Nishida, 1987; Nicol and Meinertzhagen, 1991).

Despite the basic similarities between ascidians and vertebrates, differences such as the absence of stem cells, neural crest cells, lamination, radial migration, and apoptosis during neural development in ascidians are obvious (Meinertzhagen and Okamura, 2001). Although the ascidian nervous system exhibits a variety of behaviors and responses, it comprises no more than several hundred of cells (Nicol and Meinertzhagen, 1991). The ascidian nervous system is composed of the central and the peripheral nervous systems. The former consists of the brain or anterior sensory vesicle derived from a-line blastomeres, the posterior sensory vesicle derived from A- and b-line blastomeres, the visceral ganglion containing motor neurons that innervate the tail, and the caudal neural tube formed by four rows of ependymal cells (Nishida, 1987; Okada *et al*., 1997; Nicol and Meinertzhagen, 1998a, b, 1991). The latter includes palps and RTENs in the trunk and epidermal neurons along the midline of the tail (Nishida, 1987; Takamura, 1998).

Despite the small cell number and relatively simple construction of the ascidian nervous system, structural or functional features to define different types of cells studied at the molecular level have not been sufficiently reported. Even neurons and ependymal cells are not clearly distinguished yet, especially at the early stage of development. To characterize individual cells and investigate the nature of the ascidian nervous system, gene markers which can identify these cells are required. Here we report four new genes zygotically expressed in subsets of the ascidian neural cells.

MATERIALS AND METHODS

Animals and embryos

Adult ascidians, *Halocynthia roretzi*, were obtained during the

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spawning season from fishermen near the Asamushi Marine Biological Station, Tohoku Univ., Aomori, Japan and the Otsuchi Marine Research Center, Ocean Research Institute, Univ. of Tokyo, Iwate, Japan. Naturally spawned eggs were artificially fertilized with a suspension of non-self sperm. Fertilized eggs were cultured at 11°C. Embryos were collected at appropriate stages by low-speed centrifugation and fixed for whole-mount *in situ* hybridization.

cDNAs and sequence analyses

cDNAs were sequenced using an automated DNA sequencer (ABI PRISM 377, Perkin Elmer Japan). A homology search for the predicted amino acid sequence was performed using the BLAST network service (NCBI).

Whole-mount *in situ* hybridization

Whole-mount specimens were hybridized *in situ* at 42°C using digoxigenin-labeled antisense probes, as described previously (Miya *et al*., 1994). Some specimens were manually sectioned with a fine razor under a light microscope to confirm the localization of hybridization signals. To render specimens transparent, they were dehydrated in ethanol and cleared in a 1:2 mixture (v/v) of benzyl alcohol and benzyl benzoate.

RESULTS AND DISCUSSION

The nervous system of an ascidian tadpole larva is relatively primitive compared with those of vertebrates. In spite of its simplicity, it is complicated enough to execute complex behaviors in the swimming larval stage. Considering the phylogenetic position of ascidians as lower chordates, analysis of the detailed architecture of the ascidian nervous system at the molecular level promises to provide fundamental information necessary to understand the development of the nervous systems of higher animals. We previously characterized a neural marker expressed in a wide range of neural tissues (Yagi and Makabe, 2001). Further analyses will require molecular markers that can distinguish subsets of neural cells.

We performed a screen of randomly selected clones from a *Halocynthia roretzi* fertilized egg cDNA library for localized messages by whole-mount *in situ* hybridization, and simultaneously did the same for early tailbud embryos in order to investigate the spatial expression patterns of zygotic transcription using maternal messages as probes (Makabe *et al*., 2001), based on the claim that approximately 90% of zygotically active genes are also functional in oogenesis in the fruit fly, nematode and sea urchin (Davidson, 1986). The early findings in that screen enabled us to isolate numerous genes that are expressed not only maternally in the eggs but also zygotically in the nervous systems of the tailbud embryos. In the present study, we report four genes, most of which encode probable neural cell-specific structural proteins, that are expressed in subsets of the nervous system cells and can serve as useful molecular markers in neuro-developmental biological studies to examine the types of cells that compose the nervous system and how they differentiate during embryogenesis.

HrZF-2 (accession no. AB047036) cDNA is 2818 bp in length and encodes a 550-amino-acid polypeptide that is a putative transcription factor carrying four C2H2 type zinc-fingers. The putative amino-acid sequence of *HrZF-2* is similar to that of mouse MEG2, which may be involved in testicular cell differentiation (Passananti *et al*., 1989). *HrZF2* mRNA signals first became evident at the middle gastrula stage but were faint throughout the presumptive neural plate cells, with a strong bilateral signal in the neurula (Fig. 1A,B,C). In the tailbud embryo, the gene was expressed in presumptive palps, RTENs, lateral regions of the sensory vesicle, lateral rows of ependymal cells in the neural tube, including pairs of motor neurons, and some dorsal and ventral peripheral neurons (Fig. 1D,E,F). The expression pattern of this gene resembled that of *HrETR-1* (Yagi and Makabe, 2001), although the *HrZF-2* signals were weaker overall.

HrCalnexin (accession no. AB047037) is 2525 bp in length and encodes a 581-amino-acid polypeptide that is a homolog of Calnexin, a calcium-binding protein reported to be involved in the assembly and/or retention of proteins in the ER (Tjoelker *et al*., 1994). The *HrCalnexin* gene was expressed in the mesenchyme and epidermis, intensely in the trunk and weakly in the tail, and also expressed in the nervous systems, in which the signals were detected in the sensory vesicle and the caudal neural tube (Fig. 1G,H,I,J,L). Examination of cross sections of the stained tail showed that this gene was expressed strongly in the dorsal ependymal cells in the neural tube, but was not or was only faintly expressed in the other cells (Fig. 1K). The most notable differences between the expression sites of this gene and those of other genes described in this report were the intense expression in the dorsal row of the neural tube and the lack of expression in peripheral neurons.

HrCellubrevin (accession no. AB077367) is 1442 bp in length and encodes a 110-amino-acid polypeptide, Cellubrevin, which is a member of the synaptobrevin family of vesicle-associated membrane proteins (McMahon *et al*., 1993). Intense expression of this gene was observed in the central nervous system in the trunk and motor neurons. The signals were also seen in the dorsal epidermal neurons of the peripheral nervous system in the early and middle tailbud embryos, which made it possible to locate the presumptive caudal neurons (Fig. 1M,N). Interestingly, there was no staining in the rest of the peripheral nervous system. This supports the idea that the dorsal and ventral epidermal neurons are somewhat different in structure and/or function.

HrDoublecortin (accession no. AB047038) is 3275 bp in length and encodes an 803-amino-acid polypeptide that is a homolog of Doublecortin, a calcium-dependent signaling protein that is strongly expressed in the mammalian fetal brain and is involved in neuronal migration in the central nervous system (des Portes *et al*., 1998). The onset of expression of this gene occurred around neurulation, following which the number of positive cells rapidly increased. The expression pattern of this gene was considerably different from those of the other genes examined: there was no expression in presumptive palps or the central region of the trunk CNS. The gene was expressed in RTENs, the dorsal

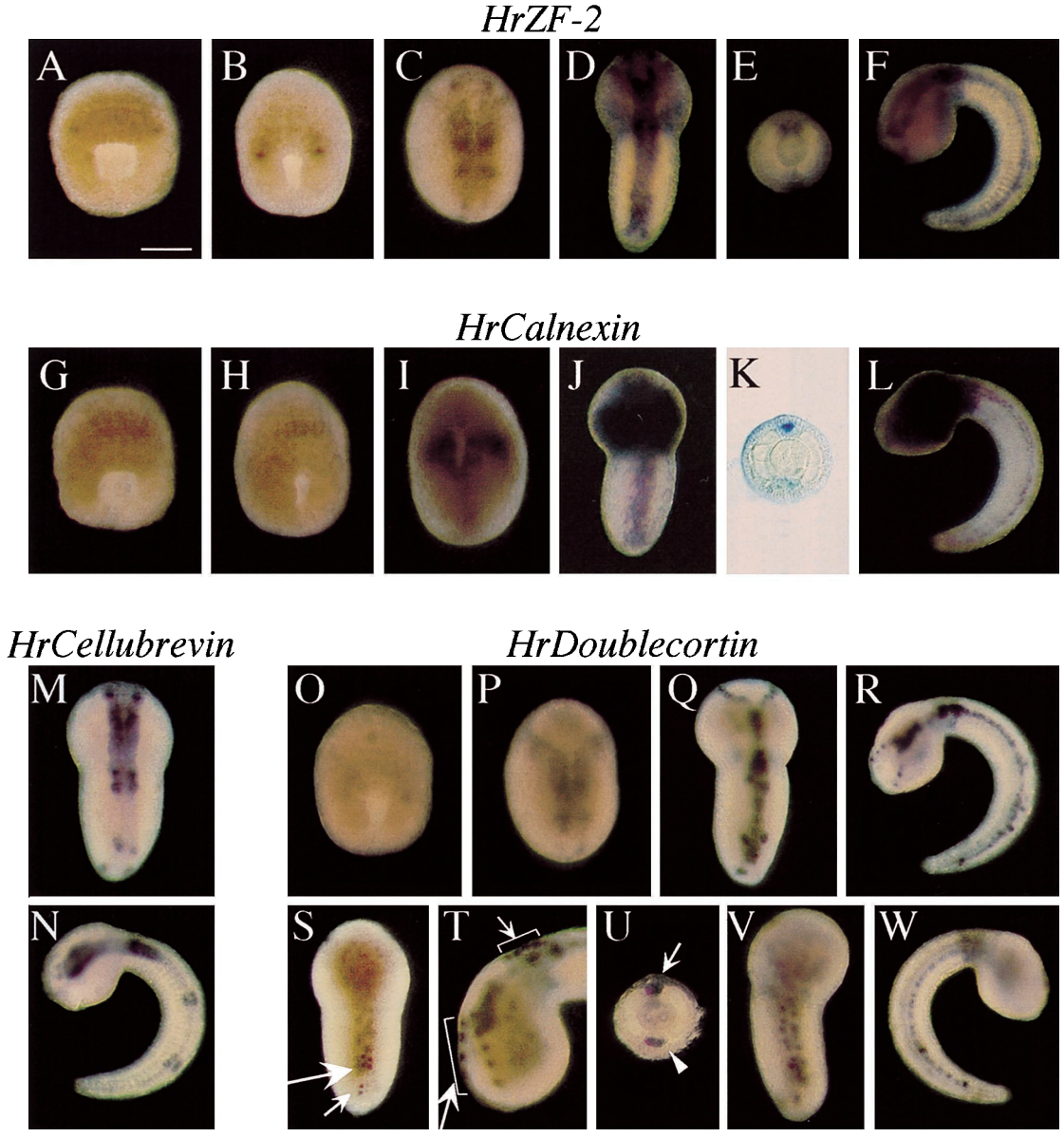

Fig. 1. Expression of *HrZF2* (**A**–**F**), *HrCalnexin* (**G**–**L**), *HrCellubrevin* (**M**,**N**), and *HrDoublecortin* (**O**–**W**) as revealed by whole-mount *in situ* hybridization. **A** A middle gastrula, ventral view, anterior is to the top. **B** A neural plate-stage embryo, dorsal view. **C** A late neurula, dorsal view. **D** An early tailbud embryo, dorsal view. **E** A cross-section of the early tailbud embryo shown in **D**, through the middle of the tail. **F** A middle tailbud embryo, dorsal view. **G** A late gastrula, **H** a neural plate-stage embryo, **I** a late neurula, **J** an early tailbud embryo, and **L** a middle tailbud embryo, dorsal view. **K** A cross-section of the early embryo shown in **J**, rendered transparent. **M** An early and **N** a middle tailbud embryo, dorsal view. **O** A neural plate-stage embryo, **P** a late neurula, **Q** an early tailbud embryo, and **R** a middle tailbud embryo, dorsal view. **S** An early tailbud embryo, ventral view. Six VCENs are stained. Signals in the endodermal strand cells are out of focus. **T** An enlarged view of the trunk of a middle tailbud embryo. Signals in epidermal neurons on the neck (DCENs) and RTENs are indicated by a short and a long arrow, respectively. **U** A cross-section of a middle tailbud embryo. Signals in the caudal neural tube and endodermal strand cells are indicated by an arrow and an arrowhead, respectively. **V** An early, and **W** a middle tailbud embryo, ventral view. Endodermal strand cells expressing *HrDoublecortin* are seen to be lined up in **W**.

sensory vesicle, the whole caudal neural tube, including motor neurons, and the peripheral neurons in the tail (Fig. 1O,P,Q,R). The signals were localized in the nuclei, which enabled us to determine the number of peripheral neurons (summarized in Fig. 2): they consisted of five bilateral pairs of RTENs (Fig. 1T, long arrow), four dorsal peripheral neurons (DCENs) (Fig. 1T, short arrow) forming a cluster flanking motor neurons, three pairs (one apart from the other two) of peripheral neurons on the dorsal side in the posterior region of the posterior region of the tail, and six peripheral neurons (VCENs) clustered as two patches on the ventral midline of the tail (four cells in an anterior and two in a posterior patch (Fig. 1S, the long and short arrow, respectively)). The origins of these peripheral neurons are, however, unclear, because *HrDoublecortin* transcripts were detectable only after the late neurula stage. Since the expression

of this gene was also observed in the endodermal strand at the tailbud stage (Fig. 1U, arrowhead), another finding made using this gene, unrelated to neurogenesis, was that intercalation of endodermal strand cells occurred between the early and middle tailbud stages: two rows of cells were observed in the early tailbud embryo, whereas only a single row was identified in the middle tailbud embryo (Fig. 1V,W).

Ishida *et al*. (1996) reported that some epidermis-specific genes were not expressed in a ring-shaped region in the anterior trunk or on the dorsal and ventral midline of the tail. This observation has been extended by showing lack of expression of the epidermis-specific *smad1/5* gene in these regions (Kobayashi and Makabe, 1999). Here we have described several neural genes expressed in these regions, suggesting that cells of the peripheral nervous system are derived from these regions. Presumptive peripheral neurons stained for *HrDoublecortin* expression seem to correspond to the larval epidermal neurons reported by Takamura (1998) as RTEN, ATEN and CEN. As for the palps, Takamura (1998) demonstrated the existence of two pear-shaped neurons in each palp; these neurons are connected with five bilateral pairs of epidermal neurons (RTENs) located in the dorso-apical region of the trunk. Our observations described above on the gene expression in palp regions did not have high enough resolution to prove a correspondence between the larval neurons and the stained cells, although the stained areas seemed to be larger than those expected for a few neurons. This is, therefore, one of the areas to be investigated in future studies.

The results obtained in this study are summarized in Fig. 2. It may be worth noting again that three of the four genes examined here encode neural cell-specific structural proteins, which are directly involved in the functions of the expressing cells. We demonstrated that the four rows of ependymal cells in the caudal neural tube differ in their gene expression patterns. In particular, *HrZF-2* was expressed in the lateral cells, as was *HrETR-1*, while *HrCalnexin* was expressed intensely in the dorsal cells but was not or was very faintly expressed in the other cells. These results may suggest that there are three kinds of cells in the neural tube with different functions at the molecular level, as also suggested by the gene expression patterns of a transcription factor and a signaling ligand (Shimauchi *et al*., 1997; Sasakura and Makabe, 2000). Moreover, *HrZF-2* and *HrDoublecortin* were expressed dominantly in the lateral and dorsal part of the sensory vesicle, respectively, as shown above. The vesicle should be functionally subdivided into even finer regions in order to establish the basis of the complex larval behavior, although such molecular evidences have not been reported. Therefore, it is now essential to isolate and characterize many genes which show differences of expression pattern in the ascidian nervous systems, and by which neural cells can be distinguished from each other in order to investigate the functions of the different neural cells.

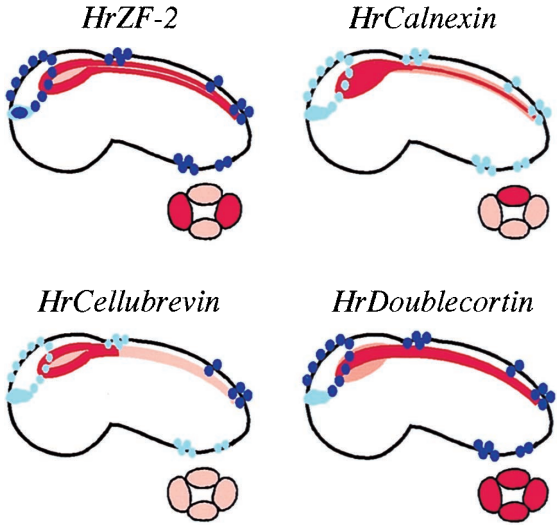

Fig. 2. Summary of the expression of the neural marker genes in the central and peripheral nervous system. Early tailbud embryos: lateral view and cross-sectional view of the neural tube through the middle of the tail are schematically drawn. The CNS and PNS are shown in pink and aqua, respectively. The regions of intense expression of each gene are colored red and blue.

ACKNOWLEDGEMENTS

We thank all members of the Asamushi Marine Biological Station of Tohoku University and the Otsuchi Marine Research Center of the University of Tokyo for their cooperation. We also thank Prof. Nori Satoh for generously allowing the use of his facility and for critical reading of this manuscript. This research was supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture, Japan (No. 14034226, No. 14011221, No. 14580710 to K. W. M.) and by the 'Research for the Future' program from the Japanese Society for the Promotion of Science (96L0040) to K. W. M.

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(Received March 12, 2002 / Accepted May 21, 2002)