DEVELOPMENTAL BIOLOGY **190,** 257–272 (1997)
ARTICLE NO. DB978708

Distinct Neuronal Lineages of the Ascidian Embryo

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The ascidian larva contains tubular neural tissue, one of the prominent anatomical features of the chordates. The cell-cleavage pattern and cell maps of the nervous system have been described in the ascidian larva in great detail. Cell types in the neural tube, however, have not yet been defined due to the lack of a suitable molecular marker. In the present work, we identified neuronal cells in the caudal neural tube of the *Halocynthia* embryo by utilizing a voltage-gated Na$^+$ channel gene, TuNa I, as a molecular marker. Microinjection of a lineage tracer revealed that TuNa I-positive neurons in the brain and in the trunk epidermis are derived from the a-line of the eight-cell embryo, which includes cell fates to epidermal and neural tissue. On the other hand, TuNa I-positive cells in the more caudal part of the neural tissue were not stained by microinjection into the a-line. These neurons are derived from the A-line, which contains fates of notochord and muscle, but not of epidermis. Electron microscopic observation confirmed that A-line-derived neurons consist of motor neurons innervating the dorsal and ventral muscle cells. Isolated A-line blastomeres have active membrane excitability distinct from those of the a-line-derived neuronal cells after culture under cleavage arrest, suggesting that the A-line gives rise to a neuronal cell distinct from that of the a-lineage. TuNa I expression in the a-line requires signals from another cell lineage, whereas that in the A-line occurs without tight cell contact. Thus, there are at least two distinct neuronal lineages with distinct cellular behaviors in the ascidian larva: the a-line gives rise to numerous neuronal cells, including sensory cells, controlled by a mechanism similar to vertebrate neural induction, whereas A-line cells give rise to motor neurons and ependymal cells in the caudal neural tube that develop in close association with the notochord or muscle lineage, but not with the epidermal lineage.

INTRODUCTION

The ascidian larva harbors a primitive tubular nervous system, which, as in the vertebrate, is derived from the neural plate. The simplicity of the larval organization and the invariable cell-cleavage pattern of this class (Conklin, 1905; Nishida, 1986) have facilitated mapping of the cell lineage for the nervous system (Nishida and Satoh, 1985; Nishida, 1987; Nicol and Meinertzhagen, 1988). The ascidian neural tube originates from three distinct lineages; a4.2, b4.2, and A4.1 in the 8-cell stage embryo (Nishida, 1987). The a4.2 blastomere gives rise to most of the anterior neural tissue, including pigment cells in the sensory organs. The other two lineages, b4.2 and A4.1, give rise to the posterior neural tube.

Regardless of the involvement of multiple lineages in the neural formation, studies on neural differentiation of the ascidian embryo have focused mainly on the lineage of the a4.2 blastomere (a-line; reviewed in Okamura *et al.*, 1993). The a-line contains cell fates to the epidermis and the anterior neural tissue, called "brain," including sensory organs and pigment cells (Nishida, 1987). The appearance of neural phenotypes from the a4.2 blastomere, including pigment cells in the brain (Nishida and Satoh, 1989) and neural action potentials, requires early cell interactions (Okado and Takahashi, 1990b). Cell interaction prior to neurulation is required for transcription of the ascidian Na$^+$ channel gene, TuNa I, in the lineage of the a4.2 blastomere (Okamura *et al.*, 1994). In addition, induction of neural action potentials and TuNa I mRNA (Inazawa *et al.*, in preparation) occurs in the a4.2-derived partial embryos following early treatment with basic FGF, one of the potent neural inducers in the amphibian embryo (Kengaku and Okamoto, 1995), sug-

0012-1606/97 $25.00

gesting that the molecular mechanism underlying induction of neural differentiation in the a-line might be similar to that of vertebrate neural induction.

In contrast to the a-line, differentiation of neural cells from b4.2 and A4.1 is poorly understood. Lineage studies have shown that these two lineages contribute to form the posterior part of the neural tube (Nishida, 1987; Nicol and Meinerthzagen, 1988). It is not clear, however, if any neuronal cells are derived from these two lineages.

Previous morphological studies suggest that the caudal neural tube contains only ependymoglial cells and axons, which originate from the neural tissue in the trunk (Katz, 1983). Our recent study showed that several cells in the neck neural tube express voltage-gated Na^+ channel mRNA (Okamura *et al.*, 1994). A4.1 contains cell fates to the posterior neural tube, endoderm, notochord, and muscle, but not epidermis. If A4.1 gives rise to neuronal cells, the mechanism of neuronal differentiation from A4.1 might be different from vertebrate neural induction in which ectodermal cells choose between two fates, neural or epidermal (Slack, 1994).

To gain further insight into the mechanism of neuronal differentiation in the ascidian embryo, we examined the lineages of neck neuronal cells. We show here that motor neurons originate from the A-line which contains cell fates to notochord and muscle, but not to epidermis.

MATERIALS AND METHODS

Animals

Halocynthia roretzi was used in most of experiments. Adults of *H. roretzi* were purchased from fishermen in the northern parts of Japan, Wakkanai and Sanriku, during December to January. Animals were maintained at 4°C in ultraviolet-light-sterilized circulating seawater. Under this condition, animals could spawn eggs and sperm until the middle of May. Spawning of eggs and sperm was induced by keeping the animals at a higher temperature, up to 12°C, under daylight. *Halocynthia aurantium* was also used for experiments of isolated cleavage-arrested blastomeres and electron microscopic observation of microinjected embryos. Adults of *H. aurantium* were obtained from fisherman in Wakkanai, the most northern part of Japan. Eggs and sperm were obtained by dissecting the gonads from adults. The methods used to rear the embryos were the same as those described before (Okamura and Shidara, 1990). Most of the results, including cell lineage, morphology, and ion channel properties, were indistinguishable between the two species, except for the cleavage-arrested A4.1 blastomere; the A4.1 blastomere of *H. aurantium* more often expressed neuronal membrane excitability after differentiation under cleavage arrest.

Lineage Analysis and Electron Microscopy

For lineage tracing, embryos were manually dechorionated with sharp tungsten needles. Denuded embryos were transferred to a 1% agarose bed (HGT agarose; Nacalai Tesque, Inc., Kyoto, Japan) in a chamber circulated with seawater on a microscopy stage. Horseradish peroxidase (HRP) was used as a lineage tracer at the initial stage of the experiments. In many cases, HRP stain was only confined to nuclei, however, and signals in the cytoplasm were too weak to detect fine structures such as neurites. In most cases, therefore, a more sensitive method using dextran–biotin was applied. Dextran–biotin (Molecular Probe, Eugene, OR) was microinjected into each blastomere of the 8- or 16-cell embryo by applying pressure (Okamura and Takahashi, 1993).

The solution used for the injection was 2.5% dextran–biotin, 0.5% dextran tetramethylrhodamine, 0.1× TE buffer, and 0.25 *M* KCl. Dextran tetramethylrhodamine was included so that the amount of the microinjected volume could be determined under fluorescent microscopy (Okamura and Takahashi, 1993). Injected embryos were transferred to filter-sterilized seawater containing diluted gonad extract from *H. aurantium*, 25 μg/ml streptomycin sulfate, and penicillin G on 0.4% agarose. Gonad extract was necessary for denuded embryos to develop into larvae with normal morphology in the normal time course (Nishida, 1987). Embryos were incubated at 8 to 10°C until they reached the stage of the swimming larvae. Approximately 50 to 80% of the microinjected embryos developed into larvae with normal morphology and displayed swimming behavior. Such larvae were selected for fixation in 1% glutaraldehyde and 5 mM Hepes, pH 7.8, for 1 hr to overnight at room temperature. They were then rinsed in phosphate buffer and incubated with avidin–biotin (Vector stain kit, Vector Laboratory) in phosphate buffer solution (PBS), 0.1% Triton X, and 0.2% bovine serum albumin overnight at 4°C. HRP staining was performed by immersing embryos in 0.003% H_2O_2, 0.5 mg/ml diaminobenzidine (DAB), in the presence of 0.1% $NiCl_2$ and 0.1% $CoCl_2$ in 10 m*M* Tris, pH 7.5. The DAB stain was continuously monitored on a dissecting microscope and stopped after 5 to 60 min by rinsing the embryos in PBS.

For observation with Nomarski optics, the stained embryos were dehydrated in methanol and transferred to the clearing solution, which was a mixture of benzylbenzoate/benzylalcohol. For electron microscopy, the larvae were fixed with 1% glutaraldehyde in PBS overnight and postfixed with 1% OsO4. Larvae were dehydrated through a graded series of ethanol and embedded in Epon 812 (TAAB) following a standard protocol.

In Situ Hybridization

Embryos of *H. roretzi* were fixed in 4% formaldehyde in 5× MEM buffer (0.5 M Mops, 10 mM EGTA, 5 mM MgSO4) on ice overnight. They were kept at −20°C in methanol. Under this condition, fixed embryos could be used for *in situ* hybridization for over 1 year. For each experiment, approximately 100 embryos were rinsed in PBS and dechorionated manually with two sharp tungsten needles on ice.

Protocols developed by Harland (1991) were used except that the embryos were permeabilized with proteinase K at a concentration of 4 μg/ml for 15 min at 37°C. pYR1 and pYT1 were used as TuNa I-specific riboprobes (Okamura *et al.*, 1994). Plasmids of pYR1 and pYT1 were linearized with Fsp1 (pYR1) and BglII (pYT1), and then digoxigenin labeled RNA probes were synthesized (DIG RNA labeling kit; Boehringer-Mannheim, Mannheim, Germany). A mixture of riboprobes transcribed from two distinct plasmids was used to increase the sensitivity of detecting TuNa I mRNA.

After staining in 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate, the embryos were dehydrated in methanol and then cleared in a bezylbenzoate/benzylalcohol mixture. They were mounted in a silicone dish and analyzed using Nomarski optics.

For sectioning, stained embryos were dehydrated in ethanol and then embedded in polyester wax (BDH Laboratory Supplies, Poole, England). Serial sections were cut at 5-μm thickness and mounted on slides. After removal of the polyester wax with absolute ethanol, the specimens were stained with 4′,6-diamidine-2′-phenylindole dihydrochloride. They were then mounted in 75% glycerol in PBS and observed with epifluorescence microscopy.

For double staining with an HRP stain of dextran–biotin and TuNa I riboprobe, microinjected embryos were fixed in 4% formaldehyde in 5× MEM buffer and then stained with HRP as described above. They were postfixed in 4% formaldehyde and immediately processed for *in situ* hybridization. The embryos were incubated in NBT and BCIP solution for 48 hr.

Cell Dissociation and Culture

Isolated blastomeres were cultured as previously described (Okamura and Shidara, 1990; Okamura *et al.*, 1994). The fertilized eggs were cultured at 9°C in sea water until the eight-cell stage. The follicular envelope was removed manually with two sharp needles and the denuded embryos were incubated in sea water. Using a fine glass needle, individual blastomeres were isolated, identified by their location in the embryo and their dark color.

For cleavage-arrest experiments, defolliculated embryos were incubated in seawater containing 2.0 μg/ml cytochalasin B (Aldrich). Cytochalasin B was dissolved in DMSO at 2 mg/ml as stock solution and diluted in seawater just before use. Isolated individual blastomeres were incubated in this solution during the period from the 32-cell stage to the neurula stage (Okado and Takahashi, 1993) and then cultured in seawater containing 0.2 μg/ml cytochalasin B and gonad extract, which was prepared as reported by Nishida (1987). Gonad extract enhanced expression of ion channels, but did not affect cell fate.

For the experiments in which partial embryos were cultured in low-Ca^{2+} seawater, eight-cell stage embryos were defolliculated as described above. Embryos were transferred to the low-Ca^{2+} artificial seawater, Ca^{2+}-free Jamarine U (Jamarine Laboratory Co., Osaka, Japan). In this solution, the cell contact of blastomeres within embryos becomes less compact and individual blastomeres could be easily isolated, as previously described by the original method (Jeffery, 1993). A4.1 blastomeres were isolated and cultured in 0.4% agarose holes until control sister embryos reached mid-gastrula stage. Cells were collected and transferred to another well containing filter-sterilized seawater until control embryos became young tadpole larvae.

Electrophysiology

Ion channel currents of cleavage-arrested blastomeres were characterized using the two-electrode voltage clamp technique as previously described (Okamoto *et al.*, 1976; Okamura and Shidara, 1990). Most of recordings were done in the artificial seawater. In a few experiments, recording solutions were Na^+-rich solution (m*M*): Na^+, 500; tetraethylammonium (TEA^+), 100; Mn^{2+}, 6; Pipes, 5, pH 7.0; and Na^+-free solution (m*M*): tetramethylammonium (TMA^+), 500; TEA^+, 100; Mn^{2+}, 6; Pipes, 5. Recording was performed at 9 ± 1°C.

RT-PCR Analysis

Cultured partial embryos or cleavage-arrested blastomeres were rinsed once in seawater and then transferred to microtubes containing guanidine–isothiocyanate solution. After incubation at room temperature for 2 hr to overnight, RNA was extracted using the acid guanidium–phenol chloroform method (Chomczynski and Sacchi, 1987). RT-PCR was performed as previously described (Okamura *et al.*, 1994). In brief, complementary DNA was reverse-transcribed by Superscript II (Gibco BRL) from half of the harvested RNA with 50 ng of oligo(dT) in a volume of 16 μl. The cDNA solution (0.8 μl) was amplified with primers labeled with [γ-^{32}P]-ATP in a total volume of 8 μl, according to the following protocol: 28 cycles of 94°C for 55 s, 54°C for 70 s, and 72°C for 60 s. For amplification from cDNAs of both *H. roretzi* and *H. aurantium*, TuNa I-specific PCR primers were NaI2 (TGTGGATTCATGGCATATGG) and NaI4c (CGTCTTTCAGTGCTTTGACAG). Cytoskeletal actin primers were Cact3 (ACAACGAACTTCGTGTAGCC)/Cact4c (TTCTTCCGGATGCATAGAGG) for *H. roretzi* and Cact6 (ACAACGAACTTCGTGTAGCC)/Cact7c (CCATCACCGGAGTCCATAAC) for *H. aurantium*. These primers can discriminate from muscle actin genes of *Halocynthia* (Y. Okamura, unpublished). Primers for an ascidian homologue of the T-gene, AST1, were ACGACAGATGAAGTTCCAACC and AACCGTCGTAGCTAAGTGGC, based on the sequence published by Yasuo *et al.* (1992). Amplifications were performed with the number of cycles yielding a log-phase increase of product, which was tested by pilot experiments for each set of primers. One fifth of the PCR products were separated on a 6% polyacrylamide gel and analyzed with a phosphoimager.

Terminology of Ascidian Nervous System

The ‘‘neck neural tube’’ in this paper indicates the tubular neural structure of the proximal tail adjacent to the border between the trunk and the tail. Recent advances in molecular cloning of ascidian counterparts of region-specific gene markers have enabled us to compare the neural tissue of the ascidian larva with that of the vertebrate (Satoh *et al.*, 1996). Comparisons of labial-related Hox genes and otx-related genes between the vertebrate and the ascidian (Katsuyama *et al.*, 1996) suggest that the most anterior neural tissue of the ascidian might correspond to the vertebrate forebrain and that the neural tube at the border between the trunk and the tail corresponds to the most anterior part of the spinal cord in vertebrates. The term ‘‘neck’’ in our paper is based on this finding.

The term ‘‘spinal cord’’ has been used to denote the caudal part of the neural tube of the ascidian larva (Nishida, 1987). As described in the present paper and others (Katz, 1983), however, the caudal part of the neural tube is only composed of ependymo-glial cells and descending motor axons, which probably originate from neck TuNa I positive neurons. Thus, the neural tube in the tail was called the ‘‘caudal neural tube’’ instead of the spinal cord in the present paper. Designations of blastomeres are based on the descriptions by Conklin (1905).

RESULTS

Both a4.2 and A4.1 Give Rise to TuNa I-Positive Neuronal Cells

TuNa I-positive cells were distributed in a wide region of the larval nervous system of *H. roretzi* (Fig. 1A), as has been shown for *H. aurantium* (Okamura *et al.*, 1994). Signals were found in the three distinct regions of the larval nervous system: (1) anterior neural tissue surrounding the pigment cells of the sensory organs, (2) neck neural tube at the border between the trunk and the tail (arrow in Fig. 1A), and (3) neurons in the epidermis of the tail and dorsal part of in the trunk. Region (1) is composed of numerous, but weak, positive signals at the anterior end of the neural tissue and, in the more posterior part, two intense bilateral signals at the ventral side and several small signals at the dorsal side. Signals in region (3) probably correspond to the epidermal neurons, identified by morphological experiments (Torrence and Cloney, 1982) in another species of ascidian. A detailed description of the location of TuNa I-positive cells in Regions (1) and (3) appear elsewhere. Region (2) contains two or three positive cells on both sides in the neck neural tube. This region appears close to the region called the ''visceral ganglion'' in other ascidians (Katz, 1983; Nicol and Meinertzhagen, 1991). TuNa I signal could not be detected in the more caudal part of the neural tube, consistent with the morphological finding, in other species of ascidians that the caudal neural tube does not contain neuronal cells (Katz, 1983).

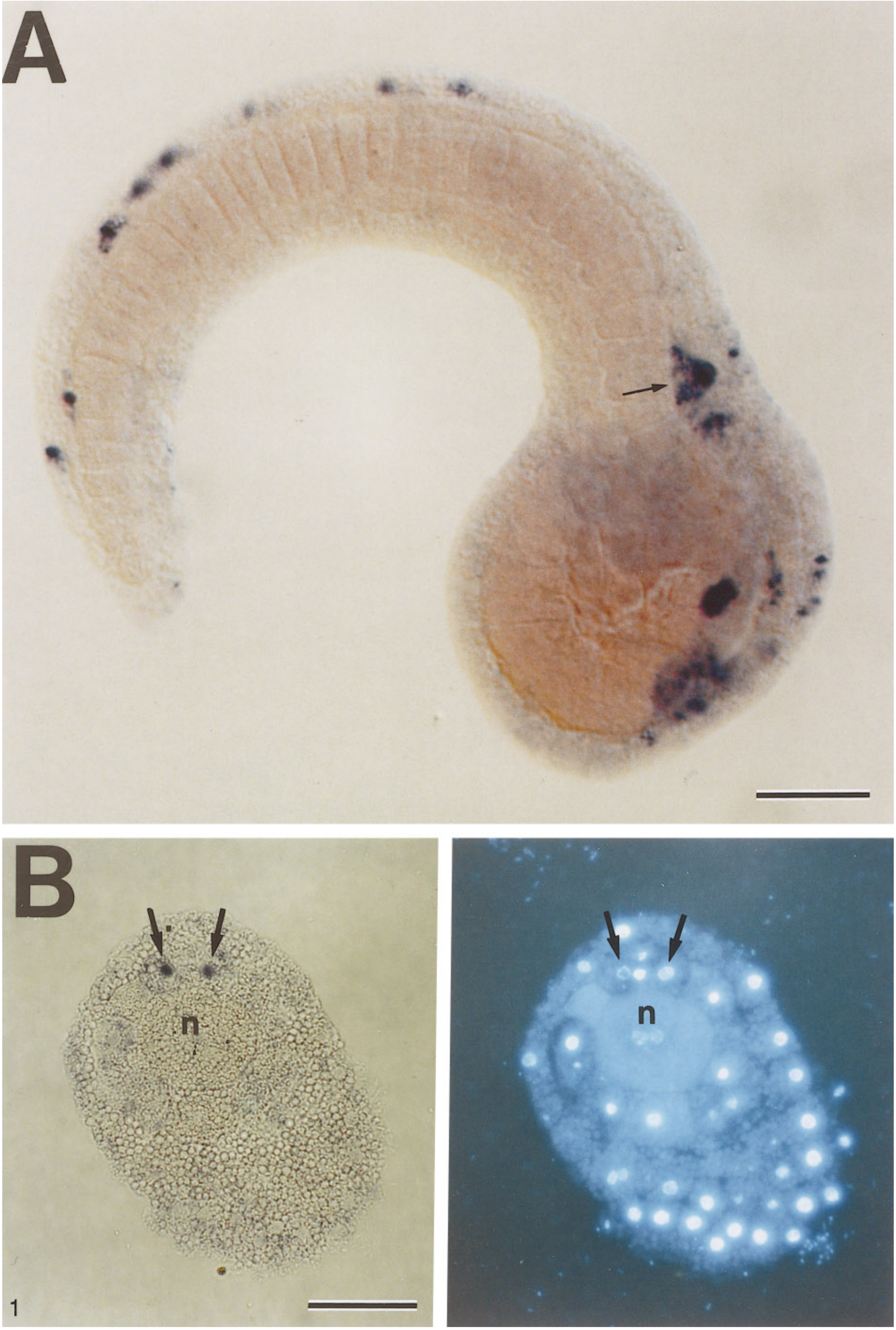
To confirm the exact location of the TuNa I-positive cells in the neck neural tube, sections were made from stained embryos (Fig. 1B). Double staining with DAPI revealed that the TuNa I-positive cells are two lateral cells out of four cells which constitute of the neural tube (Fig. 1B, right). Lineage studies (Nishida, 1986; Nicol and Meinertzhagen, 1988) indicate that the neck neural tube is not derived from the a4.2 blastomere, that the dorsal midline cells are derived from b4.2, and that the other three cells are derived from A4.1. Thus, the TuNa I-positive cells at the neck neural tube are most likely derived from the A4.1 blastomere.

To see if any neuronal cells are derived from the A-line, the lineage tracer molecule, dextran–biotin, was microinjected into the A-line blastomeres of either the 8-cell or the 16-cell embryo (Fig. 2A) and cultured until the injected embryos developed into swimming larvae. When the A4.1 or A5.2 blastomere was microinjected, the neural tube extending from the trunk to the tail, muscle cells, notochord, endoderm, and trunk lateral cells were stained (Fig. 2C). Closer examination with increased sensitivity in Ni^{2+} and Co^{2+} in the HRP staining revealed a neurite-like structure which extends from the neural tube onto the muscle cells (Figs. 2C and 2D, arrowheads), suggesting that the A-line gives rise to motor neurons. This structure was observed independent of whether the left or right cell was microinjected, suggesting that both blastomeres equally contribute in the formation of these neurons. In addition, the neurite-like structure on the ventral muscle cell was found only when an A5.2 blastomere was microinjected. When an a4.2 blastomere was microinjected, only anterior neural tissue surrounding pigment cells and the trunk epidermis were stained (Fig. 2B), consistent with a previous study (Nishida, 1987). In these embryos, no neurite-like structure in the tail neural tube or on muscle cells was observed, suggesting that motor neurons are not derived from the a-line. When b4.2 was microinjected, several cells in the neck neural tube were stained in accordance with a previous study (Nishida, 1987), but no fine neurite-like structure was observed (data not shown). When A5.1 was microinjected, part of the neural tube was stained, but no neurite-like structure could be detected (data not shown). We cannot ignore the possibility, however, that a neuronal cell, derived from an A5.1 blastomere, forms a synapse on the adjacent dorsal muscle, which might not be observed at the light microscopic level.

To prove that A5.2 cells give rise to motor neurons, electron microscopic observation was performed on the larva in which an A5.2 cell was microinjected with dextran-biotin (Figs. 3A and 3B). A cross-section of a microinjected tadpole larva at the proximal tail revealed HRP signal in axon-like structures of the neural tube (Fig. 3B). Closer examination of the uninjected larva with a more preserved ultrastructure (Fig. 3D) confirmed that these structures are motor axons, because synapses were formed on dorsal muscle cells. This is consistent with previous reports; axons make synapses with the dorsal muscle cells in the tail in other species of ascidians (Katz, 1983; Torrence, 1983; Tannenbaum and Rosebluth, 1972), and acetylcholine-sensitive spots are distributed along the tail (Ohmori and Sasaki, 1977). The same section had a crescent-like structure (indicated by arrow in Fig. 3A) between the notochord (n) and endodermal strand (es), which corresponds to the neurite-like structure observed under light microscopy (Fig. 2D, arrowheads). The crescent-like structure is a part of a motor neuron, because a synapse on ventral muscle cells was observed in an electromicrograph of the corresponding region in an uninjected larva (Fig. 3C). The finding that A5.2 gives rise to motor neurons strongly suggests that TuNa I-positive cells in the neck neural tube are motor neurons.

To confirm that TuNa I-positive cells in the neck neural tube are derived from A5.2, *in situ* hybridization was performed on the embryos in which dextran–biotin was microinjected into A5.2. The a4.2 cells of other embryos were also microinjected as controls. When a4.2 cells were microinjected, TuNa I staining coincided with a HRP-positive neuron in the sensory vesicle (arrowhead in Fig. 4A) and a putative epidermal neuron in the trunk (large arrow in Fig. 4B), but not neck TuNa I-positive cells. On the other hand, when A5.2 was microinjected, TuNa I stain coincided with the HRP-positive cells in the neck (arrow in Fig. 4C). We concluded that the A-line gives rise to TuNa I-positive neurons in the neck neural tube, and that these neurons are probably motor neurons.

FIG. 1. Whole-mount *in situ* hybridization of *H. roretzi* young tadpole larva with a TuNa I-specific probe. (A) Lateral view of a whole-mount *in situ* hybridization of *H. roretzi* young tadpole larva with a TuNa I-specific riboprobe. Arrow indicates TuNa I-positive cells in the neck neural tube. (B, left) Another larva was perpendicularly cut at the level of the neck region, i.e., the proximal end of the caudal portion, as indicated by the arrow in Fig. 1A. Two of four neural tube cells at this level are TuNa I positive (arrows). The cells are symmetrically located abutting on the notochord (n). (B, right) Nuclear staining of the same section as in the left picture. Bar, 50 μm.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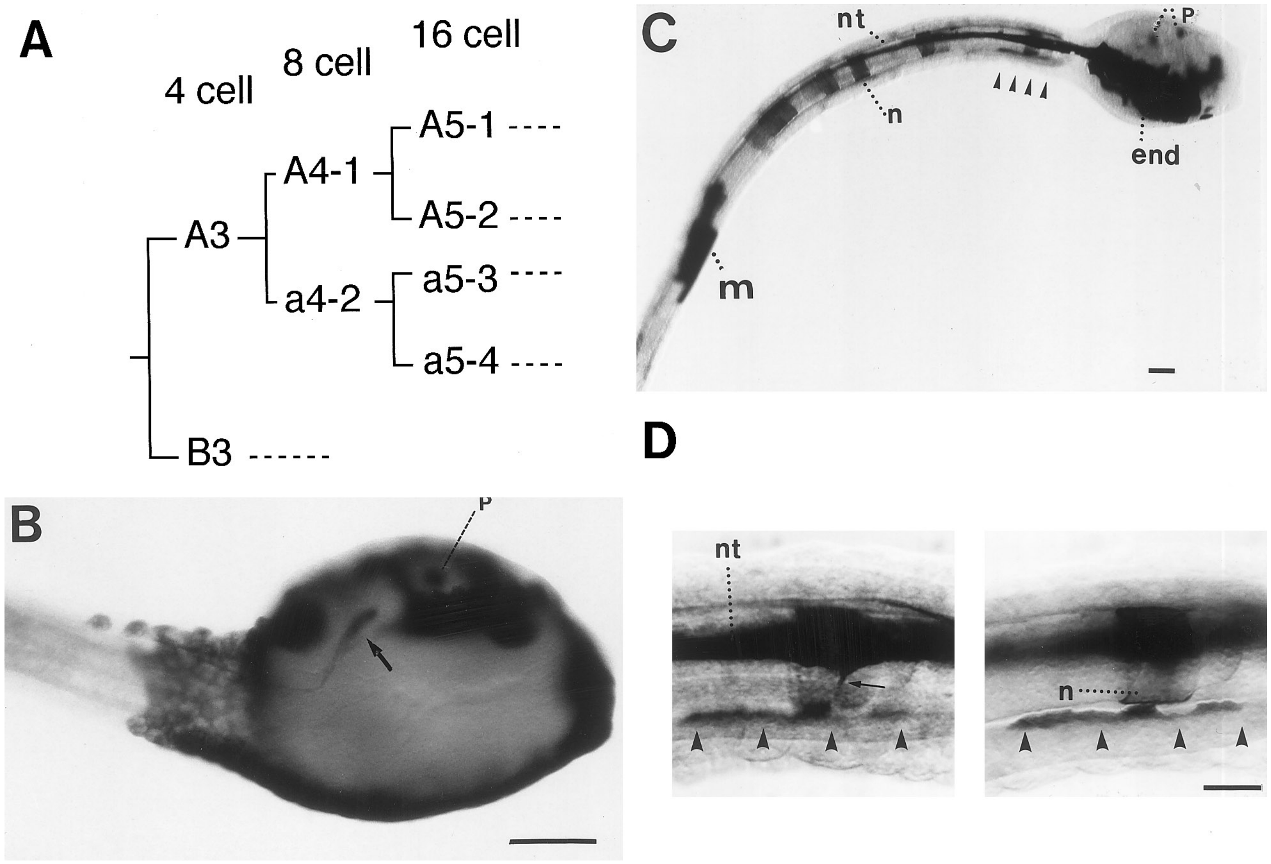


FIG. 2. Lineage tracings of the a-line and A-line cells. (A) Summary of the cell-cleavage pattern from the 4-cell stage to the 16-cell stage following descriptions by Conklin (1905). (B) A lateral view of the trunk part of a *Halocynthia* embryo in which an a4.2 blastomere was microinjected with dextran–biotin at the 8-cell stage. The arrow indicates a neuron posterior to the sensory pigment cell (P). Bar, 50 μm. (C) An example of the top view of a *Halocynthia* embryo in which A5.2 was microinjected with dextran–biotin at the 16-cell stage. Arrowheads denote a putative axon terminal of a motor neuron. Bar, 40 μm. (D) Magnified view of the putative axon terminal of the same embryo shown in C. Two pictures with different focus are shown. A branch from the neural tube, indicated by the arrow, is continuous with a structure (arrowheads) on a ventral muscle cell. Bar, 40 μm. m, muscle; nt, neural tube; n, notochord; and e, endoderm.

Expression of TuNa I in the A-line Requires Signals from the A-Line Cells, whereas Expression in the A-Line Does Not Require Other Lineage Cells

Partial embryos in which A4.1 pairs were deleted from eight-cell embryos do not transcribe the TuNa I gene (Okamura *et al.*, 1994), indicating that TuNa I-positive neurons derived from a4.2 and b4.2 blastomeres require signals from A-derived cells for differentiation. To determine if the A-derived neuronal cells require cell interactions from other lineage cells, TuNa I transcription was examined from the A-derived partial embryos. The a4.2 blastomere and the A4.1 blastomere were isolated in filter-sterilized seawater and cultured on an agarose bed. The a-derived partial embryos always differentiated into a ball composed of epidermal cells, which was surrounded by the tunic, an epidermis-specific extracellular substance (arrowheads in Fig. 5, left). RT-PCR from the a-derived partial embryos revealed that TuNa I RNA was not transcribed (Fig. 5, a4.2), in accordance with a previous study (Okamura *et al.*, 1994).

When A4.1 was isolated from the eight-cell embryos and was allowed to develop until the hatching larval stage, it often developed into the typical elongated shape without the tunic, suggesting that epidermal cells were not produced (Fig. 5, right). The A4.1-derived partial embryo always contained a few large clear cells with vacuoles, characteristic of notochord cells, consistent with other work (Nakatani and Nishida, 1994). RT-PCR with RNA isolated from five A4.1-derived partial embryos indicated that TuNaI gene transcription occurred in these embryos (Fig. 5). No TuNa I signal was obtained in the reaction in which only the reverse-transcriptase was omitted (data not shown). Another neuronal marker gene, TuNa II (Okamura *et al.*, in preparation), was also detected in those samples (data not shown). These findings indicate that neuronal differentiation in the A-line does not require signals from other blastomeres and this contrasts with neuronal differentiation in the a-line which requires cell interactions with other lineages (Okamura *et al.,* 1993).

Isolated and Cleavage-Arrested A4.1 Blastomeres Have Neuronal Excitability Distinct from That of Neurally Differentiated a4.2 Blastomeres

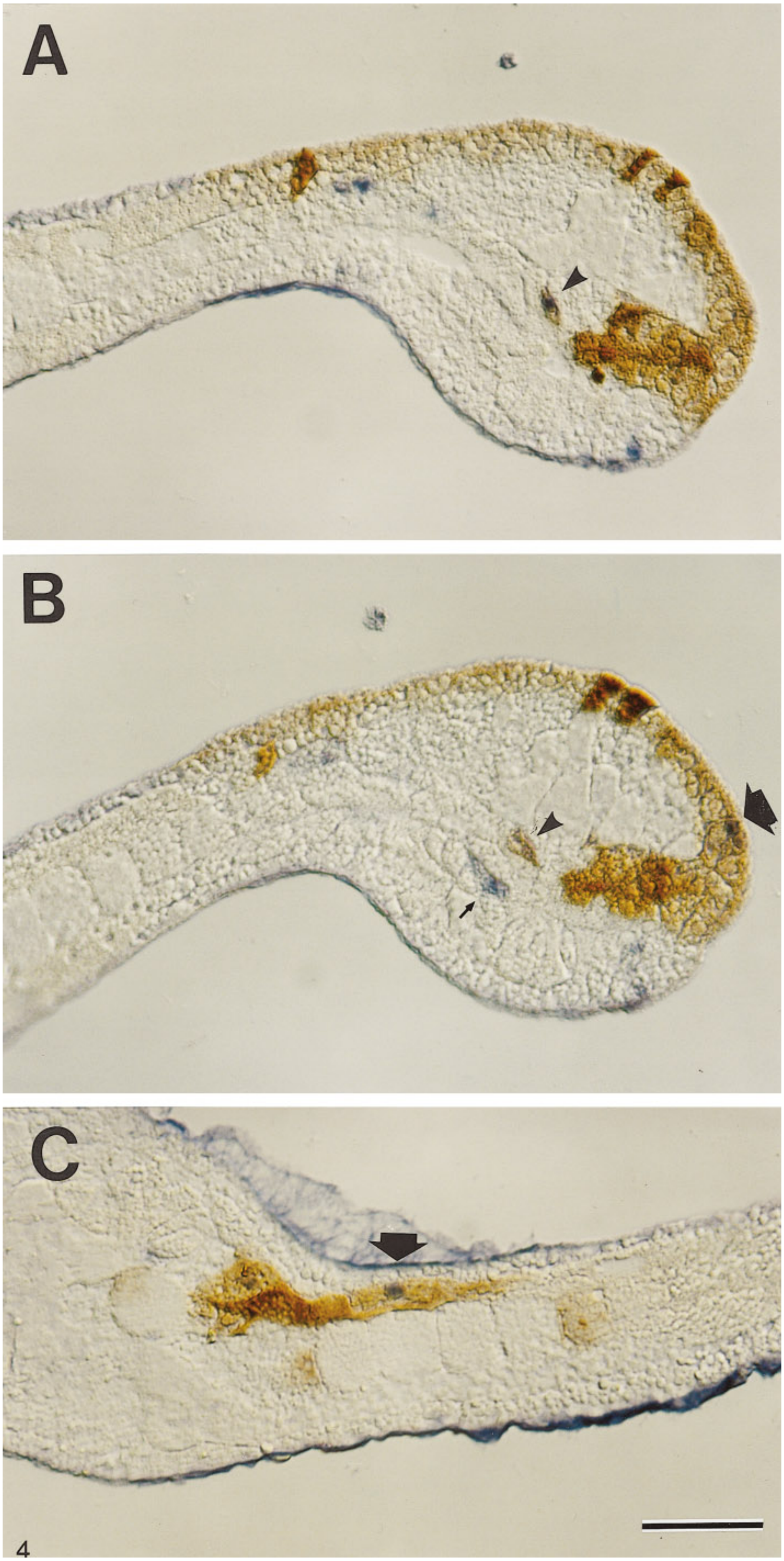
To test if the A-line cells have the potency of differentiating into excitable cells, A4.1 blastomeres were isolated and cultured under cleavage arrest, followed by electrophysiological analysis (scheme shown in Fig. 7A). Cleavage-arrested A4.1 blastomeres showed two types of electrical properties. Most blastomeres differentiated into a nonexcitable type in which no voltage-gated ion channel could be detected (12 of 17). In a few cases, however, active membrane excitability, due to inward and transient outward currents (5 of 17) in low-Ca^{2+} solution were observed (Fig. 6A). By changing the main external cation from Na^+ to TMA^+, the inward current was diminished, indicating that the inward current was due to voltage-gated Na^+ channels (Fig. 6D). The outward current was due to a class of K^+ channels with fast-decaying kinetics and resistance to TEA^+. This class of K^+ current is probably identical to the A-channel-like K^+ current, which was previously reported in cleavage-arrested differentiated A3 and A4.1 blastomeres (Okado and Takahashi, 1990a). In normal seawater, a slow inward current due to Ca^{2+} channels was also found in the neuronal type differentiation of the A4.1 blastomere. The pattern of ion channel expression in the A4.1 blastomere is similar to that of a previous report in which inward Ca^{2+} currents and transient outward currents are expressed (Okado and Takahashi, 1990a), although Na^+ currents were not carefully studied in that paper. Coexpression of the Na^+ current, the Ca^{2+} current, and the outward K^+ current suggests that A4.1 differentiated into a neural-type cell, because ascidian muscle cells do not express Na^+ channels (Miyazaki *et al.,* 1972). Different properties of K^+ channels expressed in cleavage-arrested a4.2 and A4.1 blastomeres suggest that A4.1 cells differentiated into a type of neuronal cell distinct from the a4.2-derived cells. A transient-type K^+ channel current could not be evoked by depolarizing the neurally differentiated a4.2 blastomere even when preceded by hyperpolarization to -120 mV (data not shown). The transient outward current of the A4.1 blastomere was only partially suppressed by 50 m*M* TEA^+, whereas the outward current, a class of a delayed rectifier K^+ channel (Shidara and Okamura, 1991), is almost completely suppressed in a4.2-derived neurons.

To test if the TuNa I gene encoding a voltage-gated Na^+ channel is induced in the cleavage-arrested A4.1 blastomeres, RT-PCR analysis for TuNa I was performed on those blastomeres. Ten A4.1 blastomeres from *H. aurantium* eight-cell-stage embryos were cultured under cleavage-arrest conditions in parallel with a4.2 blastomeres neurally induced by early treatment with protease, subtilisin, or uninduced a4.2 blastomeres. In this experiment, *H. aurantium* was preferentially used because neuronal excitability in cleavage-arrested A4.1 blastomeres was more frequently observed than in *H. roretzi.* Cytoplasmic actin was detected in all of the neurally induced or uninduced a4.2 blastomeres and A4.1 blastomeres. Reduction of cytoplasmic actin expression is seen in isolated neurally induced a4.2 cells, due to down-regulation by neural induction in the a-lineage (Y. Okamura, unpublished data). TuNa I expression was not observed in uninduced a4.2 blastomeres, which differentiate into epidermal cells (Fig. 7Bi). By contrast, the TuNa I signal was observed in both neurally induced a4.2 blastomeres (Fig. 7Biii) and isolated A4.1 blastomeres (Fig. 7Bii), verifying the neuronal differentiation of the A4.1 blastomere at the gene expression level.

Induction of TuNa I in a4.2 by Cell Contact to A4.1 Does Not Correlate with Neuronal Differentiation of A4.1

Previous work has shown that the a4.2 blastomere can be induced to express neuronal membrane excitability and TuNa I mRNA following cell contact to the A4.1 blastomere (Okado and Takahashi, 1990b; Okamura *et al.,* 1994). The finding that A4.1 can differentiate into neuronal cells by itself under cleavage-arrest conditions raises the possibility that induction of TuNa I in the a4.2 blastomere is mediated by homogenetic induction (Slack, 1994). To test this idea, we performed *in situ* hybridization of TuNa I for cleavage-arrested blastomeres in which a4.2 and A4.1 cells were cultured in contact to each other. The TuNa I signal was always detected in the a4.2 blastomere (8 of 8), whereas the TuNa I signal in A4.1 was detected only in some cases (2 of 8). The neuronal differentiation of a4.2 was independent of whether the A4.1 blastomere expresses TuNa I (Fig. 8). Thus, the neural inducing ability in the A4.1 blastomere does not require neuronal specification of A4.1 itself, and

FIG. 4. Double staining of TuNa I *in situ* hybridization and a lineage tracer. Nomarski differential images of sections of young tadpole larvae in which an a4.2 blastomere (A, B) and an A5.2 blastomere (C) were microinjected with dextran–biotin. (A) Arrow indicates an a-derived neuron positive for both TuNa I RNA signal (blue with alkaline phosphatase staining) and the lineage tracer (brown with HRP staining). (B) Another section of the same embryo which is approximately 5 μm apart from the section in (A). Note that a trunk epidermal neuron (large arrow) is also derived from the a-line cell. The same neuron as indicated in A is seen in this section (arrowhead). Another TuNa I-positive neuron on the other side is derived from the other a-blastomere (small arrow). (C) The arrow indicates a TuNa I-positive neuron in the neck neural tube. Bar, 50 μm.



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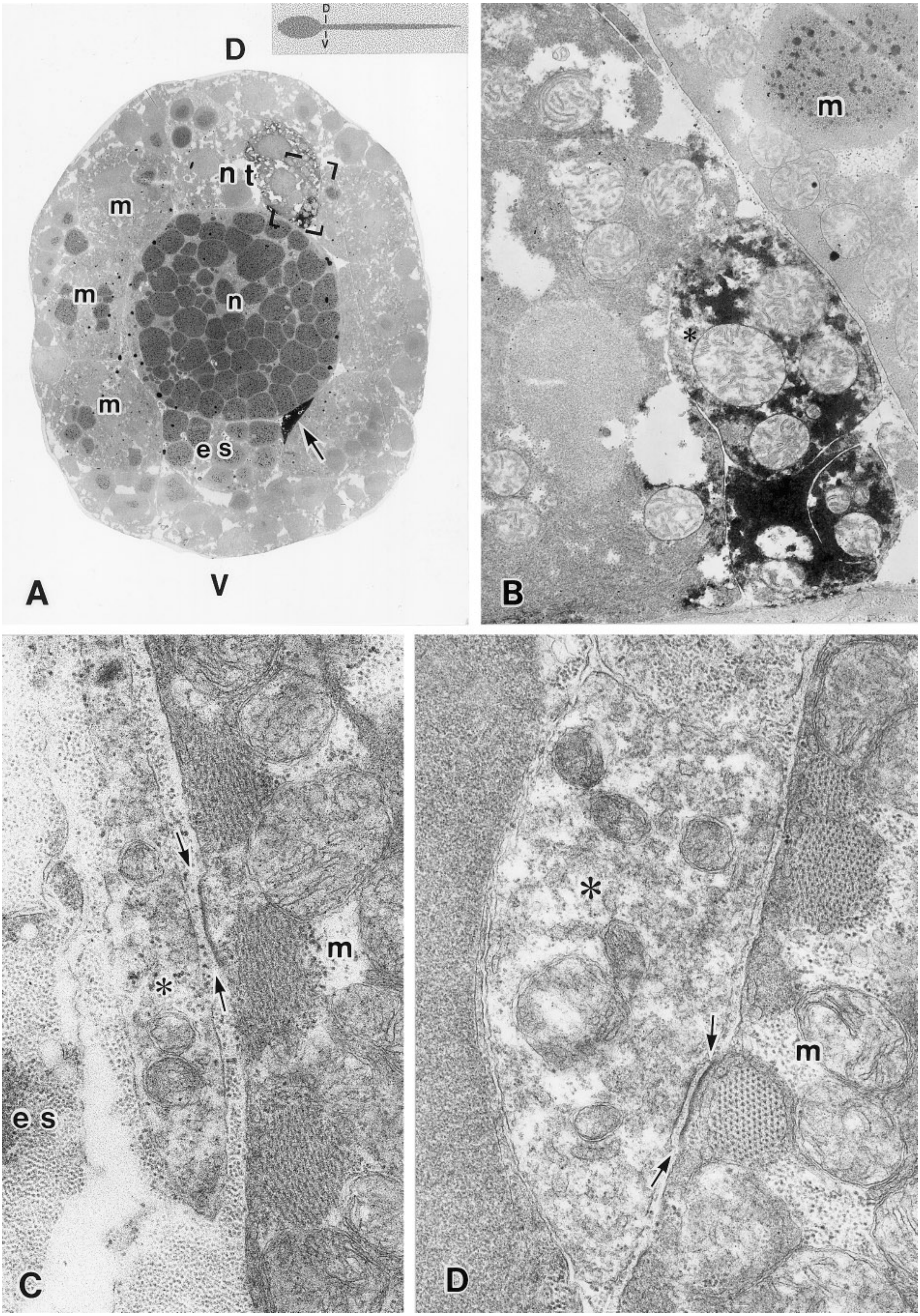
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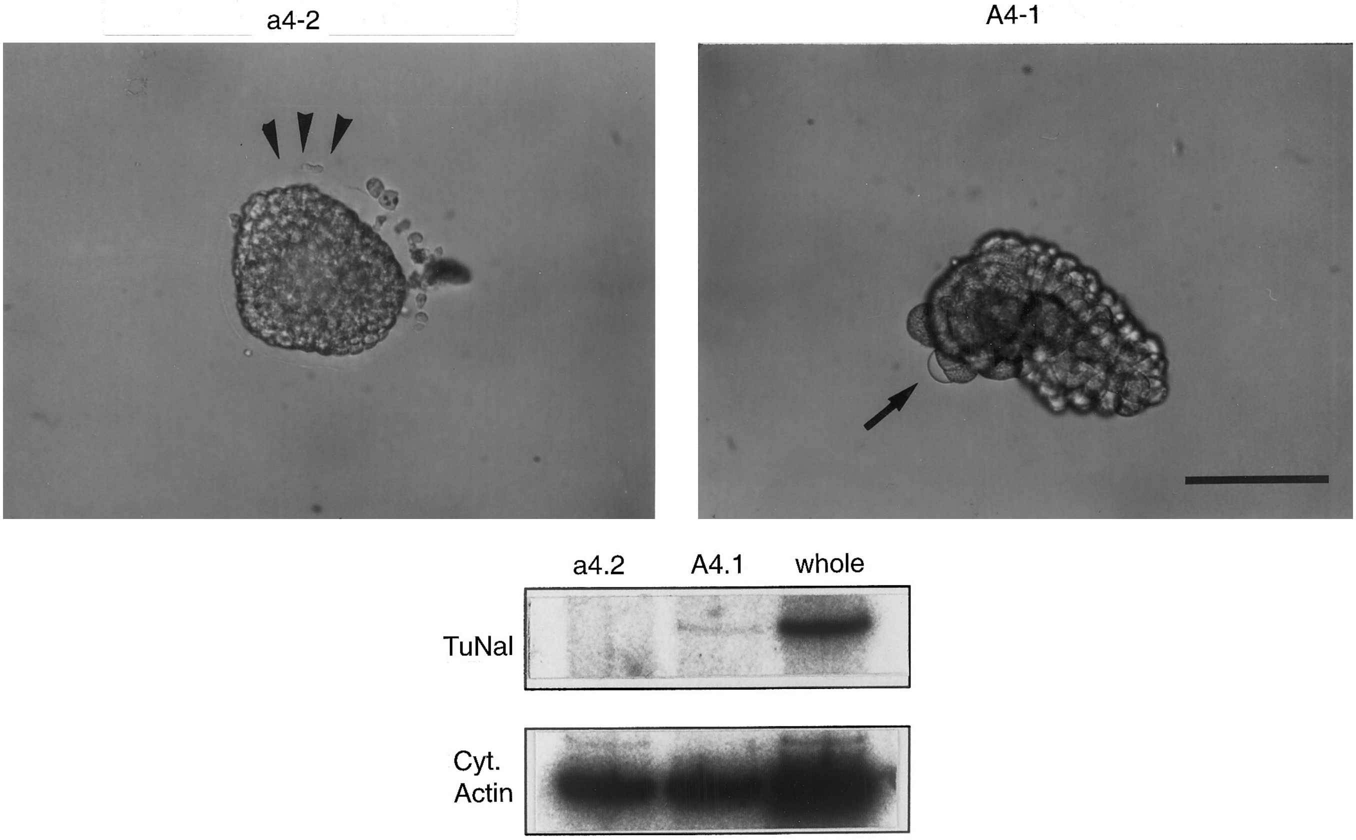

FIG. 5. Test of TuNa I gene expression in partial embryos derived from the a- and the A-line blastomeres. Pictures of partial embryos derived from a4.2 and A4.1 are shown at the top. Arrowheads indicate the tunic, the extracellular substance of epidermal cells in the a-derived partial embryo. The arrow indicates a vacuole, a characteristic feature of the notochord cell in the A-derived partial embryo. RT-PCR results of TuNa I and cytoplasmic actin from eight partial embryos for each lineage are shown below. ''Whole'' indicates the result from control embryos which were cultured as a whole embryo after defolliculation at the eight-cell stage. Bar, 50 μm.

induction of neuronal differentiation from a4.2 by A4.1 is not mediated by a mechanism of homogenetic induction.

Expression of TuNa I in the A-Line Does Not Change When Cell Contact Is Decreased before Gastrulation

The A4.1 blastomere includes cell fates of notochord, endoderm, trunk lateral cells, and muscle cells, in addition to caudal and neck neural tube tissue (Nishida, 1987). It has been shown that notochord differentiation from the A4.1 blastomere requires cell interactions within the A-line blastomeres occurring at the critical period between the 32- and 64-cell stage (Nakatani and Nishida, 1994). This critical cell interaction occurs just before the cell division in which the two lineages of notochord and neural tube are separated from each other, thus raising the possibility that the early cell interactions within the A-

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FIG. 3. Electron microscopic pictures taken from *H. aurantium* tadpole larva. es, endodermal strand cell; m, muscle cell; nt, neural tube. (A) Low-power view (×1350) of a cross-section cut at the level of the neck region as indicated by an arrow in the schematic drawing (top). An A5.2 blastomere was microinjected with dextran–biotin at the 16-cell stage and stained for HRP using the avidin–biotin complex method. Arrow indicates an HRP-stained axon making a crescent appearance in profile. Dorsal side is up. (B) High-power view (×15,000) of the region indicated by rectangle in A. Descending axons are positive for the injected HRP (asterisk), which diffusely deposits the cytoplasm except for mitochondria. (C and D) Putative descending axon terminals (asterisk) from uninjected tadpole larva sectioned at the level of the neck region as shown in A. Axon terminal in C corresponds to the region indicated by the stained crescent (arrow) in A. D corresponds to the region marked by the rectangle in A. These axons are in contact with the ventral (C) or dorsal (D) muscle cell. Magnification, ×33,000. Membrane thickening at the putative synapse sites is indicated by arrows. Synaptic vesicles and mitochondria are abundantly distributed.

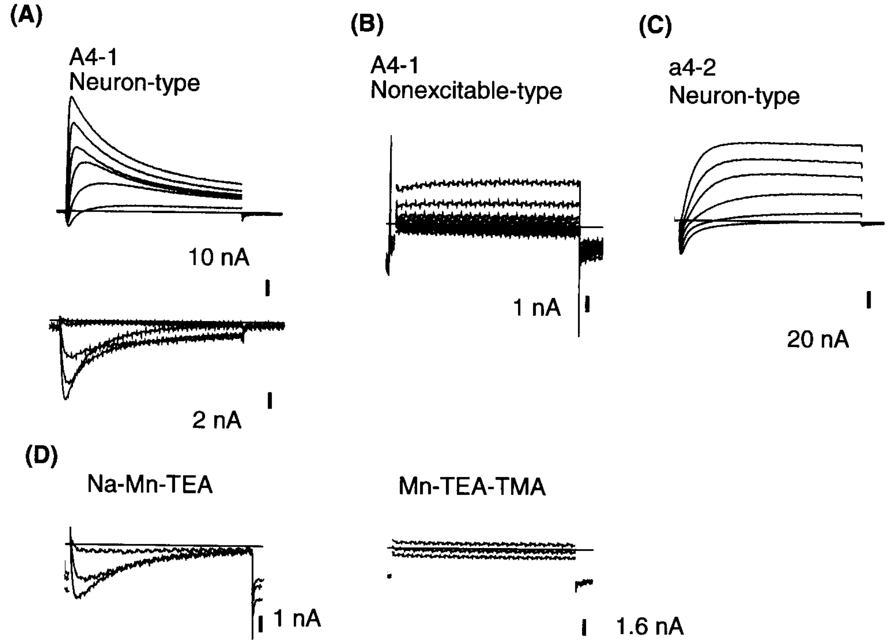

FIG. 6. Electrical properties of the a-line and the A-line cells under cleavage arrest suggest two lineages give rise to distinct types of neuronal cell. (A) An example of current traces from neuron-type cells from cleavage-arrested A4.1 blastomere. Voltage steps (mV) were to −17, −7, 4, 14, 24, and 44 in upper traces and −58, −48, −38, −33, and −28 in lower traces. Test pulses were preceded by hyperpolarization to −125 mV for 3 sec to remove slow inactivation. (B) An example of current traces from nonexcitable type from cleavage-arrested A4.1 blastomere, Voltage steps (mV) were to −58, −48, −38, −28, −18, −8, 13, and 43. (C) Neuron-type cells from cleavage-arrested a4.2 blastomere cultured in contact with A4.1 blastomere. Voltage steps (mV) were to −25, −14, −3, −7, and −18. Current traces of (A), (B), and (C) were recorded in the artificial seawater. (D) Current traces recorded from an A4.1 blastomere in the Na^+-rich solution (left) and in the solution where Na^+ was replaced by the organic cation, TMA^+ (right). Mn2+ was contained in the solution in order to inhibit voltage-gated Ca^{2+} channel currents. Voltage steps (mV) were to −28, −38, and −18. Holding potentials (mV) are −79 for all traces.

line blastomeres are also required for differentiation of neural tissue in the A-line.

To test this possibility, the A4.1 blastomere was isolated and cultured in low-Ca^{2+} seawater to prevent tight cell contact, until normal sister embryos reached the mid-gastrula stage. Partial embryos were returned to normal seawater until they developed to the stage of young tadpole larvae. Most of such treated partial embryos develop into a mass of cells in which two separate regions could be observed; one was the aggregate of round large cells and the other was that of small cells (Fig. 9A). The large cells with clear cytoplasm, the typical morphology of notochord cells, were detected in only 1 case of the 8 partial embryos. On the other hand, large cells with clear cytoplasm were found in all control partial embryos ($n = 10$). Inhibition of cell contact was analyzed by quantifying mRNA levels of the ascidian brachyury gene, AST1 (Yasuo and Satoh, 1994), expression of which is correlated with notochord induction occurring at the early phase between 32- to 64-cell stages (Nakatani *et al.*, 1996). The quantification of gene expression using the RT-PCR method revealed that AST1 mRNA expression significantly decreased in the partial embryos cultured in low-Ca^{2+} seawater compared with the control partial embryos (17%, as the ratio against cytoplasmic actin quantified by the phosphoimager). This indicates that cell contact was decreased in the partial embryos, although not enough to completely abolish notochord formation. Residual notochord induction in low-Ca^{2+} seawater might have been caused by the incomplete dissociation under these conditions because we avoided the use of a protease for dissociation treatment, which has been shown to induce neuronal phenotypes in the ascidian embryo (Okado and Takahashi, 1990). In contrast with the decrease in the AST expression, the amount of TuNa I transcript in the low-Ca^{2+} treated partial embryos was not reduced, compared with that in seawater (117%, as the ratio against cytoplasmic actin). This suggests that TuNa I expression was not reduced by decreasing cell contact. Another neuronal marker, TuNa II (Y. Okamura, unpublished data), was also equally detected between two samples. In another experiment, the whole embryo was cultured in Ca^{2+}-free seawater between the 16-cell stage and the late gastrula stage. The TuNa I mRNA level decreased by 30% (data not shown), consistent with the notion that a-derived TuNa I-positive neurons require tight cell contact over a critical time period before neurulation (Okamura *et al.*, 1994). These data indicate that neural differentiation in the A-line does not occur through the same events as for notochord induction and

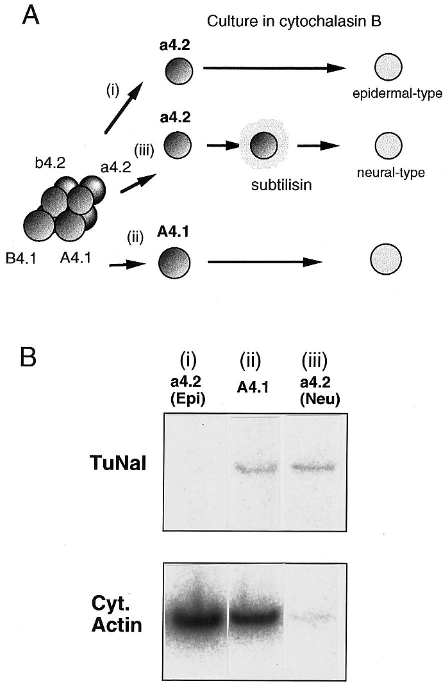

FIG. 7. RT-PCR analysis of isolated blastomeres under cleavage arrest. (A) Scheme of the experiment with cleavage-arrested blastomeres: an isolated a4.2 blastomere (i), an isolated A4.1 blastomere (ii), an isolated a4.2 blastomere neurally induced by transient treatment with a protease, subtilisin (iii). (B) Results of RT-PCR with TuNa I primers and cytoplasmic actin primers. RNA was isolated from 10 cleavage-arrested blastomeres which were cultured until control sister embryos hatched for each preparation. Fragments with size of 353 bp for TuNa I and 212 bp for actin were analyzed on polyacrylamide gel.

also suggests that neural differentiation in the A-line does not strongly depend on tight cell contact before neurulation. This finding is in contrast with the neuronal differentiation from the a-line which does require tight cell contact (Okado and Takahashi, 1990).

DISCUSSION

Utilization of the neuronal Na$^+$ channel gene, TuNa I, as a molecular marker enabled the identification of neurons in the proximal part of the caudal neural tube. Lineage tracing experiments showed that these neurons, including motor neurons, are derived from the blastomere, A4.1, which does not contain cell fates to epidermis, but contains cell fates to become notochord and muscle. This finding will provide the basis for understanding the molecular mechanisms of early neuronal differentiation in the ascidian embryo.

Identification of Neuronal Cells in A-Derived Neural Tube

In situ hybridization of tracer-microinjected embryos showed that neuronal cells in the neck neural tube are derived from the A5.2 blastomere. The presence of synaptic terminals on the ventral muscle cells suggest that neck neuronal cells include motor neurons. Serial sectioning of TuNa I-stained embryos indicate that there are two or three neck neuronal cells on each side of the neural tube (T. Okada, unpublished observation). The ascidian neural tube is composed only of four cells at a cross-section, as has been reported for other ascidians (Katz, 1983). All TuNa I-positive neurons observed at the neck region are bilateral pairs. The lateral position of the neurons at the neck neural tube is similar to that of the vertebrate motoneurons, which are located ventrolaterally.

Electrophysiological study of *H. roretzi* revealed that there are areas with many acetylcholine receptors along the muscle cells (Sasaki and Ohmori, 1977). One of the areas that had intense acetylcholine responses (Sasaki and Ohmori, 1977) is close to the site that was shown by microinjection of dextran–biotin, approximately 50 to 100 μm downstream from the border of the trunk and tail (Fig. 2D). This supports the notion that the observed structure on the muscle is the motor nerve ending. The neurite on the ventral muscle cell might be identical to the axonal structure, which was reported to be between the endoderm and the ventral muscle in *Dendrodoa*, another species of ascidians (Katz, 1983).

Previous electrophysiological studies did not demonstrate Na$^+$ currents in the isolated A4.1 blastomere after culture under cleavage-arrest conditions (Okado and Takahashi, 1990a). This discrepancy might be due to the difference in the culture conditions between the two experiments. In the present work, extract of gonads from *H. aurantium* was maintained in the culture solution, because this extract was a prerequisite to raise denuded embryos with normal morphology and enhance maturation of differentiation such as epidermis-specific tunic or ion channel expression (see Materials and Methods; Nishida, 1986). The excitability type composed of a transient outward current plus a Na$^+$ current found in cleavage-arrested A4.1 blastomeres does not represent notochord or endoderm differentiation because cleavage-arrested precursors of the notochord and endoderm isolated from A-line cells of the 32-cell embryo did not show this type of excitability (data not shown) and intracellular recording from notochord cells of a tadpole larva shows that the notochord is electrically nonexcitable (Y. Okamura, unpublished observation).

Two Distinct Neuronal Lineages in the Ascidian

Microinjection of a lineage tracer, *in situ* hybridization with TuNa I gene, and electrical recording from isolated, cleavage-arrested blastomeres showed that distinct neural lineages give rise to distinct types of neurons. The a4.2 cells give rise to epidermal neurons in the trunk epidermis, pigment cells, and numerous small neurons around the sensory organ, and a pair of large neurons at the ventral neural tube close to the sensory vesicle. Neurites are not observed on the muscle, suggesting that motor neurons are not derived from a4.2 cells.

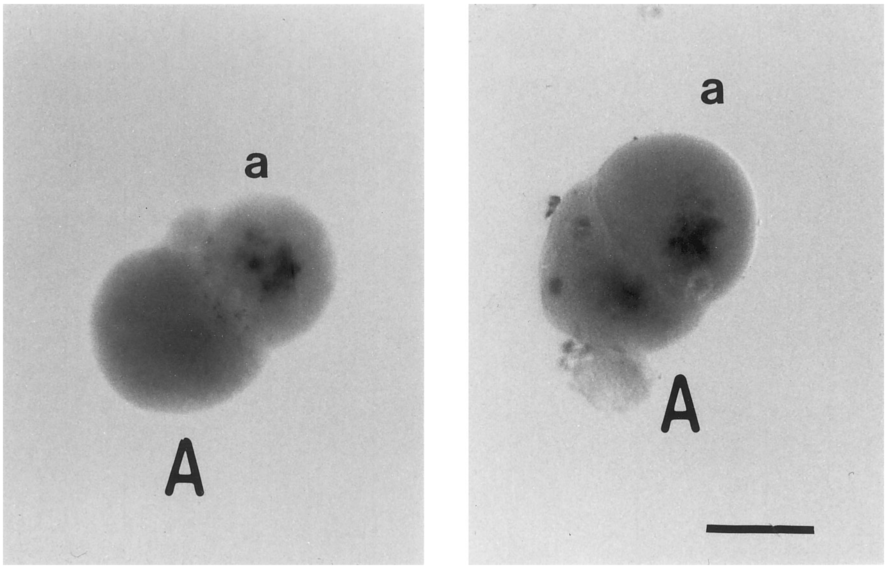

FIG. 8. Two patterns of *in situ* hybridization of a/A pairs under cleavage-arrest. "a" and "A" denote a4.2 and A4.1 blastomeres, respectively. The left sample is the pair in which only a4.2 expresses TuNa I signal. In the right sample, both blastomeres express the TuNa I signal.

In the a-line, cells fated to become both the epidermal and neural tissue share the same precursor up to the 110-cell stage. Critical cell interactions for neural differentiation occur between the 32-cell stage and mid-gastrula stage (Okado and Takahashi, 1990b; Nishida and Satoh, 1989). Isolated a4.2 blastomeres develop into a ball of epidermal cells surrounded by the tunic (Fig. 5) and never express the TuNa I gene. The b-line also contains cell fates to epidermis (Nishida, 1987) and putative sensory neurons in the epidermis (T. Okada, unpublished observation). The presence of common ancestor cells between the epidermal and neural cells in the a- and b-lineages is similar to vertebrate neural induction from the ectoderm (reviewed in Okamura *et al.*, 1993). Vertebrate neural induction has been studied with the basic concept of cell-fate selection, which occurs between neural or epidermal cells (Slack, 1994). In the amphibian embryo, suppression of epidermal fate and induction of neural differentiation are coupled, as is suggested by the effects of BMP-4, a gene of the TGF-β family (Wilson and Hemmati-Brivanlou, 1995).

In contrast, the A-line does not include cell fates to epidermis. The A5.2 blastomere of the 16-cell stage embryo gives rise to endoderm, notochord, trunk lateral cells, and muscle cells, as well as cells of neural tube tissue. The epidermal cell marker never appears in isolated A4.1 pairs (Nishikata *et al.*, 1987; Ueki *et al.*, 1994). The absence of the epidermal lineage from the A-line suggests that the determination of neuronal cells in the A-line might be different from those of the a-line and the b-line.

Two experiments suggest that neuronal differentiation from the A-line does not require intimate cell–cell interactions. First, isolated, cleavage-arrested A4.1 blastomeres can differentiate to express neuronal phenotypes as revealed by active electrical membrane properties and ion channel gene expression. Second, inhibition of tight-cell contact of A4.1-derived partial embryos in low-Ca^{2+} seawater did not significantly decrease the level of mRNA of neuronal ion channels. This contrasts well with the differentiation of notochord cells, most of which are also derived from A4.1 blastomeres. Dissociation of the A-line cells during early stages inhibited expression of notochord-specific gene, AST1, but not TuNa I expression (see Fig. 9). This suggests that factors controlling notochord induction and neuronal differentiation of the A-line are distinct.

There might be two possible explanations regarding the mechanisms for neuronal differentiation in the A-line. One possibility is that neuronal differentiation in the A-line occurs dependent on segregation of maternal factors during early cell division. According to this idea, some neural-activating factor is segregated into the presumptive neural region of the A-line or, alternatively, a putative neural-inhibiting factor is excluded from the presumptive neural region of the A-line. In the latter case, by analogy to the case of BMP-4 in vertebrate neural induction (Slack, 1994; Wilson and Hemmati-Brivanlou, 1995), some putative neural-suppressing factor such as BMP-4-like molecule may be segregated out from blastomeres of neural precursors of the A-line during early cell cleavage, which may then direct these cells to select default pathway of neural cell fate. This cannot explain, however, why neuronal differentiation only occurs in restricted cases of isolated A4.1 blastomeres under cleavage-arrest conditions (see description related to Fig. 6 under Results). Isolated A4.1 blastomeres should always dif-

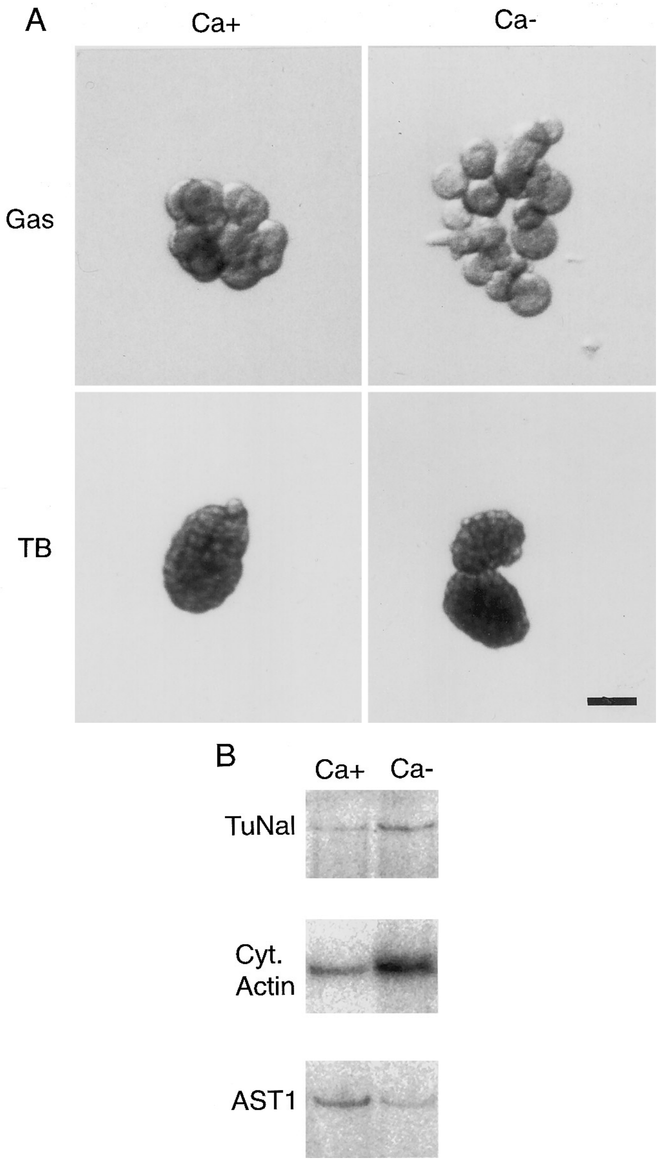

FIG. 9. TuNa I mRNA in A-derived partial embryos cultured in a low concentration of extracellular Ca^{2+}. (A) Images of A-derived embryos cultured under low-Ca^{2+} seawater (Ca^{-}) during the 16-cell stage to the late gastrula stage were taken during the incubation under low-Ca^{2+} conditions at the early gastrula stage (Gas) and after return to normal seawater at the tailbud stage (TB). The A-derived embryos cultured in normal seawater are also shown (Ca^{+}). (B) Quantitative RT-PCR detection of TuNa I, cytoplasmic actin, and ascidian brachyury, AST1. For RNA extraction, eight samples were pooled for embryos prepared in low-Ca^{2+} seawater. Ten samples were pooled for control partial embryos. TuNa I was amplified for 28 cycles, actin for 22 cycles, and AST1 for 25 cycles; with this number of cycles, each gene was amplified linearly. Amplified products were 353 bp for TuNa I, 171 bp for actin, and 247 bp for AST1. Scale bar, 40 μm.

ferentiate to express neuronal phenotypes if segregation of maternal factors causes neural differentiation.

Another more likely possibility is that a diffusible inducing substance is released to act in an autocrine fashion. It has recently been reported that the A-line cells express ascidian counterparts of LIM gene (Wada *et al.,* 1995) and goosecoid (personal communication to Dr. Saiga), which are known to be involved in activities of Speman's organizer of amphibian embryos, suggesting that, in the ascidian embryo, the A-line cells function like the Speman's organizer. Only the A-line can induce neural action potentials from the a-line and the b-line blastomere (Okado and Takahashi, 1990; 1993) and formation of pigment cells from the a-line blastomere (Nishida and Satoh, 1989). By isolating individual blastomeres from the 110-cell stage embryo, it has been shown that neural tube precursor blastomeres of the A-line have the ability to induce pigment cells in the brain sensory organs (Nishida, 1991). The presence of a neural inducing ability in the blastomere fated to become a neural cell leads us to speculate that differentiation of neuronal cells from the A-line occurs by a mechanism in which the neural-inducing substance is released in an autocrine manner. Further careful isolation and recombination experiments of individual blastomeres of the A-line will be required to determine whether neuronal differentiation in the A-line is governed by segregation of maternal factors or by an autocrine mechanism.

Neural differentiation from the A-line cells in the ascidian embryo may be evolutionarily correlated with the formation of the caudal neural tissue of the vertebrate embryo. It has been shown that floor plate cells in the caudal neural tube share the precursors with caudal mesoderm cells such as notochord and muscle in the chick (Catala *et al.,* 1996). This situation is similar to that for the A-line, in particular regarding A5.1, which gives rise to ventral neural tube cells and the notochord. It may be a tempting hypothesis that the common ancestral animal of both the vertebrate and the protochordate had two distinct lineages of the central nervous system: one is the induction of neural cells from the ectodermal cells which show fates to epidermis similar to the a-line, and the other is the lineage associated with mesodermal cells such as notochord and muscle, similar to the A blastomeres of the ascidian. Perhaps, the second lineage extended in the ascidian, resulting in most of caudal neural tube cells such as floor plate cells and motor neurons being derived from this lineage, whereas, along the way of the vertebrate evolution, the first lineage became dominant and the second lineage only survived for the formation of part of caudal neural tube cells. More comparative lineage studies in the protochordates and the primitive vertebrates may be required to test this hypothesis.

Neural Patterning and Neuronal Differentiation in the Ascidian Larva

TuNa I-positive neurons are located in the neck neural tube but are not found in the more caudal region of the neural tube. A similar pattern of gene expression has been obtained for genes putatively encoding other voltage-gated ion channels (T. Okada and Y. Okamura, unpublished observation). By referring to the lineage map of *H. roretzi* (Nishida, 1987), it is most likely that the A8.15 blastomere of the 110-cell embryo gives rise to the ventrolateral neuronal cells in the neural tube. The A8.15 blastomere, however, also gives rise to more caudal cells of the neural tube which are negative for TuNa I expression. This suggests that regional specification of TuNa I-positive neurons at the neck region should occur after the 110-cell stage. How is neuronal differentiation restricted at the anterior part of the neural tube? Heterogeneity in the cell population in the neural tube along the anterior–posterior axis has been shown by expression of a labial group Hox gene, *Hr-Hox* (Katsuyama *et al.,* 1995), and a LIM-class homeobox gene, *Hrlim* (Wada *et al.,* 1995). The location of TuNa I-positive neuronal cells in the neck seems to correspond to the level along the anterior–posterior axis where *Hr-Hox* is expressed in the neural tube, although *Hr-Hox* is expressed in the dorsal plate cell of the neural tube and not in the lateral neural tube cells. Cells expressing *Hrlim* in the neck neural tube appear to be identical to the TuNa I-positive neuronal cells in the neck neural tube (Wada *et al.,* 1995). Localized expression of these transcription factors might determine the site of neuronal cells along the anterior–posterior axis of the neural tube.

On the other hand, the pax homologue of the ascidian, *HrPax-37,* is expressed in a reiterated pattern in the dorsal neural tube, reminiscent of the segmental structure of the vertebrate neural tube (Wada *et al.,* 1996). In the ancestral vertebrate, the neural tube might contain motor neurons at each segment. The mechanisms for inducing the neurons were probably abbreviated at the caudal part of the neural tube during ascidian evolution. In fact, the larvaceans, the more ancestral type of the protochordates, show segmental localization of neuronal cells innervating muscle cells (Bone, 1989). Identification of ascidian homologues for genes known to be involved in specifying neuronal cells in the vertebrate neural tube, such as hedgehog-related genes, might shed light on this problem.

ACKNOWLEDGMENTS

We thank Mr. M. Fukuda (Kyorin University School of Medicine) for his assistance with analysis using the electron microscopy, Ms. A. Umeda for technical assistance, Dr. B. Eggen (State University of New York at Stony Brook) for critical reading of the manuscript, and colleagues for comments on the manuscript and helpful discussions. We also thank Dr. H. Okamoto for continuous support throughout this work. This work was supported by research grants from the Human Frontier Science Program, PRESTO from Japan Science and Technology Corporation, and to one of the authors (Y.O.).

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Received for publication June 2, 1997
Accepted August 5, 1997