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Supportive Role of Cellular Bridge of Neurons Expressing a Highly Polysialylated Form of NCAM (NCAM-H) at the Initial Stage of Migration of LHRH Neurons

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ABSTRACT—The early stage of cell migration from the olfactory placode to the forebrain was studied immunohistochemically in chick embryos to investigate the nature of early migrated cells and the role of these cells in the sebsequent migration of luteinizing hormone releasing hormone (LHRH) neurons. The initial cells migrating from the olfactory placode stained strongly positive for the highly polysialylated neural cell adhesion molecule (NCAM-H), but were negative for LHRH. These migrating NCAM-H immunoreactive cells were observed on embryonic day (ED) 2.5 as a bulge with a few cells from the base of the placode. By ED 3, they formed a wide cellular strand and developed into a cellular bridge between the olfactory placode and the ventro-rostral surface of the forebrain. These migrating cells are neurons because they stained positive for growth associated protein-43 (GAP-43), microtubule associated protein (MAP) 2, and MAP 5. Some of these cells seemed to migrate caudally along the ventro-lateral surface of the forebrain. LHRH-immunoreactive cells were not detected in the olfactory epithelium until after ED 3.5, which was one day after formation of the cellular bridge. Then, LHRH-immunoreactive cells appeared and began to cross the cellular bridge. The outgrowth of the olfactory nerve axon bundles from the olfactory epithelium was detected at around the same time when LHRH-immunoreactive cells first appeared. These olfactory nerve axons expressed NCAM-H, GAP-43, and MAP 5, as assessed by immunochemistry. After bundle formation, the olfactory nerve appeared to provide the migratory routes for LHRH neurons. These results suggest that the cellular bridge formed by the NCAM-H-immunoreactive neurons plays an important supportive role for LHRH neurons at the initial stage of their migration in chick embryos.

INTRODUCTION

Recently, we found that after complete placodectomy, LHRH neurons in the olfactory-forebrain axis fail to develop on the operated side in newt and chick embryos (Akutsu *et al.*, 1992; Murakami *et al.*, 1992). Labeling of the precursor cells of LHRH neurons in the olfactory placode with a carbocyanine dye (DiI) in early chick embryos revealed the sequential appearance of DiI-labeled cells expressing LHRH in the olfactory nerve, the rostral forebrain and septo-preoptic area (Murakami and Arai, 1994a). These results provide direct evidence for the migration of LHRH neurons from the nasal region to the forebrain.

The olfactory placode is thought to be the source of different types of neurons migrating toward the forebrain. Other than LHRH neurons, neurons expressing somatostatin (Murakami and Arai, 1994b) or growth hormone releasing hormone (Parhar and Sakuma, 1995) have been identified as migrating cells from the olfactory placode. Furthermore, a group of cells derived from olfactory placode has been suggested to participate in the induction of neocortical development (De Carlos *et al.*, 1995).

In our previous study, we have shown that the appearance of highly polysialylated NCAM (NCAM-H)-immunoreactive (ir) cells precedes that of the LHRH neurons in the normal development of the olfactory-forebrain axis (Miyakawa *et al.*, 1994). The NCAM-ir neural elements have been postulated to form a scaffold which is necessry to guide the migratin of LHRH neurons in the mouse embryo (Schwanzel-Fukuda *et al.*, 1992).

In the present study, we investigated the cellular nature of the NCAM-ir cells, and the role of these cells in the migration of LHRH neurons in chick embryos. Our results show that the NCAM-ir cells are neurons and that the bridge formed by these cells appears to guide the subsequent migration of LHRH neurons.

MATERIALS AND METHODS

Tissue preparation

Fertilized eggs from White Leghorn chickens obtained from a

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commercial source were incubated in a humid chamber at 37.6°C. At different times of incubation from day 2.5 to 8 about 400 embryos were fixed for 24 hr in either Bouin's solution without acetic acid or in phosphate-buffered 4% paraformaldehyde. Embryos were staged according to Hamburger and Hamilton (1951).

The first group of fixed tissues was dehydrated in graded alcohol solutions and embedded in paraffin. Serial 6-8 μm sections were cut and mounted on albumin-coated slides. The second group of fixed tissues was immersed overnight in 20% sucrose containing 0.1 M phosphate- buffered saline (PBS), and then frozen in liquid nitrogen. Serial cryostat sections of 10 μm and 16 μm were mounted on albumin-coated slides for immunofluorescent staining and for staining by the avidin-biotin peroxidase complex (ABC) method, respecively.

Immunohistochemical procedures

Monoclonal antibodies used in this study were anti-NCAM-H (Mab12E3, raised in our laboratory, Seki and Arai, 1991, dilution 1:5,000), anti-LHRH (LRH13, HAC-MM02-MSM84, gift from Dr.K.Wakabayashi at GunmaUniversity, Maebashi, Japan, dilution 1:2,000), anti-growth associated protein-43 (GAP-43) (Sigma Chemical Co., dilution 1:3,000), anti-microtubule associated protein 2 (MAP 2) (Sigma Chemical Co., dilution 1:2,000), anti-MAP 5 (Sigma Chemical Co., dilution 1:10,000) and anti-α-tubulin (Amersham, dilution 1:10000).

Immunocytochemical staining was performed by the ABC method using a commercial kit (Vector laboratories, Burlingam, CA). The cryostat sections or deparaffinized sections were treated with 0.3% H_2O_2 in methanol to suppress background staining resulting from endogenous peroxidase activity and rinsed in PBS. The sections were incubated with respective primary antibodies for 24 hr at 4°C. Then the sections were incubated with biotinylated secondary antibodies (anti-mouse IgM for anti-NCAM-H, anti-mouse IgG for other primary antibodies) for 1 hr and with ABC complex for 1.5 hr at room temperature. The sections were visualized by application of 3'3-diaminobentidine tetrahydrochloride (DAB) and H_2O_2. The sections were counterstained with methyl green. After the DAB reaction for LHRH immunostaining, some specimens of ED6 embryos were double-labeled for anti-α-tubulin and visualized by the alkaline

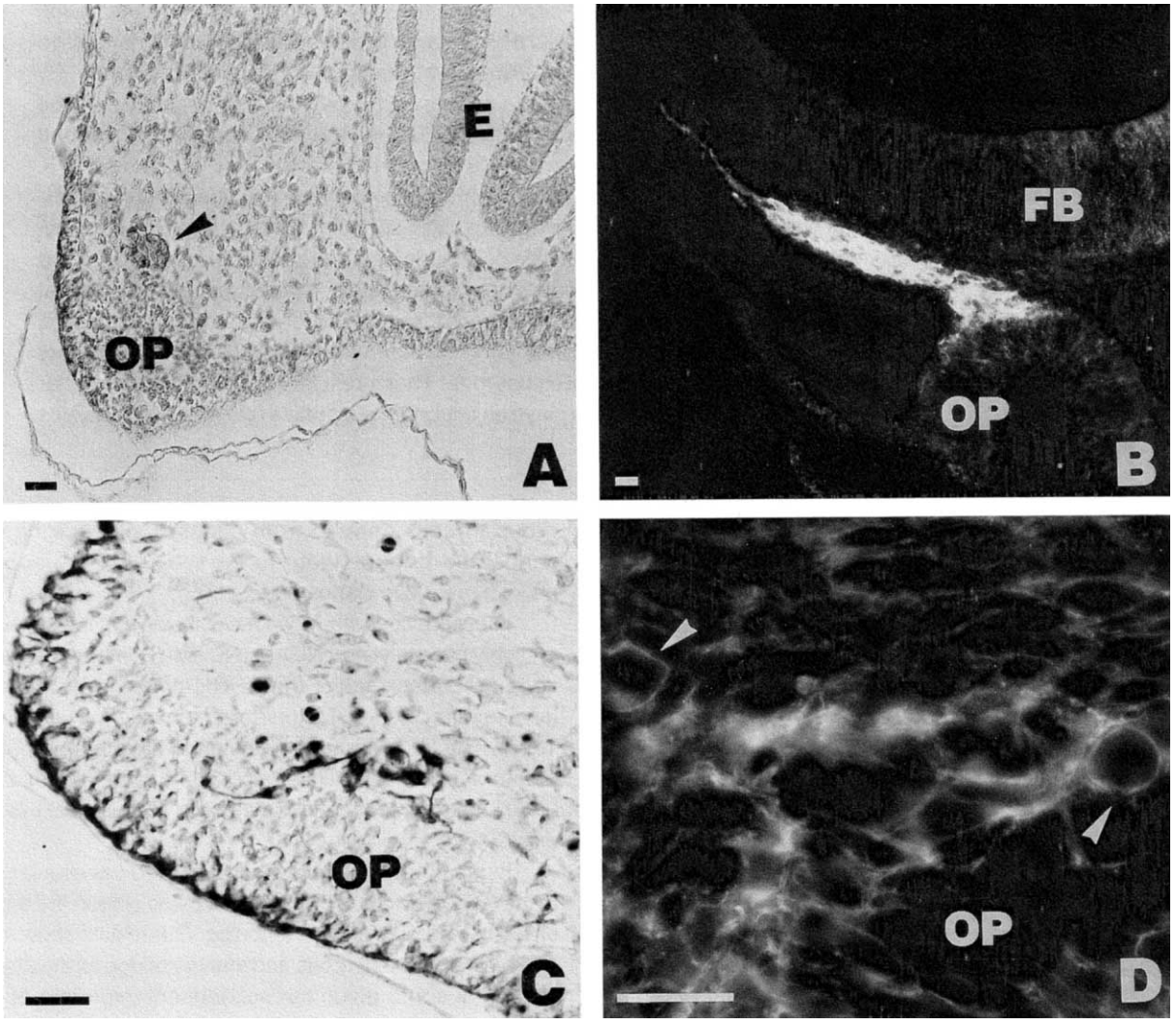

Fig. 1. Initial appearance of migrating cells from the olfactory placode (**A**) and the formation of the cellular bridge to the forebrain (**B**). (**A**) NCAM-H-ir cells (arrowhead) bulged out from the olfactory placode at ED 2.5 (60 hr incubation, stage 14). (**B**) Cellular bridge formed by NCAM-H-ir cells with long process extending toward the rostral forebrain at ED 3 (stage 17). Immunofluorescence staining. (**C**) α-tubulin-ir cells at the border of the olfactory placode and mesenchyme at ED 2.5 (63 hr incubation, stage 15). (**D**) Rhodamine-phalloidin binding to fibrous actin of the migrating cells from the olfactory placode at ED 3 (stage 17). The contour of some cells (arrowheads) are clearly visible. Sagittal sections. Rostral is to the left, and dorsal is to the top. OP, olfactory placode; FB, forebrain; E, eye. Scale = 20 μm.

phosphatase reaction using a commercial kit (Vector laboratories, Burlingam, CA).

For immunofluorescence histochemistry, cryostat sections rinsed with PBS were incubated with primary antiserum for 24 hr at 4°C. These sections were incubated with FITC-labeled second antibody (dilution 1:100) or rhodamine-labeled second antibody (dilution 1:50) for 3 hr at room temperature. For double staining, a mixture of two kinds of primary antisera and corresponding secondary antibodies was applied. Some sections were also stained for fibrous actin with rhodamine-labeled phalloidin (Molecular Probes Inc., dilution 1:200). Processed samples were examined with an epifluorescent microscope (Carl Zeiss, Axioplan). Staining was negative in all cases where only the secondary antibody was applied (controls).

RESULTS

At ED 2.5 (stage 14-16), the olfactory placode was observed as localized thickening of the head ectoderm around the rostral tip. In most embryos at this stage, a small aggregate of NCAM-H-ir cells was found to bulge from the olfactory placode epithelium and move toward the nasal mesenchyme (Fig. 1A). In some embryos, however, NCAM-H-ir migrating cells and the bulging structure were not apparent, suggesting that ED 2.5 may be the critical period for the appearance of NCAM-H-ir cells.

Shortly after this stage and by ED 3.0 (stage 17), a small aggregate of migrating cells formed a cellular strand and developed into an NCAM-H-ir cellular bridge between the olfactory placode and the ventral surface of the brain (Figs. 1B and 5A). Processes that extend from the NCAM-H-ir migrating cells in the cellular strand were found to arrive at the ventral surface of the forebrain. These fibrous components were also located between the cell cords of the migrating cells. At this stage, however, the growth of the olfactory nerve axons and LHRH-ir cells were not detected.

The migrating NCAM-H-ir cells were bipolar or fusiform and strongly immunoreactive for α-tubulin in the soma and processes (Fig. 1C). Binding of rhodamine-labeled phalloidin was found to delineate the cytoplasm of NCAM-H-ir migrating cells, suggesting the presence of fibrous actin in the inner cell surface (Fig. 1D). Importantly, the NCAM-H-ir migrating cells were immunoreactive for GAP-43, MAP 2 and MAP 5 (Fig. 2). This finding indicates that these migrating cells are of neuronal nature. At late ED 3 (stage 20), MAP 5 immunoreactivity became stronger in the fibrous processes of the NCAM-H-ir migrating cells in the strand.

Approximately one day after formation of the cellular bridge between the olfactory epithelium and the forebrain, a small number of LHRH-ir cells were first found on ED 3.5 (stage 18) in the olfactory epithelium. These cells were especially prominent in the rostral and medial edge of the olfactory pit (Fig. 3A). These LHRH-ir cells were also immunoreactive to NCAM-H (Fig. 5B). From late ED 3 to ED 4 (stage 20-21), a small number of LHRH-ir cells were observed in the cellular bridge (Fig. 3B). Occasionally, few LHRH-ir cells were found in the thin ectoderm of the peripheral region of the medial, rostral and caudal olfactory pit. In addition, a few LHRH-ir cells located perpendicular to the olfactory epithelial cells were observed (Fig. 3B). The LHRH-ir cells increased in number by ED 6 (Fig. 3C). At ED 6, LHRH-ir cells have become a major component of the migrating cell population.

Axons seemed to grow out from the olfactory epithelium at ED 3.5 (stage 18-20). However, it was difficult to distinguish axons from migrating cells by the immunohistochemical staining pattern because migrating cells were also immunoreactive to NCAM-H, GAP-43 or MAP 5. On ED 4 (stage 22), distinct axon bundles of the olfactory nerve were observed by the immunohistochemistry for GAP-43 (Fig. 4), NCAM-H and MAP 5. Afterwords, olfactory nerve bundles continued to show strong immunoreactivity for NCAM-H, GAP-43 and MAP 5. As development progressed, the distance between the olfactory epithelium and the forebrain became longer, and the cell density in the cellular bridge decreased significantly as a whole. The cellular bridge between the olfactory epithelium and the forebrain was gradually replaced by the growing NCAM-H-ir olfactory nerve bundle (Fig. 5C). However, a large number of NCAM-H-ir migrating cells were still seen in the medial part of the olfactory nerve bundle. Thin elongated cells immunonegative for GAP-43 and MAP 2 appeared on ED 5 (stage 26) and were found to be associated with the olfactory nerve axons (figure not shown).

After the formation of thick olfactory axon bundles, LHRH-ir and NCAM-H-ir cells were seen to be attached to the NCAM-H-ir axon bundles (Fig. 5C), as if crawling along the nerve fibers (Fig. 5D). Occasionally, in a few sagittal or horizontal sections of embryo from ED 2.5 to ED 3.5 (Fig. 2C-F), a caudal branch of NCAM-H positive migrating cells from the olfactory placode seemed to go in the caudal direction. The cells of caudal branch seemed to be similar to the migrating cells in the rostral portion since these cells also showed positive staining for GAP-43, MAP 2, MAP 5 in addition to NCAM-H (Fig. 2). However, LHRH-ir cells were not found in the caudal branch, and the caudal branch was not found in the embryos older than ED 4.

DISCUSSION

The close association between migrating LHRH neurons and NCAM-expressing neural elements has been observed in several recent studies (Murakami *et al.*, 1991; Schwanzel-Fukuda *et al.*, 1992; Daikoku *et al.*, 1993). In particular, NCAM-H is strongly expressed by the migrating neurons and the olfactory nerve, which provides a migratory route for the LHRH neurons in chick embryos (Murakami *et al.*, 1991).

In the present study, we found that the emergence of NCAM-H-ir cells from the olfactory pit epithelium preceded the initial appearance of LHRH neurons and outgrowth of the olfactory nerve. Similar observations have been reported previously in the mouse embryo. The cellular nature of these NCAM-ir cells, however, was not characterized (Schwanzel-Fukuda *et al.*, 1992).

The cellular aggregation of the NCAM-H-ir migrating cells resulted in formation of a cellular bridge between the olfactory pit epithelium and the forebrain. These cells of the bridge were

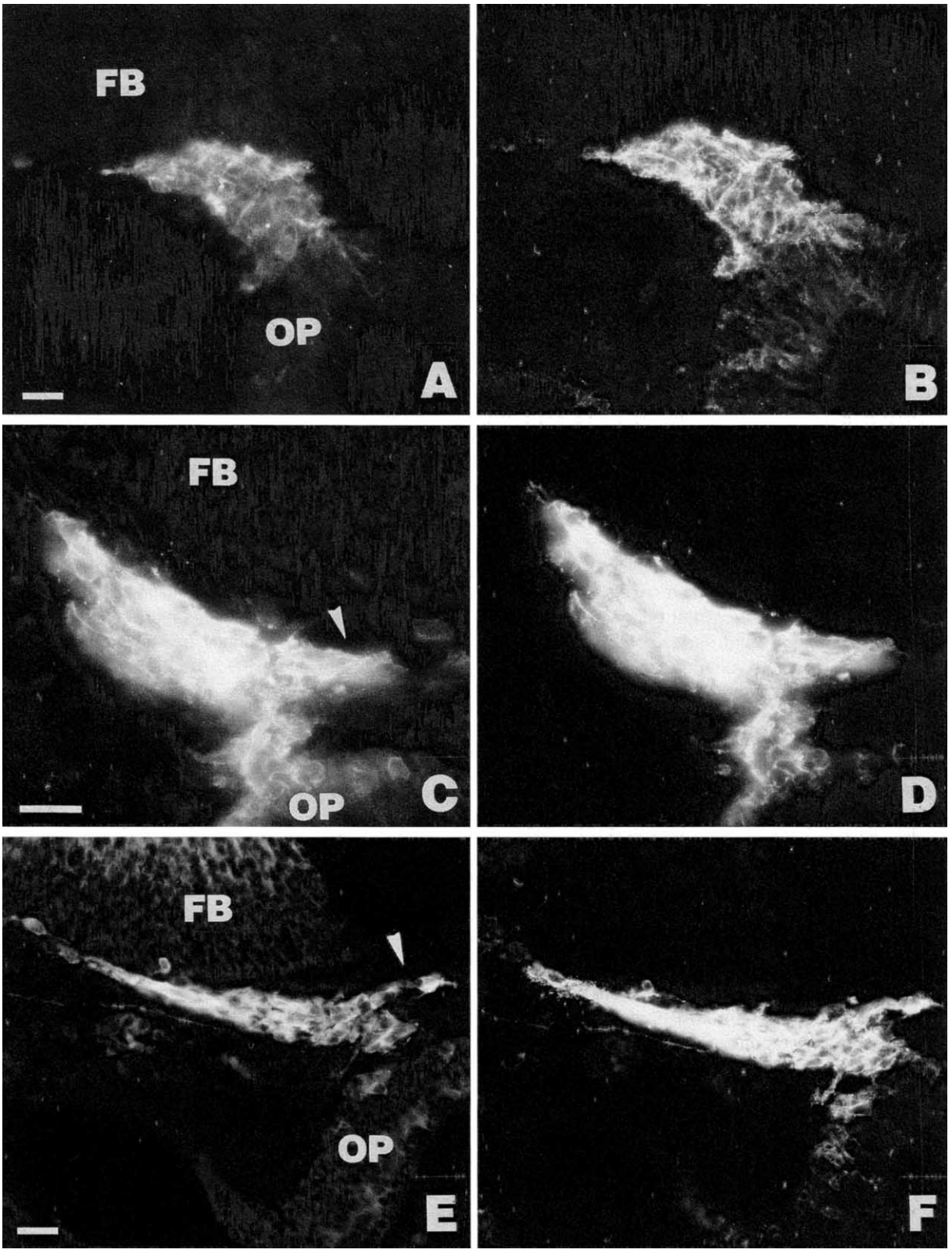

Fig. 2. Migrating cells are neuronal in cellular nature. (**A**, **B**) Double staining for GAP-43 (**A**) and NCAM-H (**B**) at ED 2.5 (65 hr incubation, stage 17). (**C**, **D**) Double staining for MAP 2 (**C**) and NCAM-H (**D**) at ED 2.5 (65 hr incubation, stage 17). (**E**, **F**) Double staining for MAP 5 (**E**) and NCAM-H (**F**) at ED 3 (stage 18). Sagittal sections from different animals. Rostral is to the left, and dorsal is to the top. A small group of migrating cells seem to go in the caudal direction (arrowheads). OP, olfactory placode; FB, forebrain. Scale = 20 μm.

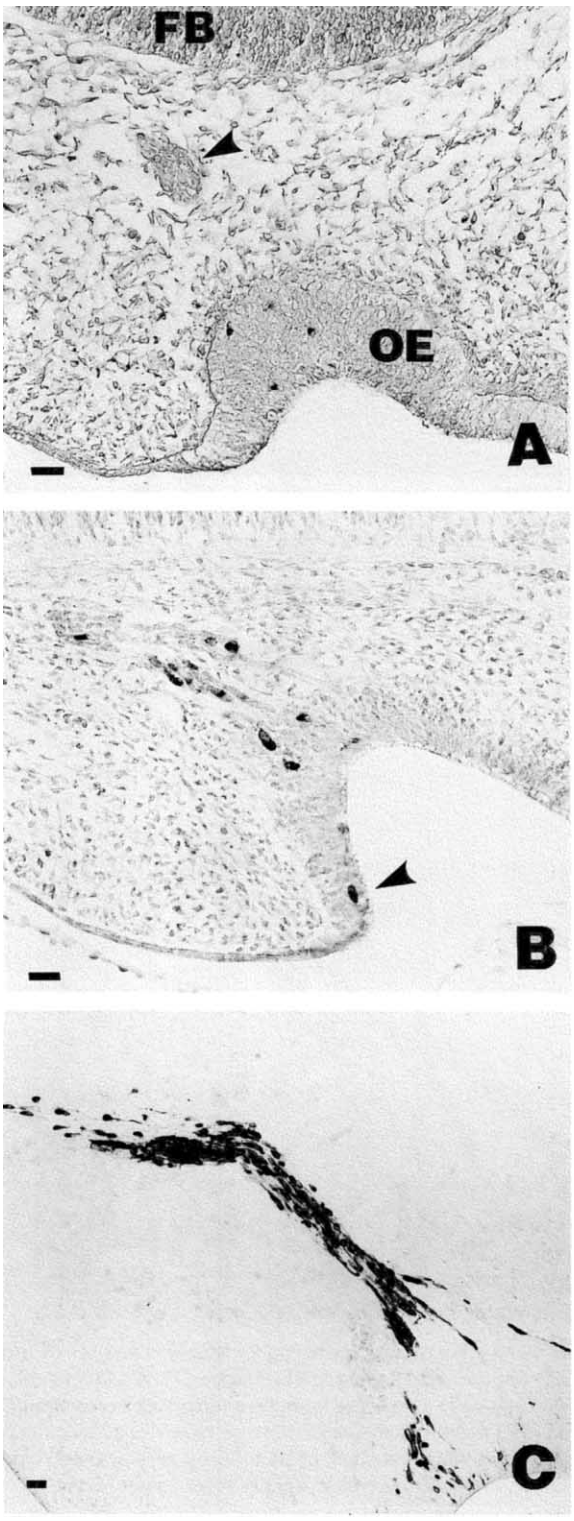

Fig. 3. The migration of the LHRH neurons. (**A**) Appearance of LHRH-ir cells in the olfactory placode on ED 3.5 (stage 19-20). Arrowhead indicates a part of the cellular strand in which no LHRH-ir cell is present. (**B**) The migratory LHRH-ir cells in the cellular strand at ED 4 (stage 24). Note the orientation of the LHRH-ir cell in the olfactory epithelium (arrowhead). (**C**) Many migratory LHRH-ir cells along the olfactory nerve at ED 6 (stage 29). Sagittal sections. Rostral is to the left, and dorsal is to the top. OE, olfactory epithelium; FB, forebrain. Scale = 20 μm.

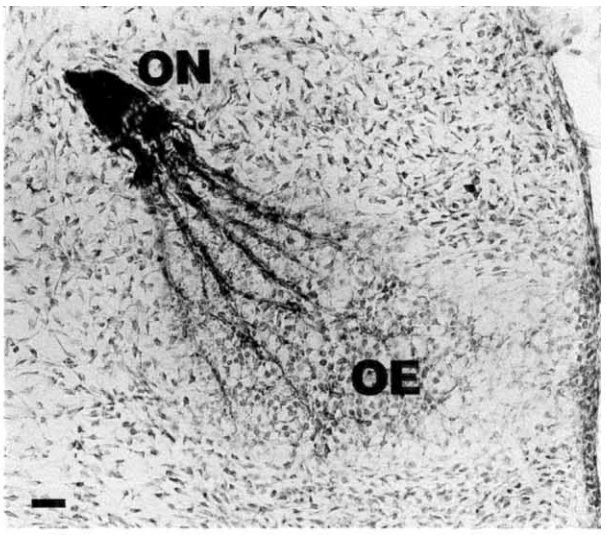

Fig. 4. Development of the olfactory nerve. GAP-43-ir olfactory nerve axons coming out from the mediodorsal aspect of the left olfactory pit at ED 4 (stage 21). Horizontal section. Rostral is to the bottom. OE, olfactory epithelium; ON, olfactory nerve axon bundle cut transversely. Scale = 20 μm.

immunoreactive for GAP-43,MAP 2 and MAP 5 in our chick embryos. Thus, these cells are neuronal in nature. Electron microscopic morphology of the migrating cells has been reported to resemble that of neuronal cells which are characterized by cell processes with many microtubules, well-developed Golgi apparatus, polyribosomes and the presence of dense core vesicles with diameter between 100 and 200 nm (Mendoza *et al.*, 1982). However, there are evidences that the migrating cells also contain ensheathing cells (Valverde *et al.*, 1992; Pellier and Astic, 1994). Using a monoclonal antibody specific for Schwann cells, Norgren *et al.* (1992) demonstrated the distinct immunoreaction in the olfactory system of the ED 5 chick embryo. The GAP-43- and MAP 2-negative elongated cells, first found at ED 5 and accompanied by the axon bundle in our study, could be the ensheathing cells. It is possible that the NCAM-H-ir neural elements consist of a mixed cell population of neuronal and glial precursor cells after ED 5.

The availability of a central projection of the olfactory nerve bundle is prerequisite for the normal migration of LHRH neurons into the forebrain. This is because when the development of the olfactory nerve axons are severely disturbed, such as in the case of incomplete olfactory placodectomy, no LHRH neurons reach the forebrain and only a small number of LHRH neurons are detected in the remnant of the olfactory epithelium and adjacent areas (Murakami *et al.*, 1995). At the very early stage, however, the time of initial appearance of LHRH neurons and axons of the olfactory nerve is almost identical. Approximately one day after the formation of the cellular bridge between the olfactory epithelium and the ventral surface of the forebrain by the NCAM-H-ir and LHRH-immunonegative migrating cells, LHRH neurons start to cross the bridge. Our immunostaining experiment using an anti-NCAM antibody

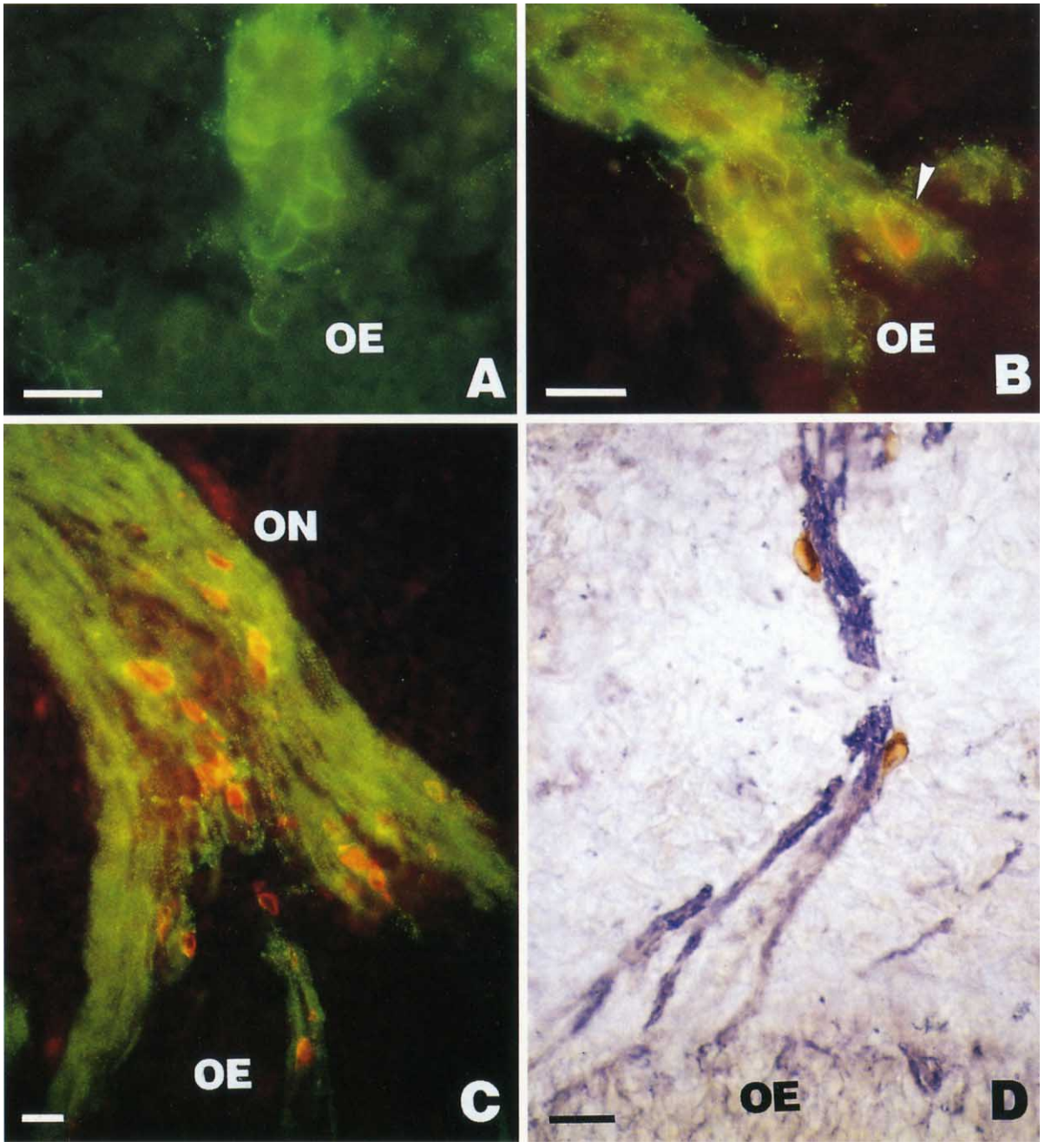

Fig. 5. NCAM-H-ir bridge for the migration of the LHRH neurons. (**A-C**) Double immunofluorescence staining for NCAM-H and LHRH (double exposure). (**A**) NCAM-H-ir cellular bridge (FITC-labeled, green) in which no LHRH neuron are detected at ED3 (stage 17). (**B**) NCAM-H-ir cells (green), and a LHRH-ir cell (rhodamine-labeled, orange, arrowhead) which is also NCAM-H-ir, emerge from the olfactory epithelium at ED 3.5 (stage 21). (**C**) At ED 6 (stage 29), NCAM-H-ir olfactory nerve axons (green) and LHRH neurons (red), which are also NCAM-H-ir, accompanying them. (**D**) Double staining for LHRH (DAB reaction, brown) and α-tubulin (alkaline phosphatase-reaction, blue). LHRH-ir cells are seen crawling along α-tubulin-rich olfactory nerve at ED 6 (stage 29). Sagittal sections. The olfactory epithelium is bottom right in these figures. Scale = 20 μm.

recognizing polysialic acid portion of the molecule (Seki and Arai, 1991) show that migratory LHRH neurons co-express NCAM-H. This finding suggest a role for the NCAM-NCAM homophilic interaction in the initial migration of LHRH neurons. Our results does not seem to be consistent with that of Schwanzel-Fukuda *et al.* (1992) since they have shown that migrating LHRH neurons do not express NCAM. However, the antibody that they used was directed against the protein domain of NCAM and not the polysialic acid portion as in our study.

NCAM has been shown to be involved in cell-cell and cell-matrix adhesions of neurons and glial cells. Of particelar relevance to our study, NCAM-H is expressed by the developing neural tissue and thought to have important

functions (e.g. cell migration) in the development of the nervous system (see Seki and Arai, 1993). In NCAM-180-null mice, in which the expression of polysialic acid is also absent, precursor olfactory bulb cells fail to migrate to the olfactory bulb from the subependymal zone at the lateral ventricle (Tomasiewicz *et al.*, 1993). The high content of polysialic acid of NCAM-H is thought to promote changes in cell interactions, thereby also affecting cell migration in the developing nervous system (Rutishauser and Landmesser, 1996).

Almost simultaneously with the start of LHRH-ir cell migration, olfactory nerve axons grow into the cellular bridge and reach the surface of the forebrain. Then, the LHRH neurons utilize the axon bundles of the olfactory nerve as a scaffold for migration. It is therefore likely that the cellular bridge formed prior to these events by the NCAM-H-ir migrating neurons plays a structural role in supporting the migration of LHRH neurons and the developing olfactory nerve axons.

It is not known as to how the NCAM-H-ir cells form this cellular bridge. At the biginning of the NCAM-H-ir cell migration, the distance between the olfactory placode and the forebrain vesicle is 2 or 3 times as long as these fusiform cells. Interestingly, we have found that especially at early stages, these migrating NCAM-H-ir cells are rich in fibrous actin and tubulin. Since these cytoskeletal components have been shown to be important for cell motility, our finding suggests that NCAM-H-ir cells at least possess motile capabilities.

Recently, the olfactory epithelium, migrating cells and olfactory nerve have been found to also express a neural chondroitin sulfate proteoglycan (6B4 proteoglycan/phosphacan) (Nishizuka *et al.*, 1996). This proteoglycan has been shown to bind adhesion molecules such as NCAM, suggesting that an interaction between NCAM-H and other extracellular matrix components could also be involved in migration and cellular bridge formation.

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