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# Immunocytochemical Investigation of a Salmonid Olfactory System-Specific Protein in the Kokanee Salmon (*Oncorhynchus nerka*)

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**ABSTRACT**—Immunocytochemical and immunoelectron microscopic localizations of a salmonid olfactory system-specific protein (N24) were investigated in the olfactory system (the olfactory epithelium, the olfactory nerve and the olfactory bulb) of kokanee salmon (*Oncorhynchus nerka*) by using a specific antiserum to N24. N24 immunoreactivities were observed in the cytoplasm of ciliated and microvillous olfactory receptor cells but were not observed in the supporting and the basal cells in the olfactory epithelium. Gold particles showing immunoreactivities for N24 were scattered in the cytoplasm of the dendrites of olfactory receptor cells. Some particles were concentrated on vesicular structures, but none were observed in the membrane of olfactory receptor cells. N24 immunoreactive axons were terminated at the glomerular layer near the mitral cells in the olfactory bulb. In an olfactory rosettectomy experiment, N24 immunoreactivity in the olfactory bulb vanished fifteenth day after the excision of the olfactory rosette. These results reveal that the olfactory receptor cells produce N24 which exists in both dendrites and axons of the olfactory receptor cells, and suggest that N24 may participate in neuromodulation in the olfactory system of kokanee salmon.

## INTRODUCTION

It is now widely accepted that information concerning unique odors characteristic of a maternal river are imprinted in juvenile anadromous salmonid fish during their downstream migration, and that adult fish evoke these memories during their homing migration (Hasler and Wisby, 1951; Hasler and Scholz, 1983). Therefore the olfactory system, including the olfactory epithelium, olfactory nerve and olfactory bulb presumably play an important role in both imprinting and discrimination of maternal river odors. However, few biochemical studies have been done to investigate basic relations between the function and the molecular components of the olfactory system in salmonids.

There are numerous examples in the literature that indicate a close relation between olfactory-specific proteins and the function of the olfactory system in higher vertebrates (Lancet, 1986; Anholt, 1989; Margolis and Getchell, 1991). Several olfactory-specific proteins or their cDNAs have been identified and used as molecular markers to study olfactory function, such as olfactory marker protein (OMP: Margolis, 1972; Kott *et al.*, 1992), odorant-binding protein (OBP: Pelosi and Maida, 1990), olfactory receptor, $G_{olf}$, type III adenylate cyclase (AC), cAMP gated channel (Reed, 1992), and Olf-1 (Wang and Reed, 1993). The immunocytochemical localization of these olfactory-specific proteins; OMP (Monti Graziadei *et al.*, 1977), OBP (Pevsner *et al.*, 1988), $G_{olf}$, AC (Menco *et al.*, 1992), and Olf-1 (Wang and Reed, 1993), have also been reported in the olfactory system of several vertebrates other than salmonids. For teleosts, one immunocytochemical study of OMP has been reported in the olfactory system of rainbow trout, *Oncorhynchus mykiss* (Riddle and Oakley, 1992). The cDNAs encoding olfactory receptors have also been identified in the channel catfish (Ngai *et al.*, 1993).

Recently, we identified a 24 kDa protein (N24) in the olfactory system of kokanee salmon, *O. nerka* (Shimizu *et al.*, 1993), the first olfactory-specific protein reported in salmonids. It was shown by Western blot analysis that the amount of N24 was higher in fish in the maternal river than those in seawater at both times of imprinting in maternal river and homing to the maternal river. In our preliminary immunocytochemical experiment, localization of N24 was observed in the olfactory epithelium. However it was recognized that more intensive immunocytochemical study of N24 in the olfactory system was required.

In the present study, we examined localization of N24 in the olfactory system of kokanee salmon by means of immunocytochemical and immunoelectron microscopic techniques. Olfactory rosettectomy was also done to investigate the role of N24 in this system.

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## MATERIALS AND METHODS

*Animals*

One-year-old kokanee salmon were reared at Toya Lake Station, Faculty of Fisheries, Hokkaido University, in outdoor tanks supplied with a continuous flow of a spring water at ambient temperature and photoperiod (fork length 15.8-20.4 cm and body weight 39.3-92.0 g). From fish anaesthetized with ethyl p-aminobenzoate, the olfactory rosette including the olfactory epithelium and the forebrain including the olfactory nerve, the olfactory bulb and the telencephalon were isolated.

*The streptavidin-biotin complex (sAB) method*

Each tissue (3 male and 3 female) was fixed with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB; pH 7.2) overnight at 4°C, rinsed with 0.1M PB containing 10% sucrose, dehydrated through graded ethanol, and embedded in paraffin (Histosec; Merck, Darmstadt, Germany). Serial sections were cut at five micron on a microtome, mounted on a gelatin coated glass slide, and air-dried at 4°C. After deparaffinization and hydration, sections were blocked with 0.1% hydrogen peroxide ($H_2O_2$) in methanol for 15 min to destroy endogenous peroxidase, and rinsed with phosphate buffer saline (PBS). Sections were incubated with normal goat serum for 15 min and then incubated with a polyclonal antiserum to N24 (Shimizu *et al.*, 1993; working dilution 1:7000) for 2 hr at room temperature (RT) in a moist chamber. After being rinsed with PBS, sections were reacted with biotinylated goat anti-rabbit immunoglobulin (IgG; Vector, Burlingame, CA, USA) for 45 min, rinsed with PBS, and then reacted with streptavidin-conjugated peroxidase (Dako, Glostrup, Denmark) for 30 min at RT. Sections were then treated with a freshly prepared diaminobenzidine tetrahydrochloride (0.5 mg/ml) solution with 0.01% $H_2O_2$ for 10 min. After being dehydrated, sections were mounted with a cover glass by Entellan neu (Merck, Darmstadt, Germany). As a control, normal rabbit serum was substituted for the anti-N24 serum. Sections adjacent to those used for the sAB method were stained with Delafield's hematoxylin and eosin.

*Indirect immunofluorescence method*

The procedures of fixation and embedding (3 male and 3 female) were similar to the sAB method. Ten micron serial sections were cut on a microtome, mounted on a gelatin coated glass slide, and air-dried at 4°C. After deparaffinization and hydration, sections were incubated with normal goat serum for 15 min and then incubated with the anti-N24 serum (working dilution 1:1000) for 2 hr at RT in a moist chamber. After being rinsed with PBS, sections were reacted with goat anti-rabbit IgG conjugated with fluorescein isothiocyanate or rhodamine (1:100, Cappel, Durham, NC, USA) for 1 hr at RT. Sections were mounted with a cover glass by 80% unfluorescence glycerol containing 1% 1,4-diazabicyclo[2.2.2]octane (Aldrich, Milwaukee, WI, USA) in PBS. Sections were examined on a Carl Zeiss LSM 410 confocal laser scanning microscope.

*Immunoelectron microscopy*

For immunoelectron microscopy, small fragments of each tissue (3 male and 3 female) were fixed with 2% PFA-1% glutaraldehyde (GA) in 0.1M PB overnight at 4°C, rinsed in 0.1 M PB containing 10% sucrose, dehydrated through graded ethanol series, and embedded in Lowicryl K4M (Polaron Equipment, Watford, England) which was polymerized at 4°C with an ultraviolet polymerizer (Dosaka EM, Kyoto, Japan). The IgG-gold technique (Ueda *et al.*, 1993) was used for immunoelectron microscopic observation. Briefly, ultrathin sections were placed for 10 min on a drop containing 0.1% bovine serum albumin (BSA) in PBS, immunoreacted with anti-N24 serum diluted at 1:10000 in 0.1% BSA-PBS for 2 hr, and reacted with goat anti-rabbit IgG-coated 15 nm colloidal gold (E•Y Labs, San mateo, CA, USA) diluted at 1:100 in 0.1% BSA-PBS for 1 hr. After rinsing first in PBS and subsequently in distilled water, the grids were stained with 5% uranyl acetate for 5 min, and examined on a Hitachi H7000 electron microscope. As a control, normal rabbit serum or PBS was substituted for polyclonal antiserum to N24.

*Conventional electron microscopy*

Small fragments of each tissue were fixed in 2% PFA-1% GA in 0.1M PB, postfixed in 1% osmium tetroxide in 0.1M PB, and embedded in epoxy resin according to standard procedures. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined using a Hitachi H7000 electron microscope operated at 75 kV.

*Olfactory rosettectomy experiment*

Forty eight fish were anaesthetized with ethyl m-aminobenzoate methanesulfonate, and then the right olfactory rosette including the olfactory epithelium was excised using a electric haemostat (Micro 1D; Mizuho, Tokyo, Japan) from the olfactory pit. After being excised, the olfactory pit was sealed with gelatin. The olfactory rosettectomized fish were returned to indoor tanks supplied with a continuous flow of a spring water at ambient temperature and photoperiod. The olfactory bulbs (3 male and 3 female) were prepared for the sAB method on 0, 1, 3, 5, 7, 11, and 15 days following the olfactory rosettectomy. Horizontal sections were used in this experiment.

## RESULTS

*The olfactory epithelium*

Figure 1 shows localizations of immunoreactivities for N24 using the sAB method (Fig. 1A) and the immunofluorescent method (Fig. 1C), Delafield's hematoxylin and eosin staining (Fig. 1B), and differential interference contract image (Fig. 1D) in the olfactory epithelium. Immunoreactivities for N24 were observed in the cytoplasm and the axon of the olfactory receptor cells (Fig. 1A, C). In particular, strong N24 immunoreactivities were detected in the dendrites of the olfactory receptor cells (Fig. 1C). In contrast, immunoreactivities for N24 were neither observed in the supporting cells nor the basal cells in the olfactory epithelium (Fig. 1A, C).

At the electron microscopic level, gold particles showing immunoreactivities for N24 were scattered in the cytoplasm of the dendrites of both ciliated (Fig. 2A) and microvillous (Fig. 2C) olfactory receptor cells. Some particles were concentrated on vesicular structures of 40 nm in size which were observed by means of the conventional electron microscopy (Fig. 2B, D). However, gold particles were rarely observed in the membrane of olfactory receptor cells. In control sections, no specific immunoreactive gold particles were seen in the olfactory epithelium.

*The olfactory bulb*

Figure 3 shows immunofluorescent localizations of immunoreactivities for N24 (Fig. 3A, C), and differential interference contrast image (Fig. 3B, D) in sagittal section of the olfactory bulb. The immunoreactivities for N24 were observed in the olfactory bulb where the olfactory nerve had penetrated (Fig. 3A). N24 immunoreactive axons were terminated at the glomerular layer near the mitral cells (Fig. 3C).

No prominent immunocytochemical differences between

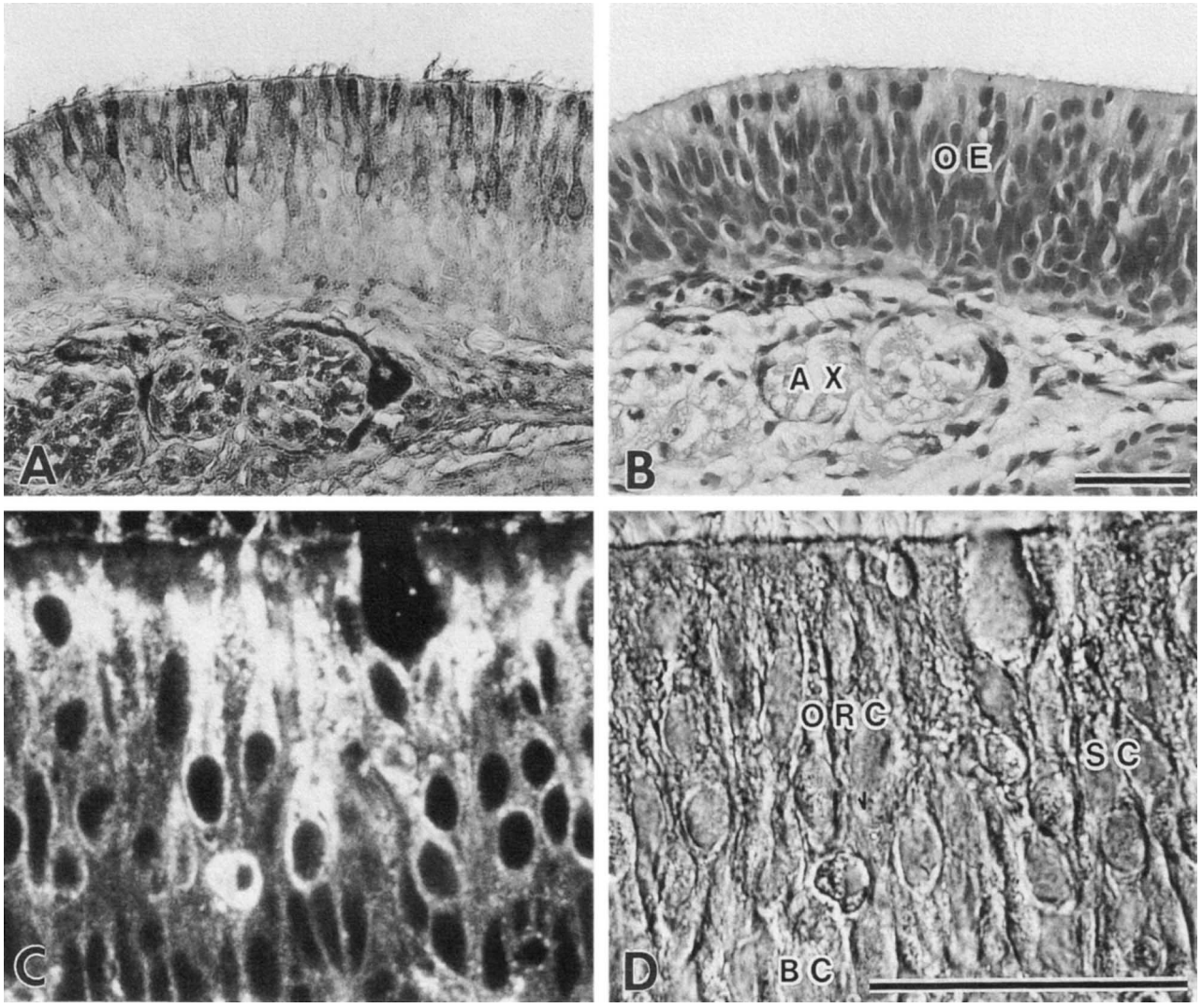


Fig. 1. Immunocytochemical localization of N24 in the olfactory epithelium of kokanee salmon using the sAB method (A) and Delafield's hematoxylin and eosin (B). Indirect immunofluorescent localizations of N24 (C), and differential interference contrast image (D) using a confocal laser scanning microscopy. AX, axons of olfactory receptor cells; BC, basal cell; OE, olfactory epithelium; ORC, olfactory receptor cell; SC, supporting cell. Bar, 50 μm.

male and female were observed in the olfactory system.

*The olfactory rosettectomy*

Figure 4 shows changes in N24 immunoreactivity using the sAB method following olfactory rosettectomy. Initially (day 0), the immunoreactivites for N24 in the horizontal sections of the olfactory bulb were observed throughout the olfactory nerve and glomerular layer (Fig. 4A). On the fifth day after the olfactory rosettectomy, N24 immunoreactivity in the right olfactory bulb began to decrease (Fig. 4B). On the seventh and eleventh day after the olfactory rosettectomy, N24 immunoreactivity in the right olfactory bulb decreased gradually, and degeneration of the olfactory nerve was observed (Fig. 4C, D). On the fifteenth day after the olfactory rosettectomy, N24 immunoreactivity in the right olfactory bulb had completely disappeared (Fig. 4E). In contrast, N24 immunoreactivity in the left olfactory bulb showed no prominent changes during this experiment (Fig. 4A-E). The olfactory rosettectomy did not show any cytological differences between male and female.

## DISCUSSION

The present study provides detailed immunocytochemical information on a salmonid olfactory system-specific protein (N24) in kokanee salmon. The distribution of N24 immunoreactive axons in the olfactory bulb are generally consistent with results of tracing experiments using cobaltlysine or horseradish peroxidase methods in other salmonids (Bazer *et al.*, 1987; Riddle and Oakley, 1992). It seemed reasonable to suppose that these axons were primary projections from the olfactory receptor cells in the olfactory epithelium (Satou, 1992).

The present immunocytochemical localizations of N24 in

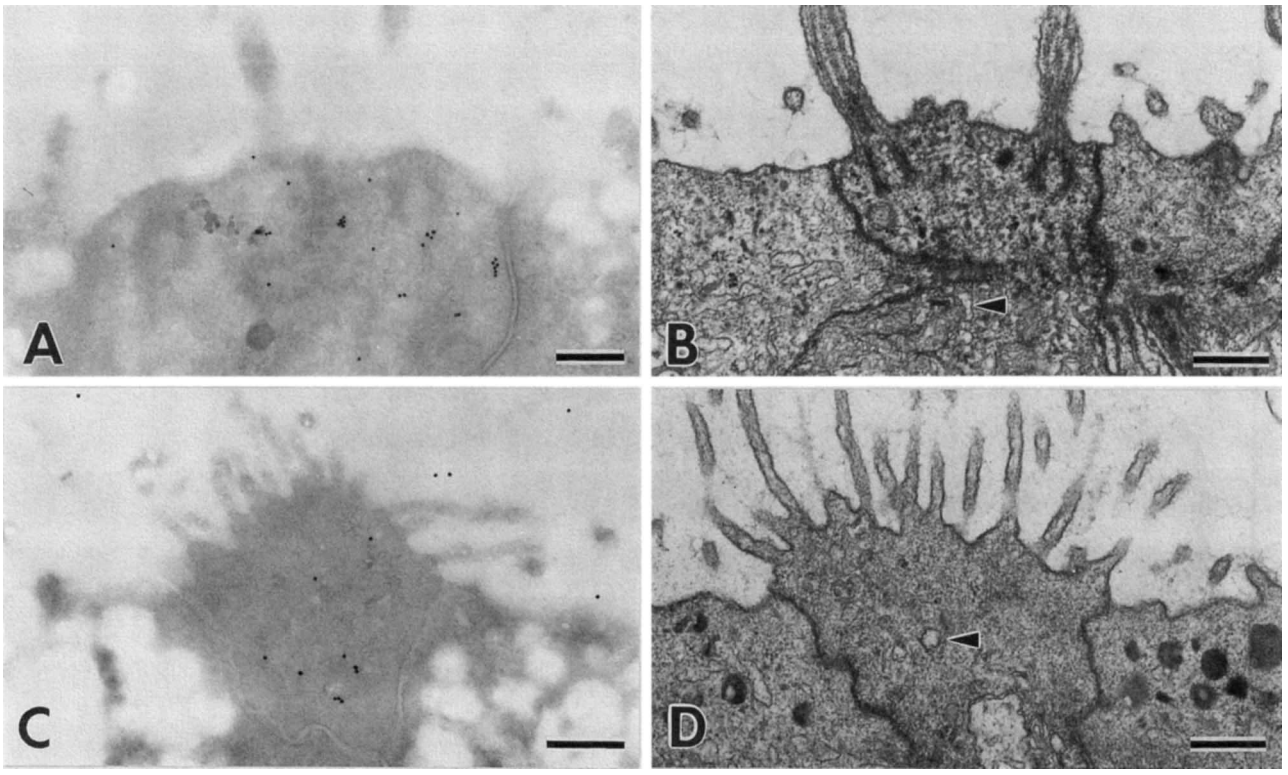


Fig. 2. Immunoelectron microscopical demonstration of N24 (A, C) and conventional electron microscopic observation (B, D) in the ciliated (A, B) and microvillous (C, D) olfactory receptor cells of kokanee salmon. Arrowheads indicate vesicular structures. Bar, 500 nm.

the olfactory system seem to be similar to the olfactory marker protein (OMP) immunoreactive sites in the olfactory system of rainbow trout (Riddle and Oakley, 1992). However, the molecular weights of N24 and OMP are about 24 kDa and 20 kDa, respectively, by SDS-polyacrylamide gel electrophoresis (Margolis, 1972; Shimizu *et al.*, 1993), so antigenic recognition polypeptide bands of N24 and OMP could be different in the olfactory system.

Two morphologically distinct olfactory receptor cell types, ciliated and microvillous olfactory receptor cells, exist in teleosts (Zeiske *et al.*, 1992). Gold particles showing immunoreactivites for N24 were observed in both cell types. Previous studies suggested that both olfactory receptor cells have separate olfactory functions; the microvillous olfactory receptor cell may play a major role in the chemical stimulus detection of socially-relevant odors and the ciliated olfactory receptor cell may play a role in feeding, recognition of the environmental conditions, and other olfaction (Wysocki and Meredith, 1987; Zielinski and Hara, 1988, 1992). N24 was involved in olfactory functions in common with the ciliated and the microvillous olfactory receptor cells.

Previous ultrastructural studies of the olfactory epithelium of rainbow trout following unilateral olfactory nerve section indicated that the olfactory nerve and receptor cell were degenerating seven days after the section, and had disappeared on the eighteenth and twenty-sixth day after this section in the olfactory epithelium (Zielinski and Hara, 1992). In the olfactory rosettectomy experiment, N24 immunoreactivity in the olfactory nerve of rostral olfactory bulb had vanished fifteenth day after the excision of the olfactory rosette. These results indicate that rapid enzymatic breakdown of N24 dose not occur in the olfactory bulb, and the time courses of actions of N24 may be the long-term type in the olfactory system. The long-term actions are representative for neuromodulators.

In the present study, N24 immunoreactivities were not localized in the membrane of olfactory receptor cells, indicating that N24 does not correlate with the membranous members of the olfactory signal transduction cascade molecules (the olfactory receptor, $G_{olf}$, type III adenylate cyclase, cAMP gated channel; Reed, 1992). In mammals, the olfactory epithelium has recently been shown to possess high concentrations and unique types of biotransformation enzymes (Banger *et al.*, 1993; Ben-Arie *et al.*, 1993). These enzymes in the olfactory epithelium have been proposed to underlie the termination of odorant signals, as well as the protection of the olfactory receptor cells against airborne toxic compounds (Burchell, 1991). In fact, these enzymes functioning as neuromodulators were expressed in the olfactory epithelium in rainbow trout (Monod *et al.*, 1994; Starcevic and Zielinski, 1995). N24 might also be linked to the olfactory signal transduction, as a neuromodulator.

Immunoreactivities for N24 were observed in the perikaryia of the olfactory receptor cells. This suggests that

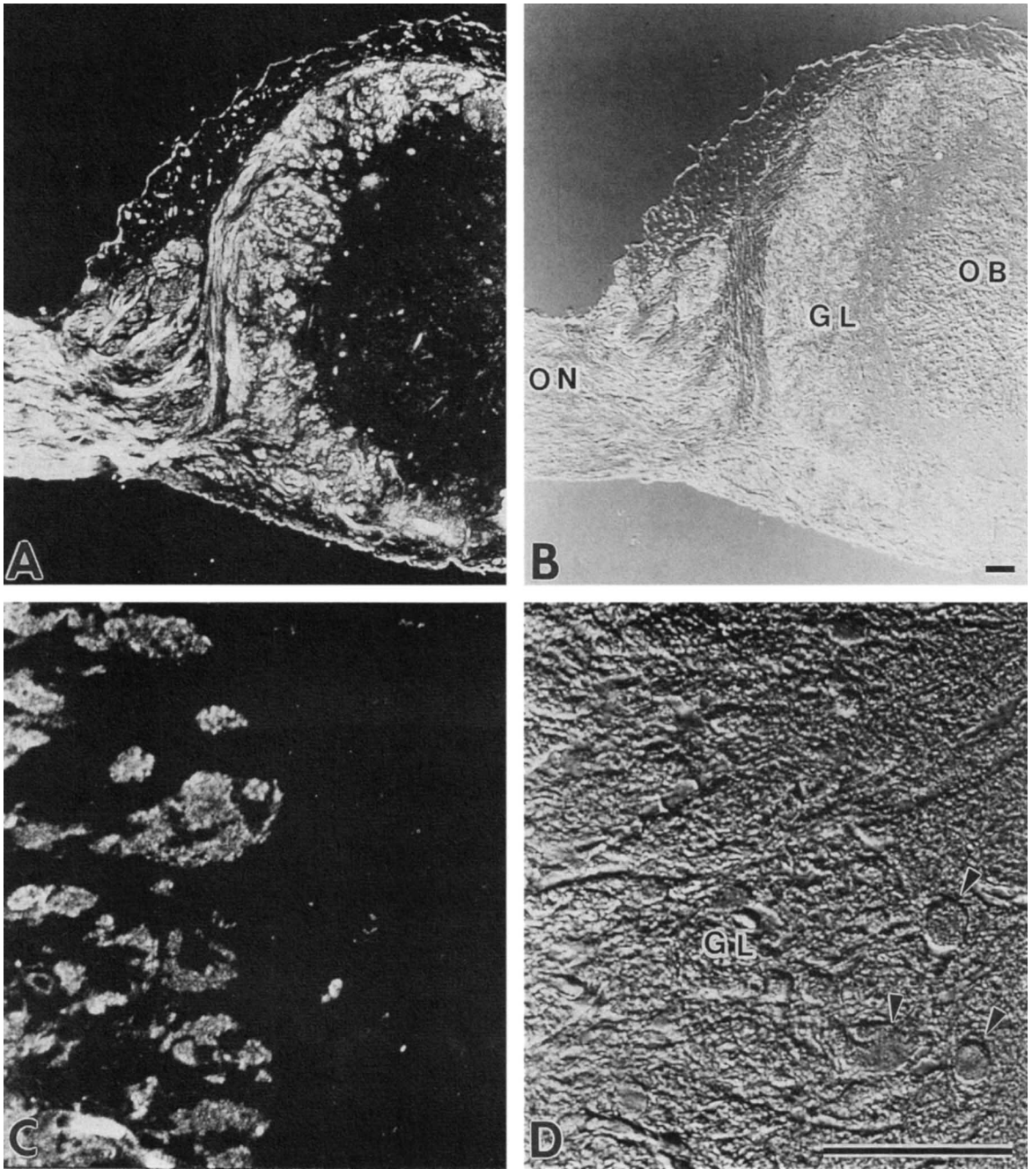


Fig. 3. Indirect immunofluorescent localizations of N24 (A, C) and differential interference contrast image (B, D) in the olfactory bulb of kokanee salmon using a confocal laser scanning microscopy. GL, glomerular layer; OB, olfactory bulb; ON, olfactory nerve. Arrowheads indicate the mitral cells. Bar, 50 μm.

N24 is produced in the olfactory receptor cells, and transported to both dendrites and axons. However, the expression of the N24 encoding gene in the olfactory receptor cell remains to be established by means of an *in situ* hybridization technique. Studies are now underway in our laboratory investigating the cDNA cloning of N24. N24 is confirmed to be a useful molecular marker for studying the olfactory functions in salmonids. Further studies would reveal cytophysiological changes in the olfactory system during salmon migration, especially at the times of imprinting and homing.

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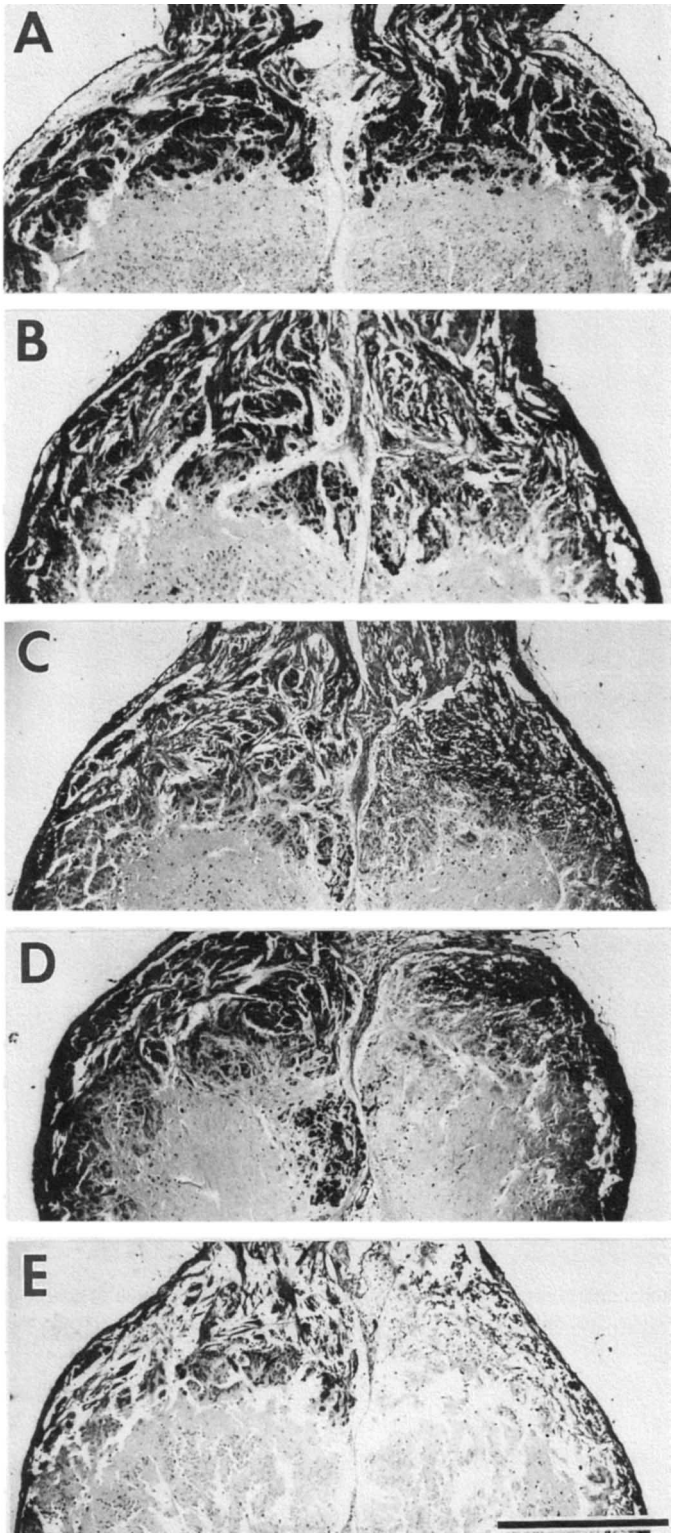


Fig. 4. Immunocytochemical changes in N24 immunoreactivity in the olfactory bulb. Horizontal section through the olfactory bulb 0 (A), 5 (B), 7 (C), 11 (D) and 15 (E) days after olfactory rosettectomy. Bar, 500 μm.

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