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ORIGINAL ARTICLE

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Expression of insulin-like growth factor family in the rat olfactory epithelium

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Abstract The goal of this study was to determine the cellular sites of insulin-like growth factor (IGF) family expression in the rat olfactory epithelium. By RT-PCR analysis, mRNAs of IGF-I, II, IGF-I receptor (IGF-IR), and IGF binding proteins (IGFBPs) 2, 3, 4, 5, and 6 were found to be expressed in the olfactory mucosa. Immunoreactivity for IGF-IR was restricted to a subset of olfactory receptor cells whose cell bodies were situated in the basal region of the olfactory epithelium. Intense IGF-I immunoreactivity was detected in the supporting cells, whereas IGF-II immunoreactivity was observed in the lamina propria, but not in the epithelium. Immunoreactivities for IGFBP-2, IGFBP-3, and IGFBP-6 were detected in olfactory receptor cells. In addition, axon bundles in the lamina propria displayed an intense reaction for IGFBP-6. IGFBP-4 immunoreactivity was restricted to the apex of the olfactory epithelium. Intense IGFBP-5 immunoreactivity was observed in Bowman's glands. These results suggest that IGF-I is secreted from supporting cells and affects its receptor- expressing olfactory cells. IGFBPs may modulate IGF-I activity via their production by Bowman's glands, olfactory cells, and supporting cells themselves.

Keywords IGF family · Immunohistochemistry · Olfactory epithelium · RT-PCR

Introduction

In the mammalian olfactory epithelium (OE), the olfactory receptor neurons (ORNs) continually die and are replaced by their progenitor, globose basal cells. A large number of growth factors play various roles in the dynamic regulation of neurogenesis, differentiation, and survival. The insulin-like growth factor (IGF) family comprises growth factors (IGF-I, IGF-II), their receptors (IGF-I receptor, IGF-II receptor), and their binding proteins (IGFBPs 1–6). IGF-I is known to stimulate proliferation of the OE cells in organotypic cultures from E19 rats (Farbman and Buchholz 1996). Moreover, when IGF-I was infused into the external naris of newborn rats, the numbers of proliferating cells in the OE and the thickness of the epithelium increased (Pixley et al. 1998). As detected by in situ hybridization, several members of the IGF family are known to be expressed in the olfactory mucosa; e.g., IGF-I mRNA was detected in the OE, whereas IGF-II mRNA was expressed in the lamina propria in the E18 rat fetus (Ayer-LeLievre et al. 1991). Bondy and Lee (1993) showed that IGF-I mRNA was lightly expressed in the olfactory mucosa of the newborn rat. In the E19 chicken embryo, IGF-IR mRNA was widely detected in the entire OE (Holzenberger and Lapointe 2000). Bondy and Lee (1993) also indicated that IGFBP-2 mRNA was heavily expressed and IGFBP-5 mRNA was lightly expressed in the olfactory mucosa of the newborn rat. As these in situ hybridization studies were done by an autoradiographic technique, the identity of the cell types expressing these IGFs is not known. Only one report using an antibody specific for the extracellular domain of the IGF-IR clarified that the antibody labeled a subset of rat ORNs (Pixley et al. 1998).

The OE consists of several cell types. Supporting cells have an apically placed cell body with a thin basal process extending to the basement membrane. ORNs have a small cell body situated in the middle of the OE and extend a single apical dendrite to the luminal surface and axon through the basement membrane into the lamina propria where axons bundle together into fascicles. Lying directly adjacent to the basement membrane are horizontal basal cells identifiable by their flattened appearance. Directly above the horizontal basal cells are the globose basal cells, which have a more rounded appearance. Bowman's gland ducts penetrate through the OE, and their acini are present in the lamina propria. In

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the present study, we examined the site of expression of IGF-I, II, IGF-IR, and IGFBPs in the adult rat olfactory mucosa by using immunohistochemistry and RT-PCR.

Materials and methods

Adult Wistar rats (98–240 g) were obtained from Sankyo Lab (Tokyo, Japan), and they were housed in polypropylene cages, kept in a room with a 12:12 h light-dark cycle and provided food and water ad libitum. The animals were killed by an overdose of Nembutal (Abbott Labs, North Chicago, Ill., USA) given by intraperitoneal injection. The heads were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) or with periodate-lysine-paraformaldehyde (PLP) for 4–6 h at 4°C. After decalcification, the paraformaldehyde-fixed specimens were embedded in paraffin and sectioned coronally at a thickness of 10 μm. PLP-fixed specimens were cryoprotected with 25% sucrose, embedded in O.C.T. compound (Tissue-Tek, Miles, Elkhart, Ind., USA), and frozen in a spray freezer (Oken, Tokyo, Japan). The tissues were then sectioned and placed on silane-coated slides. A chicken antibody specific for the α-chain of the IGF-IR, and monoclonal antibodies against IGF-I, and IGF-II were purchased from Upstate Biotechnology (Lake Placid, N.Y., USA). Goat polyclonal antibodies specific for IGFBP-1, -2, -3, -4, -5, and -6 were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). The paraformaldehyde-fixed, paraffin-embedded sections were incubated with either anti-IGF-IR or IGF-I antibodies (1:50 dilution) for 1 h at 37°C. The PLP-fixed, cryosections were incubated with either anti-IGF-II or one of the IGFBP antibodies (1:50 dilution) for 1 h at 37°C, and then stained with labeled streptavidin-biotin (LSAB kit, Dako, Kyoto, Japan). For IGF-IR, indirect staining using rabbit anti-chicken IgY HRP conjugate (Upstate) was performed. The immunoreactive product was visualized by use of diaminobenzidine (DAB). As a negative control, PBS was used instead of the primary antibody.

For RT-PCR analysis, the olfactory mucosa was dissected from adult rats, and total RNA was isolated by using TRIzol reagent (Life Technologies, N.Y., USA). RNA (5 μg) from each tissue was reverse-transcribed to cDNA by using oligo (dT) primers (Life Technologies), and the resulting cDNA was subjected to 35 cycles of PCR with a PCR kit (Quiagen, Tokyo, Japan). The primers used were the following: IGF-I receptor, 5'-CGGCATTGACATCCGCAACG-3' and 5'-GGCAGACGTCACAGAATCGAT-3' (939-bp fragment, 150–1089, which includes the α-subunit coding region; accession number M27293); IGF-I, 5'-GTGGACGCTCTTCAGTTCGT-3' and 5'-GCTTCCTTTTCTTGTGTGTCGATAG-3'(261-bp fragment, 202–463, M15480); IGF-II, 5'-ACACCTGGAGACAGTCCGCG-3' and 5'-GGTCTTTGGGTGGTAACACG-3'(146-bp fragment, 394–540, M29880); IGFBP1, 5'-CTGTCCTTCCAGGTTCGCGT-3' and 5'-AAATGGTGTGCTCCGGAGTAT-3'(848-bp fragment, 205–1053, NM013144); IGFBP2, 5'-TCACACTATGTCCCATATGCCG-3' and 5'-GCCTCAAACTGCCATATCCG-3'(548-bp fragment, 81–629, M58559); IGFBP3, 5'-GTCCCCAGCACAVATCGCG-3' and 5'-TCTTTTGTGCAAAATAAGGCGTA-3'(471-bp fragment, 519–990, NM012588); IGFBP4, 5'-TCCACCCCCATTTATCGAC-3' and 5'-CCCACCCAACACCCGGTACG-3' (620-bp fragment, 3694–4294, L08276); IGFBP5, 5'-AACGAAAAGAGCTACGGCGA-3' and 5'-TGAAAGTCCCCATCGACGTAC-3' (481-bp fragment, 851–1332, NM012817); and IGFBP6, 5'-TAATGCTGTTGTTCGCTGCG-3' and 5'-CAGCAGGGACCACGGCGATT-3' (552-bp fragment, 49–601, NM013104). The PCR products were electrophoresed through a 1% agarose gel and visualized by ethidium bromide staining.

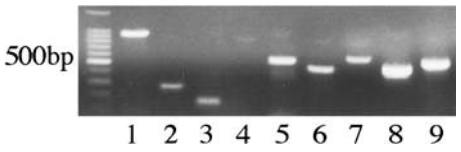

Fig. 1 RT-PCR for insulin-like growth factor (IGF) family expression in rat olfactory mucosa. The samples were amplified for 35 cycles. *Lane 1*: IGF-IR (939 bp), *lane 2*: IGF-I (261 bp), *lane 3*: IGF-II (146 bp), *lane 4*: IGFBP-1(848 bp), *lane 5*: IGFBP-2 (548 bp), *lane 6*: IGFBP-3 (471 bp), *lane 7*: IGFBP-4 (620 bp), *lane 8*: IGFBP-5 (481 bp), *lane 9*: IGFBP-6 (552 bp). Transcripts of IGF-IR, IGF-I, IGF-II, and IGF-binding protein (IGFBP) 2, 3, 4, 5, 6 are detectable. The estimated sizes for these PCR amplimers match their expected sizes. The first lane shows a100-bp molecular weight ladder

Results

When RT-PCR for 35 amplification cycles was performed on the olfactory mucosa of adult rats, amplified products of the expected sizes were found for IGF-IR, IGF-I, -II, IGFBP-2, -3, -4, -5 and –6. IGFBP-1 mRNA was not detected in the olfactory mucosa (Fig. 1).

Sections of adult rat olfactory mucosa were immunoreacted with anti-IGF-IR, IGF-I, IGF-II, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5, or IGFBP-6 antibodies. IGF-IR immunoreactivity was observed in the ORN subpopulation both in the nasal septum and turbinates. The distribution of the reactive ORNs within the nasal cavity was uniform (Fig. 2A). At higher magnification, immunoreactive cells were observed in a subset of the ORN, and their cell bodies were located in the basal region of the OE. The dendrites of IGF-IR immunoreactive cells had reached the lumen of the nasal cavity (Fig. 2B). IGF-I immunoreactivity was densest in the supporting cells, in which the apical cytoplasm was strongly immunoreactive (Fig. 2C). IGF-II immunoreactivity was observed in the lamina propria, but not in the epithelium (Fig. 2D).

Immunoreactivity for IGFBP-1 was not detected in the olfactory mucosa (Fig. 3A). The lighter, diffuse staining seen in the OE represents non-specific background staining because it was also observed in control specimens treated with PBS instead of the antibodies (not shown). Immunoreactivity for IGFBP-2 was found in the ORNs and was intense in the more apically situated cells. Moreover, ORN dendrite and dendritic knobs were apparently immunoreactive (Fig. 3B). IGFBP-3 immunoreactivity was observed in the ORNs. The apical cytoplasm of supporting cells was weakly reactive (Fig. 3C). Strong IGFBP-4 immunoreactivity was observed at the apex of the OE, including dendritic knobs, cilia of ORNs, and microvilli of supporting cells (Fig. 3D). IGFBP-5 immunoreactivity was observed in the ducts and acini of Bowman's glands. In addition, low-level immunoreactivity was detected in supporting cells and possibly in ORNs (Fig. 3E). IGFBP-6 immunoreactivity was observed in the ORNs and their axons in

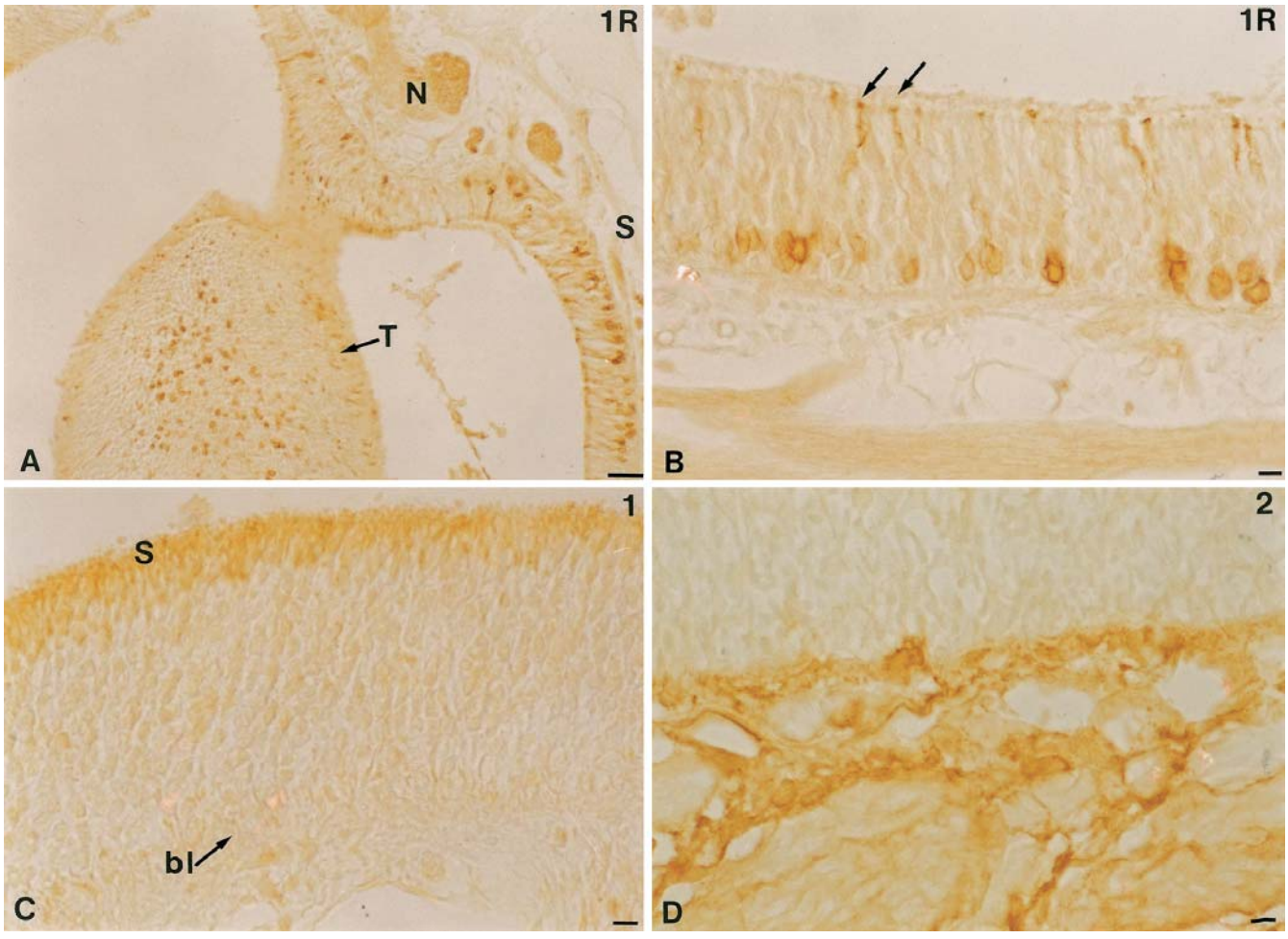

Fig. 2A–D Photomicrographs of coronal sections through the rat nasal cavity. Expression of IGF-IR (**A**, **B**), IGF-I (**C**), and IGF-II (**D**) detected by immunohistochemical means. **A–C** 4% paraformaldehyde-fixed, paraffin sections; **D** periodate-lysine-paraformaldehyde (PLP)-fixed, cryo-sections. **A** IGF-IR reactive cells scattered through the OE in the nasal septum (*S*) and turbinates (*T*). Nerve bundles (*N*) are also immunoreactive. **B** Higher magnification shows strongly labeled cells are basally located olfactory receptor neurons (ORNs). IGF-IR immunoreactivity is also present in the ORN dendrites and dendritic knobs (*arrows*). **C** IGF-I immunoreactivity is present in the supporting cells (*S*). *bl* basal lamina. **D** IGF-II immunoreactivity is found in the lamina propria. *Bars*: 50 μm in **A**, 10 μm in **B–D**

the lamina propria. Basally situated ORNs were more intensely stained than the apically situated ones. The apical cytoplasm of the supporting cells was also stained (Fig. 3F). Moreover, antibodies used in the present study did not stain the cells that had features morphologically consistent with those of basal cells.

Discussion

In the present study, IGF-IR, IGF-I, IGF-II, and IGFBPs 2–6 were expressed in the adult rat olfactory mucosa as determined by both RT-PCR and immunohistochemistry. IGFBP-1 was not expressed in the olfactory mucosa as judged by either RT-PCR or immunohistochemistry, though it was reported to be localized in dividing liver cells (Mohn et al. 1991). Although the presence of mRNAs of IGF-IR, IGF-I, IGF-II, IGFBP-2, and IGFBP-5 in the olfactory mucosa has already been detected in embryonic and newborn rats and chicken (Ayer-LeLievre et al. 1991; Bondy and Lee 1993), the present study revealed their expression also in adult rat specimens. The immunohistochemical distribution for IGF-IR is in agreement with that reported by Pixley et al. (1998), i.e., IGF-IR positive cells were basally-located ORNs, not the neuronal precursor, globose basal cells. The ligand for IGF-IR, IGF-I, was detected in the supporting cells. It may promote the differentiation of olfactory cells, although a role for IGF-I in the olfactory epithelium was reported to be proliferation of the globose cells (Pixley et al. 1998). In fact, in other neural tissues, IGF-I stimulates mitosis in sympathetic neuroblasts (DiCiccico-Bloom and Black 1988), and promotes neurite outgrowth in sensory, motor and sympathetic neurons (Aizenman and de Vellis 1987). It was reported that the activity of IGF-II was also mediated via IGF-IR (Holzenberger and Lapointe 2000), but their co-localization was not observed in the olfactory mucosa.

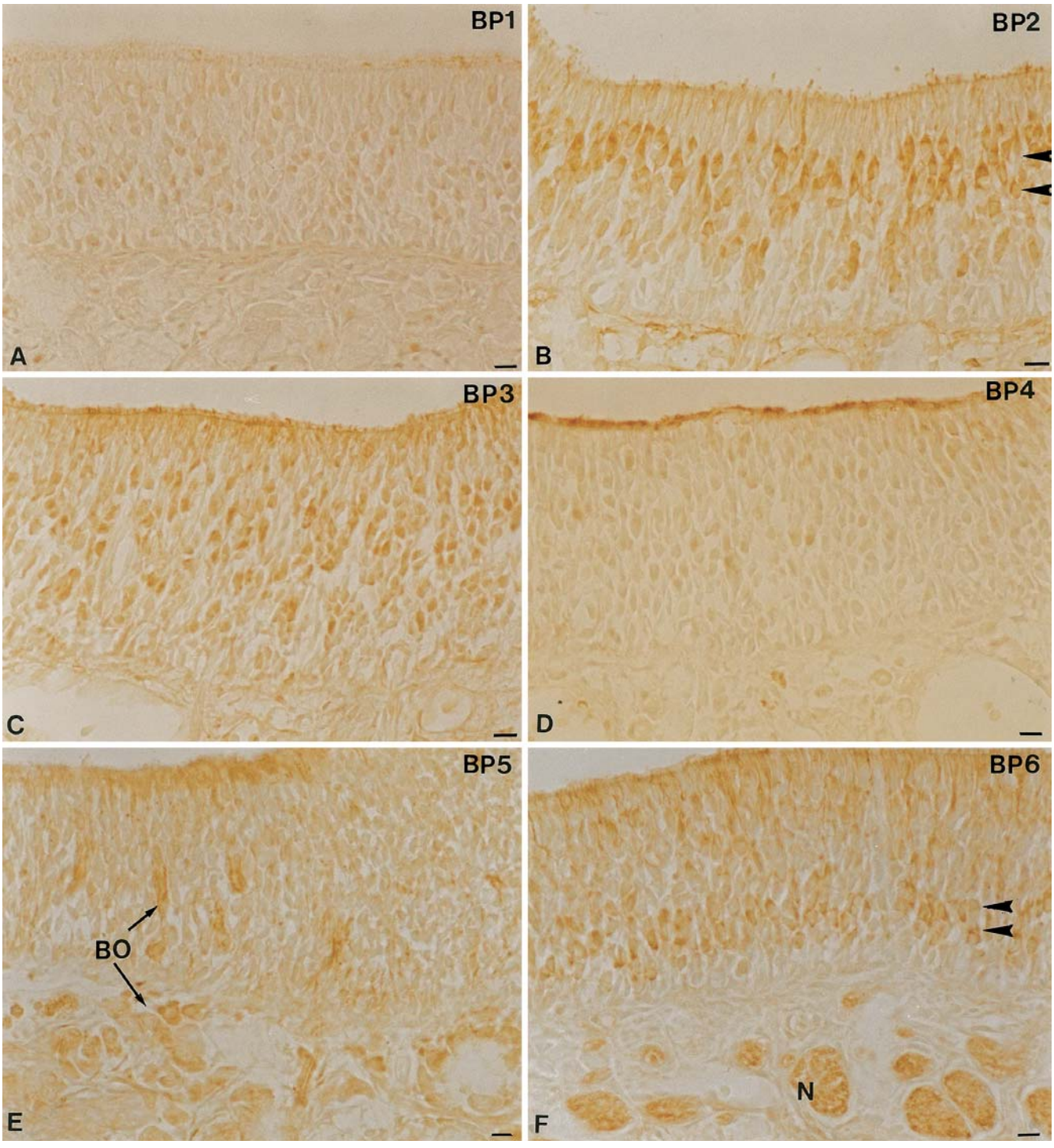

Fig. 3A–F Photomicrographs of coronal sections through the rat nasal cavity. Expressions of IGFBP-1 (**A**), IGFBP-2 (**B**), IGFBP-3 (**C**), IGFBP-4 (**D**), IGFBP-5 (**E**), and IGFBP-6 (**F**) examined by immunohistochemical means. PLP-fixed, cryosections. **A** IGFBP-1 immunoreactivity is absent. **B** IGFBP-2 immunoreactivity is seen in the ORNs situated more apically (*arrowheads*). **C** IGFBP-3 immunoreactivity is seen in the ORNs and apical cytoplasm of supporting cells. **D** IGFBP-4 immunoreactivity is restricted to the apex of the OE. **E** IGFBP-5 immunoreactivity is seen in Bowman's glands (*BO*) and apical cytoplasm of supporting cells. **F** IGFBP-6 immunoreactivity is seen in ORNs situated more basally (*arrowheads*). Nerve bundles (*N*) and apical cytoplasm of supporting cells are also immunoreactive. *Bars*: 10 µm

Differential labeling of two populations of ORNs was observed: IGFBP-2 was intensely expressed in more apically situated ORNs, whereas IGFBP-6 was expressed in more basally located ones. Strotmann et al (1994, 1996) showed that, based on odorant-receptor expression, a definite vertical zonal organization of ORNs exists. Each laminar zone contains ORNs that express a distinct group of odorant receptors. On the other hand, it is well known that GAP-43-positive ORNs are immature, more basally located ORNs; whereas olfactory marker protein-positive ORNs are mature neurons, which occupy the

middle layer of the OE (e.g., Schwob et al. 1995). The vertical distribution of IGFBP-2- and IGFBP-6-expressing ORNs may reflect intrinsic differences in cell populations or age-related processes.

IGF-IR-expressing ORNs extended their dendrites to the lumen, where they had access to the nasal mucus, whose contents may promote the differentiation or survival of these cells. In support of this function of mucus is the evidence that mucus contains dopamine (Lucero and Squires 1998), which was shown to induce the differentiation of ORNs and olfactory cell lines (Coronas et al. 1997; Feron et al. 1999). The olfactory secretions found on the surface are derived mainly from Bowman's glands, with a few from supporting cells and none from ORNs (Getchell et al. 1984; Farbman 1992). Recently, it was reported that Bowman's glands display intense immunostaining for glial cell line-derived neurotrophic factor (GDNF), suggesting secretion of this factor to the luminal surface (Buckland and Cunningham 1999). In the present study, the presence of IGFBP-5 immunoreactivity in the duct and acini of Bowman's glands would indicate that this binding protein contributed largely to the olfactory mucus. The presence of IGF-I, and IGFBP-3, -5 and -6 immunoreactivities in the supporting cells also suggests that these proteins become components of mucus. In addition the IGFBP-4 found at the apex of the OE may also become a component of mucus. In the case of humans, IGF-I and IGFBP-2, -3, and -4 were detected in the olfactory mucus by Western blot analysis, suggesting their secretion from the supporting cells and/or Bowman's glands (Federico et al. 1999). Since the IGFs are complexed with high-affinity IGFBP to target the IGF action to specific cell addresses (Bondy and Lee 1993; Roghani et al. 1991), IGFBPs from supporting cells, olfactory cells, and Bowman's glands bind IGFs in both autocrine and paracrine manners.

IGFBP-6 is unique among the IGF-binding proteins in that its affinity for IGF-II is much greater than that for IGF-I (Roghani et al. 1991). Immunoreactivity for IGFBP-6 was detected in the olfactory axon bundles in the lamina propria where IGF-II immunoreactivity was also found. Moreover, it is likely that IGFBP-6 is transported via axons to the olfactory bulb, where IGFs are known to promote growth (Bondy and Lee 1993).

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