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Differential Spatial and Temporal Expression of Two Type III Intermediate Filament Proteins in Olfactory Receptor Neurons

James D. Gorham,* Edward B. Ziff,* and Harriet Baker†
*Howard Hughes Medical Institute
Department of Biochemistry
and Kaplan Cancer Center
New York University Medical Center
New York, New York 10016
†Laboratory of Molecular Neurobiology
The Burke Rehabilitation Center
Cornell University Medical College
White Plains, New York 10605

Summary

Olfactory receptor neurons (ORNs) do not express the typical neuronal intermediate filament proteins (IFPs), the neurofilament triplet proteins. Immunocytochemical evidence shows that ORNs coexpress vimentin and peripherin but distribute them differently. Specifically, ORNs contain vimentin in dendrites, cell bodies, and axons, but not in terminals in glomeruli; peripherin is present in axons, but excluded from dendrites, cell bodies, and terminal glomeruli. In adult rats, ORN axon fascicles are variably stained with antisera for peripherin; in juvenile rats, staining of fascicles is uniform. Staining with antibody to vimentin is uniform in both adult and juvenile ORN axon fascicles. The unusual pattern of IFP expression and intracellular sorting may have implications for the unique plastic and regenerative capacities of these neurons.

Introduction

Olfactory receptor neurons (ORNs) are unique among mammalian neuronal populations in that they undergo continual neurogenesis, replacing dying neurons from stem cells and reestablishing connections within the CNS (Graziadei and Monti Graziadei, 1978). A differentiated ORN is bipolar, with its cell body nestled in a specialized columnar epithelium, the olfactory epithelium, located high in the nasal vault. Its single dendrite extends toward the nasal cavity, while its unmyelinated axon penetrates the epithelial basal lamina, traverses mesenchymal tissue, enters the cranium via foramina in the cribriform plate, and terminates within the olfactory bulb (OB) of the CNS in a tufted structure called a glomerulus. To establish the appropriate connections, therefore, the axon must pass through several different tissues, penetrate the CNS, and accurately identify the correct second order neurons. The ability of ORNs, but not other kinds of neurons, to repopulate and reinnervate is probably mediated in part by the unique molecular components expressed by these special neurons. Indeed, several proteins have been identified in ORNs in adults that are either unique to them (Jones and Reed, 1989; Margolis, 1988) or are expressed in other neuronal types only during development (Schwob et al., 1986; Schoenfeld et al., 1989; Miragall et al., 1988; Verhaagen et al., 1989).

Intermediate filaments (IFs) are 10 nm cytoskeletal filaments of largely undetermined function, which radiate from the nuclear membrane to the cytoplasmic surface (for review, see Steinert and Roop, 1988). Although different cell types contain morphologically similar IFs, these IFs are formed from subunit proteins (IFPs), which are expressed in tissue-specific patterns. The various IFPs share a basic similarity in protein structure: a well-conserved α helical "rod" domain thought to be intimately involved in filament formation is flanked by two "variable" domains, the "head" and "tail," presumed to be important for tissue-specific functions. Classifications for the subunit IFPs have been defined on the basis of similarities of primary sequence and gene structure (Steinert and Roop, 1988). The acidic and basic keratins comprise the type I and II IFPs, respectively, and are exclusive to cells of epithelial lineage. The type III IFPs include vimentin, desmin, and the glial fibrillary acidic protein (GFAP) and are expressed in cells in the mesenchymal, myogenic, and glial lineages, respectively. Neurons in general express the type IV IFPs, known as the neurofilament triplet proteins (NFTPs), NF-L, -M, and -H, for low, middle, and high molecular weights, respectively (Steinert and Roop, 1988).

Recent work has identified a novel type III IFP, peripherin, which is expressed exclusively in a limited set of neurons (for review, see Greene, 1989). Specifically, this protein is expressed in all neurons derived from the neural crest (i.e., peripheral neurons), in all neurons derived from the neural tube, which project axons to the periphery (i.e., visceral and somatic motoneurons, cranial nerves), and in a subset of neurons whose domains lie entirely within the CNS (Leonard et al., 1988; Parysek and Goldman, 1988; Brody et al., 1989; Parysek et al., 1988). In the neuronal types thus far examined (Escurat et al., 1990; Troy et al., 1990; Gorham et al., 1990), expression is initiated in development at or around the time of terminal differentiation (axonogenesis) and continues thereafter. Neuronal precursor cells express the "mesenchymal" IFP vimentin transiently (Cochard and Paulin, 1984); vimentin expression ceases and is replaced by the expression of the NFTPs and, for some neurons, peripherin. During development, therefore, these neurons selectively eliminate the expression of one type III IFP and activate the expression of a more specialized type III IFP as well as the type IV IFPs. This transition from a "precursor" type III IFP (vimentin) to a more specialized type III IFP can be generalized to the development of muscle and glia as well (Tokuyasu et al., 1984; Pixley and de Vellis, 1984).

The IFP composition of ORNs has been a subject of

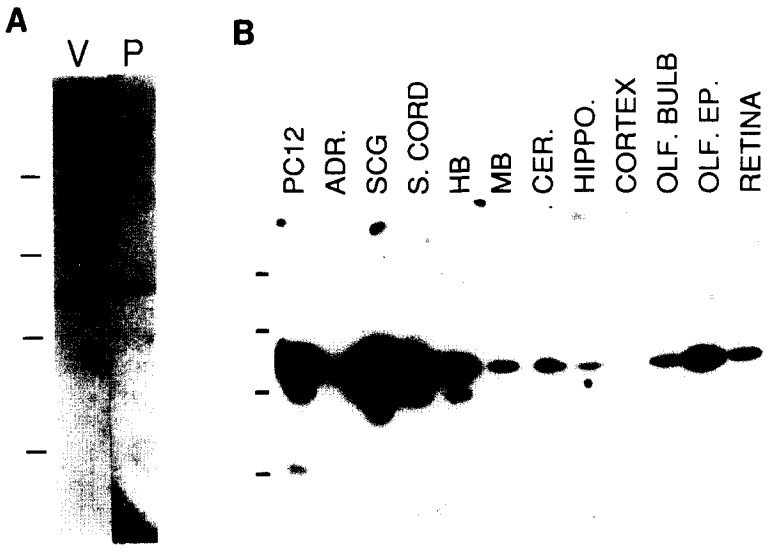

Figure 1. Western Blot Analysis Using Antibodies to Peripherin and Vimentin

(A) Protein extracts (25 μg) from OB were loaded in each lane. Following electrophoresis and transfer to a nitrocellulose filter, the filter was marked with ink and cut in half. Lane V was probed with anti-vimentin (V9), followed by ^{125}I-labeled anti-mouse IgG. Lane P was probed with anti-peripherin (anti-ppn), followed by ^{125}I-labeled protein A. The filter was reconstructed prior to autoradiography by lining up ink marks. (B) Protein extracts (25 μg) from various tissues were loaded in each lane, electrophoresed, transferred, and probed with anti-ppn, followed by ^{125}I-labeled protein A. PC12, PC12 cells (no nerve growth factor); ADR., adrenal gland; SCG, superior cervical ganglion; S. CORD, spinal cord; HB, hindbrain; MB, midbrain; CER., cerebellum; HIPPO., hippocampus; CORTEX, cerebral cortex; OLF. BULB, OB; OLF. EP., olfactory epithelium. Markers, 97.4, 66.2, 42.7, and 31.0 kd.

some controversy. Several reports have shown that ORNs are atypical in that they do not demonstrate expression of the NFTPs (Schwob et al., 1986; Vollrath et al., 1985; Ophir and Lancet, 1988; Talamo et al., 1989). Rather, it was reported that rat ORNs contain a protein, which, by immunochemical criteria, appears to be vimentin (Schwob et al., 1986). Recently, the detection of peripherin by immunocytochemistry in rat embryonic ORNs was reported (Escurat et al., 1990). Due to the high degree of sequence similarity between peripherin and vimentin (55% identity at the amino acid level; Leonard et al., 1988), it is not clear whether the two reports detected the same or different proteins. Furthermore, two other laboratories, each using independently generated antisera, have failed to detect peripherin in ORNs (Troy et al., 1990; Talamo et al., 1989).

The work described endeavors to determine whether ORNs express vimentin, peripherin, or both and, therefore, to resolve the discrepancies outlined above. As ORNs do not express the NFTPs, the function(s) of which may be important for cytoskeleton stabilization (Morris and Lasek, 1982), an understanding of the composition of IFs in these unique neurons may yield insights into their capacities for regeneration and plasticity.

Results

For peripherin analysis, an affinity-purified rabbit polyclonal antibody (anti-ppn) directed against a bacterially produced peripherin-containing fusion protein (Gorham et al., 1990) was employed. Previous studies demonstrated no cross-reactivity of this antibody with the other IFPs, including vimentin (Gorham et al., 1990). Vimentin was detected using a commercially available mouse monoclonal antibody (V9). In Western blot analysis of OB protein preparations (Figure 1A), both antibodies recognized single bands on SDS-PAGE. Although the proteins peripherin and vimentin have very similar molecular masses (55–58 kd), a difference in migration of the two bands in Figure 1A was detectable. The vimentin-reactive band migrated slightly more rapidly (lane V), consistent with previous findings (Huc et al., 1989; Parysek and Goldman, 1987). The antibodies employed for this study therefore recognize distinct proteins in OB.

Further Western blot analysis of a variety of brain regions confirmed the identity of the band recognized by anti-ppn in OB. The band in OB protein preparations comigrated with bands recognized in preparations from PC12 cells and a variety of CNS and PNS regions (Figure 1B). The lower molecular weight bands seen in some lanes probably represent products of degradation. Consistent with what is known about peripherin distribution (Leonard et al., 1988; Parysek and Goldman, 1988; Brody et al., 1989), anti-ppn detected high levels of protein in superior cervical ganglion, spinal cord, and hindbrain and lower quantities in the other neuronal regions investigated. The intensity of the peripherin-reactive band in OB was comparable with that seen in midbrain, cerebellum, or retina. A preparation of olfactory epithelium was strongly positive for peripherin, but dissection of this structure undoubtedly included significant levels of contamination from sympathetic and other peripheral fibers.

Immunocytochemical analysis of nonolfactory tissues confirmed the specificities of the IFP antibodies used. In a section through the hindbrain (Figure 2A), anti-ppn recognized neuronal cell bodies in the dorsal motor nucleus of the vagus as well as several fiber tracts, probably fasciculus cuneatus and tractus solitarius (arrows), consistent with the demonstration of peripherin expression in first order neurons (Leonard et al., 1988; Brody et al., 1989). In a similar section (Fig-

Figure 2. Anti-ppn and V9 Recognize Different Structures in Hindbrain

(A) Peripherin, stained by anti-ppn, is detected in neuronal cell bodies of the dorsal motor nucleus of the vagus (X) and in several fiber tracts, probably fasciculus cuneatus and tractus solitarius (arrows). (B) In an adjacent section, vimentin, stained by V9, is detected in ependymal cells (arrow) lining the 4th ventricle, as well as in vascular endothelial cells (arrowheads). Detection was by peroxidase with 3,3'-diaminobenzidine-tetrahydrochloride as substrate (see Experimental Procedures). Bar, 200 μm.

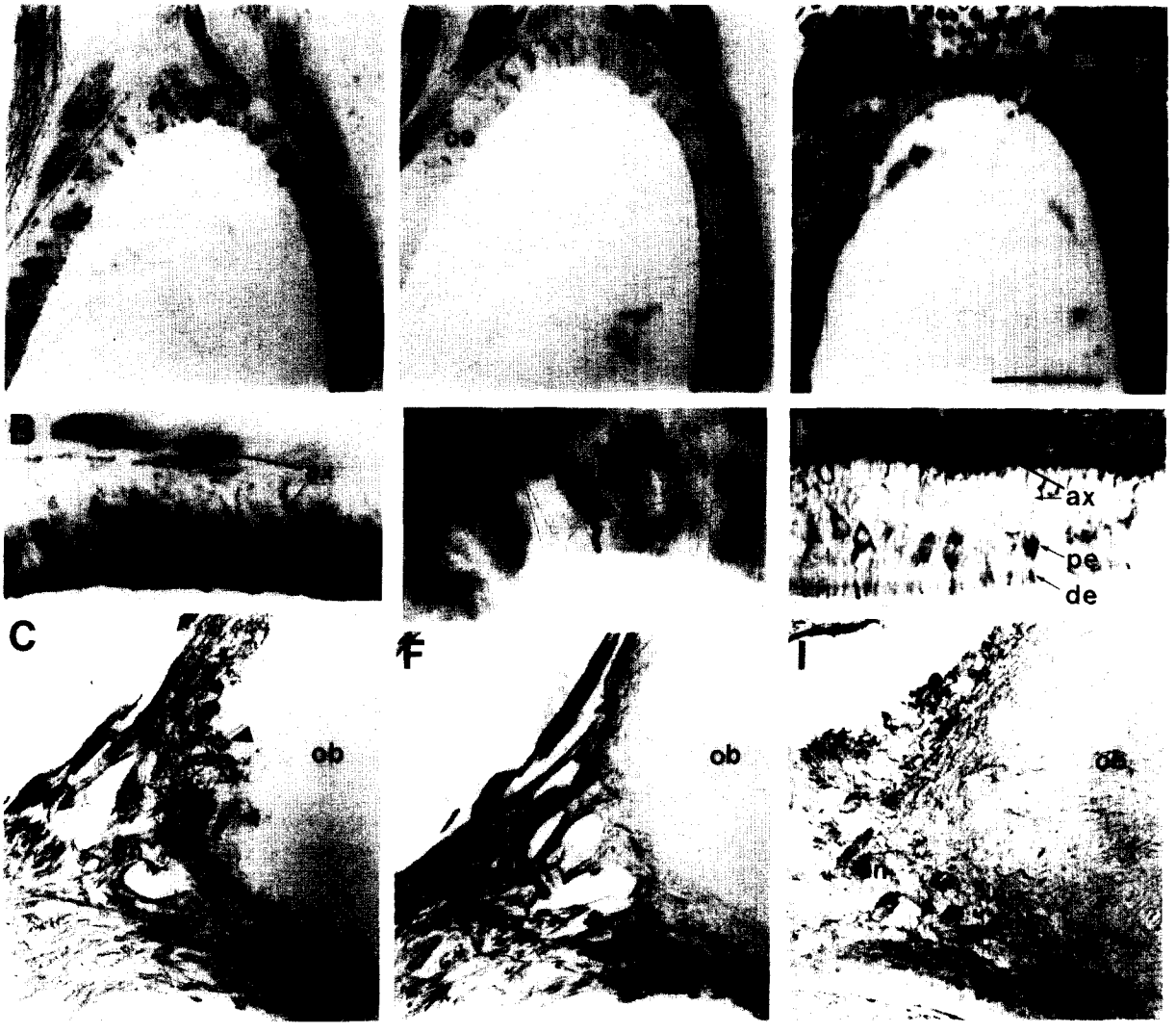

Figure 3. ORNs, Identified with an Antibody to OMP, Express Both Vimentin and Peripherin in Overlapping but Nonidentical Cellular Domains

(A, D, and G) Low magnification views of adjacent sections of olfactory mucosa, including neuron perikarya within olfactory epithelium (oe) and axons within the lamina propria (lp). (B, E, and H) High magnification views of olfactory epithelium showing details of individual neurons, including dendrite (de), perikaryon (pe), and axon (ax). Axons penetrate the epithelial basal lamina and join other axons in olfactory fascicles within the lamina propria (e.g., longer arrow in [B]). (C, F, and I) Axons enter the OB (ob) via the olfactory nerve (on) and make their way around the bulb, enveloping it.

(A–C) All domains of ORNs are identified with antibody to OMP, including the dendrites, perikarya, and full lengths of axons, including the portions residing within glomeruli (arrowheads in [C]).

(D–F) Only some of the ORN neuronal domains contain peripherin, as visualized by anti-ppn. (D) The vast majority of sections surveyed show no staining within olfactory epithelium, but considerable staining within the lamina propria. (E) Occasional ORNs contain peripherin in perikarya and dendrites, demonstrating that the lamina propria staining is neuronally derived (see Discussion). In a high magnification view of a small cluster of particularly intensely stained ORNs, peripherin can be seen in dendrites, perikarya, and axons, some of which (arrowheads) can be seen exiting the epithelium and entering the lamina propria. In contrast to this small cluster, most ORNs did not stain for peripherin in the dendrite or perikaryon. The photo is a composite to show all neuronal parts, which were in several planes of focus. (F) ORN axons within the olfactory nerve stain for peripherin, but lose immunoreactivity when they enter glomeruli.

(G–I) Vimentin is distributed differently. (G) V9 immunoreactivity is seen within the olfactory epithelium and the lamina propria, as well as in chondrocytes deep to the olfactory epithelium. Olfactory epithelium consistently showed a "reticular" pattern to its staining, due, perhaps, to low levels of expression of vimentin in supporting cells within the olfactory epithelium. (H) Vimentin can be detected in ORN dendrites, perikarya, and axons. (I) Vimentin is detected in the olfactory nerve as well as in some glomeruli. At this age (P6), there is still considerable vimentin immunoreactivity in radial glia within the OB, partly obscuring the distinction between ORN axons and the deeper OB layers.

Bar, 200 μm (A, C, D, F, G, and I), 64 μm (E), or 50 μm (B and H).

ure 2B), V9 reacted with ependymal cells intensely (arrow) and vascular endothelium lightly (arrowheads), but did not detect any neurons or neuropil.

The expression of peripherin and vimentin in ORNs was addressed. To label ORNs in their entirety, antibody to olfactory marker protein (OMP; Margolis, 1972), a 19 kd cytoplasmic ORN-specific protein of unknown function (Margolis, 1988), was employed. In a rat at postnatal day 6 (P6), anti-OMP labeled bipolar neurons in the olfactory epithelium (Figure 3A). Dendrites, perikarya, and axons were all immunoreactive (Figure 3B). In the OB (Figure 3C), anti-OMP stained afferent ORN axon fascicles in the olfactory nerve as well as their terminal arborizations within glomeruli (arrowheads). At P6, glomeruli are small and not as developed as in more mature animals (Pomeroy et al., 1990; compare Figure 3C with Figure 5A).

Anti-ppn stained only portions of ORNs. In a P6 rat, afferent axons in nerve bundles stained, while the cellular domains residing within the olfactory epithelium, including the dendrite, perikaryon, and proximal part of the axon, showed no or only faint reactivity (Figure 3D). Rarely (<1.0%), scattered ORNs could be found that were reactive in the dendrite, perikaryon, and proximal part of the axon (Figure 3E; arrowheads indicate axons exiting the olfactory epithelium and entering the lamina propria). The fields selected (Figures 3D and 3E) considerably overrepresent the proportion of ORNs that showed immunoreactivity within the olfactory epithelium; most olfactory epithelium was immunonegative, while ORN axons in the lamina propria were positive. Scattered sympathetic fibers in the lamina propria also stained (data not shown). In OB (Figure 3F), ORN axons within the olfactory nerve were intensely stained, while the arborizations within glomeruli were unstained (compare the relative extents of staining for OMP [Figure 3C] versus peripherin [Figure 3F] within the bulb).

V9 also stained ORNs, but revealed a different pattern of immunoreactivity. Vimentin reactivity in the olfactory receptor axons was intense (Figure 3G); chondrocytes and other connective tissue were also immunoreactive. In contrast to peripherin, vimentin reactivity was seen in cell bodies and dendrites in many ORNs, though some ORNs showed fainter reactivity (Figure 3H). Olfactory axons entering the OB were immunoreactive, but reactivity within glomeruli was variable (Figure 3I). This latter observation was better seen in adult bulb (see Figure 5C), since at P6 considerable vimentin reactivity could still be detected in radial glia throughout the bulb (Figure 3I), thus obscuring the distinction between the olfactory nerves and the deeper layers.

The differential localization of peripherin to postepithelial axonal domains was detected in adult olfactory mucosa. The midline nasal septum of an adult mouse, covered on both sides by olfactory epithelium, was decalcified (see Experimental Procedures) and immunostained. Anti-OMP stained axons in the lamina propria (Figure 4A, arrows) and cell bodies and dendrites within the epithelium (Figure 4A, asterisks). In sharp contrast, anti-ppn stained only the axons and failed to stain the cell bodies or dendrites (Figure 4B). In adult rat, similar patterns were seen, but morphology was severely compromised, presumably resulting from the more extensive decalcification required. (Patterns of immunostaining seen with juvenile mouse epithelium were similar to those seen with juvenile rat epithelium.)

Expression in adult rat OB was examined. Anti-OMP (Figure 5A) intensely and uniformly stained axons within the olfactory nerve layer (ONL) and their terminals within glomeruli. Anti-ppn (Figure 5B) stained afferent axons in the ONL, but failed to stain terminals within glomeruli. The axons themselves were not uniformly stained. Intensely stained axon fascicles (arrow in Figure 5B) could be seen interspersed among more lightly stained fascicles, giving the ONL a "streaky" appearance. In contrast, P6 ORN fascicles were uniformly stained for peripherin (Figure 3F), suggesting that there is a developmental transition from uniform to nonuniform peripherin expression. In adult bulb, V9 intensely and uniformly stained axons within the ONL (Figure 5C). Some glomeruli manifested vimentin reactivity throughout (arrowhead), while others showed none at all (short arrow) (Figure 5C). A typical glomerulus (longer arrow) seemed to be partially vimentin immunoreactive: reactivity was strong at the region nearest the ONL, where afferent axons enter, but subsequently faded in the more distal aspect of the glomerulus.

Confirmation of the staining patterns for peripherin and vimentin was obtained using an antibody directed toward a peptide derived from the C-terminal tail of peripherin (Aletta et al., 1988; Troy et al., 1990) and a polyclonal anti-vimentin (Sarria et al., 1990), which yielded results identical to those obtained with anti-ppn and V9, respectively.

The expression of other IFPs also was examined. The ONL is composed of two major components: the ORN axons themselves and the specialized glial cells that ensheath them. Although these cells arise from the olfactory placode (reviewed in Doucette, 1990), they maintain characteristics of CNS astrocytes, such as the expression of central-type GFAP (Barber and Lindsay, 1982). Antibody to GFAP can therefore be used specifically to mark these cells. In adult OB, anti-GFAP stained astrocytic cells in all layers of the bulb (Figure 6A). Within the ONL, immunoreactive processes tended to be markedly branched and kinked and radiated in all directions, including perpendicular to the pial surface of the bulb (i.e., orthogonal to the long axis of ORN axons; Figure 6B). Additionally, within the ONL, anti-GFAP stained the cell bodies of the ensheathing cells (Figure 6C). In a section through the hindbrain (Figure 6D), anti-GFAP stained a population of typical astroglial cells. A monoclonal antibody directed against the light subunit of the neurofilament triplet NF-L showed no reactivity in the ONL or in glomeruli in the OB (Figure 6E), consistent with previ-

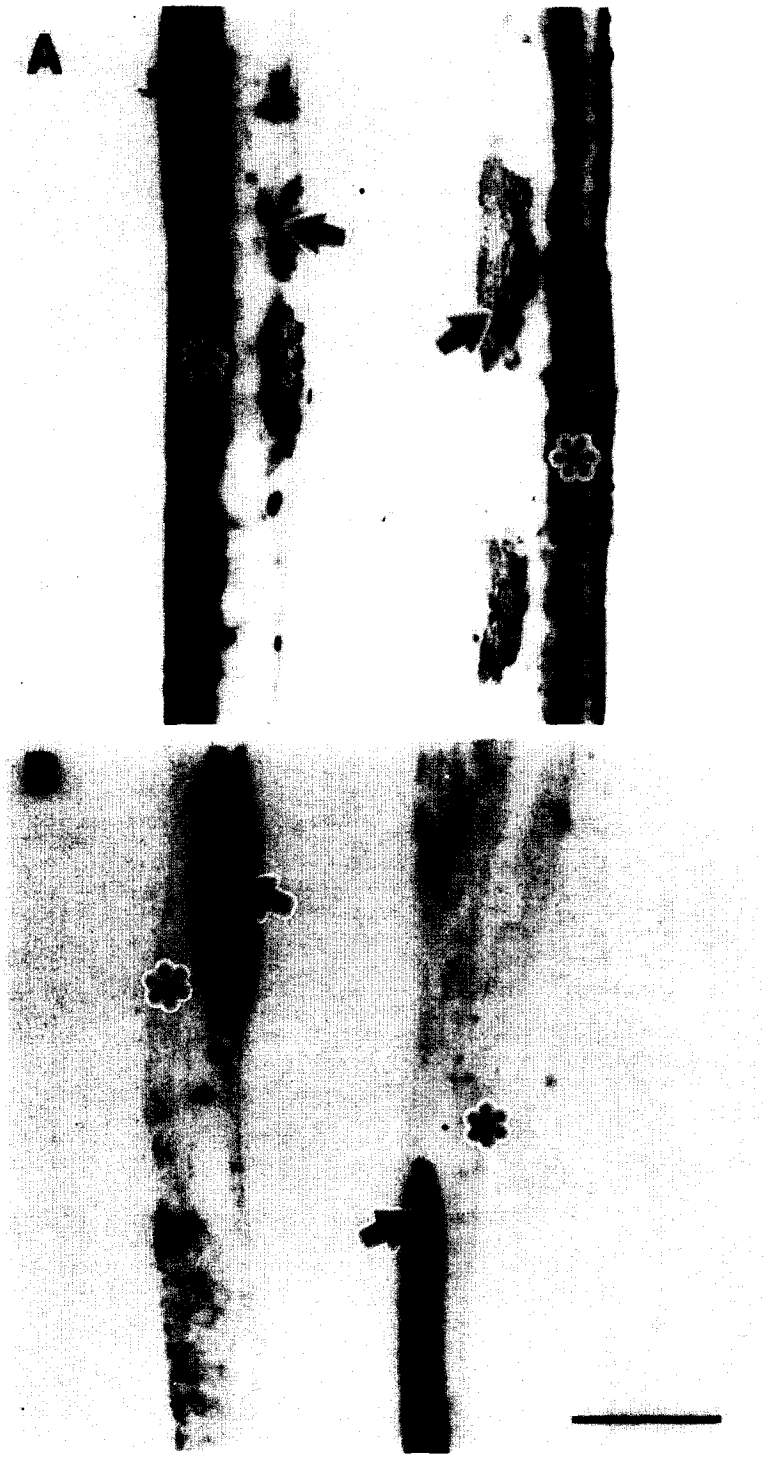

Figure 4. Peripherin Is Excluded from ORN Epithelial Domains in the Adult Rodent

The adult murine nasal septum, lined on either side by olfactory epithelium, has been decalcified, sectioned, and immunostained. (A) Anti-OMP stains ORN axons in the lamina propria (arrows) and cell bodies and dendrites in the epithelium (asterisks). (B) Anti-ppn, by contrast, stains axons in the lamina propria, but not cell bodies or dendrites in the epithelium. Bar, 100 μm.

ous reports noting the lack of NF-L in ORNs (Schwob et al., 1986; Talamo et al., 1989). The antibody was capable of recognizing fibers in other neuronal regions, such as the hindbrain (Figure 6F), in which spinal tract of the trigeminal nerve and other fibers (arrowheads) were intensely labeled. Staining of the ONL using anti-ppn (Figure 6G) resulted in a markedly different pattern than staining using anti-GFAP. Processes stained with anti-ppn were aligned in a parallel orientation with respect to the pial surface and to each other. They were thin and unbranched, and no cell bodies in this layer were stained. In a hindbrain section, anti-ppn stained mostly or only the fibers of the spinal tract of the trigeminal nerve (Figure 6H), consistent with the classification (albeit not entirely accurate) of peripherin as a marker for neurons with projections to or from the periphery. Taken together, these results indicate that the anti-ppn immunoreac-

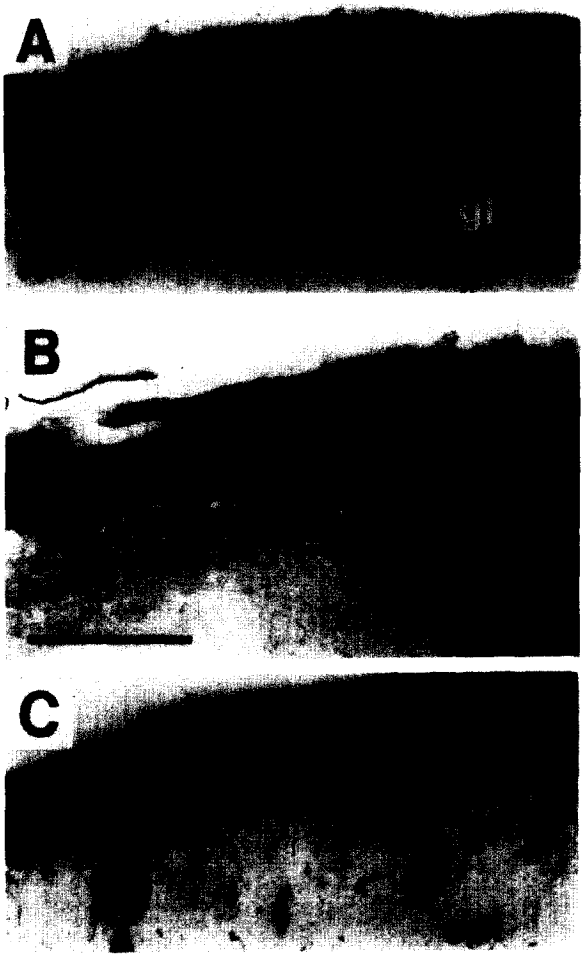

Figure 5. Differential Expression Patterns of OMP, Peripherin, and Vimentin in the Adult OB

Afferent ORN axons course through the ONL (onl), terminating in glomeruli (glomerular layer [gl]), and synapsing on mitral cell dendrites, which approach glomeruli from the external plexiform layer (epl). (A) Anti-OMP stains all domains of ORNs within the bulb, including axons in the ONL and termini in glomeruli. Note that dendrites of mitral cells (epl) are OMP negative, demonstrating the specificity of the antibody for ORNs. (B) Anti-ppn stains axons within the ONL, but glomeruli remain unstained. Some axon fascicles (arrow) are more intensely labeled than others, giving the ONL a streaky appearance. (C) Vimentin immunoreactivity within the ONL is intense and uniform. Glomerular staining is variable. Some glomeruli (arrowhead) are almost completely stained, while others (short arrow) are almost completely unstained. Most glomeruli (longer arrow) stain at the region adjacent to the ONL, where ORNs enter, but are immunonegative in deeper regions. Bar, 200 μm.

tivity within the ONL is derived from the ORN axons and not their ensheathing cells (see Discussion), and it is not the result of artifactual cross-reactivity with other IFPs found in the nervous system. Likewise, in previous work, Schwob et al. (1986) convincingly showed that vimentin immunoreactivity was derived from ORN axons and not glia.

Electron microscopic immunogold analysis was employed to determine if the immunoreactive material seen at the light microscopic level (Figures 3D–3I; Figure 5) was associated with morphologically identifiable IFs. In OB sections, both anti-ppn (Figures 7a–7c) and V9 (Figures 7d–7f), visualized by appropriate secondary antibody conjugated to 5 nm gold particles, decorated 10 nm filaments located in the ONL. As axolemma was not well preserved, the decorated filaments could not be definitively identified as axonal in origin. A previous study using electron microscopy combined with immunoperoxidase labeling did show that vimentin was contained within ONL axons (Schwob et al., 1986). The peroxidase technique obscures ultrastructural morphology however. In the glomerular layer, no labeling was detected with either antibody, confirming light microscopic results (data not shown). When control, nonspecific rabbit or mouse IgG was used as the primary antibody, no labeling was detected in the ONL (data not shown).

Discussion

In the work presented here, the IFP composition of ORNs has been characterized by immunocytochemical analysis. In the context of previous conflicting reports concerning this subject (see Introduction), great care was taken to ensure that the antibodies used were indeed specific for their intended antigens. Vimentin and peripherin were independently detected using a monoclonal (V9; Experimental Procedures) and an affinity-purified polyclonal (anti-ppn; Gorham et al., 1990) antibody, respectively. Each antibody recognized single bands in Western blot analysis of OB extracts, which migrated similarly but not identically in SDS-PAGE (the vimentin-reactive band migrated slightly more rapidly, as expected). The band identified in OB extracts by anti-ppn comigrated with the single band in extracts from PC12 cells, PNS, or CNS regions. Immunocytochemical analysis in hindbrain showed no cross-reactivity between the antibodies, as they labeled different structures in adjacent sections. Finally, results were corroborated with a polyclonal antibody to vimentin and an antibody generated against a peptide derived from the C-terminal tail of peripherin. Regarding the previous conflicting results concerning peripherin in ORNs, the most likely explanation is the sensitivity of the detection technique employed. The level of peripherin in ORNs appears to be below the threshold for detection by fluorescence (Talamo et al., 1989; Troy et al., 1990), but not for detection by amplification and peroxidase deposition (this work; Escurat et al., 1990). Indeed, using either antibody for peripherin, the intensity of peripherin staining in ORNs was always less than that seen in other peripheral neurons scattered throughout the tissue section (data not shown). The signal was nevertheless readily distinguishable from background and could be completely inhibited by preincubation of the antibody with peripherin fusion protein (data not shown).

The major conclusions drawn from the work are that ORNs express both vimentin and peripherin, but differentially regulate their expression both tempo-

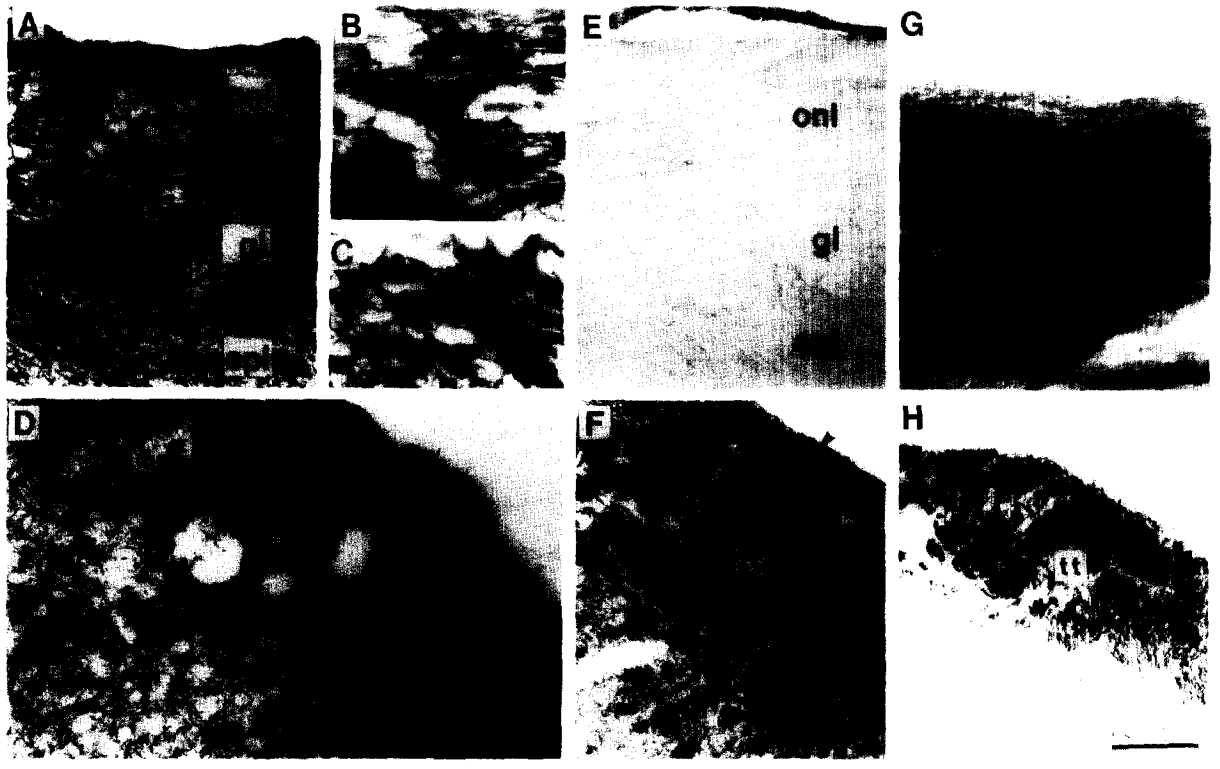

Figure 6. Other IFPs in the OB and Hindbrain

(A–D) A monoclonal antibody directed against the IFP of glia (GFAP) stains astroglial-like cells in all layers of the bulb (A) and in hindbrain (D). Within the ONL (onl), immunoreactive processes of the GFAP-expressing specialized olfactory-ensheathing cells (Barber and Lindsay, 1982; Doucette, 1990) are branched and kinked and project in any orientation, including orthogonal to the pial surface (B). Immunoreactive cell bodies within the ONL are also detected (C, arrowheads). (E and F) A monoclonal antibody directed against the light neurofilament subunit (NF-L) fails to stain ORN axons (E), but intensely stains axon fascicles in hindbrain (F). (G) In comparison with the GFAP staining, anti-ppn stains thin, unbranched axon fascicles, oriented parallel to the pial surface and to one another. No cell bodies are stained. (H) In the hindbrain, anti-ppn stains a restricted set of axon fascicles, the spinal tract of the trigeminal nerve (tt). Arrowheads in (F) indicate examples of axons that contain NF-L, but not peripherin. gl, glomerular layer; epl, external plexiform layer. Bar, 100 μm (A and E), 50 μm (B, C, and G), or 200 μm (D, F, and H).

rally, during development, and spatially, distributing them differently in the neuronal cellular compartments. In younger rats (P6), ORN axon fascicles stained for anti-peripherin in roughly equivalent intensities. In adult rats (6 weeks), some ORN axon fascicles demonstrated noticeably higher anti-peripherin immunoreactivity in comparison with adjacent fascicles on the same section. In contrast, the intensity of vimentin staining was uniform from fascicle to fascicle at P6 or 6 weeks. It would appear then that late, but not early, in development, ORNs differ from one another in the amount of peripherin, but not vimentin, protein produced. The nonuniformity of adult olfactory nerve stained with anti-ppn may be due to continual neurogenesis within the olfactory system. That is, the darker fascicles may represent regenerating olfactory axons, containing a relatively high concentration of peripherin, in a background of more mature axons, expressing relatively less peripherin. This interpretation implies a specific function for peripherin related to process growth. Relevant to this hypothesis, Oblinger et al. (1989a, 1989b) have found that, immediately following axotomy, dorsal root ganglion neurons undergoing intense axonal restoration simultaneously repress NFTP expression and activate peripherin expression.

As summarized in Figure 8, both vimentin and peripherin can be detected in the portion of the axon demarcated proximally by the basal lamina of the olfactory epithelium and distally by the entrance of the axon into the glomerulus. While peripherin in the vast majority of ORNs seems to be contained within these boundaries, vimentin distribution is less constrained, as it can be detected in cell bodies, dendrites, the axonal portions proximal to the basal lamina, as well as in the proximal (i.e., more superficial) aspect of most glomeruli. The remote possibility that the peripherin immunoreactivity in the ONL is derived not from ORN axons per se but rather from their associated ensheathing cells is ruled out by the available evidence. First, in many sections, a few ORNs contained peripherin within perikarya, dendrites, and axons, demonstrating that ORNs indeed can and do synthesize peripherin. Second, the pattern of peripherin immunoreactivity resembled not at all the pattern seen with anti-GFAP (which specifically stains the en-

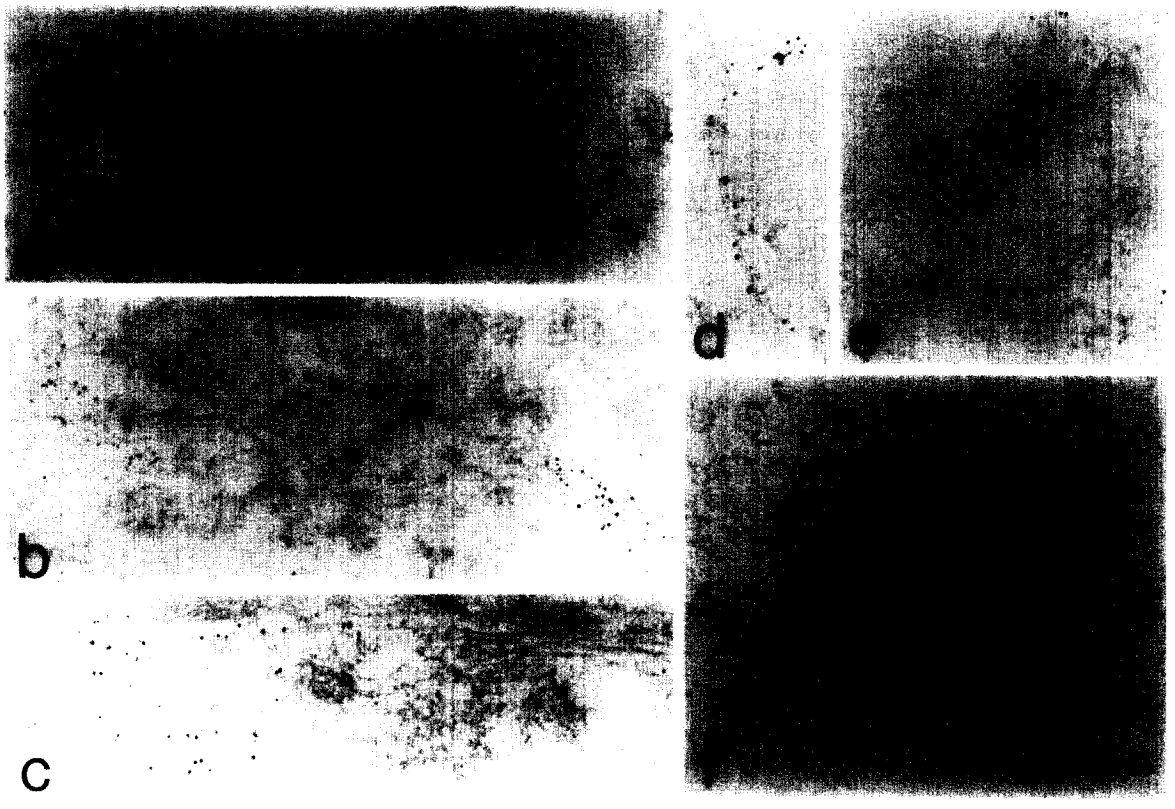

Figure 7. Antibodies to Peripherin or Vimentin Decorate IFs in the ONL

Antibody to peripherin (anti-ppn; [a-c]) or antibody to vimentin (V9; [d-f]), identified by the appropriate gold particle-conjugated secondary antibody, can be seen to decorate IFs in the ONL. In all panels, the average size of the particle is 5 nm. Direct double-label analysis was precluded by the impermeability of the tissue to larger diameter (15 nm) gold particles. Bar, 250 nm.

sheathing cells; see Results for details). Third, in in situ hybridization studies, peripherin mRNA was not detected in ONL in the bulb, but was easily detected in other regions in the brain (unpublished data). Fourth, despite extensive investigations (Leonard et al., 1988; Parysek and Goldman, 1988; Escurat et al., 1990), there is as yet no report of peripherin expression in any nonneuronal (e.g., glial) cell type, adrenal chromaffin cells excepted.

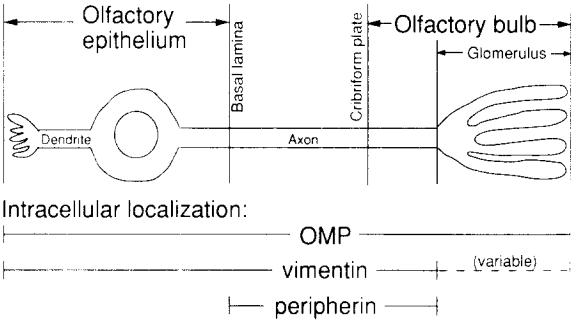

Figure 8. Diagrammatic Representation of ORN Domains Containing Vimentin and/or Peripherin

This diagram represents an idealized ORN. The dendrite, perikaryon, and proximal part of the axon reside within the olfactory epithelium. After exiting the epithelium by penetrating the basal lamina, the axon passes through peripheral tissue and enters the CNS (OB) via fenestrations in the bony cribriform plate. The axon terminates in several branches within a glomerulus, where it synapses upon mitral, juxtaglomerular, and periglomerular neurons. The subcellular localizations of OMP, vimentin, and peripherin are indicated. Domains are not drawn to scale.

Another possible explanation for the limited peripherin distribution is that the antibodies used recognize some but not all forms of peripherin, and the various forms are themselves differentially distributed. In particular, phosphorylation of peripherin within ORNs might affect the ability of anti-ppn to recognize endogenous peripherin. This concern is not trivial, since phosphorylated NFTPs are preferentially located in axons, as compared with cell bodies and dendrites (Sternberger and Sternberger, 1983), and, moreover, many antibodies to the NFTPs are sensitive to the state of phosphorylation of their antigen (Sternberger and Sternberger, 1983; Lee et al., 1987). This phosphorylation occurs in a repeated C-terminal motif unique to the type IV IFPs (Julien and Mushynski, 1983). For the following reasons, we believe that if peripherin phosphorylation indeed occurs in ORNs it is not acting as an impediment to antibody-antigen recognition. First, peripherin, a type III IFP, lacks the type IV C-terminal motif and is therefore probably not a substrate of a neuro-filament-specific kinase. Second, anti-ppn was generated to a bacterially produced and therefore unphosphory-

lated peripherin fusion protein (Gorham et al., 1990); it nevertheless recognized without difficulty ORN axonal peripherin. Finally, identical results were obtained with the antibody to a C-terminal peptide (Aletta et al., 1988; Troy et al., 1990); in sympathetic neurons, phosphorylation occurs on the N-terminus, not the C-terminus, of peripherin (Huc et al., 1989).

What are the possible functions of peripherin and vimentin in ORNs? The fact that ORNs establish a mechanism for differential distribution of these two related proteins implicates differential functions for them. The preferential placement of vimentin in the dendrites and cell bodies suggests that some vimentin-specific function is subserved here. Georgatos and Blobel (1987b) showed that vimentin can bind to the nuclear lamin B in vitro and suggested that a complex containing lamin B might act as an initiation site for IF polymerization (Georgatos and Blobel, 1987a). Recent results by Djabali et al. (1991) show that, in vitro and in a neuroblastoma cell line, lamin B also binds peripherin; interaction with lamin B may be a general property of type III IFPs (Georgatos et al., 1987). ORNs present the unusual case in which two type III IFPs are expressed in the same cell, yet one is excluded from the space around the nucleus. Extrapolating from the results presented here, little or no peripherin–lamin B interaction would be expected in ORNs, yet vimentin–lamin B interaction remains a very real possibility. Further understanding of the differential biochemical properties of type III IFPs may yield insight into the physiological consequences of the differential distribution.

The differential distribution also has implications for cellular control of IF polymerization and dynamics. IFs can be seen in cultured cells to extend from the nuclear membrane to the plasma membrane. Consequently, new subunits may be added at the proximal end, in a "nuclear organizing center," with concomitant radial displacement of the rest of the filament, at the distal end, or at sites in between (side-to-side incorporation) (Albers and Fuchs, 1989; Angelides et al., 1989; Sarria et al., 1990; for review see Steinert and Liem, 1990). Results here suggest that, in ORNs, peripherin subunits are not incorporated at a nuclear organizing center, since peripherin can be detected only at some distance from the nucleus. Probably, unincorporated subunits are transported anterogradely down the axon to become incorporated into IFs in axons that have left the epithelium. Some component of the basal lamina or extraepithelial milieu may be providing the cue leading to peripherin subunit incorporation (see below). We were unable to determine conclusively in ultrastructural studies whether peripherin and vimentin coincorporate into IFs in ORNs or whether separate peripherin and vimentin IF networks exist. The tissue proved to be quite impermeable to antibody conjugated to large (15 nm) gold particles, precluding direct double-label analysis. However, vimentin and peripherin subunits readily form copolymers in vitro (K. Djabali and M.-M. Portier, personal communication), as do vimentin and desmin or GFAP subunits (Steinert et al., 1981; Quinlan and Franke, 1983). Since most identifiable IFs were gold decorated when antibody to either peripherin or vimentin was used, it is probable that most IFs in ORN axons are composed of both vimentin and peripherin.

What is the nature of the mechanism(s) by which ORNs restrict peripherin from the intraepithelial domains and peripherin/vimentin from the intraglomerular domains? The spatial organization of peripherin could be a property intrinsic to the ORN, or it may be a result of extrinsic signals communicated to the ORN domains from adjacent components found in the various microenvironments. For example, ensheathing cells found in the lamina propria and the ONL of the bulb could be providing a signal(s) directing the establishment of peripherin filaments in adjacent axons; conversely, the exclusion of peripherin from the intraepithelial neuronal domains could be a result of a signal(s) derived from the adjacent nonneuronal olfactory epithelial cells. The exclusion from glomeruli may reflect a more generalizable phenomenon, since both vimentin and peripherin are excluded from this domain. The microtubule-associated protein MAP1B is also expressed in ORN axons, but is excluded from glomeruli (Schoenfeld et al., 1989; J. D. G. and H. B., unpublished data). A general property of neurons is the exclusion of neurofilaments from synaptic regions, which may allow for greater plasticity therein (Roots, 1983). Current models invoke a Ca^{2+}-dependent mechanism of action: synaptic activity leads to high local Ca^{2+} concentration, activating Ca^{2+}-dependent cysteine proteases (calpains), which degrade, among other substrates, IFPs (Fischer et al., 1986; Perides et al., 1987) and microtubule-associated proteins (Siman and Noszek, 1988). A similar protease mechanism may be operative in the OB glomerulus, an anatomically specialized region of high synaptic activity; unfortunately, nothing is known about the distributions or activities of calpains in the OB (see Hamakubo et al., 1986). Clearly, further experimental analysis is needed to evaluate these various hypotheses.

In summary, ORNs express two type III IFPs, one (vimentin) characteristic of neuronal precursors, the other (peripherin) of differentiating and differentiated neurons. In adult tissue, coexpression of two type III IFPs has been described in some skeletal and smooth muscle cell types (vimentin$^+$ desmin$^+$; Granger and Lazarides, 1979; Frank and Warren, 1981) and in some astrocyte populations (vimentin$^+$ GFAP$^+$; Shaw et al., 1981), but in these cases the intracellular distributions of the two isotypes cannot be distinguished using the light microscope. The unusual isotype expression combination in ORNs (vimentin$^+$ peripherin$^+$ NFTP$^-$), combined with the differential distribution, may confer upon ORNs properties uniquely suited among neurons to the processes of regeneration and axon growth.

Experimental Procedures

Antisera

Peripherin was detected using an affinity-purified polyclonal rabbit antibody (anti-ppn) generated against an E. coli fusion protein, as described (identical to anti-per; in Gorham et al., 1990). Vimentin was detected with the monoclonal antibody V9 (ICN). For confirmation of immunocytochemical results, peripherin was detected with a rabbit polyclonal antibody generated against a C-terminal peptide (kind gift of Dr. L. Greene, Columbia University; Aletta et al., 1988; Troy et al., 1990), and vimentin was detected with a rabbit polyclonal antibody specific for rodent vimentin (kind gift of Dr. R. Evans, University of Colorado Health Sciences Center; Sarria et al., 1990). OMP was detected using a goat antibody (Margolis et al., 1972). NF-L and GFAP were detected using the monoclonal antibodies NR4 and GA5, respectively (Boehringer Mannheim).

Western Blot Analysis

PC12 cell lysates were prepared as in Gorham et al. (1990). Dissected rat tissue was rapidly frozen on dry ice. Frozen tissue was sonicated for 20 s in 0.5% SDS, 0.4 M NaCl, 0.025 M $NaPO_4$, 1% Aprotinen (Sigma), 1 mM phenylmethylsulfonyl fluoride (Sigma) and boiled for 3 min. Following centrifugation (10,000 rpm, 10 min), the protein content of supernatants was determined according to the method of Lowry. Western blotting was performed as in Gorham et al. (1990). Anti-ppn (1:400) was detected using ^{125}I-labeled protein A (1:1000; NEN), and V9 (1:10,000) was detected using ^{125}I-labeled anti-mouse IgG (1:1000; NEN).

Immunocytochemistry

Rats, purchased from Charles River, were deeply anesthetized with sodium pentobarbital and perfused transcardially with saline followed by 4% buffered (0.1 M $NaPO_4$ buffer [pH 7.2]) paraformaldehyde. Adult (~6 weeks postnatal) brains, including OBs, were removed from the cranium and postfixed in 4% buffered paraformaldehyde for 1 hr. Heads containing the olfactory mucosa were decalcified for 2–4 days in 0.1 M EDTA, 0.05 M $NaPO_4$ buffer (pH 7.2) at 4°C with several changes of solution. The decalcification process resulted in a significant loss of morphological integrity. Mouse heads decalcified more rapidly and therefore were used to examine adult olfactory epithelium (Figure 4). For better preservation of rat olfactory epithelium, rats at P6, an age at which the rat snout can be sectioned without prior decalcification, were also used. P6 rat olfactory epithelium is relatively mature. P6 rats were also cardiac perfused, but with less pressure and volume, in accordance with their smaller body weights. At least three animals from each age were examined. Tissues were equilibrated to 30% sucrose overnight at 4°C. Adult bulb and brain sections were obtained as freely floating sections on a sliding microtome at 30 μm. P6 rat and decalcified adult mouse sections were obtained on a cryostat at 25 μm and melted directly onto gelatin-coated slides.

Staining was performed essentially as in Baker et al. (1989). Briefly, for floating sections, primary antibodies were incubated overnight at the following dilutions: anti-ppn (1:1000), anti-peripherin peptide (1:6000), V9 (1:100,000), rabbit anti-vimentin (1:6000), anti-OMP (1:35,000), NR4 (1:100), and GA5 (1:100). For staining premounted sections, primary antibodies were incubated at 5-fold higher concentrations. Bound antibodies were detected using appropriate biotinylated secondary antibodies and the ABC complex (Vector Labs, Burlingame, CA) and visualization by reaction with 3,3'-diaminobenzidine-tetrahydrochloride (Aldrich). In some experiments, anti-ppn was preincubated for 3 hr at room temperature with 10 μM purified E. coli fusion protein containing either peripherin (Gorham et al., 1990) or an unrelated sequence (Vosatka et al., 1989).

Electron Microscopy

Immunoelectron microscopy was carried out according to Wu et al. (1990). Briefly, adult OBs were fixed and sectioned on a sliding microtome as for light microscopy. Floating sections were incubated sequentially at room temperature in 0.1 M PBS (pH 7.2), 1% BSA, 0.2% Triton X-100 for 1 hr, either anti-ppn (1:50) or V9 (1:2000) for 12–16 hr, and then goat anti-rabbit IgG conjugated to 5 nm gold or goat anti-mouse IgG conjugated to 5 nm gold (Jensen Life Science Products) for 12–16 hr. For control sections, equivalent concentrations (2 μg/ml) of nonimmune rabbit or mouse IgG fraction were used as the primary antibody. The specimens were then fixed in 2% glutaraldehyde, postfixed in 1% OsO_4, dehydrated, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and then observed.

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