

Neurofilament Antibodies and Spiral Ganglion Neurons of the Mammalian Cochlea

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ABSTRACT

The spiral ganglia of the cat, gerbil, mouse, rat, and human were immunohistochemically stained with various monoclonal neurofilament antibodies. Three antibodies to the 200-kD neurofilament protein (R-3, Dräger et al., '84; ICN anti-200, clone NE14, Debus et al., '83; RT-97, Wood and Anderton, '81) labeled the somata of type II spiral ganglion neurons but not those of type I ganglion neurons. In the extreme base of the cochlea of cats, mice and rats, there was intense labeling of a few (< 0.5% of the total ganglion population) large neurons resembling type I ganglion neurons.

Several other neurofilament antibodies (Amersham anti-68, Amersham and ICN anti-160, and SMI-32) did not specifically label type II ganglion neurons but instead labeled all neurons of the spiral ganglion. These two patterns of labeling prompted us to investigate the cause for this difference. Because antibodies against the 200-kD neurofilament protein preferentially labeled type II neurons and because 200-kD neurofilament is highly phosphorylated, we treated cochlear tissue with alkaline phosphatase in order to remove phosphate groups. This treatment eliminated the intense labeling of type II neurons with R-3, ICN anti-200, and RT-97, but had no effect on the intense labeling of ganglion cell bodies observed with the other neurofilament antibodies tested. This evidence suggests that labeling occurs because of the cytoplasmic presence of phosphorylated 200-kD neurofilament protein in type II ganglion neurons. Populations of neurons may thus differ in their neurofilament epitopes and monoclonal antibodies can be used to mark such differences.

Key words: auditory system, immunocytochemistry, phosphorylated neurofilament, sensory system, monoclonal antibodies

Spiral ganglion neurons provide afferent input to the central nervous system (CNS) from receptor cells in the cochlea of mammals. Two populations of ganglion neurons have been defined by comparing cell body characteristics such as size, myelination, ultrastructural components, and staining properties (e.g., Munzer, '31; Suzuki et al., '63; Kellerhals et al., '67; Spoendlin, '73). These somatic distinctions also correspond to differences in peripheral innervation (Kiang et al., '82; Ginzburg and Morest, '83; Berglund and Ryugo, '87; Brown, '87). The larger type I neurons represent 90–95% of the population and contact inner hair cells (IHCs) by way of myelinated radial fibers, whereas the smaller type II neurons constitute the remaining population and contact the more numerous outer hair cells (OHCs) by way of unmyelinated outer spiral fibers. These observations are consistent for many species despite differ-

ences in techniques and descriptive criteria. Thus it may be concluded that the two populations of ganglion cells provide separate afferent pathways in the auditory system of mammals.

The most definitive feature of type II neurons is their innervation of OHCs. It is difficult, however, to determine this relationship for individual spiral ganglion neurons using the light microscope (Kiang et al., '82, '84) or the electron microscope (Kimura, '86), and virtually impossible to determine the peripheral innervation for large samples of spiral ganglion neurons. In contrast, the cell bodies are

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TABLE 1. Total Counts of Type II Cells Identified by Staining Entire Cochleas of Norway Brown Rats with Basic Dye or NF Antibodies

Stain	Sections counted	Average no. of type II cells		Average no. of type I cells		% type II cells based on	
		Raw count	Corrected count	Raw count	Corrected count	Raw count	Corrected count
Toluidine blue (n = 2)	Every 10th	1586	1137	21,648	14,305	6.8%	7.4%
RT-97 (n = 2)	All	1742	1235	—	—	7.5%*	7.9%
ICN anti-200 (n = 1)	Every 4th	1709	1197	—	—	7.4%*	7.7%

*Calculated with the type I count obtained for the cochleas stained with toluidine blue, because type I cells do not stain intensely with the antibodies and thus are not always unambiguously visible for counting. n = number of cochleas.

accessible for population studies and the type II ganglion neurons have been distinguished in the light microscope using basic dyes (e.g., Kellerhals et al., '67; Keithley and Feldman, '79), protargol staining (Kiang et al., '84), and preferential uptake of taurine (Ryan and Schwartz, '83). In addition, electron microscopists have noted that a small proportion of spiral ganglion cells contain abundant neurofilaments (Nishimura et al., '65; Thomsen, '66; Kellerhals et al., '67), and neurofilament antibodies have been shown to preferentially and intensely stain type II cell bodies (Berglund and Ryugo, '86; Romand et al., '88).

In the present study, we applied immunocytochemical staining methods using neurofilament antibodies to further characterize type II spiral ganglion neurons. First, we sought to determine whether neurofilament antibodies can be employed to reliably label the somata of type II neurons in all regions of the cochlea across several species of mammals. Second, we wanted to examine how antibodies directed against the three neurofilament subunits (the 68-kD, 160-kD, and 200-kD proteins) would stain spiral ganglion neurons. Finally, we wanted to know whether immunostaining of spiral ganglion neurons would provide insight into their biological function.

METHODS

Preparation of specimens

Cochleas of cats (*Felis catus*), rats (*Rattus norvegicus*, Norway brown), gerbils (*Meriones unguiculatus*), and mice (*Mus musculus*, CD-1) were examined. The animals were anesthetized prior to intracardiac and subsequent intracochlear perfusion with saline, followed by fixative. Bouin's fixative provided optimal staining and was used in most cases. In some initial experiments with the RT-97 antibody, we employed other fixatives (95% ethanol, a 2.5% glutaraldehyde-1.0% paraformaldehyde mixture, 4% paraformaldehyde, and 10% formalin). A human cochlea from a 45-year-old male who died from cardiac arrest and had no history of auditory disorder was perfused with Bouin's fixative within 6 hours postmortem (made available to us courtesy of the Otopathology Laboratory, Massachusetts Eye and Ear Infirmary). All cochleas were left in the initial fixative overnight and then transferred to 10% formalin for 1–5 days. They were decalcified by drilling with stone burrs and/or immersion in 10% EDTA. The decalcified cochlea was immersed in buffered 30% sucrose for 24 hours and then OCT compound for another 24 hours before it was sectioned at 10–15 μ m in a cryostat. Sections of 15- μ m thickness or less ensure that cell bodies are cleaved to allow penetration of the antibodies to the intracellular space. The sections were collected on double-subbed (chrome-alum) slides and further affixed to the slides by a 1-hour immersion in 10% formalin.

Primary antibodies

The following monoclonal NF antibodies were employed: RT-97 (antibody to 200-kD protein, obtained from Dr. John Wood, Sandoz, London); R-3 (antibody to 200-kD protein, courtesy of Dr. Ursula Dräger, Dept. Neurobiology, Harvard Medical School); commercially available antibodies: to 68-kD protein, Amersham (clone NR4, Debus et al., '83); to 160-kD protein, Amersham and ICN Biochemicals (clone NN18, Debus et al., '83); to 200-kD protein, ICN Biochemicals (clone NE14, Debus et al., '83); and SMI-32, specific to cell bodies, Sternberger-Meyer (Sternberger and Sternberger, '83). The following dilutions were employed: RT-97: 1:100-1600; R-3: undiluted supernatant; Amersham anti-68: 1:25; Amersham anti-160: 1:20-100; ICN anti-160: 1:25; ICN anti-200: 1:1000; and SMI-32: 1:750.

Tissue staining

In some cochlear sections, we identified type I and type II cell bodies by staining with the basic cell stain, toluidine blue. After photographing the stained cells for purposes of later comparison of the same calls stained by antibodies as previously described (Berglund and Ryugo, '86), we conducted the antibody incubations. Other cochleas, several of which were sectioned in entire series, were processed only with the antibodies. Tris-buffered saline (0.1 M Trizma, pH 7.5) was routinely used as the buffer. Briefly, the primary antibody was applied for 45 minutes at room temperature or overnight at 4°C. Triton X-100 detergent (0.02%) was added to the primary antibody solution in some cases (see Table 2). After washing with buffer, the secondary antibody (goat antimouse IgG or IgM, heavy and light chain-specific, conjugated with rhodamine isothiocyanate or peroxidase; Cooper Biomedicals) was applied for 35 minutes. Some sections were viewed by epifluorescence (rhodamine). Others (peroxidase) were subsequently reacted with the chromogen 3,3'-diaminobenzidine (Sigma Chemicals; 0.05% DAB in 0.1 M Tris with 0.01% H₂O₂; 8 min.) to provide a permanent label. We treated some tissue with the *E. coli* enzyme, alkaline phosphatase (Sigma Chemicals, Type III-N), for 3 hours at 37°C before staining with antibodies. Phosphatase treatment removes phosphate groups from isolated NF proteins (Carden et al., '85) and from tissue (Lee et al., '86).

Counts of neurons and morphometric analysis

Type II neurons were counted in rat tissue prepared by basic dye and antibody methods to determine whether the type II cell bodies defined by the antibody method represented approximately the same proportion of ganglion cells in the entire cochlea as has been previously defined by Nissl criteria (e.g., Keithley and Feldman, '79). The somata of type I and type II neurons, identified by their Nissl charac-

TABLE 2. Staining Patterns of Spiral Ganglion Neurons in Different Mammals After Cochlear Tissue Is Reacted With Various Monoclonal NF Antibodies and Dependence of These Patterns on Detergent and Phosphatase Treatment

Antibody	Species	Standard	Treatment/Labeling	
			0.02% Triton-X-100 added to primary antibody incubation	Preincubation with alkaline phosphatase
Phosphate-sensitive				
RT-97 (J. Wood)	Rat	IIs (6)	IIs (4)	none (3)
	Cat	IIs (2)		none
	Mouse	IIs (5)		
	Gerbil	IIs		none
	Human	IIs		none
ICN Biochem. anti-200	Rat	IIs (3)	Is, IIs	none
	Cat	IIs		none
	Mouse	IIs (2)		
	Gerbil	IIs		
R-3 (U. Dräger)	Rat	IIs	IIs (2)	none
	Cat		IIs (2)	none
	Mouse		IIs	
Phosphate-insensitive				
Amersham anti-68	Rat	none	Is, IIs	Is, IIs
	Cat		Is, IIs	Is, IIs
	Gerbil			Is, IIs
Amersham anti-160	Rat	none (2)	Is, IIs	Is, IIs
	Cat		Is, IIs	Is, IIs
ICN Biochem. anti-160	Rat	none	Is, IIs	Is, IIs
SMI-32 (Sternberger-Meyer)	Mouse	none	Is, IIs	Is, IIs

IIs—type II cell bodies are intensely stained and > 99.7% of type I cell bodies are not; Is, IIs—all spiral ganglion cell bodies are stained with approximately equal intensity; none—no spiral ganglion cell bodies are stained; numbers in parentheses indicate the number of animals when $n > 1$.

teristics, were counted in every 10th section of serial sections of two rat cochleas stained with the basic dye, cresyl violet. Neurons were included in the counts if their nuclei were visible in the section (raw count). Nuclei were counted rather than nucleoli because the heterochromatin visible in the nucleus was sometimes mistaken for the nucleolus and thus resulted in counts that differed by 10–20% when two microscopists counted the number of type I and type II cells in the same section. When nuclei were counted, there was < 5% difference in two independent counts. Cell bodies intensely stained with NF antibodies were counted in several other complete series of rat cochleas for comparison with the counts of Nissl-identified type II neurons. All raw counts were corrected for split-cell error by the Abercrombie correction, with the assumption that the nuclei were spherical [i.e., corrected count = (raw count \times section thickness)/(section thickness + nuclear diameter); Abercrombie, '46]. Corrected counts facilitated comparisons with other studies.

Morphometric analyses of cell-body silhouette area and average process diameters of immunoperoxidase-stained cells in cat were made with the aid of a computerized planimeter. Individual neurons were divided into cell body, peripheral process, and central process as previously described (Berglund and Ryugo, '87). Average diameters for the processes were determined for 10- μ m segments adjacent to the cell body. Similar measurements for individual type I and type II neurons labeled with horseradish peroxidase (HRP) by injection into the auditory nerve of cats were available for comparison from another study (Ryugo et al., '86).

Optical densitometry

The intensity of antibody stain was evaluated by optical densitometry measurements conducted on a Macintosh computer with "Image" software (NIH). Samples of cells from three different animals (2 cats and 1 rat) were

included in the analysis. The microscope slides were viewed on a video camera at a magnification of 580 \times . A cytoplasmic "patch" of approximately 2–4 μ m² was outlined on the video image with a mouse control and the densitometry of this patch was averaged by the computer and output as an intensity value on a scale of 0–256 (white = 0; black = 256) units. At the start of each session, the absolute intensity value for one and the same tissue section was calibrated numerically with respect to its subjectively determined lightest and darkest areas. In this way, all densitometry measurements could be reliably reproduced, even though absolute intensity varied slightly from one tissue section to another. As further checks of the procedure, the intensity values for several cytoplasmic patches in the same cell were tested for reproducibility of the average intensity measurement and patches for the same cells were tested during different sessions. All of these control procedures produced < 5% variation.

RESULTS

The cell bodies of type II neurons of cats and rodents were recognized by their small size and their relatively light cytoplasm surrounding a darker staining nucleus (Fig. 1, left panels). After washing the tissue to remove the toluidine blue, the sections were restained with RT-97, a monoclonal antibody directed against the 200-kD NF protein (Fig. 1, right panels). The individual somata of type II neurons as defined by basic dye criteria were unequivocally those same neurons that stained intensely when reacted with RT-97 (compare left and right panels of Fig. 1). These staining procedures demonstrated that type II neurons were identifiable in cats, gerbils, mice, and rats. Antibody concentrations from 1:100 to 1:1,600 were equally effective in producing the intense staining. Since type II neurons appear dark against a relatively unstained background, they are much easier to locate and characterize in immuno-

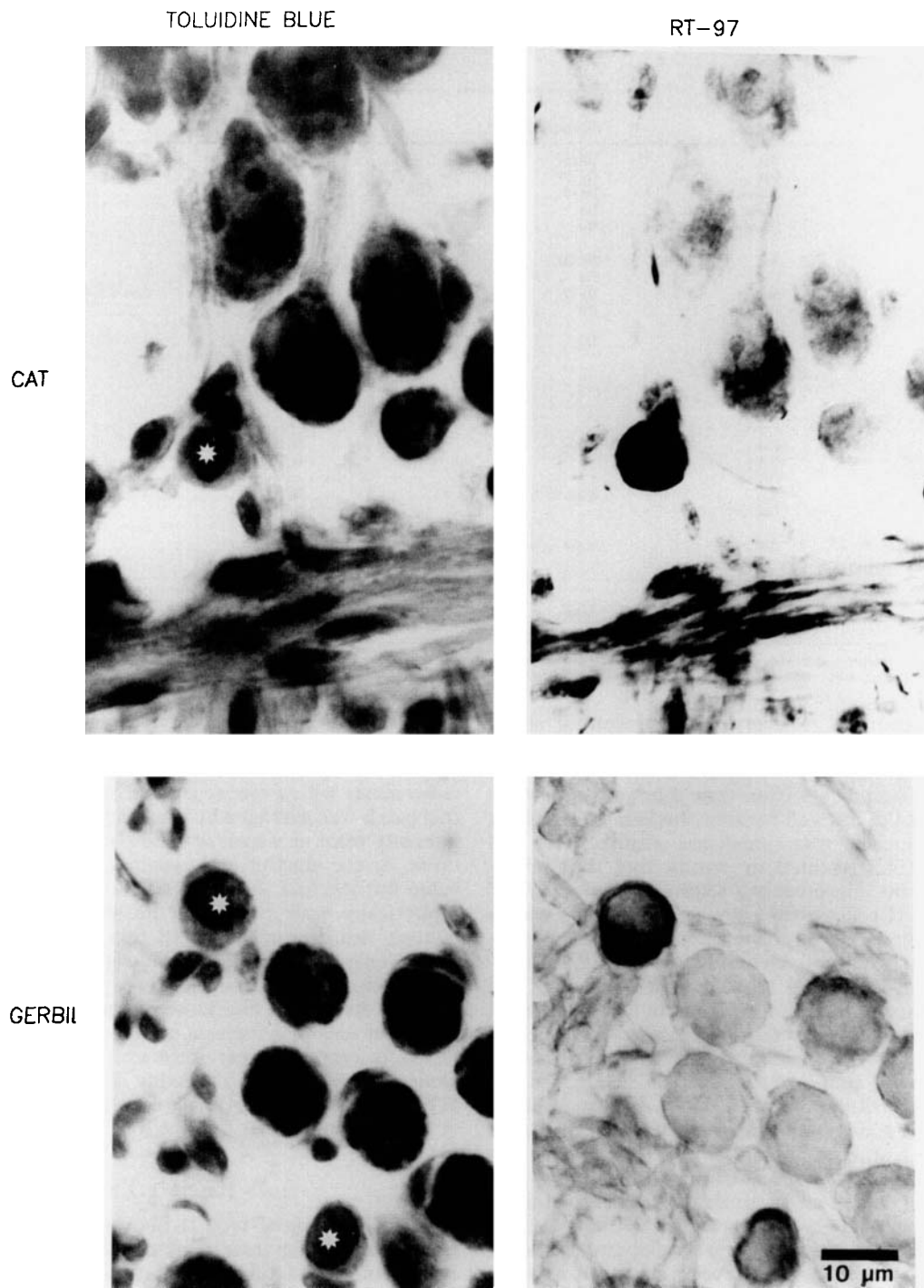


Fig. 1. Double staining of type II spiral ganglion neurons in the cat and gerbil. In the left panel, cochlear tissue has been stained with the basic dye, toluidine blue, and type II ganglion cells (asterisk) have been identified by their small size and pale-staining cytoplasm. The larger and more numerous neurons are type I ganglion cells. In the right

panel, this tissue has been washed free of toluidine blue and then treated with the neurofilament antibody, RT-97. The antibody is made visible by use of a secondary antibody conjugated to peroxidase and treated with DAB. Except in the human, type II neurons were identified by this double staining technique.

cytochemically labeled tissue than in toluidine blue stained tissue.

Cell counts from toluidine blue stained or immunostained tissue (Table 1) yielded the following numbers: In rats, type II neurons represented 7.4% of the ganglion

population in toluidine blue stained tissue and 7.7–7.9% of the population in immunostained tissue (range of 1,137–1,235 for 5 cochleas). Immunostained neurons numbered 3,342 in a cat cochlea, 557 in a mouse cochlea, and 1,214 in a gerbil cochlea. When compared to the number of corre-

sponding type I neurons, the resulting percentages are consistent with the 5–10% previously reported for type II neurons in these particular species.

Somatic shape of type II neurons

The central and peripheral processes adjacent to the cell bodies of type II neurons were often visible when stained by RT-97 and could be followed for some distance within the spiral ganglion (Fig. 2). Outside the ganglion, all processes were heavily labeled so that it was difficult to follow any individual fiber. In the vicinity of the cell body, the stained processes revealed cell "polarity" or shape. If a line is drawn to connect the two processes, the cell body is divided into two pieces that may be equal or unequal in size. When this line bisects the cell body, the cell is described as "bipolar" and when the line divides the cell body into two unequal pieces (as in the case where the two processes are located close to each other on one side of the cell body), the cell is considered to be "pseudomonopolar." Type II neurons, whether immunostained by RT-97 or labeled by HRP following extracellular injections into the nerve, are mostly pseudomonopolar in cats (as shown in Fig. 3) and mostly bipolar in rodents (Fig. 2). Cell body shape, therefore, is not a universal indicator of type II neurons.

Silhouette areas of cell bodies and diameters of central and peripheral processes were measured in cat spiral ganglia stained by RT-97 immunoperoxidase methods. These values were compared with those of HRP-labeled neurons whose peripheral processes were traced to their termination under IHCs or OHCs (Ryugo et al., '86). A type II neuron, as defined by its innervation of OHCs (Fig. 3, bottom left) or by its immunostaining to RT-97 (Fig. 3, bottom right), has a relatively small cell body with a pseudomonopolar shape. It exhibits a central process whose diameter in the vicinity of the cell body is approximately equal to that of its peripheral process. The central process diameter divided by the peripheral process diameter produces a ratio that segregates the two types of spiral ganglion neurons: Type I neurons have an average ratio of 4, whereas type II neurons have a ratio approximately equal to 1. When the values for 30 intensely immunostained neurons are compared to a population of ganglion neurons traced to hair cells, the data points for immunostained neurons are clustered with those of type II neurons (Fig. 3, top). These data are consistent with the idea that the immunostained neurons innervate OHCs.

RT-97 staining of the human cochlea

Cryostat sections were collected through the middle turn of one human cochlea and subjected to antibody staining. In this sample, 32 of 820 ganglion cells (4%) were intensely labeled by RT-97. One well-labeled neuron appeared pseudomonopolar and had processes of roughly equal caliber (Fig. 2, bottom). Postmortem effects, however, impaired analysis: The disintegration of cell borders coupled with obvious shrinkage and vacuolation of cell bodies prevented measurements of cell body size, and the erosion of Nissl characteristics obscured cell type identification. Furthermore, peripheral processes of labeled neurons could not be traced to the organ of Corti in this material. Nevertheless, the intense immunolabeling was restricted to small neurons that appeared to represent a small proportion of the ganglion cell population. Hence, the antibody method appeared to mark type II neurons in the human.

Optical density measurements

In our nonhuman specimens, small (type II) cell bodies were darkly labeled by RT-97 in all regions of the cochlea, whereas large (type I) cell bodies were typically unlabeled or lightly labeled. Occasionally, a large cell body was observed that was moderately or darkly stained. We selected random sections from each turn of two cochleas and analyzed every ganglion cell with a visible nucleus using a computerized algorithm for cytoplasmic optical density. This method allowed us to quantify our subjective estimates of "lightly" versus "darkly" labeled immunoreactive cells and to investigate the distribution of staining in the spiral ganglia. A unimodal distribution of staining intensity would suggest that type II neurons represent one end of the continuum for a single immunoreactive population, whereas a bimodal distribution would indicate that two populations of immunoreactive cells could be defined.

Two distinct peaks of "light" and "dark" antibody label are found in the cochleas of each cat (Fig. 4). One cat displays a peak at approximately 85 O.D. units and a second peak at approximately 205 O.D. units (Cat 1R, 10 sections with 575 cells). The other cat displays peaks at approximately 105 and 215 O.D. units (Cat 2L, 6 sections with 207 cells). The peaks having low optical density (light antibody label) are composed of neurons with large cell bodies and are interpreted as representing type I neurons. The peaks having high optical density (intense antibody label) are composed of neurons having small cell bodies and are interpreted as representing type II neurons. The trough at 150 O.D. units was selected as the division between the two populations. The darkly labeled cells constituted 7.6% of the ganglion cells in Cat 1R and 10.8% of the cells in Cat 2L.

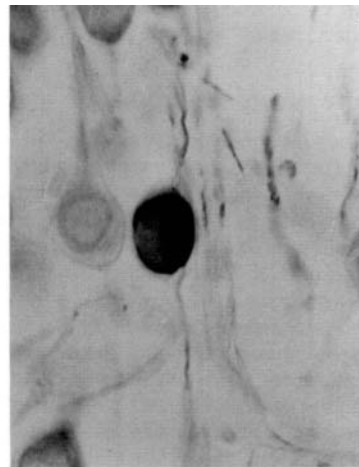
Staining features in the extreme base of the cochlea

In rats, cats, and mice, the extreme basal region of the cochlea (also called the hook region in cats) exhibited unique staining properties. That is, in contrast to the rest of the cochlea, a very small number of large neurons were intensely stained with RT-97 (Fig. 5). In some cases, these large neurons displayed process diameters resembling those of type I ganglion cells (e.g., Fig. 3, arrow). In one rat, 36 heavily labeled large cells were located in the cochlear base; in the entire cochlea, there were 1839 labeled small cells. Large cells thus represented 1.92% of the total immunoreactive population. In a second rat, 60 such basal neurons were labeled where there were also 1,639 labeled small cells; in this case, large cells were 3.6% of the immunoreactive population. These large immunostained neurons of the extreme basal region represent a small percentage of the total number of immunostained neurons (2–4%) and a very small proportion of type I neurons (< 0.3%).

Optical density measurements reveal two intensity peaks from the apical turn, upper middle turn, lower middle turn, and upper basal turn, but only a single peak in the lower basal turn (or hook) of the cochlea (Fig. 6). The extreme base contains few lightly labeled neurons; roughly half of the neurons are moderately (140–170 O.D. units) stained and the remainder are darkly (> 180 O.D. units) stained. Optical density measurements were also compared to cell body silhouette area for these different cochlear regions (Fig. 7). Using 150 O.D. units as the dividing point, in all



MOUSE



CAT



HUMAN

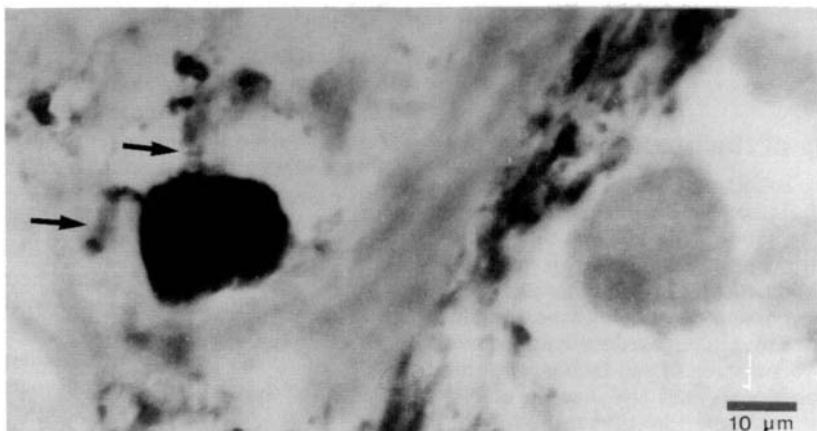


Fig. 2. Type II neurons labeled by RT-97 (immunoperoxidase) in several species. Notice that all intensely labeled neurons exhibit central and peripheral processes, marked by arrows in the human, whose diameters are roughly the same. The constellation of features (e.g.,

small somatic size, 5–10% of the population, uniform process diameters, and Nissl appearance when double labeled) are typical for type II neurons.

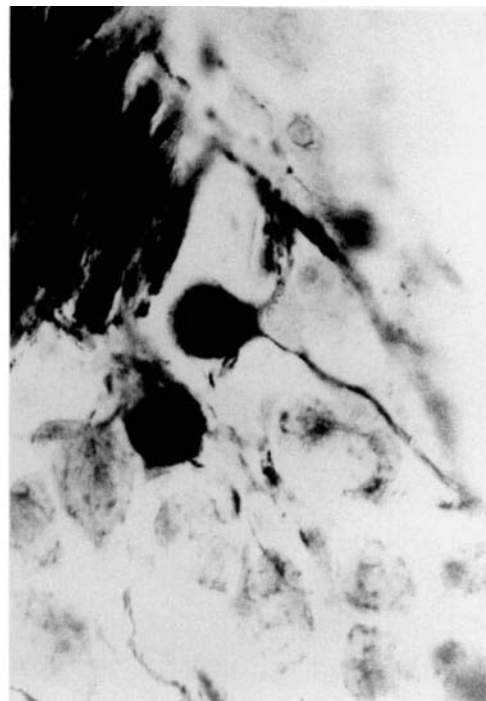
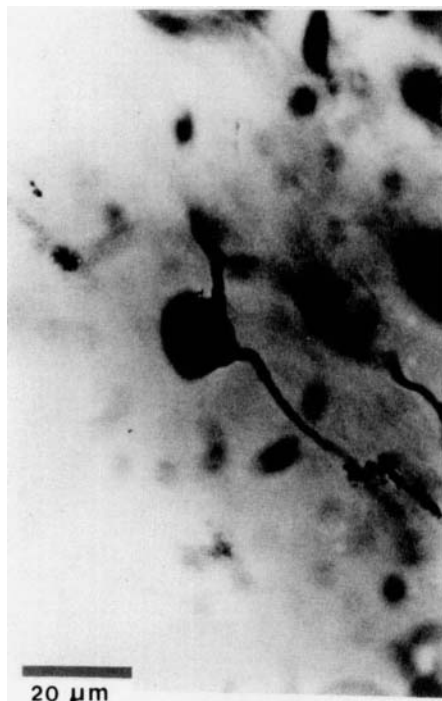
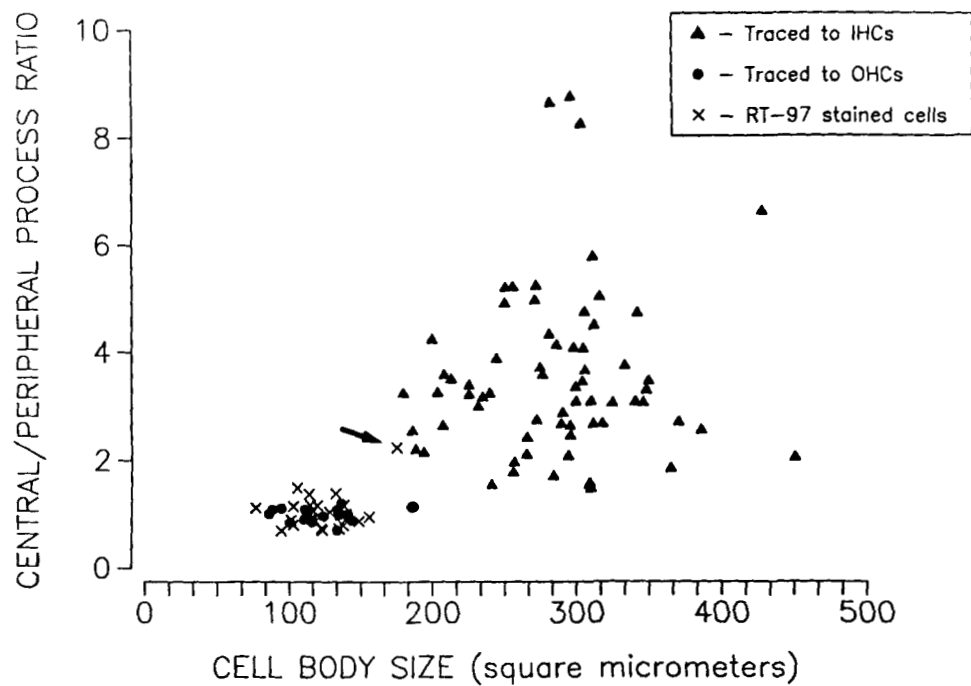


Fig. 3. Neurons intensely stained with RT-97 are compared with HRP-labeled type I and type II neurons whose peripheral processes have been traced to IHCs and OHCs, respectively. Bottom left: Type II neuron labeled with HRP. Bottom right: Two neurons intensely labeled with RT-97 but whose processes could not be traced to receptors. All 3 neurons are approximately the same size. Top plot: Cell body size is plotted against the ratio of central process diameter divided by peripheral process diameter for neurons traced to IHCs (n = 66), to OHCs

(n = 16), and those immunostained with RT-97 (n = 30). Two populations of points are revealed: The more scattered population (filled triangles) in the upper right represents type I neurons and the more circumscribed cluster in the lower left represents type II neurons (filled circles, x's). One RT-97 labeled cell from the base of the cochlea (arrow) has characteristics of a type I neuron. IHC = inner hair cell; OHC = outer hair cell.

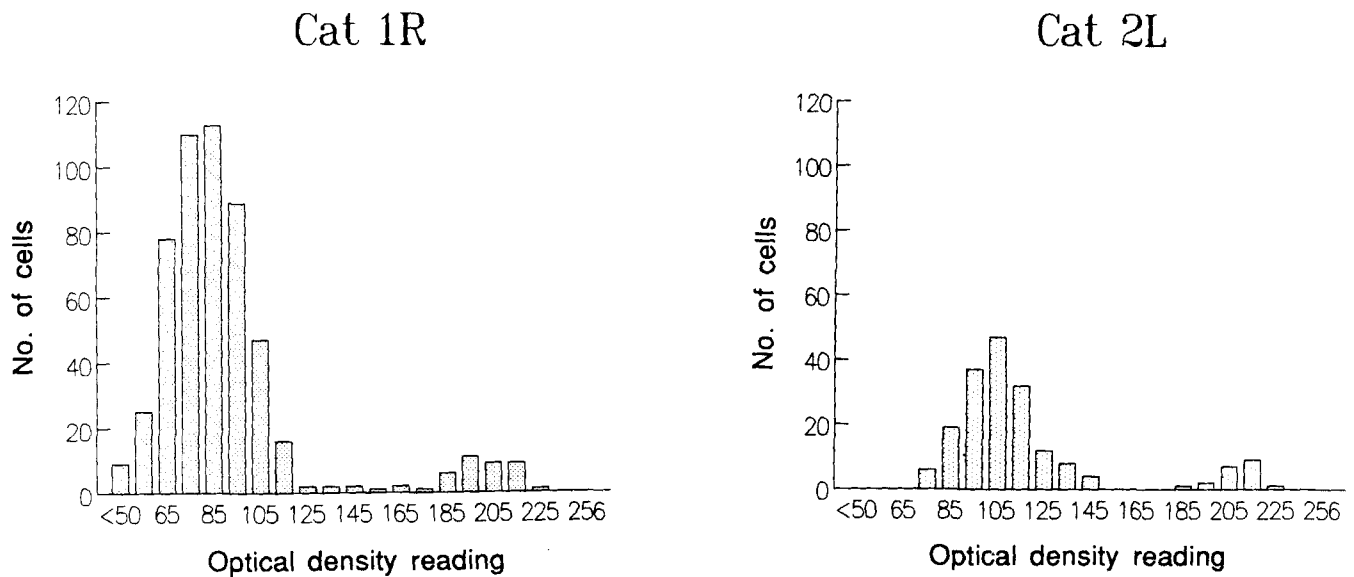


Fig. 4. Histograms representing the distribution of cytoplasmic optical density measurements for spiral ganglion neurons in 2 cats whose cochleas were treated with RT-97. Each histogram contains a major peak of low optical density (type I neurons) and a minor peak of high optical density (type II neurons).

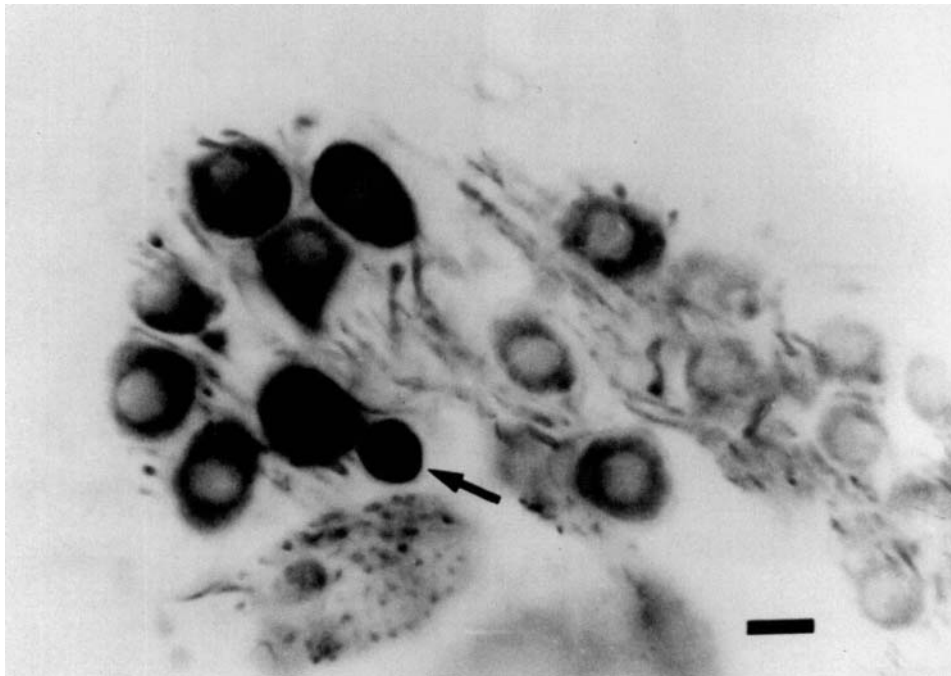


Fig. 5. Spiral ganglion neurons in the extreme base of a rat cochlea immunostained with ICN anti-200. Throughout the rest of the cochlea, this antibody preferentially stains type II neurons. In the base, however, both large and small neurons can be intensely stained and

some large neurons remain unstained. The arrow indicates a small darkly stained neuron, presumably a type II cell. This staining pattern in the base is reliable but not readily explainable. Scale bar equals 10 μ m. In this and the following figures, ICN = ICN Biochemicals.

regions except the extreme base, the light and dark staining cell populations differed with respect to cell body size ($p < 0.001$, Student's *t*-test, 2 tailed). Throughout most of the cochlea, small cells were darkly stained by RT-97 and represented 5–10% of the sample, whereas in the extreme base, there were large and small labeled cells that represented approximately 50% of the population. Although we do not know the significance of this staining pattern in the

extreme base, it is consistent across individual animals and different species.

Labeling with other neurofilament antibodies

We applied several other monoclonal antibodies raised against the three different neurofilament proteins (68-kD, 160-kD, and 200-kD) to cochlear tissue (see Table 2). These

Cat 2L

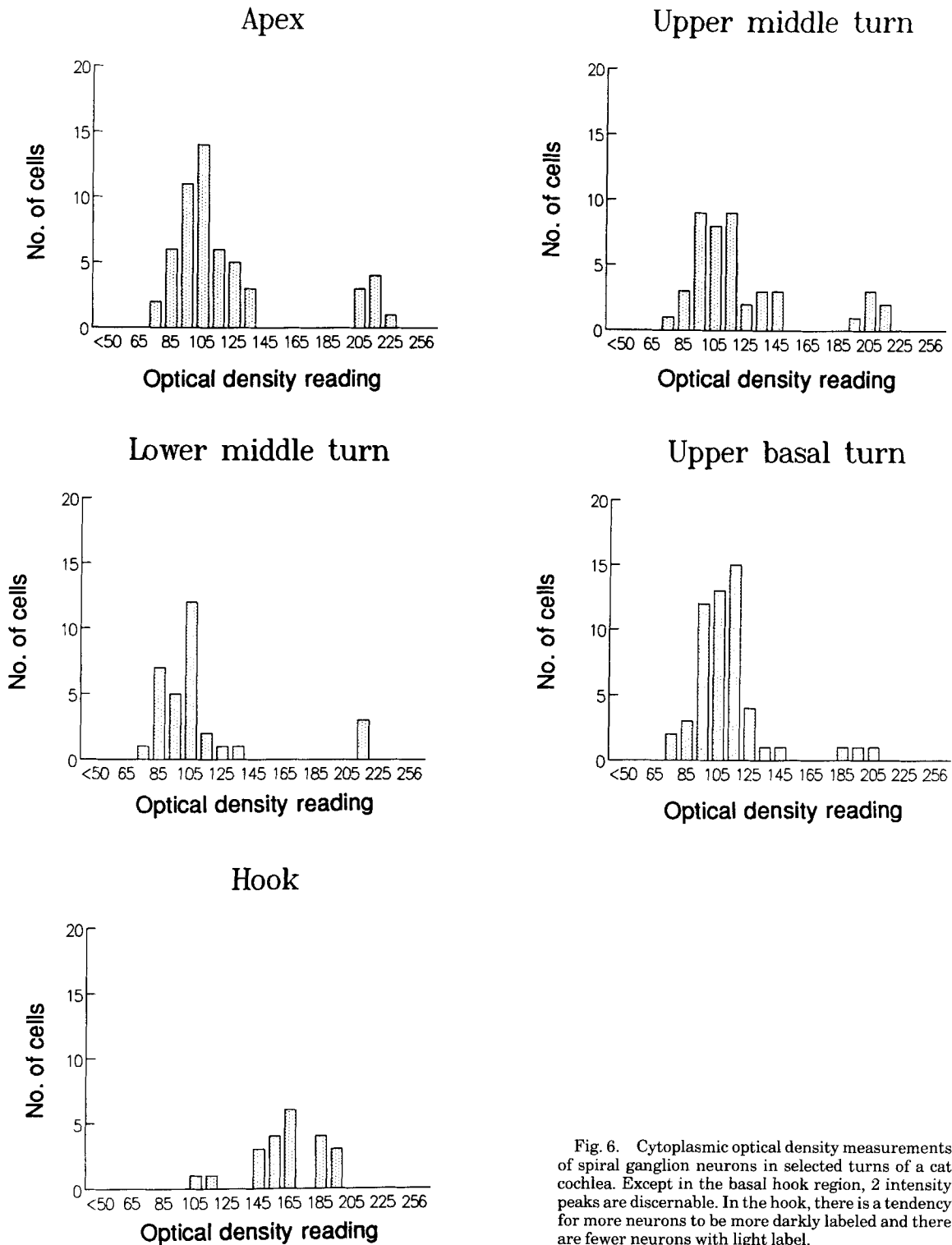


Fig. 6. Cytoplasmic optical density measurements of spiral ganglion neurons in selected turns of a cat cochlea. Except in the basal hook region, 2 intensity peaks are discernable. In the hook, there is a tendency for more neurons to be more darkly labeled and there are fewer neurons with light label.

experiments produced two distinct labeling patterns. One pattern produced by antibodies raised against the 200-kD neurofilament protein (ICN anti-200 and U. Dräger's R-3) labeled only the small ganglion cells and was indistinguishable from that obtained with RT-97 (Fig. 8). That is, double-labeling experiments demonstrated that type II

neurons defined by Nissl criteria were the same as those intensely stained by the antibodies, and in one rat cochlea ICN anti-200 stained 1,197 type II neurons, 7.7% of the ganglion population. Within the ganglion, very few fibers were labeled, whereas outside of the ganglion all primary fibers appeared labeled. Optical densitometry of neurons

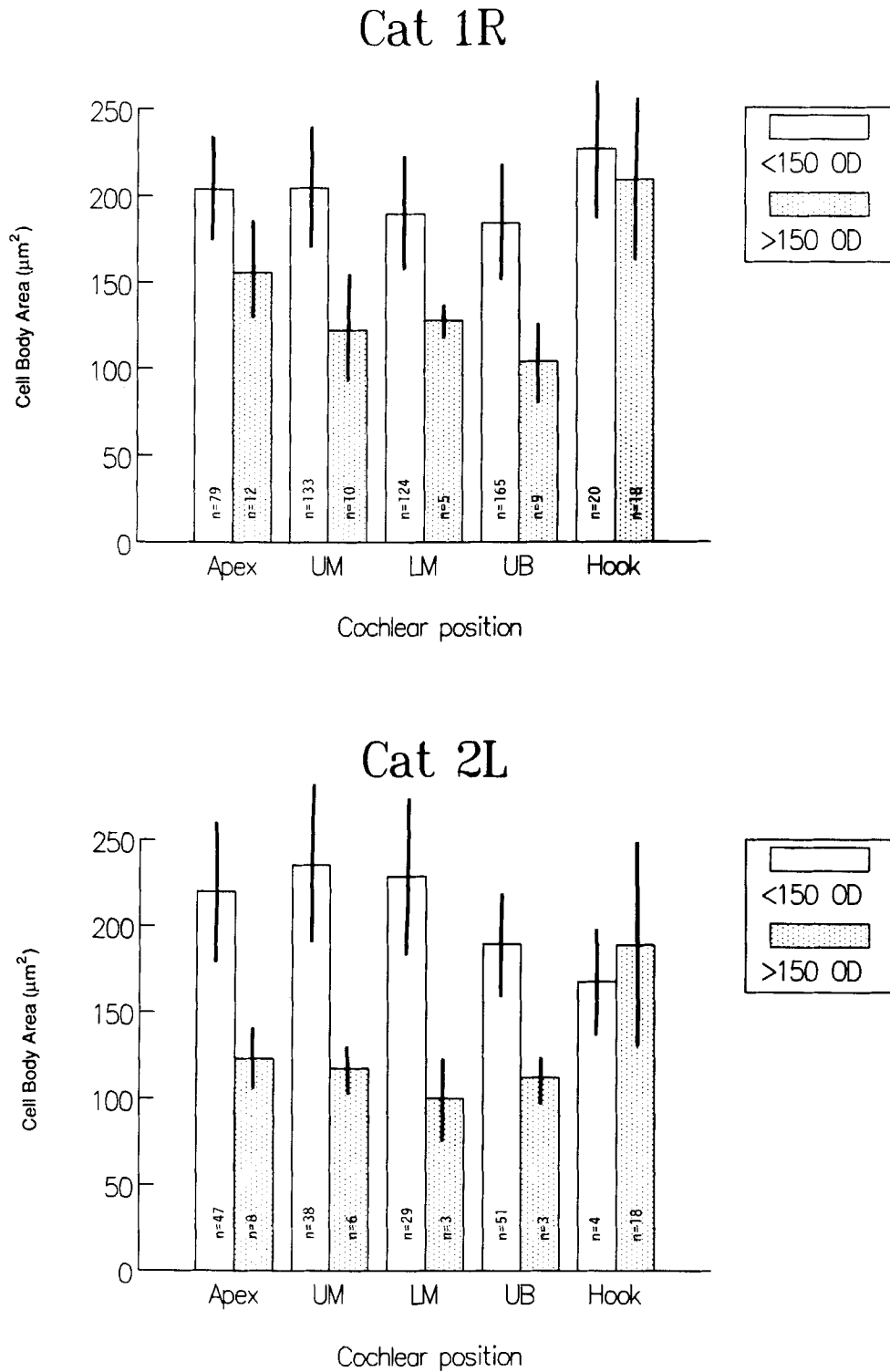


Fig. 7. Histograms demonstrating the relationship of optical density label, cell body area, and cochlear position. Neurons in the apex, upper middle (UM), lower middle (LM), and upper base (UB) are

separated into 2 groups on the basis of both cell body area and darkness of staining. In the basal hook, staining intensity does not correspond to cell body area. Bars indicate standard deviation.

stained with ICN anti-200 revealed a bimodal distribution of "lightly" and "darkly" stained cells in all regions of the cochlea except the extreme base. In the base, 13 large neurons were darkly stained.

The other pattern was produced by antibodies Amersham anti-68 and clone NN18 (Amersham anti-160 and ICN anti-160) that stained the cytoplasm of all neurons in the spiral ganglion (Fig. 9). These antibodies also labeled all

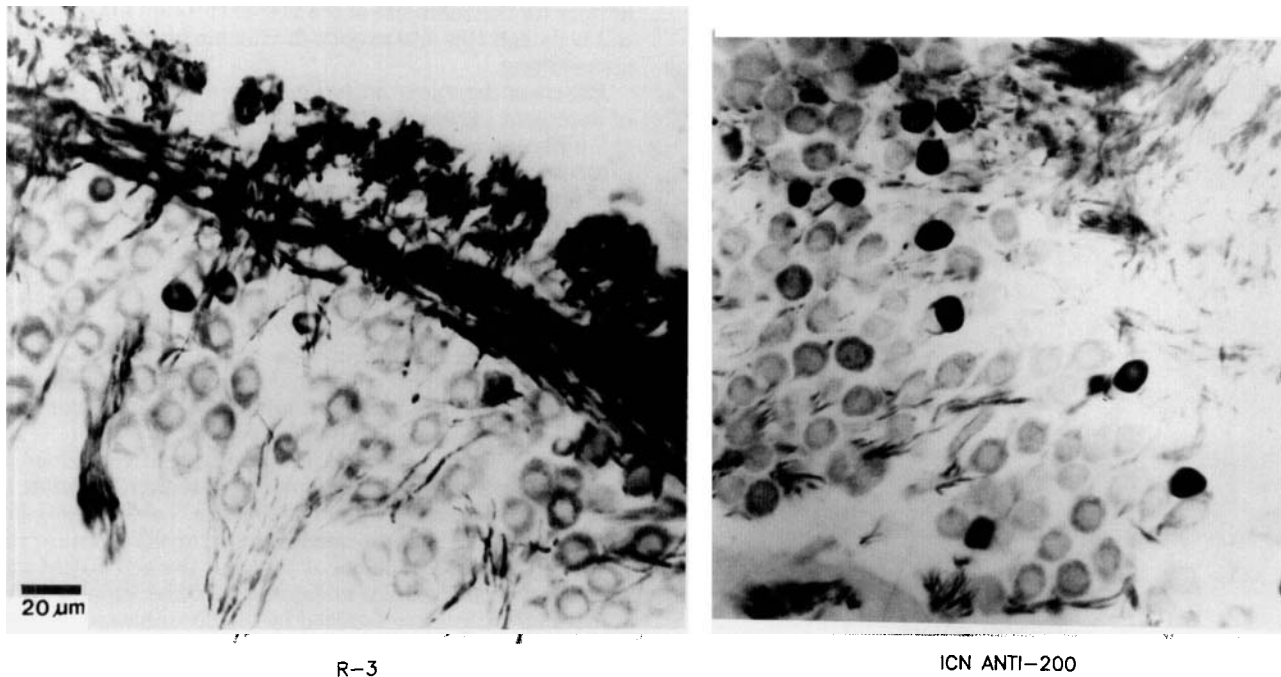


Fig. 8. Photomicrographs of cochlear tissue immunostained with R-3 (left) and ICN anti-200 (right). These monoclonal antibodies are directed against the 200-kD neurofilament protein, and intensely stain type II cell bodies and some fibers in the spiral ganglion. This labeling profile is similar to that exhibited by RT-97.

fibers of the cochlea including those within the ganglion. Antibody SMI-32 (Sternberger-Meyer anti-200) labeled all cell bodies in the spiral ganglion but displayed little fiber staining. Thus we divided our antibodies into two sets on the basis of their somatic staining patterns.

In additional experiments, one in a rat and one in a mouse, we applied members of the above two sets of antibodies to separate but serially adjacent tissue sections of the same spiral ganglion. Again, the staining pattern was repeated: RT-97, R-3, and ICN anti-200 darkly stained type II neurons, whereas ICN anti-160, Amersham anti-160, and SMI-32 uniformly stained the cytoplasm of all spiral ganglion neurons. The processing of tissue from the same animals under identical experimental conditions, except for the primary antibody, reinforced our confidence in the reliability of these immunoreactive differences. The most parsimonious explanation for our observations is the presence of a true difference in antigenicity of the neurofilament components.

Phosphatase treatment

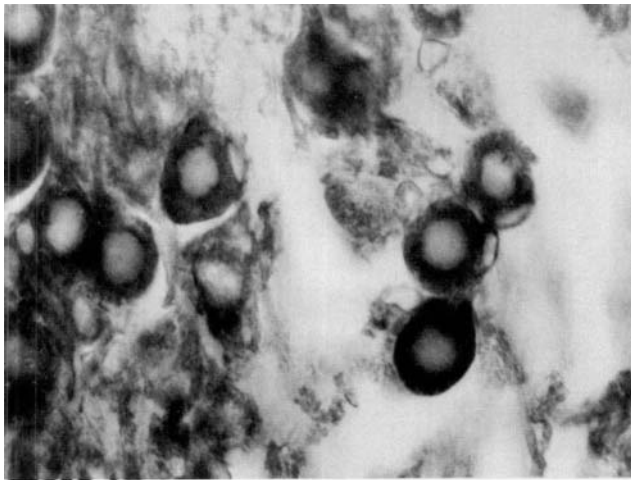
Because the 200-kD neurofilament protein is highly phosphorylated (Julien and Mushynski, '82; Lee et al., '87; Dahl et al., '88), we tested whether our antibodies retained the same labeling patterns for spiral ganglion neurons after phosphate groups were removed by treating cochlear tissue with alkaline phosphatase. Alkaline phosphatase enzymatically dephosphorylates tissue (McComb et al., '79). The three antibodies that preferentially and intensely labeled type II somata displayed no labeling of spiral ganglion cell bodies after phosphatase treatment (Fig. 10). Cochlear fibers were still labeled but reduced in intensity. Control tissue from the same cochleas incubated with either RT-97, R-3, or ICN anti-200 but not subjected to pretreatment with alkaline phosphatase produced intensely stained type

II somata. The elimination of staining following phosphatase treatment implies that the above three antibodies depend on the presence of phosphates in the epitopes and most likely have affinity for one or more phosphorylated epitopes of the 200-kD neurofilament protein in the fixed tissue (Lee et al., '86).

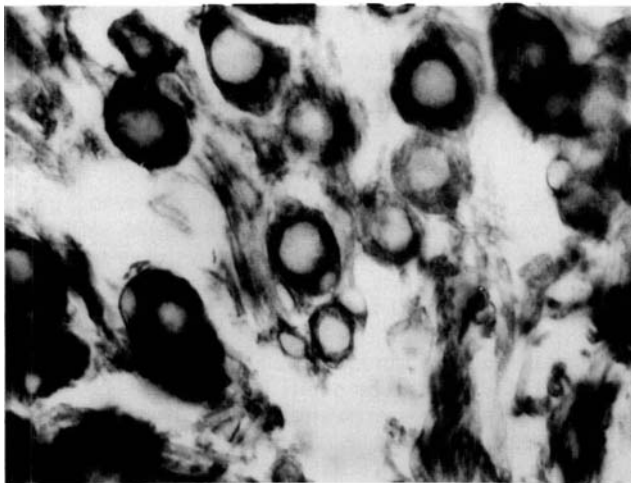
The other neurofilament antibodies tested (Amersham anti-68, ICN and Amersham anti-160, and SMI-32) labeled all ganglion cell bodies, even after the tissue was pretreated with alkaline phosphatase. The intensity of label was approximately the same in phosphatase-treated and phosphatase-untreated sections. Use of tissue from the same cochleas controlled for differences due to variability among animals or experimental conditions. This latter group of antibodies appears to be insensitive to phosphatase.

Other protocols

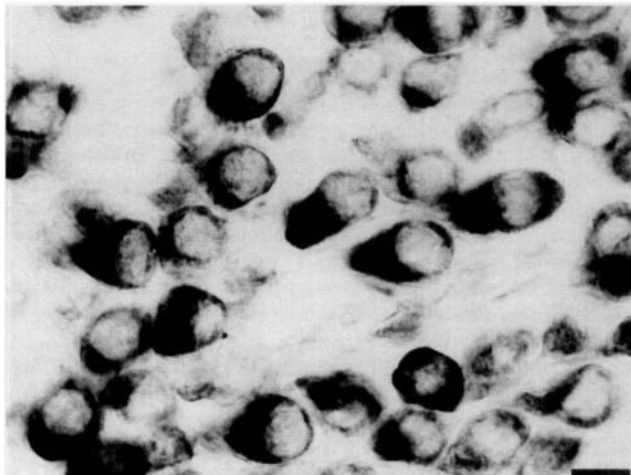
RT-97 and the effects of fixation. With Bouin's fixative, RT-97 produced a labeling pattern that was constant from animal to animal and for each species investigated. Paraformaldehyde and formalin produced less consistent labeling patterns: Some cochleas displayed slight fiber and no somatic staining, others exhibited heavy label in fibers and in most ganglion somata. If the tissue was fixed with a solution containing glutaraldehyde, all staining was diminished. Glutaraldehyde also appeared to disrupt staining by protargol. Using Bouin's fixative, attempts to double label type II neurons with RT-97 and the protargol stain (Berglund and Ryugo, '86) in either order, were unsuccessful. The double labeling results suggest that the two different stains may interact with the same molecular target such that labeling with one may mask the target, making it inaccessible for the other label. Protargol and certain phosphatase-sensitive neurofilament antibodies may have



AMERSHAM ANTI-160



SMI-32



affinity for the same site of the 200-kD protein in the type II cell body and this site may be modifiable by glutaraldehyde crosslinking.

Effects of detergent on immunostaining. The presence of detergent (Triton X-100) produced variable results with the different neurofilament antibodies (see Table 2). The greatest variability occurred with the phosphatase-sensitive antibodies. RT-97 was unaffected by the presence or absence of detergent, whereas R-3 labeled type II somata more intensely when detergent was used; type I somata remained unlabeled. In the case of ICN anti-200, however, detergent treatment resulted in both types of ganglion somata being labeled. Apparently, the epitope recognized by ICN anti-200 is masked in type I cells in the absence of detergent. These data imply that the phosphorylated antigens are different epitopes and reveal the difficulty in interpreting immunocytochemical results.

In contrast, all of our phosphatase-insensitive antibodies required detergent in the primary incubation solution in order to label spiral ganglion cell bodies. Labeling was not restricted by the somatic membrane or myelin because the cryostat sections typically cut through the cell bodies and exposed the intracellular components to the staining solutions. The antigens recognized by the phosphatase-insensitive antibodies appear to be unmasked by the detergent.

DISCUSSION

In the present study, we have applied basic dyes, immunocytochemistry, and horseradish peroxidase histochemistry to cochlear tissue in several mammalian species. Our results provide evidence that the small spiral ganglion neurons, defined as type II neurons by the criteria of cytologic appearance (e.g., Spoendlin, '73; Keithley and Feldman, '79; Kiang et al., '84; Spoendlin and Schrott, '88) and peripheral innervation (Kiang et al., '82; Berglund and Ryugo, '87; Brown, '87), represent the same population that is preferentially and intensely stained by antibodies directed against the phosphorylated 200-kD neurofilament protein. It is our contention that we have a selective marker for type II neurons in the mammalian cochlea. Furthermore, the immunostaining of a small population of small ganglion cells in the human cochlea imply that humans also have type II neurons that innervate OHCs.

Double-labeling experiments directly confirmed that individual ganglion somata defined as type II by Nissl criteria were exclusively stained with the antibodies RT-97, ICN anti-200, and R-3. Morphometric analysis of these immunostained neurons further revealed that they display the same cell body size and process diameter characteristics as those labeled with HRP and reconstructed to their terminations against OHCs. There is also a correspondence between our counts of immunostained spiral ganglion neurons and counts of type II neurons by other investigators. The

Fig. 9. Photomicrographs of cochlear tissue immunostained with monoclonal neurofilament antibodies in rat (Amersham anti-68 and anti-160) and in mouse (SMI-32). When detergent was used in the primary antibody incubation, the cytoplasm of both large and small neurons was highly reactive; when tissue was incubated in alkaline phosphatase prior to antibody treatment, there was no apparent change in the intensity of labeling. These observations imply that the antibodies have a similar affinity for antigens in the somata of type I and type II ganglion cells.

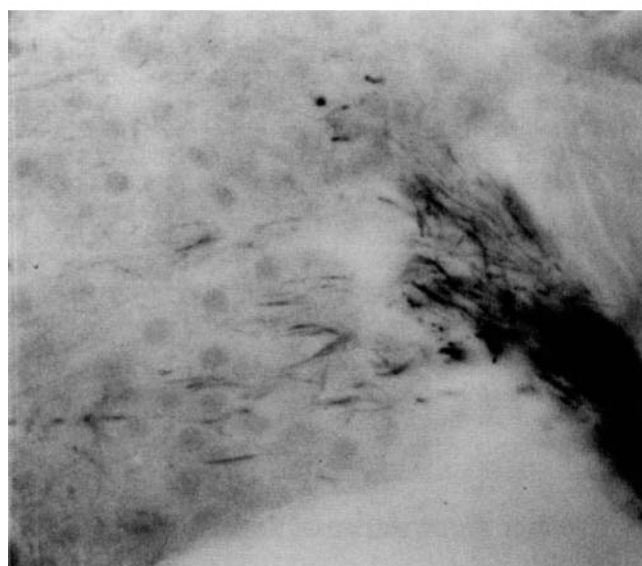
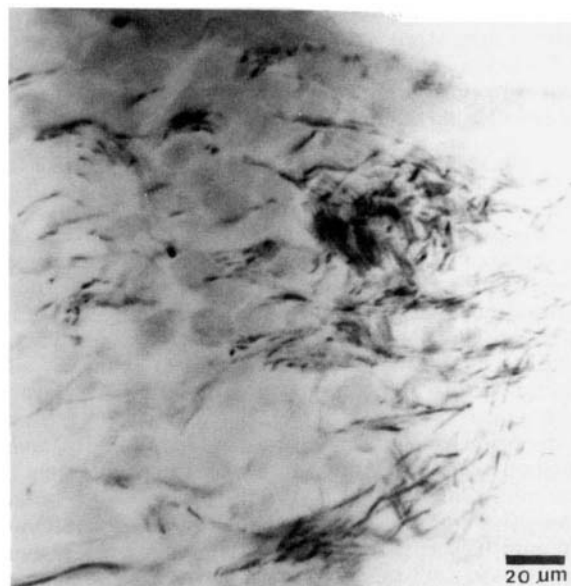
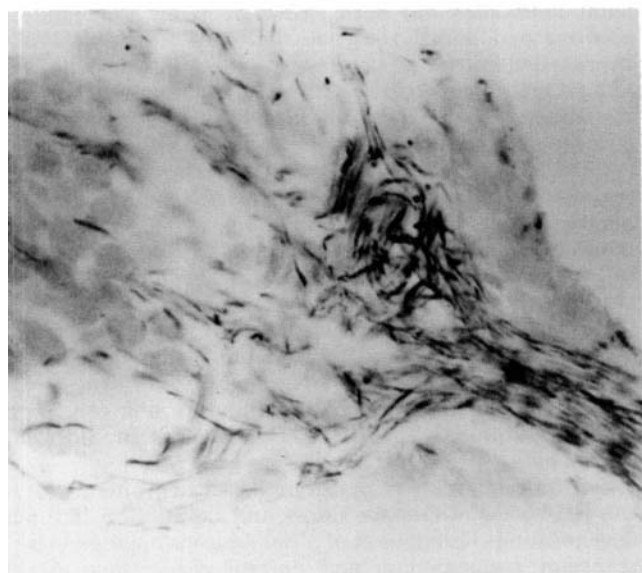
ICN α -200

Fig. 10. Photomicrograph of cochlear tissue from rat incubated with alkaline phosphatase and then treated with 3 antibodies that normally label only the somata of type II neurons (top left and bottom right and left). All somatic labeling was eliminated, although some fibers were still intensely labeled. The control tissue, subjected to the

same conditions but without phosphatase pretreatment, and treated with RT-97, shows the usual intense label of somata of type II neurons (top right). The phosphatase treatment, which removes phosphate groups, suggests that these antibodies recognize a phosphorylated antigen in the cell bodies of type II neurons.

immunostained ganglion neurons in the rat represent 7–8% of the total ganglion population, which is in agreement with the number of type II neurons reported by Keithley and Feldman ('79). In the cat, Gacek and Rasmussen ('61) reported an average of approximately 52,000 myelinated auditory nerve fibers that are the central axons of type I spiral ganglion neurons. If we use this value, we find that cells labeled with RT-97 represent 5.8% of the ganglion population, a percentage that corresponds to the 4–7%

found with protargol staining (Kiang et al., '84) and electron microscopy (Spoendlin, '71). In the mouse, our count of immunostained ganglion cells is consistent with a count of tunnel basal fibers (presumed to be outer spiral fibers) in silver-stained cochleas (Ehret, '79). The percentage of immunostained neurons in the middle turn of a human cochlea (4%) approximates the 6% found in counts of small ganglion cells using the electron microscope (Ota and Kimura, '80). Overall, the proportion of antibody-

labeled ganglion neurons is consistent with that reported for type II neurons using different methods.

Staining in the basal region of the cochlea

Although large neurons were usually unlabeled by RT-97, ICN anti-200, and R-3, a few were moderately or heavily labeled in the basal (high frequency) region of the cochlea. These labeled large neurons represented less than 4% of the total number of immunostained ganglion neurons and less than 0.3% of the total ganglion cell population. We do not know how to categorize these ganglion neurons because they have the histologic appearance of type I neurons and the immunostaining features of type II neurons. There is, however, a precedent for puzzling observations in the mammalian cochlea. Over 25 years ago, Rosenbluth ('62) proposed a now outdated type I and type II dichotomy of spiral ganglion neurons in rats using the electron microscope, where small granular neurons were distinguished from large filamentous neurons. Since type II neurons have subsequently been described as small and filamentous (e.g., Thomsen, '66; Spoenclin, '73), Rosenbluth, who concentrated his study in the cochlear base, may have observed two kinds of type I neurons and failed to detect the relatively infrequent type II neurons. Alternatively, the large immunostained neuron may correspond to that which gave rise to the "giant fiber" projecting to a group of IHCs in the basal region of neonatal rat and cat cochleas (Perkins and Morest, '75), or it may be a type I neuron in a degenerating state, akin to the type III neuron described in pathologic cochleas (Spoendlin, '73). Additional studies are needed to clarify the categories of spiral ganglion cells and the possible variations in peripheral innervation patterns.

Type I and type II neurofilament immunostaining

Two groups of neurofilament antibodies produced two separate patterns of immunostaining in the spiral ganglion. This result merits further discussion because it indicates that antibody labeling does not always completely coincide with the distribution of neurofilaments. Although the visibility of cytoplasmic neurofilaments is one of several criteria for distinguishing type II from type I spiral ganglion neurons (Spoendlin, '71; Kiang et al., '84), there are no quantitative data that address relative density, and both ganglion types are known to contain neurofilaments (Adamo and Daigneault, '73). The group of phosphatase-insensitive antibodies (Amersham anti-68, Amersham anti-160, and ICN anti-160) labeled all ganglion cell bodies and processes in a manner consistent with neurofilament distribution as revealed in electron micrographs (Adamo and Daigneault, '73; Ota and Kimura, '80). In contrast, the group of phosphatase-sensitive antibodies (RT-97, R-3, and ICN anti-200) preferentially labeled type II neurons in their entirety and the processes of type I neurons outside the ganglion. This differential immunostaining was produced even when using adjacent sections from the same tissue and where histological treatment was identical except for the primary antibody. Consequently, our results appear to be due to the presence of different epitopes (of the phosphorylated 200-kD neurofilament protein) or different access to these epitopes. The virtual "all-or-none" immunostaining that distinguishes type II from type I cell bodies clearly emphasizes the cell type distinctions. Although we cannot rule out differences in the relative amount of neurofilaments present between the two ganglion cell types, the

results do argue against the notion that preferential labeling of type II neurons is due solely to a greater density of neurofilaments.

In the dorsal root ganglia, immunostaining has been linked to the relative quantity of neurofilament proteins. For example, axon diameter is correlated with the number of axonal neurofilaments (Lasek et al., '83), the amount of transport of neurofilament proteins (Oblinger et al., '87), and cell body size (Ramón y Cajal, '09). Since all axonal proteins are supplied from the synthetic centers in the cell body, cell bodies giving rise to large axons are expected to contain more neurofilament proteins and thus, to stain more intensely with neurofilament antibodies. The increased quantity of somatic neurofilament proteins has been proposed to account for the more intense staining of large rather than small dorsal root ganglion neurons by the neurofilament antibody RT-97 (Lawson et al., '84). Overall, these results indicate important differences in the immunocytochemical and hence, molecular properties of neurons in the spiral and dorsal root ganglia.

Possible significance of neurofilament label

There is evidence that neurofilament immunostaining of some neurons in the central and peripheral nervous systems may indicate that the labeled neurons are degenerating, or at least manifesting a response to distal axon trauma. The application of phosphate-sensitive neurofilament antibodies has been shown to stain spinal motor neurons and dorsal root ganglion cells following sciatic nerve crush (Moss and Lewkowicz, '83; Goldstein et al., '87) and retinal ganglion cells following axotomy (Dräger and Hoffbauer, '84). Under normal conditions, retinal ganglion cells are not stained and fewer dorsal root ganglion cells are stained. Electron micrographs of neurons undergoing degeneration have depicted an increase in somatic neurofilaments (Guillery, '70; Raisman and Matthews, '72). A build-up of phosphorylated neurofilament proteins in the cell bodies of axotomized neurons could explain the somatic staining in these particular pathologic cases (Moss and Lewkowicz, '83; Dräger and Hoffbauer, '84; Goldstein et al., '87), perhaps resembling the effects of enhanced somatic staining following colchicine treatment (e.g., Yen and Fields, '81). Although neurofilament immunostaining can be related to pathologic conditions, this relationship does not always hold.

The accumulation of phosphorylated neurofilaments in the cell bodies of reactive (Moss and Lewkowicz, '83) and degenerating (Koliatsos et al., '89) neurons appears to be a transient phenomenon, and normal populations of cell bodies stain with other monoclonal antibodies to phosphorylated epitopes of the 200-kD proteins (Trojanowski et al., '86). Populations of presumably healthy neurons stain reliably with RT-97, including many of the cell bodies of the dorsal root ganglia, the mesencephalic nucleus of V, the superior (or jugular) ganglion (Lawson et al., '84), and a majority of those in the vestibular ganglia (unpublished observations). It is clear that certain populations of normal neurons are distinguishable from other populations simply by the abundance of epitopes in their somata, which are reactive with certain phosphorylated 200-kD neurofilament antibodies.

The intense staining of the subpopulation of type II spiral ganglion neurons by RT-97, R-3, and ICN anti-200 most likely results because these three antibodies are directed against phosphorylated determinants of the 200-kD pro-

tein. A number of antibodies have been shown to be specific to such determinants (e.g., Carden et al., '85) and our dephosphorylation of cochlear tissue using alkaline phosphatase is consistent with the idea that the presence of phosphate groups on the 200-kD neurofilament protein is required for antibody recognition (Lee et al., '86). One concern was that our antibodies cross-reacted with phosphorylated determinants of other proteins, specifically tau proteins, a family of heat-stable microtubule-associated phosphoproteins (Ksiezak-Reding et al., '87; Nukina et al., '87). We applied an anti-tau antibody (kindly supplied by Dr. K. Kosik, Kosik et al., '86) to our cochlear tissue and found that the tau antibody did not specifically label type II ganglion cells under our experimental conditions. Overall, there are complex interactions between cross-reactivity and epitope masking by histological procedures (e.g., fixation, detergents), which complicate identifying the structural substrate that accounts for antibody staining patterns.

If one accepts that the pattern of immunostaining in the cochlea is due to the presence of phosphorylated 200-kD neurofilament epitopes, then the question is how to account for that pattern. For type I ganglion cells, only the processes some 100 μm away from the cell body stain, whereas the cell bodies and processes of type II ganglion cells stain intensely throughout. Not only do we need to understand why there is differential staining within individual type I cells but also why the two ganglion cell populations stain differently.

With respect to the question of differential staining within a neuron, the 200-kD neurofilament protein undergoes considerable post-translational phosphorylation as it is transported from the cell body to the axon (Nixon and Lewis, '86); therefore, axons but not somata might immunostain. It has been proposed that one of the primary functions of neurofilaments is mechanical support (Lasek and Brady, '82; Nagel, '88) or they may be involved in mediating intracellular transport of substances and organelles along axons (Lasek, '82). These cellular mechanisms would produce normal variations in the level of phosphorylation according to location and type of neurofilament protein, and consequently, variations in site and intensity of immunostaining. Antibodies do in fact distinguish various neurofilament isoforms, even for the same neurofilament protein (Lee et al., '87). Different subpopulations of neurons and different compartments within a neuron may contain different isoforms of the same neurofilament protein (Goldstein et al., '83).

With respect to the differential staining of the ganglion cell population, the contrast between the myelinated type I cell body and the unmyelinated type II cell body might underlie important cytostructural requirements for the two ganglion cell populations and be revealed by differences in neurofilament composition. For example, the selective presence of phosphorylated neurofilament proteins in the somata of type II ganglion neurons might be necessary for the immediate assembly of neurofilaments, related to stabilizing the cytoskeleton. Ultimately, different task-dependent neurofilament proteins would confer functional versatility for neurofilaments in diverse biological systems. Whatever the functional significance for these differences might be, the fact remains that different neuronal populations can be identified by using monoclonal neurofilament antibody markers.

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