Lectin Labeling of Sprouting Neurons

I. Regional Distribution of Surface Glycoconjugates

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ABSTRACT   Well-defined ferritin-conjugated lectins were used to map glycoconjugates on the surface of sprouting neurons from rat superior cervical ganglion (SCG) and spinal cord (SC). The cultured neurons were exposed to the markers and processed for electron microscopy, and the number of ferritin particles per unit area of plasmalemma was measured in three different regions: perikaryon, neuritic shaft, and growth cone. Three different binding patterns are observed for different lectin: equal receptor density throughout the plasmalemma of the growing neuron (e.g., *Ricinus communis* agglutinin I in SCG neurons), gradual decrease (e.g., wheat-germ agglutinin in SCG and SC neurons) and gradual increase (e.g., *Ricinus communis* agglutinin II in SC neurons) in the density of lectin receptors as one moves from the perikaryon to the growth cone. Furthermore, lectin receptor densities differ in the two types of neurons analyzed. We can conclude that the plasmalemma of the growth cone has biochemical properties different from those of the perikaryon, and that the neuron's structural polarity is expressed in its surface glycoconjugates. This phenomenon may be related to the growth cone's special functional properties and to the process of expansion of the plasma membrane.

The developing neuron has entered a new and critical phase of its differentiation when it starts forming processes. Neuritic components, including vast amounts of new plasma membrane, have to be synthesized. The axon has to find its way to and recognize an appropriate target cell for synapse formation in order to ensure its own and its parent neuron's further survival. The plasmalemma of the growing axon, largely composed of newly synthesized membrane, is likely to be involved in the mechanisms of guidance and target recognition (14, 21). Thus, it is not surprising that the sprouting axon's plasmalemma is different from that of the perikaryon, as indicated by freeze-fracture observations on growing neurites in culture (16) and freeze-fracture and saxitoxin binding data on axons growing in vivo (23–25).

It is of particular interest to investigate glycoconjugates of the growing neuron's plasmalemma because of the widely accepted role of cell surface saccharides in mechanisms regulating cellular differentiation and development (e. g., references 11 and 12). This paper presents the results of such an investigation. The data have been generated by the use of well-defined lectin-ferritin conjugates to map quantitatively surface carbohydrates in various domains of neuronal plasmalemma at the electron microscope level. The studies were carried out on neurons sprouting in culture because only in this system are

cell surfaces freely exposed for experimental manipulations such as washing, enzymatic digestion, and labeling. Some of the results presented here have been reported elsewhere in abbreviated form (17–19).

MATERIALS AND METHODS

Tissue Culture

Two types of rat nerve tissues were used: superior cervical ganglia (SCG), removed just before or right after birth of the pups, and spinal cord (SC) dissected from 14.5- to 15.5-d-old fetus. Each type of tissue was placed in tissue culture at the age at which it would exhibit maximal neuronal sprouting capacity. Both tissues were stripped free of connective tissue ensheathment and cut into small pieces, and from SC segments the dorsal halves were removed to produce small explants containing mainly anterior horn. The tissue pieces were either grown as explants or dissociated with trypsin (230 U/mg protein; 190 U/ml, 30 min at 36°C, Worthington Biochemical Co., Freehold, N. J.), spun through a cushion of 4% bovine serum albumin (BSA) in Eagle's minimal essential medium, and then seeded into culture dishes. Collagen-coated Aclar wells (33C Aclar, gauge 5, Allied Chemical Co., Morristown, N. J.) were used as culture dishes, according to the method of Bunge and Wood (2). The neurons were grown for 3–7 d in vitro in Eagle's minimal essential medium containing 25% human placental serum, 10% chick embryo extract ultrafiltrate, nerve growth factor (calibrated to produce maximal outgrowth in SCG neurons), and 6 mg/ml glucose, but no antibiotics. At the time of labeling, all explants were characterized by a broad halo of vigorous neuritic outgrowth, and dispersed neurons were also found to have produced at least one long neurite tipped by a growth cone. Small pieces of

cerebellum from newborn rats were also grown in the medium described above and treated in the same way as the SC and SCG explants.

Lectin-Ferritin Conjugates

Affinity-purified lectins were conjugated to ferritin with glutaraldehyde and then purified according to the method of Maylié-Pfenninger and Jamieson (9). In brief, ^{125}I-labeled lectin and ferritin were exposed to a very low concentration of glutaraldehyde in the presence of hapten sugar. The resulting conjugate mixture was first loaded on a mucin peptide affinity column, and the lectin-containing conjugates were eluted with the hapten sugar. They were then chromatographed on a Bio-Gel A5m column (Bio-Rad Laboratories, Richmond, Calif.) to isolate the protein peak that contained only one ferritin molecule per conjugate. The ratio of lectin to ferritin in this fraction was estimated by measuring ferritin-specific absorbancy at 310 nm and counting radioactivity to assess the amount of lectin present. On the average, lectin and ferritin were present in a 1:1 ratio. The lectins used for this study and their specificities are listed in Table I.

Labeling Procedure and Processing for Electron Microscopy

Before the experiments, all cultures were rinsed for at least 45 min in a gentle but continuous stream of medium, in which serum and embryo extract had been replaced by 1% BSA to remove nonspecifically adsorbed glycoconjugates from cell surfaces. This washing was critical to avoid patches of heavily labeled, fuzzy material on cell surfaces. The procedure was tested in washout experiments with ^{125}I-labeled serum glycoproteins as follows: The serum glycoproteins were isolated from human placental serum by affinity chromatography on a concanavalin A–Sepharose 4B column and eluted with 0.4 M glucose in phosphate-buffered saline (PBS), pH 6.5. The eluted glycoproteins were radioiodinated by the lactoperoxidase–glucose oxidase method (6) to produce a specific radioactivity of 3.2×10^7 dpm/mg protein. Collagen-coated culture dishes, with three explants each or without tissue, were exposed to 4.3×10^6 dpm ^{125}I-glycoprotein in the complete tissue culture medium for 30 min at 0°C. Each culture was then subjected to a specific number of rinses with BSA medium, drained at the appropriate time, and counted in a gamma counter. The counts presented are averages of three sets of cultures. For comparison, concanavalin A was also labeled with ^{125}I and then purified on a mucin peptide column. The eluted, active lectin had a specific radioactivity of 3.1×10^6 dpm/mg protein. Culture dishes with or without explants were first rinsed as described above and then exposed at 0°C to 1 mg/ml concanavalin A in BSA medium for 15 min. They were then rinsed three times for 5 min each with BSA medium, drained, and subsequently counted in a gamma counter. The results presented are averages of three sets of cultures in each case.

After the rinsing, which was well tolerated by the cultures, they were either cooled and exposed to the lectin-ferritin conjugate (in the presence of 1% BSA; cf. below) for 15 min at 0°C before being fixed, or aldehyde-fixed and then labeled with the lectins. Additionally, several aldehyde-prefixed cultures were digested before lectin labeling with neuraminidase (0.6 U/ml in PBS, pH 6.8 from C. perfringens; Boehringer Mannheim Biochemicals, Indianapolis, Ind., or type VI, Sigma Chemical Co., St. Louis, Mo.) for 30 min at 36°C, in the presence of 1% BSA. The neuraminidase-treated cultures were again rinsed with PBS-BSA and subsequently labeled with the lectins, as described below.

The fixative (1.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, with 0.4 mM calcium chloride and 120 mM glucose added) was infused slowly into the culture dishes over a period of 10 min at room temperature (cf. reference 15). The fluid in the dishes was then completely replaced by fresh fixative, in which the tissue was allowed to sit for 20–40 min at room temperature. In the cases of

lectin labeling before aldehyde fixation, the cultures were subsequently washed with arsenate buffers, osmicated, and processed for embedding as described below. In those cases where lectin labeling followed aldehyde fixation, the cultures were gently rinsed with several changes of 1 mM glycine in PBS to quench remaining aldehyde groups, and subsequently with PBS containing 1 mM glycine and 1% BSA.

Labeling of prefixed cultures with lectin-ferritin conjugates was carried out in the PBS-BSA solution for 15 min at room temperature. The presence of BSA during the labeling step is crucial for keeping ferritin-dependent nonspecific binding to a minimum (9). The marker was applied at concentrations that were found to be saturating for isolated pancreatic cells (10). These concentrations are listed in Table I. In addition, a few cultures were exposed to considerably lower or higher lectin concentrations (50 μg/ml and 500 μg/ml wheat-germ agglutinin [WGA]). After the labeling step, the cultures were gently rinsed three times for 5 min each with PBS-BSA. Control experiments were done in the same way except that PBS-BSA used for rinsing, as well as the lectin-ferritin solution, contained 0.2 M hapten sugar (Table I). After labeling and rinsing, cultures were refixed with the aldehyde solution described above with the hope of anchoring the cell surface markers covalently to some amino groups that may still have been left on the cell surface. After 20 min of aldehyde fixation, cultures were gradually transferred into 0.2 M arsenate buffers, pH 7.3, with 150 mM glucose added; the initial buffer contained 2 mM $CaCl_2$, whereas the second rinse contained 5 mM $CaCl_2$. A solution containing 1% OsO_4 in 0.2 M arsenate buffer (pH 7.3), 10 mM calcium chloride, and 50 mM glucose was used for gentle osmication for 20 min at room temperature. After extensive rinsing with sodium chloride (0.17 M in water), the cultures were treated with a solution of 0.5 g of uranyl magnesium acetate in 100 ml of the same saline for 20 min, rinsed with sodium chloride solution, and then rapidly dehydrated in a graded ethanol series and embedded in Epon 812.

After polymerization, the Aclar culture well was simply peeled off the Epon disk, and the embedded culture was examined under the phase-contrast light microscope. Areas exhibiting vigorous neuritic outgrowth and spread-out growth cones were selected, cut out, and remounted on Epon blanks for thin sectioning. Sections were cut in parallel to the culture substratum with emphasis on even section thickness (silver interference color, ~60 nm). Sections were stained with uranyl acetate and lead citrate.

Quantitative Analysis of Lectin Binding Sites

The goal of this study was to obtain reliable numbers for the density of lectin binding sites per unit area of membrane. Assuming even and known section thickness, the number of ferritin particles seen along a perpendicularly cut stretch of membrane should be directly proportional to the actual density of lectin binding sites. Therefore, perpendicularly cut membrane areas were randomly selected (see below) and photographed at a primary magnification of 25,000 or 26,000. In these electron micrographs, enlarged 2.5 to 2.6 times, all cross-sectioned membrane areas showing clear-cut unit membrane structure were designated, their lengths measured, and the associated ferritin particles counted under a magnifying glass. To calculate membrane areas, the measured lengths were multiplied by the "effective section thickness," S_e. We assumed that the 10-nm-diameter iron core of a ferritin particle was unlikely to be visible in the electron microscope if its center fell into the most superficial 5 nm on either side of the section because at least half of it would be cut away. Accordingly, the estimated section thickness was reduced by 2×5 nm in order to obtain the value for S_e (50 nm). Thus, visible particles per unit area of membrane could be calculated.

Occasionally, the plasmalemma of a certain cellular region was seen not only sectioned perpendicularly but, a little farther on, cut tangentially so that an en-face view of the membrane and its label was obtained. In these cases, areas could be measured directly and the associated ferritin particles counted. The resulting density values were then compared with those obtained from counts and mea-

surements on adjacent, perpendicularly cut membrane regions. In the five cases where this comparison could be made, the values obtained from tangentially cut membranes were consistently higher by an average factor of 1.49. Membrane areas seen *en face* are likely to be oriented in the section at an oblique angle and, consequently, membrane areas appear smaller (thus, density values higher) in the transmitted electron beam. However, this error cannot be greater than a few percent in the areas selected, so the source for the density difference must be sought in the analysis of cross sections. It is likely that a significant number of ferritin particles cannot be seen in cross section because of superimposition, and because the membranes are not perfectly smooth and thus particles that lie in concave plasmalemmal regions may be obscured. To account for these errors in the measurement of lectin receptor density, we chose to adjust all particle density measurements obtained from perpendicularly sectioned membrane areas by the correction factor indicated above, × 1.49.

To obtain statistically meaningful data, we collected samples as follows: Except where indicated, at least two to three completely independent experiments were carried out for each lectin and each tissue. For random sampling, in each experiment all perpendicularly cut plasmalemma of each domain, encountered in at least seven different cells and at least three different sections (to compensate for slight variations in section thickness), was photographed for quantitative analysis. Such sampling resulted in the analysis in each experiment of an average area of 0.65 μm^2. Because average values for the different experiments turned out to be similar, all density measurements were pooled and the mean value and standard error of the mean were calculated. To assess the significance of differences in lectin binding of different cellular regions, the pooled data for the plasmalemmal domains were compared with the t test.

RESULTS

General Features of the Labeling of Cultured Neurons

The mapping of surface components of cultured cells is complicated by a number of problems that have rarely been dealt with in detail but are of great importance if reliable quantitative results are to be obtained.

CELL SURFACE CONTAMINATION: Cell surfaces, especially those of nerve cells that are grown in very rich media, are coated with a number of extrinsic glycoconjugates that originate from serum components and additives (such as embryo extract) of the medium and, possibly, further substances that may have been released by the cells. It is critical that the vast majority, if not all, of these adsorbed glycoproteins be removed from the cell surfaces before lectin labeling is attempted. In a series of experiments we have measured the washout from the cultures of a group of medium glycoproteins, glucosyl/mannosyl glycoproteins isolated from human placental serum. The resulting curve in Fig. 1 describes the removal of the ^{125}I-labeled glycoproteins from the cultures during a sequence of five rinses carried out over a period of 150 min. After the fourth rinse with medium containing 1% BSA as the only protein, a medium that we have found to be very effective, residual radioactivity in the culture dishes reaches a constant level just above that of tissue culture dishes without nerve cells in them. This curve also shows that a single rinse or two rinses would result in incomplete desorption of serum glycoproteins. For an estimate of the contribution that adsorbed serum glycoproteins (remaining after five rinses) make to total lectin binding, we have compared the amount of residual serum glycoprotein with that of ^{125}I-concanavalin A binding. Results are shown in Table II. The amount of labeled proteins (serum glycoprotein or lectin) bound to the nerve cells can be calculated from the comparison of empty and tissue-containing dishes. The respective values for serum glycoprotein and concanavalin A are 16 and 196 ng. We do not know the predominant glycoprotein species of our serum glycoprotein fraction, but assume that the average glycoprotein has a molecular weight in the neighborhood of 100,000. On a molar basis, and assuming average binding of one lectin molecule to one gly-
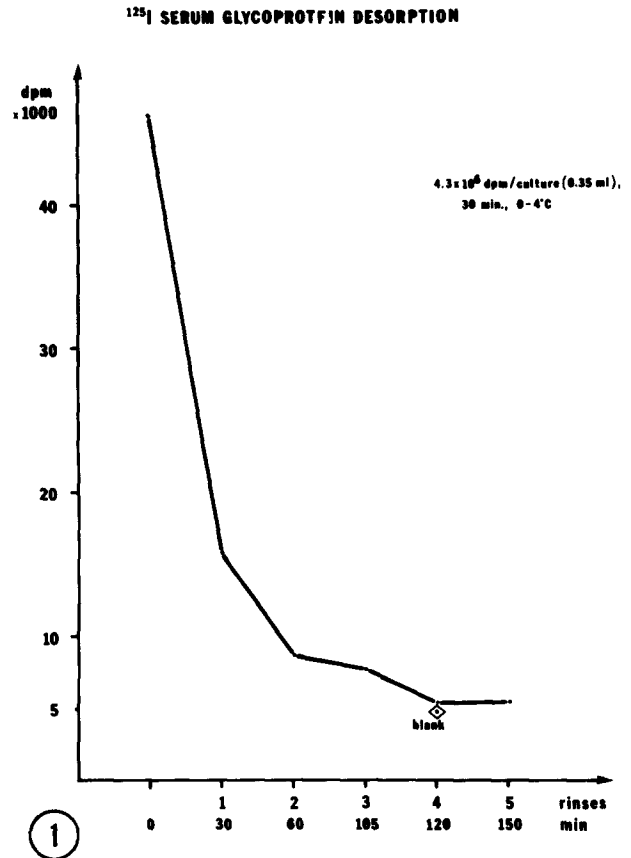

FIGURE 1 Residual ^{125}I-labeled serum glycoproteins in nerve tissue cultures as a function of rinsing with medium containing 1% BSA (cf. text). The values are averages of three cultures for each point. The point in the diamond (*blank*) was obtained from collagen-coated but tissue-free culture dishes. Note that a plateau is reached only after four to five rinses with BSA medium.

TABLE II

Adsorbed Serum Glycoproteins vs. Con A Binding Sites in Nerve Tissue Cultures

	^{125}I-Serum glycoprotein*		^{125}I-Con A	
	dpm	*ng*	*dpm*	*ng*
Empty dishes‖	4,992¶	156	1,665¶	532
Dishes with tissue‖	5,513¶	172	2,278¶	728
Difference	521	16‡	613	196§

* Glucosyl/mannosyl glycoprotein isolated on a Con A-Sepharose column from human placental serum. 45,000–900,000 mol wt.

‡ Estimated average molecular weight of majority of glucosyl/mannosyl glycoproteins in serum, 100,000; corresponding amount in picomoles, 0.16.

§ 104,000 mol wt; corresponding amount in picomoles, 1.89.

‖ All dishes are collagen-coated; dishes with tissue contain three explants each.

¶ Counts per dish, mean of three dishes.

coprotein molecule, we estimate that adsorbed glucosyl/mannosyl glycoproteins contribute ~8% of the total number of concanavalin A binding sites. Considering that many of the serum glycoproteins may be trapped in intercellular spaces rather than adsorbed to freely exposed membrane areas (which are the ones used for quantitative analyses of lectin-ferritin binding), the contribution of adsorbed medium glycoprotein to lectin binding sites measured on the membrane is likely to be well below 10%.

On the basis of these data, the cultures used for the quantitative lectin binding analysis presented here have been rinsed in a continuous stream of medium containing 1% BSA for a period of 45 min. During this continuous rinse the medium in the culture dish is exchanged at least 20 times, which results in considerably more extensive washing than the maximum analyzed in the washout experiments. The addition of BSA to the rinsing medium seems to improve significantly the effectiveness of the rinsing procedure.

LECTIN CONCENTRATION AND NONSPECIFIC BINDING: It is not possible to study biochemically the concentration dependence of lectin binding to nerve tissue cultures because the neurons' geometry and the cultures' irregular growth patterns do not permit the use of DNA, protein, or lipid as a bias for the determination of the amount of exposed cell surface. Therefore, lectin-ferritin conjugates have been applied at concentrations that are saturating for isolated pancreatic cells (10). In one experiment, ferritin-WGA (F-WGA) has been applied at three different concentrations (50, 200, and 500 $\mu g/$ ml) to neurons of the SCG. Yet, no differences in labeling density have been found electron microscopically, indicating that the saturating lectin concentrations determined for the pancreatic cells are saturating for the neurons as well.

Nonspecific binding of the conjugates is determined by incubation of cultures with lectins in the presence of 0.2 M hapten sugar (cf. Table I). For all lectins used in this study, nonspecific binding is very low, not measurable with our electron microscope method ($<25/\mu m^2$). Examples of the lectin labeling are shown in Figs. 2–4. Fig. 2 shows a small process of an SCG neuron, labeled with F-WGA. The iron cores of the ferritin particles are seen to form a uniform layer at close proximity to the plasmalemma's trilaminar structure. Fuzzy, slightly electron-dense material that can often be seen in insufficiently rinsed cultures is not present in this picture or in any of the preparations used for this study. A control preparation, SCG labeled with F-WGA in the presence of N-acetylglucosamine, is shown in Fig. 3. No ferritin particles can be seen attached to the cell membrane. For further demonstration of the specificity of lectin binding in nerve tissue cultures, Fig. 4 shows an example of ferritin–Ricinus communis agglutinin II (F-RCA II) binding to a culture of rat cerebellum. Neuronal elements, as well as processes of a nonneuronal cell, probably a macrophage, can be seen. Although the membrane of the putative macrophage is heavily labeled with the conjugate, the neurites are completely free of the marker.

LABELING OF LIVE AND ALDEHYDE-PREFIXED NEURONS: To avoid redistribution artifacts, it is necessary to slow down or stop the lateral movement of membrane glycoconjugates by low temperature (0°C) or aldehyde before labeling. Cooling to 0°C does not seem to alter neuronal ultrastructures, especially not that of cytoskeletal elements or membranes. Although glutaraldehyde does not react with the carbohydrate residues of glycoconjugates, it is conceivable that fixation before lectin labeling influences the results by changing the configuration of membrane glycoproteins. Therefore, the labeling with F-WGA of fresh and glutaraldehyde-prefixed neurons was compared quantitatively. The results are indicated in Table III. Glutaraldehyde prefixation leads to a small, marginally significant decrease in the density of lectin binding sites (13%). Prolonged exposure to lectin at 36°C is expected to lead to a decrease in the density of binding sites because of internalization and/or capping-like rearrangement of the membrane-bound ligand. The observed decrease in binding sites

over a period of 15 min, however, is surprisingly small and, in fact, may not be significant (confidence level, $P < 0.10$). The meaning of this result is discussed in more detail in the companion paper (20).

IDIOSYNCRASIES OF NEURONS IN CULTURE: Primary nerve tissue cultures are the only neuronal system in which the labeling and surface mapping of different parts of neuronal plasmalemma are possible. However, the unnatural microenvironment in vitro may be altering the neuron's expression of glycoconjugates. Although this question cannot be fully answered at present, we can compare lectin binding of neurons that were grown in explant culture with those grown after dissociation by trypsin. The results of such experiments involving SCG neurons and the lectins WGA, RCA I, and RCA II are presented in Table IV. No significant differences in surface labeling of exposed neurites were found. Therefore, we combined data derived from explant cultures and dissociated neurons.

Although the culture system is ideal for surface labeling studies, it should be noted that even in the dispersed neuron cultures very close cellular associations may occur, especially those between supporting cells and neurites. It is not infrequent to see very thin glial processes, sometimes $<0.2 \mu m$ thick, that wrap neurites or perikarya. Such cellular processes would escape light microscopic detection and, thus, may mislead the investigator in studies with fluorescent cell surface probes.

## Lectin Binding Sites on Sprouting Neurons from the SC

WGA: Results of F-WGA labeling of spinal cord anterior horn neurons are illustrated in Figs. 5–7, and the quantitative results are presented in Table V. A moderate density of label is observed in the various plasmalemmal regions of the neuron: perikaryon, neuritic shaft, and nerve growth cone. The label tends to be somewhat clustered. This is also true for the binding of the other lectins to SC neurons (cf. Fig. 11), but not for lectin binding of SCG neurons treated identically (cf. Figs. 8 and 9). Only quantitative analysis reveals that there is a decrease of 28% in the density of binding sites as one moves from the perikaryon to the distal end of the neurite. Quantitative results are consistent within and between experiments of the same type, as indicated by the relatively small standard errors (this is true for all lectins). Note also the similarity of data from closely related but not identical experiments, as shown in Tables III, IV, and VI (unfixed vs. prefixed labeling; explant vs. dissociated-cell cultures).

RICIN I: As indicated in Table V, the density of F-RCA I binding sites on the perikaryon of SC neurons is similar to that of WGA. However, as one moves from the perikaryon to the growth cone, only a minor decrease in lectin binding sites can be observed. In the t test, the significance of this decrease is marginal at best ($P < 0.10$). F-RCA I binding was also studied after neuraminidase treatment of aldehyde-prefixed spinal cord neurons. A more than twofold increase in RCA I binding was observed, thus revealing a consistent and high density of RCA I sites throughout the neuronal plasmalemma, at $\sim 2,900/\mu m^2$.

SOYBEAN AGGLUTININ (SBA) AND RICIN II: These two lectins have the same monosaccharide specificity, N-acetylgalactosamine, and galactose, but distinguish sugar linkages and/ or their immediate environment (see, e.g., references 7, 8, 10). The labeling of spinal cord neurons is a further strong indicator of this fact. Whereas F-SBA binding is not measurable with

the electron microscope (Fig. 14), F-RCA II binding is significant, albeit low (Figs. 10 and 11). The regional binding pattern of RCA II is particularly remarkable. Contrary to WGA and RCA I, RCA II binding increases by >100% from the peri-karyon to the growth cone. This increase is highly significant. Pretreatment with neuraminidase leads to a nearly fivefold increase of F-RCA II binding at the perikaryon. In the neur-aminidase experiments, preterminal RCA II binding sites are
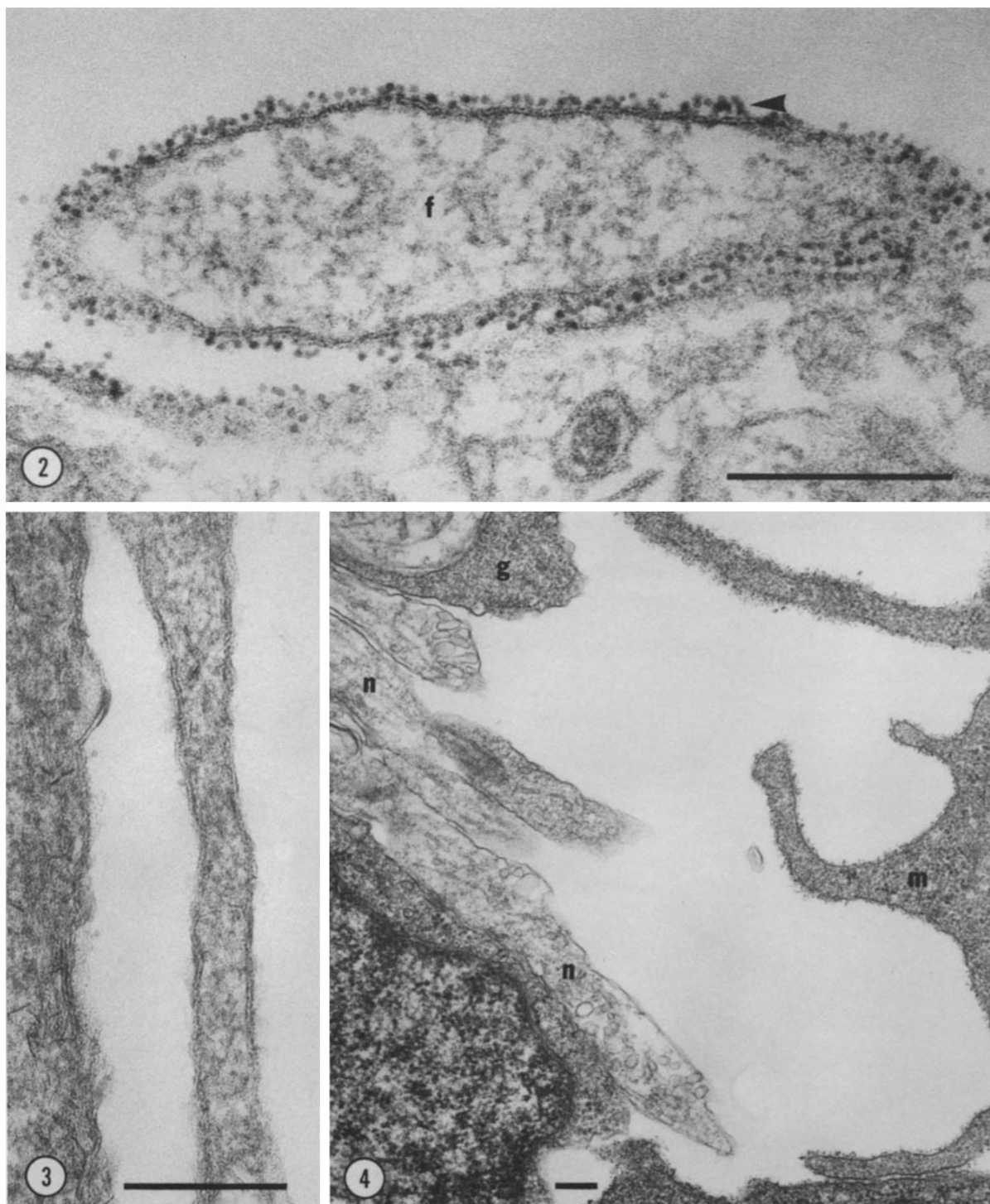
FIGURES 2 and 3   High-power electron micrographs of labeling experiment with F-WGA and control carried out in the presence of 0.2 M GlcNAc. In the experimental case (Fig. 2), a uniform layer of electron-dense ferritin particles (arrowhead) can be seen covering a growth cone filopodium (f); labeling was carried out in the cold, before fixation. The small neuronal process in the control experiment (Fig. 3) was labeled after aldehyde fixation; it is completely free of the label. Bar, 0.2 μm. Fig. 2, × 181,000. Fig. 3, × 130,000.

FIGURE 4   Cellular processes in the distal outgrowth of an explant culture of rat cerebellum, labeled with F-RCA II after aldehyde prefixation. Note that neuronal (n) and glial processes (g) are not labeled, whereas processes of a putative macrophage (m) exhibit a heavy layer of the marker. Bar, 0.2 μm. × 33,300.

also seen to increase in density as one moves toward the growth cone (by a total of 23%), but this increase is not nearly as dramatic as for the terminal RCA II binding sites. Line 7 in Table V shows the difference in lectin binding sites between neuraminidase-treated membranes and controls. Although this difference increased by 25% for RCA I between perikaryal and growth cone plasmalemma, it is constant for RCA II binding sites. Electron micrographs of F-RCA II labeling of spinal cord neurons are shown in Figs. 10 and 11. Figs. 12 and 13 show labeling with the same lectin after neuraminidase pretreatment.

Lectin Binding Sites on Sprouting Neurons from the SCG

For comparison with SC neurons, the binding of three lectins—WGA, RCA I, and RCA II—was also studied on sprouting principal neurons from the SCG. Results are illustrated in Figs. 8 and 9 and listed in Table VI.

WGA: F-WGA labels these neurons at high density in all plasmalemmal regions. However, a small but significant decrease in the density of lectin binding sites can be observed as one moves from the perikaryon to the nerve growth cone. In all these areas labeling density is considerably higher and more even than in SC neurons (Figs. 8 and 9, to be compared with Figs. 5 and 7).

RICIN I AND RICIN II: F-RCA I and F-RCA II bind at moderate to high density to all plasmalemmal regions of the sprouting SCG neurons. The binding patterns are very different from those found on SC neurons. Whereas RCA I binds highly uniformly to the plasmalemma of SCG neurons, RCA II's receptors exhibit a small, marginally significant decrease in

density as one moves from the perikaryon to the nerve growth cone.

Binding of F-SBA to SCG neurons will be dealt with in a forthcoming communication.

DISCUSSION

Region-specific Membrane Composition in the Growing Neuron

The various control experiments performed in this study indicate that, under appropriate conditions, lectin receptors can be mapped quantitatively in the various plasmalemmal regions of the sprouting neuron. Among the most important parameters are knowledge of the surface marker (binding specificity and ligand-to-marker ratio) and removal of medium glycoconjugates from the surfaces of the cells to be examined. In these experiments, ferritin-to-lectin ratio is ~1:1, and wash-out studies indicate for the model case of concanavalin A that the contribution of medium glycoproteins to measured lectin binding sites does not exceed 10%. Furthermore, nonspecific binding is so low that it cannot be measured with the electron microscope and, therefore, amounts to a few percent or less of observed binding. Because of the large sample sizes, the data can be used for accurate comparison of lectin binding sites in different membrane regions and on different types of neurons. In addition, an attempt has been made to generate absolute values for the density of lectin binding sites per unit area of membrane. Our density figures are thought to be realistic in relative as well as in absolute terms (a) because the entire binding site analysis was strictly confined to truly cross-sectioned membrane areas and (b) because values obtained from cross-sectioned membrane regions could be compared in several cases with those from tangentially sectioned membrane areas of the same cellular element. Control experiments show that glutaraldehyde prefixation may slightly decrease the number of available lectin binding sites on the membrane, at least in the case of WGA. The most likely explanations for this decrease are steric hindrance resulting from cross-linking of membrane glycoproteins and/or the attachment to membrane glycoproteins of glutaraldehyde molecules and glycine, which was used as a quenching reagent. For consistency, the data presented in the Tables V and VI are derived from cultures that were first glutaraldehyde-fixed and then lectin-labeled. The last point dealt with in our control experiments is the influence of the culture environment on lectin receptor density. Although it is impossible to claim that the lectin receptor densities described in the cultured neurons are identical to those present in vivo, we can exclude alterations of membrane

TABLE III

WGA Labeling of Live and Glutaraldehyde-prefixed Nerve Growth Cones

	Binding sites*		Δ	P‡
	per μm^2	n		
Glutaraldehyde-prefixed	2,280 ± 90	20		
			13%	<0.025
Fresh, 0°C	2,610 ± 120	15		
			12%	<0.10
Fresh, 36°C§	2,300 ± 110	8		

Data are from a set of three SCG cultures, derived from the same litter of rat pups, labeled with the same F-WGA preparation, but processed differently.
* Mean ± SEM, with number (n) of lengths of membrane analyzed.
‡ Confidence level in t test.
§ Cultures were rinsed, exposed to a saturating concentration of F-WGA for 5 min, and then incubated for 15 min in the absence of the conjugate (chase). All these steps were carried out at 36°C. Aldehyde fixation followed the chase.

TABLE IV

Influence of Cell Dissociation on the Density of Lectin Receptors on Neuritic Shafts and Nerve Growth Cones*

| Lectin	Location	Binding sites‡				Δ Explants − dissociated§	
		Explants		Dissociated			
		per μm^2	n	per μm^2	n	%	P
WGA	Growth cone	2,450 ± 190	8‖	2,220 ± 70	30¶	9	ns
RCA I	Shaft	2,330 ± 70	23¶	2,290 ± 70	34¶	2	ns
	Growth cone	2,380 ± 50	17¶	2,290 ± 80	24¶	4	ns
RCA II	Shaft	1,470 ± 90	23‖	1,410 ± 150	12¶	4	ns

* Principal neurons from rat SCG.
‡ Mean ± SEM, followed by n, number of membrane lengths analyzed.
§ Difference of density in explant cultures and dissociated neurons, in % of explant values. P = confidence level in t test.
‖ Data from one experiment.
¶ Data from two experiments.
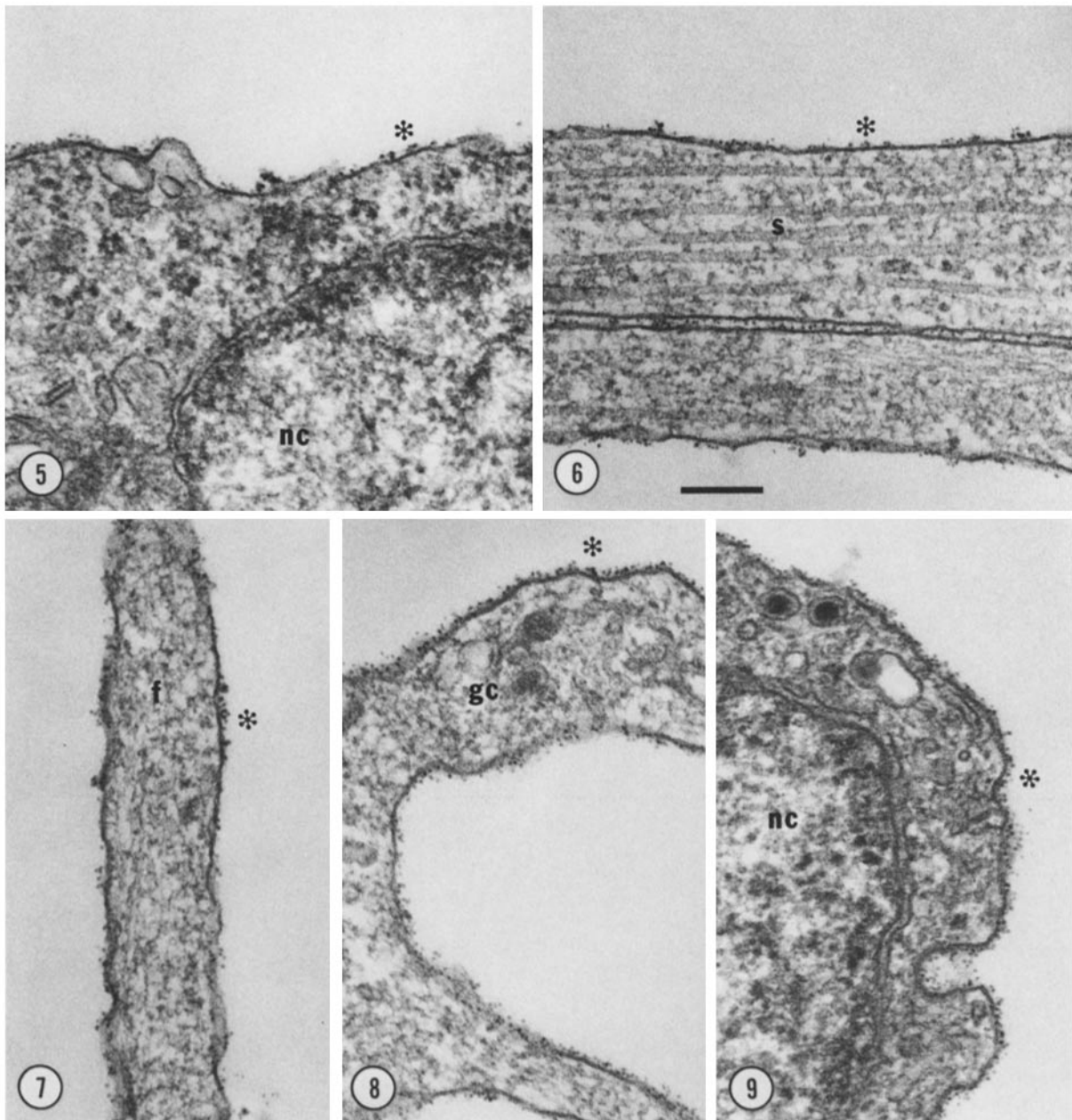
FIGURES 5-7   Spinal cord anterior horn neurons labeled with F-WGA after aldehyde prefixation. Note the relatively sparse, apparently uniform labeling of perikaryon (Fig. 5; nc, nucleus), neuritic shaft (Fig. 6, s) and growth cone filopodium (Fig. 7, f). Only extensive quantitative analysis reveals that the growth cone exhibits 28% fewer binding sites than the perikaryon. Asterisks indicate cross-sectioned membrane areas such as those used for quantitation. Bar, (applies to Figs. 5-9), 0.2 μm. × 61,100.

FIGURES 8 and 9   Growth cone (gc) and perikaryon (nc, nucleus) of SCG principal neuron labeled with F-WGA. Note high, apparently uniform density of lectin receptors and compare with sparse labeling on SC neurons (Figs. 5-7). Neurons were labeled following aldehyde fixation. Asterisks indicate cross-sectioned membrane regions. × 61,100.

glycoconjugates that would interfere with normal neuronal function because cultured neurons are known to form synapses and to become myelinated in a fashion indistinguishable from that observed in vivo (see, e.g., reference 1). Nevertheless, we have attempted to shed some light on this problem by comparing neuritic and growth cone lectin receptors in explant and dissociated-neuron cultures. In all four cases examined, lectin receptor densities were not influenced by cell dissociation. This result raises confidence in the biological significance of our data.

A further problem of surface mapping studies with ferritin-ligand conjugates is steric hindrance. We cannot exclude the possibility that a given glycoconjugate has several binding sites for the same lectin but that, for steric reasons, only one binding site is labeled with our electron-dense marker. However, the labeling of neighboring glycoconjugates in the membrane is unlikely to be affected by steric hindrance because of the relatively low receptor densities observed: Even if arranged at a density of 2,000/μm² in a single plane, uniformly distributed ferritin particles would occur at a center-to-center spacing of ~25 nm. Yet, the diameter of the entire ferritin molecule is ~12 nm (900,000 mol wt; see reference 3).

TABLE V

Lectin Binding Sites on the Plasmalemma of Growing Neurons Isolated from the Anterior Horn Region of Rat Spinal Cord

SC	Perikaryon*		Neuritic shaft*		Growth cone*		ΔPK-GC‡	
	sites/μm^2	n	sites/μm^2	n	sites/μm^2	n	%	P
1. WGA	1,210 ± 130	7	910 ± 80	22	880 ± 80	13	28	0.025
2. SBA	<10		<10		<10		—	—
3. RCA I	1,330 ± 90	32	1,230 ± 90	30	1,110 ± 110	23	(16)§	<0.10
4. RCA II	420 ± 60	12	510 ± 110	9	850 ± 90	12	−103	<0.0005
5. Neuram., RCA I‖	2,830 ± 120	16	2,990 ± 110	16	2,980 ± 140	15	—	ns
6. Neuram., RCA II‖	2,060 ± 230	7	2,340 ± 140	12	2,530 ± 120	16	−23	<0.05
7. 6 − 4¶	1,630		1,830		1,680		—	
8. 5 − 3	1,500		1,760		1,870		−25	

* Mean ± SEM, rounded to the third decimal, with number (n) of lengths analyzed (see Materials and Methods).
‡ Difference between perikaryon (PK) and growth cone (GC), in % of binding sites at perikaryon; negative values indicate increase towards growth cone. P, significance in Student's t test.
§ Confidence level inadequate.
‖ Neuraminidase pretreatment.
¶ Comparison of neuraminidase-treated and control membranes.

TABLE VI

Lectin Binding Sites on the Plasmalemma of Growing Principal Neurons from the Superior Cervical Ganglion of the Rat

SCG	Perikaryon*		Neuritic shaft*		Growth cone*		ΔPK − GC‡	
	sites/μm^2	n	sites/μm^2	n	sites/μm^2	n	%	P
1. WGA	2,560 ± 120	18	2,230 ± 60	25	2,220 ± 70	30	13	<0.01
2. RCA I	2,500 ± 170	12	2,300 ± 50	57	2,320 ± 50	41	—	ns
3. RCA II	1,650 ± 140	19	1,450 ± 80	35	1,420 ± 100	19	14§	<0.10

* Mean ± SEM, rounded to the third decimal, with number (n) of lengths analyzed (see Materials and Methods).
‡ Difference between perikaryon (PK) and growth cone (GC), in % of binding sites at perikaryon; negative values indicate increase towards growth cone. P, significance in Student's t test.
§ Confidence level inadequate.

The first two conclusions to be drawn from the studies presented here are (a) that perikaryon, neuritic shaft, and growth cone exhibit different sets of lectin receptors on their surface and (b) that different lectins produce very different binding profiles along the axis of the sprouting neuron. In neurons of the anterior horn region of the spinal cord, the following picture emerges: (a) Some carbohydrate residues are evenly distributed. This holds for preterminal (masked by neuraminidase-sensitive N-acetylneuraminic acid [NANA]) and terminal galactosyl residues (line 5 in Table V) and for neuraminidase-sensitive NANA linked to RCA II receptors (predominantly GalNAc; line 7 in Table V). (b) Other lectin receptors decrease in density as one moves from the perikaryon to the growth cone. The foremost example is WGA, whose primary receptor in this material is GlcNAc. WGA binds to only a fraction of NANA residues, if any, as concluded from the comparison of the large number of neuraminidase-sensitive residues (lines 7 and 8 in Table V) and the much lower density of WGA binding sites present in the growth cone region. RCA I binding sites, galactosyl residues, also appear to belong to the decreasing-density category, although the difference observed is small and only marginally significant. (c) Yet another class of superficially exposed carbohydrate residues increases in density as one moves from the perikaryon to the growth cone. These include RCA II receptors, which seem to consist predominantly of GalNAc residues and, at best, a fraction of Gal residues because many more of the latter saccharide moieties are recognized by RCA I. RCA II receptors are twice as frequent in the growth cone as in the perikaryon. They can be increased in number very substantially by neuraminidase pretreatment. Preterminal and terminal RCA II receptors combined also increase toward the growth cone, but only by 23%, a rise that can be accounted for by the dramatic increase in terminal RCA II receptors alone (cf. lines 4, 6, and 7 in Table

V). Gal-linked NANA (line 8 in Table V) also appears to belong to the category of carbohydrate residues that increase in density in proximo-distal direction. Interestingly, SBA, which has the same monosaccharide specificity as RCA II (GalNAc and Gal), does not bind in significant quantity to the plasmalemma of the sprouting SC neuron. Clearly, SBA labels receptors whose configuration is different from those recognized by RCA II (cf. reference 10).

To facilitate the overall understanding of these data, the results are presented graphically in Fig. 15. This figure also includes the density of intramembranous particles observed in SC neurites by freeze-fracture (16). Because the lectin binding data can be used not only as relative figures but as absolute density values, they can be compared directly with the data on intramembranous particles. It is evident from Fig. 15 that lectin binding site densities do not parallel in any way the density profile of intramembranous particles, the putative morphological equivalents of protein and glycoprotein clusters spanning the membrane (for review, see, e.g., reference 13). It is also evident that lectin binding sites are far more numerous than intramembranous particles in growth cone plasmalemma. Attempts to interpret this result will have to take into consideration glycolipids as likely determinants of cell surface chemistry.

The lectin binding studies furnish proof for the hypothesis, suggested by the earlier freeze-fracture studies, that the growth cone membrane is chemically different from that of the perikaryon and proximal neuritic shaft of the same neuron. Thus, cellular polarity is also reflected in cell surface chemistry. This finding has to be viewed in light of the fact that neuronal plasmalemma is expanding very rapidly during neuritic growth, and that addition of certain new components to the plasma membrane occurs predominantly in the growth cone area, whereas other membrane components appear to be added primarily at the perikaryon (19, 20, 22). The addition of lectin
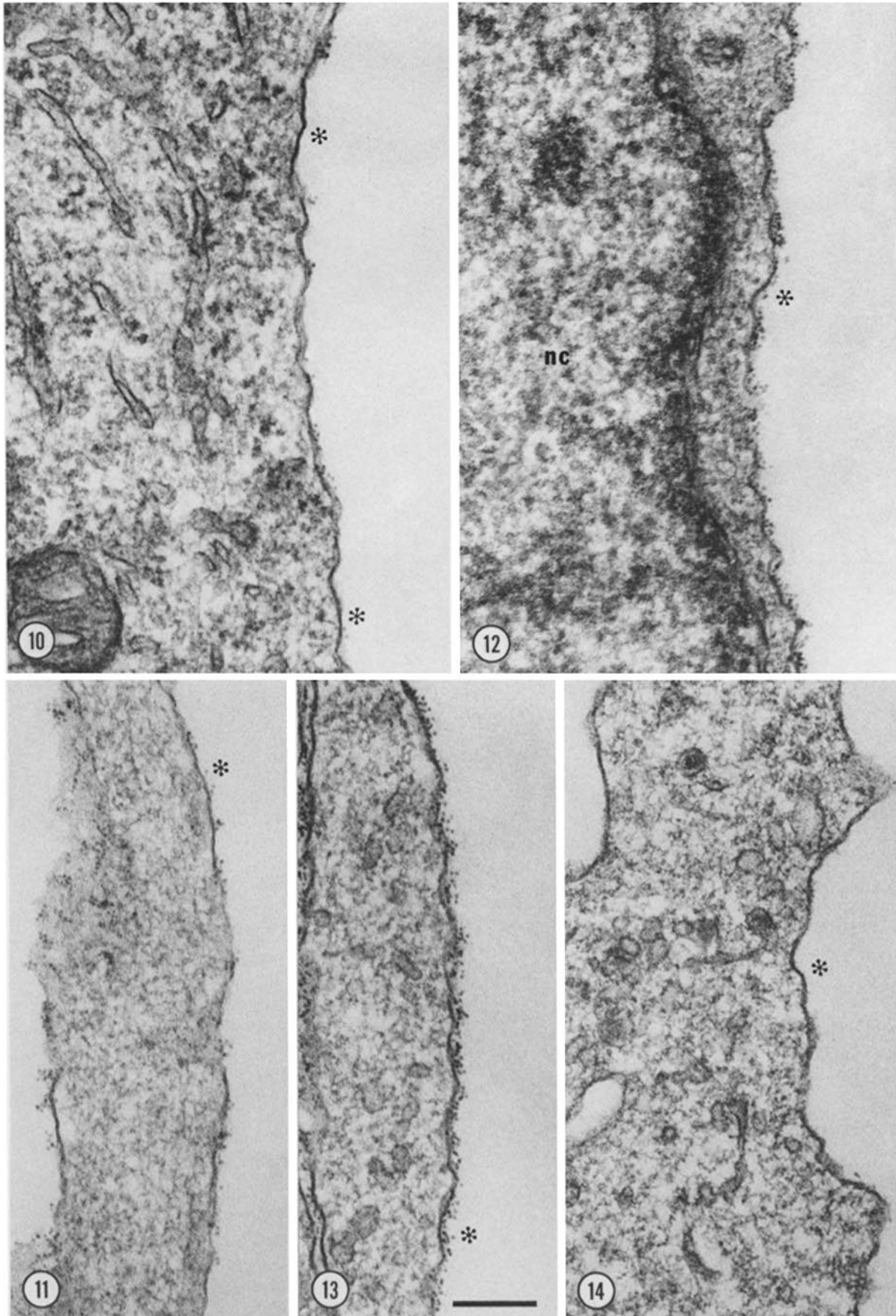
FIGURES 10–14 Labeling of spinal cord anterior horn neurons with F-RCA II and F-SBA. All samples were aldehyde-fixed before labeling. In normal binding studies there are only few RCA II receptors present on the cell surface, but they are about twice as frequent on the growth cone (Fig. 11, a filopodium) as on the perikaryon (Fig. 10). However, neuraminidase digestion before lectin labeling greatly enhances the number of RCA II binding sites both on perikarya (Fig. 12; nc, nucleus) and growth cone filopodia (Fig. 13). Fig. 14 shows part of a growth cone labeled with F-SBA. No labeling is observed although SBA and RCA II have the same monosaccharide specificity. Asterisks indicate areas of cross-sectioned membrane. Bar, 0.2 μm. × 74,100.
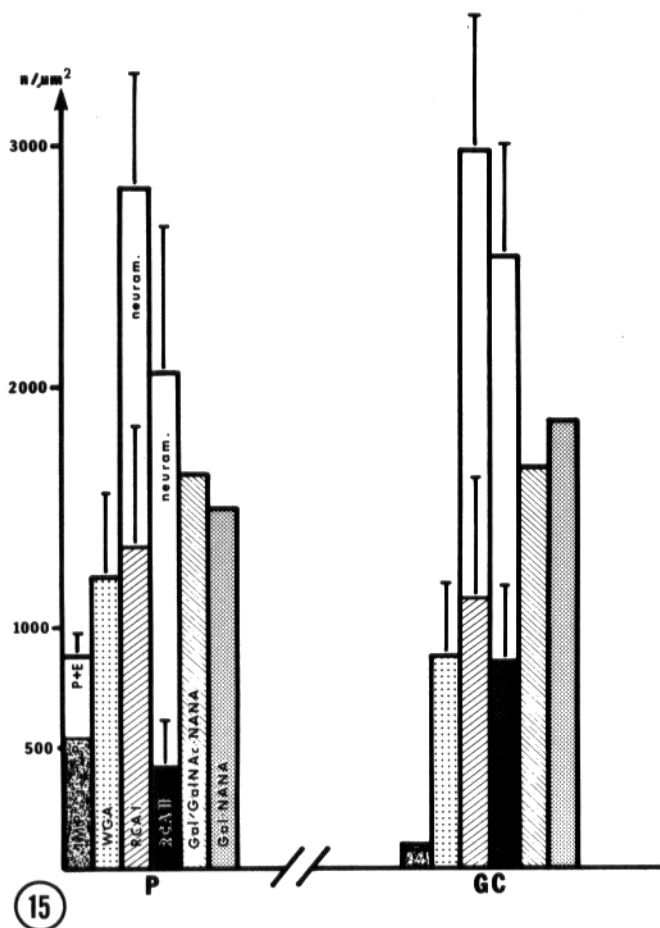
FIGURE 15 Graphic representation of the densities of lectin receptors and of intramembranous particles (IMPs; 16) in the plasmalemma of perikaryon (P) and growth cone (GC) of sprouting neurons from spinal cord anterior horn. Note the substantial differences for some of the parameters investigated, especially IMPs, RCA II, and WGA. neuram., Digestion with neuraminidase before labeling. For further description, see text.

that this hypothesis is indeed justified and that there are highly significant and complex differences in membrane composition between the growth cone and more proximal regions of neuronal plasmalemma, especially the perikaryon (cf. also reference 25). Those glycoconjugates that occur at increased density in the growth cone region—many of them possibly glycolipids—are the most likely partners in receptor-ligand interactions with cells in the axon's microenvironment, especially potential target cells.

Type-specific Membrane Glycoconjugates in the Growing Neuron

Comparison of lectin labeling of SC and SCG neurons leads to the following conclusions: As in SC neurons, lectin binding in SCG neurons does not parallel the distribution of intramembranous particles and follows different density profiles. This confirms in a peripheral neuron that the nerve growth cone exhibits distinctive membrane properties and further strengthens the view that certain glyconjugates are involved in functions specific to the growth cone. When the two types of neurons are compared, it is also evident that their lectin binding properties are significantly different, confirming studies by others (4, 5) and our own preliminary reports (14, 18, 19). This result may suggest that surface saccharides are involved in the signaling of the neuron's identity to its environment and/or in other cell-cell interactions that discriminate among neuronal types. An extensive analysis of differential carbohydrate residues on different types of neurons will be the topic of a forthcoming communication (Pfenninger and Maylié-Pfenninger, manuscript in preparation).

receptors that occur at significantly higher density in the growth cone than anywhere else in the neuron would thus be expected to take place predominantly at the growth cone, whereas lectin receptors that are sparser at the growth cone would be added to the plasma membrane primarily at the level of the perikaryon, just as seems to be the case for intramembranous particles (16, 22, 23). Saccharide residues that are evenly distributed throughout the neuron would be added at the growth cone as well as at the perikaryon and, maybe, even at the shaft. However, this reasoning is hypothetical for the time being, because similar density gradients of lectin receptors could also be created by region-specific glycoconjugate modification on the cell surface, by glycosyl transferases and glycosidases, or by active lateral transfer of specific glycoconjugates within the membrane.

A further important aspect of these data is concerned with the fact that the nerve growth cone is the leading edge of a cellular process that finds its path to an appropriate target region and forms a synapse with a highly selected target cell. Being aware of this phenomenon, one is immediately tempted to conclude that growth cone plasmalemma is endowed with specific functional and, therefore, biochemical properties (e.g., references 14 and 21). We report in this paper for the first time

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