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DISTRIBUTION AND MOBILITY OF LECTIN RECEPTORS ON SYNAPTIC MEMBRANES OF IDENTIFIED NEURONS IN THE CENTRAL NERVOUS SYSTEM

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ABSTRACT

The distribution and mobility of concanavalin A (Con A) and *Ricinus communis* agglutinin (RCA) receptors (binding sites) on the external surfaces of Purkinje, hippocampal pyramidal, and granule cells and their attached boutons were studied using ferritin-lectin conjugates. Dendritic fields of these cells were isolated by microdissection and gently homogenized. Cell fragments and pre- and postsynaptic membranes were labeled with the ferritin-lectin conjugates at a variety of temperatures, and the distribution of lectin receptors was determined by electron microscopy. Both classes of these lectin receptors were concentrated at nearly all open and partially open postsynaptic junctional membranes of asymmetric-type synapses on all three neuron types. Con A receptors were most concentrated at the junctional membrane region, indicating that the mature neuron has a specialized nonrandom organization of carbohydrates on its outer surface.

Lectin receptors located on postsynaptic junctional membranes appeared to be restricted in their mobility compared to similar classes of receptors on extrajunctional membrane regions. Labeling with ferritin-RCA and -Con A at 37°C produced clustering of lectin receptors on nonjunctional surfaces; however, Con A and RCA receptors retained their nonrandom topographic distribution on the postsynaptic junctional surface. The restricted mobility of lectin receptors was an inherent property of the postsynaptic membrane since the presynaptic membrane was absent. It is proposed that structures in the postsynaptic density may be transmembrane-linked to postsynaptic receptors and thereby determine topographic distribution and limit diffusion of specialized synaptic molecules. Specialized receptor displays may play an important role in the formation and maintenance of specific synaptic contacts.

It has been proposed that specialized molecules of the cell surface may play a role in the formation and maintenance of synaptic connections (3, 9, 37). Although this hypothesis is attractive, it will

be necessary to identify, isolate, and characterize these specialized molecules to understand the interactions which bond the pre- and postsynaptic membranes at the synapse.

Plant lectins have proved a powerful tool in the identification and characterization of cell surface receptors on a wide variety of cells including neural cells and other cells of central nervous system origin (25). Recent reports have described the distributions of concanavalin A (Con A)- and *Ricinis communis* I agglutinin (RCA)-binding sites on nerve endings, dendritic membranes, and on synaptic junctions from whole brain (5, 11, 20). In addition, immobilized plant lectins have been used to isolate specific glycoproteins from synaptic membranes by affinity chromatography (13, 45). To date, however, studies employing lectins as probes for synaptic membrane glycoproteins have used mixtures of different cell types. It is not yet known whether specific neurons have similar or very distinct carbohydrates on their surfaces and at their synapses.

In the present study we describe a method for localizing two different classes of saccharide residues on the external surfaces of particular neuron types in brain with ferritin-lectin conjugates. To avoid mixed cell populations, we have chosen to examine the Purkinje cells in the cerebellum and the granule and pyramidal cells in the hippocampus of the adult rat. Each of these cell types has its cell bodies organized in a discrete layer, receives a few well-defined inputs, and sends its dendrites into a zone essentially free of dendrites from other neurons. In tissue slices, the layers of cell bodies and neuropil can be readily isolated by microdissection. Thus, a specific population of cell membranes can be isolated and examined for possible differences in the arrangement of glycocomponents.

Since ferritin-lectin conjugates do not readily penetrate into tissue slices or synaptic clefts, a means is required to separate the pre- and postsynaptic membranes so that they are accessible to the ferritin-lectin conjugates. We have found that large, well-preserved fragments of dendritic membranes with boutons attached can be prepared by gentle homogenization of neuropil. At the same time many synaptic clefts are opened, permitting access of the ferritin conjugates. Using these dendritic fragments, we have determined the arrangements and mobilities of surface carbohydrates in synapses between identified pre- and post-synaptic elements.

Previously, it was proposed (2, 10) that proteins on the external surface of the postsynaptic membrane may be restricted in mobility due to transmembrane anchoring by components in the postsynaptic density. The presence of open synaptic clefts in our preparations provided an opportunity to examine the mobilities of Con A- and RCA-binding sites on the postsynaptic surface. We have been able to demonstrate a restricted mobility of Con A- and RCA-binding macromolecules within the synaptic region compared to similar receptors in other areas of the postsynaptic membrane.

MATERIALS AND METHODS

Preparation of Ferritin-Lectin Conjugates

Purified Con A (Calbiochem, San Diego, Calif.) was coupled to purified ferritin (6 × recrystallized, Miles Laboratories, Inc., Elkhart, Ind.) by either of two methods. In the first method, Con A was conjugated to ferritin in the presence of 0.05% glutaraldehyde (Polysciences Inc., Warrington, Pa.) essentially as previously described (28) at a Con A:ferritin ratio of 1:2 by weight. After coupling, excess glutaraldehyde was removed by filtration on a Sephadex G-25 column (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) and the conjugate was separated from free ferritin and inactive ferritin-Con A by affinity chromatography on Sephadex G-100. The ferritin-Con A conjugate was eluted with 0.2 M α-methyl-D-glucoside (an inhibitor of Con A), concentrated in dialysis tubing against aquacide (Calbiochem), and dialyzed extensively at 4°C against 0.4 M NaCl, 50 mM phosphate buffer (pH 6.5) to remove excess α-methyl-D-glucoside. The second method was a modification of the procedures of Kishida et al. (17), which uses prederivatized ferritin in a two-step coupling reaction. Ferritin was diluted into 7% (vol/vol) glutaraldehyde at a final protein concentration of 2 mg/ml. After the first step of the reaction, excess glutaraldehyde was removed by gel filtration and the derivatized ferritin was concentrated. In the second step, glutaraldehyde-derivatized ferritin was coupled to Con A at the same ratio by weight as in the first method. After purification by affinity chromatography on Sephadex G-100, the active conjugate was separated from unconjugated Con A on an agarose column (A-1.5 m; Bio-Rad Laboratories, Richmond, Calif.) (28). When this latter procedure was followed, the conjugate preparation agglutinated erythrocytes at a lower protein concentration (typical titers were at least 1:1,200), and there was less nonspecific binding to membranes in the presence of inhibitory saccharides.

RCA_I was purified from castor beans according to the method of Nicolson and Blaustein (27). RCA_I, the 120,000-agglutinin, was coupled to ferritin, and the conjugate was further purified by agarose affinity chromatography as previously described (28). Immediately

before use, ferritin-lectin conjugates were dialyzed against 150 mM NaCl in 50 mM sodium phosphate buffer (pH 7.0). Because of the loss of activity and the tendency of these two lectin conjugates to aggregate with time, all membrane-binding experiments were conducted within 3–4 days of lectin conjugation.

Tissue Preparation

Tissue samples were obtained from 100- to 150-day-old albino rabbits. After decapitation, the brains were placed immediately in 0.1 M sodium phosphate buffer (pH 7.1) and cooled to 2–3°C. The hippocampus and cerebellum were quickly dissected out and cut into slices of 1 mm thickness as previously described (22). Alternatively, slices of about 300–500 μm were cut with a Sorvall tissue chopper (DuPont Instruments-Sorvall, Newtown, Conn.). The slices were moistened with phosphate buffer and the areas selected for study were cut out with the aid of a dissecting microscope. The areas were: the granule cell layer and outer two-thirds of the molecular layer of the dentate gyrus, the pyramidal cell layer of hippocampus regio superior, and the outer part of the molecular layer of the cerebellum. About 10 mg wet weight of each area was homogenized (2% wt/vol) in phosphate buffer by three to four manual passes in a tight-fitting Dounce homogenizer (Kontes Co., Vineland, N. J.). The homogenates were centrifuged at 2,000 *g* for 4 min at 4°C, and the pellet was resuspended in sodium phosphate buffer at a final concentration of 0.8–1.0 mg protein/ml. These preparations contained large synaptic membrane fragments and were evaluated as targets for the binding of ferritin-lectin conjugates.

Incubation Conditions

Labeling of brain tissue particulate fractions with ferritin-lectins was carried out at a membrane protein concentration of 0.4–0.5 mg/ml at concentrations of lectin conjugates which yielded maximal labeling (0.5–1.0 mg/ml final conjugate concentration). The incubation medium was 150 mM NaCl in 50 mM sodium phosphate buffer, pH 7.0. A few rabbit erythrocytes were included in each incubation mixture to serve as an internal control since their Con A and RCA receptor distributions are well documented (23). Controls with the appropriate lectin-binding inhibitors (0.1 M α-methyl-D-glucoside for Con A and 0.1 M lactose for RCA) were included in each experimental group to evaluate nonspecific binding. In most cases, the reaction was carried out for 30 min and was terminated by the addition of 5 vol of buffer to each reaction mixture. The suspension was centrifuged at 300 *g* for 4 min (4°C) and the pellet was resuspended in buffer made 4% (vol/vol) with glutaraldehyde. In a few cases, fixative was added directly to the sample. Samples were fixed for 4–5 h in glutaraldehyde and were post-fixed in 2% (wt/vol) osmium tetroxide in Caulfield's buffer (7) at 4°C for 2 h. The samples were then resuspended in a small volume (30–60 μl) of warm 4% (wt/vol) agar to disperse the particles evenly and carry them through the embedding procedure. The agar-embedded samples were stained *en bloc* overnight with 2% (wt/vol) uranyl acetate in Kellenberger's buffer (15), dehydrated in a graded ethanol series and propylene oxide, and embedded in Epon-Araldite. Samples were sectioned, stained with lead citrate (32, 39), and examined in a Siemens II A electron microscope (Siemens Corp. Medical/Industrial Groups, Iselin, N. J.).

RESULTS

The homogenates of granule, pyramidal, and Purkinje cells contained numerous large membrane vesicles, many of which had one or more boutons attached, some unidentified membrane fragments, and synaptosomes which commonly included a small postsynaptic membrane fragment. Approximately half of the total asymmetric synapses were partially opened, so that a portion of the postsynaptic membrane with an underlying postsynaptic density (PSD) was unapposed by the presynaptic membrane. About 25% of the total asymmetric synapses observed were completely open, that is, only the postsynaptic portion was present.

In each sample a few erythrocytes were included as an internal control to monitor the consistency of binding patterns from experiment to experiment. In agreement with other reports (23, 24), erythrocytes bound both lectin conjugates uniformly over their outer membrane surface. The conjugates used in these experiments produced consistent binding patterns for several days after conjugation, and all erythrocytes in each sample showed indistinguishable patterns of lectin binding. Labeling was specific because the binding of RCA-ferritin was completely abolished by lactose (0.1 M) and that of Con A-ferritin by α-methyl-D-glucoside (0.1 M).

Ferritin-Con A Binding

Ferritin-Con A bound to the dendritic membrane surface of all three neuron types to similar extents and in similar distributions. Few ferritin-lectin molecules bound to extrajunctional regions, and what did bind was found in randomly distributed clusters or patches (Figs. 1 and 2). In contrast, the junctional area of the postsynaptic membrane (i.e., the portion overlying the PSD) was studded with molecules of ferritin-Con A. Ferritin-lectin molecules formed a closely-packed row along the postsynaptic junctional membrane in most cases, whereas the amount and density of conjugate bound to extrajunctional membrane

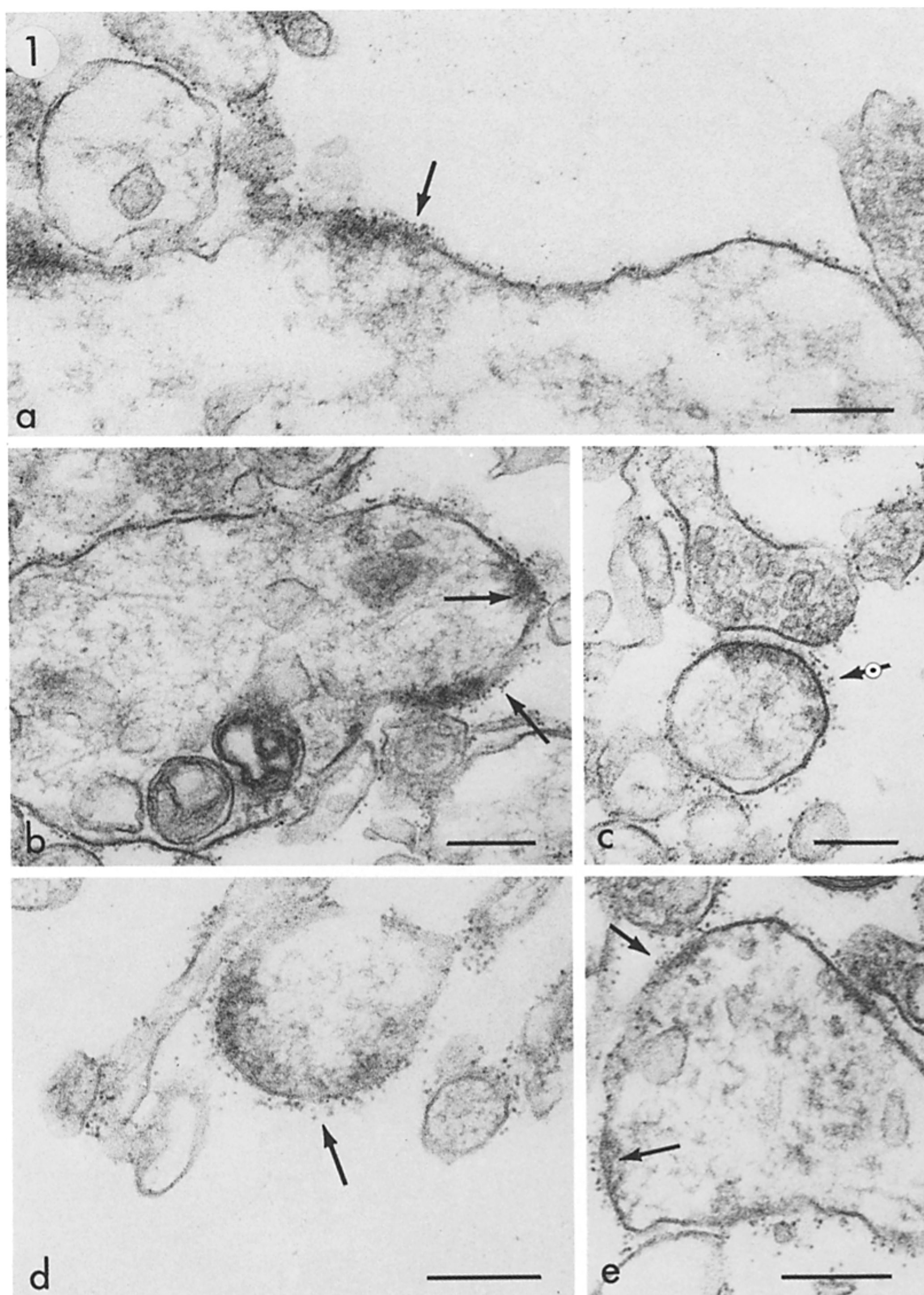

FIGURE 1 Ferritin-Con A conjugate binding to dendritic membrane of pyramidal cells (*a*, *b*, *c*) and granule cells (*d*, *e*) (4°C). Junctional regions (arrows) display numerous ferritin particles. Outside the junctional region, binding is reduced. Bar is 0.2 μm.

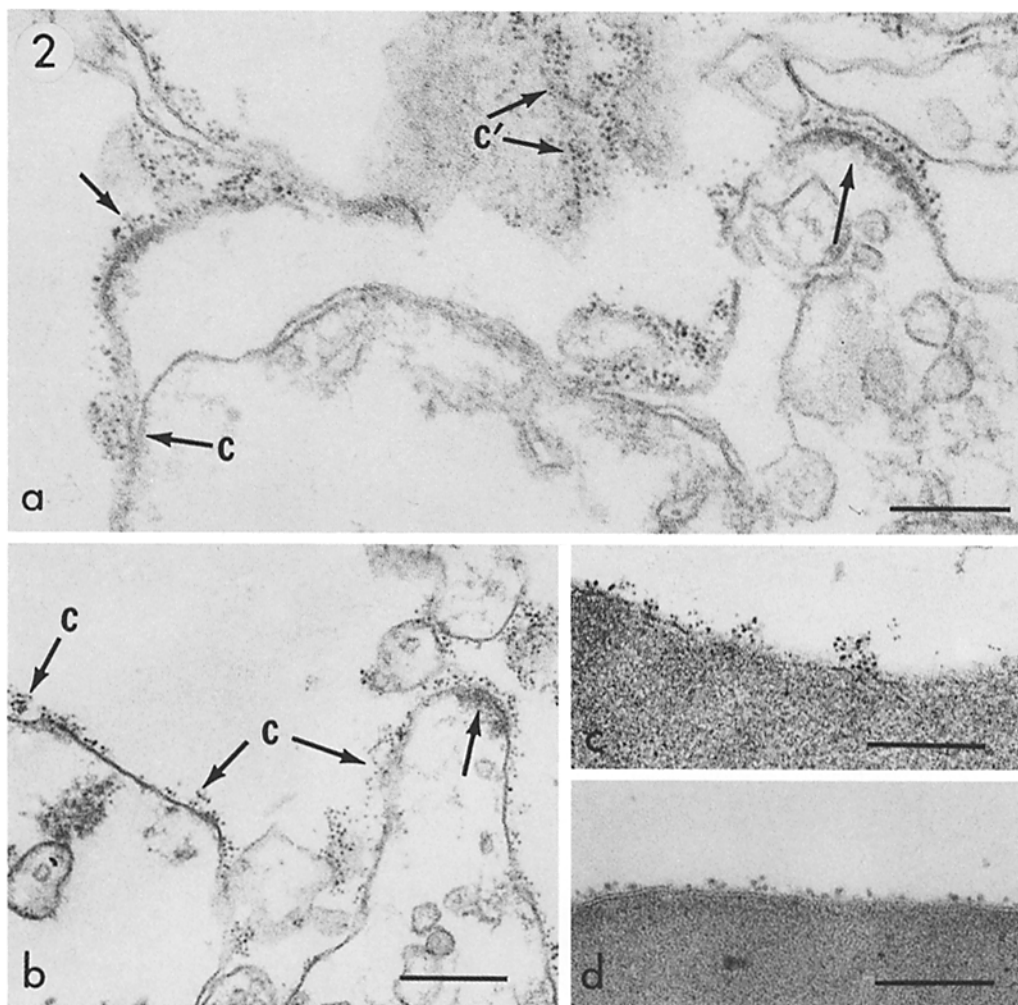

FIGURE 2 Ferritin-Con A conjugate binding to dendrites of Purkinje cells (*a*) or granule cells (*b*) at 37°C. Conjugate bound to the junctional region is dense and linear (arrows), whereas that on the extrajunctional surface tends to be organized in clusters. A cluster is seen adjacent to the linear array in a junction (*a*, arrow *C*) (*c*) and clusters are seen in a surface view of a membrane (arrows *C*′). At 4°C, erythrocytes show uniform binding (*d*) and at 37°C the conjugate appears in aggregates (*c*). Bar is 0.2 μm.

varied and was very rarely found in arrays characteristic of postsynaptic regions. Occasionally, ferritin-Con A was observed within the clefts of intact synaptic junctions. This phenomenon appeared more frequently when incubations were carried out at 37° than at 4°C.

About 95% of 130 bare postsynaptic sites bound ferritin-Con A, and more than 85% of 160 partially opened synapses bound significant quantities of conjugate. Approximately the same quantities of conjugate were bound to dendritic surfaces at 4° and 37°C.

While temperature did not markedly affect the quantity of ferritin-lectin conjugate that was bound, the distribution of bound ferritin-Con A varied. When incubations were carried out at 37°C, Con A conjugate molecules that were bound outside the synaptic junction region often appeared clustered (Fig. 2*a*, *b*), similar to the patterns observed on the surfaces of adjacent erythrocytes (Fig. 2*c*). However, no such aggregation could be seen when the tissue was labeled with ferritin-Con A at 4°C. These results indicate that extrajunctional binding sites for Con A are mobile

and undergo temperature-dependent ligand-induced aggregation (25, 26). In contrast, ferritin-Con A molecules were linearly arrayed along the junctional postsynaptic membrane surface at either temperature, suggesting a relative lack of lateral mobility. In a few cases, this inherently linear arrangement of binding sites could be seen to change to clustered or aggregated outside the boundary of the PSD at 37°C (Fig. 2*a*).

The dense linear arrangement of Con A-binding sites which characterized the junctional postsynaptic membrane was not found elsewhere when incubations were carried out at 4°C. Presynaptic membranes and myelin membrane surfaces bound the conjugate the same way as did extrajunctional postsynaptic membranes, while ferritin-Con A did not bind to outer mitochondrial membranes.

Ferritin-RCA Binding

Ferritin-RCA molecules were bound randomly along the extrajunctional dendritic membrane and presynaptic membranes at 4°C (Figs. 3 and 4). The extrajunctional dendritic (Figs. 3*a*, 4*a,c*) and presynaptic (Fig. 4*b,f*) membranes generally bound more ferritin-RCA than -Con A conjugates; however, the three cell types displayed little difference in the density or distribution of RCA-binding sites. About three to five × more ferritin-RCA than ferritin-Con A conjugate bound to erythrocytes.

About 85% of the bare postsynaptic junctional membranes and 60% of those which were partially exposed bound significant amounts of ferritin-RCA conjugate over background. Some postsynaptic junctional membranes bound as much ferritin-RCA as Con A conjugate (Figs. 3*a*, 4*a*), but others bound much less (Figs. 3*c*, 4*b, c, d*). Portions of the granule cell dendrite membranes sometimes bound particularly small amounts of ferritin-RCA conjugate (Fig. 4). However, as was the case for ferritin-Con A binding, no striking differences among neuron types were evident in the binding of ferritin-RCA. Myelin did bind ferritin-RCA conjugate, whereas mitochondria did not. Capillaries bound very large quantities of conjugate.

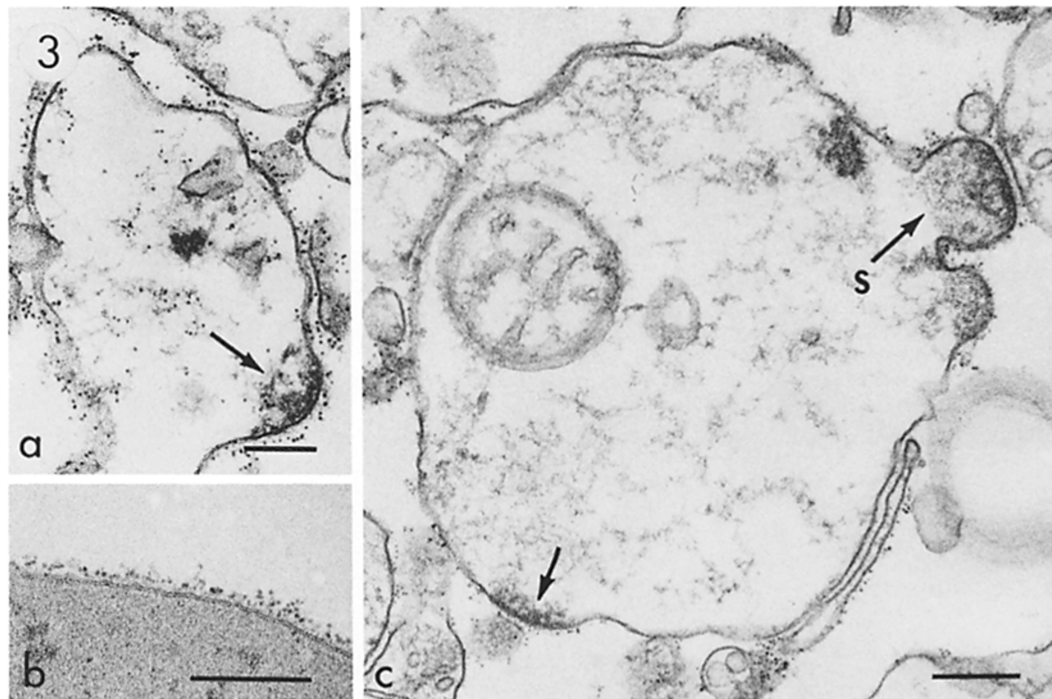

FIGURE 3 Ferritin-RCA conjugate binding to pyramidal cell dendritic surfaces (*a*, *c*). Postsynaptic sites are designated by arrow. The small spine-like protuberance shown in *c* (*S*) was characteristic of this sample. The post-synaptic site in (*a*) shows more binding than that in (*c*). Binding to the surface of a erythrocyte is shown for comparison (*b*). Bar is 0.2 μm.

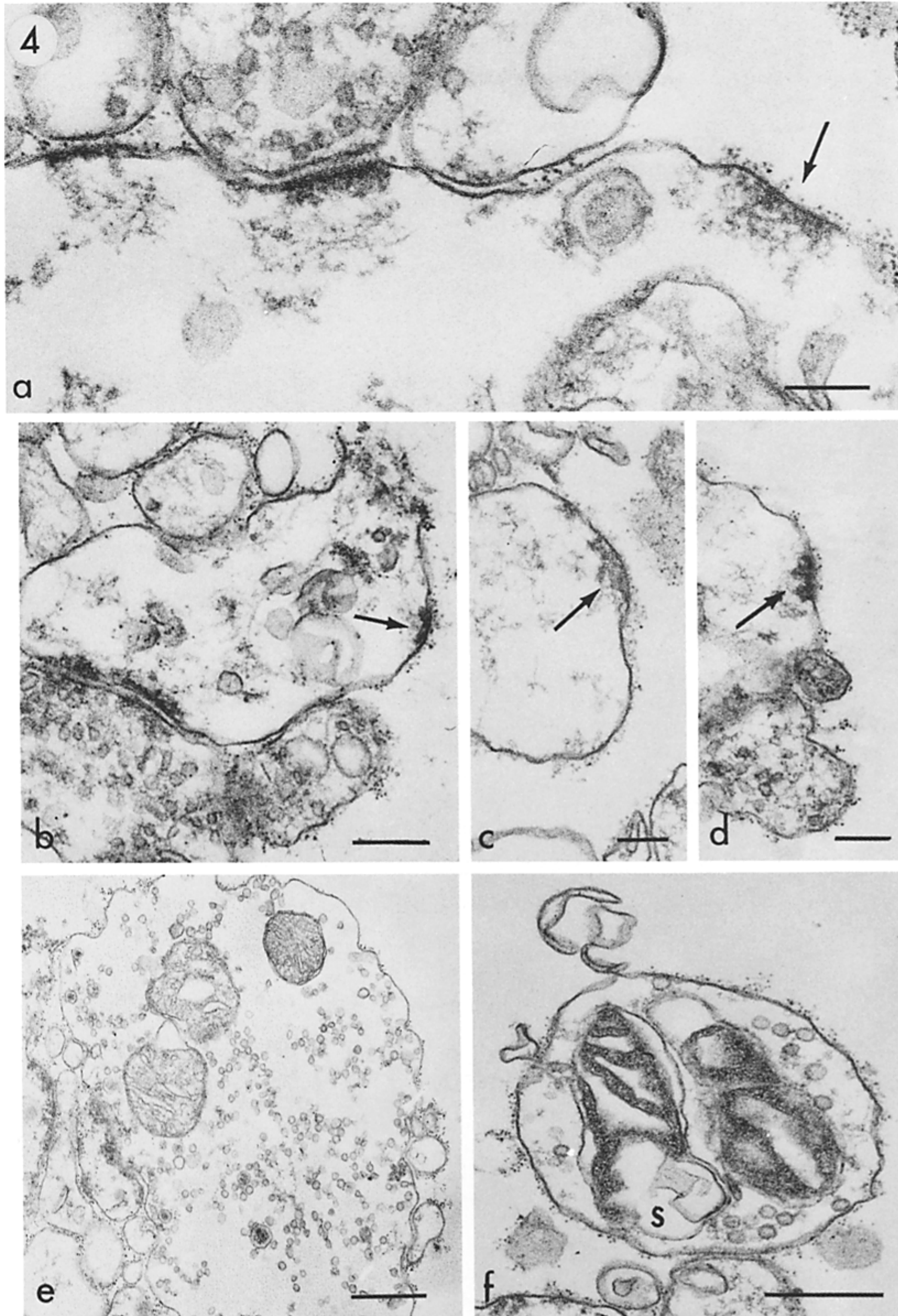

FIGURE 4 Ferritin-RCA conjugate binding to granule cell dendrites (*a–d*), the mossy fiber synapse of the granule cell (*e*) and a bouton bound in the molecular layer (*f*). The postsynaptic sites (arrows) in (*a*) and (*b*) show moderate concentrations of bound conjugate, while those in (*c*) and (*d*) show very little binding. The extrajunctional membranes in (*a*) and (*c*) possess moderate quantities of bound conjugate. The outer surface of the mossy fiber ending in (*e*) shows a scattered array of conjugate while the synaptosome (*S*) in (*f*) shows heavy binding. Bar is 0.1 μm in *a–d* and 0.5 μm in *e* and *f*.

In some cases, a budding or blebbing of the presynaptic or postsynaptic membrane was seen where there was no underlying cytoplasmic structure. Invariably, these areas lacked label. These blebs are probably lipid-rich domains which could have been artifactually created during membrane preparation or fixation procedures. They may lack bound conjugate because lectin receptors are unable to redistribute laterally into these regions.

DISCUSSION

The present study demonstrates the presence of Con A- and RCA-binding sites on the outer surface of granule, pyramidal, and Purkinje cell dendrites, and on the outer surface of attached boutons. The binding sites were particularly concentrated on the postsynaptic junctional membrane at asymmetric-type synapses. While the ferritin-Con A conjugates bound at relatively high densities to all such membranes, the binding of ferritin-RCA was more variable.

Nearly all postsynaptic junctional membranes from all three neuron types bound ferritin-Con A or -RCA conjugates, indicating that the postsynaptic membrane surfaces of asymmetric synapses were particularly rich in D-mannosyl- and D-galactosyl-like residues. Of the two classes of lectin receptors, Con A receptors were the most concentrated at the junctional region compared to extrajunctional areas. Extrajunctional surfaces of the postsynaptic membranes displayed marked reductions in the quantities of Con A conjugates bound compared to junctional regions. The differential organization of lectin receptors along the neural cell surface indicates that the membrane of the mature neuron has a highly specialized pattern or display of carbohydrates along its surface. This receptor pattern or display could be of importance in determining where synapses form on the neuronal surface.

In order for distinct receptor patterns to be established on cell surfaces characterized by fluid surface membranes (35), the mobility of surface molecules must be under control to limit their lateral diffusion (26). In the case of lectin receptors on neural cells, the extrajunctional postsynaptic Con A-binding sites seem to be more mobile than those in the synapse regions. Ferritin-Con A conjugates induced clustering of their receptors at some points along the extrajunctional membrane surface at 37°C, but not at 4°C. Matus et al. (20) have also reported that extrajunctional lectin-binding sites are relatively mobile. In contrast, Con A receptors were not clustered at 37°C in junctional regions of the postsynaptic membranes. In the junctional regions of postsynaptic membranes, ferritin-Con A conjugates were always arrayed in a dense monolayer. The restricted mobility of Con A-binding sites on this portion of the postsynaptic surface appeared to be an inherent property of this membrane, since it was demonstrable even when the presynaptic membrane was absent. It has previously been noted that at intact synapses, intramembranous particles seen in the postsynaptic membrane are immobile (18). Most likely, this too is an inherent property of the postsynaptic membrane.

Although the mechanism that restrains the mobilities of postsynaptic receptors in the synaptic regions is unknown, the underlying PSD may be responsible for the comparatively restricted mobility of Con A-binding sites at the synaptic junctional surface. We have previously suggested that the PSD could serve to anchor receptors for neurotransmitters and other unique constituents of the postsynaptic membrane (2, 10), and this hypothesis is strengthened by the recent demonstration in the PSD of a protein similar to tubulin (12, 40, 41). Neurofilaments also appear to be a part of the PSD (44). In other cellular systems, microtubules and microfilaments have been suggested as important cytoskeletal components involved in the transmembrane regulation of surface membrane receptor mobility (4, 26, 29, 30, 38, 42) and they may play a similar role in neurons. The presumptive glycoproteins which bind the lectins may even extend from the PSD through the postsynaptic membrane, similar to the transmembrane glycoproteins found in erythrocyte membranes (33). Alternatively, however, it may be that the Con A receptors in the postsynaptic membrane overlying the PSD may self-associate as does bacteriorhodopsin in the purple membrane of *Halobacterium* (6, 26, 37). Additional experiments are necessary to distinguish between the transmembrane control and self-association hypotheses or a combination of the two.

The physiological role of the postsynaptic junctional lectin-binding components is at present unknown. It could be that they are involved in the specification of synapses, but their distribution was similar on all the dendritic membranes which were examined. Of course, lectins would not be expected to show the same specificities of binding as neural recognition components, and it is known that Con A and RCA will recognize several classes

of membrane glycoproteins (25). In this respect, lectins will bind to a variety of membrane glycocomponents on the basis of their carbohydrate composition. Alternatively, the lectin receptors present at the synaptic junction could play a functional role as postsynaptic receptors or critical enzymes in excitatory synaptic transmission. For example, Michaelis et al. (21) reported that Con A inhibits the high-affinity binding of glutamate to synaptic membranes, and Mathers and Usherwood (19) have shown that Con A prevents the desensitization of glutamate receptors on insect muscle fibers.

A more likely possibility is that the lectin-binding sites are involved in synaptic adhesion (11). In many other systems, membrane surface glycocomponents have been shown to be involved in cell-sorting events and intercellular adhesion (14, 34, 43). After an afferent nerve makes contact with an appropriate postsynaptic site, a synaptic junction forms which in mature synapses is sufficiently strong to resist dissociation by denaturing agents, extremes of pH, chelators, and relatively harsh detergent treatments (8, 9, 16). These linkages may make use of the lectin-binding glycocomponents to form covalent or very strong noncovalent bonds. For this purpose, highly distinctive classes of carbohydrates between neuron types may not be necessary. We would hypothesize that during synaptogenesis these lectin-binding glycocomponents are inserted along with the coated vesicles which fuse into the membrane and form the PSD (1, 31). These coated vesicles are derived from the Golgi apparatus (31) where they may acquire their carbohydrate. It may be that the coated vesicles bring surface carbohydrates to the postsynaptic membrane surface and that these molecules serve as templates for presynaptic membranes to become securely bonded to the postsynaptic membrane.

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