

Distribution of Glycine Receptors at Central Synapses: An Immunoelectron Microscopy Study

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ABSTRACT The distribution of receptors for a neurotransmitter was investigated cytochemically for the first time in the central nervous system, at synapses established on cells of the ventral horn of the rat cervical spinal cord. Three monoclonal antibodies (mAb's) raised against glycine receptors were used. Immunofluorescent staining already showed discontinuous labeling at the surface of neurons, and immunoenzymatic electron microscopy further revealed that the antigenic determinants were confined to the postsynaptic membrane and concentrated at the level of the synaptic complex. More specifically, one mAb directed against the receptive subunit of the oligomeric receptor recognized an epitope on the extracellular side of the plasma membrane, whereas two other mAb's bound to the cytoplasmic face. Epitopes for the last two mAb's were more accurately localized with protein A-colloidal gold, using an intermediate rabbit anti-mouse immunoglobulin serum. (a) In addition to the presence of gold particles in areas facing the presynaptic active zone (visualized with ethanolic phosphotungstic acid), the labeling extended beyond this zone for ~50–60 nm, which corresponds to the width of one presynaptic dense projection. (b) The distances between the mid membrane and the gold particles were different for the two mAb's (with means of 21.7 ± 8.5 nm and 29.8 ± 10.4 nm, respectively). The data suggest that one of the recognized epitopes is close to the plasma membrane, whereas the second protrudes into the cytoplasm. Our results indicate that the receptor is a transmembrane protein which has a restricted spatial distribution on the postsynaptic neuronal surface.

Little is known about the organization of receptors for neurotransmitters at central synapses, owing, primarily to the lack of specific high-affinity ligands. In the peripheral nervous system, α -bungarotoxin has been used as a cytochemical marker for acetylcholine receptors localized at the endplate (15, 16). Thus, in the central nervous system, the regional distribution of receptors has only been studied, using agonistic substances, either by light microscopy autoradiography (e.g., for opioid receptors [1]) or by electron microscopy, as in the case of opiate (11), benzodiazepine (18), and muscarinic cholinergic (14) receptors. Despite a statistical analysis of the distribution of reduced silver grains, the precise distribution of the labeled molecules with respect to the plasma membrane was difficult to assess due to limitations of the autoradiographic technique. We show in this paper that a direct determination of receptor's distribution, such as that for glycine, is now possible with the help of immunochemistry.

In the spinal cord, and several other regions of the central

nervous system, glycine is involved in inhibitory synaptic transmission (20); it produces an increase of the postsynaptic chloride conductance (32) and this effect is selectively antagonized by strychnine (20). Recently, the glycine receptor was solubilized in the presence of detergent (22) and after purification on a strychnine affinity column, shown to be an oligomeric glycoprotein with a total molecular weight of $246,000 \pm 6,000$ (23). Gel electrophoresis revealed three associated polypeptides, strychnine being incorporated irreversibly into the 48-kD subunit upon UV illumination. More important for this work, monoclonal antibodies (mAb's)¹ have been prepared against the affinity-purified receptor protein (24). Three of them, which have been used in this work, recognize the denatured receptor. Two of these mAb's, GlyR 2b and GlyR 5a, react with a single protomere, the 48- and

¹ Abbreviations used in this paper: mAb, monoclonal antibody; PB, phosphate buffer.

the 93-kD subunits, respectively. The third one, GlyR 7a reacts with the 93-, and to a lesser extent, the 48-kD proto-meres.

With these immunoglobulins, we have obtained the following: (a) a direct visualization of glycinergic synapses on the somata and dendrites of central neurons; (b) evidence that the glycine receptor is concentrated at postsynaptic sites and that they are mostly distributed co-extensively with the postsynaptic density; (c) information about the transmembrane location of epitopes, recognized by the antibodies used; and (d) indications concerning a possible difference in the relative distances of the two cytoplasmic epitopes from the bilayer center.

MATERIALS AND METHODS

Fixation Procedures

Rats (Fischer 344) were anesthetized with pentobarbital (30 mg/kg) and perfused intracardially for 30 min with solutions which best allowed the three mAb's to be visualized. The fixatives were as follows. For light microscopy, 4% paraformaldehyde in 0.12 M phosphate buffer (PB) at a pH of 7.4 was preferred. For electron microscopy, its composition was 2% paraformaldehyde and 0.2% glutaraldehyde in PB followed by paraformaldehyde (2%) alone in the same buffer. The brain was then removed and kept overnight in the same fixative. The concentration of glutaraldehyde was lowered in an attempt to reduce the extent of cytoplasmic protein cross-linking (7) and to minimize the loss of antigenicity. Nevertheless, the antigenic determinant recognized by mAb GlyR 2b was still destroyed by this technique. Thus when this mAb was used, rats were perfused for 30 min with 1% tannic acid, 0.75% paraformaldehyde, and 0.1% glutaraldehyde in 0.12 M PB (11) and washed with PB. In this case, the cervical spinal cord was rapidly removed and serial 50- μ m-thick sections were cut and immediately incubated for binding (see below).

Immunofluorescence

After washing in PB, the spinal cords were freeze sectioned and slices were incubated overnight at room temperature with the mAb's diluted (1:50) in phosphate-buffered saline (PBS) and for a further 2 h with tetramethylrhodamine-coupled sheep-antimouse IgG (Sam-Tritc, Cappel Laboratories, Cochranville, PA). Sections (30- μ m-thick) were viewed with a Zeiss microscope equipped with epifluorescence optics and selective Zeiss filters for rhodamine.

Immunoelectron Microscopy

For electron microscopy, the spinal cord was sectioned (50- μ m-thick) in ice cold PBS with a vibratome and incubated with the mAb overnight. In the case of fixation with tannic acid, required for mAb GlyR 2b, the tissue was postfixated for 2 h (11) with 4% paraformaldehyde.

IMMUNOPEROXIDASE STAINING: The "Vectastain" (Vector Laboratories, Inc., Burlingame, CA) immunoperoxidase staining procedure was used. After incubation with the mAb, the sections were kept for 1 h with the diluted biotinylated horse anti-mouse IgG, washed and incubated for 2 h with the avidin and biotinylated horseradish peroxidase reagent, specially prepared to form ideal complexes for immunoperoxidase staining ("Vectastain ABC" reagent). The enzymatic reaction was carried out using 0.01% hydrogen peroxide and 0.5% diaminobenzidine tetrahydrochloride, in 0.05 M Tris buffer, pH 7.4. The reaction time was checked under light microscope, and ranged from 2 to 5 min.

IMMUNOGOLD LABELING: Penetration of gold particles was achieved during the process of fixation by a hypoosmotic shock which produces a disruption of plasma membranes and elution of most cytosolic proteins (5). The same fixative as the one above was used, except that the 0.12 M PB was replaced by 5 mM PB (5). The cervical cord was immediately removed and sectioned with a Vibratome, and the slices were treated overnight with the selected mAb (1:50). Subsequently, the sections were immersed for 2 h in a rabbit anti-mouse IgG (Nordic Immunological Laboratories), treated with protein A adsorbed on 5.5 ± 1.4 nm colloidal gold particles (27). However, it should be pointed out that the penetration of labeling in the tissue did not exceed 5–10 μ m.

Specific Visualization of the Synaptic Complex

After immunogold labeling, the sections were dehydrated in graded ethanol and incubated in ethanolic phosphotungstic acid for 2 h at 60°C (30, 31), which

selectively stains pre- and postsynaptic differentiations (2). The slices were then successively immersed in two changes of acetone and embedded flat in araldite. Sections (0.5 μ m thick), without additional stains, were observed with the electron microscope (Philips EM 400) operating at 100 KV.

For all other electron microscope observations, ultrathin light gray (500 Å) sections were poststained and observed with the electron microscope operating at 80 kV. Quantitative analysis of distances between the gold particles and the membrane were achieved with micrographs which unambiguously showed the bilayer configuration of the postsynaptic membrane, and were made with a final magnification of 220,000. Coordinates of the lipidic layer (midpoint) and of the nearest point of the periphery of each particle were entered via a graphic tablet (Houston Instrument, Austin, TX) and computed by a connected micro-computer (Apple II).

RESULTS

Data obtained with mAb's GlyR 2b, 5a, and 7a, which recognize the 48-kD, the 93-kD, and both subunits, respectively, were essentially the same at the light microscopic level, although their respective epitopes had different relationship with the plasmalemma, as shown by an ultrastructural approach.

Discontinuous Immunofluorescence Staining on the Surface of Neurons

As illustrated in Fig. 1, the immunofluorescence staining was detected at the periphery of the soma and dendrites of cells and was not continuous (Fig. 1, A–C). As the focus was changed, the labeling appeared to be distributed in discrete patches (Fig. 1 D), irregular in shape and with an apparent diameter varying from 0.4 to 2 μ m (the smaller ones may represent the postsynaptic side of individual boutons and the larger ones that of clustered terminals). Also, the size of the stained areas were not randomly distributed on all cell types: more specifically, they were smaller on large (20–30 μ m) cells, presumably motoneurons, of lamina IX than on medium-sized (10–20 μ m) neurons of lamina VIII (according to the classification of Rexed [26]). In contrast, no labeling was obtained in control experiments with nonspecific mouse IgG.

Subcellular Localization of Receptors by Immunoperoxidase Techniques

Staining was observed at the postsynaptic level when the tissues were processed by the avidin–biotin technique. When present, the reduced diaminobenzidine–OsO₄ precipitate always faced terminals containing a pleiomorphic population of vesicles (although all synapses of this type were not labeled). Such heterogeneity, which was also observed for the other mAb's, is shown in Fig. 2 A for the mAb GlyR 5a. At higher magnification, as in Fig. 2 B, the postsynaptic membrane was heavily labeled on its cytoplasmic leaflet, the staining being predominant at the level of the synaptic complex itself. This structure was identified (21) by the presence of presynaptic dense projections, by a constant distance (here 15 nm) between the pre- and postsynaptic membranes and by the presence of uneven electron-dense material within the cleft (the postsynaptic differentiation itself was obscured by the diaminobenzidine–OsO₄ electron-dense precipitate). Some staining was also often observed on the adjacent cytoplasmic membrane, and on elements of the cytoskeleton, owing presumably to a translocation of the enzymatic reaction product and to its nonspecific adsorption. A similar pattern of labeling was also obtained with the mAb GlyR 7a, as illustrated in Fig. 2 C. In contrast, the mAb GlyR 2b (which only recognizes an epitope on the 48-kD subunit) stained the synaptic cleft. As

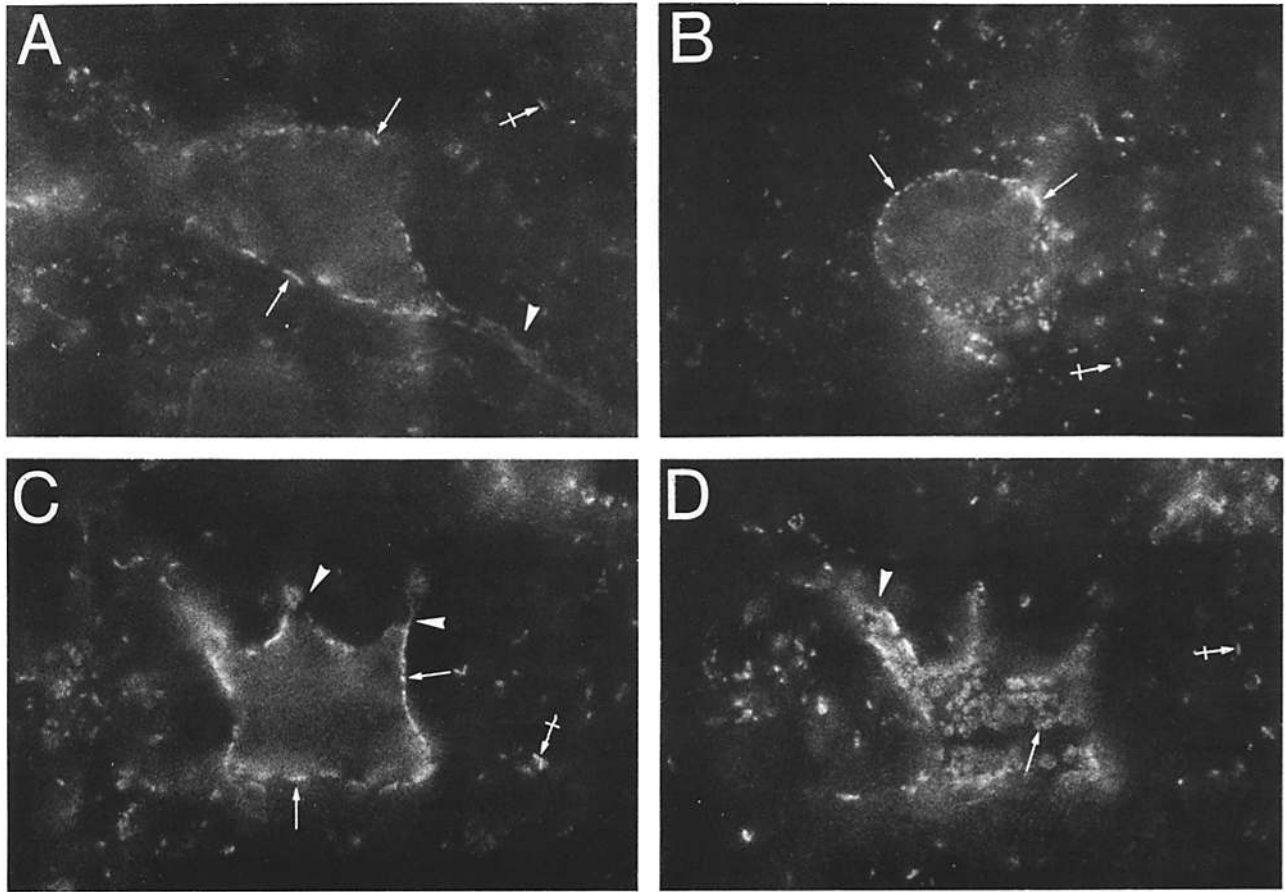


FIGURE 1 Discontinuous distribution of glycine receptors. The three mAb's selected for this work, revealed with indirect immunofluorescence, produce a discrete pattern of fluorescence which is distributed over the perikarial (arrows) and the dendritic (arrowheads) surfaces of large multipolar neurons of the central horn of the rat cervical cord. (A–C) Transversal sections labeled with mAb's GlyR 5a, 7a, and 2b, respectively. (D) Tangential view of the same neuron as in C showing that receptors are localized at discrete patches on the plasma membrane (arrows). Stained patches (crossed arrows) on the background are presumably due to labeling of dendritic synapses. $\times 1,120$.

shown in Fig. 2D, it was in fact very difficult in this case to determine the exact location and extent of the labeling and the size of the synaptic complex, mainly because the enzymatic reaction took place in a narrow space. Furthermore, the density was quite variable from one synapse to another, and when the reaction was intense, the presynaptic membrane could also be labeled.

Localization of Antigens by Immunogold Labeling

The problem of immunolocalization by enzymatic reaction was successfully overcome using colloidal gold particles as a marker in the case of mAb's GlyR 7a and 5a, although this method failed for mAb GlyR 2b. As illustrated in Fig. 3, A and C, gold particles were found close to the cytoplasmic leaflet of the membrane and were predominantly localized at the level of synaptic complexes, co-extensively with the postsynaptic differentiation. Very occasionally, particles (Fig. 3E) were seen on the same side and at the same distance from the plasmalemma, but more laterally and not in sufficient amounts for quantification.

The distribution of the epitopes was even more accurately defined when the active zones were specifically stained with ethanolic phosphotungstic acid, so that the outer margins of the synaptic complexes could be determined, taking as an index the most peripheral dense projections, as in Fig. 3, B and D. On these 0.5- μm -thick sections, the apparent density

of gold particles was increased, and this material supported the notion that receptors are mostly concentrated at the level of the postsynaptic differentiation. As seen in Fig. 3, B and D, the labeling fades progressively after the last dense projections, and completely disappears ~ 50 nm away from them.

Gold particles linked to the antireceptor mAb's GlyR 7a and 5a tend to be arranged in narrow bands, each of them at a different distance from the plasmalemma (Fig. 3F). The distance frequency histograms reported in Fig. 4 show that the particles associated with mAb GlyR 7a and 5a were observed at 21.7 ± 8.5 nm ($n = 219$) and 29.8 ± 10.4 nm ($n = 451$), respectively, from the lipidic portion of the membrane. This configuration indicates that the binding sites recognized by these two mAb's have different spatial relationships with the membrane.

DISCUSSION

Our observations were restricted to cells of the ventral horn of the cervical spinal cord located in laminae VIII and IX of Rexed (26); in the latter, at the light microscope level, neurons with larger somata could be identified as motoneurons.

Synaptic Localization of Glycine Receptors

Immunoreactive epitopes of this receptor are distributed in patches, and when observed with an electron microscope,

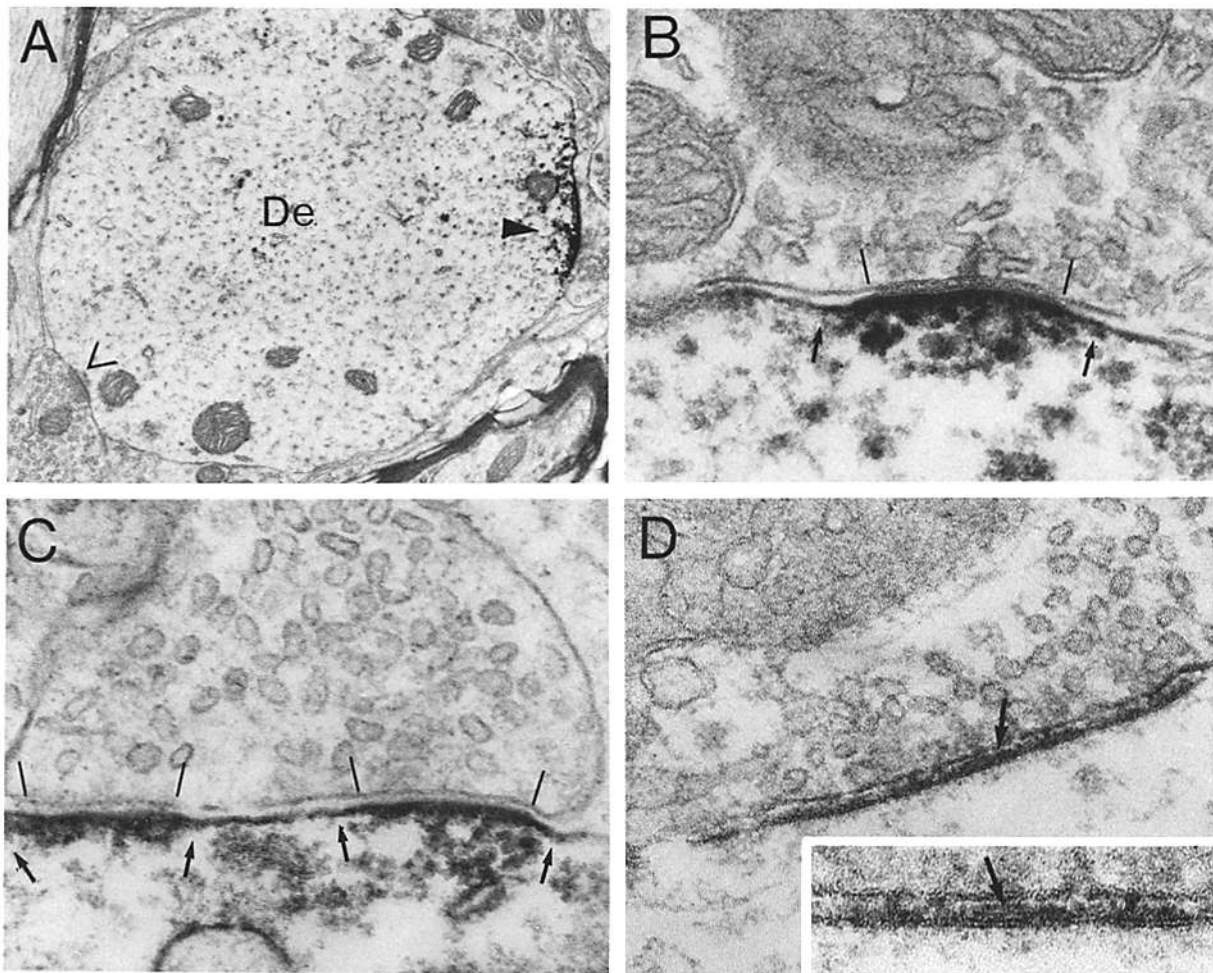


FIGURE 2 Localization of receptors at the synaptic complex. mAb binding sites were revealed using the biotin-avidin immunoperoxidase technique. (A) Survey micrograph of a dendrite (De) contacted by synapses which are either unstained (open arrowhead) or stained (black arrowhead) by GlyR 5a. (B and C) Higher magnification of synaptic complexes stained with mAb's GlyR 5a and 7a, respectively; both contacts are established by terminals that contain a population of pleiomorphic vesicles. Note that the enzymatic activity is mostly concentrated at the postsynaptic side of the synaptic complex and slightly extends (arrows) beyond the limits of the synaptic complex (indicated between bars). (D) Transverse section of an active zone showing that the antigenic determinant recognized by mAb GlyR 2b is localized on the extracytoplasmic side of the synaptic plasmalemma (arrow). The postsynaptic nature of the recognized epitope (arrow) is further illustrated by the inset. (A) $\times 17,400$; (B-D) $\times 90,000$; (inset) $\times 165,000$.

they appear to be concentrated at the postsynaptic side of the synaptic complex in front of the presynaptic site of neurotransmitter release (i.e., the active zone [4]). This structure is comparable to that of the neuromuscular endplate where a high density of receptors is located in juxta-neural regions of the postsynaptic membrane while deep portions of the junctional fold contain very low densities of them (15, 16, 25). Up to now, studies at other synapses in the central nervous system had left open the question of the location of receptor in relation to the sites of neurotransmitter release. For instance, mAb's raised against the β -adrenergic complex of erythrocyte bind throughout the whole length of rat cerebellum and frog hippocampal dendrites, with aggregation at only some postsynaptic sites (29). Similarly, using autoradiographic techniques, hindered by the fact that reduced silver grains are not exactly located at the radioactive sources, only a fraction of muscarinic cholinergic receptors (14) and benzodiazepine receptors (18) were found to be associated with synapses in rat hippocampal neurons; and in the case of

opioid receptors in the neostriatum, no more than 7% of the binding sites were clearly related to synaptic junctions (11).

Extension of the Receptor Matrix

The size of the receptor matrix in relation to that of the postsynaptic differentiation is quite difficult to assess, mainly because of the hazy border of this structure; but we consistently find with immunogold labeling that receptors extend slightly beyond the limits of the synaptic complex, this situation being appropriate for exocytotic events occurring at the periphery of the active zone. In any case, the high concentration of receptors in front of the active zone is compatible with the notion that particles observed by cryofracture to be aggregated at the level of the postsynaptic density are receptors for neurotransmitters (10, 17). This concept was primarily based on evidence that, at the electroplaque, postsynaptic intramembrane particles are receptor proteins and/or ion channels (12).

On the basis of indirect evidence, it has been proposed (6) that receptors involved in the generation of a glycine-mediated

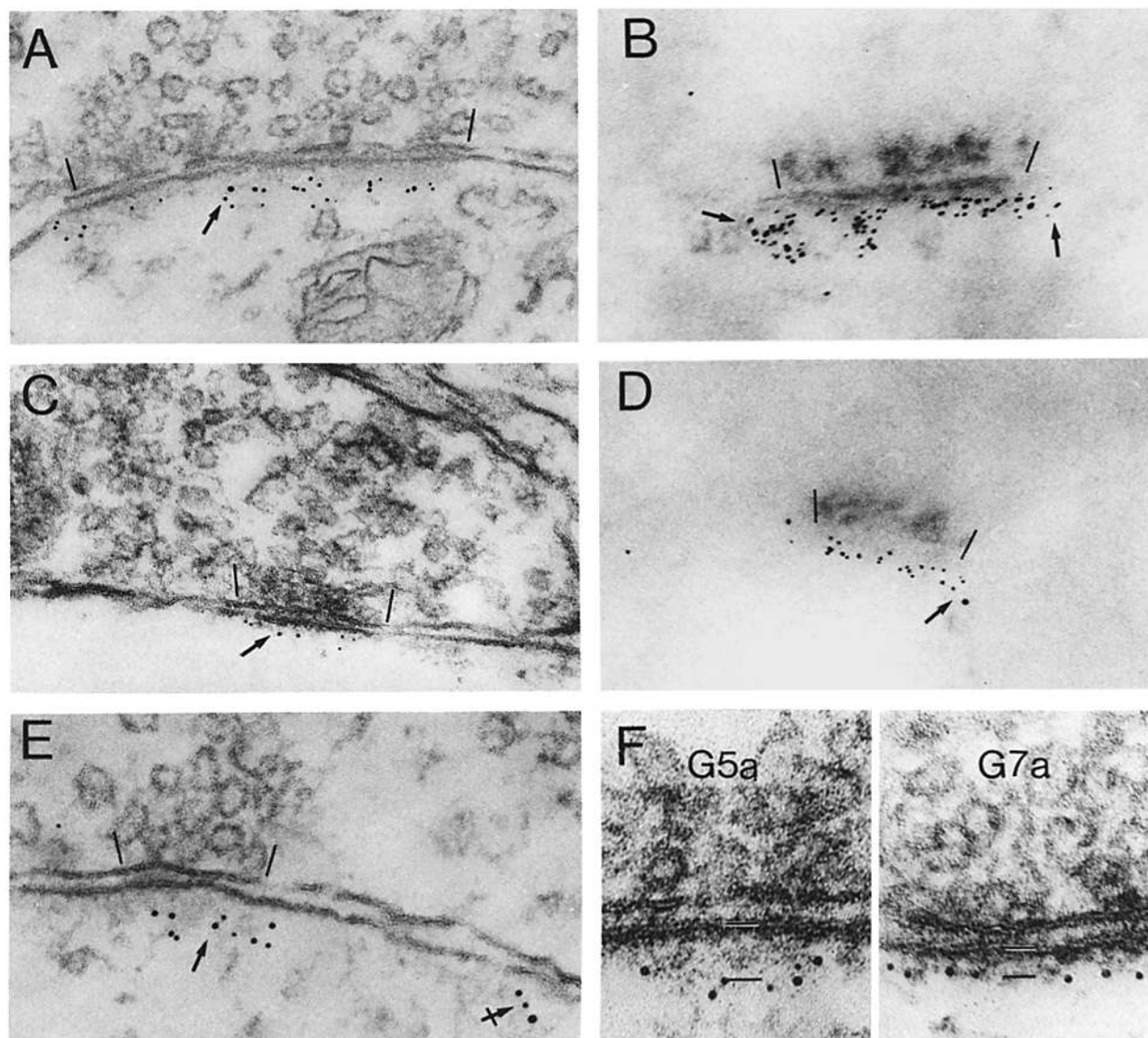


FIGURE 3 Extension of the matrix of glycine receptors labeled with immunogold techniques. (A) Gold particles (arrows) linked to mAb GlyR 5a are associated with the intracellular side of the postsynaptic membrane and are clustered within the limits of the synaptic complex (shown by bars). (B) Transversal view, contained from a semi-thin section ($0.5 \mu\text{m}$) of the synaptic complex, specifically stained with ethanolic phosphotungstic acid; note the high concentration of receptors facing the active zone (between bars); some of them are also present outside this region (arrows). (C and D) Evidence that epitopes recognized by mAb GlyR 7a have the same distribution as those for mAb GlyR 5a; same staining technique as for A and B. (E) Scant gold particles (crossed arrow), as they are occasionally seen away from those associated with the postsynaptic differentiation (arrow). (F) Transverse sections of active zones showing that distance between gold particles and membranes (underlined by small bars) is larger for mAb GlyR 5a than for GlyR 7a. (A–D) $\times 100,000$; (E) $\times 154,000$; (F) $220,000$.

inhibition of the Mauthner cell are widely distributed and include regions located outside the postsynaptic membrane directly facing the presynaptic release sites. Labeling of the postsynaptic membrane, away from the postsynaptic differentiation was indeed found in this material but only occasionally, and the fact that some gold particles were adjacent to the synaptic junctions exhibiting glycine receptor determinants confirms the specificity of this extrajunctional labeling. However, even though it is difficult to achieve full immunohistochemical reactions in tissue pieces, and although it may well be that the techniques used for this work are not sensitive enough to detect sites of low antigen concentrations (3), such receptors, if really present, exist at best in low amounts; they could correspond to the aggregates of particles spread out around

postsynaptic densities, as described at other central synapses (10).

Implications for the Structure of the Receptor

One mAb, GlyR 2b, directed against the 48-kD subunit which harbors the strychnine antagonist binding site of the glycine receptor (8, 9), recognizes an extracytoplasmic membranous determinant. More interesting is the situation of the two other mAb's, which once indirectly labeled with colloidal gold have different relationships with the plasmalemma. For GlyR 5a, which binds to the 93-kD protomer, the distance to the center of the membrane is very comparable to that obtained with the same technique in the peripheral nervous system for antibodies directed against extracellular determi-

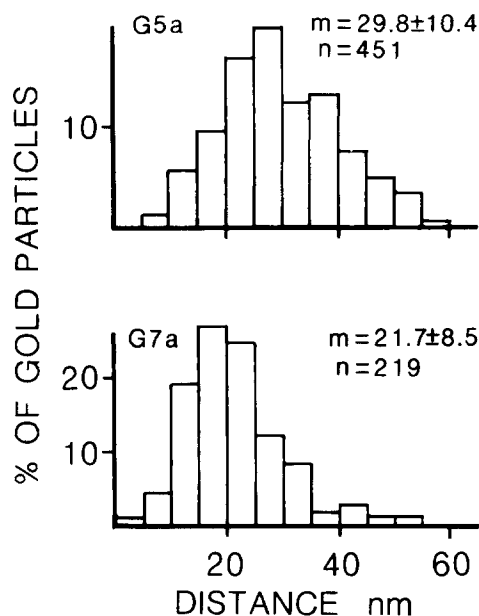


FIGURE 4 Frequency histograms of the distances between the membrane and gold particles associated with mAb GlyR 5a (G5a, upper diagram) and with mAb GlyR 7a (G7a, lower diagram). The mean values (m) were statistically different (Student's t test, $P < 1/1,000$).

nants of the acetylcholine receptor (28) known to extend 5–6 nm beyond the membrane surface (13). This interspace is also comparable with that reported for the mAb's which bind to the cytoplasmic acetylcholine receptor-associated 43-kD protein (19, 28). In contrast, measurements of labeling associated with GlyR 7a indicate that the determinant recognized is closer to the surface of the membrane; on blots, this immunoglobulin gives only trace reactivity with the 48-kD subunit (24) and probably reacts merely with the 93-kD subunit when used as a morphological marker.

In conclusion, our antibody binding experiments clearly establish the glycine receptor as a transmembrane protein. In addition, they demonstrate that at least some important antigenic determinants of the largest glycine receptor polypeptide are localized intracellularly. Whether these determinants fulfill special functions (e.g., receptor clustering or anchoring to the cytoskeleton) remains to be determined.

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