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 [11]) 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 ± 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) 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

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

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

With these immunoglobulins, we have obtained the following: (a) a direct visualization of glycine receptor 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.

**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 μm (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 μm) cells, presumably motoneurons, of lamina IX than on medium-sized (10–20 μm) 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 pre- and postsynaptic 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 translational 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.
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. × 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. 3 E) 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 etherolic 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,
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 $\beta$-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
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 immunochromical 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 determini-
nients 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 antigentic 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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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 < 1/1000).

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