GLYCINE-SPECIFIC SYNAPSES IN RAT SPINAL CORD

Identification by Electron Microscope Autoradiography

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On the basis of neurophysiological and biochemical studies, it has been suggested that the amino acid, glycine, is a major inhibitory transmitter in the mammalian spinal cord (1, 23). The evidence in support of a role for glycine as a neurotransmitter includes the demonstration that glycine (*a*) mimics the action of naturally occurring inhibitory transmitter when it is iontophoretically applied to motor neurons (5, 6, 24), (*b*) is released after dorsal root stimulation (12), (*c*) is reduced in concentration when inhibitory interneurons are selectively destroyed (7), and (*d*) is taken up into brain slices and a distinct population of synaptosomes by a glycine-specific high-affinity transport system (2, 16, 17, 22). This uptake into specific synaptosomes can be demonstrated only in those regions of the central nervous system in which glycine is iontophoretically active and present in high concentration.

Electron microscope autoradiography has been used in attempts to identify synaptic terminals possessing high affinity uptake systems for glycine (10, 11). These autoradiographic studies of [^{3}H] glycine uptake in vitro and in vivo have shown silver grains over nerve terminals with flattened vesicles (9, 14, 15). However, previous reports have not (*a*) provided quantitative evidence for selective labeling of synapses, (*b*) quantitatively analyzed the topographical distribution of labeled synapses, or (*c*) demonstrated that the label was in glycine and not in protein or metabolites.

In the present investigation, synapses on rat ventral horn cells were labeled by [^{3}H]glycine in vivo and were identified by electron microscope autoradiography. Our findings indicate that (*a*) the grain density is greatest in synaptic terminals, (*b*) a high proportion of axosomatic and proximal axodendritic synapses take up [^{3}H]glycine, (*c*) many of the labeled terminals have elliptical and pleomorphic vesicles, and (*d*) [^{3}H]glycine is not metabolized significantly under the conditions of our experiment.

MATERIALS AND METHODS

Microinjection Technique and Fixation

Sprague-Dawley rats weighing 150–300 g were anesthetized with chloral hydrate (40 mg/kg body weight), the lumbar spinal cord was exposed, and a small dural puncture 1.2 mm from midline was made over the L5 cord level with a fine sharpened needle. By means of a micromanipulator, a micropipette (tip diameter 15 μm) was advanced perpendicularly 1.6 mm into the ventral horn. 0.4 μl [^{3}H]glycine (10 Ci/mmol, 4.5 $\times$ 10^6 dpm/μl, New England Nuclear, Boston, Mass.) in 0.1 M phosphate buffer (pH 7.3) was injected over 5 min, using a screw-driven apparatus. The calculated concentration of [^{3}H]glycine at the site of injection was 2.1 $\times$ 10^{-2} mM. All animals were sacrificed 10 min after the beginning of the injection. Groups of animals were studied in three ways. Autoradiography was done on four female rats perfused with 2.5% glutaraldehyde in 0.1 M cacodylate buffer with 3% sucrose (Group I). By using a dissecting

microscope, the site of injection could readily be recognized *in situ* in the fixed animals by means of the small dural puncture. The cord was sectioned transversely at this level with a razor blade, taking care to reproduce the perpendicular course of the micropipette. 1-mm sections for embedding were then cut from the transected cord specimens.

Biochemical studies were done on six male rats. Three were perfused with fixative (Group II), and three were decapitated and their spinal cords were removed without fixation and frozen immediately in dry ice-isopentane (Group III).

Autoradiography

For autoradiography (13, 20), the spinal cords of Group I animals were postfixed in OsO_4, dehydrated in alcohol, and embedded in Epon 812. Thick sections (1.5 μm) were dipped in Kodak NTB-2 (Eastman-Kodak Co., Rochester, N. Y.); thin sections were covered with Ilford L4 emulsion. Light and electron microscope autoradiographs were developed in Kodak D19, stained with toluidine blue or uranyl acetate-lead citrate, respectively, and examined in a Zeiss photomicroscope III or AEI 801 electron microscope. Electron microscope autoradiographs were made from samples which were trimmed back from the injection site and yet showed sufficient labeling on the light microscope autoradiographs to assure satisfactory labeling. Electron microscope autoradiographs of the same tissue over three exposure times (20, 25, and 34 days) were used to analyze the distribution of silver grains. For grain density analysis, large motor neurons of the ventral horn were arbitrarily chosen and the complete margin of the cell body and surrounding neuropil were photographed at × 4,000. These negatives were enlarged to × 14,000 on 11 × 14 in Kodabromide paper and used to construct montages. A 500-point clear plastic grid (8 × 10 in) was used to analyze the montages in terms of unit areas of six anatomic compartments: (*a*) axosomatic and (*b*) axodendritic synaptic terminals (presynaptic compartments); (*c*) cell bodies and (*d*) dendrites of motor neurons (postsynaptic compartments); (*e*) axons; and (*f*) glia. Montages of two different motor neurons at each exposure time were photographed; the total unit area of the ventral horn analyzed was 12,000 μm^2 (Tables I and II). A clear plastic mask with a series of concentric circles of increasing radii was placed over each developed grain; the center of the smallest circle that could circumscribe the grain was punched through the micrograph and the hole was considered the grain location (20).

Determination of Radioactivity Surviving Fixation

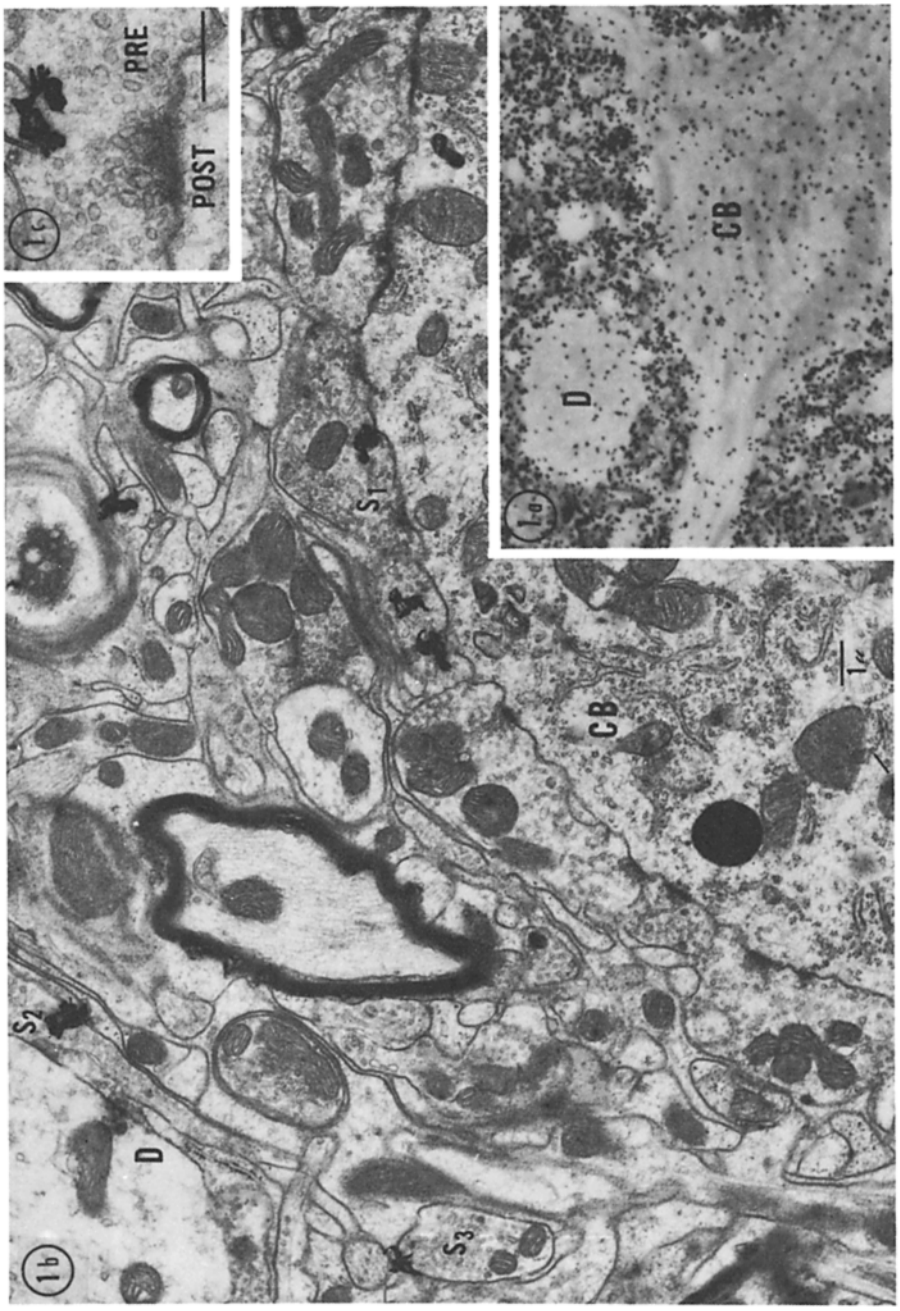
For biochemical studies (Groups II and III) sections of spinal cords were homogenized in 10 vol ice-cold 10% trichloroacetic acid (TCA) and allowed to stand for 0.5 h on ice before spinning at 27,000 *g* for 10 min. The supernates were decanted and the pellets resuspended in 10% TCA. This procedure was repeated four times. The pellets were solubilized by the addition of 1 ml Protosol (New England Nuclear, Boston, Mass.). Samples of supernatant fractions and precipitates were placed in 10 ml of LSC-complete (Yorktown) and counted in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instruments, Inc., Downers Grove, Ill.). Samples of the supernates from TCA-extracted spinal cords (Group III) were analyzed by thin-layer chromatography in three solvent systems: (*a*) *n*-butanol-acetic acid-water (4:1:1) on thin-layer plated cellulose (Eastman-Kodak); (*b*) *n*-butanol-*n*-propanol-20% ammonia (2:2:1); and (*c*) ethanol-water (7:3) on silica gel F254 (EM Laboratories, Inc., Elmsford, N. Y.).

RESULTS

Binding of [^{3}H]Glycine by Fixation

Spinal cord frozen 10 min after microinjection showed that less than 2% of label was precipitated by cold 10% TCA; therefore, in these studies only a small amount of isotope is incorporated into newly synthesized protein. Thin-layer chromatography of tissue extracts in three solvent systems showed that 98% of the labeled material had the same R_f value as glycine, not its metabolites. However, after perfusion with glutaraldehyde, approximately 63% of the isotope was precipitated by TCA, indicating that glutaraldehyde binds [^{3}H]glycine to tissue proteins. Thus, the autoradiographs can be inter-

FIGURE 1 A Light autoradiogram showing distribution of [^{3}H]glycine grains in ventral horn. Silver grains are abundant in neuropil; focal accumulations are present at margins of motor neuron cell body (*CB*). Note paucity of radioactivity over cell body and dendrite (*D*). Section (1.5 μm) stained with toluidine blue. × 1,000. B, Electron microscope autoradiogram with orientation similar to A. [^{3}H]Glycine is present in nerve terminals ($S_{1,2,3}$). The axosomatic synapse (S_1) contains three grains. Label is not present over motor neuron cell body (*CB*) or dendrite (*D*), or over axons. One silver grain (upper middle) is not within a synaptic compartment. × 15,000. C, High resolution electron micrograph of axosomatic synapse shows presynaptic (*PRE*) and postsynaptic (*POST*) elements. A silver grain is present over presynaptic compartment. Note elliptical and pleomorphic vesicles. × 30,000. Scale = 1 μm.


1b
D
S2
S3
S1
CB
1c
PRE
POST
1d
D
CB

preted as showing the localization of free [^{3}H]glycine.

Light Microscope Autoradiography

Autoradiograms showed dense accumulations of silver grains in the neuropil of the ventral horn with a concentration of grains around the cell bodies of motor neurons (Fig. 1 *a*). The perikary of the ventral horn cells showed low levels of autoradiographic activity.

Electron Microscope Autoradiography

Silver grains were located predominately over axodendritic and axosomatic synaptic terminals (Figs. 1 *b*, 2), with the great majority of labeled synapses containing elliptical and pleomorphic vesicles (Fig. 1 *c*). Other tissue elements were lightly labeled, but small axons and glia showed more autoradiographic activity than the postsynaptic compartments. Montages of the three different exposure times were quantitatively analyzed and showed a fairly constant ratio of grain density between the presynaptic terminals and all other compartments; in particular, the grain density in the presynaptic elements was more than three times that of the postsynaptic compartments (Table I). Presumably, this difference reflects the high affinity transport system for glycine in nerve terminals. Glial, dendritic, and axonal processes were present which were both larger and smaller than the synaptic terminals; the density of labeling bore no apparent relationship to the volume of the underlying structures. Analysis of variance on the 34-day exposure data (Table II) demonstrated a significant difference at the 1% level of significance ($P < 0.01$) among the grain densities of these six compartments. The Studentized range test showed that in the 5% level of significant ($P < 0.05$) there was a real difference in grain densities between the synaptic terminals and the other four compartments (cell bodies, dendrites, axons, glia) which showed no significant differences among themselves. In the selected regions of the neuropil which were studied, 40% of all synapses showed silver grains (Table I). 49% of the counted axosomatic synaptic terminals (225) were labeled with [^{3}H]glycine while 37% of the counted axodendritic synapses (575) contained silver grains. The labeled axodentritic terminals were usually on dendrites greater than 2 μm in diameter. These results would appear to reflect the actual number of labeled synapses, because over the three exposure periods the percentage of labeled synapse rose rapidly between 20 and 25 days and then leveled off between 25 and 34 days (Table I).

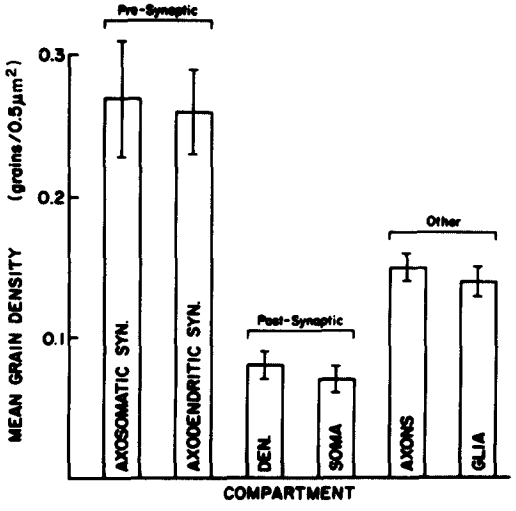


FIGURE 2 Histogram (34-day exposure) showing the grain densities (grains/0.5 μm^2) of [^{3}H]glycine in six anatomic compartments: axosomatic nerve terminals; axodendritic terminals; dendrites; soma; axons, glia. The grain densities are greatest in synaptic terminals. Note difference between presynaptic and postsynaptic compartments. The vertical lines through the top of each bar indicate the standard error of the mean grain density.

DISCUSSION

Binding of [^{3}H]Glycine at Uptake Sites

This study takes advantage of two properties of glutaraldehyde. First, when used in vivo for perfusion, this fixative preserves structural relationships so that ultrastructural features and topographical relationships of synaptic connections can be optimally visualized; in addition, this fixative binds free amino acids to tissue proteins (19), an advantageous action in experiments designed to localize sites of uptake of amino acid transmitters (8, 11, 18).

After microinjection into the ventral horn, [^{3}H]glycine diffuses locally and is rapidly transported into cellular elements presumably by both low and high affinity uptake systems (2, 22). It has been suggested that the high affinity uptake system is responsible for transport of glycine into a unique population of synaptosomes (2, 10), while low affinity transport serves general metabolic functions. At high concentrations (2.0 mM) of glycine, entry is primarily by the low affinity transport system; at lower concentrations (1×10^{-6} M),

TABLE I

Percent Total Area, Grain Densities, and Percent Total Label in Each Compartment over Three Exposure Times

Anatomic compartment	Exposure time: 20 days Total area	20 days Grains/ 0.5 μm^2	20 days Labeled synapses	25 days Total area	25 days Grains/ 0.5 μm^2	25 days Labeled synapses	34 days Total area	34 days Grains/ 0.5 μm^2	34 days Labeled synapses
	%		%	%		%	%		%
Axodendritic terminals	9.3	0.06	11	6.4	0.24	35	11.4	0.26	37
Axosomatic terminal	4.6	0.08	27	6.9	0.19	47	4.7	0.27	49
Dendrites	18.2	0.016		10.1	0.06		17.9	0.08	
Cell bodies	27.4	0.015		35.2	0.07		29.9	0.07	
Axons	15.4	0.03		23.3	0.12		19.8	0.15	
Glia	25.2	0.03		18.1	0.12		16.3	0.14	
Total labeled terminals			15			39			40

TABLE II

Grain Density Analysis in Six Anatomic Compartments

Sample grain density	AD	AS	Dendrites	Soma	Axon	Glia
1	0.20	0.42	0.05	0.14	0.17	0.11
2	0.22	0.06	0.06	0.05	0.14	0.13
3	0.55	0.14	0.08	0.07	0.10	0.22
4	0.06	0.13	0.11	0.03	0.11	0.13
5	0.19	0.30	0.02	0.00	0.20	0.11
6	0.23	0.57	0.10	0.05	0.10	0.12
7	0.25	0.21	0.05	0.07	0.14	0.07
8	0.24	0.15	0.05	0.13	0.23	0.16
9	0.47	0.06	0.03	0.08	0.11	0.13
10	0.35	0.41	0.20	0.09	0.11	0.15
11	0.23	0.05	0.09	0.08	0.19	0.15
12	0.18	0.32	0.10	0.04	0.22	0.18
13	0.19	0.58	0.11	0.03	0.08	0.12
14	0.19	0.20	0.04	0.03	0.08	0.12
15	0.31	0.33	0.06	0.10	0.20	0.25
16	0.27	0.35	0.15	0.14	0.21	0.13
Mean grain density	0.26	0.27	0.08	0.07	0.15	0.14
SEM	0.03	0.04	0.01	0.01	0.01	0.01
99% confidence interval	0.18–0.34	0.17–0.37	0.05–0.11	0.04–0.10	0.12–0.18	0.11–0.17

Each sample consisted of one placement of the 500 point grid on the montage. The mean grain density, standard deviation and standard error of mean were calculated from the 16 individual sample grain densities of each compartment. These results were used for obtaining standard deviation at 34 days where N(16) represents the number of placements of the grid.

glycine is taken up by means of high affinity uptake. Autoradiographs were taken from sites away from the center of injection where the concentration of [^{3}H]glycine was necessarily less than 2×10^{-2} mM. In these areas, there was selective accumulation of isotope in synaptic terminals presumably due to the high affinity transport system for glycine.

Uptake into Synaptic Terminals

In this quantitative study, spinal synaptic terminals show preferential labeling, particularly as

compared to postsynaptic compartments. In our investigation, [^{3}H]glycine was taken up into 40% of synapses in the neuropil of ventral horns. These results can be contrasted with the in vitro studies of Iversen and Bloom which showed that[^{3}H]glycine was selectively taken up into 27.9 ± 1.8% of nerve terminals in whole spinal cord homogenates (10). The authors suggested that the population of synaptosomes labeled with [^{3}H]glycine represents those terminals which normally use this amino acid as a transmitter. The higher value obtained in our study may reflect the fact that we studied synapses only in the ventral horn rather than whole spinal cord (10). Although grain densities were greatest in synaptic terminals, glia and axons also showed labeling by [^{3}H]glycine. In studies by others (8, 9, 11, 18), glial cells have been shown to be capable of accumulating putative amino acid transmitters; the significance of this finding is uncertain, but these cells may function to remove released glycine and thus regulate transmitter levels around synapses. The high level of labeling of small axons was unexpected; one possibility is that these labeled axons may be axons of glycinergic inhibitory interneurons.

Spatial Distribution of Inhibitory Inputs

On the basis of neurophysiologic investigations, it has been suggested that synaptic inputs from different sources may be differentially distributed on various portions of the soma-dendritic receptive surface (3). Although the synaptic organization of the ventral horn shows considerable overlap between sites of central, afferent, and intrinsic connections (4, 21), recent physiologic studies have shown that specific inputs do have specific patterns of distribution. Burke et al. (3) have investigated the reversal potentials of different types of inhibitory inputs and have shown different susceptibilities to intracellular chloride concentrations; for example, the reversal potential for the group Ia inhibitory postsynaptic potential (IPSP) is different from that for the recurrent or Renshaw IPSP. On the basis of these studies, they suggested that the inhibitory nerve terminals of interneurons generating the group Ia IPSP are close to or on the soma of motor neurons (3) and thus more effectively control impulse initiation in the initial segment (21). The present autoradiographic study shows that a substantial proportion of axosomatic and proximal axodendritic terminals on motor neurons are labeled with [^{3}H]glycine. We interpret this observation as support for a distinct spatial distribution for at least one population of inhibitory inputs; these synapses, labeled by [^{3}H]glycine, are in an optimal location for mediating postsynaptic inhibition.

SUMMARY

Glycine, an inhibitory transmitter in spinal cord, is taken up into specific nerve terminals by means of a unique high-affinity uptake system. In this study, [^{3}H]glycine was directly microinjected into rat ventral horn in vivo and electron microscope autoradiography used to localize the label in various anatomic compartments. Quantitative analysis showed that [^{3}H]glycine labeled a high proportion of axosomatic and axodendritic synapses which presumably act to inhibit spinal motor neurons.

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