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Immunocytochemical Characterization of Glycine and Glycine Receptors

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Antibodies against the glycine postsynaptic receptor and against glycine conjugated to BSA with glutaraldehyde were used to immunocytochemically localize and characterize glycinergic neurons in auditory nuclei of the brainstem. Intense glycine receptor immunoreactivity is found throughout the cochlear nucleus, appearing as puncta often present around cell bodies. In the superior olivary complex, the most intense labeling is present in the lateral superior olive. Electron microscopic studies show that immunoreactivity is restricted to the synaptic region. With mAb GlyR 2 antibody, which recognizes the ligand binding subunit of the glycine receptor, reaction product is present in the synaptic cleft; while mAb GlyR 7 antibody, which recognizes an associated protein, shows reaction product on the cytoplasmic side of the postsynaptic membrane. Labeling is restricted to specific populations of synapses, and in the cochlear nucleus it is often associated with terminals containing flattened synaptic vesicles. Glycine immunoreactivity is seen in cell bodies and in puncta, which resemble presynaptic terminals. Most intense cell body labeling is in the medial nucleus of the trapezoid body. These cells project to the lateral superior olive where heavy glycine receptor labeling was seen. In the cochlear nucleus several populations of glycine immunoreactive cell bodies are found, with most being in the dorsal cochlear nucleus. A population of glycine immunoreactive cells in the dorsal cochlear nucleus appears similar to a population of GABA immunoreactive cells identified in an earlier study. To determine if the same cells are labeled with both antibodies, a double label study was done using rabbit anti-glycine and guinea pig anti-GABA. This confirmed that some cells in the dorsal cochlear nucleus are immunoreactive for both glycine and GABA. Similar results were seen in the cerebellum where Golgi cells were glycine immunoreactive, whereas other GABA containing cells were not labeled.

For several years glycine (gly) has been considered to be a major inhibitory neurotransmitter in the brainstem and spinal cord. The first indication of its possible neurotransmitter role came from the work of Curtis and Watkins (1960) who found that gly potently depressed neuronal firing when applied to

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spinal neurons. Distribution studies showing gly to be enriched in certain areas of the spinal cord and low in some areas of the brain, led Aprison and Werman (1965) to first propose that gly may be a neurotransmitter. This was followed by several physiological studies (Bruggencate and Engberg, 1968; Curtis, Hosli, Johnston, and Johnston, 1968; Kelley and Krnjević, 1969; Werman, Davidoff, and Aprison, 1968) which further strengthened the case for gly being a spinal cord inhibitory neurotransmitter and identified strychnine as a powerful antagonist of gly (Curtis, Duggan, and Johnston, 1971; Curtis, Hosli, and Johnston, 1968). Since this time many studies have been done throughout the central nervous system (CNS) implicating gly as a neurotransmitter. Most of these have been iontophoretic analyses to determine the effects of applied gly or strychnine; biochemical studies to measure the distribution of free gly; and uptake studies to determine the presence of a high affinity uptake system for gly. Use of ^{3}H-strychnine binding and autoradiography has also provided information on the distribution of gly receptors. However, an immunocytochemical marker, which would allow the quick and precise localization of glycinergic neurons, was not available. Two recent developments have made the immunocytochemical identification of glycinergic neurons and synapses now possible: the purification of the gly postsynaptic receptor and production of monoclonal antibodies against its subunits (Graham, Pfeiffer, Simmler, and Betz, 1985; Pfeiffer, Graham, and Betz, 1982; Pfeiffer, Simmler, Grenningloh, and Betz, 1984) and the successful development of selective antibodies against small molecule neurotransmitters pioneered by Pelletier, Steinbusch, and Verhofstad (1981) for serotonin and by Storm-Mathisen et al. (1983) for GABA and glutamate. We have used antibodies against the gly receptor and antibodies against gly conjugated to BSA with glutaraldehyde in our characterization of glycine in the auditory nuclei of the brainstem. Previous biochemical, physiological and autoradiographic studies have indicated that gly is a major inhibitory neurotransmitter in these nuclei (Wenthold and Martin, 1984). Similar studies using antibodies against gly and the gly receptor have recently been reported for other areas of the CNS (Campistron, Buijs, and Geffard, 1986; Pourcho and Goebel, 1985; Triller, Cluzeaud, Pfeiffer, Betz, and Korn, 1985; Van Den Pol and Gorcs, 1986).

Immunocytochemical Analysis of the Glycine Receptor

The glycine postsynaptic receptor has been purified from rat spinal cord, and monoclonal antibodies have been made against its subunits (Pfeiffer, Graham, and Betz, 1982; Pfeiffer et al., 1984). The receptor complex is composed of three subunits with molecular weights of 48,000, 58,000, and 93,000 with the 48,000 subunit containing the strychnine binding site. The functions of the other polypeptides, which co-purify with the ligand binding subunit, are not known. It was recently shown that the 93,000 subunit is a

peripheral membrane protein while the two other subunits are integral membrane proteins (Schmitt, Knaus, Becker, and Betz, 1986). We have used two antibodies in our immunocytochemical studies, mAb GlyR 2 which binds to the 48K polypeptide and mAb GlyR 7 which binds to the 93K polypeptide. At the light microscopic level similar distribution patterns are seen with the two antibodies. However, at the electron microscopic level, mAb GlyR 2 shows a deposition of reaction product in the synaptic cleft while reaction product with mAb GlyR 7 is found at the cytoplasmic side of the postsynaptic membrane (Triller et al., 1985; Wenthold and Altschuler, unpublished observation). While both antibodies are useful in light microscopic analyses, the use of mAb GlyR 2 is limited in ultrastructural studies because of its loss of binding to fixed material. Staining with this antibody was obtained only with weak fixatives.

Analysis of auditory nuclei shows the heaviest immunoreactivity obtained with either antibody located in the lateral superior olive (LSO). At the light microscopic level, reaction product is punctate and is often seen outlining unlabeled cell bodies (see Figure 1A). Such staining is most apparent where axosomatic synapses are prevalent, such as in the ventral cochlear nucleus (Altschuler, Betz, Parakkal, Reeks, and Wenthold, 1986). At the electron microscopic level, the reaction product obtained using mAb GlyR 7 is seen on the cytoplasmic side of the postsynaptic membrane of some synapses (see Figure 1B) [Altschuler et al., 1986]. In the AVCN, reaction product is often apposed by terminals containing flattened synaptic vesicles. It remains to be determined if all synapses with flattened vesicles contain gly receptor immunoreactivity and if such labeling is limited to synapses with flattened vesicles.

Immunocytochemical Analysis of Glycine

Whereas antibodies against the gly postsynaptic receptor can be used to identify glycinergic synapses, they cannot be directly applied to determining the neurons giving rise to the presynaptic glycinergic terminals. Since no selective enzyme markers are known for glycinergic neurons, one approach is to identify neurons which contain high levels of free gly, analogous to that which has been successfully used to identify GABAergic neurons (Storm-Mathisen et al., 1983). Unlike GABA, which is largely restricted to GABAergic neurons, gly is a ubiquitous amino acid associated with many cellular functions, in addition to being a neurotransmitter. However, biochemical analyses have shown that gly is much more concentrated in areas of the CNS where it appears to be a neurotransmitter than in other areas. For example, the concentration of gly is more than 10 fold higher in spinal cord than in hippocampus (Berger, Carter, and Lowry, 1977). In our studies we sought to determine if free gly could be determined immunocytochemically and if levels

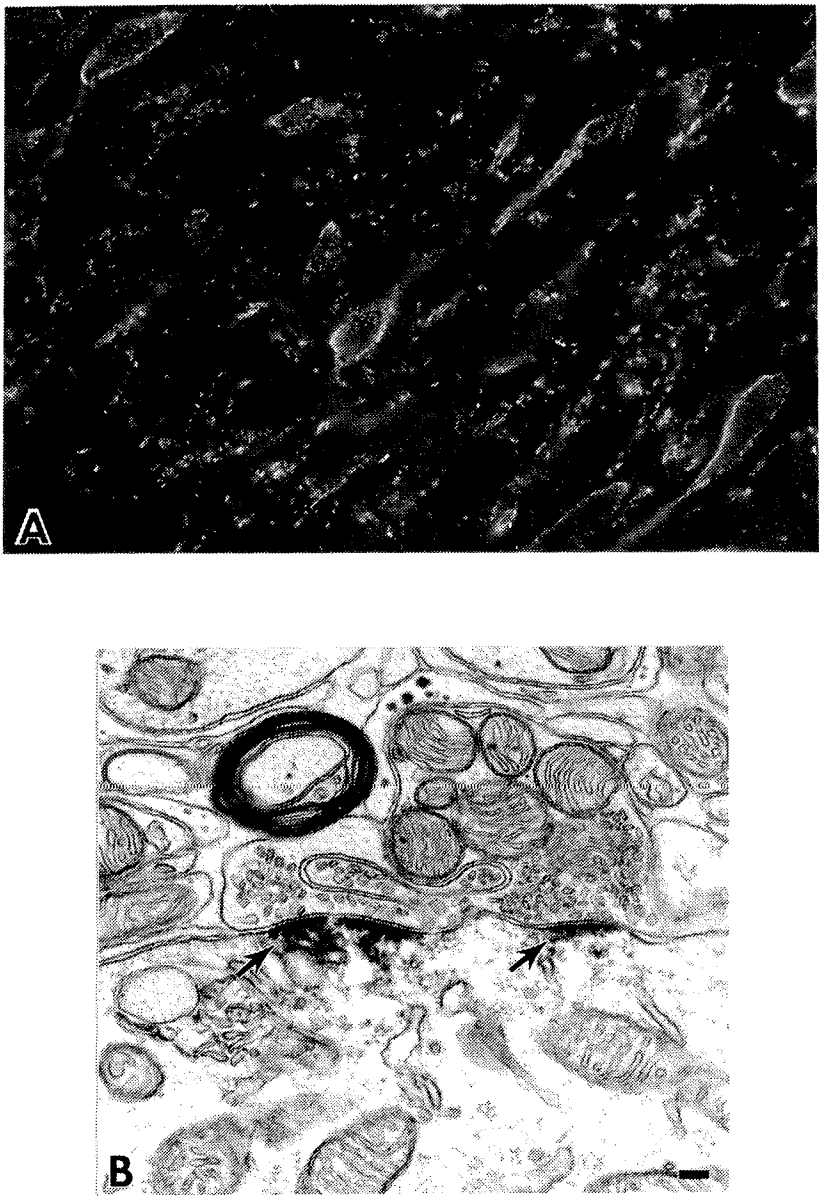

Figure 1: Gly receptor immunoreactivity localized by immunofluorescence in the LSO (A) and at the ultrastructural level with immunoperoxidase (arrows) in the anteroventral cochlear nucleus (B) of the guinea pig. Bar = 0.2 microns.

of gly in neurons are related to a neurotransmitter role for gly.

Antibodies were made against gly conjugated to BSA with glutaraldehyde (Wenthold, Altschuler, Huie, Parakkal, and Reeks, 1986; Wenthold, Huie, Altschuler, and Reeks, 1987). Affinity purification of the antibodies was done by passing serum through a column of immobilized gly-ovalbumin conjugate and eluting the bound antibodies with weak acid. This fraction was then passed through a column of immobilized GABA-BSA conjugate and the unbound fraction was used for immunocytochemistry. Specificity of the antibodies was determined by immunoblot analysis using ovalbumin conjugates of amino acids. Affinity purified antibodies showed a very slight interaction with conjugates of alanine and beta-alanine. A minor interaction with GABA was detected only with high concentrations of primary antibody. These cross-reactions were not eliminated by additional affinity chromatography, and preincubation of antibody with conjugates of these amino acids did not change the immunocytochemical staining pattern. Antibody preincubated with conjugates of gly gave no specific immunocytochemical staining. Animals were perfused with 4% paraformaldehyde and 0.25% glutaraldehyde, and immunocytochemical analyses were done on 50 micron thick vibratome sections.

Analysis of auditory nuclei using affinity purified anti-gly antibodies showed the most intense immunoreactive cell bodies in the medial nucleus of the trapezoid body (MNTB) (see Figure 2A). The morphological characteristics of these labeled cells suggest that they are the principal neurons of this nucleus which project to the ipsilateral LSO. In the LSO intense punctate and fiber labeling were seen. Intense labeling was also seen in the LSO using the gly receptor antibodies (see Figure 1A). These findings are consistent with this pathway being glycinergic, and support previous pharmacological (Moore and Caspary, 1983) and autoradiographic (Zarbin, Wamsley, and Kuhar, 1981) studies which also indicated that it is glycinergic. Intense gly immunoreactive cells were found in the superficial dorsal cochlear nucleus, whereas scattered, less heavily labeled cells were present in the deeper layers of the DCN (see Figure 2B). In the VCN, small immunoreactive cells were found mostly in the granule cell cap, and scattered large immunoreactive cells were most abundant in the caudal AVCN (Wenthold, Altschuler et al., 1986; Wenthold, Huie et al., 1987). Punctate immunoreactive labeling was also present throughout the cochlear nucleus. This resembled labeled presynaptic terminals and was often seen adjacent to unlabeled cell bodies in the VCN (see Figure 3).

The gly immunoreactive neurons in the superficial DCN were similar to GABA immunoreactive neurons which we described previously (Wenthold, Zempel, Parakkal, Reeks, and Altschuler, 1986). To determine if these neurons contain both immunoreactivities, a double label study was done using rabbit anti-gly and guinea pig anti-GABA antibodies. These studies showed that many neurons in the superficial DCN contain both immunoreactivities

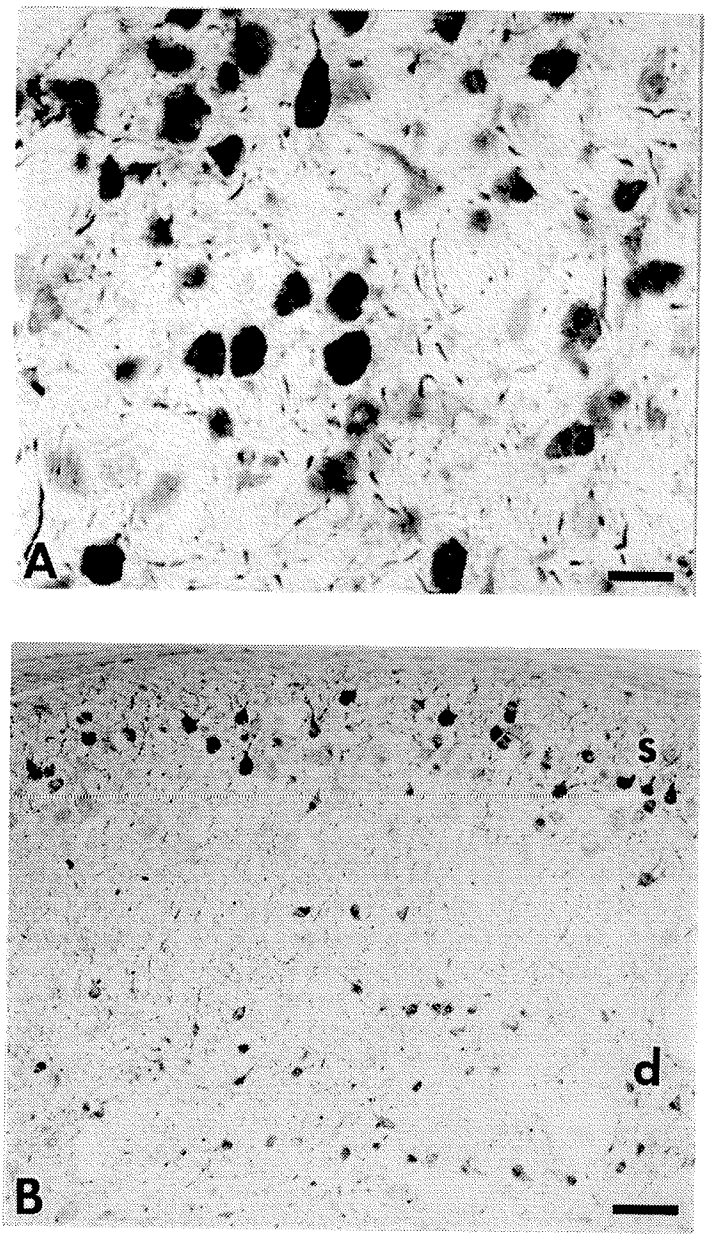

Figure 2: Gly-immunoreactive cell bodies in the MNTB (A) and DCN (B) of the guinea pig. In the DCN intense cell labeling is seen in the superficial layers (s) and scattered more lightly labeled cells are present in the deeper layers (d). Primary antibody used at 1/200 dilution. Bar = 20 microns (A), 60 microns (B).

(Wenthold, Altschuler et al., 1986; Wenthold, Huie et al., 1987). The presence of gly immunoreactivity in GABAergic neurons does not appear to be limited to the DCN. Golgi cells in the cerebellum—which appear to be GABAergic by several criteria including immunoreactivity for GABA and glutamate decarboxylase—also contain gly immunoreactivity (see Figure 4). Scattered immunoreactive puncta and fibers present in the cerebellar granule cell layer (see Figure 4B) may originate from the labeled Golgi cells.

Discussion

We discuss here two potentially useful immunocytochemical markers for the characterization of glycinergic neurons and synapses. The gly receptor is the first CNS neurotransmitter receptor to be extensively characterized immunocytochemically. In both the spinal cord and brainstem, the gly receptor is concentrated at the postsynaptic site of some synapses, presumably those which release gly. Since few studies have been done on the immunocytochemical localization of other CNS receptors, it can not yet be determined if the gly receptor distribution is typical of CNS receptors. Some

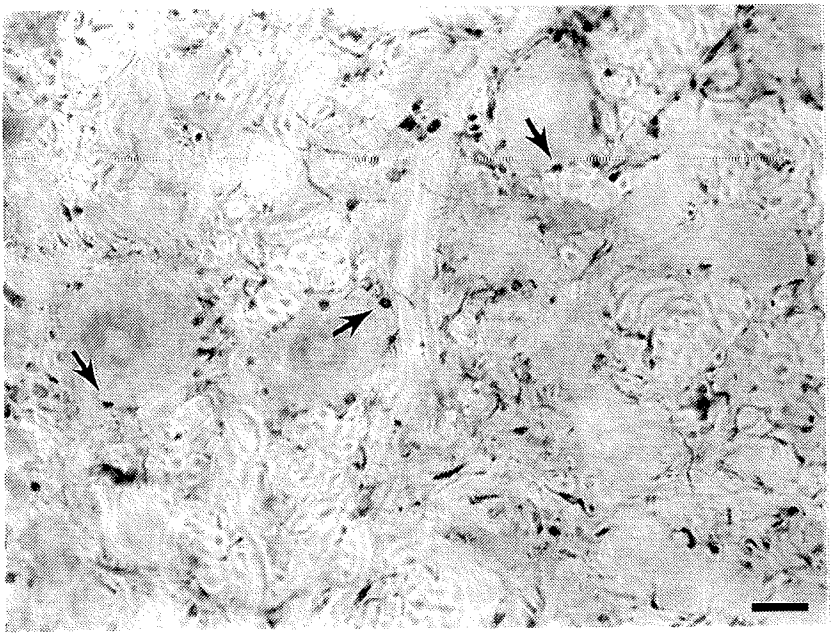

Figure 3: Punctate gly-immunoreactive labeling (arrows) in the guinea pig posteroventral cochlear nucleus. Such labeling may represent gly-immunoreactive presynaptic terminals. Primary antibody used at 1/60 dilution. Bar = 8 microns.

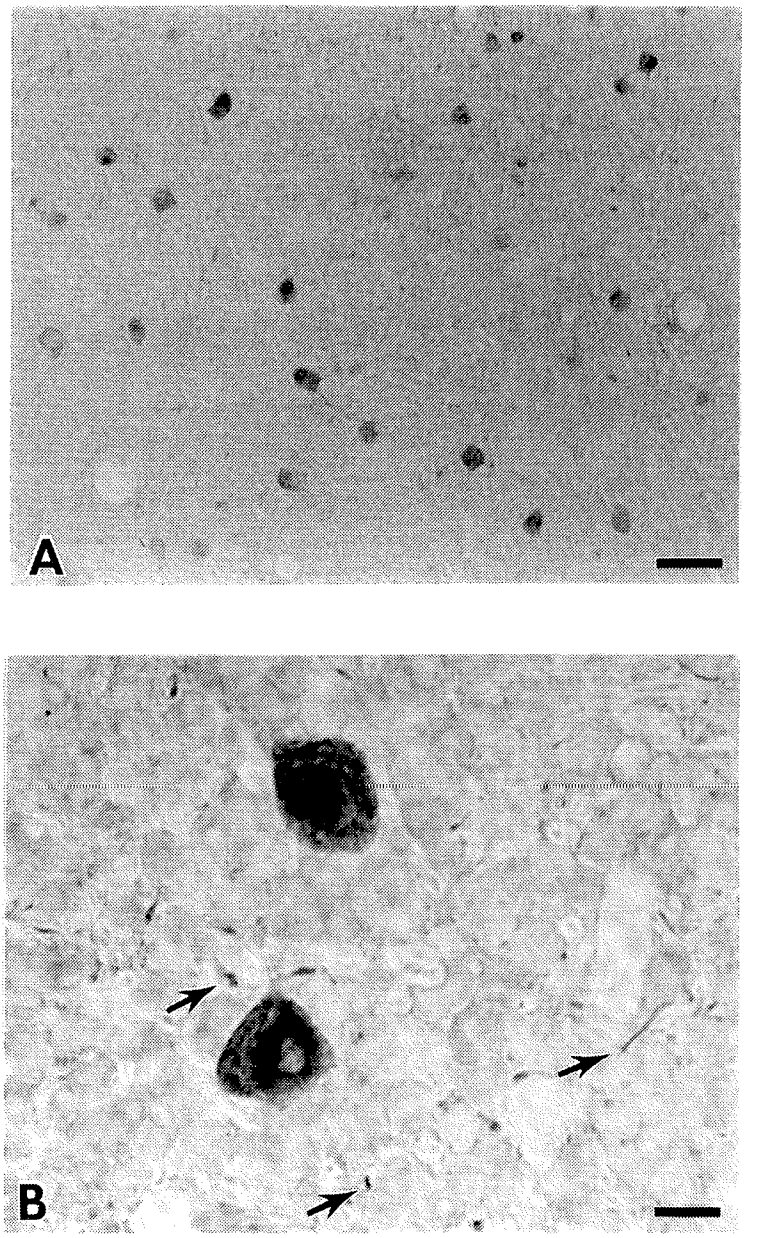

Figure 4: Gly-immunoreactive Golgi cells in the guinea pig cerebellum. At a higher magnification (B), immunoreactive fibers and puncta (arrows) can also be discerned. Primary antibody used at 1/60 (A) and 1/200 (B) dilution. Bar = 50 microns (A), 10 microns (B).

studies suggest that other receptors are localized at relatively high concentrations at sites other than the postsynaptic membrane. For example, the beta adrenergic receptor is concentrated at postsynaptic membranes, but significant receptor immunoreactivity is also present throughout the neuron (Strader et al., 1983). The GABA/benzodiazepine receptor, identified with selective antibodies, was reported to be on both pre- and postsynaptic membranes in the rat substantia nigra (Mohler et al., 1986).

The direct localization of a neurotransmitter postsynaptic receptor could be a specific indicator for identifying a neurotransmitter at a particular synapse, if the postsynaptic receptor is sufficiently concentrated at the postsynaptic site. In fact, the postsynaptic receptor may be the only specific immunocytochemical marker for amino acid neurotransmitters such as gly, glutamate, and aspartate. Studies on the gly postsynaptic receptor show a specific and discrete localization which is consistent with the results of other studies directed at identifying glycinergic synapses. For immunocytochemical studies the use of mAb GlyR 2 would be preferred since it has been shown to recognize the strychnine binding subunit of the receptor. However, this particular antibody apparently binds to a site which is readily affected by fixation, thereby limiting its use, especially in electron microscopy. While mAb GlyR 7 recognizes another polypeptide of the gly receptor complex, it is not yet known if this polypeptide is only and always associated with the gly receptor. Using this antibody we observed staining of intracellular organelles in a few populations of brainstem neurons. Such staining may suggest that these organelles are related to receptor breakdown or synthesis. This seems unlikely, because it would indicate that only small percentages of neurons containing gly receptors use such a mechanism.

Gly immunoreactivity shows a pattern of staining in the brainstem consistent with earlier studies. Some populations of cell bodies are intensely labeled, and punctate labeling, which may be immunoreactive presynaptic terminals, is found in areas where the gly receptor is also localized. However, the localization of gly is less selective than that of the gly receptor. Since gly serves functions in neurons in addition to being a neurotransmitter, some or all of the substance localized may reflect gly in a non-neurotransmitter role. Other substances are also recognized by the anti-gly antibodies. The immunoblot data show that our antibody weakly recognizes alanine and beta-alanine, so these, or other immunologically-related molecules, may in some cases be detected immunocytochemically in addition to gly.

The finding that gly immunoreactivity is present in some GABAergic neurons warrants further study. One explanation is that gly, or an immunologically related molecule, is concentrated in such neurons in a function unrelated to neurotransmission. If so, it is interesting that the gly immunoreactive staining is limited to only some populations of GABAergic neurons. Another explanation is that both GABA and gly are present in these

neurons and that both function as neurotransmitters. This possibility can be further addressed by determining if the gly receptor and GABA or GAD immunoreactivities are present at the same synapse.

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