

Localization of Glycine Neurotransmitter Transporter (GLYT2) Reveals Correlation with the Distribution of Glycine Receptor

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Abstract: We studied by immunocytochemical localization, the glycine neurotransmitter transporter (GLYT2) in mouse brain, using polyclonal antibodies raised against recombinant N-terminus and loop fusion proteins. Western analysis and immunocytochemistry of mouse brain frozen sections revealed caudal-rostral gradient of GLYT2 distribution with massive accumulation in the spinal cord, brainstem, and less in the cerebellum. Immunoreactivity was detected in processes with varicosities but not cell bodies. A correlation was observed between the pattern we obtained and previously reported strychnine binding studies. The results indicate that GLYT2 is involved in the termination of glycine neurotransmission accompanying the glycine receptor at the classic inhibitory system in the hindbrain. **Key Words:** Glycine—Neurotransmitter—Transporter—Immunocytochemistry. *J. Neurochem.* **64**, 1026–1033 (1995).

Amino acids are known to function as neurotransmitters in the brain (Davidson, 1976; Kontro et al., 1980). The most significant amino acids in this respect are glutamic acid, acting as a stimulatory neurotransmitter, and GABA and glycine as inhibitory neurotransmitters (Grenningloh et al., 1987; Nicolls and Attwell, 1990). Termination of neurotransmission by these amino acids proceeds via removal of secreted amino acids from synaptic clefts by a rapid sodium-dependent uptake system (Kanner, 1989). Recently, several cDNAs encoding neurotransmitter transporters were cloned. Only a few were shown to be neuronal, presumably acting in the classic reuptake systems (Clark et al., 1992; Amara and Kuhar, 1993). Glycine has at least two distinct functions as a neurotransmitter in the brain. The first involves inhibitory action mediated by glycine receptors on the postsynaptic membranes (Grenningloh et al., 1987). The second is the modulation of the glutamate NMDA receptor in excitatory postsynaptic cells (Johnson and Ascher, 1987). These observations as well as the distribution of glycine binding sites in the brain indicate that glycine action may not be limited to only the hindbrain (Py-

cock and Kerwin, 1981). Recently, cDNAs encoding the glycine neurotransmitter transporters GLYT1a, GLYT1b (GLYT2 and GLYT1 according to the nomenclature in Borowsky et al., 1993), and GLYT2 have been cloned and sequenced (Guastella et al., 1992; Liu et al., 1992a, 1993a; Smith et al., 1992). All belong to the family of the Na⁺/Cl⁻-dependent transporters with 12 hydrophobic membrane-spanning domains (Liu et al., 1992b, 1993b). The difference between GLYT1a and GLYT1b is only a few amino acids in the N-terminus. Their transcripts may result from alternative splicing and/or different promoters and their mRNAs are expressed in a tissue-specific manner (Liu et al., 1993a; Borowsky et al., 1993). On the other hand, the sequence of GLYT2 revealed that it is only remotely related to the other glycine transporters and contains a relatively long N-terminus with up to eight potential phosphorylation sites (Liu et al., 1993a). Use of specific antibodies against the various glycine transporters may shed light on their function in different locations in the brain.

MATERIALS AND METHODS

Preparation of antibodies against the glycine transporter (GLYT2)

Recombinant fusion proteins for antibody production were prepared as follows: A 700-bp *EcoRI-HindIII* encoding the N-terminus and a 400-bp *EcoRI-HindIII* encoding the loop were generated by polymerase chain reaction from the rat GLYT2 cDNA. For the expression of fusion proteins between the maltose-binding protein and the corresponding amino acid sequences of GLYT2, the DNA fragments were cloned in frame into the plasmid pMAL-c (New England Biolabs). The expressed protein was purified on 12% sodium dodecyl sulfate (SDS) polyacrylamide gel. Each fusion pro-

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Abbreviations used: GLYT, glycine neurotransmitter transporter; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

tein eluted from the SDS gel was injected into two guinea pigs using a procedure similar to that described previously for rabbits (Nelson, 1983). The antibodies (2 ml) were purified on an Affi-Gel 10 column containing the corresponding pMAL fusion protein and subsequently passed through an Affi-Gel (Bio-Rad) column containing only the maltose binding protein. For immunohistochemistry, the affinity-purified antibodies were used at a dilution of 1:150 or 1:30. For western analysis, mouse brains were removed from the head and dissected into their specified parts. The various brain parts were homogenized in SDS sample buffer, and after sonication for 2 min, the homogenate was centrifuged for 15 min in a microfuge. The supernatants were diluted to equal protein concentrations and loaded onto a 9% polyacrylamide gel. The proteins were electrotransferred from the gel onto an ECL nitrocellulose membrane (Amersham). Membranes were processed according to manufacturer's instructions in phosphate-buffered saline (PBS) containing 0.1% Tween 20. For western analysis the purified antibody against N-terminus was used at a dilution of 1:1,000.

Immunocytochemistry

For immunocytochemistry, adult mice (60 days old, BALB/cJ, Jackson Laboratories) were deeply anesthetized with avertin and perfused with a 20-ml solution containing 0.9% NaCl. Then they were perfused with a solution containing 0.9% NaCl, 4% paraformaldehyde, and 0.5% zinc salicylate, pH 6.5 (1 ml/g of body weight). Alternatively, the perfusion was performed with PBS and subsequently with a solution containing PBS, 4% paraformaldehyde, 0.1% glutaraldehyde (pH 7.5). Brains were then further fixed for 1 h in the given fixative at room temperature and cryoprotected in a solution containing 30% sucrose and 0.9% NaCl, pH 6.5 (or 30% sucrose/PBS) for 2 days at 4°C. Frozen sections (20 μ m) were cut on a cryostat microtome and allowed to dry at room temperature for 2 days on microscope slides. Sections were then rehydrated by washing three times for 10 min in 0.5 M Tris-HCl (pH 7.6), blocked 1 h in the same buffer containing 5% goat serum and 0.5% Triton X-100, and incubated with the primary antibody for 6 h or overnight at room temperature in a solution containing 0.5 M Tris-HCl (pH 7.6), 4% normal goat serum, and 0.1% Triton X-100. Sections were then incubated for 2 h at room temperature with the biotinylated secondary anti-guinea pig antibody followed by incubation with avidin-biotin-horse-radish peroxidase complex in the same buffer but without Triton. After each incubation, sections were washed three times for 10 min in 0.5 M Tris-HCl (pH 7.6). Finally, the sections were developed with a DAB substrate kit (Vector), dehydrated, and mounted with Permount (Fisher).

RESULTS

We chose to use fusion proteins for obtaining specific affinity-purified antibodies. Antibodies against GLYT2 N-terminus as well as against the potential glycosylated loop were used in this work. In all cases immunoreactivity was abolished by adding the specific fusion protein, but not the maltose binding protein, into the incubation mixture. The addition of fusion proteins containing sequences of other parts of the transporter had no effect on the staining. Immunoreactivity was found to be localized in gray matter processes and varicosities without a detectable presence

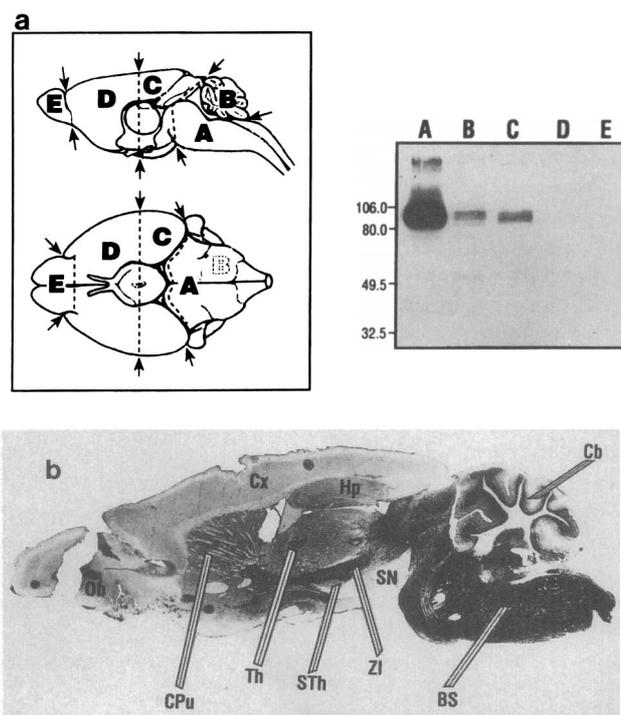


FIG 1. Caudal-rostral gradient of GLYT2 distribution in mouse brain. **a:** Western analysis of GLYT2 in the various parts of mouse brain as schematically depicted in the left side of the figure. Aliquots (20 μ g of the protein per line) of crude SDS extracts from different parts of the mouse brain were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with antibodies against the N-terminus. **b:** A photomicrograph of a sagittal section of GLYT2 immunoreactivity distribution in the mouse brain. BS, brainstem; Cb, cerebellum; Cx, cortex; CPu, caudate putamen; Hp, hippocampus; Ob, olfactory bulb; SN, substantia nigra; STh, subthalamic nucleus; Th, thalamus; ZI, zona incerta. Western blots and immunocytochemistry were performed as described in Materials and Methods.

in cell bodies. To exclude treatment artifacts, the same sections were probed with the antibody against the noradrenaline transporter that clearly stained the locus ceruleus neurons (Jursky et al., 1994). In contrast to the immunostaining with GLYT2 antibodies, the anti-noradrenaline transporter stained not only processes but also cell bodies. Western analysis using protein extracts from different parts of the mouse brain revealed a single band at \sim 90 kDa (Fig. 1a). The position of the immunoreactivity on the western blot is in close agreement with the one reported for the isolated transporter from spinal cord and brainstem (López-Corcuera et al., 1991, 1993) as well as the predicted size calculated from the open reading frame (Liu et al., 1993a). The apparent diffused band on the gel may have resulted from the glycosylation of the protein or its hydrophobicity. These two properties have an opposite influence on the migration of proteins in gels. Immunocytochemistry using both fixation procedures gave similar results. However, the method using zinc salicylate resulted in higher sensitivity of the immuno-

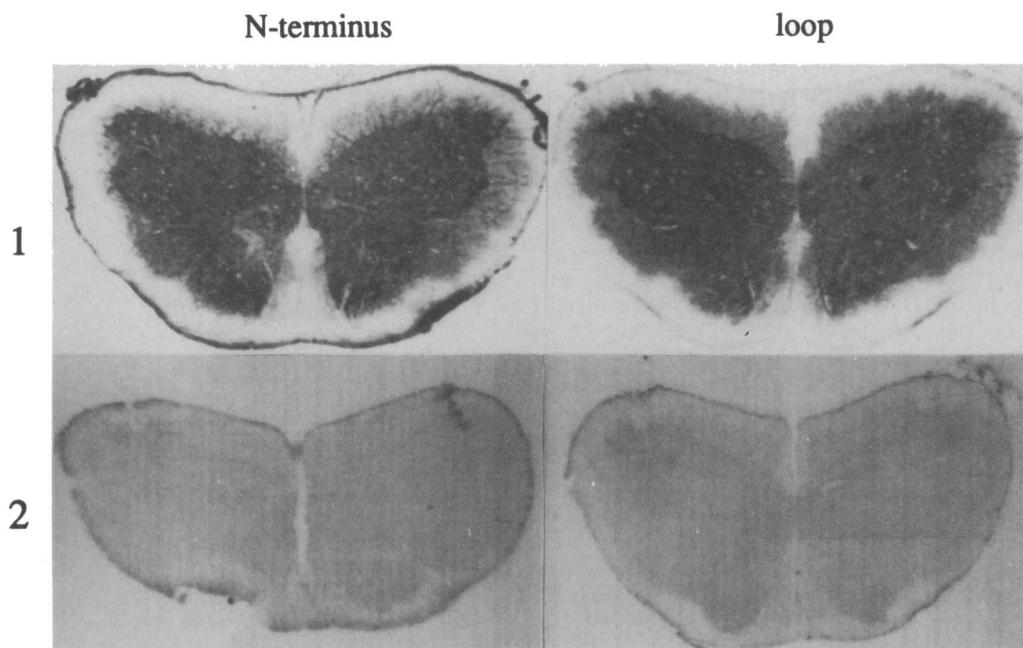


FIG. 2. Inhibition of immunoreactivity with the specific fusion protein. Antibody against the N-terminus as well as loop were preincubated 1 h at room temperature with fusion proteins and then used for immunocytochemistry. **1:** pMAL protein, no effect on staining. **2:** Specific fusion proteins, complete loss of immunoreactivity.

staining but exhibited a slightly increased background. To see some of the rostral staining the sections were developed longer, even if the staining in the spinal cord reached a saturated level. Therefore, GLYT2 appeared to be more abundant in midbrain and diencephalon from the immunostaining results in comparison with western blot (Fig. 1). The specificity of the immunocytochemical staining was verified by competition with the corresponding fusion protein. As shown in Fig. 2, addition of the fusion protein during incubation with the antibody abolished the staining of the sections. In addition, we analyzed five different antibodies raised against different neurotransmitter transporters and each of them gave a unique pattern in the brain slices (Jursky et al., 1994). Taking these experiments together with the antibody used in this study being raised against a unique amino acid sequence leaves little doubt that the immunocytochemical staining represents immunoreactivity with GLYT2 (Liu et al., 1993a).

An immunocytochemical stain of GLYT2 in a sagittal section of rat brain is shown in Fig. 1b. As was observed for the glycine receptor (Araki et al., 1988), a caudal-rostral gradient in the distribution of the transporter is noticed. The distribution of GLYT2 is almost superimposable with that obtained for the glycine receptor traced by [^3H]strychnine (Zarbin et al., 1981; Frosthalm and Rotter, 1985; Cortes and Palacios, 1990; White et al., 1990). The following pattern was obtained in coronal sections moving from back to frontal parts of the brain: In the spinal cord, the whole

gray matter showed a high level of GLYT2 immunoreactivity with weaker staining protruding to the white matter (Fig. 3a). It was apparent in some sections that immunoreactivity was slightly greater in the dorsal horn than in the other parts of the spinal cord. In the cerebellum, immunoreactivity was detected in fibers with frequent varicosities mostly in the granular layer and fewer in the molecular layer and deep cerebellar nuclei (see Fig. 5). In the brainstem, except for the substantia nigra pars reticulata and the superficial gray layer of the superior colliculus, all the gray matter showed a moderate or strong presence of GLYT2. At the medulla, the highest level of signal was detected in the hypoglossal, the gracile, the cuneate nuclei, and all parts of the spinal trigeminal nucleus (Fig. 3b). A high level of immunoreactivity was also observed in the medullary reticular formation. In the midbrain and pons, the highest concentration of GLYT2 was visible in facial, vestibular, and cochlear nuclei (Fig. 3c). Strong immunoreactivity was present in the inferior colliculus, the superior colliculus (except for the superficial gray layer), the tegmental area, central gray (including central gray matter), pontine reticular formation, and in the lateral lemniscus (Fig. 3d). In the thalamus, relatively strong GLYT2 immunolabeling was obtained in the pretectal nucleus, the parafascicular nucleus, the periventricular fiber system, the ventral lateral geniculate nucleus parvocellular, the zona incerta, the field of Forell, and the areas surrounding the mammillothalamic tract (Fig. 3e). Coronal section revealed a clear stain of the U-shaped intralaminar

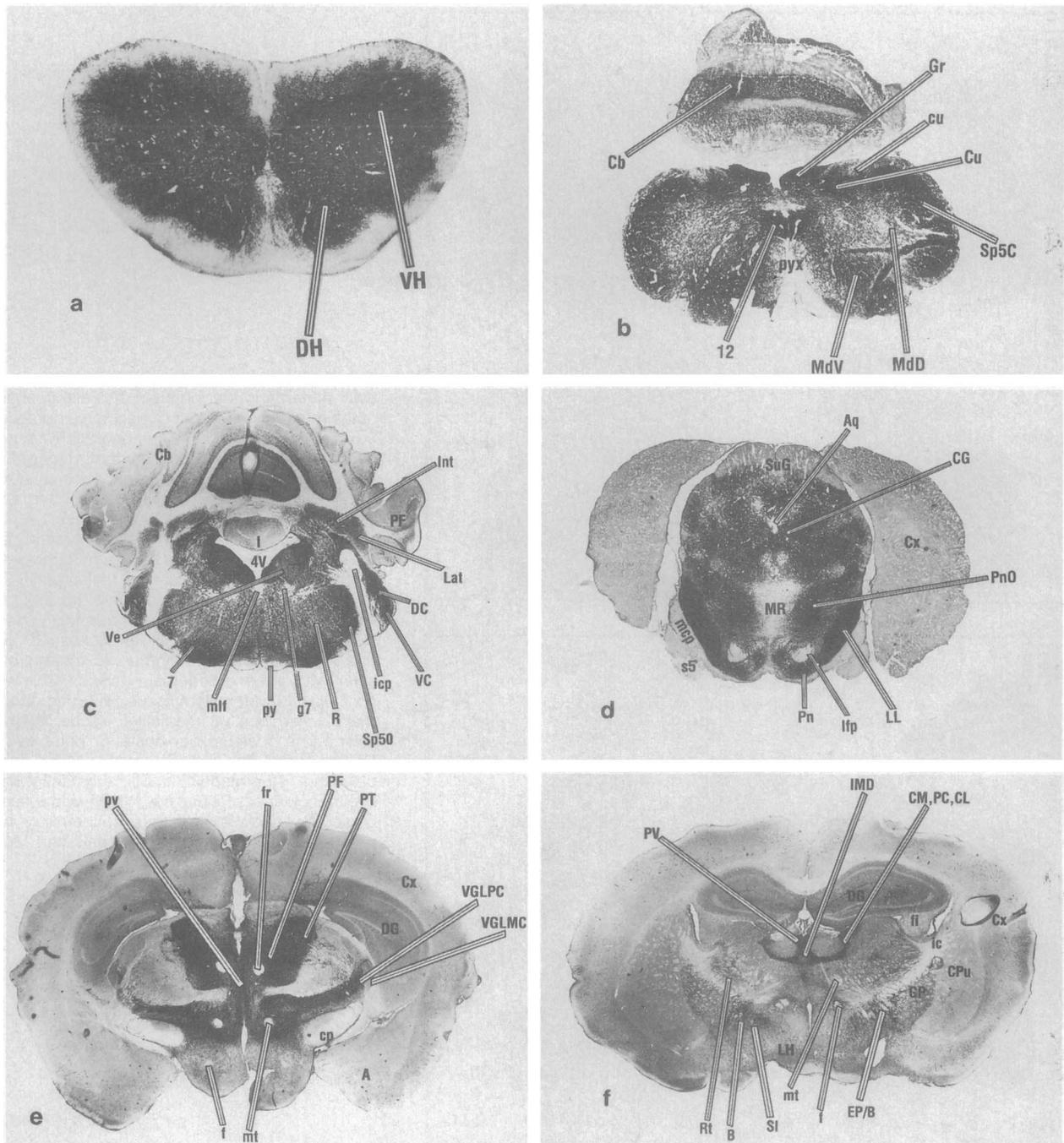


FIG. 3. Distribution of GLYT2 immunoreactivity in coronal brain sections. **a:** Spinal cord: DH, dorsal horn; VH, ventral horn. **b:** Medulla: Cb, cerebellum; Cu, cuneate nucleus; cu, cuneate fasciculus; Gr, gracile nucleus; MdD, medullary reticular field dorsal; MdV, medullary reticular nucleus ventral; pyx, pyramidal decussation; Sp5C, spinal trigeminal nucleus caudal; 12, hypoglossal nucleus. **c** and **d:** Midbrain and pons: Aq, aqueduct; CG, central gray; Cx, cerebral cortex; DC, dorsal cochlear nucleus; g7, genu of facial nerve; Int, interposed cerebellar nucleus; icp, inferior cerebellar peduncle; Lat, lateral cerebellar nucleus; Ifp, longitudinal fasciculus pons; LL, lateral lemniscus; mcp, middle cerebellar peduncle; mlf, medial longitudinal fasciculus; MR, median raphe nucleus; R, reticular formation; SUG, superficial gray layer superior colliculus; Pn, pontine nuclei; PnO, pontine reticular nucleus oral; py, pyramidal tract; s5, sensory root trigeminal nerve; Sp50, spinal trigeminal nucleus oral; VC, ventral cochlear nucleus; I, cerebellar lobule; 4V, fourth ventricle; 7, facial nucleus. **e** and **f:** Diencephalon: A, amygdala; B, basal nucleus Meynert; CL, centrolateral nucleus; CM, central medial nucleus; Cx, cerebral cortex; cp, cerebral peduncle; cpu, caudate putamen; DG, dentate gyrus; EP, entopeduncular nucleus; f, fornix; fi, fimbria hippocampus; fr, fasciculus retroflexus; IMD, intermediodorsal nucleus; PC, paracentral nucleus; PF, parafascicular nucleus; mt, mammillothalamic tract; pv, periventricular fiber system; PT, pretectal nucleus; Rt, reticular thalamic nucleus; SI, substantia innominata; VGLMC, ventral lateral geniculate nucleus magnocellular; VLGP, ventral lateral geniculate parvocellular. Immunohistochemistry was performed as described in Materials and Methods.

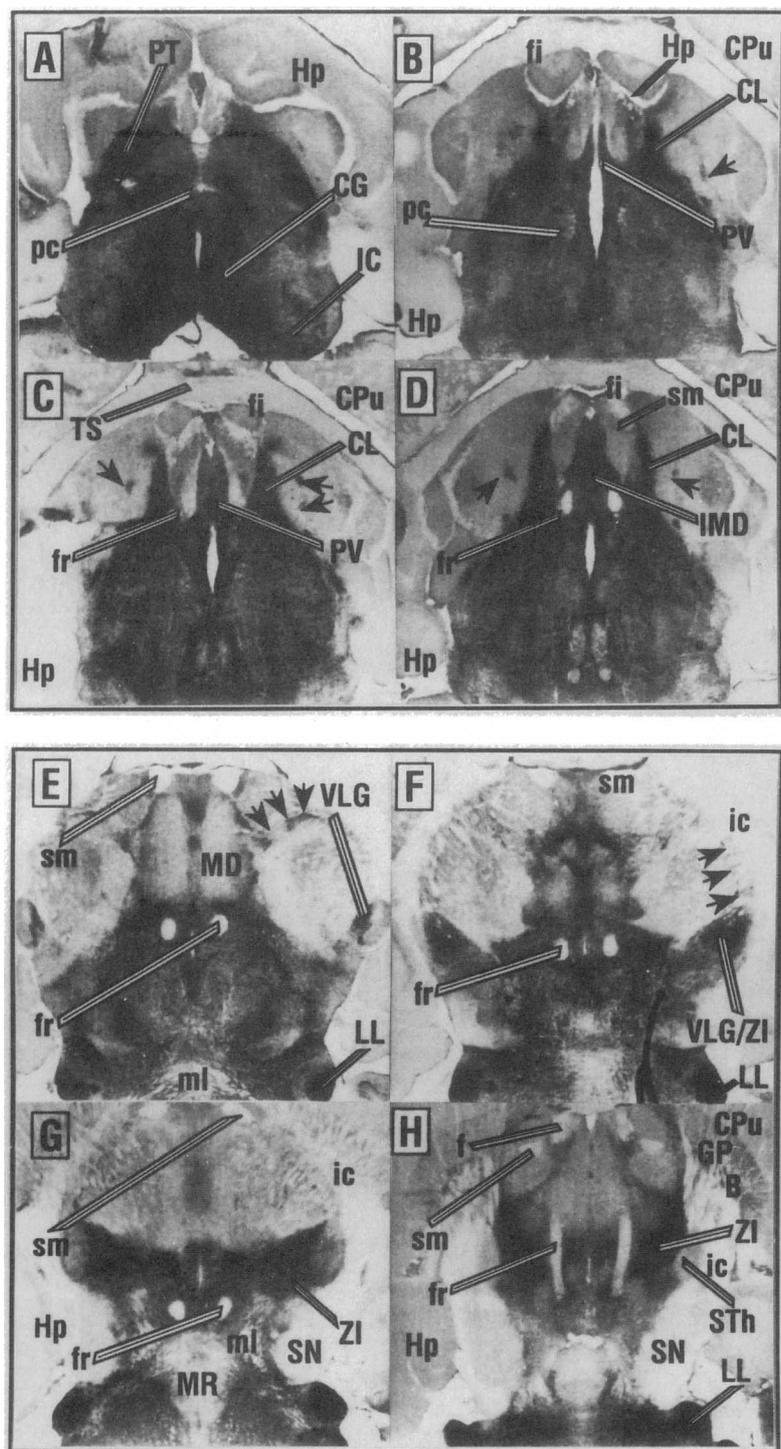


FIG. 4. Horizontal view of GLYT2 immunoreactivity patterns in the midbrain and diencephalon of the mouse brain. **A-H:** Consecutive dorsoventral sections. Main GLYT2 immunoreactivity present in the rostral brainstem (midbrain) merges to the thalamus through the intralaminar nuclei (most rostral staining pattern in B-F) and zona incerta (ZI; G and H). The area of basal (B), entopeduncular nucleus (EP; H) is indicated, and the arrows show complex GLYT2 immunoreactivity patterns in ventral posterior thalamus (B-F). CG, central gray; CL, central lateral thalamic nucleus; CPu, caudate putamen; f, fornix; fi, fimbria hippocampus; fr, fasciculus retroflexus; GP, globus pallidus; Hp, hippocampus; ic, internal capsule; IMD, intermediodorsal nucleus; IC, inferior colliculus; LL, lateral lemniscus; MD, mediodorsal thalamic nucleus; ml, medial lemniscus; MR, median raphe nucleus; pc, posterior commissure; PT, pretectal nucleus; PV, paraventricular nucleus; sm, stria medullaris; SN, substantia nigra; TS, triangular septal nucleus; VLG, ventral lateral geniculate nucleus. Immunocytochemistry was performed as described in Materials and Methods.

nuclei as well as T-shaped intermediodorsal and paraventricular thalamic nuclei. Localized immunoreactive structures were observed in some parts of the ventroposterior thalamus, and the narrow area along the reticular nucleus appears to be following the way of the external medullary lamina (Figs. 3f and 4). GLYT2 immunoreactivity was not present in the subthalamic

nucleus. The level of the GLYT2 in the hypothalamus was very low and present mostly in the lateral hypothalamic area. In the basal ganglia, immunoreactivity was localized mainly in the entopeduncular nucleus basal nucleus and supraoptic decussation and less in the globus pallidus and areas corresponding mostly to the substantia innominata, the ventral pallidum, and the

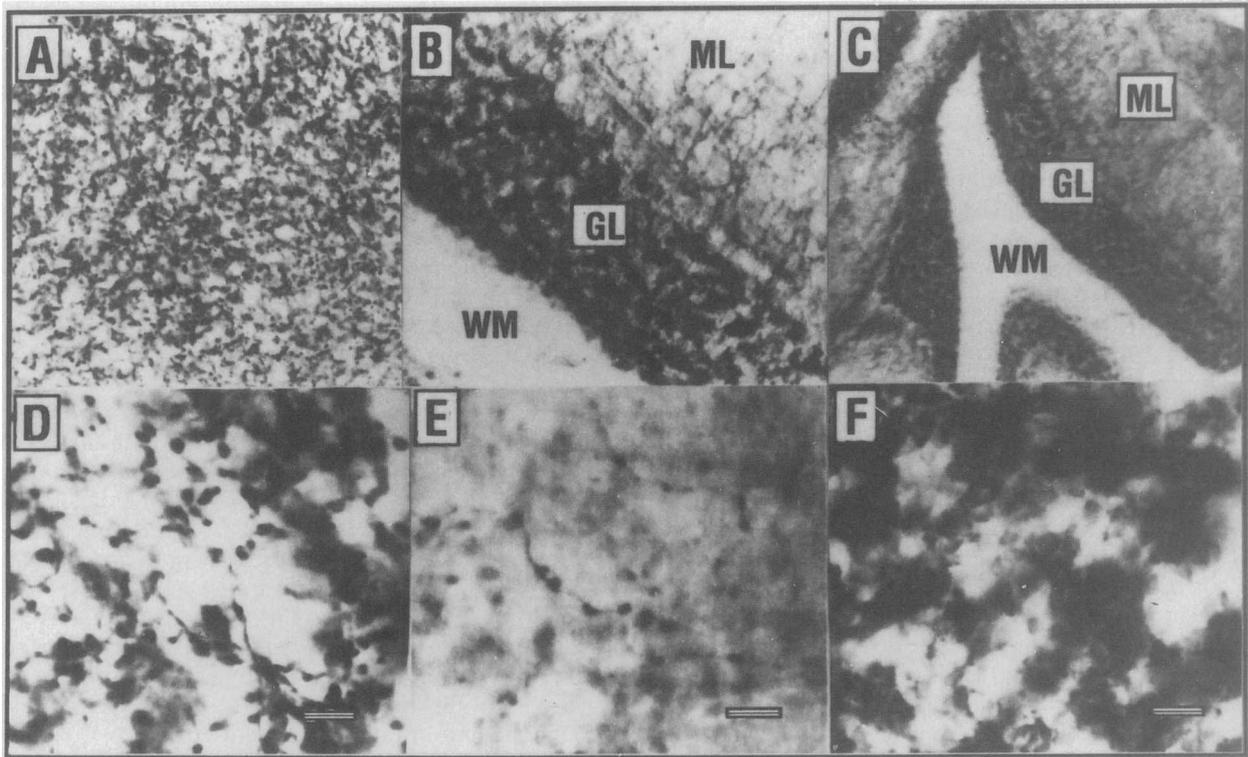
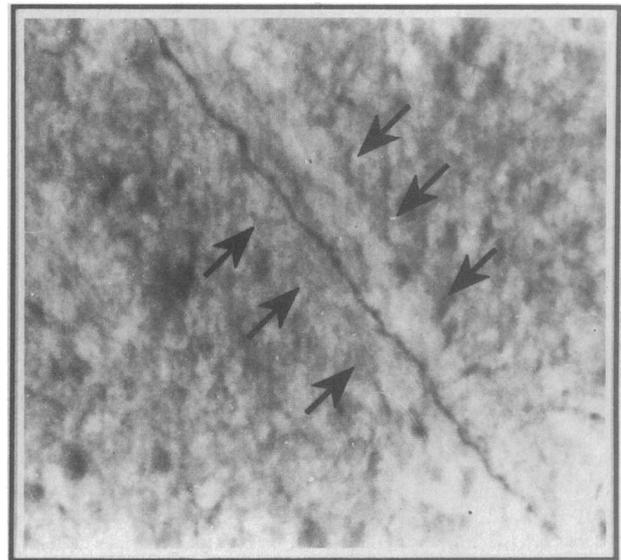


FIG. 5. High-power light microscopic analysis of GLYT2 immunoreactivity in the cerebellum and internal capsule. **Top:** Immunoreactivity is localized in fibers, with frequent varicosities mainly in granular layer (GL) (**A**, **B**, and **D**), deep cerebellar nuclei (**C** and **F**), and molecular layer (ML) (**A**, **B**, and **E**). Immunoreactivity is clearly absent from the cerebellar white matter (bar = 10 μ m). **Bottom:** GLYT2 immunopositive axon traversing internal capsule. Arrows delineate the border of the capsula interna fasciculus. Immunohistochemistry was performed as described in Materials and Methods.



fundus striati. In all cases immunoreactive spots were found on fibers with varicose shape (Fig. 5). In the cerebral cortex, weak immunoreactivity was detected along the border of the corpus callosum and cortex. Stained processes entering deeper layers in the forebrain in areas of the frontal and cingulate cortex were detected. In the hippocampus, GLYT2 staining was localized in the subicular areas, the dentate gyrus, and between stratum radiatum and stratum lacunosum moleculare. In the septum, GLYT2 immunoreactivity was

found in the medial part, continuing to the diagonal band and in the triangonal septal nucleus. GLYT2 immunoreactivity was not detected in the olfactory bulb (Fig. 1).

DISCUSSION

The presence of GLYT2 in a concentration gradient from the spinal cord to the thalamus is in correlation with glycine receptor immunohistochemistry and

strychnine binding studies (Zarbin et al., 1981; Frostholm and Rotter, 1985; Araki et al., 1988; Cortes and Palacios, 1990; Triller et al., 1990; White et al., 1990). This correlation was also observed in recent *in situ* hybridization with GLYT2 probes in the rat brain (M. J. Luque, N. Nelson, and G. Richards, in preparation). There are some deviations from the general correlation between the distribution of GLYT2 and strychnine binding sites. Whereas GLYT2 is present mostly in the granular layer of the cerebellum (Fig. 5B, C), the strychnine-sensitive α 1-glycine receptor immunoreactivity was localized mainly in the cerebellar molecular layer (Araki et al., 1988; Malosio et al., 1991). *In situ* hybridization data showed expression of GLYT2 in Golgi neurons, which implicates the function of GLYT2 in Golgi cells (M. J. Luque, N. Nelson, and G. Richards, in preparation). Relatively high strychnine binding was reported in the superficial gray layer of the superior colliculus, the substantia nigra pars reticulata, and the subthalamic nucleus, but we did not detect GLYT2 in those regions (Figs. 3 and 4). Anatomical and physiological studies indicated a possible role for glycine neurotransmission in the substantia nigra (Pycocock and Kerwin, 1981). GABA agonist muscimol increases the firing rate of dopamine-containing cells in the zona compacta after intravenous injection into the rat. This paradoxical excitatory action of GABA is explained via proposed GABA inhibition of nigral glycinergic inhibitory interneurons and the suggestion that the nigrostriatal pathway is under the tonic inhibitory influence of glycine and GABA (Guastella et al., 1992). The presence of GLYT2 immunoreactivity in the zona compacta indicates the possible function of GLYT2 in this process. A direct inhibitory pathway from the frontal cortex to the lateral hypothalamus, which may use glycine as a neurotransmitter, has been described (Ohta and Oomura, 1979; Kita and Oomura, 1982). This pathway is of particular interest because no long glycinergic axons have been identified in the CNS and glycine is usually found to be associated with interneurons. We detected GLYT2 immunoreactivity in the frontal cortex, the lateral hypothalamus, and, in addition, found rare GLYT2 immunopositive axons in striatal internal capsule fascicles (Fig. 5, bottom).

Two additional variants of glycine transporters (GLYT1a and GLYT1b) were cloned and sequenced (Guastella et al., 1992; Liu et al., 1992a, 1993a; Smith et al., 1992). They probably result from a differential splicing of a single gene (Borowsky et al., 1993; Liu et al., 1993a). The distribution of mRNA encoding GLYT1 in the various brain parts suggested its involvement in modulating NMDA receptors rather than terminating glycine neurotransmission by the glycine receptor (Liu et al., 1992a; Smith et al., 1992). However, this hypothesis has no direct experimental evidence. Further immunocytochemical studies with specific antibodies to GLYT1a and GLYT1b may shed light on the various functions of these transporters.

Little is known about the glycine inhibitory function in the supraspinal sites, where the major action of glycine is expected to be neuromodulatory. In comparison with the strychnine binding data and GLYT2 *in situ* hybridization, we detected a very low level of the GLYT2 in many rostral parts of the brain. Characteristic morphology (Fig. 5) of the GLYT2 immunoreactivity suggests the same function of the GLYT2 in the brainstem and supraspinal sites. Therefore, the distribution of the GLYT2 might provide important information about the glycine inhibitory function in the rostral parts of the brain.

Glycine functions as a neurotransmitter with glycine receptors and a neuromodulator with NMDA receptors (Grenningloh et al., 1987; Johnson and Ascher, 1987). Both functions can be terminated by sodium-dependent uptake systems. In addition, these uptake systems may function in controlling the level of glycine outside and inside cells of particular tissues. In certain physiological or pathological conditions, transporters may function in calcium-independent transmitter release (Attwell et al., 1993). A constant supply of glycine is also required for sustaining protein synthesis in the various cells. The three variants of glycine transporters together with the general glycine transport systems may cooperate in fulfilling the above functions. We propose that the main function of GLYT2 is in the termination of glycine neurotransmission in the hindbrain. This function may be supported in some brain parts by GLYT1a or GLYT1b. Moreover, we observed colocalization of the GABA transporter GAT4 and the glycine transporter GLYT1 in specific locations in the hindbrain (F. Jursky and N. Nelson, unpublished data). Indeed, there are several immunological and physiological evidences that GABA and glycine coexist in some neurons and modulate the release of each other (Triller et al., 1987; Raiteri et al., 1992). Therefore, glycine transporters may function in multiple uptake reactions and cooperation among them may be required for the termination of neurotransmission, neuromodulation, and even neurosecretion.

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