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Molecular Biology of Glycinergic Neurotransmission

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Abstract

Glycine is a major inhibitory neurotransmitter in the spinal cord and brainstem of vertebrates. Glycine is accumulated into synaptic vesicles by a proton-coupled transport system and released to the synaptic cleft after depolarization of the presynaptic terminal. The inhibitory action of glycine is mediated by pentameric glycine receptors (GlyR) that belong to the ligand-gated ion channel superfamily. The synaptic action of glycine is terminated by two sodium- and chloride-coupled transporters, GLYT1 and GLYT2, located in the glial plasma membrane and in the presynaptic terminals, respectively. Dysfunction of inhibitory glycinergic neurotransmission is associated with several forms of inherited mammalian myoclonus. In addition, glycine could participate in excitatory neurotransmission by modulating the activity of the NMDA subtype of glutamate receptor.

In this article, we discuss recent progress in our understanding of the molecular mechanisms that underlie the physiology and pathology of glycinergic neurotransmission.

Index Entries: Glycine receptors; glycine transporters; glycine metabolism; NMDA receptor; spinal cord; brainstem; myoclonus.

Introduction

Several lines of evidence indicate that glycine is a major inhibitory neurotransmitter in posterior areas of the vertebrate central nervous system (CNS). First, glycine presents an uneven distribution throughout the nervous system, with higher concentrations in the cytoplasm and terminals of neurons in the spinal cord and brainstem (Campistron et al., 1986; Ottersen et al., 1987). Second, glycine is released following neuron depolarization (Mulder and Snyder, 1974). Third, glycine binds and activates a postsynaptic receptor generating inhibitory postsynaptic potentials as a result of increasing chloride conductance (Werman et al., 1967). The postsynaptic action is specifically antagonized by the alkaloid strychnine (Curtis et al., 1968). Finally, the synaptic action of glycine is terminated by an efficient high-affinity uptake mechanism located in presynaptic and glial plasma membranes (Neal and Pickles, 1969)

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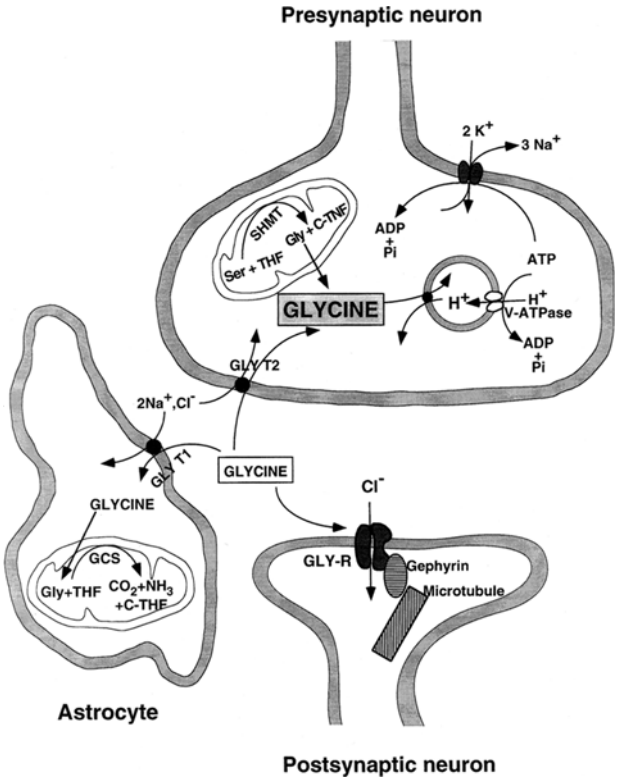

Fig. 1. Schematic diagram of a glycinergic axodendritic synapse. Glycine is synthesized in the mitochondria of presynaptic terminals by the enzyme SHMT, accumulated in synaptic vesicles by an H^+-dependent transport protein, and released to the synaptic cleft on depolarization. Inhibitory postsynaptic potentials are generated by the chloride current produced by activation of the glycine receptor (GlyR). The synaptic action of glycine is terminated by Na^+- and Cl^--dependent transporters located in the presynaptic plasma membrane (GLYT2) and in the glial plasma membrane (GLYT1). Glycine is degraded in astroglial mitochondria by the glycine cleavage system (GCS).

(Fig. 1). Inhibitory glycinergic neurotransmission plays an important role in the processing of sensory and motor information. For instance, glycinergic interneurons in the spinal cord provide the major inhibitory feedback that coordinates the firing pattern of motoneurons during movement (Werman et al., 1967). Glycine is also an important neurotransmitter in the processing of auditory information in the cochlear nuclei, the superior olivary complex, and the inferior colliculus (Wenthold and Hunter, 1990). In addition, inhibitory glycinergic neurons are involved in processing visual information in the retina (Pourcho and Goebel, 1990).

More recently, a role for glycine has been suggested in excitatory glutamatergic neurotransmission as a coagonist or modulator of the NMDA type of glutamate receptor (Johnson and Ascher, 1987). Although controversy exists about the physiological meaning of this action of glycine, externally applied glycine has been found to potentiate NMDA-evoked neuronal activity not only in vitro, but also in vivo.

Localization of Glycine in the CNS

Elevated concentrations of glycine are present in neurons that use it as a transmitter, at least at the inhibitory pathways. An uneven distribution of glycine was found by biochemical methods in the CNS of vertebrates, with particularly high levels in the gray matter of the spinal cord and strikingly smaller concentrations in the rostral brain (Aprison and Daly, 1978). More recently, the availability of specific antibodies for glycine conjugates has provided a detailed picture of the anatomical localization of glycine at the cellular and subcellular levels (Pourcho and Goebel, 1985; Campistron et al., 1986; Ottersen et al., 1987; van den Pol and Gorcs, 1988). In the spinal cord, immunoreactive cell bodies and axons are located in the dorsal and ventral horns. In particular, glycine-like immunoreactivity (GlyLI) is very high in the Renshaw cells (Ottersen and Storm-Mathisen, 1987). Motoneurons are negative for GlyLI, but are surrounded by immunoreactive bouton-like dots apposed to their surfaces, covering 26–42% of the motoneuron somatic surface (Shupliakov et al., 1993). In the dorsal horn, the highest GlyLI is concentrated in laminae III and IV. Interestingly, most of the immunoreactive neurons in this area also contain GABA (Todd and Sullivan, 1990), suggesting that both amino acids could act as cotransmitters, especially taking into consider-

ation that both glycine and GABA receptors also colocalize in neuronal membranes postsynaptic to these neurons (Todd et al., 1996). Immunoreactive axons can be found throughout the white matter, although fewer are present in the dorsal funiculus. In the brainstem, a high density of GlyLI is also found in cell bodies, axons, and terminals of several nuclei, especially in the auditory system. For instance, at least two populations of immunoreactive cells have been described in the dorsal cochlear nucleus: medium-size neurons concentrated in the superficial layers (probably cartwheel neurons) and small neurons in the deeper layers of this nucleus. In the ventral choclear nucleus, GlyLI is particularly high in large cells concentrated near the auditory nerve root (Wenthold et al., 1987, 1988). In the superior olivary complex, GlyLI is high both in neurons of the medial nucleus of the trapezoid body, which project to the lateral superior olive (LSO), and in a population of neurons of the LSO that project to the inferior colliculus (Wenthold et al., 1987; Aoki et al., 1988). Another area with glycine-containing neurons is the cerebellum, where GlyLI is selectively localized in the cell bodies of the Golgi neurons as well as in their terminals in the glomeruli, around the terminals of the mossy fibers (Ottersen et al., 1987, 1988). GlyLI has also been shown in the retina and is concentrated mainly in the amacrine cells (Pourcho and Goebel, 1985, 1990), as well as in some bipolar cells (Davanger et al., 1991).

Metabolism of Glycine

Theoretically, the high concentrations of glycine in glycinergic neurons could be achieved by a high rate of synthesis, slow degradation, an efficient accumulative reuptake process, or by a combination of these mechanisms. Studies using radioactive precursors suggest that much of the glycine synthesis in the CNS is derived from *de novo* synthesis from glucose through serine. Serine is converted to glycine by the enzyme serine hydroxymethyltransferase (SHMT), a pyridoxal-5′-phosphate-dependent enzyme that uses tetrahydrofolate as an acceptor of the methylene group and requires manganese ions for activity. This enzyme presents two isoforms, one mitochondrial and the other cytosolic. It has been suggested that glycine synthesis from serine occurs in the mitochondria, whereas the conversion of glycine to serine is a cytosolic process. The distribution of the mitochondrial SMHT activity in brain shows some correlation with the distribution of glycine (Aprison and Daly, 1978), although detailed immunocytochemical studies have not been performed. The primary structures of cytosolic and mitochondrial SHMT of rabbit and human are known (for review, see Alexander et al., 1994). The human cDNA for the mitochondrial enzyme encodes a mature protein of 474 residues, whereas the cytosolic one encodes a 483-residue protein (Garrow et al., 1993). These isozymes share a high degree of identity to each other (63%), but are encoded by different genes, which map on chromosome 12 and 17, respectively (Garrow et al., 1993).

The degradation of glycine in the CNS seems to occur mainly by the glycine cleavage system (GCS), which is associated with the inner mitochondrial membrane and consists of four protein components (named P-, H-, T-, and L-protein) (Okamura-Ikeda et al., 1992). P-protein catalyzes the pyridoxal phosphate-dependent decarboxylation of glycine and the transfer of the remaining methylamine moiety of glycine to the lipoyl prosthetic group of the H-protein. The T-protein catalyzes the release of ammonia from the intermediate attached to the H-protein and transfers the methylene group to tetrahydrofolate. Finally, the L-protein is a lipoamide dehydrogenase that reoxydates the dihydrolipoic acid on the H-protein, and is common to GCS and to the three α-ketoacid dehydrogenase complexes, i.e., pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and branched-chain α-ketoacid dehydrogenase. All the components are synthesized cytoplasmically as precursor forms, transported into mitochondria, and assembled in the complex after processing (Otulakowski and

Robinson, 1987). cDNA clones for all the components of the GCS have been obtained from different species, but the complex is best characterized in chicken liver. Although the brain GCS has not been studied in detail at the molecular level, it seems that the characteristics of the complex are similar to those of the complex in the liver. The primary structure of chicken P-protein consists of 970 amino acid residues and has pyridoxal phosphate at Lys-704 (Kume et al., 1991). The decarboxylase activity of the P-protein is very low and requires H-protein as a regulatory protein for the expression of its activity (Hiraga and Kikuchi, 1980). Chicken H-protein is composed of 125 amino acids with lipoic acid at Lys-59, and the T-protein is composed of 376 amino acids (Okamura-Ikeda et al., 1992), but the binding site of tetrahydrofolatefolate is not known.

Primary defects in GCS cause a group of metabolic disorders named nonketotic hyperglycinemia. These diseases, which develop early in infancy, are characterized by severe neurological alterations, such as lethargy, hypotonia, myoclonus, and generalized seizures. Enzymatic analysis showed that most patients with nonketotic hyperglycinemia had a defect in either P-protein or T-protein (Hayasaka et al., 1983), rendering high levels of glycine in cerebrospinal fluid.

The anatomical localization of GCS in the nervous system has been performed by immunocytochemistry with an antibody to P-protein (Sato et al., 1991a). The enzyme is confined to the astrocytes and the intensity of immunostaining varies in different brain regions, with the strongest staining located in the hippocampus, cortex, cerebellum (Bergmann glia), and retina (Müller cells). The weakest staining was found in the brainstem and spinal cord. Thus, the distribution of GCS could account, in part, for the regional differences in glycine concentrations: High activity of GCS seems to be associated with low levels of glycine. High activity of the GCS in astrocytes would also explain the low cytoplasmic concentration of glycine that has been determined by immunocytochemistry in these cells (Ottersen et al., 1990).

Nevertheless, neither SHMT nor GCS appears to be a good marker for glycinergic neurons. The factor that seems to play a central role in the accumulation of glycine in the cytoplasm of glycinergic neurons is the reuptake mechanism, which would permit the neuron to reuse the glycine, thus avoiding part of the glial degradation. This concentrative task is carried out by a sodium-and-chloride-dependent transport process, which shows a cellular distribution that matches perfectly with specific areas of neurons having a high cytoplasmic glycine content (*see below*) (Zafra et al., 1995a; Spike et al., 1997).

Release of Glycine

The vesicle hypothesis, describing quantal release of neurotransmitter, provides an understanding of the main mode of neurotransmitter release in the nervous system. Calcium entry into the presynaptic terminal leads to a fusion of neurotransmitter-containing vesicles with the terminal membrane and release of neurotransmitter to the synaptic cleft. After acting on postsynaptic receptors, the transmitter is inactivated either by enzymatic conversion to inactive species in the extracellular space, or removed from the extracellular space by a transporter-mediated reuptake into the nerve terminals or adjacent glial cells. For several amino acid neurotransmitters and monoamines, a considerable amount of work has proven the existence of a vesicular mechanism of release, although much less experimental work has been done for glycine (Maycox et al., 1990). In addition, a number of studies have suggested the existence of another nonvesicular mechanism of release (Adam-Vizi, 1992; Attwell et al., 1993). In certain conditions, both physiological and pathological, the transport system could run backward, pumping transmitter out of cells and serving as a calcium-independent, nonvesicular mechanism for neurotransmitter release. This mechanism could be of great importance in the modulation of the NMDA receptor activity in several areas of the brain, especially in those where no glycinergic termi-

nals have been found, and hence the vesicular mechanism of release would be improbable.

In any case, release and inactivation of the neurotransmitter during the synaptic transmission involves the participation of two distinct transport systems, one located in the membrane of the synaptic vesicles, which accumulates glycine inside the vesicles, and the other in the plasma membrane, which regulates the concentration of glycine in the synaptic cleft. The kinetic properties, energy dependence, substrate specificities, and inorganic ion requirements are clearly different in these two types of transport systems.

Vesicular Transport

In recent years, energy-dependent uptake of glycine and other transmitters, such as glutamate, GABA, monoamines, and acetylcholine, has been demonstrated in synaptic vesicle preparations from mammalian CNS (Maycox et al., 1990). The affinity of the vesicular transporters for amino acids is low, in the millimolar range, whereas for monoamines, it is in the low-micromolar range. The highest levels of glycine uptake have been observed in synaptic vesicles isolated from spinal cord (Kish et al., 1989). GABA and glycine seem to share the same vesicular transporter, since they show mutual competitive inhibition in the lower millimolar range (Christensen et al., 1990; Burger et al., 1991), although Kish et al. (1989) did not find any evidence of competition. Vesicular transport is a secondary active process driven by the proton electrochemical gradient created by a proton pump that belongs to the vacuolar class of ATPases (V-ATPases) (Fig. 1). The V-ATPases are clearly distinguished from the well-known mitochondrial and plasma membrane ATPases. Although the precise subunit composition of V-ATPases is still unclear, they have been shown to consist of at least eight subunits with a molecular mass exceeding 500 kDa (Nelson, 1993). Mechanistic studies have suggested that vesicular GABA/glycine transport depends on both the $\Delta\Psi$ and the ΔpH components of the proton electrochemical gradient. In contrast, transport of glutamate uses mainly the $\Delta\Psi$ component, and transport of monoamines, although it uses both $\Delta\Psi$ and ΔpH, is maximal at high ΔpH (Maycox et al., 1990). The molecular nature of the GABA/glycine transporter remains unknown. Nevertheless, the GABA transporter has been functionally reconstituted in proteoliposomes (Hell et al., 1990). This step could represent a tool for purification of the protein, a procedure that is hindered by the limited amount of protein in the vesicles. Recently, two mechanistically related vesicular amine transporters, one from CNS (SVAT or VMAT2) and the other from adrenal chromafin cells (CGAT or VMAT1), have been cloned (Erickson et al., 1992; Liu et al., 1992). The sequences of these two proteins are closely related with an overall identity of 62% and a similarity of 78%. Analysis of the hydrophobic moment predicts 12 transmembrane domains, a structure also predicted for plasma membrane transporters (*see below*). Since the deduced primary structure predicts no signal sequence, the amino- and the carboxyl-terminus are presumed to be in the cytoplasmic face of the membrane, whereas a big loop located between putative transmembrane domains 1 and 2, which contains three potential sites for *N*-glycosylation, would reside in the lumen of the vesicle. In addition, Rand and coworkers (Alfonso et al., 1993) have identified the product of the gene *unc-17* in *Caenorhabditis elegans* as a putative vesicular transporter for acetylcholine (VAChT). This protein of 532 residues is 37% identical to VMAT1 and 39% to VMAT2. By homology screening, a cDNA has been isolated for the *Torpedo* protein (43% identity to VMAT1 and VMAT2) (Varoqui et al., 1994), as well as for the human and rat VAChTs (Erickson et al., 1994; Roghani et al., 1994). All the vesicular transporters identified so far show no sequence homology with the plasma membrane transporters, but they do show homology to a class of bacterial drug resistance transporters (Edwards, 1993). Although cDNAs for the vesicular transport of neurotransmitter amino acids have not been identified to date, the similar mechanistic characteristics and the

homology data of the cloned vesicular transporters suggest that these proteins could also belong to the same gene family.

Plasma Membrane Transport

Biochemical and Mechanistic Studies

Specific high-affinity transport systems for glycine have been identified in nerve terminals and glial cells (K_m from 20 to 100 μ*M*) (Johnston and Iversen, 1971; Logan and Snyder, 1972; Kuhar and Zarbin, 1978; Mayor et al., 1981; Zafra and Giménez, 1986; Fedele and Foster, 1992). Glycine is accumulated against its concentration gradient through the plasma membrane using the electrochemical gradient of sodium and chloride as a driving force (Kuhar and Zarbin, 1978). The uptake process is electrogenic (positive charge moving inward) with a stoichiometry of 2 Na^+ and 1 Cl^- for 1 glycine zwitterion (Aragón et al., 1987; Zafra and Gimenez, 1988). Since the potential energy of the sodium electrochemical gradient is a power function of the coupling coefficient, a coefficient greater than one imparts a thermodynamic advantage for uphill transport. Taking into consideration the Gibbs free energy changes accompanying these ion movements, the minimum value of the extracellular glycine concentration that can be obtained by the operation of the carrier has been calculated by Attwell et al. (1993) to be 0.2 μ*M*.

A glycine transporter was purified to apparent homogeneity by sequential chromatography on phenyl-Sepharose, wheat germ agglutinin-Sepharose, and hydroxyapatite columns, followed by 5–20% sucrose gradient fractionation. The pure transporter of about 100 kDa was reconstituted into liposomes, retaining the absolute dependence on sodium and chloride gradients, the electrogenicity, the glycine affinity, the substrate specificity, and the sensitivity to group-selective modifiers characteristic of the native transporter (López-Corcuera et al., 1991, 1993). About 30% of the mass of this transporter consists of glycosidic residues that can be removed with PNGaseF, an enzyme that hydrolyzes most types of N-linked carbohydrate groups from glycoproteins at a point between the di-GlcNAc core and the asparagine to which the carbohydrate is linked. The glycine transporter-linked carbohydrates are predominantly tri- or tetra-antennary complex N-linked oligosaccharides containing sialic acid residues (Núñez and Aragón, 1994).

Identification of Glycine Transporter cDNAs

Partial sequencing of the purified GABA transporter by the Kanner's group allowed the synthesis of oligonucleotide probes that were used to clone the GABA transporter (Guastella et al., 1990). By following an expression cloning strategy, Pacholczyk et al. (1991) isolated cDNA for the human noradrenaline transporter. The sequence was found to be very similar to that of the GABA transporter, with an absolute amino acid sequence identity of 48%, increasing to 68% when conservative substitutions were included in the comparison. The design of probes from the conserved regions of these proteins led to the identification of new members of this gene family by a number of laboratories. These proteins have now been classified into two families: the sodium- and chloride-dependent neurotransmitter transporter family, including transporters for GABA, catecholamines, serotonin, proline, and glycine; and the glutamate transporter family (Uhl and Hartig, 1992; Amara and Kuhar, 1993; Kanai et al., 1994). In addition, a subfamily of orphan transporters has been identified (Uhl et al., 1992; Liu et al., 1993b).

To date, two different glycine transporters (GLYT1 and GLYT2) have been cloned (Guastella et al., 1992; Smith et al., 1992; Liu et al., 1993a). GLYT1 presents three isoforms (GLYT1a, 1b, 1c) produced by a single gene (Borowsky et al., 1993; Kim et al., 1994; Adams et al., 1995). Isoforms 1a and 1b originate from transcription initiated at alternate promoters, whereas isoform 1c is an alternative splicing variant of a 54 amino acid exon in the amino-terminal tail of the 1b transcript (Adams et al.,

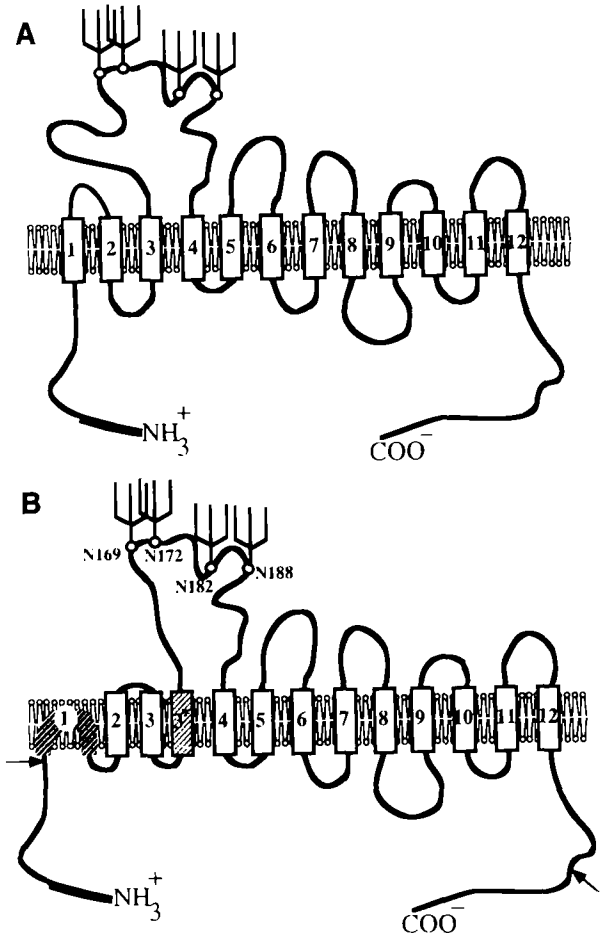

Fig. 2. Schematic model for the secondary structure of glycine transporter GLYT1. **(A)** Theoretical model derived from hydropathic profiles. Predicted transmembrane domains are represented by rectangles included in the lipidic bilayer. Putative glycosylation sites are represented as branched twigs. The thicker line in the amino-terminus indicates variability in GLYT1 isoforms. **(B)** GLYT1 topologic model derived from experimental data. Hydrophobic domain 1 would be a re-entrant loop, and hydrophobic domains 2 and 3 would have a reversed orientation. Part of the glycosylated loop could form a transmembrane segment (3*), unpredicted in the theoretical model. Glycosylation occurs at N169, N172, N182, and N188. Amino acids located at the amino- and carboxyl-termini of GLYT1 (arrows) are not necessary for transport activity (according to Olivares et al., 1994, 1995, 1997).

1995). The three proteins GLYT1a, GLYT1b, and 1c are essentially identical except in their amino-terminal sequences (Fig. 2). These isoforms show no difference in their uptake properties. Actually, when the differential amino-terminal residues are eliminated by site-directed mutagenesis, the resultant truncated proteins retain the kinetics and energetic parameters of the intact protein (Olivares et al., 1994). However, the isoforms display distinct patterns of expression in the CNS and peripheral tissues, suggesting specialized functions for each (Borowsky et al., 1993). The fourth transporter, GLYT2, is generated from a separate gene and displays 48% amino acid sequence identity with GLYT1 (Liu et al., 1993a). GLYT1 and GLYT2 can be pharmacologically distinguished by the sensitivity of the GLYT1 isoforms to sarcosine (*N*-methylglycine) (Liu et al., 1993a).

Localization of Glycine Transporters

The existence of four transporters for glycine is somewhat surprising and raises the question regarding what the physiological meaning of this diversity could be. The detailed anatomical localization gives us some clues. Several studies using *in situ* hybridization and immunocytochemistry techniques define the populations of cells that express mRNA and protein, respectively, for the different glycine transporters (Guastella et al., 1992; Smith et al., 1992; Borowsky et al., 1993; Adams et al., 1995; Jursky and Nelson, 1995; Luque et al., 1995; Zafra et al., 1995a,b). Quantitative analysis by Northern and Western blot techniques reveals that both GLYT1 and GLYT2 (mRNA and protein) are mainly expressed in caudal areas of the brain, although GLYT1 is also expressed in moderate levels in forebrain areas. These data indicate a clear association of both proteins with the inhibitory glycinergic neurotransmission and suggest additional functions for GLYT1, at least in regions devoid of inhibitory glycinergic neurotransmission. More precise immunocytochemical analysis has been performed both by light and electron microscopy (Zafra et al., 1995a). Whereas GLYT1 is expressed in general by astrocyte-like perikarya and processes both in gray and white matter, GLYT2 is found almost exclusively in axons and especially in

terminal boutons. The distribution of GLYT2 is in good agreement with the distribution of the inhibitory glycine receptor, as reported by immunocytochemistry (Araki et al., 1988; van den Pol and Gorcs, 1988; Wenthold et al., 1988) and strychnine binding studies (Zarbin et al., 1981; Jursky and Nelson, 1995), and is found in presynaptic elements of synapses thought to use glycine as an inhibitory neurotransmitter. As mentioned above, GLYT2 is expressed only in neurons with a high cytoplasmic content of glycine. The highest levels of GLYT2 are found in the dorsal and ventral horn of the spinal cord, in the auditory system, and in the nuclei of the cranial nerves. All these areas are known to be rich in glycine receptors. The reverse is also true: Areas without glycine receptor are also devoid of GLYT2. For instance, neither the receptor nor GLYT2 is found in the cerebral hemispheres. One notable exception is the retina that expresses glycine receptor, but not GLYT2. Instead, a subpopulation of retinal cells (amacrine neurons) expresses GLYT1. The neuronal localization of GLYT1 in the retina is atypical, since in the rest of the brain it has only been localized in the glial elements. GLYT1 is abundantly expressed in the delicate glial processes that surround various types of neurons in the spinal cord, pons/medula, and midbrain areas, and moderate to high expression is found in the different nuclei of the thalamus, hypothalamus, and olfactory bulb (Zafra et al., 1995a). In general, those caudal regions that express GLYT1 are known to have inhibitory glycine receptors, indicating that glial cells play an important role in the regulation of the extracellular levels of glycine in the glycinergic synapses. Nevertheless, the expression of GLYT1 in caudal areas is also more widespread than that of the strychnine-sensitive glycine receptor. For instance, the inferior olive has very abundant GLYT1 expression, but rather few glycine receptors.

Although the antibodies for GLYT1 are not able to detect a clear expression of GLYT1 in the plasma membrane of neurons, with the exception of the amacrine neurons in the retina, the existence of a neuronal form of the protein cannot be discarded. It is possible that some of the three isoforms of the GLYT1 are not detected by these antibodies (Zafra et al., 1995a). In fact, several studies at the mRNA level (*in situ* hybridization) have shown a moderate to high expression of GLYT1 mRNA in neurons, not only in spinal cord, brainstem, or cerebellum, but also in forebrain regions, such as the cortex, the hippocampus, the thalamus, the hypothalamus, or the olfactory bulb (Smith et al., 1992; Borowsky et al., 1993; Zafra et al., 1995b), areas where no inhibitory glycinergic neurons have been found. Nevertheless, there are some discrepancies in the literature with respect to the distribution of GLYT1 mRNA, since some authors did not find evidence of neuronal expression (Guastella et al., 1992; Adams et al., 1995). This is probably owing to a difference in the isoform specificity of the probes used in the studies and could be solved with the development of isoform-specific antibodies. On the basis of the mRNA distribution, Smith et al. (1992) suggested that GLYT1 could play a role in modulating the activity of the NMDA receptor. However, the levels of GLYT1 protein in areas rich in NMDA receptors, such as the corpus striatum, the hippocampus, or the cortex, are rather low. Nevertheless, as discussed above, the GLYT1 mRNA distribution suggests the existence of a neuronal form of GLYT1 that has not yet been detected at the protein level. Thus, it cannot be excluded that this hypothetical neuronal GLYT1 could be associated with the NMDA receptor-mediated glutamatergic neurotransmission (*see below* for further discussion). Other possible explanations also exist, such as the association of GLYT1 with nonstrychnine-sensitive glycine receptors. In this respect, the distribution of the β-subunit of the glycine receptor mRNA (Malosio et al., 1991b) shows similarities with that of GLYT1 mRNA (Borowski et al., 1993; Zafra et al., 1995b). Additional experimental work is necessary to solve this important issue.

Structure–Function Relationships of Glycine Transporters

The tentative structural model based on the analysis of hydropathy plots of the amino acid sequences of the sodium- and chloride-dependent transporters predicts 12 transmembrane α-helical domains, with both amino- and carboxyl-termini oriented toward the cytoplasm, and a large, glycosylated, extracellular loop separating putative transmembrane domains 3 and 4 (Fig. 2). All the glycine transporters share the structural features indicated, although GLYT2 has an exceptionally long N-terminus (Liu et al., 1993a).

Some studies on the structure–function relationships and topology of the glycine transporter GLYT1 have been performed. Using antibodies against the terminal regions of this transporter, it has been experimentally shown that both amino- and carboxyl-termini of the protein are intracellularly located (Olivares et al., 1994; Zafra et al., 1995a), as predicted from the hydropathy plots. The topology of the rest of the protein has been studied by glycosylation scanning mutagenesis and by in vitro translation techniques. These studies confirm a 12-transmembrane domain structural model, but support an important topological rearrangement in the amino-terminal third of the protein (Olivares et al., 1997). By examining the effect of tunicamycin, a glycosylation inhibitor, and the effect of the disruption of the putative glycosylation sites of GLYT1 by site-directed mutagenesis, it has been possible to gain further insight into the role of *N*-glycosylation in its function. Progressive mutation of the predicted *N*-glycosylation sites produces a progressive decrease in transport activity and in protein size, indicating that the four putative glycosylation sites located between putative transmembrane domains 3 and 4 (Fig. 2) are actually glycosylated. *N*-glycosylation of the GLYT1 is not indispensable for transport activity itself, but unglycosylated GLYT1 remains in the intracellular compartment. Thus, it appears that the carbohydrate moiety of the glycine transporter GLYT1 is necessary for trafficking the protein to the plasma membrane (Olivares et al., 1995). Recently, it has been demonstrated that extensive parts of the amino- and carboxyl-termini of the GABA transporter are not required for proper function of the transporter (Mabjeesh and Kanner, 1992). In contrast, a large part of the carboxyl-terminus of GLYT1 is necessary to avoid retention in the endoplasmic reticulum, progression to the Golgi apparatus, and complete glycosylation (Fig. 2) (Olivares et al., 1994).

Regulation of the Glycine Transporters

Despite rapid progress in this field, there is currently very little information available on the regulatory possibilities of the neurotransmitter transporters by hormones and second messengers. All the glycine transporters have putative phosphorylation sites for several kinases, suggesting that neuronal or glial carriers may be regulated by diverse signal transduction systems. The phorbol ester phorbol 12-myristate 13-acetate (PMA) causes a concentration- and time-dependent inhibition of the glycine transporter GLYT1 both in glioma cells and in COS cells transiently transfected with an expression vector of GLYT1 (Gomeza et al., 1995). PMA-induced inhibition was suppressed by staurosporine, a PKC inhibitor. These data suggest that high-affinity glycine uptake can be regulated by second messenger systems. The closely related GABA transporter is similarly inhibited by phorbol esters acting through PKC (Gomeza et al., 1991). The exact mode of GLYT1 regulation by PKC remains to be elucidated. Although direct phosphorylation is possible, as demonstrated for the glial glutamate transporter GLT1 (Casado et al., 1991, 1993), other alternative mechanisms cannot be ruled out. Thus, the effect observed on transport activity could be indirectly mediated by changing the activity of ion channels or the (Na^+-K^+)ATPase function, and hence, altering ion concentrations both inside and outside the cell (Clark and Amara, 1993).

In addition to second messenger systems, several lipids have been shown to play a role in regulating the transport of glycine. Arachidonic acid and other unsaturated fatty acids have been shown to inhibit the high-affinity glycine transport into glial cells by a mechanism that involves alterations in the lipid domain surrounding the carrier (Zafra et al., 1990).

The Glycine Receptor

Biochemical Studies

The inhibitory effects of glycine on neurons in the spinal cord and some other areas of the CNS were established in the 1960s. Since then, a large number of electrophysiological, pharmacological, biochemical, immunological, and molecular studies have converted the glycine receptor (GlyR) in the best-understood molecule taking part in glycinergic neurotransmission (for review, *see* Betz, 1990; Betz et al., 1994). These studies indicate that the glycine receptor is a chloride channel with a maximal pore size of 5.2 Å, which is activated by glycine and other agonists (β-alanine, taurine). Strychnine, a convulsive alkaloid from the plant *Strychnos nux vomica*, is the most potent antagonist (K_i 5–10 nM). The first step toward a biochemical characterization was its solubilization with detergents from synaptic membrane fractions of rat spinal cord and its posterior purification by Betz and colleagues using affinity purification over an agarose affinity matrix derivatized with 2-amino strychnine (Pfeiffer et al., 1982). The affinity-purified glycine receptor consists of two glycosylated integral membrane-spanning polypeptide subunits of 48- (α-subunit) and 58-kDa (β-subunit), respectively. Photoaffinity labeling of affinity-purified GlyR in the presence of [^{3}H]strychnine indicates that the binding site for strychnine is mainly in the α-subunit and to a much lesser extent on the β-subunit (Graham et al., 1983; Ruiz-Gómez et al., 1990). A peripheral membrane protein of 93 kDa, named gephyrin, copurifies with the GlyR (Pfeiffer et al., 1982; Schmitt et al., 1987). Gephyrin has been shown by immunocytochemical methods to be present in the cytoplasmic face of the glycinergic postsynaptic membranes (Triller et al., 1985). Studies with purified gephyrin have shown that this protein binds to tubulin with high affinity (Kirsch et al., 1991). Moreover, inhibition of the synthesis of gephyrin with antisense oligonucleotides or disruption of the cytoskeleton with specific drugs in neuronal cultures inhibits the accumulation of both gephyrin and GlyR in postsynaptic membrane specializations, emphasizing the importance of this protein in the biogenesis of the glycinergic synapse (Kirsch et al., 1993; Kirsh and Betz, 1995). Reconstitution of α- and β- subunits into liposomes resulted in the formation of functional receptors, and intramolecular crosslinking experiments suggested that the native glycine receptor is a pentameric structure (proposed stoichiometry α3β2) (Langosch et al., 1988). Purification of the α- and β-receptor subunits was followed by their molecular cloning.

Cloning and Localization of GlyR

Screening cDNA and genomic libraries has resulted in the identification of several variants of the α-subunit. Three α-subunits (α1, α2, α3) are known from rat, and human, (Grenningloh et al., 1987, 1990a; Kuhse et al., 1990a). The rat α2 presents an allelic variant (named α2*) with a single amino acid exchange at position 167, a variation that alters the pharmacology of the α2* subunit, making it resistant to strychnine (Kuhse et al., 1990b). An additional α-subunit has been identified from mouse and rat (α4) (Matzenbach et al., 1994). Moreover, both α1 and α2 present two isoforms generated by alternative splicing (α1ins, α2ins) (Kuhse et al., 1991; Malosio et al., 1991a). All the α-subunits are highly homologous, with an identity ranging from 80 to 99% (Matzenbach et al., 1994). To date, a single β-subunit has been identified. The predicted amino acid sequence exhibits 47% identity to the α1-subunit (Grenningloh et al., 1990b).

The high affinity and specificity of strychnine for GlyR allowed the study of the receptor localization by light microscopic autoradiographic techniques using [^{3}H]strychnine as a ligand. Binding of radiolabeled strychnine predominates in caudal areas of the CNS, in such regions as the dorsal and ventral horns of the spinal cord, the cuneate, hipoglossus, cochlear, or facial nuclei, and different sensory and motor nuclei of the trigeminal nerve, among others. In contrast, negligible binding occurs in the forebrain and cerebellum. More recently, the availability of probes for the different subunits of GlyR has allowed a detailed analysis of the expression patterns of their respective mRNAs by *in situ* hybridization histochemistry. In addition, several antibodies with different specificities have also been raised and used for immunocytochemistry studies (Triller et al., 1985). In general, it can be concluded that the differential expression of the α-subunit variants contributes to a much higher regional and developmental heterogeneity of GlyR than previously anticipated by binding studies. In adult animals, the expression of α1 mRNA and protein overlaps with that of [^{3}H]strychnine, i.e., spinal cord, brainstem, nuclei, colliculi (Araki et al., 1988; van den Pol and Gorcs, 1988; Wenthold et al., 1988; Malosio et al., 1991b; Sato et al., 1991b), and retina (Pourcho and Goebel, 1985; Grünert and Wässle, 1993). With the exception of the spinal cord, the expression of α1 is only postnatal. However, high levels of α2-subunit expression are observed in spinal cord during the embryonic and neonatal periods, but this is undetectable in the spinal cord of adult animals. Nevertheless, significant expression of α2 is still seen in many adult brain regions, including the deeper layers of the cortex, the hippocampus, and the thalamus among others (Malosio et al., 1991b; Naas et al., 1991). The GlyR α3-subunit is expressed at low levels in the cerebellum, the olfactory bulb, and the hippocampus. The β-subunit is abundantly expressed throughout the entire brain and spinal cord, and accumulates since embryonic stages (Malosio et al., 1991b).

Structure–Function Relationships

The primary structure of all these GlyR subunits, as determined by cDNA sequencing, is predicted to share a common transmembrane topology and significant sequence homology with nicotinic, serotonin type 3, and $GABA_A$ receptor proteins (Fig. 3) (Grenningloh et al., 1987). The amino-termini of these mature proteins display 50–70% sequence identity. In the carboxyl-termini of these polypeptides, there are four highly conserved hydrophobic segments (M1–M4). Although it has commonly been considered that these segments would form membrane-spanning α-helices, the tridimensional structure of the nicotinic receptor, recently resolved by Unwin (1993), suggests that only the M2 segment displays an α-helix conformation. A number of studies on the nAChR and other members of this gene family indicate that a pentameric arrangement of the M2 segment generates the ionic pore.

The structure of three functionally important regions of the GlyR has been studied:

1. The ligand binding pocket;
2. The ionic channel, and
3. Sequences involved in oligomerization and clustering of the receptor.

1. Photoaffinity labeling of the glycine receptor with [^{3}H]strychnine (Graham et al., 1983; Ruiz-Gómez, et al., 1990), and heterologous expression of the cloned subunits (Schmieden et al., 1989) revealed that both the strychnine and glycine binding sites are located in a pocket formed by a stretch of amino acids preceding M1 on the α-subunits. The structure of this binding site has been further detailed by comparing the pharmacological profile of the different α-subunits, by site-directed mutagenesis, and by analysis of the naturally occurring mutations. These techniques have yielded a model of the ligand pocket where agonist binding involves multiple interactions with at least three different domains within the extracellular region of the α-subunit (Fig. 3) (Schmieden et al., 1992; Rajendra et al., 1995a,b). This three-loop model appears to be conserved across the ligand-gated ion channel superfamily, since a similar ligand-binding site structure has been

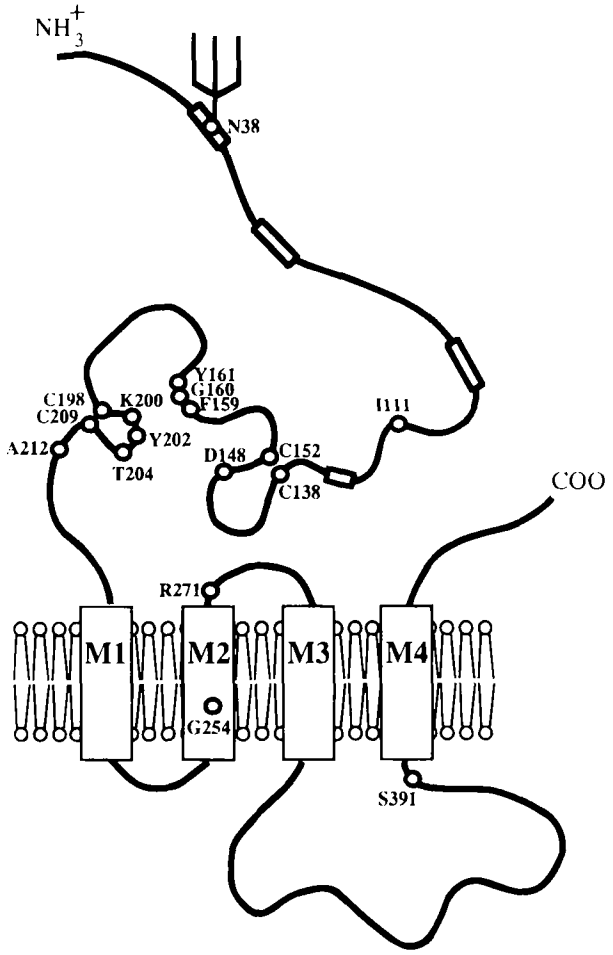

Fig. 3. Schematic structure of the GlyR α1-subunit showing functionally important residues. Rectangles M1–M4 represent transmembrane domains. G254 occupies the most constricted part of the M2-lined chloride channel. Dashed rectangles at the amino-terminal part of α1 represent motifs involved in assembly of subunits. Glycosylation occurs at residue N38, and phosphorylation at residue S391. Loop 2 binding domain contains residues F159, G160, and Y161, which form a β-turn. The loop three binding domain is formed by a putative disulfide bonding of C198 and C209, and contains the residues K200, Y202, and T204. Residues equivalent to loop 1 of the nicotinic receptor have not been identified for GlyR. However, other residues involved in ligand binding are I111 and A212, which mediate taurine activation, as well as D148, in the conserved cysteine loop, which mediates strychnine binding.

reported for the $GABA_A$ and nicotinic receptors (Devillers-Thiéry et al., 1993).

2. The mechanism of ion flux through GlyR channels has been analyzed by several authors by applying patch-clamp techniques to cultured neurons, to spinal cord slices, and to heterologous expression systems. The conductance properties of the GlyR depend on the subunit composition of the oligomers. Thus, the native adult GlyR adopts an elementary main-state conductance of 42–48 pS plus a number of other conductance states (Hamill et al., 1983; Takahashi et al., 1992; Bormann et al., 1993). Homooligomers of α1-, α2- and α3-subunits have main state conductances of 86, 111, and 105-pS, respectively. The β-subunit alone is not able to form channels, but when it is coexpressed with any of the α-subunit, it increases the whole-cell currents and changes the main state conductance to 44–54 pS, values similar to those of the native GlyR (Bormann et al., 1993). Variations in the conductance properties that occur during ontogenic development can also be explained by a change in the subunit composition of GlyR owing to the transition from the expression of α2- to α1-subunits that takes place during the second postnatal week (Takahashi et al., 1992).

   In the GlyR, as in the nAChR, M2 segments of the five subunits that constitute the receptor line the wall of the ionic pore. This is supported by experiments with the channel blocker cyanotriphenylborate (CTB), a compound that blocks α1-homo-oligomers, but not α2-homo-oligomers. By exchanging the M2 segment of the α1 with that of the α2, the resistance to CTB is also transferred. In fact, the sensitivity to CTB of α1 resides in Gly254, which is located in the inner part of M2 (Fig. 3) (Rundström et al., 1994). This position, which seems to occupy the most constricted part of the channel, is also critical for the kinetics of the channel opening. A similar inhibitory mechanism has been suggested for picrotoxinin, an alkaloid that blocks the chloride current in the GABA receptor as well as in homooligomeric GlyR generated by heterologous expression of α-subunits (Pribilla et al., 1992). However, more detailed studies on the action mechanism of picrotoxininin suggest that the binding site is not in the channel, but in the extracellular domain (Lynch et al., 1994). In addition, glutamate residues located at position 290 and 297 of the β-subunit seem to play

an important role in the main state of conductance adopted by GlyRs and in the specific selection of Cl^- vs cations by the channel (Bormann et al., 1993).

3. Assembly of the different subunits to form a functional receptor with the correct stoichiometry and neighborhood relationships must involve specific interactions through recognition sites on the individual subunits. Studies on the nAChR have identified assembly motifs within the extracellular domain of its subunits (Hall, 1992). These types of motifs have also been identified in the GlyR subunits. The assembly behavior of α-subunits is clearly different from that of β-subunits. Coexpression in *Xenopus* oocytes of α2- and α1-subunits of GlyR gives rise to heterooligomers with variable stoichiometry. This experiment suggests that the different α-subunits cannot mutually distinguish each other, and their combination only depends on the relative abundance of α1- vs α2-polypeptides. However, when α- and β-subunits are coexpressed, the α/β heterooligomers have an unvariant (3:2) stoichiometry, independent of the relative abundance of each subunit. Several assembly motifs have been identified within the amino-terminus by exchanging residues diverging between α- and β-subunits (Fig. 3). Exchange of two or three of these motifs in the β-subunit by the corresponding motif of the α1-subunit converts the assembly behavior from β-type to α-type (Kuhse et al., 1993).

   As mentioned above, an important requirement for the correct oligomerization and clustering of functional GlyRs in the postsynaptic plasma membrane is the interaction with the associated protein gephyrin. GlyR interacts with gephyrin throughout an 18 amino acid motif located in the intracellular loop between M3 and M4 of the β-subunit (amino acids 394–411). When this motif is transferred by mutational insertion to a homologous position of the GlyR α1 subunit or to the $GABA_A$ receptor β1-subunit, which normally does not bind gephyrin, a gephyrin binding site is created in these subunits (Meyer et al., 1995).

Regulation of the Glycine Receptor

Recent studies have suggested that a variety of neurotransmitters that regulate intracellular second messenger levels may affect the efficacy of synaptic transmission by modulating the phosphorylation of ion channels (Huganir and Greengard, 1990; Raymond et al., 1993). GlyR, like other members of the ligand-gated ion channel family, presents consensus sites for protein phosphorylation. The α-subunit of GlyR is phosphorylated in intact neurons in response to the presence of either PKC or PKA activators. The activation of these two kinases has opposite effects on the function of GlyR in the spinal cord. Whereas activation of PKA enhances glycine responses (Song and Huang, 1990; Vaello et al., 1994), activation of PKC decreases currents elicited by glycine (Uchiyama et al., 1994; Vaello et al., 1994). Nevertheless, in hippocampal neurons, PKA has no effect on glycine-induced currents, and PKC has a stimulatory effect. These differences have been attributed to the presence of different isoforms in the different areas of the CNS (Schönrock and Bormann, 1995). In the purified GlyR from spinal cord, phosphorylation mediated by PKC in the α-subunit seems to occur in a cytoplasmic serine residue (Ser-391) close to the putative fourth transmembrane domain (Ruiz-Gómez et al., 1991). The phosphorylation site for PKA is not known. However, the only consensus site for this kinase is an eight amino acid insert present in the alternatively spliced form α1ins (Malosio et al., 1991a). The dual mechanism of regulation of GlyR by PKC and PKA provides a potential instrument for the integrated modulation of the glycinergic function by extracellular messengers acting on the neuron.

In the last few years, it has become apparent that phosphorylation is a common mechanism for regulation of the functionality of several other ligand-operated ion channels (Huganir and Greengard, 1990). For instance, phosphorylation of muscle and neuronal nAChR by PKC increases the rate of desensitization to agonists of these receptors. Also the $GABA_A$ receptors are phosphorylated by PKA and PKC. However, results on the physiological effects of phosphorylation of $GABA_A$ receptors are complex and sometimes contradictory, perhaps because of the different subunit composition of the receptor in the different areas of the brain (Raymond et al., 1993).

Recently, the bivalent metal ion zinc has been described as a new modulator of the glycine receptor. Zinc, which is stored in synaptic vesicles and coreleased with neurotransmitter in diverse types of terminals (Assaf and Chung, 1984), is known to modulate an extensive array of voltage-and ligand-gated ion channels, including the $GABA_A$ receptor (Smart, 1992) and the NMDA receptor (Hollmann et al., 1993). The effect of zinc on GlyR is biphasic, with low concentrations of zinc (below 10 μ*M*) potentiating glycine responses and higher concentrations (over 100 μ*M*) inhibiting the response. The response to zinc is observed both in homo-oligomeric receptors formed by α-subunits and in α/β-heterooligomers (Bloomenthal et al., 1995; Laube et al., 1995). Analyses of chimeric GlyR have determined that the zinc binding site is located in the amino-terminal region of the α-subunits (Laube et al., 1995).

Molecular Basis of Glycinergic Pathology

GlyRs play a crucial role in motor and sensory function. A decrease in CNS GlyR has been associated with inherited myoclonus, a widespread group of neurologic disorders characterized by hyperexcitability, muscular spasticity, and myoclonus. Electromyographic and behavioral features of myoclonus resemble subconvulsive strychnine poisoning (Floeter and Hallet, 1993). Mouse, bovine, equine, and human forms of these diseases have been identified.

The spontaneous recessive mutation *spastic* (*spa*) is a prototype of myoclonus in the mouse. *Spa* homozygotes develop a severe neuromuscular disease characterized by an exaggerated startle response, rapid tremor, myoclonus, rigidity, abnormal gait, and impaired righting reflex. Pharmacological and electrophysiological studies indicate that the binding of [^{3}H]strychnine (White and Heller, 1982) and the glycine-mediated chloride conductance (Biscoe and Duchen, 1986) are drastically reduced in *spastic* mice. The *spa* mutation, linked to mouse chromosome 3, is caused by a LINE-1 transposable element insertion in the gene encoding the β-subunit of GlyR (Kingsmore et al., 1994; Mülhardt et al., 1994). This insertion produces reduced expression levels (Kingsmore et al., 1994), aberrant splicing of β-subunit mRNA (Mülhardt et al., 1994), or both. Interestingly, the spastic phenotype was corrected after the gene for the rat β-subunit had been introduced into mice carrying the *spa* mutation, proving the causal relationship between GlyR β-gene mutation and motor disease (Hartenstein et al., 1996). A phenotype similar to *spa* is produced by the murine autosomal-recessive mutation *spasmodic*, linked to chromosome 11. In this case, a missense mutation in the amino-terminus of the α1-subunit (A52S) produces a reduced agonist sensitivity in GlyR expressed in vitro (Ryan et al., 1994; Saul et al., 1994). The *spastic* and *spasmodic* phenotypes normally are not lethal and do not manifest until the second postnatal week, when the neonatal GlyR, which is a homooligomer composed of α2-subunit, is replaced by adult GlyR composed of α1- and β-subunits (Becker et al., 1988; Hoch et al., 1989; Bormann et al., 1993). A related, and more severe, disease is found in the mutant mouse *oscillator*, characterized by fine tremor and muscle spasms that begin at 2 wk of age and progressively worsen, resulting in death by 3 wk of age. *Oscillator* is caused by a microdeletion in the gene encoding the α1-subunit. The deletion produces a frameshift, resulting in loss of the third cytoplasmic loop and M4 segment (Buckwalter et al., 1994).

In humans, hereditary hyperekplexia, or startle disease, is a rare autosomal-dominant neurological disorder that is characterized, as in the *spa* mouse, by an exaggerated startle reflex accompanied by increased muscle tone in infancy (Floeter and Hallet, 1993). Hyperekplexia has been reported to be associated with the substitution by an uncharged amino acid (leucine or glutamine) of arginine 271 in the α1-subunit of GlyR (Shiang et al., 1993). This residue is located at the extracellular terminus of the channel-forming M2 segment (Fig. 3). Voltage-clamp recording of the heterologously

expressed mutants dramatically reduced the agonist binding, the glycine-induced whole-cell current, and the elementary chloride conductance (Langosch et al., 1994; Rajendra et al., 1994). These mutations also transform the binding of GlyR agonists β-alanine and taurine into competitive antagonists (Rajendra et al., 1995a). These data not only help to understand the molecular basis of this disease, but also give some clues to the structure of GlyR, indicating that residue R271 could contribute directly to the ligand binding site, or alternatively, there could be an allosteric coupling between the binding site and the channel domain of GlyR, which is disrupted by mutations in R271 (Langosch et al., 1994; Lynch et al., 1994; Rajendra et al., 1995a).

A novel mutation of the α1-subunit (N244A) has recently been associated with a form of human startle disease (Rees et al., 1994). Interestingly, this form of the disease, as in the *spasmodic* mouse, is inherited in an autosomal-recessive manner. Thus, mutations in α1-subunit can display recessive as well as dominant inheritance, resulting from different point mutations.

The Glycine Binding Site on the NMDA Receptor

Pharmacology and Structure of the Glycine-Binding Site

Glutamate, the major excitatory amino acid in the CNS, activates several ionotropic receptors that are classified according to their pharmacology into AMPA, kainate, and NMDA receptors (Gasic and Hollmann, 1992). The NMDA receptor is formed by pentameric associations of two types of subunits, the NR1, which presents several splice variants, and any of the four NR2 subunits (A, B, C, or D) (Monyer et al., 1992; Moriyoshi et al., 1991). The NMDA receptor has received special attention, since this is involved in synaptic plasticity, learning, development, and neuronal death (Nakanishi, 1992). The NMDA receptor is unique among the ligand-gated ion channels in its calcium permeability and its voltage dependency regulated by magnesium, and also in requiring two coagonists, glutamate and glycine, for activation of the channel (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). Addition of concentrations of glycine as low as 0.1 μ*M* are sufficient to restore normal NMDA responses in electrophysiological experiments. The glycine site on the NMDA receptor is distinguishable from the inhibitory glycine receptor by its insensibility to strychnine. Numerous agonists, antagonists, and partial agonists have been found in the last few years (for review, *see* Leeson and Iversen, 1994). The actions of glycine on the NMDA receptor are mimicked by other small amino acids, such as (*R*)-serine and (*R*)-alanine, and antagonized by HA-966, 7-chlorokynurenic acid, and zinc. The binding site of glycine interacts allosterically with other sites of the receptor. For instance, glycine reduces the binding of antagonists of the glutamate recognition site, but enhances [^{3}H]glutamate binding. Conversely, L-glutamate and the polyamines spermine and spermidine increased the [^{3}H]glycine binding affinity. Moreover, glycine increases the ability of glutamate to promote the binding of MK-801 to the ion channel (Leeson and Iversen, 1994).

The availability of cDNAs for the different NMDA receptor subunits has permitted further studies to be carried out that focus on understanding the role of the glycine site in the receptor function. If NR1 is expressed alone in transfected cells, it forms a high-affinity glycine binding site detected by radioligand binding (Grimwood et al., 1995), but it does not form ion channels detectable by voltage-clamp recordings. However, the expression of NR2 alone does not produce channels gated by glutamate or glycine binding. Nevertheless, when both subunits are expressed in combination, there is a dramatic increase in the amplitude of the current and in the glycine affinity (Nakanishi, 1992). These experiments suggest that the glycine binding site is in NR1, although the site is modulated by the presence of NR2 subunits. Recent studies by site-directed muta-

genesis support this possibility. To date, five sites in the NR1 primary sequence have been found where amino acid exchanges affect glycine efficacy. Two of these are located in the distal third of the extracellular amino-terminal region of NR1. The first contains the motif phenylalanine-uncharged amino acid-tyrosine (F390, Y392), which is also found in the glycine binding site of the inhibitory GlyR. The second region involves F466 and the adjacent charged amino acids D463 and K465. It has been suggested that both the aromatic ring of F466 and the carboxyl group of D463 could contribute to the binding by interacting with the amino moiety of glycine (Kuryatov et al., 1994; Wafford et al., 1993). Another residue involved in the formation of the glycine binding pocket is R505, also in the amino-terminal domain of NR1 (Hirai et al., 1996). In addition, substitution of cysteines 402 and 418 disrupts the cooperation of glycine and glutamate in NMDA receptor channel activation (Laube et al., 1993). Finally, the extracellular loop connecting M3 and M4 also seems to contribute to the glycine binding pocket with two sites: V666-S669 and F735-F736 (Kuryatov et al., 1994; Hirai et al., 1996).

Physiological Role of the Glycine Site

The functional significance of the glycine effect still remains obscure. Measurements of the glycine in the CSF indicate that it is normally present at concentrations above 10 μ*M*, a concentration that should chronically saturate the glycine site. In this respect, published results are somewhat contradictory. Whereas various reports indicate that a full response can be elicited when glutamate or NMDA is applied to single neurons in brain slices or in intact brain of anesthetized animals without adding exogenous glycine, others have found a potent enhancement of NMDA responses by externally applied glycine. For instance, at the mossy fiber inputs to the cerebellar granule neurons, glycine potentiates glutamate responses (D'Angelo et al., 1990). Furthermore, intracerebral administration of the agonist (*R*)-serine increases the potency of NMDA to induce seizures in mice (Singh et al., 1990), and glycine potentiates NMDA-dependent LTP in vivo (Thiels et al., 1992), suggesting that the glycine site may not normally be saturated by glycine in the intact brain. Glycine also potentiates NMDA-evoked neuronal activity in the rat spinal cord and thalamus in vivo (Salt, 1989; Budai et al., 1992). The same conclusion can be obtained from an experiment that shows that local injections of glycine or (*R*)-serine into mouse brain lead to increased levels of cGMP in cerebellum, a response known to be elicited by NMDA receptor activation (Rao et al., 1990). The effects of glycine and (*R*)-serine were blocked by HA-966 (Rao et al., 1990). The reasons for the reported discrepancies in the glycine effect on the NMDA receptor function are not known. However, several explanations have been suggested. For instance, it is known that different NMDA receptors differ in their sensitivity to glycine. The affinity of the receptor from cerebellar neurons is more than four times lower than that from cortical neurons (Priestley and Kemp, 1993), and the affinity of heterooligomeric receptors containing NR1/NR2A is 10 times lower than that of receptors formed by NR1/NR2B or NR1/NR2C (Wafford et al., 1993). In addition, measurements in CSF may not accurately reflect the actual concentration of glycine in the synaptic cleft. As discussed above, the activity of transport systems in presynaptic nerve endings or in adjacent glial cells could maintain microenvironments with low concentrations of glycine (0.2 μ*M*) (Attwell et al., 1993). Moreover, other factors may modulate the glycine affinity in vivo. For instance, extracellular calcium enhances the glycine affinity for NMDA (Gu and Huang, 1994). Thus, it is possible that after intense neuronal activity, when the extracellular calcium is depleted in the synaptic cleft (Wadman et al., 1985), the reduction in glycine affinity could provide a negative feedback mechanism for regulating the NMDA-mediated excitability of the neuron (Gu and Huang, 1994).

If, finally, it ensues that glycine plays a physiological role in the regulation of the NMDA receptor activity, other questions can be raised:

Is glycine released together with glutamate at the synaptic cleft? How could this regulation happen in those regions with high expression of NMDA receptor, but no glycinergic terminals? One possibility, as indicated above, could involve the glycine transporters working in the opposite direction (Attwell et al., 1993). They would release the neurotransmitter to the synaptic cleft in a regulated way, and thus the uptake/release balance could finally modulate the NMDA receptor. As the synaptic terminal depolarizes, the intracellular sodium concentration increases to a level that may be able to reverse the operation of the glycine carrier (Aragón et al., 1988). It is known that depolarization with veratridin or potassium can evoke calcium-independent release of various neurotransmitters from synaptosomal and brain slice preparations (Adam-Vizi, 1992). The carrier-mediated release of amino acid neurotransmitters has been more extensively studied for GABA and glutamate. In retina horizontal cells, the glutamate-induced depolarization release of [^{3}H]GABA is largely independent of extracellular calcium, inhibited in a sodium-free medium, and blocked by the transporter inhibitor nipecotic acid (Schwarz, 1982; Yazulla and Kleinschmidt, 1983; Ayoub and Lam, 1984). Voltage-clamp experiments have proven that the amount of GABA released by the backward operation of the GABA transporter is high enough to stimulate the postsynaptic GABA receptor in the apposed bipolar cell (Schwarz, 1987). Depolarization-evoked release (by glutamate) of GABA by reversal of the transporter has also been shown in cultured neurons (Pin and Bockaert, 1989) as well as in vivo during epileptogenesis (During et al., 1995). Evidence also exists for carrier-mediated release of glutamate in situations of brain anoxia or ischemia (Szatkowski and Attwell, 1994). GABA and glutamate can also be released from glial cells by reverse operation of the carrier (Szatkowski et al., 1990; Gallo et al., 1991). Whether a similar mechanism exists for the release of glycine with physiological relevance in the regulation of the NMDA receptor is unknown. Other regulatory possibilities can be considered; for instance, instead of a release mechanism, the extracellular concentration of glycine could be controlled by regulation of the uptake. As discussed above, the carrier can be inhibited by activation of PKC (Gomeza et al., 1995) and by arachidonic acid (Zafra et al., 1990). Alternatively, the control point could be at the NMDA receptor itself, by changes in the affinity for glycine as a function of neuronal activity (Gu and Huang, 1994).

Independently of the physiological role of the glycine site on the NMDA receptor activity, what is clear at the moment is the potential of this site as a target of pharmacological manipulation, and thus therapeutic modification of the physiological and pathological processes mediated by the NMDA receptor (Kemp and Leeson, 1993).

Conclusions and Perspectives

Biochemical, immunological, electrophysiological, and molecular cloning studies performed during the last decade have contributed to the elucidation of the molecular nature of a variety of proteins involved in inhibitory glycinergic neurotransmission. Among them, the best characterized is the glycine receptor, a glycine-activated chloride channel formed by a pentameric association of various types of subunits belonging to the ligand-gated channel superfamily. The subunit composition of pentamers and, hence, the characteristics of the receptor differ regionally and developmentally. The synaptic action of glycine is terminated by a family of plasma membrane transporters localized in the presynaptic terminal or in the adjacent glial plasma membrane, and that belong to the sodium- and chloride-dependent neurotransmitter transporter family. Despite the important advances in the characterization of all these proteins, some basic aspects of the molecular biology, cell biology, and physiology of glycinergic neurotransmission remain, however, uncertain. For instance, although it is known that gephyrin plays an essential role in the clustering and correct localization of the

receptor in the postsynaptic membrane, nothing is known about the organization of the presynaptic element and the reason why a glycinergic terminal contacts precisely a glycinoceptive postsynaptic element. Equally unclear is the release mechanism, the contribution of synaptic vesicle components, and the role of transporters in this process. Also, the contribution of glycine to the NMDA receptor function requires further clarification.

Nevertheless, all these studies have unraveled an unexpected diversity of glycine receptors and transporters. Challenges, such as the expansion of primary and secondary receptor and transporter structures to the atomic level tertiary structures, a complete description of the active sites of these proteins, and its elements for regulation and interaction with other cellular components, represent promising directions for future research in this field. Advanced knowledge at the molecular level of these structural features will assist the development of novel approaches for studying and therapeutic modification of glycinergic neurotransmission.

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