Glycine transporters: essential regulators of synaptic transmission

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Abstract

Glycine is a major inhibitory neurotransmitter in the mammalian CNS (central nervous system). Glycinergic neurotransmission is terminated by the uptake of glycine into glycinergic nerve terminals and neighbouring glial cells. This uptake process is mediated by specific Na^+/Cl^--dependent GlyTs (glycine transporters), GlyT1 and GlyT2. GlyT1, in addition, is thought to regulate the concentration of glycine at excitatory synapses containing NMDARs (*N*-methyl-D-aspartate receptors), which require glycine as a co-agonist. We have analysed the physiological roles and regulation of GlyT1 and GlyT2 by generating transporter-deficient mice and searching for interacting proteins. Our genetic results indicate that at glycinergic synapses, the glial transporter GlyT1 catalyses the removal of glycine from the synaptic cleft, whereas GlyT2 is required for the re-uptake of glycine into nerve terminals, thereby allowing for neurotransmitter reloading of synaptic vesicles. Both GlyT1 and GlyT2 are essential for CNS function, as revealed by the lethal phenotypes of the respective knockout mice. Mice expressing only a single GlyT1 allele are phenotypically normal but may have enhanced NMDAR function. GlyT2 is highly enriched at glycinergic nerve terminals, and Ca^{2+}-triggered exocytosis and internalization are thought to regulate GlyT2 numbers in the pre-synaptic plasma membrane. We have identified different interacting proteins that may play a role in GlyT2 trafficking and/or pre-synaptic localization.

Introduction

Neurotransmission at chemical synapses proceeds with high spatial resolution and extraordinary speed. At individual synaptic sites, the post-synaptic response to transmitter released from the pre-synaptic nerve terminal lasts only for milliseconds. These rapid kinetics require an effective clearance of transmitter molecules from the synaptic cleft. In the CNS (central nervous system), pre-synaptically released neurotransmitters are removed through both diffusion and transporter-mediated re-uptake into nerve terminals and adjacent glial cells. In the present paper, we report on the *in vivo* functions, disease aspects and putative trafficking mechanisms of the mammalian transporter for the amino acid glycine: GlyT1 (glycine transporter 1) and GlyT2.

In caudal regions of the adult CNS, glycine is a major inhibitory neurotransmitter used by many interneurons [1]. Upon release from glycinergic pre-synaptic terminals, glycine activates strychnine-sensitive post-synaptic GlyRs (glycine receptors) [2]. The agonist-induced opening of these ligand-gated anion channels leads to an influx of chloride ions into the post-synaptic cytoplasm. The resulting hyperpolarization raises the threshold for neuronal firing and thereby inhibits the post-synaptic cell.

Besides serving as an inhibitory neurotransmitter, glycine is also important at excitatory glutamatergic synapses. Here, glycine acts as an essential co-agonist of glutamate at ionotropic NMDARs (*N*-methyl-D-aspartate receptors) [3]. Recent studies have shown that superfusion with 0.5–20 μM glycine causes a potentiation of NMDAR currents in slice preparations [4]. Furthermore, higher concentrations of glycine ($\geqslant$100 μM) have been found to 'prime' NMDARs for internalization triggered by the activating agonist glutamate [5]. In conclusion, glycine has both co-agonist and regulatory functions at NMDAR-containing excitatory synapses.

GlyTs are members of the Na^+/Cl^--dependent transporter family

GlyTs belong to a large family of Na^+/Cl^--dependent transporter proteins that includes transporters for monoamines [5-hydroxytryptamine (serotonin), noradrenaline and dopamine] and γ-aminobutyric acid [6]. These polytopic membrane proteins have 12 transmembrane domains connected by six extracellular and five intracellular loops. The large second extracellular loop connecting transmembrane domains 3 and 4 is multiply N-glycosylated, and the N- and C-termini are located intracellularly. cDNA cloning has identified two GlyT subtypes, GlyT1 and GlyT2, which share approx. 50% amino acid sequence identity but differ in pharmacology and tissue distribution [7]. Both GlyTs exist

Key words: glycine receptor, glycine transporter, neurotransmission, *N*-methyl-D-aspartate receptor, synaptic transmission.

Abbreviations used: CNS, central nervous system; GCS, glycine cleavage system; GlyR, glycine receptor; GlyT, glycine transporter; IPSC, inhibitory post-synaptic current; NMDAR, *N*-methyl-D-aspartate receptor; PKC, protein kinase C.

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in multiple variants: alternate promoter usage generates three GlyT2 isoforms (a, b and c) that differ in their N-terminal regions, whereas alternative splicing of three N-terminal (a, b and c) and two C-terminal (d and e) exons results in different variants of GlyT1. GlyT2 is a larger protein than GlyT1 due to a unique extended N-terminal domain of approx. 200 amino acids.

GlyT-mediated glycine uptake is energetically coupled with the transmembrane sodium gradient maintained by the Na^+/K^+-ATPase. Extracellular binding of glycine together with Na^+ and Cl^- is thought to induce a conformational change that switches the transporters from an 'outward' to an 'inward' facing state [7]. This exposes the glycine-binding site to the cytosol, thereby allowing release of bound glycine and ions. The 'empty' transporter then can return to an outward facing conformation. Roux and Supplisson [8] have shown that the stoichiometry of substrate/ion co-transport is 3 Na^+/Cl^-/glycine for GlyT2, whereas GlyT1 has a transport stoichiometry of 2 Na^+/Cl^-/glycine. This difference in ionic coupling implies that, under physiological conditions, the driving force available for uphill glycine transport by GlyT2 is much higher than that by GlyT1. Thus GlyT2 has a higher capacity for maintaining millimolar intracellular versus submicromolar extracellular glycine levels than GlyT1. In contrast, its lower Na^+ transport stoichiometry facilitates GlyT1 to operate in a reverse uptake mode, e.g. neurotransmitter release from the cytosol into the extracellular space, upon changes in substrate or ion concentration gradients or membrane potential [8]. This is thought to allow for Ca^{2+}-independent glycine release in synaptic regions.

GlyT1 and GlyT2 differ significantly in their expression patterns. The GlyT1 gene is expressed throughout most regions of the CNS [9–11]. Intense GlyT1 immunoreactivity is found in astrocytes and, in addition, in selected neuronal subpopulations, such as some glutamatergic neurons in the hippocampus [9,12]. Furthermore, GlyT1 is also found in non-neuronal tissues, e.g. liver, pancreas and intestine. In contrast, GlyT2 displays an exclusively neuronal expression in CNS regions rich in glycinergic synapses, e.g. spinal cord, brain stem and cerebellum [9,10]. GlyT2 immunoreactivity is seen only in glycinergic neurons, where it is juxtaposed to GlyR-containing post-synaptic specializations [13]. Immunoelectron microscopy indicates that GlyT2 is enriched in the plasma membrane of glycinergic nerve terminals, but excluded from active zones where neurotransmitter release occurs [14].

Knockout mice define GlyT isoform functions at inhibitory synapses

As GlyT2 and GlyR immunoreactivities show punctate co-localization in CNS sections, it was previously thought that GlyT2 is responsible for the clearance of pre-synaptically released glycine at inhibitory synapses. However, due to overlapping expression patterns of GlyT1 and GlyT2 in caudal regions of the CNS, GlyT1 might also contribute. In an attempt to clarify the *in vivo* functional roles of these transporters, we have generated knockout mice deficient in GlyT1 or GlyT2 [15,16]. Both homozygous knockout lines are externally normal at birth; no changes in brain anatomy or the expression of other synaptic proteins could be detected. However, electrophysiological recordings from hypoglossal motoneurons in the brain stem revealed abnormal glycinergic inhibitory currents in the mutant animals. In GlyT1-deficient mice, increased chloride conductances, consistent with a tonic activation of GlyRs by elevated extracellular glycine concentrations, were observed [15]. Furthermore, spontaneous IPSCs (inhibitory post-synaptic currents) had longer decay time constants than those in wild-type mice. Apparently, GlyT1 has a crucial role in lowering extracellular glycine levels at glycinergic synapses. In contrast, glycinergic IPSCs recorded from neurons of GlyT2-deficient mice displayed markedly reduced amplitudes compared with those from wild-type mice [16]. This reflects a reduced glycine content of synaptic vesicles and hence insufficient glycine release. Apparently, GlyT2 is not important for clearing glycine from glycinergic synapses but is required for glycine uptake into the pre-synaptic cytosol and hence efficient refilling of synaptic vesicles with glycine. In summary, GlyT1 and GlyT2 have complementary functions at glycinergic synapses: GlyT1 eliminates glycine from the synaptic cleft and thereby terminates glycine neurotransmission, whereas GlyT2 enhances its efficacy by providing cytosolic glycine for vesicular release [16].

GlyT knockout mice are models of human neurological diseases

The perturbations of glycinergic neurotransmission observed in GlyT-deficient mice have fatal consequences for these animals. Newborn GlyT1 knockout mice die on the day of birth [15]. During the short period of postnatal survival, the mutant pups display severe motosensory deficits characterized by lethargy, hypotonia and hyporesponsivity to tactile stimuli as well as a severe depression of rhythmic breathing. Recordings of neuronal activity in the brain stem circuitry responsible for generating the respiratory rhythm disclosed a slowed and irregular pattern that was normalized upon application of the GlyR antagonist strychnine [15]. Apparently, the loss of GlyT1 results in glycinergic over-inhibition due to the sustained activation of GlyRs in the presence of high levels of extracellular glycine. GlyT2-deficient mice also die postnatally, but death occurs only during the second postnatal week and after developing an acute neuromotor disorder, whose symptoms are entirely different from those seen upon GlyT1 deletion [16]. Here, diminished glycinergic neurotransmission caused by the loss of GlyT2 results in hyperexcitability accompanied by muscular spasticity, impaired motor co-ordination and tremor.

Notably, the symptoms observed in each line of mice are similar to those associated with a group of human hereditary diseases, which develop in early postnatal life or during adolescence. Hyperglycinergic GlyT1 knockout mice present symptoms similar to those of glycine encephalopathy, a

disease often associated with mutations in the mitochondrial GCS (glycine cleavage system) that degrades excess intracellular glycine [17,18]. Hypoglycinergic GlyT2 knockout animals resemble patients suffering from hyperekplexia. This neuromotor disorder results in exaggerated startle responses and, in severe cases, a 'stiff baby syndrome' and has been shown to involve mutations of the $\alpha 1$ and β subunit genes of the GlyR [19]. Notably, in several patients diagnosed with those illnesses, no defects in GCS or GlyR genes have been found [17,20]. This indicates that glycine encephalopathy- and hyperekplexia-like syndromes can be caused through different genetic mechanisms and suggests that some as yet unclassified forms of these diseases may involve mutations in the human GlyT genes.

Modulation of NMDARs by GlyT1

As outlined above, changes in synaptic glycine concentrations alter the activity of NMDARs, which require glycine as an essential co-agonist. The first evidence that GlyT-dependent processes contribute to the regulation of effective glycine levels at NMDAR-containing excitatory synapses came from uptake inhibition studies. Both GlyT1- and GlyT2-specific inhibitors have been found to enlarge NMDAR currents in spinal cord [21]. Similarly, in hippocampal slice preparations, partial inhibition of GlyT1 caused a facilitation of NMDAR responses, resulting in enhanced long-term potentiation [22]. These observations are consistent with GlyT1 activity preventing saturation of the glycine-binding site of synaptic NMDARs. Recent genetic studies corroborate this interpretation. Heterozygous knockout mice carrying only one functional GlyT1 allele are phenotypically normal [15,23,24] although [^{3}H]glycine uptake into brain membrane fractions prepared from such animals is reduced to approx. 50% of that obtained with wild-type samples [15,23]. Notably, in brain slices prepared from the heterozygous animals, NMDAR-evoked post-synaptic currents are significantly larger than in wild-type, and glycine fails to potentiate current amplitudes [23,24]. Furthermore, behavioural studies indicate changes in the pharmacology of pre-pulse inhibition, a desensitization of the acoustic startle response that has been associated with NMDAR function [23]. Together, these findings underline an essential regulatory role of GlyT1 in glutamatergic neurotransmission.

GlyT regulation and interacting proteins

The results presented above show that GlyT1 and GlyT2 have essential functions at both inhibitory and excitatory synapses. Therefore changes in transport activities and/or surface numbers of GlyTs might profoundly affect the efficacy of neurotransmission. Indeed, GlyTs have been found to be regulated through multiple mechanisms. Stimulation of PKC (protein kinase C) by phorbol esters induces an endocytosis of both GlyT1 and GlyT2 [25,26]. However, mutation of all consensus PKC phosphorylation sites in GlyT1 does not abolish this down-regulation [27], suggesting that additional PKC substrates must be involved. Also, rapid membrane depolarization in the presence of extracellular Ca^{2+} has been shown to stimulate the incorporation of GlyT2 into the plasma membrane of spinal cord synaptosomes, whereas conditions favouring sustained Ca^{2+} influx enhance GlyT2 internalization [28]. These and other results indicate that regulatory protein interactions are crucial for GlyT insertion and retrieval from the plasma membrane.

Biochemical and transfection experiments have disclosed physical and functional interactions between GlyTs and the pre-synaptic SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) protein syntaxin 1A. Co-expression of GlyT1 or GlyT2 with syntaxin 1A results in a reduction of GlyT numbers on the cell membrane [29]. In addition, syntaxin 1A has been found to be essential for constitutive and Ca^{2+}-triggered insertion of GlyT2 into, but not its retrieval from, the plasma membrane of neurons [28]. We have employed yeast two-hybrid screening to identify additional binding partners of GlyT2. These include Ulip6, a member of the collapsin response mediator protein family [30] that has been implicated in endocytosis, and the PDZ domain protein syntenin-1 [31]. Interestingly, syntenin-1 also binds syntaxin 1A, revealing a close network of interactions between GlyT2 and these proteins, which may have an important role in the intracellular trafficking and/or pre-synaptic localization of GlyT2 [7].

Conclusions and perspectives

The studies summarized above have provided major insights into the physiological roles of GlyTs in the mammalian CNS. GlyT1 is essential for regulating glycine concentrations at synaptic receptors. At glycinergic synapses, it shortens the duration of the post-synaptic response by lowering glycine concentrations at inhibitory GlyRs, and its inactivation or blockade results in overinhibition. In addition, GlyT1 prevents saturation of the glycine-binding site of NMDARs, thereby allowing for glycine potentiation of excitatory glutamatergic neurotransmission. GlyT2, in contrast, is uniquely designed for neurotransmitter recycling at inhibitory glycinergic synapses, and loss of GlyT2 function generates a severely hyperexcited state.

The clear dichotomy of GlyT subtype functions at central synapses may provide the basis for future therapeutic approaches, which selectively target these membrane proteins. A severe and widespread psychiatric disorder that is thought to involve NMDAR hypofunction is schizophrenia. Schizophrenic patients show enhanced motor activity, cognitive deficits and an increase in stereotyped behaviours. Rather similar symptoms can be induced in healthy humans and rodents by NMDAR blockers such as ketamine and phenylcyclidine [32]. Notably, the effects of these blockers can be reversed upon inhibition of GlyT1 [33]. Therefore it is thought that GlyT1 inhibitors might be beneficial in the therapy of schizophrenic patients [34]. In addition, they may be useful in hyperexcitability syndromes, pain treatment and narcosis, whereas GlyT2 antagonists may be of value for increasing the general muscle tone.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 269 and SPP 1172), European Community (TMR ERBFMRXCT9), Fonds der Chemischen Industrie, Lundbeck Foundation, Novo Nordisk Foundation, Carlsberg Foundation and the Danish Medical Research Council.

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Received 22 August 2005