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# Cloning and expression of a glycine transporter from mouse brain

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We have isolated a cDNA clone from a mouse brain library encoding the glycine transporter (GLYT). *Xenopus* oocytes injected with a synthetic mRNA accumulated [$^3$H]glycine to levels of up to 80-fold above control values. The uptake was specific for glycine and dependent on the presence of $Na^+$ and $Cl^-$ in the medium. The cDNA sequence predicts a highly hydrophobic protein of 633 amino acids with 12 potential transmembrane helices. The predicted amino acid sequence has 40–45% identity to the GABA, noradrenaline, serotonin and dopamine transporters. This implies that all of these neurotransmitter transporters may have evolved from a common ancestral gene that diverged into the GABA, glycine and catecholamine subfamilies at nearly the same time.

Neurotransmitter; Transport; Glycine; Cloning; Expression

## 1. INTRODUCTION

Synaptic transmission involves the release of a transmitter into the synaptic cleft, interaction with a postsynaptic receptor, and subsequent removal of the transmitter from the cleft [1–4]. The majority of the transmitters are removed from the cleft by rapid sodium-dependent uptake systems in the plasma membrane of the presynaptic cells. This re-uptake system is catalyzed by transporters specific for the various neurotransmitters in the brain [5–7]. Amino acids are known to function as neurotransmitters in the brain. The most important amino acids in this respect are glutamic acid acting as a stimulatory neurotransmitter and glycine which functions as an inhibitory neurotransmitter. A glycoprotein exhibiting sodium and chloride-dependent glycine transport activity has been identified and purified from brain synaptosomes [8–10]. Recently the purified transporter was reconstituted into phospholipid vesicles that exhibited sodium and chloride-dependent glycine transport activity [10].

The cDNA encoding the GABA transporter was cloned, sequenced and expressed in *Xenopus* oocytes [11,12]. Subsequently, other cDNAs encoding neurotransmitter transporters of the catecholamine subfamily were cloned and expressed [13–17]. They exhibited significant sequence homology with the GABA transporter and were highly homologous to each other. In this work we report on the cloning and expression of the glycine transporter from mouse brain. Its sequence suggests that this transporter represents a distinct subfamily of transporters related to the other two known subfamilies of GABA and catecholamine transporters.

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## 2. EXPERIMENTAL

### 2.1. *Screening the brain cDNA library and sequencing*

A mouse brain λZAP cDNA library (Stratagene) was screened under low stringency with $^{32}$P-labeled cDNA encoding a catecholamine transporter of bovine adrenal medulla ([18] and unpublished). The pBluescript was excised from the positive phages and analyzed by dot blot and Southern hybridizations [19]. Synthetic RNA was produced from one of the clones and was identified as a glycine transporter by expression in *Xenopus* oocytes as described previously [11]. The cDNA of this clone was sequenced by the dideoxy chain termination method following serial deletions by exonuclease III [20,21]. Further sequencing for verification was performed with synthetic oligonucleotide primers. Sequences were aligned and analyzed by DNAstar and GCG software packages.

### 2.2. *Northern blot analysis*

Mouse brain was dissected to its various parts and RNA was prepared from the different brain parts as well as from whole brain and liver as previously described [19]. About 10 μg of total RNA (5 μg from the spinal cord) were applied onto an RNA denaturing agarose gel, and following electrophoresis and transfer onto a nylon filter was probed by nick translated $^{32}$P-labeled GLYT cDNA. The filter was washed at 65°C as previously described [19].

### 2.3. *Expression in* Xenopus *oocytes*

The synthetic RNA was obtained by transcribing the pBluescript containing the GLYT cDNA with an RNA synthesis and capping kit from Stratagene. Oocytes were surgically removed from frogs and defolliculated by collagenase treatment. After recovery for 24 h the oocytes were injected with 50 nl medium containing 2–10 ng synthetic RNA. After two or three days they were assayed for glycine transport. Prior to uptake assay the oocytes were preincubated for 15 min in 1 ml solution containing 100 mM KCl and 10 mM HEPES (pH 7.5). The transport reaction was initiated by the addition of a solution containing 100 mM NaCl, 10 mM HEPES (pH 7.5), about 0.1 μCi of [$^3$H]glycine and the specified amounts of cold glycine. At the end of a 45-min incubation period, the oocytes were washed three times with 1.5 ml of the same buffer in which the radioactive glycine was ex-


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cluded. Individual oocytes were solubilized in 50 μl of 10% SDS, and the radioactivity was measured by scintillation counting. Five oocytes were used for each experimental point and the data are expressed as the average uptake per hour.

## 3. RESULTS

The clone encoding the glycine transporter was isolated from a mouse brain cDNA library by hybridization with $^{32}$P-labeled cDNA encoding a catecholamine transporter cloned from a bovine adrenal library ([18] and unpublished data). Over forty clones were collected and one was determined to contain the glycine transporter by expression in *Xenopus* oocytes. The clone contains a cDNA insert of 3.2 kb with an open reading frame of 1,899 bp. The region of the first ATG resembles the consensus sequence described by Kozak [22] and was assigned as the putative translation initiation site. The sequence predicts a protein of 633 amino acids with a molecular mass of 70,785 (71 kDa) that is somewhat larger than the GABA and catecholamine transporters [11–17]. Hydrophobicity analysis is superimposable with that of the other transporters, and reveals 12 potential hydrophobic segments that could form transmembrane domains. Therefore, the tentative structural model of the glycine transporter is similar to the proposed membrane topologies of the GABA and catecholamine transporters [11,13]. The external loop after the third transmembrane helix contains 9 and 20 more amino acids than the noradrenaline and GABA transporters, respectively (Fig. 1). It also contains an additional putative glycosylation site to those of the GABA and noradrenaline transporters.

The amino acid sequence of the glycine transporter is 45, 43, 40 and 42% identical to the respective amino acid sequences of the GABA, noradrenaline, dopamine and serotonin transporters [11–17]. The three catecholamine transporters are highly homologous among themselves. Therefore it is likely that the glycine, GABA and catecholamine subfamilies of transporters


Fig. 1. The amino acid sequence of the mouse glycine transporter aligned with the human GABA and noradrenaline transporters. The amino acid sequence of the mouse glycine transporter (GLYT) was deduced from the cDNA sequence of the cloned gene (to be disposed in GeneBank). The amino acid sequences of the human GABA transporter (GABAT) [12] and noradrenaline transporter (NET) [13] were previously published. Identical amino acids are indicated by (*) and conservative amino acid replacements by (:). Putative transmembrane domains are underlined.

branched out from a common ancestral gene at approximately the same time. The sequence comparisons of these three subfamilies also open up a new avenue of research toward the definition of the various binding sites in the different transporters which may lead to a better understanding of their mechanism of action.

*Xenopus* oocytes injected with GLYT synthetic RNA accumulated up to 80 times as much glycine as water-injected oocytes, uninjected oocytes, or oocytes injected with GABA-transporter RNA. Transport of other amino acids such as alanine, serine, threonine, proline, tyrosine, valine, isoleucine, phenylalanine, aspartic acid, glutamic acid and GABA could not be detected and cold amino acids (except for glycine) or betaine at 100 $\mu$M concentration did not inhibit glycine transport. Sarcosine inhibited glycine uptake with a $K_i$ of about 50 $\mu$M (not shown). Fig. 2 shows the rate of [$^3$H]glycine uptake into *Xenopus* oocytes injected with a synthetic mRNA of the glycine transporter at various glycine concentrations. The Eadie–Hofstee plot revealed a Michaelis constant ($K_m$) of 25 $\mu$M, and two other experiments gave $K_m$ values of 18 and 20 $\mu$M. The mean value of about 20 $\mu$M is within the range of values reported for the affinity of glycine uptake in brain [10]. Thus the affinity of the brain glycine transporter is about 80-fold higher than the liver transporter and exhibits a much higher specificity to glycine [23]. It was reported that glycine transport in brain slices and in solubilized and reconstituted membrane vesicles is dependent on a $Na^+$ gradient and the presence of chloride in the external medium [8–10]. In the GLYT-RNA injected oocytes the glycine uptake was absolutely dependent on the electrochemical gradient of sodium (Fig. 3). Potassium, lithium and choline were inactive as cation replacement. Glycine uptake was also dependent on the presence of specific anions. Chloride was the most effective and gluconate was less than 5% as effective as chloride. On the other hand, nitrate supported about 25% of the activity obtained with chloride. Glycine uptake at various chloride concentrations revealed that about 23 mM of the anion were required for half maximal activity of the transporter (Fig. 4).

In contrast with the other transporters little is known of the location and pharmacology of the glycine transporter. Due to its high affinity to strychnine, the strychnine-sensitive glycine receptor provided the bulk of the data on neurotransmission by glycine [24–26]. Northern blot with cDNA encoding the strychnine binding subunit of the glycine receptor revealed its presence in the spinal cord and brainstem but not in the cerebellum [27]. Northern blot analysis of RNA from different parts of the mouse brain with labeled cDNA encoding the glycine transporter gave strong signals with cerebellum, medulla, pons, spinal cord, and weak signals with frontal cortex and whole brain (Fig. 5). A related transcript was also identified in the kidney and to a lesser extent in the liver (not shown). There is an apparent discrepancy between the absence of the glycine receptor

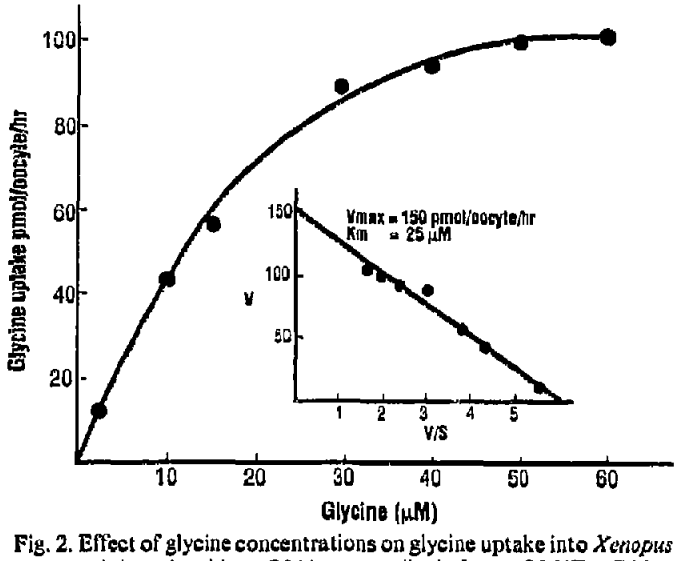


Fig. 2. Effect of glycine concentrations on glycine uptake into *Xenopus* oocytes injected with mRNA transcribed from GLYT cDNA. [$^3$H]Glycine uptake was assayed as described in section 2, with mRNA-injected and -uninjected oocytes at room temperature for 45 min. The values obtained with uninjected oocytes were subtracted from the corresponding injected samples. Eadie–Hofstee analysis is described in the insert of the figure.

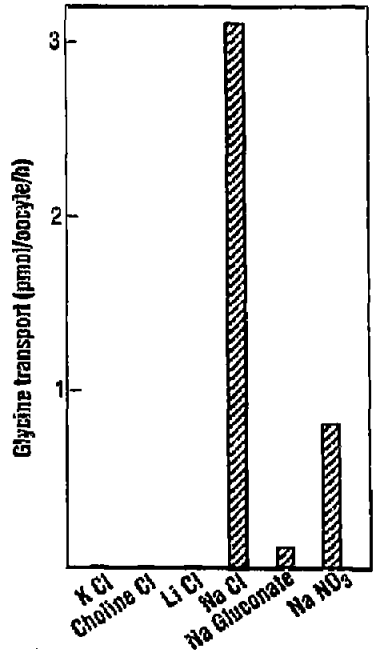


Fig. 3. Effect of various anions and cations on glycine uptake by GLYT mRNA-injected *Xenopus* oocytes. Oocytes were prepared and assayed as described in the legend to Fig. 2 except that the assay buffer was changed according to the different ions tested. For the assay of cations NaCl was replaced by 100 mM of KCl, LiCl or choline chloride during the uptake period. During the preincubation, for the various anions, the 100 mM KCl was substituted with 100 mM potassium gluconate, and for the uptake medium the NaCl was replaced by sodium gluconate or sodium nitrate. The glycine concentration was 2 $\mu$M in all the assays.

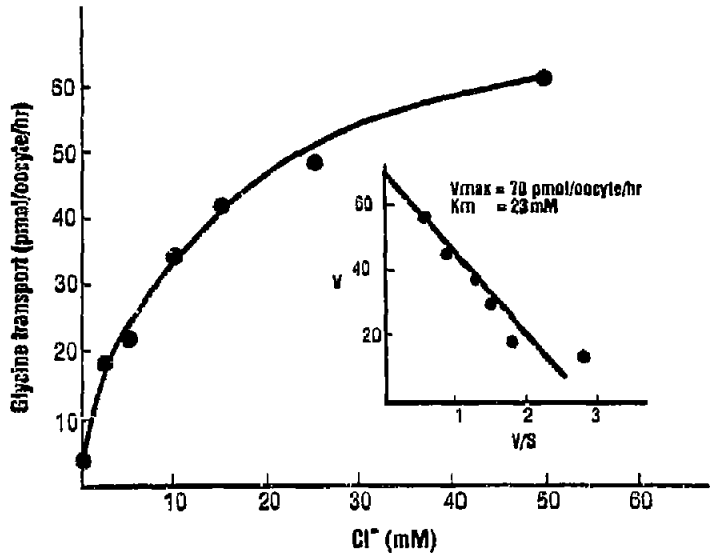


Fig. 4. Effect of chloride concentrations on glycine uptake by GLYT mRNA-injected *Xenopus* oocytes. Oocytes were prepared and assayed as described in the legend to Fig. 2 except that 100 mM sodium gluconate replaced the NaCl. The indicated amounts of NaCl were added to the assay displacing equivalent amounts of sodium gluconate. The glycine concentration in the assay was kept at 10 $\mu$M.

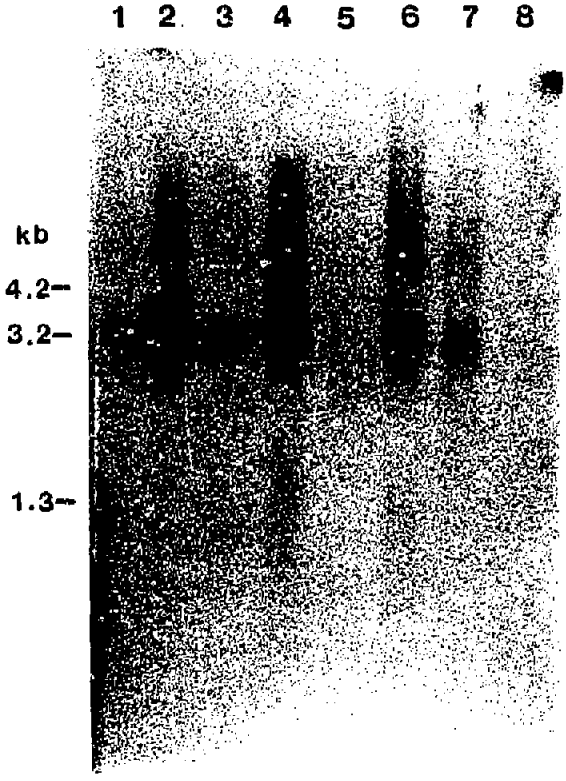


Fig. 5. Localization of glycine transporter mRNA in various tissues. Northern hybridization was performed as described in section 2. The size of the hybridized RNA was determined by second hybridization with labeled cDNA of GABA transporter (4.2 kb) [11,12], the bovine adrenal proteolipid [12,40] and molecular weight standards. The source of the RNA was: (1) whole brain, (2) cerebellum, (3) frontal cortex, (4) medulla, (5) thalamus, (6) pons, (7) spinal cord, (8) liver.

in the cerebellum and the strong signal obtained with the glycine transporter cDNA. This can be due to the transporter functioning in the cerebellum in another system that does not involve the strychnine sensitive glycine receptor. Recently, a new role for glycine in allosteric regulation of the NMDA receptor has been proposed [28]. This will require a glycine uptake system in brain parts that do not contain the strychnine sensitive glycine receptor. Alternatively the cDNA of the glycine transporter may hybridize with a closely related transporter (e.g. the glutamate transporter) that could be present in high amounts in the cerebellum. This obstacle of Northern hybridization applies to all other Northern analyses performed with the other neurotransmitter transporters.

## 4. DISCUSSION

Neurotransmitter transporters are a vital part of brain neurotransmission. They are present not only in the presynaptic terminals but also in glial cells where they function in the removal of the transmitters that escaped the synaptic clefts [29,30]. However, every transporter is highly specific to the neurotransmitter it transports across the membrane. The cloning of the cDNA encoding the GABA transporter [11,12], and later the catecholamine transporters [13] have defined a novel family of membrane transporters. The cloning of the glycine transporter reported in this work extended the family to amino acid neurotransmitters. Only recently cDNAs encoding amino acid transporters

in mammalian cells were cloned and sequenced [31–33]. Analysis of the transport activity of an ectopic mouse retrovirus receptor revealed that the original activity of the protein was a transport of cationic amino acids across plasma membranes [31,32]. This transporter contained 14 putative transmembrane helices and may be related to arginine and histidine permeases of *Saccharomyces cerevisiae* [34,35]. Very recently the first neutral amino acid transporter was cloned from a rat kidney cDNA library [33]. It encodes a protein of about 70 kDa with a potential 4 transmembrane helices. Expression in *Xenopus* oocytes revealed that it transports a variety of neutral amino acids.

The glycine transporter is highly specific for glycine and will not transport any other amino acid. Even alanine that is structurally closest to glycine is not taken up by GLYT mRNA-injected oocytes. Moreover, all the amino acids tested did not inhibit glycine transport into the mRNA-injected oocytes. Recently we cloned more than ten genomic and cDNA clones encoding proteins of the neurotransmitter–transporter gene family. We estimate that there are more than fifty different transporters functioning in mammalian brain. A search in the GenBank also revealed two related genes of *Drosophila melanogaster* and *C. elegance*. Therefore the family of neurotransmitter transporter genes is rooted in the early development of the nervous system and its specificity may define specific brain functions.

The cloning and expression of a glycine transporter may provide an opportunity to look for specific pharmacological substances affecting the glycine re-uptake system. Glycine receptors were implicated in the pathogenesis of spasticity [36,37] and the loss of motor control associated with different human diseases [38,39]. The study of a cloned glycine transporter may complement the search for a cure of these diseases.

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