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Alternative splicing generates two isoforms of the α2 subunit of the inhibitory glycine receptor

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The inhibitory glycine receptor (GlyR) is a ligand-gated chloride channel protein which displays developmental heterogeneity in the mammalian central nervous system. Here we describe 2 novel cDNA variants of the rat GlyR α2 subunit and demonstrate that alternative splicing generates these 2 isoforms. The deduced protein sequences (α2A and α2B) exhibit 99% identity with the previously characterized human α2 subunit. In situ hybridization revealed expression of both α2A and α2B mRNAs in the prenatal rat brain, suggesting that these variant proteins may have a role in synaptogenesis. Heterologous expression in *Xenopus* oocytes showed that the more abundantly expressed α2A subunit forms strychnine-sensitive ion channels which resemble human α2 subunit GlyRs in their electrophysiological properties.

Glycine receptor; Alternative splicing; Receptor heterogeneity; Brain development

1. INTRODUCTION

The inhibitory glycine receptor (GlyR) is a ligand-gated chloride channel which antagonizes depolarization of the postsynaptic membrane in spinal cord and other regions of the vertebrate central nervous system [1]. Sedimentation and crosslinking experiments have revealed a pentameric structure of this membrane protein which is assembled from ligand binding α subunits of 48 kDa and homologous β polypeptides of 58 kDa [2–6]. The ensemble of these subunits forms an anion selective membrane channel which opens upon binding of the agonistic amino acids glycine, taurine or β-alanine [7].

Immunological and molecular cloning data have disclosed heterogeneity of GlyR α subunits during development [8–10]. In spinal cord of adult rats, α1 and α3 subunit genes are expressed [5,9] whereas α2* mRNA is predominantly found in newborn animals [10]. In addition to this heterogeneity resulting from different GlyR α subunit genes, a splice variant of the α1 subunit has been described which originates from alternative splice acceptor site selection, thus producing a novel potential phosphorylation site within the predicted cytoplasmic loop region of this protein [11]. Here we report the characterization of 2 rat α subunit cDNAs which are highly homologous to the previously described human α2 [12] and rat α2* [10] sequences. These α2 variants are generated by alternative splicing of 2 homologous exons, and differ in the primary structure of their extracellular domains. In situ hybridization with radiolabelled probes specific for α2A and α2B showed that these 2 isoforms are expressed during early brain development.

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2. MATERIALS AND METHODS

2.1. *RNA isolation*

RNA was isolated from rat spinal cord and cortex using the method of Cathala [13]. Poly(A)$^+$ RNA was enriched by chromatography on oligo(dT) cellulose.

2.2. *Construction and screening of cDNA libraries*

Two cDNA libraries were constructed from mRNA isolated from brain and spinal cord of newborn (P0) rats. The brain library was obtained by cloning size-fractionated cDNA into the lambda phage vector lambda gt10 while cDNA synthesized from spinal cord poly(A)$^+$ RNA was directionally cloned into lambda ZAP II according to the protocols supplied with cDNA cloning systems from Pharmacia and Stratagene. Size fractionation of cDNAs was achieved either by gel filtration on a Sepharose CL 4B column (brain cDNA), or by separating the cDNA in an agarose gel followed by electroelution (spinal cord cDNA). From the spinal cord cDNA, 3 independent sublibraries were constructed which contained insert sizes of 6–10 kb (I), 2–6 kb (II) and 0.5–2 kb (III), respectively.

2.3. *Isolation and sequencing of GlyR cDNAs*

A ^{32}P-labelled *Xho*I fragment (1794 bp) of the rat GlyR α2* cDNA [12] was used to screen about 2×10^5 plaque forming units each, of the

lambda gt10 P0 rat brain cDNA library, the spinal cord P0 library II, and an adult rat brain library [14]. Screening was performed using stringent conditions for hybridization (50% formamide, 5 × SET, 42°C) and washing (0.2 × SSC, 0.1% (w/v) SDS, 60°C) of screening filters [9]. After exposure to Kodak X-Omat films we identified one positive clone in the P0 brain library (clone α2A-I), 2 in the spinal cord library (clones α2AII and α2AIII), and one in the adult brain library (clone α2B). After plaque purification, the *Eco*RI fragments of these clones were recloned into a Bluescript vector (Stratagene) or excised from the lambda vector ZAPII using helper phage functions, resulting in clones pGRα2I, II, III and pGRα2B. The nucleotide sequences of these clones were determined for both strands with the chain termination method [15] using specific primers.

2.4. *Isolation and characterization of genomic fragments of the α2 gene from mouse*

A mouse genomic library in lambda EMBL3 (a kind gift from Dr K. Chowdhury, Max-Planck-Institut, Göttingen, FRG) was screened with the radiolabelled 830 bp *Pvu*II fragment of clone huB1 [12], which contains most of the coding sequence for the extracellular region of the human GlyR α2 subunit. Hybridization was performed in 5 × SSPE at 60°C, and filters were washed twice for 20 min in 5 × SSPE at 60°C. From a number of positive clones, one was selected by hybridization with a ^{32}P-labelled oligonucleotide probe corresponding to positions 788–807 of the rat α2A cDNA sequences. Within several subcloned DNA fragments, a *Hin*dIII-*Eco*RI restriction fragment of 2 kb was identified, which hybridized with oligonucleotides specific for α2A and α2B. Using these oligonucleotides and a T7 primer, about 550 bp of the DNA sequence of this genomic fragment was determined.

2.5. *In situ hybridization*

Preparation of sections from rat brain and in situ hybridization procedures were as described [16]. Specific antisense oligonucleotides used were for α2A: 5′-GTGGTTTCTGTGACCGATCCAAAAC-TGTTGATAAAAATATTGCAA-3′ and for α2B: 5′-GTAGTTT-CTGCTATTGACCCAAAGCTGTTGATAAATATGTTGCAG-3′.

3. RESULTS

To further investigate the developmental and regional heterogeneity of GlyR α subunits, we constructed and screened cDNA libraries of spinal cord and brain of newborn and adult rats with an α2* cDNA probe. This approach led to the isolation of several overlapping cDNAs encoding 2 isoforms of the rat α2 GlyR subunit. Three of the clones isolated contained *Eco*RI fragments spanning a sequence of about 3.2 kb. This sequence codes for a putative polypeptide of 452 amino acids (Fig. 1) which displays about 99% amino acid sequence identity with the human α2 subunit [12] and differs from the previously identified α2* subunit [10] at only a single amino acid position (pos. 167).

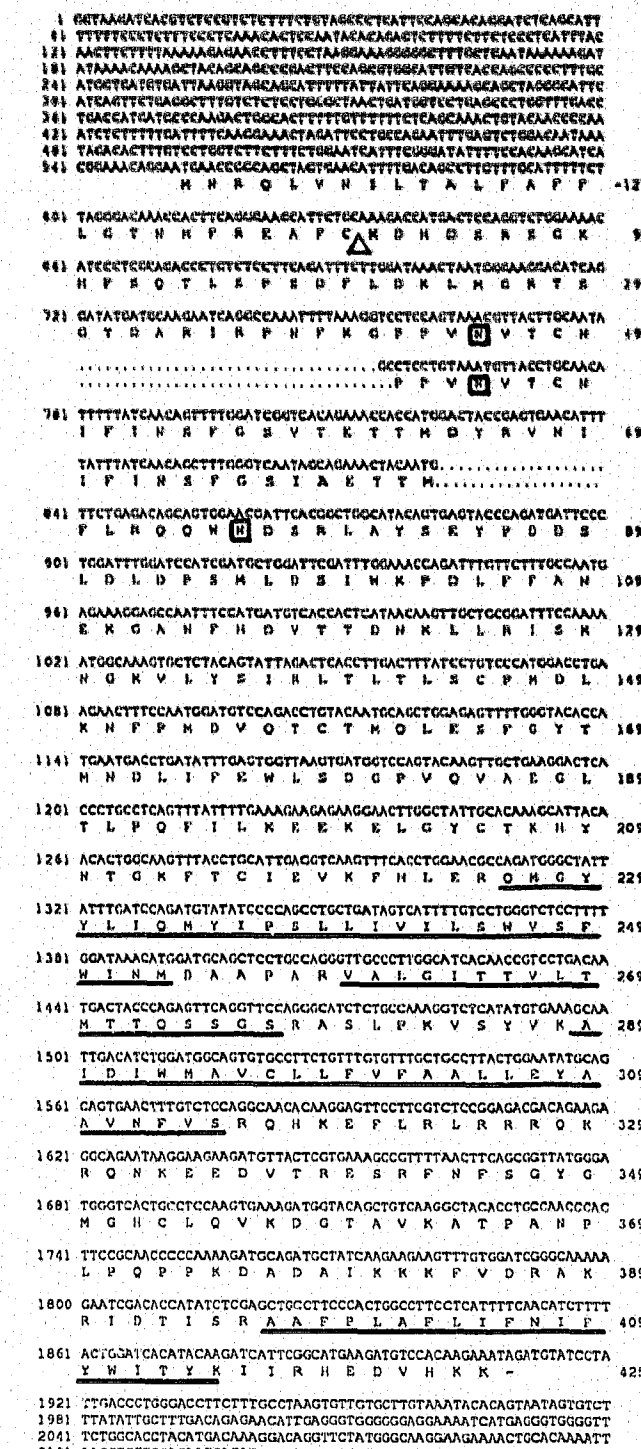


Fig. 1. Nucleotide and deduced amino acid sequences of the GlyR α2A and α2B subunit variants from rat. The complete sequence is shown for α2A, and only the divergent region for α2B (the α2B cDNA spans nucleotides 662–2235 of clone α2A). Deduced amino acid residues are indicated in the single letter code below the nucleotide sequences. The putative signal peptidase cleavage site is marked by an arrow. Proposed transmembrane spanning regions M1 to M4 are underlined, and putative extracellular *N*-glycosylation sites are boxed. An asterisk denotes the 3′-terminal stop codon. Numbering of nucleotides is indicated on the left and of amino acid residues on the right side of the figure.

Moreover, this cDNA contained a 5'-nontranslated region of 551 bp and a 3'-nontranslated region of 1250 bp. At positions 3143-3148, a putative polyadenylation signal AATAAA was found, indicating that this cDNA (α2A) might be almost complete at its 3'end.

Another hybridization-positive clone (α2B) was isolated from a rat brain cDNA library [14] whose sequence proved to be identical to the above-mentioned clones with the exception of a continuous short sequence region, where identity dropped to about 79%. Out of 22 amino acid positions encoded by this diverging part of the DNA sequence of α2B, only 2 residues were different between both variants. In α2B, an isoleucine is found at position 58 and an alanine at position 59, instead of valine and threonine residues, respectively, in the rat α2A and α2* [10] and human α2 [13] proteins. Interestingly, the same isoleucine and alanine residues are conserved at the corresponding positions of the rat α1 [6] and α3 [9] subunits.

To investigate whether the difference between the sequenced cDNAs originates from an alternative splicing event, we isolated the corresponding region from the mouse genome. An 2 kb *Eco*RI-*Hind*III fragment subcloned from a recombinant lambda phage was found to contain 2 variants of the putative exon 3 (Y. Maulet and B. Matzenbach, unpublished data) of the α2 gene, which were identical to the sequences found in the rat cDNA clones α2A and α2B (Fig. 2). These variant exons are separated by a short intervening sequence of 91 bp, and both are surrounded by proper splice acceptor and donor sequences (Fig. 2).

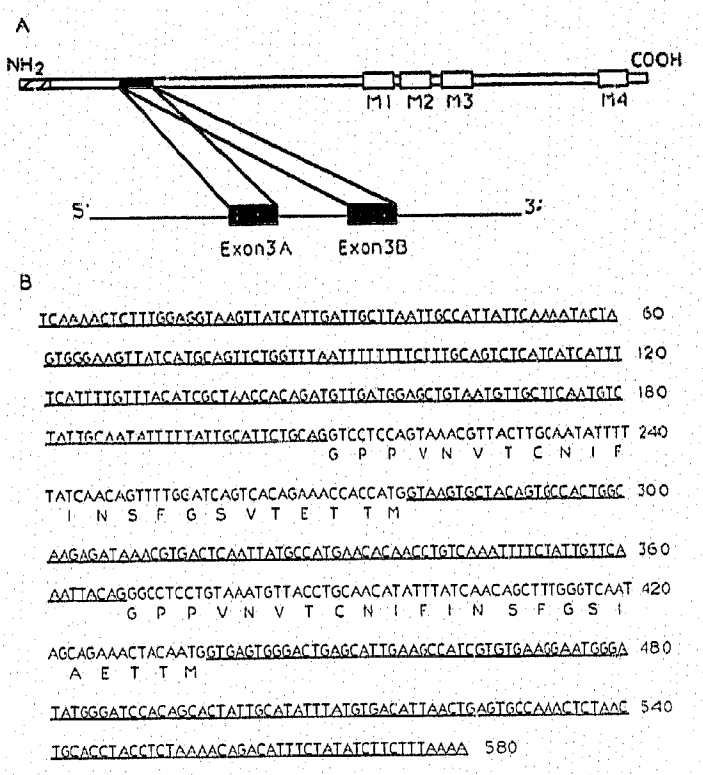
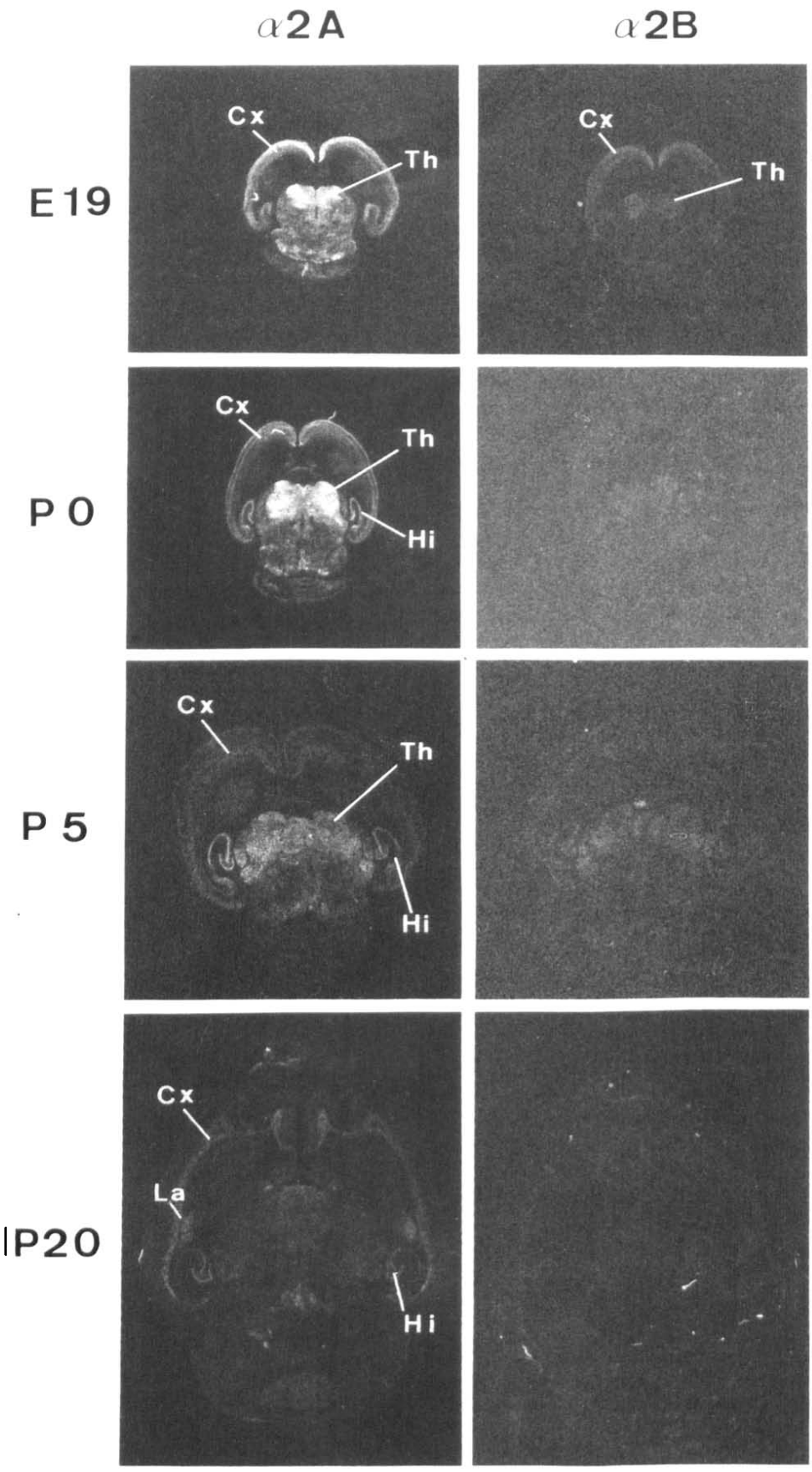


Fig. 2. (A) Schematic diagram of the position of exon 3 encoded amino acid residues in the primary structure of the GlyR polypeptide. Residues encoded by exons 3A or 3B are indicated by a black box; M1 to M4 shows positions of putative transmembrane regions of the GlyR. The signal peptide sequence is indicated by hatches. (B) Nucleotide sequence of the two exon 3 variants of the mouse α2 gene. Exonic regions are indicated by the encoded amino acid sequences, and intronic sequences are underlined.

Two 45 bp antisense oligonucleotide probes specific for exons 3A and 3B were used to perform in situ hybridization experiments. Rat brain sections of different developmental stages were incubated with the radiolabelled oligonucleotides, and hybridizing RNA sequences located by autoradiography. These experiments demonstrated the expression of the α2A sequence in various higher brain regions during pre- and postnatal development (Fig. 3). Unexpectedly, α2A mRNA was strongly expressed in cortex, thalamus and hippocampus at embryonic day 19 (E19) and at birth (P0). Expression of α2B transcripts was also mainly seen at prenatal day E19. At this stage, the regional distribution of both sequences appeared to be quite similar, although hybridization signals were significantly lower for the α2B mRNA. At later postnatal stages, α2A expression was clearly decreasing and α2B transcripts were barely detectable, although a low basal level of expression may still persist (Fig. 3). To investigate the pharmacological properties of the rat GlyR α2 polypeptide, in vitro synthesized α2A RNA was injected into *Xenopus* oocytes. After 2-5 days, the cells were analyzed for glycine responses by electrophysiology in the voltage clamp mode. Membrane currents were seen at glycine concentrations $< 100\ \mu$M, with half-maximal responses being obtained at about 250 μM, a value similar to those required for gating human α2 and mutated rat α2* GlyR proteins (data not shown; see refs [10,12]. Strychnine blocks the opening of channels formed by the rat and human α1 and the human α2 subunits at concentrations of 20-100 nM [10,12,17]. A similar inhibition of chloride currents was observed here upon expression of α2A mRNA in the oocyte system indicating that the α2A polypeptide forms strychnine-sensitive GlyR channels (data not shown).

4. DISCUSSION

The identification of 2 variants of the GlyR α2 subunit resulting from alternative exon selection corroborates alternative splicing as an important mechanism to generate GlyR heterogeneity. The conservation of splicing pathways in mouse and rat demonstrated in this paper suggests a crucial role of minor sequence variations in GlyR function. Interestingly, the novel exon 3B of the α2 gene identified here codes for amino acid residues which are conserved in rat α1 [5] and α3 [9] subunits at identical positions, thus supporting further the evolutionary conservation


α2A
α2B
E19
P 0
P 5
P20
Cx
Th
Hi
La

of this subunit domain. Comparison of our data with alternative splicing events reported for other ligand gated ion channel proteins [18-21] raises the question whether similar exon variations might exist in GlyR α1 and α3 subunit genes. Indeed, alternative splicing cassettes have recently been shown to be conserved in the kainate/AMPA class of glutamate receptors and found to determine the functional properties of the resulting channels [22]. If so, alternative exon usage may constitute a general mechanism to provide plasticity of synaptic connections, a function which probably evolved before subunit diversification via gene duplication.

In a previous report, we have analyzed a GlyR subunit sequence termed α2* which is highly expressed in the spinal cord of newborn, but not adult, rats [10]. As shown above, α2* and α2A cDNA sequences are identical except for an A to G substitution at position 1132, which results in a glycine residue at position 167 in the α2A protein instead of a glutamate in the α2* polypeptide. Besides, a further nucleotide exchange was found in the 5'nontranslated region (pos. 371 G/C). Thus, the PCR amplification experiments in our earlier studies [10] did not distinguish between α2A, α2B and α2* subunits, and all these α2 sequences might indeed be expressed at early stages of spinal cord development. This assumption is supported by in situ hybridization experiments with α2A and α2B specific oligonucleotides on spinal cord sections which produced clear hybridisation signals for both probes at early postnatal stages (M.L. Malosio, unpublished data). As demonstrated previously by site-directed mutagenesis, the glutamate residue at position 167 of the α2* subunit causes a loss of a strychnine sensitivity of the receptor upon expression in *Xenopus* oocytes [10]. We therefore had proposed that the α2* sequence might correspond to the ligand-binding subunit of the biochemically characterized neonatal GlyR, which displays only low affinity binding of the antagonist strychnine [8]. As the sequences of the α2A and α2* cDNAs are identical besides 2 nucleotide substitutions in the 5'nontranslated and the coding regions, it seems unlikely that 2 genes of nearly identical sequence exist in the rat genome. We therefore speculate that α2* and α2 mRNAs may represent allelic variations of the same α2 subunit gene. This would be one possible explanation for the isolation of α2* and α2A cDNAs, which now is under investigation in our laboratory.

The in situ hybridization experiments with brain sections from pre- and postnatal rats performed here revealed strong signals in higher brain regions. Cortical layers in forebrain were labelled by both the α2A and α2B probes already at E19. During further development, α2B transcripts appeared to be down-regulated, whereas α2A gene expression remained well detectable beyond early postnatal stages and was still seen in the hippocampus and thalamus of P20 rats. Thus, the α2A and α2B subunit variants may have different roles during synapse formation in the mammalian brain.

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Fig. 3. In situ hybridization of GlyR subunit mRNAs in horizontal sections of rat brain using antisense oligonucleotide probes specific for α2A and α2B GlyRs. Hybridizations to sections from a late prenatal (E19) and 3 postnatal (P0, P5 and P20) stages are shown. Cx, cortex; Hi, hippocampus; La, lateral amygdaloid nucleus; Th, thalamus. Exposure on X-Omat AR film was for 8 weeks.

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