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Glycine receptor subunits expression in the developing rat retina

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ABSTRACT

Background and methods: Glycine receptor (GlyR) consists of two α (1–4) and three β subunits. Considerable evidence indicates that the adult retina expresses the four types of α subunits; however, the proportion of these subunits in adult and immature retina is almost unknown. In this report we have studied mRNA and the protein expression of GlyR subunits in the retina during postnatal rat development by Real-Time qRT-PCR and western blot.
Results: mRNA and protein expression indicated a gradual increase of the α1, α3, α4 and β GlyR subunits during postnatal ages tested. The mRNA β subunit showed higher expression levels (~3 fold) than those observed for the α1 and α3 subunits. Very interestingly, the α2 GlyR subunit had the highest expression in the retina, even in the adult.
Conclusions: These results revealed the expression of GlyR at early postnatal ages, supporting its role in retina development. In addition, our results indicated that the adult retina expressed a high proportion of the α2 subunit, suggesting the expression of monomeric and/or heteromeric receptors. A variety of studies are needed to further characterize the role of the specific subunits in both adult and immature retina.

1. Introduction

Glycine action is mediated by its postsynaptic receptor (GlyR), which belongs to the Cys-loop family of ionotropic receptors. GlyRs are formed by α (α1- α4) and β subunits, and they are present mainly as an heteromer formed by two α and three β subunits (Grudzinska et al., 2005), which interact with the membrane through the protein gephyrin (Kneussel and Betz, 2000). In brain and spinal cord, there are well established studies regarding an enrichment of the α2 and α4 mRNA expression at early stages of development with low levels of the α1 and α3 GlyR isoforms (Malosio et al., 1991; Harvey et al., 2000). Interestingly, the opposite expression pattern occurs in the mature structures where very low levels of the α2 and α4 transcripts are detected, and the expression of the α1 transcript predominates (Malosio et al., 1991).

In the adult mammalian retina, glycine action is mainly restricted to the inner plexiform layer (IPL), and immunohistochemical studies have revealed the presence of all α (1–4) subunits (Grünert, 2000; Haverkamp et al., 2003, 2004; Heinze et al., 2007; Wässle et al., 2009). Interestingly, the presence of glycine (Fletcher and Kalloniatis, 1997) and their transporters (Glyt1 and Glyt2) have been reported at early retinal postnatal ages (Salceda, 2006), before synapses formation. Besides, there is evidence that signaling by GlyR α2 plays a role in retinal photoreceptor cells development (Young and Cepko, 2004). In spite of these studies, scarce information exists for the expression of GlyR subunits during retina development. Therefore, we analyzed the expression of α1-4 and β subunits during postnatal development of the rat retina by

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quantitative real–time PCR and western blot.

2. Experimental procedures

2.1. Experimental animals

Experiments were performed with postnatal 0–30 day old animals (P0–P30) and adult (P60-70) Long-Evans rats. The animals were maintained under normal dark-light conditions (12:12 h) and allowed free access to food and water. All animals were handled according to the Mexican Institutes of Health Research (DOF. NOM-062-Z00-1999) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 80–23, revised 1996). The protocol was approved by the Institutional Committee for the Care and Use of Laboratory Animals which belongs to the Institute of Cellular Physiology of the Autonomous National University of Mexico (CICUAL Comité Institucional para el Cuidado y Uso de los Animales de Laboratorio del Instituto de Fisiología Celular de la Universidad Nacional Autónoma de México) Protocol Number RSS18-14; this was done in accordance to the Office of Laboratory Animal Welfare (OLAW), Assurance Number A5281-01.

All efforts were made to minimize animal suffering, and to reduce the number of rats used.

2.2. Quantitative real-time PCR

RNA extraction from one rat retina per experiment was carried out with TRIZOL® and incubated at room temperature (RT) for 5 min. Then, 200 μl chloroform were added and the sample was vigorously shaken, incubated at 4 °C for 15 min, and centrifuged at 12,000 × g (4 °C, 15 min). Total RNA was precipitated in the presence of 1–2 isopropanol (v/v) by incubating at RT for 30 min and centrifugating at 12,000 × g for 10 min. The resulting pellet was washed three times with 75% ethanol and the ethanol residues were removed by evaporation. Purified RNA was suspended in 50 μl nuclease-free water (Cleaver Scientific). Concentration and purity of RNA were evaluated in the NanoDrop Spectrophotometer (Thermo Scientific).

cDNA was synthesized with the Revert Aid First Strand cDNA Synthesis kit (Thermo Scientific). cDNA amplification was performed in the presence of the SYBR Green Master Mix from Applied Biosystems (Foster City, CA, USA), in the StepOne PCR system (Applied Biosystems, Foster City, CA, USA). The PCR conditions were as follows: Holding step was 50 °C (2 min) and 95 °C (2min); Cycling was 40 cycles at 95 °C (15 s) and melting temperature (TM) of primers (1 min), followed by a melting curve to test the specificity of the reactions. The relationship between the Ct (cycle threshold) and mRNA levels was determined by calibration curves with sequential dilutions of known cDNA concentration per each gene. The initial concentration for each gene at each developmental age was calculated by plotting Ct vs. the log concentration of cDNA. Also, PCR efficiency needed for the relative quantification by comparative Pfaffl method (Pfaffl, 2001) was calculated from the plotted curves. For each GlyR gene, the mean of three reactions was used to calculate the corresponding expression level. The negative controls, 'no reverse transcription' and 'no template', were included with samples.

2.3. Oligonucleotides

For the α1-α3 and β GlyR subunits, the oligonucleotide primers employed were those described by Aroeira et al. (2011). The α4 GlyR subunit was amplified by using the Prime Prime Time® qPCR Primers (Rn. PT.56a. 12226027; Exon location 7–8) from Integrated DNA Technologies (Coralville, IA, USA). For the α4 GlyR subunit we also used the following primer sequence: 5′-AGCAGCAAATA-CAAACAGCAG-3′ and 5′-TCCTCACCATGACAACTCAGA-3′,and for the 18S gene sequence 5′-TACCACATCCAAGGAAGGCAGCA-3′ and 5′-TGGAATTACCGCGGCTTGCTGGCA -3′(T4 Oligo) (Irapuato, Gto. Mexico).

2.4. Western blot

Retinae (6 for P0–P15 and 3 for P30 and adult for each experiment) were homogenized in a lysis buffer 1:4 (p/v) in RIPA-Tris buffer (mM: EGTA 2; NaCl 316; Na_2MoO_4 20; NaF 50; Tris-HCl 20; Na_3VO4 100, PMSF 100 and EDTA 100; 0.1% of leupeptin and aprotinin; SDS 0.2% and Triton X-100 2%) and maintained under constant shaking for 1 h at 4 °C. Subsequently, the sample was centrifuged for 30 min at 17,000 × g and the supernatant (100 μg of protein) was denatured in Laemmli's sample buffer (Laemmli, 1970), resolved through 10% SDS polyacrilamide gels and electroblotted to PVDF membranes. Molecular weight markers were Precision Plus Protein Standards Kaleidoscope from Bio-Rad (161–0375). Blots were stained with Ponceau S to confirm that protein loading was the same in all lanes. Membranes were soaked in Tris-buffered saline (TBS) to remove the Ponceau S and incubated for 2 h in TBS containing 5% dried skimmed milk, 2% BSA, and 0.1% Tween 20 to block the nonspecific protein binding sites. Afterwards, membranes were incubated for 14 h at 4 °C with the primary antibodies (Table 1) diluted in BSA 0.25%, Tween 20 0.1%, thimerosal 0.01% in TBS buffer. Then they were washed and incubated with the secondary antibodies (Table 1). Protein loading was normalized to actin using a mouse monoclonal primary antibody (1:800; MAB1501 Millipore-Corp.) and an anti-mouse horseradish peroxidase-conjugated as secondary antibody (1:15,000 NA934VS, Healthcare Life Sci.). The protein was detected using a Chemiluminescent HRP Substrate (Immobilon Western Chemiluminescent HRP Substrate, Millipore Corp.) according to the manufacturer's instructions. The blots were subjected to a densitometry analysis and data were analyzed using Graph Pad Prism5 software (San Diego, CA, USA). The relative expression between the alpha subunits was obtained considering as 100% the addition of the optical densities from the four adult alpha subunits in the same western blot. To confirm the quality of antibodies used, we established the standardization conditions, using extracts from rat spinal cord or mouse retina.

2.5. Statistical analysis

All data are presented as the average mean ± SEM. One way ANOVA was performed, followed by the Dunnett's *post hoc* test, using the GraphPad Prism 6 program.

3. Results

3.1. 1qPCR

In order to determine the expression levels of the GlyR during the rat retina development, qPCR experiments were carried out at different postnatal stages of the rat. Data showed a progressive increase of the α1 GlyR mRNA expression during rat retina development (P5, 0.38; P7, 0.48 pg), showing a significant growth from P12 (P12, 1.56 pg) to the adult (3.14 pg) (Fig. 1A).

In contrast to that observed in the spinal cord, the α2 GlyR did not seem to be exclusive of early stages of the rat retina development. The α2 mRNA expression was high since the first postnatal week (P5, 3.24 pg) and it remained almost constant up to P15 (3.1 pg), increasing considerably in the adult retina (5.7 pg) (Fig. 1B).

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