Developmental expression of glycine receptor subunits in rat cerebellum

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Abstract

The distribution of the glycine receptor subunits α1–3 and β in the developing rat cerebellum was studied from postnatal day 1 to adulthood by means of quantitative RT-PCR and immunohistochemistry. qRT-PCR of postnatal cerebella indicated the presence of mRNA for each subunit, with a relative expression of α2 > α3 > α1 > β. The immunohistochemistry indicated a strong α2 signal in the Purkinje cells, internal and external granular layers. The α1–3 subunits had weak signals in the Purkinje cells and molecular layer. The α1 subunit was expressed at a low level and was also found in the white matter. The function of these receptors in neuronal and glial plasma membranes in early postnatal development remains to be determined.

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1. Introduction

Fast inhibitory neurotransmission in the central nervous system undergoes marked developmental changes as a consequence of a "genetic switch" that represses the expression of glycine receptors (GlyRs) while at the same time it promotes the expression of γ-aminobutyric acid (GABA) receptors (Carpenter et al., 1988). This switch was initially observed when studying, in Xenopus laevis oocytes, the GABA A and Gly receptors expressed after injecting mRNAs isolated from rat brain at different stages of development. Furthermore, a functional heterogeneity of GlyRs was found in the developing spinal cord where two different populations of GlyRs were distinguished by the size of their mRNAs and by the functional properties of the receptors expressed (Akagi et al., 1989; Morales et al., 1994). Molecular cloning of the genes encoding the GlyR subunits (α1–4 and β) allowed a determination of their individual contribution to the receptors in the brain and spinal cord as well as in several areas of the embryonic rat brain (Akagi et al., 1989; Charrier et al., 2006; García-Alcocer et al., 2001).

Activation of Gly and GABA A receptors during early development increases Cl– conductance and excitatory potentials (Chen et al., 1996; Flint et al., 1998; Virginio and Cherubini, 1997). The ionic changes induced by GlyRs in immature cells bring about the release of Ca2+, and it generates molecular signals necessary for proper neuronal development (Flint et al., 1998). The importance of the GlyRs during ontogeny was clearly revealed when its antagonist strychnine was administrated to pregnant rats. This induced severe neurotoxicity and abnormalities, such as anencephaly and aplasia (García-Alcocer et al., 2005).

In the rat brain embryo, we previously detected mRNA transcripts coding for the β subunit as early as E8 whereas transcripts coding for the α1–3 subunits were expressed from E13 onwards (García-Alcocer et al., 2001). At developmental stage E13, cerebellar differentiation is incomplete, and the role of GlyRs early during ontogeny is still unknown. In this work we report the expression of the mRNAs for four GlyR subunits during early postnatal cerebellar development when the functional cerebellar circuits are formed. In addition, using immunohistochemistry we observed the distribution of the GlyR subunits.
2. Methods

The cerebellum of P1, P12, P18 and P60 Wistar rats sacrificed by cervical dislocation was dissected out (Altman and Bayer, 1995; Paxinos and Watson, 1986), placed immediately in liquid nitrogen and stored at −80 °C until processed.

2.1. RNA preparation

Samples (100–200 mg) of dissected cerebellum (total cortex), from the different ages were homogenized using an Ultra Turrax T18 (IKA Labortechnik), and total RNA was extracted (Chomczynski and Sacchi, 1987). The integrity of the RNA was confirmed by gel electrophoresis and its concentration was measured by spectrophotometry (Amersham Pharmacia Biotech). RNA isolations were performed in triplicate from pools of three cerebellum of each age. The RT was performed with Superscript II Reverse Transcriptase enzyme (Invitrogen, Cat. No. 18064-014) and the DNA was amplified with Taq DNA Polymerase (Invitrogen, Cat. No. 11615-010).

2.2. Real-time quantitative RT-PCR (qRT-PCR)

The expression levels of mRNA for rat GlyR subunits (α1, α2, α3 and β) were determined in samples of the RNA isolated from different ages. The cDNA was synthesized and pooled in microtiter plates; each well containing were determined in samples of the RNA isolated from different ages. The relationship between cycle number (Ct) and mRNA levels, primers (Table 1) were calibrated by using serial dilutions of cDNA. Data for each developmental stage were collected from two or three independently synthesized cDNA samples and each amplification was carried out in duplicate. qRT-PCR reactions were performed with TaQurate GREEN Real-Time PCR Master Mix enzyme (Epipetec Technologies, Cat. No. TM04600), using Taq polymerase in 1 min at 94 °C; 50 cycles of PCR at 94 °C for 20 s, 55–59 °C for 8 s, and 72 °C for 20 s; a dissociation program of 1 cycle and a final incubation at 40 °C for 5 min.

The target gene mRNA level was quantified using a point of standard curve in each experiment. The relative transcription level of the target gene was obtained after the 2AACt was calculated for three independent experiments by duplicate, and the mean of the results was obtained after the standard deviations. The relative transcription level of the target gene was transcribed to the highest extent and at the same level of transcription from P1 to P60. Transcription of the α3 subunit clearly decreased during postnatal development, whereas the α1 subunit was expressed at constant levels during development. The α2 subunit mRNA was always the least expressed, with statistical significance in comparison with the α1–3 subunits.

2.3. Data analysis

Ct values were analyzed using the 2AACt method, as described in the user Bulletin 2 for the ABI Prism 7700 “Sequence Detection System: Relative Quantitation of Gene Expression Comparative Ct Method” (product no. 4303859 Applied Biosystems). As control we used a standard curve for each subunit.

2.4. Immunohistochemistry

Rats were anesthetized with sodium pentobarbital (40 mg/kg, IP) and intracardially perfused with 4% paraformaldehyde–saline phosphate buffer (pH 7.4). The cerebellum were removed rapidly after decapitation, postfixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and then cryoprotected overnight in 30% sucrose in 0.1 M phosphate buffer. Specimens were embedded in tissue-freezing medium, and 12-μm coronal sections were obtained over super frost slides using a cryostat (Leica CM 1850). The cerebellum were examined with reference to the brain atlas of Paxinos and Watson (Paxinos and Watson, 1986).

For immunostaining glycine receptor subunits, the following polyclonal antisera were used: anti-α1 (goat anti-mouse sc-17279), anti-α2 (rabbit anti-human sc-20133), anti-α3 (goat anti-human sc-17282), and anti-β (goat anti-human sc-17285). Endogenous peroxidase was blocked by 1 h immersion in 1% H2O2, followed by 1 h incubation in 5% nonfat milk to block unspecific sites. Tissue sections were incubated overnight with the antisera (1:50) and then with biotin-conjugated goat anti-rabbit antibody (1:500) or peroxidase-conjugated donkey anti-goat antibody (1:500) for 2 h. Avidin–horseradish peroxidase conjugates were added, and the color was developed with diaminobenzidine and H2O2. The sections were examined with an Axiosstar Zeiss microscope and scanned through an MRC Axiocam for digitization.

3. Results

3.1. Quantitative RT-PCR

Fig. 1 shows the copy numbers for the four GlyR subunits and the α-tubulin cDNA. The results indicate that α2 GlyR subunit was transcribed to the highest extent and at the same level of transcription from P1 to P60. Transcription of the α3 subunit clearly decreased during postnatal development, whereas the α1 subunit was expressed at constant levels during ontogeny. The β subunit mRNA was always the least abundantly expressed and it decreased throughout development. The α-tubulin transcript showed a transient drop at P18. In summary, during postnatal development the relative abundance of the mRNAs coding for the GlyR subunits is: α2 > α3 > α1 > β, with statistical significance in α2 compared with the subunits.

Table 1

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Fig. 1. Expression of glycine receptor subunit mRNAs during development. qRT-PCR shows the relative expression (number of copies) of mRNAs for α1–3 and β GlyR subunits (means ± S.D.). The panel shows an agarose gel with the amplicons generated for each subunit at P1, P12, P18, and P60.
α3 subunit in P60 whereas the β subunit was weak throughout the postnatal development.

4. Discussion

The objective of this study was to determine the developmental distribution of the GlyR subunits (mRNA and proteins). The expression of GlyRs in the brain and spinal cord is regulated by a maturation process whose molecular bases are still unknown (Akagi and Miledi, 1988; Carpenter et al., 1988; Kotak et al., 1998; Morales et al., 1994).

To study the relative level of expression of the α1–3 and β GlyR subunit mRNAs during development of the cerebellum, we carried out qRT-PCR using RNA from four postnatal stages. Although the structural organization of the cerebellum is not completed until the second week after birth (Kirsch et al., 1993), we found that the four GlyR subunits were expressed in all stages studied.

The role and mechanisms of glycine neurotransmission during early cerebellar development are not completely known, although it may contribute important developmental cues, as is the case in the spinal cord interneuron differentiation (McDearmid et al., 2006).

We observed the expression of the GlyR subunits in the cerebellum from P1 to P60, a period related to the arborization of Purkinje neurons (Mason and Gregory, 1984). GlyRs are expressed in these cells as well as in the external (from P1 to P18) and internal granular layers. It is known that during early development the ionotropic neurotransmission causes both excitation and inhibition (Kawa, 2003; Polina et al., 2007). In our experiments, granular cells expressed all four GlyR subunits during early development, suggesting that inhibitory transmission in these cells could be generated by glycine as well as GABA receptors (Dugue et al., 2005). Our results revealed the expression of the subunits in the different layers of the cerebellum and especially in the Purkinje and granular cells. More experiments are necessary to determine how the receptors are assembled and anchored to the plasma membrane in the cells with very weak expression of the β subunit.

Acknowledgements

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References