Expression of GABAC subunits during rat cerebellum development

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Abstract

In the present study, we provide evidence for the expression of all three GABAC receptor ρ subunits through development of the rat cerebellum. Injection of cerebellum mRNA into frog oocytes gave rise to the expression of both GABA A and GABA C receptors. qRT-PCR of RNA isolated from postnatal developing cerebella showed that the expression of each ρ subunit is relatively low, with a relative comparative expression of ρ3 > ρ1 > ρ2. In situ hybridization and immunohistochemistry revealed a limited distribution of GABA C receptors in the Purkinje and Golgi neurons whereas electron microscopy detected the ρ1 and ρ2 subunits in the soma and dendritic tree of the Purkinje cells.

The expression of GABA C receptors in the cerebellum adds a new dimension to the regulation of GABAergic neurotransmission and suggests further experiments to determine their functional consequences.

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In the adult central nervous system (CNS), the neurotransmitter γ-aminobutyric acid (GABA) is widely distributed, mediating inhibitory synaptic transmission and regulating the excitatory activity of neurons. The importance of GABAergic neurotransmission in the cerebellum is highlighted by the fact that four of five types of cerebellar cortex neurons: Purkinje, stellate, basket and Golgi, all release GABA [10,14].

GABAA and GABAB receptors are widely distributed in the cerebellum, where they play a central role in the inhibition of synaptic transmission [10,17,20]. On the other hand, GABA C receptors are present mainly in the retina [16], where they are expressed in the bipolar neurons and upon activation they generate bicuculline-resistant, non-desensitizing Cl− currents. Nevertheless, strong evidence indicates that GABA C receptors are also expressed in several populations of brain neurons, where they may play significant functional roles, acting either alone or forming heteromeric receptors in combination with GABAA subunits [3,9,15]. Thus, the unique functional and pharmacological properties of the GABA C receptors [4,16,21,22] may give rise to synaptic characteristics not previously identified.

GABA C receptors are formed by functional homo- or hetero-pentamers composed of ρ1, ρ2 and/or ρ3 subunits [7]. Although the distribution of the ρ subunit mRNAs in the adult brain has been studied using RT-PCR and in situ hybridization, information on the developmental regulation of the receptor in the brain is scattered and incomplete [3,8,15,18]. Alakuijala et al. [1] described the distribution of the three GABA C subunits during postnatal development of the superior colliculus, lateral geniculate nucleus and hippocampus evidencing a temporal regulation of the genes encoding the GABA C subunits.

Further evidence of the presence of GABA C receptors in the bovine cerebellum was obtained by means of RT-PCR, in situ hybridization and GABA-current recordings [15]. More recently [12] GABA C currents were recorded from Purkinje neurons, suggesting the existence of at least three populations of ionotrophic GABA receptors: GABA A, GABA C and heteromeric receptors formed by GABA A and GABA C receptors. Therefore a full description of the cellular distribution of the subunits that form the GABA C receptor now becomes necessary. In this study, we have combined functional expression of mRNA in frog oocytes, real-time quantitative RT-PCR (qRT-PCR), in situ hybridization, immunohistochemistry and electron microscopy to determine the developmental expression of the three GABA C receptor ρ subunits in the rat cerebellum.
Wistar rats were anesthetized and sacrificed in compliance with protocols approved by the UNAM ethics committee. Cerebella were isolated, placed immediately in liquid nitrogen, and stored at $-80^\circ$C until processed.

100–200 mg of resected rat cerebellum at different ages (P1, P12, P18 and P60, $n=3$), were processed using the Chomczynski and Sacchi (1987) method [5]. For each RNA extraction we used three cerebella of each stage, each preparation was repeated at least three times. Reverse transcription was performed with protocols approved by the UNAM ethics committee. Cerebella were isolated, placed immediately in liquid nitrogen, and stored at $-80^\circ$C until processed. The brains were rapidly removed and fixed overnight in 5% paraformaldehyde in PBS. The fixed tissues were stored in sucrose 30%/PBS at 4$^\circ$C. Sagital sections were cut at 10 m cryosections using the cryostat, thaw mounted onto superfrost slides and stored at $-60$ or $-80$ mV.

Expression levels of GABA$_C$ receptor subunit mRNAs ($\rho_1$, $\rho_2$ and $\rho_3$) were determined for the cerebellar RNA isolated from each age. The cDNA was synthesized and pooled into microtiter tubes containing cDNA from 100 $\mu$g of total RNA. To determine the relationship between cycle number ($C_t$) and expression of each mRNA subunit, primers (Table 1) were calibrated by using serial dilutions of cDNA. In all cases, data from three independently synthesized cDNA samples were collected and each amplification was carried out in duplicate. Reactions were performed with Tqurate GREEN Real Time PCR Master Mix enzyme (EPICENTRE TECHNOLOGIES, No. cat. TM046400) using $\alpha$-tubulin as standard control. PCR amplifications were generated in an ABI Prism 7700 Sequence Detection System (Applied Biosystems). An amplification plot for each sample was generated showing the increase in fluorescence with each amplification cycle. The negative control (no reverse transcription) consistently showed no increase in fluorescence.

$C_t$ values were analyzed using the $2^{-\Delta\Delta C_t}$ method, as described in the user Bulletin 2 for the ABI Prism 7700 Sequence Detection System: Relative Quantification of Gene Expression Comparative $C_t$ Method (Applied Biosystems, product no.4303859). As a reference control, we used the sum of values obtained from all subunits under examination; data are presented as number of copies of mRNA of each GABA$_C$ subunit for postnatal days 1, 12, 18 and 60.

Whole adult cerebella were fixed in 3.5% paraformaldehyde in phosphate buffer saline (PBS), preserved in Superfrost Plus® media, frozen immediately and stored at $-80^\circ$C. In situ hybridization was performed on 12 $\mu$m cryosections using the method described by the manufacturer (Roche). The $\rho_1$–$\rho_3$ probes correspond to the region encoding the large intracellular loop of each subunit, where the DNA nucleotide sequence is quite divergent. These fragments were obtained by RT-PCR and cloned into the plasmid pT7 (Novagen). Identity of the cloned fragments was corroborated by DNA sequencing. No hybridization was detected with the sense probe.

P1, P12, P18 and P60 male Wistar rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and decapitated. The brains were rapidly removed and fixed overnight in 5% paraformaldehyde in PBS. The fixed tissues were stored in sucrose 30%/PBS at $4^\circ$C overnight, immersed in tissue freezing medium and frozen at $-80^\circ$C. Sagital sections were cut at 10 m on a cryostat, thaw mounted onto superfrost slides and stored at $-20^\circ$C. Sections on the glass slides were treated with methanol containing 0.3% $H_2O_2$ for 30 min, PBS for 10 min, 3% non-fat dry milk in PBS for 1 h and incubated overnight with antibodies against each of the GABA$_C$ subunit, diluted 1:100 in PBS; $\rho_1$ (Santa Cruz, sc-16879), $\rho_2$ (Santa Cruz, sc-30254) and $\rho_3$ (Santa Cruz, sc-22362). After rinsing three times with PBS for 15 min, sections were treated with secondary antibody (diluted

Table 1

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Fragment size</th>
</tr>
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<tbody>
<tr>
<td>Rho1S</td>
<td>5'-TGGACACGCGCTACAGTGACGG-3'</td>
<td>209</td>
</tr>
<tr>
<td>Rho1A</td>
<td>5'-AAGCAGCTGGAGAAAATGTAC-3'</td>
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</tr>
<tr>
<td>Rho2S</td>
<td>5'-AGAACCACCTACCTAAGTCG-3'</td>
<td>248</td>
</tr>
<tr>
<td>Rho2A</td>
<td>5'-AATACTTCTAGAGGCAAGGAAT-3'</td>
<td></td>
</tr>
<tr>
<td>Rho3S</td>
<td>5'-TGATGGGTAGACTGAGTGAC-3'</td>
<td>193</td>
</tr>
<tr>
<td>Rho3A</td>
<td>5'-CGGGGAAAGACAAATCTCTAGTGG-3'</td>
<td></td>
</tr>
<tr>
<td>TubS</td>
<td>5'-CCAGATCTGCAAATGGAAGAC-3'</td>
<td>522</td>
</tr>
<tr>
<td>TubA</td>
<td>5'-GCTCCTATTGCTACATGAGGC-3'</td>
<td></td>
</tr>
</tbody>
</table>

The table shows the primer sequences and the size of the corresponding amplification products for GABA$_C$ subunits as well as for the isoform-6 of human tubulin, used for quantitative PCR.

![Fig. 1](image-url)

Fig. 1. Functional expression in X. laevis oocytes. (A) GABA-currents were mainly non-desensitizing due to activation of predominant GABA$_A$ receptors. (B) The GABA-currents activated by 1 $\mu$M GABA were mostly bicuculline-resistant and blocked by TPMPA. Oocytes were voltage-clamped at $-60$ mV (A) or $-80$ mV (B).
Fig. 2. qRT-PCR of GABAC subunits. Three animals from separate litters were used for each age, and each data point was obtained in triplicate. For each subunit, the number of copies of mRNA is plotted from three separate experiments ± S.E. Inset shows an agarose gel of the PCR products of ρ1, ρ2, ρ3 and tubulin.

1:1000 in PBS, Santa Cruz, sc-2949) and developed with 3-3′-diaminobenzidine (DAB) tetrachloride with 0.02% hydrogen peroxide in PBS.

Male adult rats (60 days old) were anesthetized with an overdose of pentobarbital injected intraperitoneally and then perfused transcardially with 0.9% NaCl and the fixative solution (4% paraformaldehyde and 1% glutaraldehyde). The whole brain was removed and the region corresponding to the vermis cerebellum was dissected, this was cut in coronal sections and fixed for 30 min in the same solution [6].

Sixty micrometers slices were washed three times in 0.1 M sodium cacodylate, pH 7.0, dehydrated in ethanol and encapsulated in gelatin, LR white resin was added and left to polymerize for 24 h at 60 °C, 500 nm slices were obtained with a glass knife to select an area that included the cerebellar cortex. Following, 60 nm slices were obtained in an ultramicrotome (MRC-MTXL) using a diamond point knife, the sections were placed in nickel grids covered with Formvar, washed twice with water and PBS and submerged in 0.5 M glycine in PBS, then washed again in PBS and incubated in 0.1% albumine and tween 20 in PBS. Unspecific sites were blocked with 5% albumine in PBS and incubated with the primary antibody (1:50 goat IgG anti-GABA ρ1 or anti-GABA ρ2, Santa Cruz Technologies); then washed three times in PBS and incubated with the secondary antibody (1:200 20 nm gold-conjugated rabbit anti-goat IgG, PELCO), finally the samples were dyed with 2% uranyl acetate and lead, rinsed and observed in the electronic microscope (JEOL JEM 1010) [19].

Injection in oocytes of cerebellum polyA+ RNA led to the expression of a large GABA_A component, averaging a current of 689 ± 116 nA (1 mM GABA) in 24 oocytes and seven independent injections. Fig. 1A, shows sample currents generated by an oocyte exposed to either 1 μM or 1 mM GABA, revealing the fast desensitizing component, which is typical of GABA_A receptors. Including 100 μM bicuculline to the medium bath while perfusing with 1 μM GABA, a concentration similar to the GABAC

Fig. 3. In situ hybridization. ρ subunit mRNAs were localized to the cortical layer in the adult cerebellum cortex. Arrows point to positively labeled Purkinje cells, arrowheads indicate the Golgi neurons positive for ρ2 and ρ3. Bar = 50 μm.

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References