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Expression of GABAρ subunits during rat cerebellum development

Carmen Mejía [a], Guadalupe García-Alcocer [b], Laura C. Berumen [b], Abraham Rosas-Arellano [a], Ricardo Miledi [a], Ataúlfo Martínez-Torres [a,*]

[a] *Instituto de Neurobiología, Departamento de Neurobiología Molecular y Celular, Universidad Nacional Autónoma de México-Campus Juriquilla, (Boulevar) Universitario 3001, Querétaro, 76230, Mexico*
[b] *Facultad de Química, Universidad Autónoma de Querétaro Centro Universitario, 76010 Querétaro, México*

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Abstract

In the present study, we provide evidence for the expression of all three $GABA_C$ 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.

Keywords: Neurotransmitter receptor; Purkinje cell; qRT-PCR

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].

$GABA_A$ and $GABA_B$ 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 $GABA_A$ 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 heteropentamers 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 ionotropic 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.

* Corresponding author. Tel.: +52 442 2381064; fax: +52 442 2381064.
E-mail address: ataulfo@inb.unam.mx (A. Martínez-Torres).

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Table 1
Nucleotide sequence of primers used for qRT-PCR of the ρ subunits

Primers	Sequence	Fragment size
Rho1S	5′-TGGACAGCAGCTACAGTGACGG-3′	209
Rho1A	5′-AAGCAGCTGGGAAAATGATC-3′	
Rho2S	5′-AGAACCATGACGCTGGATGG-3′	248
Rho2A	5′-AACTATGTAGAAGGCAGGAAT-3′	
Rho3S	5′-TGACGGTGAGACTGACGTGGAC-3′	193
Rho3A	5′-CGGGGAAGACAATTCTAGAGTAGG-3′	
TubS	5′-CCAGATGCCAAGTGACAAGACC-3′	522
TubA	5′-GCCTCATTGTCTACCATGAAGGC-3′	

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

Wistar rats were anesthetized and sacrified in compliance with protocols approved by the UNAM ethics committee. Cerebella were isolated, placed immediately in liquid nitrogen, and stored at −80 °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 the Superscript II Reverse Transcriptase (Invitrogen, No. cat. 18064-014) and the cDNA was amplified with Taq DNA Polymerase (Invitrogen, No. cat. 11615-010). PolyA$^+$ RNA from adult cerebella was selected by affinity chromatography using an oligo (dT) cellulose column.

Frogs were obtained from Nasco (Fort Atkinsons WI) and oocytes removed and processed as previously described [4,15]. The two-microelectrodes voltage-clamp technique was performed as previously described [4,15,16]. GABA and/or bicuculline were applied by bath perfusion while the oocyte plasma membrane was held at −60 or −80 mV.

Expression levels of $GABA_C$ receptor subunit mRNAs (ρ1, ρ2 and ρ3) were determined for the cerebellar RNA isolated from each age. The cDNA was synthesized and pooled into microtiter tubes containing cDNA from 100 μg 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 TAQurate GREEN Real Time PCR Master Mix enzyme (EPICENTRE TECHNOLOGIES, No. cat. TM046400) using α-tubulin as standard control. PCR amplifications were generated in an ABI Prism 7000 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 $2_T^{-\Delta\Delta C}$ 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 °C. *In situ* hybridization was performed on 12 μm cryosections using the method described by the manufacturer (Roche). The ρ1–ρ3 probes correspond to the region encoding the large intracellular loop of each subunit, where the DNA nucelotide 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 °C overnight, immersed in tissue freezing medium and frozen at −80 °C. Sagital sections were cut at 10 μm on a cryostat, thaw mounted onto superfrost slides and stored at −20 °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; ρ1 (Santa Cruz, sc-16879), ρ2 (Santa Cruz, sc-30254) and ρ3 (Santa Cruz, sc-22362). After rinsing three times with PBS for 15 min, sections were treated with secondary antibody (diluted

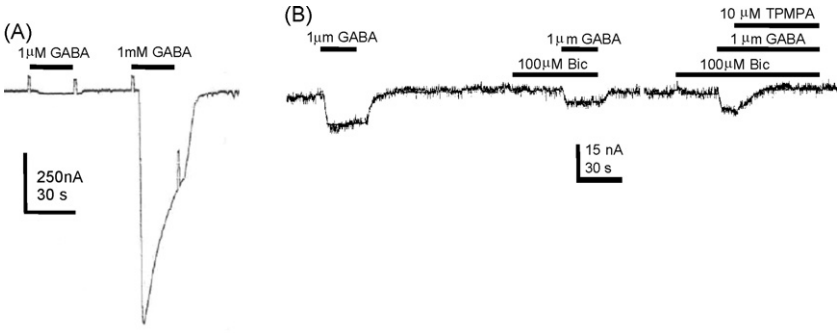

Fig. 1. Functional expression in *X. laevis* oocytes. (A) GABA-currents were mainly non-desenistizing due to activation of predominant $GABA_A$ receptors. (B) The GABA-currents activated by 1 μM GABA were mostly bicuculline-resistant and blocked by TPMPA. Oocytes were voltage-clamped at −60 mV (A) or −80 mV (B).