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GABAρ expression in the medial nucleus of the trapezoid body

Daniel Reyes-Haro, Abraham Rosas-Arellano, María Alejandra González-González, Ernesto Mora-Loyola, Ricardo Miledi, Ataúlfo Martínez-Torres*

Departamento de Neurobiología Celular y Molecular, Instituto de Neurobiología, Universidad Nacional Autónoma de México, Campus Juriquilla, Boulevard Juriquilla, 3001, Juriquilla, Querétaro, Qro. CP 76230, Mexico

HIGHLIGHTS

- ▶ GABAρ subunits are involved in GABA mediated currents.
- ▶ GABAρ3 is expressed before hearing onset (PN8–PN10) but not in adult stage.
- ▶ GABAρ1 and GABAρ2 are expressed in adult stage but not before hearing onset.

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ABSTRACT

The Calyx of Held (CoH) synapse is the largest synapse in mammals. It is located in the medial nucleus of the trapezoid body (MNTB) and forms part of the auditory pathway. Modest GABAergic signaling is present in the CoH before hearing onset, when glutamatergic transmission predominates. In mice, after postnatal day 12, the absolute strength of glycinergic transmission increases markedly, while GABAergic signaling remains constant. The persistent GABAergic transmission in the MNTB is mediated by a slowly desensitizing component. In this study we recorded GABA-mediated responses from postsynaptic principal neurons (PPNs) of the MNTB and found that they are sensitive to TPMPA, suggesting the involvement of GABAρ subunits. RT-PCR and immunohistofluorescence in the MNTB confirmed GABAρ expression in PPNs. Interestingly, GABAρ3 was present only before hearing onset, and there was a switch to GABAρ1 and GABAρ2 expression in adult animals.

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1. Introduction

The Calyx of Held (CoH) is part of the auditory pathway specialized for sound localization and requires high-frequency signal transmission. This process involves the globular bushy neurons of the anterior ventral cochlear nucleus that project their axons to postsynaptic principal neurons (PPNs) of the contralateral MNTB and form the glutamatergic CoH [22,27]. The CoH is an axosomatic synapse where a giant glutamatergic presynaptic terminal contacts a single PPN and glial cells [13,17]. During postnatal development of MNTB, a switch from $GABA_A$ to glycine receptors occurs, and the absolute strength of glycinergic transmission increases markedly after hearing onset while GABAergic signaling is relatively constant [3]. GABAergic neurotransmission modulates transmitter release through $GABA_B$ receptors and is thought to mediate tonic inhibition through $GABA_A$ receptors at this synapse [3,7,24].

Inhibition mediated by GABA receptors is fundamental for auditory processing, and several of the 19 $GABA_A$ subunits (α_{1-6}; β_{1-3}; γ_{1-3}; δ; ε; θ; π and ρ_{1-3}) were detected in the brainstem. GABAρ subunits have been found to be expressed in neurons of several nuclei of the brainstem, particularly in the olive, reticulate, cuneiform, dorsal vagal, and *tractus solitarius* [12,18,26]. GABAρ subunits were originally described in the mammalian retina, and due to their peculiar functional and pharmacological properties several studies have explored their distribution in various areas of the brain. These subunits form homo- or heteromeric receptors that do not desensitize upon activation by the agonist, are insensitive to the classic $GABA_A$ antagonist bicuculline, are selectively activated by CACA, and are blocked by TPMPA [11].

In the present work, we studied the properties of PPNs of MNTB and observed that GABA responses have two kinetically different components; one desensitizes rapidly whereas the second does not desensitize in the presence of the agonist. This observation suggested the presence of a combination of classic $GABA_A$ subunits along with a component mediated by GABAρ. Here, we provide

Abbreviations: TPMPA, (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; aCSF, artificial cerebrospinal fluid; CoH, Calyx of Held; CACA, cis-4-aminocrotonic acid; D-APV, D-(−)-2-amino-5-phosphonopentanoic acid; MNTB, medial nucleus of the trapezoid body; MBSC, Mouse Brain in Stereotaxic Coordinates; PPN, postsynaptic principal neuron; TTX, tetrodotoxin.

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

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evidence that GABAρ subunits may be responsible for the non-desensitizing component of the GABA responses in PPNs of MNTB. Our results also suggest that GABAρ3 is present before hearing onset early during postnatal development, whereas GABAρ1 and GABAρ2 are expressed in adults.

2. Materials and methods

2.1. Preparation of brainstem slices and electrophysiological recordings

All experiments were conducted according to the guidelines of the National Institutes of Health Guide for Care and use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the Universidad Nacional Autónoma de México. NMRI and CD-1 mice strains were used for this study. These strains have a similar genetic background [2] and recent studies reported that the expression of GABAρ subunits does not show significant variations [19,20]. NMRI mice (Charles River) from 8 to 10 postnatal days of age (PN8–10) were used for slice preparations. Slices, artificial cerebrospinal fluid (aCSF), internal solutions, and patch micropipettes were prepared and used as previously described [13,17]. Briefly, the PPNs of MNTB were identified using light microscopy in transverse brainstem slices (160 μm) and were recorded with the patch-clamp technique using the whole-cell recording configuration [5]. Only one neuron was tested for every slice, from different animals. Current signals were amplified with a triple EPC10 (HEKA), filtered at 3 kHz, sampled at 10 kHz, and recorded using TIDA software (5.19). Chemicals were obtained from Sigma–Aldrich (St Louis, MO, USA) or Tocris Cookson (Ballwin, MO, USA) if not otherwise indicated. Slices were superfused with oxygenated aCSF with tetrodotoxin (TTX, 1 μM) to minimize the indirect effect of neuronal electrical activity. Likewise, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 25 μM), D-2-amino-5-phosphonovaleric acid (D-APV 50 μM), and strychnine (1 μM) were added to the aCSF to block ionotropic glutamate and glycine receptors. Patch micropipettes with a resistance of 3–5 MΩ were pulled from thin-walled borosilicate glass (o.d., 1.5 mm; i.d., 0.87 mm; Hilgenberg) using a P2000 laser-based micropipette puller (Sutter Instrument Co.). GABA was applied at least 3 times on each recorded neuron and no significant desensitization was observed (less than 10%; $p \geq 0.67$). TPMPA or Gabazine (alone or combined) were added to the aCSF and preincubated 1 min before agonist and during GABA application. Statistical analysis was performed using Origin 7.0 software (Origin Laboratories). The results are expressed as mean ± S.E.M. if not otherwise stated. Comparisons were made using Student's *t*-test, and a p value < 0.05 was considered significant.

2.2. Western blot and immunohistofluorescence

The expression of GABAρ subunits was investigated in CD1 mice of PN9 and adult stage (30 g). We have previously confirmed the specificity of antibodies of GABAρ1 and GABAρ2 [20], whereas the specificity for GABAρ3 was tested in this study by Western blot assays (Fig. 2B). Brainstem slices containing the MNTB were obtained from 10 CD1 mice of PN9, proteins were isolated by homogenization and centrifugation, then resolved by 12% PAGE, electrotransferred, and exposed to anti-GABAρ3 (Santa Cruz Biotechnology sc-28793); the second antibody was goat anti-rabbit IgG-AP (Santa Cruz Biotechnology sc-2034).

Immunohistofluorescence was performed in PN9 and adult mice. The animals were anesthetized and perfused transcardially with saline (0.9% NaCl) and fixative solutions (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). The brain was removed

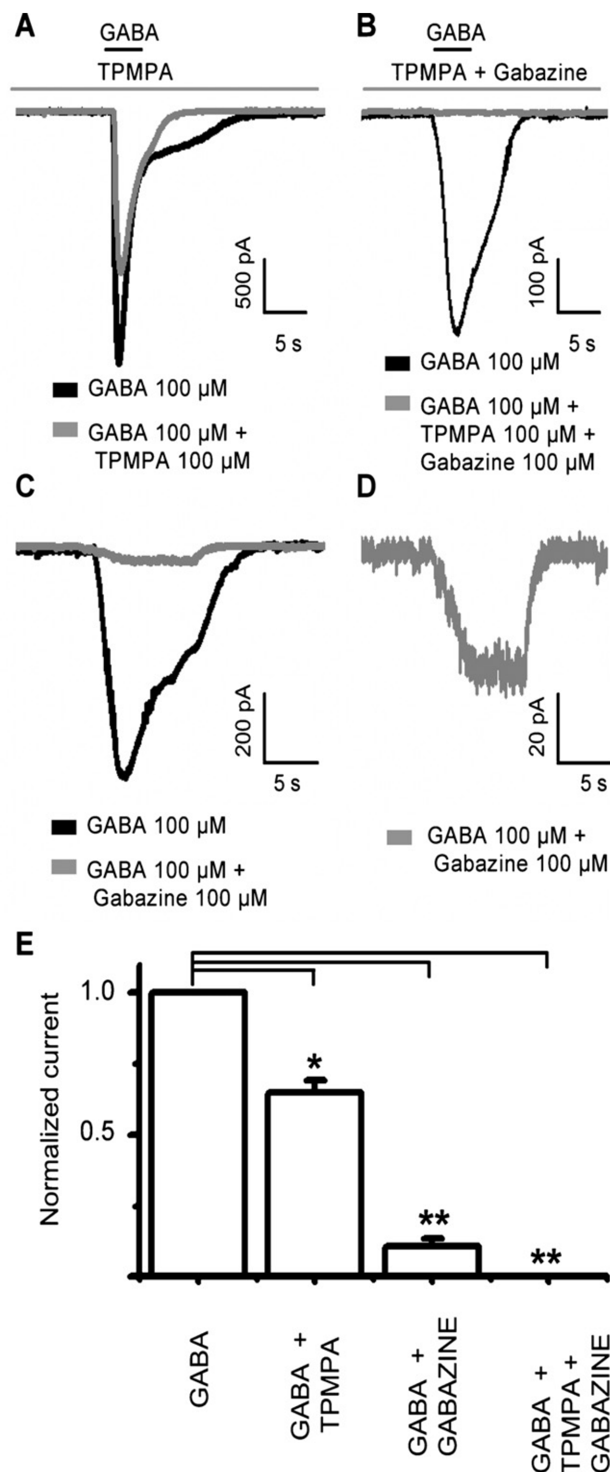

Fig. 1. GABA-mediated responses are sensitive to TPMPA antagonism. (A) The GABA- (100 μM) mediated responses ($n = 8$) were reduced when TPMPA (100 μM; $n = 8$) was added to the aCSF. (B) The GABA-evoked response (100 μM) was abolished when TPMPA (100 μM) was combined with Gabazine (100 μM) ($n = 3$). (C) The GABA-evoked response (100 μM) was significantly reduced when Gabazine (100 μM) alone was added to the aCSF ($n = 4$). Nevertheless, a small remaining current was observed. The unblocked fraction of the GABA-evoked current is amplified in (D) and these data are summarized in the histogram (E). Data are shown as mean ± S.E.M. Asterisks indicate significant differences, $p < 0.05$ (*) or $p < 0.01$ (**).

and cryoprotected, and 30-μm sections were obtained in a cryostat (Leica CM1850). To identify the MNTB, we rely on three criteria: coordinates of Mouse Brain in Stereotaxic Coordinates (MBSC) [15], shape of the nucleus, as well as shape and size of

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