

Distribution of the GABA_A receptor complex β 2/3 subunits in the brain of the frog *Rana pipiens*

María Isabel Aller^a, Skirmantas Janusonis^b, Katherine V. Fite^b, Arsenio Fernández-López^{a,*}

^aDepartamento Biología Celular y Anatomía, Universidad de León, Campus de Vegazana s/n, 24071 León, Spain

^bDepartment of Psychology, University of Massachusetts at Amherst, Amherst, MA 01003, USA

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Abstract

This report describes the distribution of labeling of the monoclonal antibody bd-17 against the β 2/3 subunits of the mammalian GABA_A receptor complex throughout the brain of the frog *Rana pipiens*. The distribution matches quite closely those in homologous brain regions as previously described for this antibody in fishes, birds, and mammals, indicating that this antibody also labels β 2/3 subunits of frog. A semiquantitative analysis of the distribution of labeling throughout the brain is based upon relative optical densities with respect to the structure showing maximal optical density in each brain, using standard illumination conditions. Comparison with distributions in birds and mammals suggests that these GABA_A receptor complex subunits are strongly conserved in vertebrate evolution and play an important role in the visual, auditory, olfactory and motor systems. © 1997 Elsevier Science Ireland Ltd.

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The neurotransmitter γ -aminobutyric acid (GABA) and its chloride channel GABA/benzodiazepine receptor complex have been described in several vertebrate species (for reviews, see [3,7]). This receptor complex is thought to be a pentameric channel. At least 15 different subunits (α 1–6; β 1–3; γ 1–3; δ and ρ 1–2) have been described in mammals so far (for reviews, see [7,8]). Additional subunit subtypes other than those found in mammals have been described in avian species such as subunits γ 4 and β 4 [2]. At present, little is known about the distribution of the GABA_A receptor complex subunits in vertebrates other than mammals. The antibody used in this study has been reported to label the GABA_A receptor complex β 2/3 subunits in mammals [4], birds [10] and fishes [1]. We report, here, for the first time the labeling of these subunits in frog, demonstrating its presence in a new group of vertebrates, amphibians.

Brains of 12 adult frogs, *Rana pipiens*, were analyzed. After anesthesia by immersion in 0.2% MS222, pH 7.4, frogs were perfused at 4°C, with 30 ml of frog saline followed by 120 ml of one of the following fixatives: 4%

paraformaldehyde, 4% paraformadehyde, 0.1% glutaraldehyde, or 4% paraformaldehyde, 0.1 M l-lysine-0.01 M sodium *m*-periodate. Brains were rapidly removed and left in fresh perfusate fixative for 6 h at 4°C and overnight in 30% sucrose, 0.5 M phosphate buffer (pH 7.4 at 4°C). Coronal sections 50 μ m thick were kept in a cryoprotectant solution (30% sucrose, 1% polyvinylpyrrolidone, 30% ethylene glycol in 0.1 M phosphate buffer, pH 7.2) at 20°C until reacted using a free-floating section method. Monoclonal antibodies against the anti-GABA_A receptor, β 2/3 chains, were used as the primary antibody (Boehringer Mannheim, clone bd-17) and goat anti-mouse biotinylated antibodies as secondary ones (Vector). To detect them, the biotin/avidin system (Vectastain® ABC kit) intensified with nickel salts was used. Control sections were obtain with the same method, but the primary antibody was omitted.

To obtain some degree of quantification, the optical densities of the sections were analyzed using an image analyzer system (VIDAS; Kontron). All sections of each animal were studied under the same conditions of illumination, the images digitized and then measured. The area of each brain showing the greatest optical density (the striatum) was defined as 100% and the same structure in the control

* Corresponding author. Tel.: +34 87 291485; fax: +34 87 291487; e-mail: dbcafl@unileon.es

brain section was defined as 0% of the relative percent optical density scale. Individual optical density values measured at $5\times$ magnification were converted to this relative scale.

The percentage optical densities from different brain areas are shown in Table 1. Fig. 1 shows different representative levels through the brain, and Fig. 2 shows the labeling in the medulla and optic tectum. Densities greater than 75%

Table 1

Structure	% optical densities (mean \pm SEM)
Telencephalon	
(1) Vomeronasal nerve (vn)	3 \pm 3
(2) Glomerular layer of olfactory bulb (g)	57 \pm 7
(3) Internal granule layer of olfactory bulb (ig)	10 \pm 3
(4) Extragranular plexiform layer and mitral cell layer of olfactory bulb (ep + m)	82 \pm 5
(5) Accessory olfactory bulb (aob)	64 \pm 6
(6) Medial postolfactory eminence (po)	45 \pm 6
(7) Dorsal pallium (dp)	47 \pm 9
(8) Medial pallium (mp)	48 \pm 9
(9) Lateral pallium (lp)	64 \pm 8
(10) Medial septal (ms)	28 \pm 4
(11) Lateral septal (ls)	8 \pm 5
(12) Pars medialis of the amygdala (pm)	24 \pm 3
(13) Nucleus accumbens (na)	51 \pm 9
(14) Striatum (st)	92 \pm 4
Diencephalon	
(15) Preoptic area (poa)	42 \pm 3
(16) Dorsal hypothalamic nucleus (dh)	41 \pm 9
(17) Ventral hypothalamic nucleus (vh)	44 \pm 8
(18) Posterior thalamic nucleus (p)	48 \pm 9
(19) Lateral thalamic nucleus posterodorsal (lpd)	56 \pm 4
(20) Lateral thalamic nucleus posteroventral (lpv)	50 \pm 5
(21) Central thalamic nucleus (c)	59 \pm 4
(22) Posterior tubercle (tp)	39 \pm 8
Brain stem	
<i>Layers labeled in the optic tectum (tect)</i>	
(23) Layer 1 (L1)	58 \pm 4
(24) Layer 2 (L2)	60 \pm 3
(25) Layer 3 (L3)	58 \pm 2
(26) Layer 4 (L4)	56 \pm 6
(27) Layer 5 (L5)	60 \pm 1
(28) Layer 6 (L6)	70 \pm 2
(29) Layer 7 (L7)	66 \pm 2
(30) Nucleus anterodorsalis tegmenti mesencephali (ad)	55 \pm 3
(31) Nucleus anteroventralis tegmenti mesencephali (av)	49 \pm 8
(32) Nucleus posterodorsalis tegmenti mesencephali (pd)	55 \pm 8
(33) Nucleus posteroventralis tegmenti mesencephali (pv)	56 \pm 8
(34) Nucleus reticularis isthmian (ris)	51 \pm 7
(35) Nucleus isthmian (is)	36 \pm 8
(36) Nucleus visceralis secundarius (visc)	73 \pm 9
(37) Nucleus interpeduncularis (ip)	35 \pm 9
(38) Nucleus laminaris tori semicircularis (tsl)	42 \pm 5
(39) Nucleus princeps of tori semicircularis (tsp)	92 \pm 1
(40) Nucleus magnocellularis tori semicircularis (tsm)	69 \pm 6
(41) Cerebellum (cer)	74 \pm 8

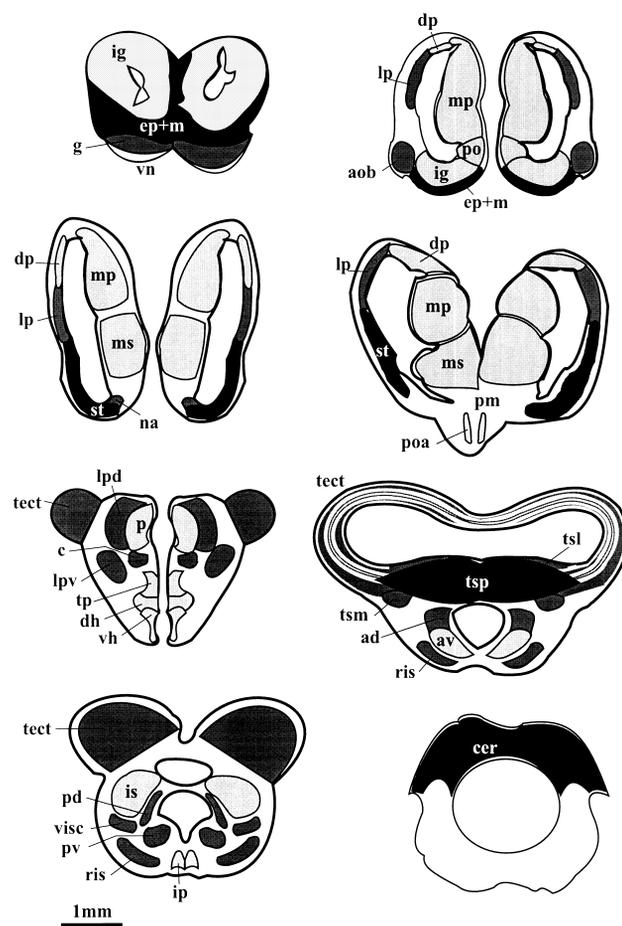


Fig. 1. Coronal sections through the frog brain from the olfactory bulb to the medulla. Abbreviations of the different structure names are indicated in Table 1. Areas $>75\%$ optical density with respect to striatum are represented in black. Areas with 50–75% are in dark gray. Areas with 25–50% are in light gray and areas with less than 25% are represented in white.

were found in olfactory bulb, striatum, torus semicircularis and cerebellum. Densities ranging between 50–75% were found in accessory olfactory bulb, lateral pallium, nucleus accumbens, lateral and central thalamic nucleus, optic tectum, nucleus tegmenti mesencephali, nucleus reticularis isthmi and nucleus visceralis secundarius. Densities between 25–50% were found in dorsal and medial pallium, septal nucleus, preoptic area, hypothalamic nucleus, posterior tubercle and nucleus isthmi.

Since the range of optical densities is not necessarily linear, caution must be exercised with regard to the values of the table since 50% of optical density does not necessarily mean 50% of labeling. The relative values described contribute to a more accurate description of labeling until a more accurate quantification is available.

The distribution of these GABA_A receptor complex subunits, shows a strong labeling in a number of sensory and motor nuclei suggesting an important role of $\beta 2/3$ subunits in both sensory and motor functions. The strong labeling in areas such as olfactory bulb, pyriform cortex, striatum, some layers of tectum opticum, torus semicircularis or cerebellum in frog, is similar to the degree of labeling reported

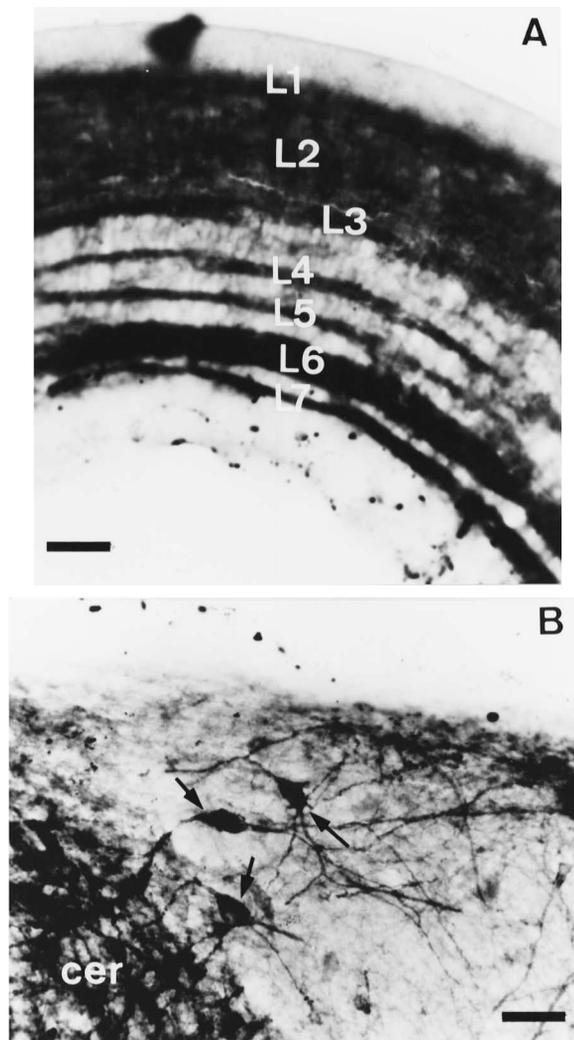


Fig. 2. (A) Displays the optic tectum, L1–L7 correspond to the layers showed in Table 1. (B) Displays neurons from medulla stained by the antibody against GABA_A receptor complex β 2/3 subunits, cer, cerebellum. Bar, (A) 0.1 mm; (B) 50 μ m.

in homologous structures in birds [10,11] and mammals [4] using the same antibody. This strong immunocytochemical labeling also matches the binding densities described either for benzodiazepine binding sites ($[^3\text{H}]$ flunitrazepam binding) or GABA_A binding sites ($[^3\text{H}]$ muscimol binding) in these areas in frogs [9], birds [10], and mammals [5]. These data also fit with those of fishes [1] except in the telencephalon, which could be due to the evaginated telencephalon of teleosts.

One of the unresolved issues in establishing homologies between amphibian and mammalian brains concerns primordial cortex. It has been suggested that amphibian dorsal pallium is homologue to mammalian isocortex; and the amphibian lateral pallium, is either a field homologue of both olfactory cortex and part of isocortex in amniotes. However, some inconsistencies in this homology exist regarding efferents [6]. We observed striking differences

in the frog telencephalon labeling in the lateral vs. the dorsal pallium, indicating that differences other than connectivity patterns exist between these two areas.

The labeling of β 2/3 subunits seems to be higher in the torus semicircularis in frog than the respective homolog nucleus mesencephalicus lateralis, pars dorsalis in birds [10], or in the mammalian inferior colliculus [4]. However, a very high GABA_A receptor complex radiolabeling ($[^3\text{H}]$ flunitrazepam binding) in these respective acoustic areas in frog [9], birds [10], and mammals [5] has been described. These facts suggest that GABA_A receptor plays an important role in the acoustic area in vertebrates and β 2/3 subunits may be more important in lower than in higher vertebrate brains which is also supported by a strong labeling of this area in fishes [1].

One major concern was to determine if this antibody, raised for mammals, labeled specifically the β 2/3 subunits of GABA_A receptor complex in a species as different from mammals as is the frog. The general agreement comparing the immunocytochemical distribution of these subunits between frogs and higher vertebrates mentioned, together with the agreement in comparing the distribution of β 2/3 subunit mRNAs of this complex in mammals [12], strongly support equivalent specificity. However, data from in situ hybridization in frogs is necessary to further confirm this specificity.

In conclusion, the present results support the hypothesis that the distribution of GABA_A receptor β 2/3 subunits is likely to have been highly conserved in phylogeny, since close matches have been observed in frogs in relation to those described in fishes, mammals, and birds using the same monoclonal antibody. The observed distributions indicate that these subunits play an important role in motor systems and in sensory systems such as olfaction, vision and audition.

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