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# Expression and distribution of metabotropic GABA receptor subtypes $GABA_BR1$ and $GABA_BR2$ during rat neocortical development

G. López-Bendito,[1,4] R. Shigemoto,[2] A. Kulik,[2] O. Paulsen,[3] A. Fairén[4] and R. Luján[5]
[1]Department of Human Anatomy and Genetics, University of Oxford, Oxford, OX1 [3]QX, UK
[2]Division of Cerebral Structure, National Institute for Physiological Sciences, Myodaiji, Okazaki 444–8585, CREST Japan Science and Technology Corporation, Japan
[3]University Laboratory of Physiology, Parks Road, Oxford, OX1 3PT, UK
[4]Instituto de Neurociencias-CSIC, Universidad Miguel Hernández, Campus de San Juan, 03550 San Juan de Alicante, Spain
[5]Centro Regional de Investigaciones Biomédicas, Facultad de Medicina, Universidad Castilla-La Mancha, Campus de Albacete, 02071 Albacete, Spain

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## Abstract

To understand the possible contribution of metabotropic γ-aminobutyric acid receptors ($GABA_BR$) in cortical development, we investigated the expression pattern and the cellular and subcellular localization of the $GABA_BR1$ and $GABA_BR2$ subtypes in the rat neocortex from embryonic day 14 (E14) to adulthood. At the light microscopic level, both $GABA_BR1$ and $GABA_BR2$ were detected as early as E14. During prenatal development, both subtypes were expressed highly in the cortical plate. Using double immunofluorescence, $GABA_BR1$ colocalized with $GABA_BR2$ in neurons of the marginal zone and subplate, indicating that these proteins are coexpressed and could be forming functional $GABA_BRs$ during prenatal development *in vivo*. In contrast, only $GABA_BR1$ but not $GABA_BR2$ was detected in the tangentially migratory cells in the lower intermediate zone. During postnatal development, immunoreactivity for $GABA_BR1$ and $GABA_BR2$ was distributed mainly in pyramidal cells. Discrete $GABA_BR1$-immunopositive cell bodies of interneurons were present throughout the neocortex. In addition, $GABA_BR1$ but not $GABA_BR2$ was found in identified Cajal-Retzius cells in layer I. At the electron microscopic level, immunoreactivity for $GABA_BR1$ and $GABA_BR2$ was found in dendritic spines and dendritic shafts at extrasynaptic and perisynaptic sites throughout postnatal development. We further demonstrated the presynaptic localization of $GABA_BR1$ and $GABA_BR2$, as well as the association of the receptors with asymmetrical synaptic junctions. These results indicate potentially important roles for the $GABA_BRs$ in the regulation of migratory processes during corticogenesis and in the modulation of synaptic transmission during early development of cortical circuitry.

## Introduction

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the cerebral cortex. The response to GABA is mediated via activation of both ionotropic and metabotropic receptors (Macdonald & Olsen, 1994; Misgeld *et al*., 1995). Metabotropic GABA receptors ($GABA_BRs$) are coupled to intracellular signal transduction mechanisms via G-proteins and mediate slow inhibitory postsynaptic potentials (Misgeld *et al*., 1995). Activation of $GABA_BRs$ can modulate multiple signal transduction pathways, mainly activating $K^+$ channels, inhibiting voltage-dependent $Ca^{2+}$ channels, stimulating phospholipase A2, inhibiting adenylate cyclase and regulating inositol phospholipid hydrolysis (Misgeld *et al*., 1995).

Two different cDNAs encoding $GABA_BRs$ have been cloned to date: $GABA_BR1$, which exist in alternatively spliced forms designated R1a–e, and $GABA_BR2$ (Bowery & Brown, 1997; Kaupmann *et al*., 1997, 1998a; Isomoto *et al*., 1998; Pfaff *et al*., 1999; Schwarz *et al*., 2000). It has been suggested that heterodimeric assembly of $GABA_BR1a/b$ and $GABA_BR2$ subtypes is required to provide fully functional receptors (Jones *et al*., 1998; Kaupmann *et al*., 1998a; White *et al*., 1998; Kuner *et al*., 1999).

Light microscopic immunocytochemistry, *in situ* hybridization and receptor autoradiographic studies have shown that $GABA_BRs$ are abundant in the cerebral cortex of adult animals (Bowery *et al*., 1987; Chu *et al*., 1990; Turgeon & Albin, 1994; Kaupmann *et al*., 1997; Bischoff *et al*., 1999; Margeta-Mitrovic *et al*., 1999; Muñoz *et al*., 2001). Electron microscopic studies revealed that $GABA_BRs$ are present in both excitatory and inhibitory synapses and also located outside the synaptic membrane specialization, at both postsynaptic and presynaptic locations (Gonchar *et al*., 2001; Kulik *et al*., 2002). Electrophysiological and pharmacological studies have also characterized pre- and postsynaptic inhibitory functions of $GABA_BRs$ in the neocortex (Howe *et al*., 1987; Deisz *et al*., 1993, 1997; Varela *et al*., 1997). Presynaptic $GABA_BRs$ are thought to suppress neurotransmitter release by depressing $Ca^{2+}$ influx via voltage-activated $Ca^{2+}$ channels, whereas postsynaptic $GABA_BRs$ decrease neuronal excitability by activating an inwardly rectifying $K^+$ (Kir3) conductance (Kaupmann *et al*., 1998b).

*Correspondence*: Dr Guillermina López-Bendito, [1]Department of Human Anatomy & Genetics, as above.
E-mail: guillermina.lopez-bendito@anat.ox.ac.uk

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Little information is available about the expression of $GABA_BRs$ in the developing neocortex. Nevertheless, several lines of evidence suggest that $GABA_BR$ play important roles during cortical development. First, $GABA_BRs$ modulate the excitatory actions of glutamate in developing cortical neurons through the regulation of glutamate-activated calcium transients (Obrietan & van den Pol, 1999) and second, specific $GABA_BR$ agonists stimulated migration of neurons (Behar *et al*., 1998), whereas specific antagonists inhibited migration (Behar *et al*., 2000).

Information regarding the distribution of $GABA_BRs$ in the cerebral cortex is a prerequisite for studies designed to elucidate the contribution of $GABA_BRs$ to developmental processes and to cortical function. However, the distribution and localization of $GABA_BR$ subtypes during corticogenesis are incompletely characterized. To better understand the mechanisms by which $GABA_BRs$ mediate the physiological effects of GABA on developing cortical neurons, the goal of the present study was to characterize the expression pattern, and the cellular and the precise subcellular localization of $GABA_BR1$ and $GABA_BR2$ in the rat neocortex during pre- and postnatal development.

## Materials and methods

Thirty-nine Wistar rats from embryonic day 14 (E14) to adulthood, obtained from the Animal House Facilities of the University Miguel Hernández in Alicante and the Department of Human Anatomy and Genetics in Oxford were used in the present study. The care and handling of the animals prior to and during the experimental procedures followed European Union and UK Home Office regulations, and were approved by the Animal Care and Use Committees of the authors' institutions.

For each developmental stage, the animals used ($n = 3$ for each stage) were from different litters and were grouped as follows: E14, E16, E18, postnatal day 0 (P0, day of birth), P3, P5, P7, P10, P12, P15, P21, P30 and P60.

### *Antibodies and controls*

Affinity-purified polyclonal antibodies against $GABA_BR1a/b$ and $GABA_BR2$ were raised in rabbits and guinea pig, respectively. The characteristics and specificity of the antibody against $GABA_BR1/b$ have been described elsewhere (Kulik *et al*., 2002). The antibody against $GABA_BR2$ was obtained from Chemicon (Temecula, CA, USA). To identify neuronal populations during cortical development, we used a monoclonal antibody, G10, against Reelin (De Bergeyck *et al*., 1998) gift of Dr A.M. Goffinet (Neurobiology Unit, University of Namur School of Medicine, Belgium), a monoclonal antibody, 12E3, against PSA-NCAM protein (the polysialylised form of the neuronal cell adhesion molecule NCAM; Seki and Arai, 1991), gift of Dr Tatsunori Seki (Department of Anatomy, Juntendo University School of Medicine, Tokyo, Japan), and a monoclonal antibody against calbindin (Chemicon, Temecula, CA, USA).

To test method specificity in the procedures for light and electron microscopy, the primary antibody was omitted or replaced with 5% (v/v) normal serum of the species of the primary antibody. Under these conditions, no selective labelling was observed. Staining patterns were also compared to those obtained by calretinin and calbindin (Chemicon, Temecula, CA, USA); only the antibodies against $GABA_BR1$ and $GABA_BR2$ labelled consistently the plasma membrane of cells. When double labelling was used, some sections were always incubated with only one primary antibody and the full complement of secondary antibodies to test for any cross-reactivity. Other sections were incubated with two primary antibodies and one secondary antibody, followed by the full sequence of signal detection. No cross-labelling was detected that would influence the results.

### *Immunocytochemistry for metabotropic GABA receptors*

#### *Light microscopic procedures*

Fetuses were collected by caesarean section after anaesthesia of the dam with an intraperitoneal (i.p) injection of a Rompun–Imalgene mixture (0.1 mL/kg body weight). Animals were deeply anaesthetized by hypothermia (E14 to P5) or by i.p. injection of Rompun–Imalgene (1 : 1, 0.1 mL/kg body weight) and then the hearts were exposed surgically for perfusion fixation (López-Bendito *et al*., 2001, 2002). After perfusion, tissue blocks containing the neocortex were dissected and washed thoroughly in 0.1 M phosphate buffer (PB, pH 7.4). Coronal, 60 μm sections were cut with a Vibratome and collected in 0.1 M PB. For the immunoperoxidase method, sections were incubated in 10% normal goat serum (NGS) in 50 mM Tris buffer (pH 7.4) containing 0.9% NaCl (TBS), with 0.2% Triton X-100, for 1 hour. Sections were then incubated for 48 hours with affinity-purified polyclonal antibodies against $GABA_BR1a/b$ or $GABA_BR2$ at a final protein concentration of 1–2 μg/mL in TBS containing 1% NGS. After washes in TBS, the sections were incubated for 2 hours in biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) diluted 1 : 50 in TBS containing 1% NGS. They were then transferred into avidin–biotin–peroxidase complex (ABC kit, Vector Laboratories, Burlingame, CA, USA) diluted 1 : 100 and left for 2 hours at room temperature. Peroxidase enzyme activity was revealed using 3,3′-diaminobenzidine tetrahydrochloride (DAB; 0.05% in TB, pH 7.4) as the chromogen and 0.01% $H_2O_2$ as substrate. Finally, the sections were air-dried and coverslipped prior to observation with a Leica DMRS photomicroscope equipped with differential interference contrast optics.

Alternate sections were used for immunofluorescence experiments using a laser scanning confocal microscope (Leica TSLCM, Austria). Primary antibodies used in combination with a polyclonal antibody against $GABA_BR1$ were: guinea pig polyclonal anti-$GABA_BR2$; monoclonal anti-PSA-NCAM, diluted 1 : 1000; monoclonal anti-Reelin antibody, G10, diluted 1 : 1000 and monoclonal anti-calbindin antibody diluted 1 : 1000. Sections were incubated overnight at 4 °C in a mixture of primary antibodies in TBS containing 1% NGS and 0.1% Triton X-100. Sections were then washed in TBS and incubated for two hours in a mixture of secondary antibodies coupled to the cyanine-derived fluorochromes Cy2 or Cy3 (Amersham, UK). These secondary antibodies were used at a dilution of 1 : 200 in TBS with 0.1% Triton X-100. After extensive TBS washes, the sections were mounted on gelatinized slides and coverslipped with Vectashield (Vector Laboratories, Burlingame, CA, USA).

Each labelling combination was analysed in sections from two to four prenatal or postnatal animals. Co-localization of $GABA_BR1$ with $GABA_BR2$ or with the neurochemical markers Reelin and calbindin was assessed by confocal laser microscopy (Leica TLSCM, Austria). For each marker, a semiquantitative estimation of the proportion of double labelled neurons was performed by analysing samples of 120–160 cells, collected in 8–10 coronal sections through the neocortex. Images were stored digitally and analysed using appropriate software supplied by the microscope manufacturer. Brightness and contrast were adjusted for the whole frame; no part of a frame was enhanced or modified in any way.

*Electron microscopic procedures*

A similar procedure to that described earlier (López-Bendito *et al.*, 2001, 2002) was used.

*Pre-embedding immunoperoxidase and immunogold methods.* Immunocytochemical reactions for the pre-embedding method were carried out as described above, but with the omission of Triton X-100 in all steps. Sections for the pre-embedding immunogold method were incubated together with those used for peroxidase reaction up to the secondary antibody stage. They were then incubated in goat anti-rabbit or goat anti-guinea pig IgG coupled to 1.4 nm gold particles (Nanoprobes Inc., Stony Brook, NY) diluted 1 : 100 in TBS containing 1% NGS. After several washes in phosphate-buffered saline (PBS), the sections were postfixed in 1% glutaraldehyde dissolved in the same buffer. They were washed in double distilled water, followed by silver enhancement of the gold particles with an HQ Silver kit (Nanoprobes Inc., Stony Brook, NY).

The peroxidase-reacted sections and the gold–silver-labelled sections were treated with $OsO_4$ (1% in 0.1 M PB), block-stained with uranyl acetate, dehydrated in graded series of ethanol and flat-embedded on glass slides in Durcupan (Fluka, Reidel-deHaën, UK) resin. Regions of interest were cut at 70–90-nm-thick sections using an ultramicrotome (Reichert Ultracut E, Leica, Austria). Ultrathin sections were contrasted and analysed in a Jeol-100 electron microscope.

## Results

### *Expression of $GABA_BRs$ during prenatal development*

To study the tissue expression of $GABA_BRs$ during prenatal cortical development we carried out pre-embedding immunohistochemical studies at different ages.

*$GABA_BR1$*

At E14, $GABA_BR1$ immunoreactivity was detected in cells bodies located in the mantle layer of the septal and ganglionic eminences (Fig. 1A–D). The intensity of immunoreactivity was strongest at the palaeocortical primordium, at the surface of the lateral and medial ganglionic eminences. Immunoreactivity at the neocortical primordium was less intense (Fig. 1C). The receptor expression level increased with development. Thus, at E16 we found strong immunoreactivity for $GABA_BR1$ in the habenula, dorsal thalamus, thalamic eminence, hypothalamus, in a circumscribed area surrounding the forming internal capsule and in the marginal zone (MZ) of the neocortical primordia (Fig. 1E). In the MZ, we could distinguish at least two populations of cells immunoreactive for $GABA_BR1$: large rounded cells located deeply in that layer, and superficial cells running horizontally (Fig. 1H). In the neocortical primordium, the cortical plate (CP) and the subplate (SP) were also immunoreactive (Fig. 1E–G). In addition, $GABA_BR1$ immunoreactivity was also present in the tangential migratory cells in the intermediate zone (Fig. 1G and I).

At E18 we found increased levels of $GABA_BR1$ immunoreactivity in the same regions as in previous ages (Fig. 1J–L). Immunoreactivity in the CP was observed in apical dendrites of SP neurons or in ascendent or descendent fibres running in this zone (Fig. 1K and L). $GABA_BR1$ immunoreactivity was also present in axons running in the intermediate zone, just below the SP (see Fig. 1K and L).

*$GABA_BR2$*

At E14, $GABA_BR2$ immunoreactivity was found at very low levels in most telencephalic areas (Fig. 2A), though it was intense in cells localized in the preplate (PP, Fig. 2A and B). The receptor expression level increased with development. Thus, at E16 we found strong $GABA_BR2$ immunoreactivity in the neocortical MZ, CP and SP (Fig. 2C–E). In addition, we observed immunolabelled axons in the intermediate zone, as well as numerous immunopositive processes in the ventricular zone (VZ, Fig. 2D and C). However, we did not detect $GABA_BR2$ immunoreactivity in cell bodies of tangentially migratory neurons in the intermediate zone.

At E18, $GABA_BR2$ immunoreactivity was strong in the neocortex (Fig. 2F–I), and found in axons running in the lower intermediate zone, as well as in dendrites crossing the CP vertically (Fig. 2G–I). The pyramidal cell layer in and above the SP was devoid of $GABA_BR2$ immunoreactivity. In the VZ, $GABA_BR2$ immunoreactivity was stronger than at earlier ages, especially in vertical processes located in the lower part, possibly related with radial glial cell processes (Fig 2G and I).

*Co-localization of $GABA_BR1$ and $GABA_BR2$*

In order to investigate whether $GABA_BR1$ and $GABA_BR2$ were coexpressed in individual neurons during development, we carried out double-immunofluorescence experiments (Fig. 3). At E16, we observed, in the cortical MZ, that around 95% of the cells immunoreactive for $GABA_BR2$ were also labelled for $GABA_BR1$ (Fig. 3), resembling pioneer neurons of the MZ (Meyer *et al.*, 1998) and SP neurons. The tangentially migratory cells located in the intermediate zone immunoreactive for $GABA_BR1$, were not labelled for $GABA_BR2$ (Fig. 3B, also see Fig. 1G).

### *Characterization of neurons expressing $GABA_BRs$*

In order to establish which neuronal populations express $GABA_BR1$ at early stages of development, we carried out double-immunofluorescence experiments for the receptor and for specific neuronal markers using confocal microscopy. We used PSA-NCAM (Fig. 4B), a specific protein of tangentially migratory neurons in the cortical primordium (Seki & Arai, 1991); calbindin (CB, Fig. 4E), a specific protein expressed in interneurons at early stages of development (Anderson *et al.*, 1997); and Reelin (Fig. 4H), an extracellular matrix glycoprotein that is synthesized and secreted by Cajal-Retzius cells (D'Arcangelo *et al.*, 1995).

We observed an intense labelling for PSA-NCAM in cells located in the intermediate zone, SP and MZ (Fig. 4B), displaying the morphology of tangentially migrating neurons. A quantitative survey showed that around 97% of the cells immunopositive for PSA-NCAM in the MZ and intermediate zone also showed $GABA_BR1$ immunoreactivity (Fig. 4C), suggesting that the receptor was expressed in the majority of the tangentially migratory cells. However, not all cells with $GABA_BR1$ immunoreactivity also expressed PSA-NCAM, indicating that the receptor might be also present in a population of nonmigratory neurons.

Using a monoclonal antibody against calbindin, we found intense labelling in cells located in the cortical MZ, corresponding to large rounded cells (Fig. 4E), resembling pioneer neurons. Quantitative studies showed that at least 83% of those cells also showed $GABA_BR1$ immunoreactivity.

Finally, in order to investigate whether the horizontal neurons expressing $GABA_BR1$ in the cortical layer I were Cajal-Retzius cells, we used Reelin (Fig. 4H). In prenatal animals, we did not detect colocalization of $GABA_BR1$ and Reelin. However, in postnatal

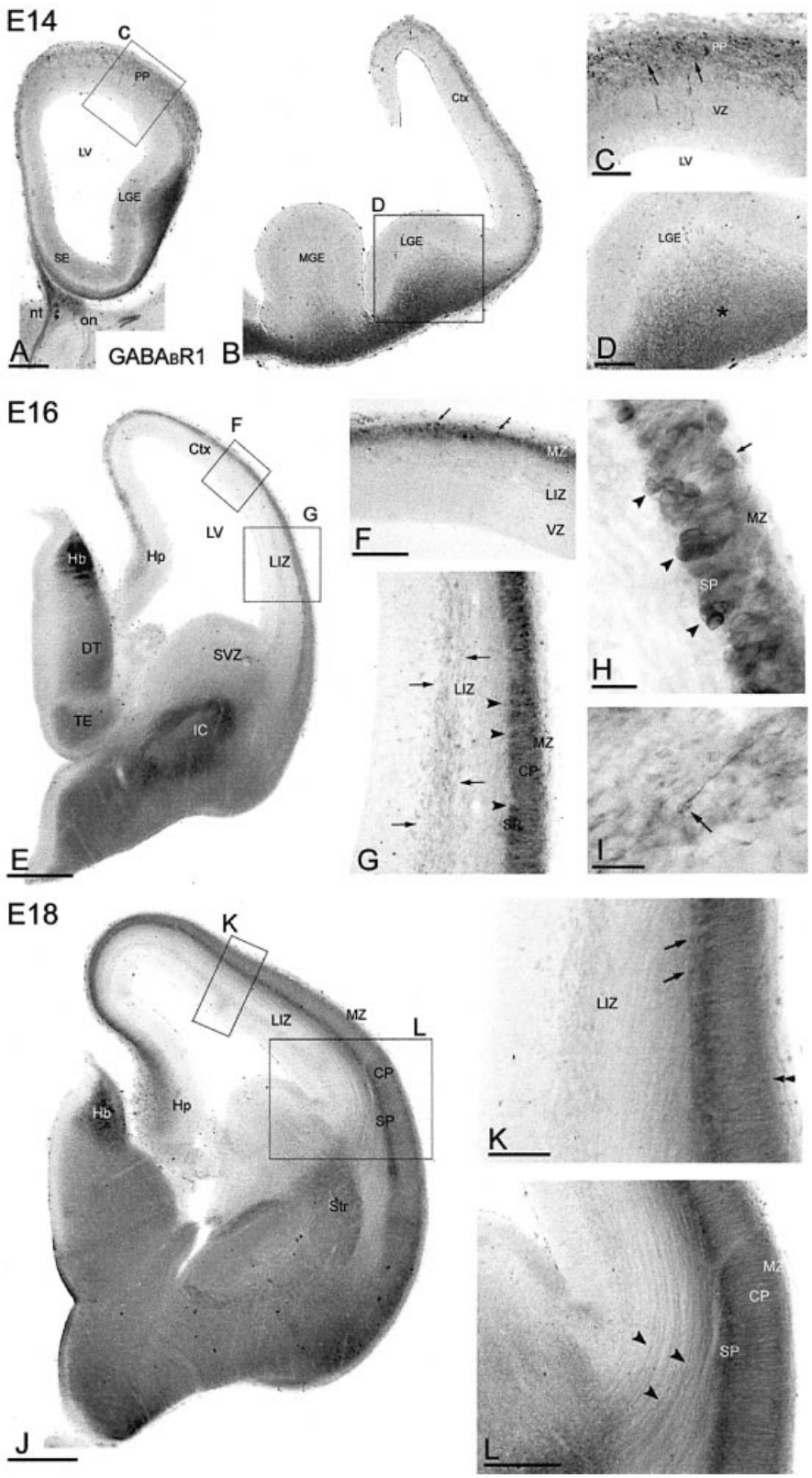


FIG. 1. Immunoreactivity for $GABA_BR1$ during prenatal development using a pre-embedding immunoperoxidase method. Rostral (A) and mid-caudal (B) sections of the telencephalon at E14 showing $GABA_BR1$ immunoreactivity in the mantle zones of the septal (SE) and of both medial (MGE) and lateral (LGE) ganglionic eminences, and in the preplate (PP) and marginal zone (MZ) of the cortical anlage. Ctx, cortex; nt, *nervus terminalis* on, olfactory nerve. (C) Detail of the telencephalic area boxed in panel A, showing strong $GABA_BR1$ immunolabelling in PP cells (arrows) and weaker labelling in the ventricular zone (VZ). (D) Detail of the LGE area boxed in panel B. The mantle layer of the LGE contained a large number of cells expressing $GABA_BR1$ (asterisk). (E) Mid-caudal coronal section of the telencephalon at E16 showing a strong $GABA_BR1$ immunolabelling in the habenula (Hb), dorsal thalamus (DT), internal capsule (IC) and cortex (Ctx). Hp, hippocampus; SVZ, subventricular zone; LIZ, lower intermediate zone. (F) Detail of the cortex boxed in panel E. $GABA_BR1$ immunolabelling was observed in large rounded cells of the cortical MZ (arrows). (G and I) $GABA_BR1$ immunoreactivity was also strong in the tangential migratory cells located in the lower part of the intermediate zone (LIZ) (e.g. arrows). (G and H) $GABA_BR1$ immunoreactivity was also detected in cells located in the subplate (SP) (arrowheads), forming groups in this cortical layer (arrowheads). (J) Mid-caudal coronal section of the telencephalon at E18 showing a strong $GABA_BR1$ immunoreactivity in the SP, CP and MZ of the neocortex, and in the tangentially migratory neurons in the LIZ. Str, striatum. (K and L) Detail of the areas boxed in panel J, showing $GABA_BR1$ immunopositive cells in the SP (arrows) and MZ (double arrowhead), as well as in axons located immediately beneath the SP (arrowhead). on, olfactory nerve; LV, lateral ventricle; DT, dorsal thalamus; IC, internal capsule; TE, thalamic eminence. Scale bars, 200 μm (A–B and L); 100 μm (C, D, F, G and K); 500 μm (E and J); 30 μm, (H and I).

animals (P2), the estimate of colocalization showed a 97% overlap for $GABA_BR1$- and Reelin-immunoreactivity in cortical layer I cells (Fig. 4I), suggesting that the neurons expressing $GABA_BR1$ are indeed Cajal-Retzius cells.

### *Expression of $GABA_BRs$ during postnatal development*

To study the tissue expression of $GABA_BRs$ during postnatal development we carried out pre-embedding immunohistochemical studies at different ages up to adulthood.

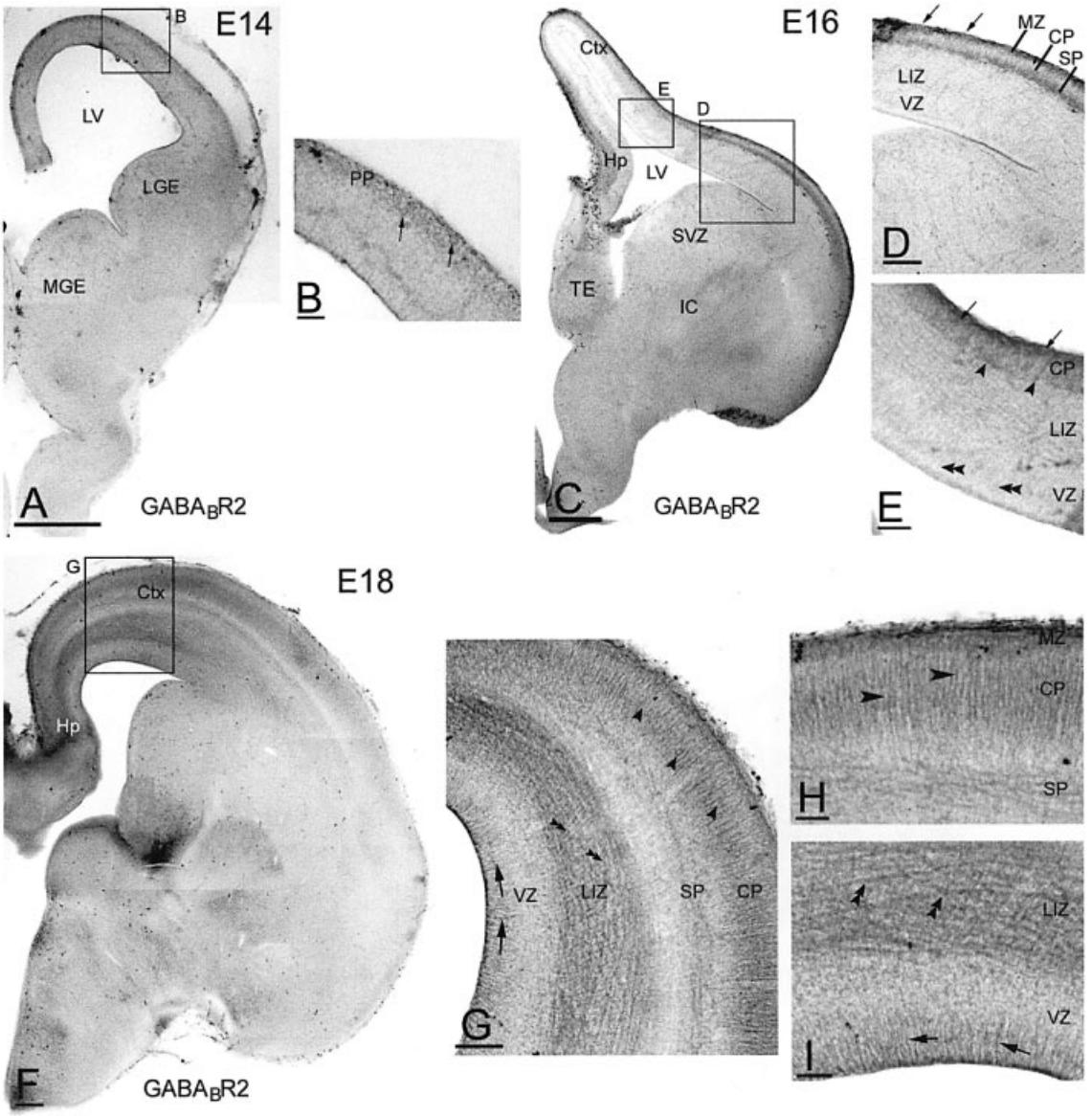


FIG. 2. Immunoreactivity for GABA$_B$R2 during prenatal development using a pre-embedding immunoperoxidase method. (A) Coronal section at E14 showing GABA$_B$R2 immunoreactivity in the preplate (PP) of the cortex, with no detectable immunoreactivity observed in other areas. (B) Detail of the PP boxed in panel A. GABA$_B$R2 immunoreactivity was observed in cells located throughout this cortical stratum. (C–E) Mid-caudal coronal section at E16 showing GABA$_B$R2 immunoreactivity in different telencephalic areas. (D and E) Detail of two cortical areas boxed in panel C, showing GABA$_B$R2 immunoreactivity in cells located in the subplate (SP, arrowheads), cortical plate (CP) and marginal zone (MZ, arrows), as well as in processes and cells distributed in the ventricular zone (VZ, double arrowheads). (F–I) Mid-caudal coronal section at E18 showing GABA$_B$R2 immunoreactivity in different telencephalic areas. (G) Detail of boxed area in panel F. Immunoreactivity was localized in CP cells (arrowheads). Axons immunoreactive for GABA$_B$R2 were detected immediately above the VZ (double arrowhead). Immunoreactivity for GABA$_B$R2 in the VZ was more evident at E18 than at earlier ages (arrows). Ctx, cortex; MGE, medial ganglionic eminence; LGE, lateral ganglionic eminence; Hp, hippocampus, DT, dorsal thalamus; LIZ, lower intermediate zone; IC, internal capsule; SVZ, subventricular zone; LV, lateral ventricle. Scale bars, 200 μm (A, C and F); 50 μm (B, E, H and I); 100 μm (D and G).

*GABA$_B$R1*

At birth, we found high expression levels in all layers of the neocortex (Fig. 5A). Thus, GABA$_B$R1 immunoreactivity was detected mainly in cortical layer I neurons (Fig. 6B) having the typical fusiform morphology and horizontal orientation of Cajal-Retzius cells. In supragranular layers, GABA$_B$R1 was observed in vertical processes most likely corresponding to the apical dendrites of layers IV–V pyramidal cells (Fig. 5A). This characteristic pattern of distribution changed during development. Thus, at around P7, we observed strong labelling in somata (Fig. 5C and F) and apical dendrites (Fig. 5E) of layer V pyramidal cells, and in layer VIb cells (Fig. 5A, F, I and N).

At P7, GABA$_B$R1 immunolabelling was found in interneurons of supragranular layers, most of them located between layers I and II/III (Fig. 5D and G). GABA$_B$R1 immunolabelling was still found in

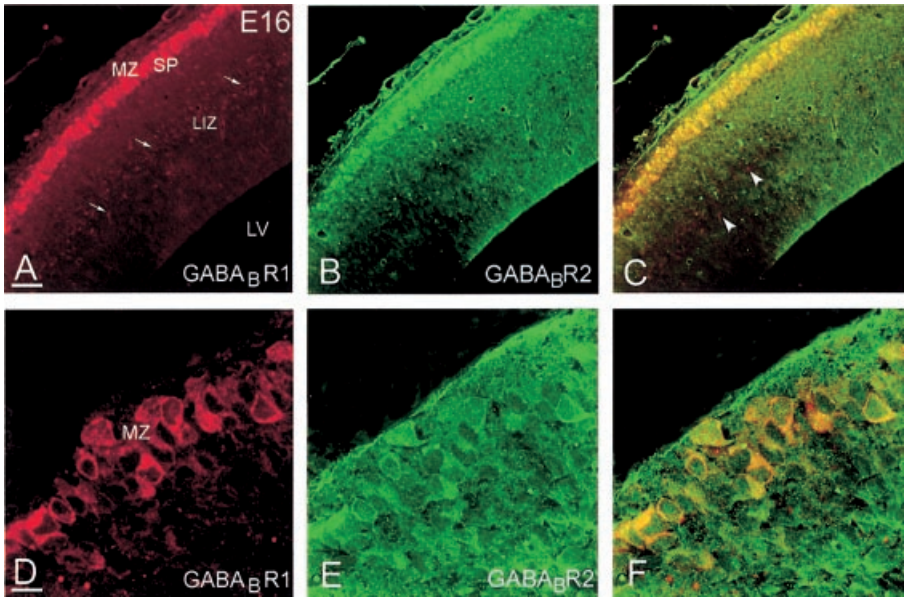


FIG. 3. Co-localization of $GABA_BR1$ and $GABA_BR2$ during prenatal development as demonstrated using double immunofluorescence. Low magnification of a neocortical region at E16 double-labelled for $GABA_BR1$ (A, red) and $GABA_BR2$ (B, green). (C) Overlay of panels A and B. Note the high degree of colocalization of these $GABA_BRs$ at the MZ. However, cells in the LIZ appear immunoreactive for $GABA_BR1$ but not for $GABA_BR2$ (arrowheads). (D–F) High magnification of MZ region labelled for both $GABA_BRs$. Virtually all cells in that region showed colocalization of the two receptor subtypes. Scale bars, 10 μm (A–F).

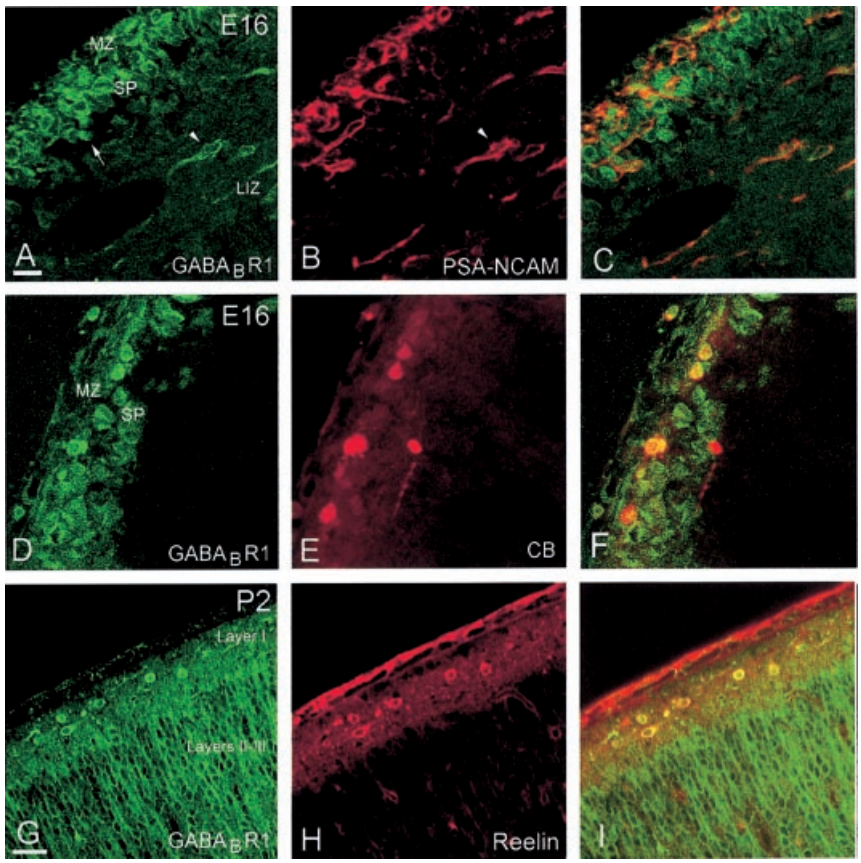


FIG. 4. Characterization of cell populations expressing $GABA_BR1$ during pre- and postnatal development. Double-immunofluorescence for $GABA_BR1$ and PSA-NCAM, calbindin and Reelin. (A–C) Immunofluorescence for $GABA_BR1$ (A, green) and PSA-NCAM (B, red) in the neocortex at E16. (C) Overlay of panel A and B. Almost all the PSA-NCAM positive cells expressed $GABA_BR1$ (e.g., arrowheads). (D–F) Immunofluorescence for $GABA_BR1$ (D, green) and calbindin (E, red), showing that almost all calbindin positive cells in the MZ were positive for $GABA_BR1$ (F, overlay). (G–I), Immunofluorescence for $GABA_BR1$ (G, green) and Reelin (H, red) in layer I of the neocortex at P2. I, Overlay of panel G and H. $GABA_BR1$ immunoreactivity was observed throughout all layers of the cortex. The specific protein of Cajal-Retzius cells, Reelin, was restricted to layer I neurons (see panel N).Virtually all cells expressing Reelin also showed $GABA_BR1$. Scale bars, 20 μm (A–I); 40 μm (G–I).

putative Cajal-Retzius cells (Fig. 5D), though at P10 the number of those neurons decreased (Fig. 5G) and disappeared shortly after that age. $GABA_BR1$ immunoreactivity was also observed in layer V pyramidal cells and their apical dendrites (Fig. 5C, E and F).

At P15, the strong $GABA_BR1$ labelling of pyramidal cell somata and apical dendrites decreased dramatically, while an intense neuropilar staining appeared in all cortical layers, especially in layer V (Fig. 5I). In addition, we also observed many $GABA_BR1$-immunoreactive interneurons with multipolar and bipolar (Fig. 5I and J) morphology distributed throughout all cortical layers (Fig. 5J).

At P21–P30, the adult levels of $GABA_BR1$ immunoreactivity in the neocortex were reached. Immunolabelling was intense in the

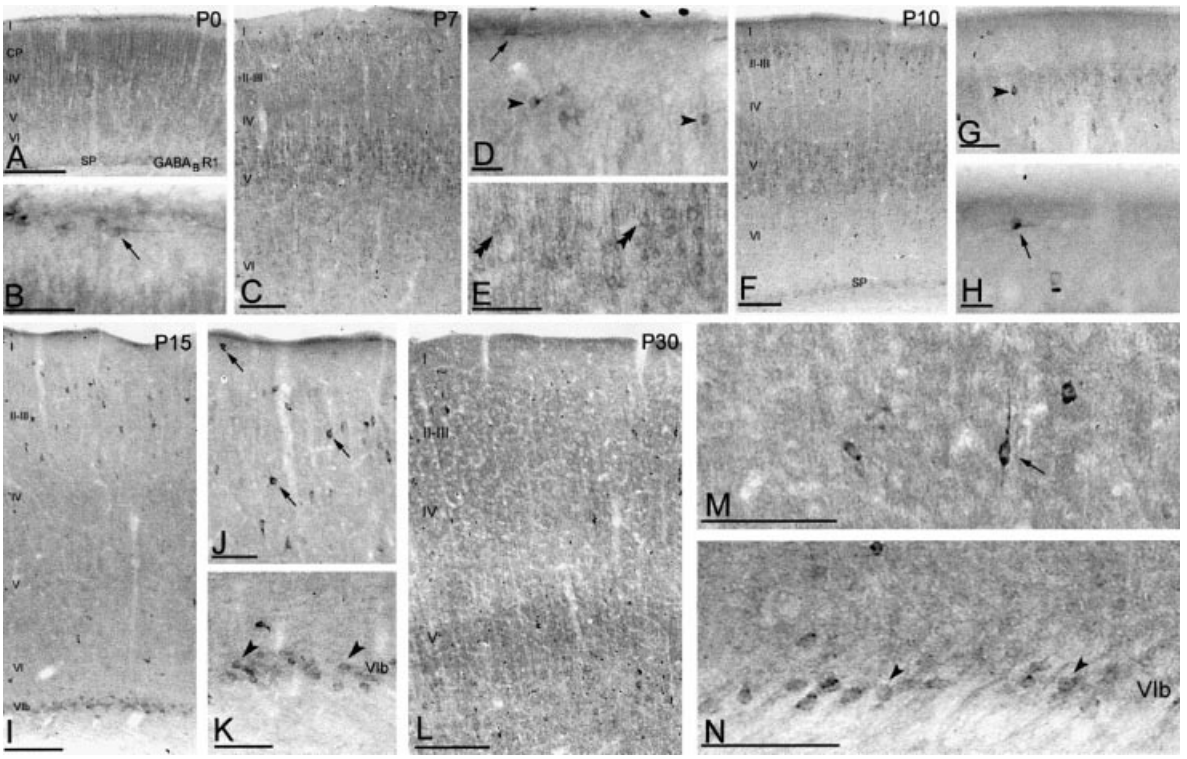


FIG. 5. Immunoreactivity for GABA$_B$R1 in the neocortex during postnatal development using a pre-embedding immunoperoxidase method. (A) At P0, GABA$_B$R1 immunolabelling was detected in somata and apical dendrites of neocortical pyramidal cells. Immunolabelling was intense in layers II/III and in cells located in layer IV. (B) Detail of the GABA$_B$R1 immunoreactivity in cells with a horizontal morphology located in layer I (arrow). (C–E) At P7, strong GABA$_B$R1 immunoreactivity was found in somata and apical dendrites of layer V pyramidal neurons (E, double arrowheads). GABA$_B$R1 immunoreactive cells were detected in layer I (D, arrow), as well as in interneurons in the upper part of layer II/III (D, arrowheads). (F–H) At P10, intense GABA$_B$R1 immunolabelling was observed in layer V cells, layer II/III interneurons (arrowheads) and in cells located in layer VIb. The number of immunopositive cells in layer I considerably decreased with age, and only a few cells could still be detected at P10 (arrow). (I–K) At P15, GABA$_B$R1 immunoreactivity was found in interneurons throughout the necortex, though particularly strong in supragranular layers (J, arrows), as well as in cells in layer VIb (K, arrowheads). A neuropilar staining was also detected throughout the neocortex. (L–N) At P30, the number of interneurons immunoreactive for GABA$_B$R1 decreased (M, arrow), although the neuropilar immunoreactivity remained similar to that at earlier ages. We also detected immunopositive cells located in layer VIb (arrowheads). Scale bars, 200 μm (A, C, F, I and L), 20 μm (B and D); 100 μm (E, G, J, K, M and N).

neuropil of layers I, II/III and V (Fig. 5L), as well as a large proportion of immunoreactive interneuron somata and dendritic processes throughout all cortical layers (Fig. 5M).

*GABA$_B$R2*

During postnatal ages, we also observed a high expression of GABA$_B$R2 in the neocortex. At P2, we found an intense GABA$_B$R2 immunolabelling in most cortical layers (Fig. 6A and B), and particularly strong in the neuropil of layer I (Fig. 6B). In supragranular layers, GABA$_B$R2 was detected in vertical processes, probably apical dendrites of infragranular pyramidal cells (Fig. 6B), though this characteristic pattern of distribution changed during development (Fig. 6). Thus, at P7 and P10, the strongest neuropilar staining was detected in layer I (Fig. 6C and D), but from P15 onwards, all layers reached similar levels of GABA$_B$R2 immunoreactivity (Fig. 6E and F). We did not detect immunoreactive somata of pyramidal cells or interneurons in any layer at any developmental age.

### *Subcellular localization of GABA$_B$Rs during pre- and postnatal development*

To investigate the precise subcellular localization of GABA$_B$R1 and GABA$_B$R2 during pre- and postnatal development we carried out electron microscopic investigations using the pre-embedding immunoperoxidase and immunogold techniques.

*GABA$_B$R1*

During prenatal development we concentrated on the SP and CP, where the intensity of immunostaining at E18 was the strongest. GABA$_B$R1 immunoreactivity was found postsynaptically in SP cells with an elongated morphology and only one dendritic process (Fig. 7) running parallel to the pial surface. In all the cases, the peroxidase reaction end-product was observed forming patches and associated with the plasma membrane (Fig. 7A and B) or intracellular organelles (Fig. 7C and D).

During postnatal development, GABA$_B$R1 immunoreactivity was associated to the plasma membrane and the endoplasmic reticulum (Fig. 8A–C) of layer I cells with an ultrastructure resembling that of Cajal-Retzius cells (Fig. 8A). Strong GABA$_B$R1 immunoreactivity was also found in the somata (Fig. 8D) and dendritic trees (Fig. 8D–F) of a subset of interneurons in all layers of the neocortex, invariably associated with the plasma membrane and intracellular organelles. Peroxidase reaction end-product for GABA$_B$R1 filled dendritic spines of pyramidal cells throughout all cortical layers (Fig. 8G and H). The dendritic shafts of pyramidal cells were also immunolabelled, but the density of the reaction product was consistently lower than that observed within the spines.

Finally, we also observed GABA$_B$R1 immunoreactivity in presynaptic elements, distributed throughout all cortical layers and

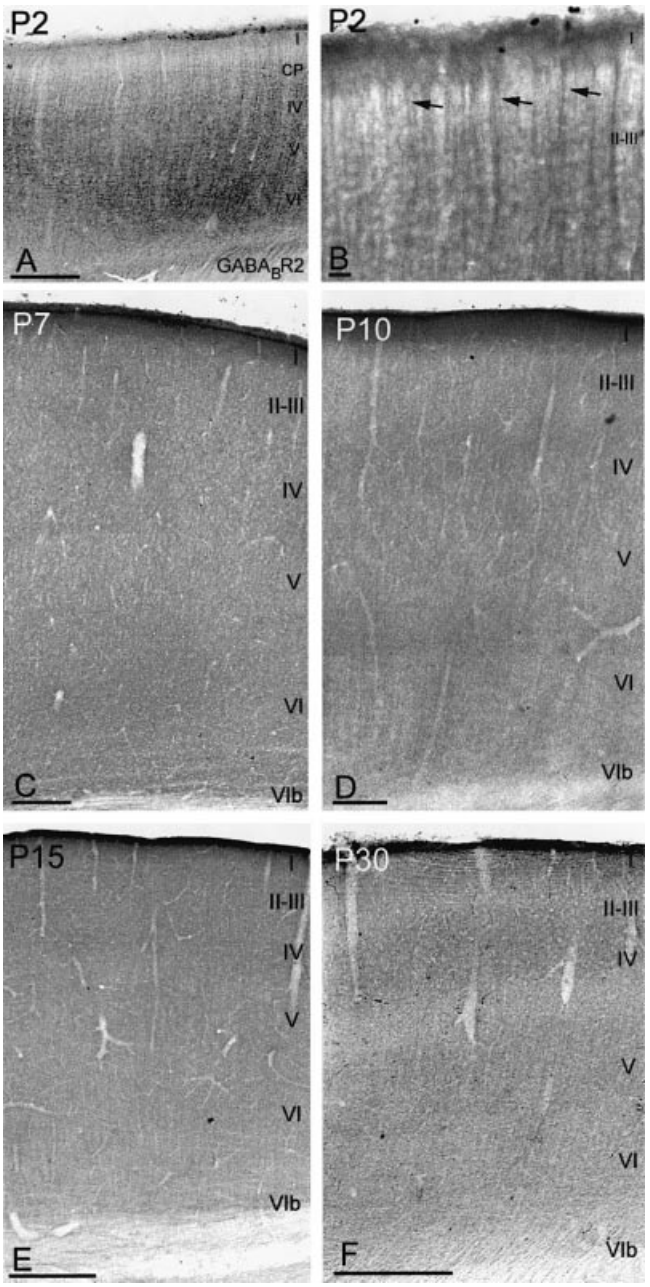


FIG. 6. Immunoreactivity for $GABA_BR2$ in the neocortex during postnatal development using a pre-embedding peroxidase method. (A) At P2, $GABA_BR2$ immunolabelling was intense in layers I and II/III and in cells located in layer IV. (B) Detail of the $GABA_BR2$ immunoreactivity in upper layers of the neocortex showing labelled apical dendrites of pyramidal cells (arrows). (C–D) At P7 and P10, $GABA_BR2$ immunoreactivity was intense in layer I. In the remaining layers, a uniform neuropilar $GABA_BR2$ immunostaining was observed. (E–F) From P15 to adulthood, $GABA_BR2$ immunoreactivity was moderate to intense throughout the neocortex. Scale bars, 100 μm (A, C and D); 20 μm (B); 300 μm (E and F, ).

postnatal ages (Fig. 8I and J). Peroxidase reaction end-product filled axon terminals establishing asymmetrical synapses (e.g., see Fig. 8I). A few immunolabelled symmetrical synaptic contacts were also observed. Immunoparticles were also found on axonal membranes and occasionally at the presynaptic active zone of putative glutamatergic synapses (Fig. 8K).

*$GABA_BR2$*

The distribution of $GABA_BR2$ immunoreactivity in the neocortex during postnatal development was almost identical to the distribution of $GABA_BR1$. Thus, peroxidase reaction end-product for $GABA_BR2$ filled dendritic spines and dendritic shafts of pyramidal cells (Fig. 9A–G). Immunoreactive dendrites established asymmetrical synaptic contacts with putative glutamatergic axon terminals (Fig. 9A, D, E and G). Immunoparticles were mostly detected on the extrasynaptic plasma membrane of pyramidal cells spines (Fig. 9H and G) and also at the edge of asymmetrical synapses (Fig. 9I). $GABA_BR2$ immunoreactivity was also detected in presynaptic elements throughout development (Fig. 9B and F). Immunoparticles were located on axonal membranes, as well as at the presynaptic active zone of putative glutamatergic synapses (Fig. 9J).

## Discussion

The present study provides the first detailed description of the expression pattern, and precise cellular and subcellular localization of $GABA_BR$ in the rat neocortex during prenatal and postnatal development. Using conventional light and confocal microscopy we have shown that $GABA_BR1$ and $GABA_BR2$ are expressed from very early stages of cortical development and are widely distributed in the neocortex during development in pyramidal and nonpyramidal cells. The colocalization of the two receptor subtypes in the same embryonic cortical cells suggests that $GABA_BR$ form heteromeric complexes and that these cells express functional $GABA_BR$ from very early stages of development. Furthermore, immunoelectron microscopy revealed an association of $GABA_BR1$ and $GABA_BR2$ with putative glutamatergic synapses and demonstrated their postsynaptic and presynaptic localization in the neocortex during development. The similar subcellular localization of the two receptor subtypes suggest that during cortical morphogenesis, functional $GABA_BR$ expressed in different neuronal compartments may play an important role in maturation and cortical organization events.

### *Expression of $GABA_BRs$ in embryonic cortical cells*

$GABA_BR1$ and $GABA_BR2$ were expressed from very early stages of neocortical development, though they differed mainly in the intensity of immunolabelling within the same brain areas. This early presence of $GABA_BR$ in the neocortex is in line with a recent study showing that embryonic cortical cells contain mRNA and protein for $GABA_BR1$, and that this receptor subtype is functional at E17 in both cortical plate and ventricular zone cells (Behar *et al*., 2001). Our data further demonstrated that $GABA_BR1$ is expressed in cortical plate cells even earlier, at E14, and showed that it is present in the tangentially migratory interneurons coming from the medial ganglionic eminence.

Previous *in vitro* studies, using neurons dissociated from immature cortical regions, demonstrated that GABA could act as a chemoattractant, stimulating embryonic nerve cell movement (Behar *et al*., 1996, 1998). The same authors reported that GABA released by cortical cells stimulates postmitotic layer II/III cells to migrate radially into the cortical plate via activation of saclofen- and picrotoxin-sensitive receptors (Behar *et al*., 2000). Taken together, these and our results suggest that both routes of neuronal migration, radial and tangential, might be modulated via activation of $GABA_BR$.

Our immunohistochemical studies have indicated that a majority of MZ and CP cells coexpress $GABA_BR1$ and $GABA_BR2$ during prenatal development. It has been suggested that the coassembly of

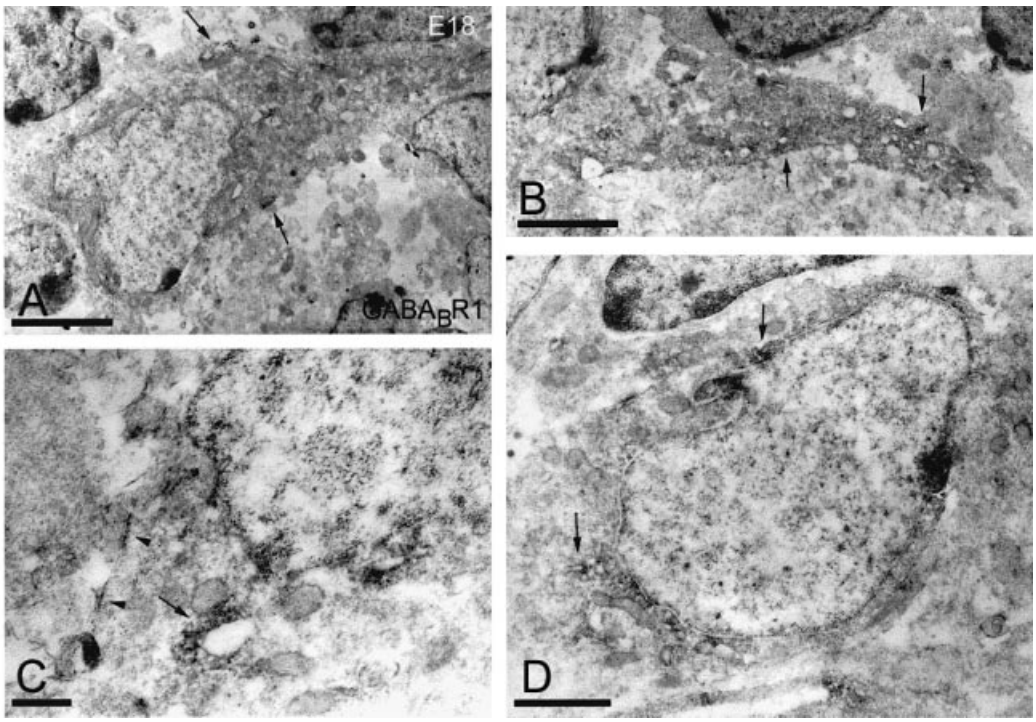


FIG. 7. Electron micrographs of the neocortex showing immunoreactivity for GABA$_B$R1 in subplate neurons at E18, using a pre-embedding immunoperoxidase method. (A–D) Peroxidase reaction end-product (arrows) was concentrated near the plasma membrane of cell bodies (A and D) and processes (B) of cells located in that stratum. GABA$_B$R1 immunoreactive synaptic contacts onto these cells were also observed (C, arrowheads). Scale bars, 2 μm (A–B); 0.3 μm (C); 10 μm (D).

GABA$_B$R1 and GABA$_B$R2 subtypes into heteromeric complexes is required for the translocation of the receptor from the synthetic site to the cell surface (Margeta-Mitrovic *et al*., 2000) and also for agonist-induced signalling (Couve *et al*., 1998; Jones *et al*., 1998; Kaupmann *et al*., 1998a; White *et al*., 1998; Kuner *et al*., 1999). Furthermore, colocalization of GABA$_B$R1 and GABA$_B$R2 has been observed in the CNS (Kaupmann *et al*., 1998a; Billinton *et al*., 2000; Gonchar *et al*., 2001; Kulik *et al*., 2002) in adult animals. However, no colocalization has been demonstrated in embryonic cortical cells yet. Therefore, our finding, that both GABA$_B$R subtypes are expressed in the MZ and CP cells, suggests that membrane-bound GABA$_B$R are functionally active at an early stage of neuronal development.

In the neocortex, the precise functional significance of GABA$_B$R during development remains unclear. Obrietan & van den Pol (1998, 1999) postulated that in embryonic cells, GABA$_B$R play a crucial inhibitory role regulating $Ca^{2+}$ rises elicited by GABA$_A$ activation and/or by inhibiting glutamate receptor-mediated $Ca^{2+}$ transients. Alterations in intracellular $Ca^{2+}$ are involved in many processes essential for neuronal development, such as growth cone motility, neurite outgrowth, neuronal migration and synapse formation (Komuro & Rakic, 1996; Mattson & Kater, 1987; Nelson *et al*., 1990). Our finding of abundant GABA$_B$R1 and GABA$_B$R2 immunoreactivity in the embryonic cortex supports a potentially important role for GABA$_B$R in a wide array of physiological processes during corticogenesis.

### *Developmental distribution of GABA$_B$Rs*

The immunohistochemical distribution pattern of GABA$_B$R1 and GABA$_B$R2 in the neocortex is consistent with earlier *in situ* hybridization (Kaupmann *et al*., 1998a; Bischoff *et al*., 1999; Durkin *et al*., 1999; Clark *et al*., 2000) and autoradiographic studies (Bowery *et al*., 1987; Chu *et al*., 1990; Turgeon & Albin, 1994).

GABA$_B$R1, but not GABA$_B$R2, was observed in Cajal-Retzius cells and the tangential migratory cells during prenatal development. Physiological responses following activation of GABA$_B$R require the coassembly of GABA$_B$R1 and GABA$_B$R2 (Jones *et al*., 1998; Kaupmann *et al*., 1998b; White *et al*., 1998; Kuner *et al*., 1999). The possible expression of homomeric GABA$_B$R1 by Cajal-Retzius cells and the tangential migratory cells in the lower intermediate zone (LIZ) suggests either that they are nonfunctional GABA$_B$R or that additional subtypes or isoforms of GABA$_B$R and/or other proteins (White *et al*., 2000) exist that have not yet been identified. The absence of functional coupling to postsynaptic ion channels (White *et al*., 1998; Martin *et al*., 1999) is supported by our preliminary electrophysiological data on Cajal-Retzius cells that revealed no activation of outward current after application of baclofen (G. López-Bendito, R. Luján and O. Paulsen, unpublished observations). Overall, the spatiotemporal expression of GABA$_B$R1 reported here in the MZ/layer I may be an important mechanism to regulate $Ca^{2+}$ dynamics, which is thought to play a role in the regulation of gene expression and control of developmental events.

Interestingly, GABA$_B$R1 showed a developmental reorganization in cortical pyramidal cells peaking around P10. Thus, during the first postnatal week, GABA$_B$R1 was located preferentially in pyramidal cell somata and dendrites projecting to layer I. At P10–P15, the receptor became more uniformly distributed on the surface of the pyramidal cells, though mainly localized in dendritic spines and associated with synapses. A similar redistribution has also been described for group I mGluRs in the same neocortical cell types during development (López-Bendito *et al*., 2002).

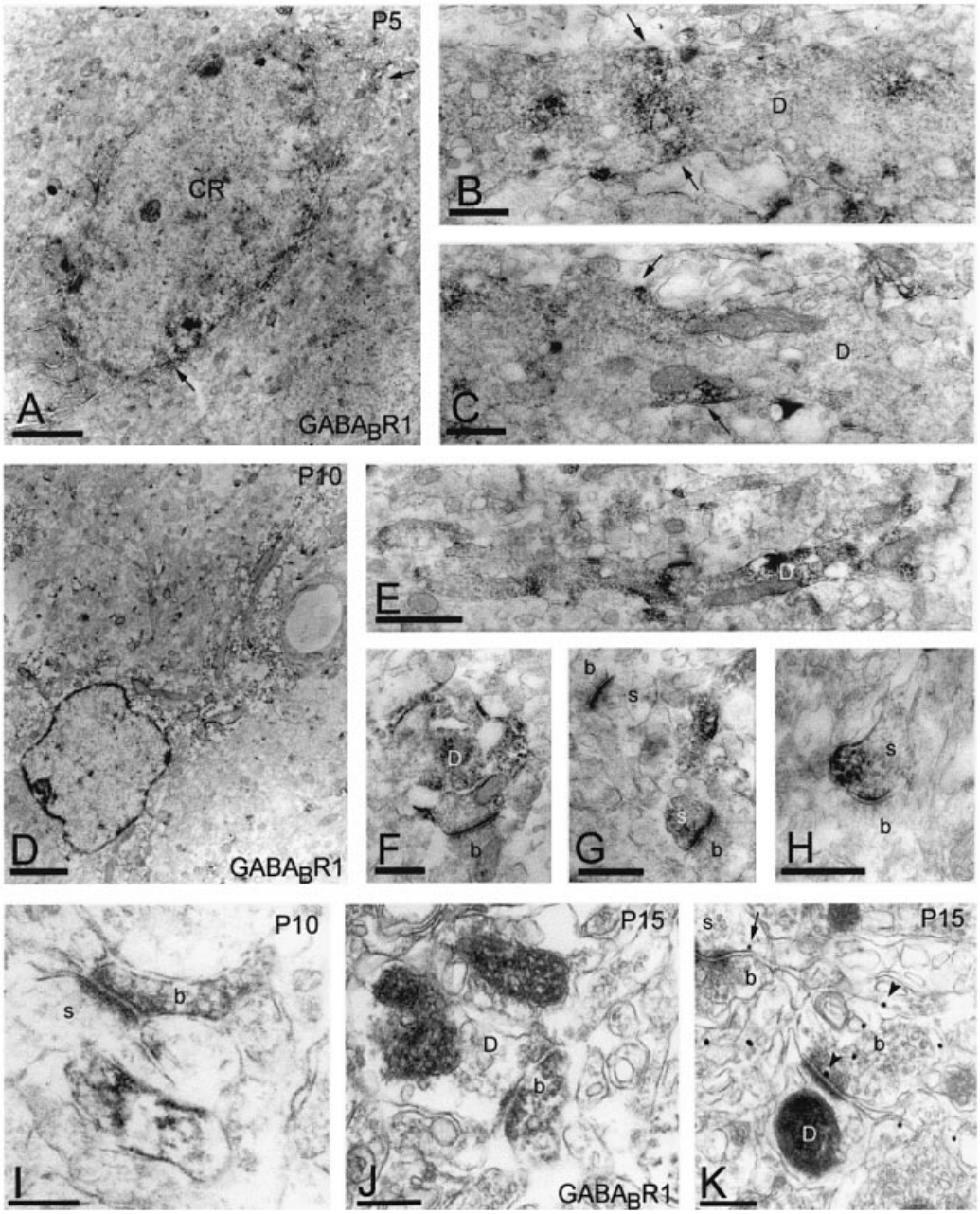


FIG. 8. Electron micrographs showing $GABA_BR1$ immunoreactivity in the neocortex during postnatal development, using pre-embedding immunoperoxidase and immunogold methods. (A–C) Presence of $GABA_BR1$ in Cajal-Retzius cells (CR) at P5. Note the large cell body and the abundant rough endoplasmic reticulum. Immunolabelling was concentrated near the plasma membrane of cell bodies and processes (arrows). (D–F) At P10, $GABA_BR1$ immunoreactivity was observed mainly in somata and dendrites (white 'D') of interneurons in layer II/III. (G and H) Dendritic spines (s) of pyramidal cells immunolabelled for $GABA_BR1$. (I and J) $GABA_BR1$ immunoreactivity in axon terminals establishing asymmetrical synapses with spines and dendritic shafts ('D') in layer I (I) and II/III (J) at P10 and P15, respectively. (K) Immunogold particles were localized on the extrasynaptic plasma membrane of dendritic spines (s, arrow) and axon terminals (b, arrowheads). White 'D' and small black 'D' show dendrite. Scale bars, 2 μm (A and D); 0.5 μm (B, C and F); E, 1 μm.

Interneurons expressed $GABA_BR$ during development in the neocortex, with the staining for $GABA_BR1$ being present in somata and extending uniformly throughout the dendritic arbour. However, although at light microscopic level the staining for $GABA_BR2$ was not detected in cell bodies of interneurons, electron microscopy showed that they expressed this subtype indeed. The $GABA_BR1$-containing interneurons showed different morphology and location throughout the neocortex, suggesting the existence of different subset of interneurons expressing $GABA_BR$. This is in agreement with findings of Gonchar *et al*. (2001) who reported that parvalbumin-, calretinin- and somatostatin-containing interneurons of the visual cortex colocalized with $GABA_BR1a/b$.

### *Subcellular localization of $GABA_B$ Rs*

The current view of the subcellular distribution of $GABA_BR$ in the CNS is mainly based on data obtained in the adult cerebellum (Kaupmann *et al*., 1998a; Fritschy *et al*., 1999; Ige *et al*., 2000; Kulik *et al*., 2002), ventrobasal thalamus (Kulik *et al*., 2002) and visual

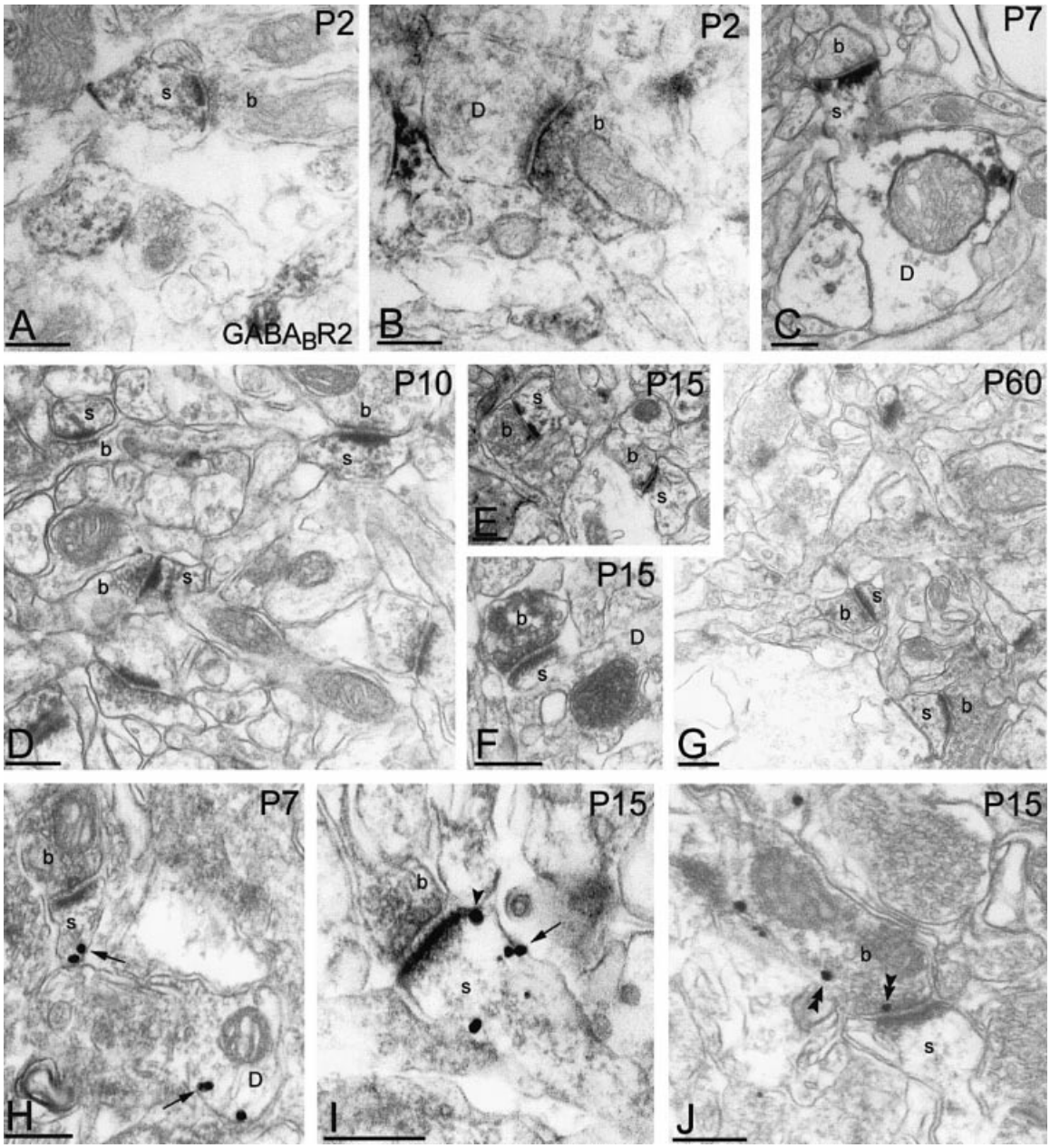


FIG. 9. Electron micrographs showing GABA$_B$R2 immunoreactivity in the neocortex during postnatal development, using pre-embedding immunoperoxidase and immunogold methods. (A and B) At P2, GABA$_B$R2 immunolabelling concentrated in dendritic spines (s) of pyramidal cells, as well as in some axon terminals (b) establishing synapses with dendritic spines or dendritic shafts ('D') in layer I. (C) Dendrite in layer II/III immunolabelled for GABA$_B$R2 at P7. Peroxidase reaction end-product concentrated near the plasma membrane and in the spine (s) attached to the shaft ('D'). (D–G) GABA$_B$R2 immunoreactivity was consistently present in dendritic spines (s) of pyramidal cells throughout the neocortex in postnatal development until adulthood, as well as in axon terminals establishing asymmetrical synapses with dendritic spines (s) or shafts ('D'). (H–I) Immunogold particles were localized in the extrasynaptic (arrows) plasma membrane of dendritic spines (s) and shafts ('D') of pyramidal cell, as well as in perisynaptic sites (I, arrowhead) associated with asymmetrical synapses. (J) Presynaptically, gold particles were also present along the plasma membrane of axon terminals (b, double arrowheads) establishing asymmetrical synapses with dendritic spines (s) of pyramidal cells. Black small 'D' indicates dendrite. Scale bars, 0.25 μm (A–J).

cortex (Gonchar *et al*., 2001). Those studies showed that GABA$_B$R1 and GABA$_B$R2 were found at extrasynaptic sites, at the edges of asymmetrical synapses and occasionally over the synaptic membrane specialization. Our finding that a high proportion of dendritic spines of cortical pyramidal cells were immunoreactive for both GABA$_B$R1 and GABA$_B$R2 is consistent with previous studies (Benke *et al*., 1999; Bischoff *et al*., 1999; Fritschy *et al*., 1999; Gonchar *et al*., 2001; Muñoz *et al*., 2001). The majority of GABA$_B$R were localized on cortical dendritic spines, mainly at extrasynaptic sites or at the edge of the synaptic specialization. The similar subcellular localization of GABA$_B$R1 and GABA$_B$R2 on neuronal elements of the neocortex presented in our study further suggests colocalization of these receptors in different subcellular compartments of pyramidal and nonpyramidal cells during development. In addition, our finding

directly demonstrated that pyramidal cells and interneurons contained both postsynaptic and presynaptic $GABA_BR$.

Several studies have shown that baclofen suppressed evoked excitatory postsynaptic potentials (EPSPs) and IPSPs, suggesting that $GABA_BR$ mediate presynaptic inhibition of excitatory and inhibitory neurotransmission in different brain regions (Pittaluga *et al*., 1987; Brugger *et al*., 1993). In the neocortex, we found $GABA_BR1$ and $GABA_BR2$ in axon terminals establishing asymmetrical synapses during cortical development. This finding is consistent with electrophysiological studies that showed depression of excitatory responses by $GABA_BR$ agonist in neocortex (Howe *et al*., 1987; Deisz *et al*., 1997; Varela *et al*., 1997).

The association of $GABA_BR$ with asymmetrical synapses, presumably glutamatergic, in adult and during development raises the question of what is the source of GABA to activate these receptors. One possible source of GABA to activate $GABA_BR$ is the spillover from neighbouring interneurons (Isaacson *et al*., 1993). Another possibility is the activation through taurine, a weak agonist at $GABA_BR$. Taurine is present in the cortex and has been suggested to act as a chemoattractant for neurons during cortical development via mechanisms involving $GABA_BR$ (Behar *et al*., 2001). Finally, $GABA_BR$ may be activated by nonsynaptically released GABA. In the neocortex, this mechanism of activation has recently been suggested for presynaptic $GABA_BR$ on pyramidal cell axon terminals (Zilberter *et al*., 1999). Regardless of the mechanism of activation of $GABA_BR$, the colocalization of $GABA_BR1$ and $GABA_BR2$ at excitatory synapses suggests that they are functional and it is likely that they are involved in developmental physiological events.

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## Abbreviations

CP, cortical plate; Ctx, cortex; DAB, 3,3′-diaminobenzidine; DT, dorsal thalamus; E, embryonic day; $GABA_BR$, metabotropic γ-aminobutyric acid receptor; Hb, habenula; Hp, hippocampus; IC, internal capsule; LGE, lateral ganglionic eminence; LIZ, lower intermediate zone; MGE, medial gangliomic eminence; MZ, marginal zone; NGS, normal goat serum; nt, *nervus terminalis*; on, olfactory nerve; P, postnatal day; PB, phosphate buffer; PBS, phosphate buffer saline; PP, preplate; PSA-NCAM, polysialylised form of the neuronal cell adhesion molecule NCAM; SE, septal eminence; SP, subplate; SVZ, subventricular zone; TB, Tris buffer; TBS, Tris-buffered saline; TE, thalamic eminence; VZ, ventricular zone.

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