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Distribution of Metabotropic GABA Receptor Subunits $GABA_{B1a/b}$ and $GABA_{B2}$ in the Rat Hippocampus During Prenatal and Postnatal Development

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ABSTRACT: Metabotropic γ-aminobutyric acid receptors ($GABA_B$) play modulatory roles in central synaptic transmission and are involved in controlling neuronal migration during development. We used immunohistochemical methods to elucidate the expression pattern as well as the cellular and the precise subcellular localization of the $GABA_{B1a/b}$ and $GABA_{B2}$ subunits in the rat hippocampus during prenatal and postnatal development. At the light microscopic level, both $GABA_{B1a/b}$ and $GABA_{B2}$ were expressed in the hippocampal primordium from embryonic day E14. During postnatal development, immunoreactivity for $GABA_{B1a/b}$ and $GABA_{B2}$ was distributed mainly in pyramidal cells, with discrete $GABA_{B1a/b}$-immunopositive cell bodies of interneurons present throughout the hippocampus. Using double immunofluorescence, we demonstrated that during the second week of postnatal development, $GABA_{B1a/b}$ but not $GABA_{B2}$ was expressed in glial cells throughout the hippocampal formation. At the electron microscopic level, $GABA_{B1a/b}$ and $GABA_{B2}$ showed a similar distribution pattern during postnatal development. Thus, at all ages the two receptor subunits were located postsynaptically in dendritic spines and shafts at extrasynaptic and perisynaptic sites in both pyramidal and nonpyramidal cells. We further demonstrated that the two subunits were localized presynaptically along the extrasynaptic plasma membrane of axon terminals and along the presynaptic active zone in both asymmetrical and, to a lesser extent, symmetrical synapses. These results suggest that $GABA_B$ receptors are widely expressed in the hippocampus throughout development and that $GABA_{B1a/b}$ and $GABA_{B2}$ form both pre- and postsynaptic receptors.

KEY WORDS: $GABA_B$; immunohistochemistry; synapse; electron microscopy; development; light microscopy; inhibition

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INTRODUCTION

γ-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the mammalian hippocampus, and its action is mediated by ionotropic and metabotropic receptors (Macdonald and Olsen, 1994; Misgeld et al., 1995; Johnston, 1996). The metabotropic GABA receptors ($GABA_B$ receptors) are coupled to second messenger systems through G-proteins, and mediate slow and prolonged inhibitory effects (Misgeld et al., 1995; Bowery et al., 2002). The activation of $GABA_B$ receptors contributes to both presynaptic and postsynaptic responses. Presynaptically located $GABA_B$ receptors modulate neurotransmitter release by depressing Ca^{2+} influx via voltage-activated Ca^{2+} channels, whereas the activation of postsynaptic $GABA_B$ receptors generally causes inhibition of adenylate cyclase, as well as activation of Kir3-type potassium channels, thereby hyperpolarizing the postsynaptic membrane (Kaupmann et al., 1998b).

Two different genes encoding $GABA_{B1}$, which exists in alternatively spliced forms designated 1a, b, c, d, and e, as well as $GABA_{B2}$, have been identified so far (Kaupmann et al., 1997; Bowery and Brown, 1997; Isomoto et al., 1998; Pfaff et al., 1999; Schwarz et al., 2000). Physiological responses following activation of $GABA_B$ receptors require the co-assembly of $GABA_{B1}$ and $GABA_{B2}$ (Jones et al., 1998; Kaupmann et al., 1998b; White et al., 1998; Kuner et al., 1999; Filippov et al., 2000).

In the adult hippocampus, $GABA_B$ receptors appear to be involved in long-term potentiation (Davies et al., 1991), in the control of the frequency and synchronization of theta rhythm (Davies et al., 1990; Mott and Lewis, 1991), as well as in the mediation of the late phase of the inhibitory postsynaptic potentials (Dutar and Nicoll, 1988; Karlsson and Olpe, 1989; Olpe et al., 1993). Despite their important role in the aforementioned hippocampal functions, little information is available about the expression of $GABA_B$ receptors in the developing hippocampus. Most of the current knowledge has been derived from autoradiographic and light microscopic immunohistochemical studies (Turgeon and Albin, 1994; Fritschy et al., 1999). During hippocampal development, $GABA_B$ receptors appear to mediate the excitatory actions of glutamate in developing cortical neurons through the regulation of glutamate-

activated calcium transients (Obrietan and van den Pol, 1999), and are involved in the modulation of neuronal migration in immature cortical regions (Behar et al., 2000; López-Bendito et al., 2003). Electrophysiological and pharmacological studies have characterized pre- and postsynaptic inhibitory functions of $GABA_B$ receptors in the immature hippocampus (Howe et al., 1987; Dutar and Nicoll, 1988), which are not present in animals that lack $GABA_{B1}$ (Prosser et al., 2001; Schuler et al., 2001). Finally, autoradiographic and light microscopic immunohistochemical studies (Turgeon and Albin, 1994; Fritschy et al., 1999) have shown that $GABA_B$ receptors are expressed at high levels in the hippocampus during postnatal development. However, a distinct subcellular localization of $GABA_B$ receptors has not been accomplished in the developing hippocampus. Only in the adult hippocampus and in the developing neocortex have recent immunoelectron microscopic studies demonstrated the localization of $GABA_{B1}$ and $GABA_{B2}$ at both pre- and postsynaptic sites (López-Bendito et al., 2002; Kulik et al., 2003).

Information regarding the distribution of $GABA_B$ receptors is crucial to elucidate the contribution of the receptors to developmental processes and hippocampal functions. Therefore, the goal of the present study was to characterize the distribution pattern as well as the cellular and the precise subcellular localization of $GABA_{B1a/b}$ and $GABA_{B2}$ in the rat hippocampus during pre- and postnatal development.

MATERIALS AND METHODS

Tissue Preparation

Thirty-nine Wistar rats from embryonic day (E) 14 to adulthood, obtained from the authors' Animal House Facilities, were used in the present study. The care and handling of the animals before 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.

Fetuses were collected by cesarean section after anesthesia of the dam with an intraperitoneal (i.p.) injection of ketamine-xylazine 1:1 (0.1 ml/kg body weight). Animals were deeply anesthetized by hypothermia (E14-P5) or by i.p. injection of ketamine-xylazine 1:1 (0.1 ml/kg body weight), and the heart was surgically exposed for perfusion fixation (López-Bendito et al., 2002). After perfusion, tissue blocks containing the hippocampus were dissected, washed thoroughly in 0.1 M phosphate buffer (PB) for several hours, embedded in 4% agarose and subsequently sectioned at 60 μm with a microtome (Leica VT1000S).

Antibodies

An affinity-purified polyclonal antibody against $GABA_{B1a/b}$ (recognizing 1a and 1b splice variants of $GABA_{B1}$ subunit) was raised in rabbit. The characteristics and specificity of that antibody (B17) have been described elsewhere (Kulik et al., 2002). An affinity-purified polyclonal antibody against $GABA_{B2}$ (B32) was raised in rabbit and its specificity was recently described (Li et al., 2001). Additionally, an affinity-purified antibody against $GABA_{B2}$ was raised in guinea pig and obtained from Chemicon (Temecula, CA). To characterize the glial cells that expressed $GABA_{B1a/b}$ during the first and second postnatal week, we used a monoclonal antibody to glial fibrillary acidic protein (GFAP), obtained from Chemicon, Temecula, CA.

Immunocytochemistry for Light and Laser Confocal Microscopy

Similar procedures to those described earlier (López-Bendito et al., 2002) were used.

Immunoperoxidase method

Sections were incubated in 10% normal goat serum (NGS) diluted in 50 mM Tris buffer (pH 7.4) containing 0.9% NaCl (TBS), with 0.2% Triton X-100, for 1 h. Sections were then incubated for 48 h with affinity-purified polyclonal antibodies anti-$GABA_{B1a/b}$ or anti-$GABA_{B2}$ at a final protein concentration of 1–2 μg/ml diluted in TBS containing 1% NGS. After several washes in TBS, the sections were incubated for 2 h in biotinylated goat anti-rabbit IgG or anti-guinea pig IgG (Vector Laboratories, Burlingame, CA) diluted 1:100 in TBS containing 1% NGS. They were then transferred into avidin-biotin-peroxidase complex (ABC kit; Vector) diluted 1:100 for 2 h at room temperature. Bound peroxidase enzyme activity was revealed using 3,3′-diaminobenzidine tetrahydrochloride (DAB; 0.05% in TB, pH 7.4) as chromogen and 0.01% H_2O_2 as substrate. Finally, the sections were air-dried and coverslipped before observation in a Leitz DMRS photomicroscope equipped with differential interference contrast optics and a digital imaging camera (Leica DC 200, Leica, Austria).

Immunofluorescence method

Sections were blocked in 10% NGS in TBS and then incubated with the mixture of primary antibodies for $GABA_{B1a/b}$ (B17) and GFAP (Chemicon) in TBS containing 1% NGS and 0.1% Triton X-100 overnight at 4°C. After washes in TBS, the sections were incubated with the mixture of secondary antibodies (anti-mouse Alexa®-488 for GFAP and anti-rabbit cyanine-derived fluorochrome Cy3 for $GABA_{B1a/b}$) made up in TBS with 0.1% Triton X-100. After extensive TBS washes, the sections were mounted on gelatinized slides and coverslipped with Glycerol/PB 0.1 M (1:1). Fluorescent signals were examined with a confocal laser scanning microscope (Leica TLSCM, Austria).

Immunocytochemistry for Electron Microscopy

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

Pre-embedding immunoperoxidase and immunogold methods

Immunocytochemical reactions for the pre-embedding immunoperoxidase 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, 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).

The peroxidase-reacted sections and the gold-silver-labeled sections were treated with OsO_4 (1% in 0.1 M PB), block-stained with uranyl acetate, dehydrated in a 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 with an ultramicrotome (Reichert Ultracut E, Leica, Austria). Ultrathin sections were contrasted and analysed in a Jeol-100 electron microscope.

Controls

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 labeling was observed. Staining pattern were also compared to those obtained by calretinin and calbindin (Chemicon); only the antibodies against $GABA_{B1}$ and $GABA_{B2}$ consistently labeled the plasma membrane of cells. When double labeling for confocal microscopy was carried out, 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-labeling that would influence the results was detected.

RESULTS

$GABA_B$ Receptors During Prenatal Development

To study the tissue expression of $GABA_B$ receptors during prenatal hippocampal development, we carried out pre-embedding immunohistochemical studies at different ages.

$GABA_{B1a/b}$

At E14, $GABA_{B1a/b}$ immunoreactivity was detected in the mantle layer of the septal and ganglionic eminences (Fig. 1A) and in the preplate of the neocortical primordium. However, the preplate of the hippocampus showed very weak $GABA_{B1a/b}$ immunoreactivity at that age. The intensity of immunostaining increased with the development of the hippocampus. Thus, at E16 we found stronger immunoreactivity for $GABA_{B1a/b}$ detected in virtually all cells located in the marginal zone (Fig. 1B,C). At E18, we found an increased level of $GABA_{B1a/b}$ expression in the marginal zone of the hippocampus (Fig. 1D,E).

$GABA_{B2}$

At E14, the subunit was expressed at very low levels in most telencephalic areas, including the preplate of the hippocampus (Fig. 1F). The intensity of immunolabeling increased with development, similar to $GABA_{B1a/b}$. Thus, at E16 we found strong $GABA_{B2}$ immunoreactivity in the hippocampal marginal zone (Fig. 1G,H). At E18, $GABA_{B2}$ was highly expressed in the hippocampus (Fig. 1I,J). However, the level of $GABA_{B2}$ expression in the marginal zone of the hippocampus was similar to that of E16 (Fig. 1I,J). In addition, we observed numerous immunolabeled axonal processes between the marginal zone and the ventricular zone (VZ) (Fig. 1J). In contrast to the distribution of $GABA_{B1}$, which was concentrated in cell bodies, overall $GABA_{B2}$ was present at the level of the neuronal processes and neuropil.

$GABA_B$ Receptors During Postnatal Development

To display the staining pattern and distribution of $GABA_B$ receptors during postnatal development of the hippocampus, we carried out pre-embedding immunohistochemical studies at different ages.

To eliminate variations due to regional differences in the development of the hippocampus, we have selected similar levels for comparisons throughout the developmental stages. The descriptions that follow are based on the hippocampal formation of coronal sections obtained frontally, in which the laterodorsal thalamic nuclei and the habenular nuclei were also present.

$GABA_{B1a/b}$

At birth (P0), we observed that $GABA_{B1a/b}$ was intensely expressed in principal cells of all hippocampal areas (Fig. 2A,B). At P7, $GABA_{B1a/b}$ immunoreactivity was also observed in pyramidal cells (Fig. 2C), as well as in glial cells. In addition, immunoreactivity for the subunit was also observed in neurons with the typical fusiform morphology and horizontal orientation of Cajal-Retzius cells located in the hippocampal fissure (Fig. 2B). In the dentate gyrus, we found strong immunoreactivity for $GABA_{B1a/b}$ in non-pyramidal cells, putative mossy cells, and putative glial cells (Fig. 2D), in contrast to what has been observed for $GABA_{B2}$ (Fig. 3). To investigate whether some cell types immunoreactive for $GABA_{B1a/b}$ in the hippocampus were glial cells, double-labeling experiments were carried out to co-localize $GABA_{B1a/b}$ and GFAP, a glial cell marker (Fig. 4A–F). Thus, at P7, some small size cells expressing $GABA_{B1a/b}$ were also labeled for GFAP in all subfields of hippocampal formation, indicating that glial cells expressed $GABA_{B1a/b}$ (Fig. 4A–F).

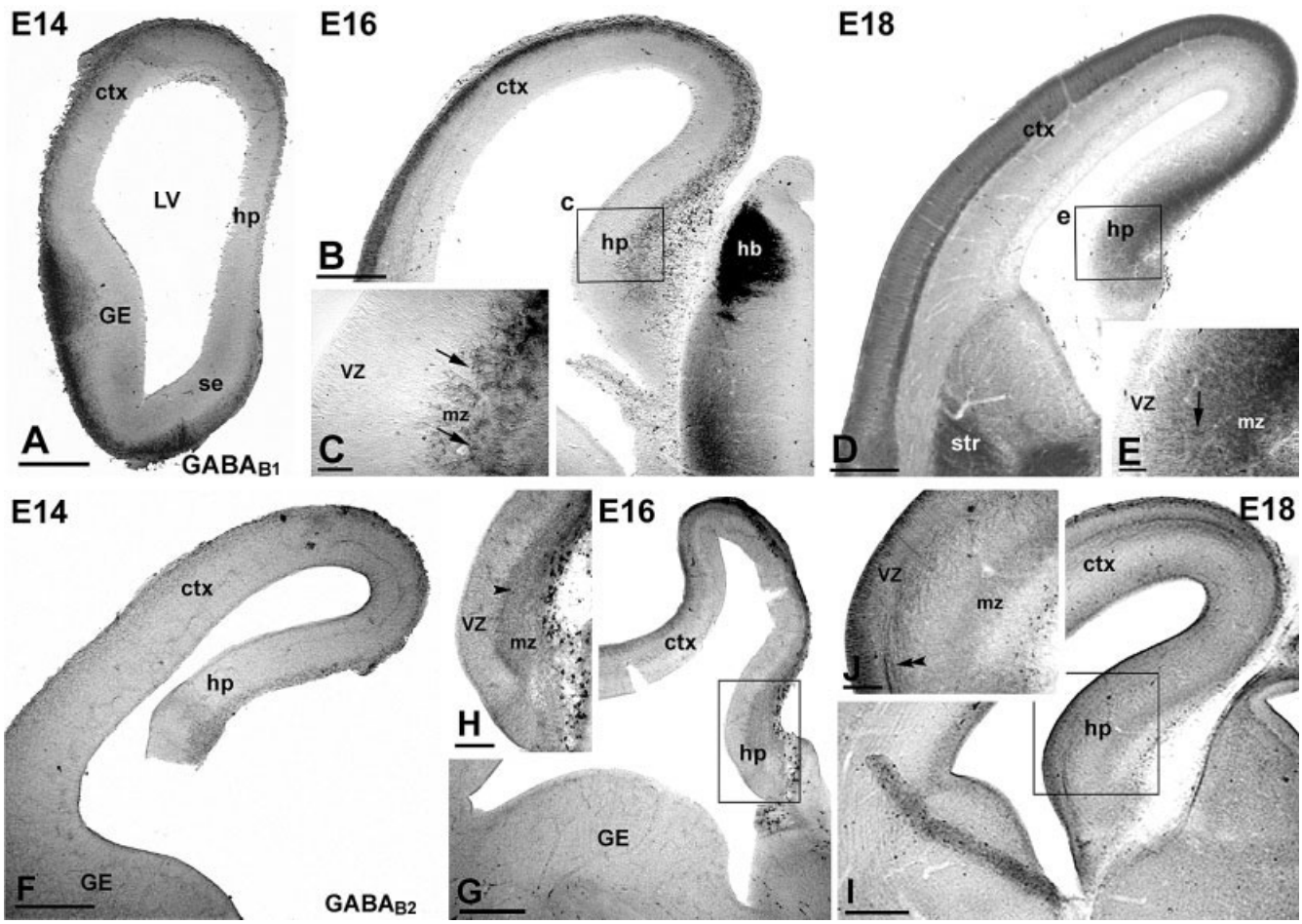


FIGURE 1. Immunoreactivity for γ-aminobutyric acid (GABA) receptors $GABA_{B1a/b}$ and $GABA_{B2}$ in the hippocampus at embryonic ages E14, E16, and E18 using a pre-embedding immunoperoxidase method (A–J). A: Rostral coronal section of the telencephalon at E14. Immunoreactivity for $GABA_{B1a/b}$ was strong in the marginal zone (mz) of the septal (se) and ganglionic eminences (GE) and very weak in the preplate of the hippocampus. B: Mid-caudal coronal section at E16 showing that $GABA_{B1a/b}$ was strongly expressed in the habenula (hb) and preplate of the cortex (ctx). C: Detail of the $GABA_{B1a/b}$ immunolabeling in cells localized in the mz of the hippocampus (e.g., arrows). The ventricular zone (VZ) was not immunoreactive for the receptor. D–E: At E18, the mz of the hippocampus expressed more $GABA_{B1a/b}$ than at earlier ages. F: Mid-caudal coronal section of E14 rat brain showing that $GABA_{B2}$ was expressed at very low levels in the preplate of the cortex (ctx) and hippocampus (hp). G–H: Mid-caudal coronal section at E16 exhibiting that $GABA_{B2}$ immunoreactivity increased at this age and was only detected in the neuropil (arrowhead). I: Mid-caudal coronal section at E18 showing that the mz of the hippocampus expressed more $GABA_{B2}$ than at earlier ages. (J) Detail of the $GABA_{B2}$ immunoreactivity in the neuropil of the hippocampal mz. The VZ was also immunoreactive for the receptor. In addition, axons immunoreactive for $GABA_{B2}$ were detected immediately between the mz and the VZ (double arrowhead). LV, lateral ventricle. Scale bars = 300 μm in A,B,D,G,I,; 50 μm in C,E; 200 μm in F,; 100 μm in H,J.

During the second postnatal week (P10), immunolabeling for $GABA_{B1a/b}$ was similar (Fig. 2E,F) to that observed during the first week of development. However, during the third postnatal week (P15), we detected a considerable decrease in immunoreactivity in glial cells throughout the hippocampus (Fig. 2G). Overall, the distribution of $GABA_{B1a/b}$ in the hippocampal formation did not change from P15 to adulthood. Thus, in the CA1 region, we detected an intense labeling in the pyramidal cell layer, and a weak labeling in the dendritic layers (Fig. 2G,I). The stratum lacunosum-moleculare of the CA1 region showed a moderate labeling for $GABA_{B1a/b}$ (Fig. 2G,I). The CA3 region exhibited a moderate to strong labeling in the dendritic layers, and a strong labeling in the stratum lacunosum-moleculare (Fig. 2G,I). In the dentate gyrus, we detected weak to moderate $GABA_{B1a/b}$ labeling (Fig. 2G–I). Finally, a subset of interneurons distributed throughout the hippocampal formation was strongly immunolabeled for $GABA_{B1a/b}$ (Fig. 2G–J).

$GABA_{B2}$

At P2, we observed an intense immunoreactivity in all regions of the hippocampal formation (Fig. 3A,B), particularly in all dendritic fields of the developing principal cells (Fig. 3B). Toward the end of the first week (P5), and during the second postnatal week (P10), immunoreactivity for $GABA_{B2}$ was most intense in the CA3 region (Fig. 3C–E), in the stratum lacunosum-moleculare of the

CA1 region (Fig. 3C,D), and in the molecular layer of the dentate gyrus (Fig. 3C,D). However, the distribution of $GABA_{B2}$ in the hippocampal formation did not change from P15 (Fig. 3F) to adulthood (Fig. 3G,H). Thus, $GABA_{B2}$ was not detected in the pyramidal cell layer in any hippocampal region (Fig. 3F–H). In the CA1 region, we detected a moderate immunolabeling for $GABA_{B2}$ in the dendritic layers (Fig. 3F–H), with the exception of the stratum lacunosum-moleculare that showed an intense labeling (Fig. 3F–H). The CA3 region exhibited a very intense labeling in the dendritic layers, particularly in the stratum lacunosum-moleculare (Fig. 3F–H). In the dentate gyrus, a moderate to intense $GABA_{B2}$ labeling was observed (Fig. 3F–H). Finally, we did not detect immunoreactivity for $GABA_{B2}$ in the stratum pyramidale, in cell bodies of nonpyramidal neurons and glial cells at any postnatal age (Fig. 3).

Subcellular Localization of $GABA_B$ Receptors During Postnatal Development

To investigate the precise subcellular localization of $GABA_{B1a/b}$ and $GABA_{B2}$ in the hippocampal formation during postnatal development we carried out electron microscopic studies using the pre-embedding immunoperoxidase and immunogold techniques at different ages.

At all ages of development, the peroxidase reaction end-product for the two receptor subunits was predominantly observed postsynaptically in dendritic spines and dendritic shafts of pyramidal cells (Figs. 5A and 6A–C), as expected on the basis of light microscopic data. In addition, from P5-P7 onward, the dense reaction product for $GABA_{B1a/b}$ and $GABA_{B2}$ was also observed in dendritic shafts of interneurons (Fig. 5C,J) and immunoreactivity for $GABA_{B1a/b}$ but not $GABA_{B2}$, was present in the somata of interneurons throughout the hippocampal formation (Figs. 5E,I and 6G,H). The somatic immunoreactivity for $GABA_{B1a/b}$ in pyramidal cells and interneurons could be ascribed to the abundance of the receptor in the endoplasmic reticulum (Figs. 5C,E,I,K and 6A,H). However, no immunoparticles for $GABA_{B1a/b}$ or $GABA_{B2}$ could be detected along the somatic plasma membrane. In both pyramidal and nonpyramidal cells, and at all postnatal ages, immunolabeling for the two receptor subunits was always localized in the extrasynaptic plasma membrane of dendrites and spines (Fig. 5A,B,D,F–H,K,L and 6B,C,E–I).

Immunoreactivity for $GABA_{B1a/b}$ and $GABA_{B2}$ was also observed at the edge of asymmetrical (Fig. 5H,K and 6C,I) and, to a lesser extent, symmetrical synapses, established by putative glutamatergic and GABAergic axon terminals, respectively. The main difference in the distribution of $GABA_{B1a/b}$ and $GABA_{B2}$ was the relative abundance of the receptors along the extrasynaptic plasma membrane and relative to the neurotransmitter release site. Thus, at P21 and P30 (Figs. 5I–L and 6H,I), the density of immunoparticles was consistently lower in dendritic shafts than that observed within the spines (Figs. 5K and 6F,I), reaching at those postnatal ages the pattern observed in adult (Kulik et al., 2003).

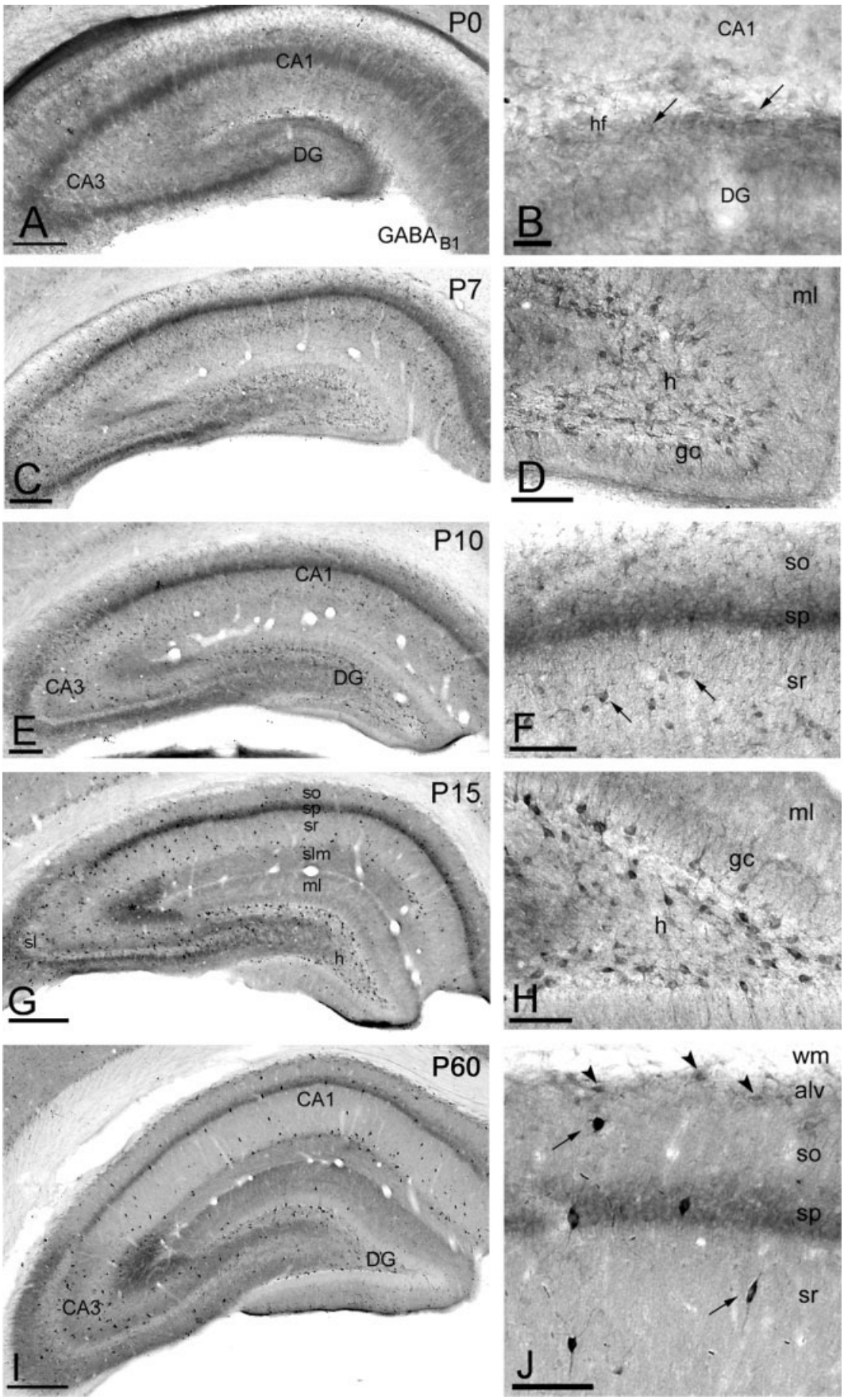
Immunoreactivity for $GABA_{B1a/b}$ and $GABA_{B2}$ was also observed in presynaptic elements distributed throughout all hippocampal subfields and postnatal ages (Figs. 5G,K and 6C,E,F,I). Immunoparticles were found along the extrasynaptic plasma membrane of axon terminals, along the presynaptic active zone in putative glutamatergic synapses (Figs. 5G,K and 6C,E,F), and to a lesser extent in putative GABAergic synapses.

In adulthood, ultrastructural localization of immunoreactivity for $GABA_{B1a/b}$ and $GABA_{B2}$ showed a similar distribution pattern in the hippocampal formation to that described during postnatal development, and virtually identical to the distribution observed from P21 onward (Kulik et al., 2003).

DISCUSSION

This study provides a detailed description of the expression pattern, and precise cellular and subcellular localization of $GABA_{B1a/b}$ and $GABA_{B2}$ in the rat prenatal and postnatal developing hippocampus. Using light and confocal microscopy we have shown that both receptor subunits were widely distributed in the hippocampus during development in pyramidal and nonpyramidal cells and that they were transiently expressed in glial cells. Furthermore, immunoelectron microscopy demonstrated a predominantly extrasynaptic localization of $GABA_{B1a/b}$ and $GABA_{B2}$ on both pre- and postsynaptic elements of the developing hippocampus, as well as an association with asymmetrical and symmetrical synapses, with the same subcellular distribution being observed in the adult hippocampus (Kulik et al., 2003). A homologous receptor distribution was reported in the developing and adult neocortex (López-Bendito et al., 2002), suggesting the importance of $GABA_B$ receptors in cortical function, and their possible role in the maturation and organization processes in the hippocampus during development.

FIGURE 2. Immunoreactivity for γ-aminobutyric acid $(GABA)_{B1a/b}$ in the hippocampus during postnatal development using a pre-embedding immunoperoxidase method at P0, P7, P10, P15, and P60. A,B: At P0, all the hippocampal areas (CA1, CA3, and dentate gyrus) were immunoreactive for $GABA_{B1a/b}$. In addition, immunopositive cells were observed at the hippocampal fissure (hf) (B, arrows), where Cajal-Retzius cells are located. C,D: At P7, an intense $GABA_{B1a/b}$ immunolabeling was found in interneurons and glial cells along all hippocampal areas, especially in the stratum radiatum (sr) of CA1 and CA3, and in the dentate gyrus, as well as putative mossy cells in the dentate gyrus (D). E,F: At P10, a similar distribution for $GABA_{B1a/b}$ as in earlier ages was observed. Subsets of interneurons expressing $GABA_{B1a/b}$ were found in the sr of CA1 (F, arrows). The number of immunolabeled glial cells decreased. G,H: At P15, strong labeling for the receptor was found in the stratum pyramidale (sp) of CA1 and in the stratum lacunosum-moleculare (slm) of CA3 (asterisk). Numerous immunolabeled interneurons and putative mossy cells were found in the hilus (h), located immediately below the granular cell layer (gc) of the DG. I,J: At P60, the receptor was expressed in interneurons located in the CA1 region (J, arrows) and other hippocampal areas. Immunolabeled cells were found also in the alveus (alv) (arrowheads), so: stratum oriens, ml: molecular layer. Scale bars = 200 μm in A,C,E; 20 μm in B; 100 μm in D,H,F,J; 400 μm G,I.


P0
CA1
DG
CA3
A
GABA$_{B1}$
CA1
hf
DG
B
P7
C
ml
h
gc
D
P10
CA1
CA3
DG
E
so
sp
sr
F
P15
so
sp
sr
slm
ml
sl
h
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CA1
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FIGURE 2

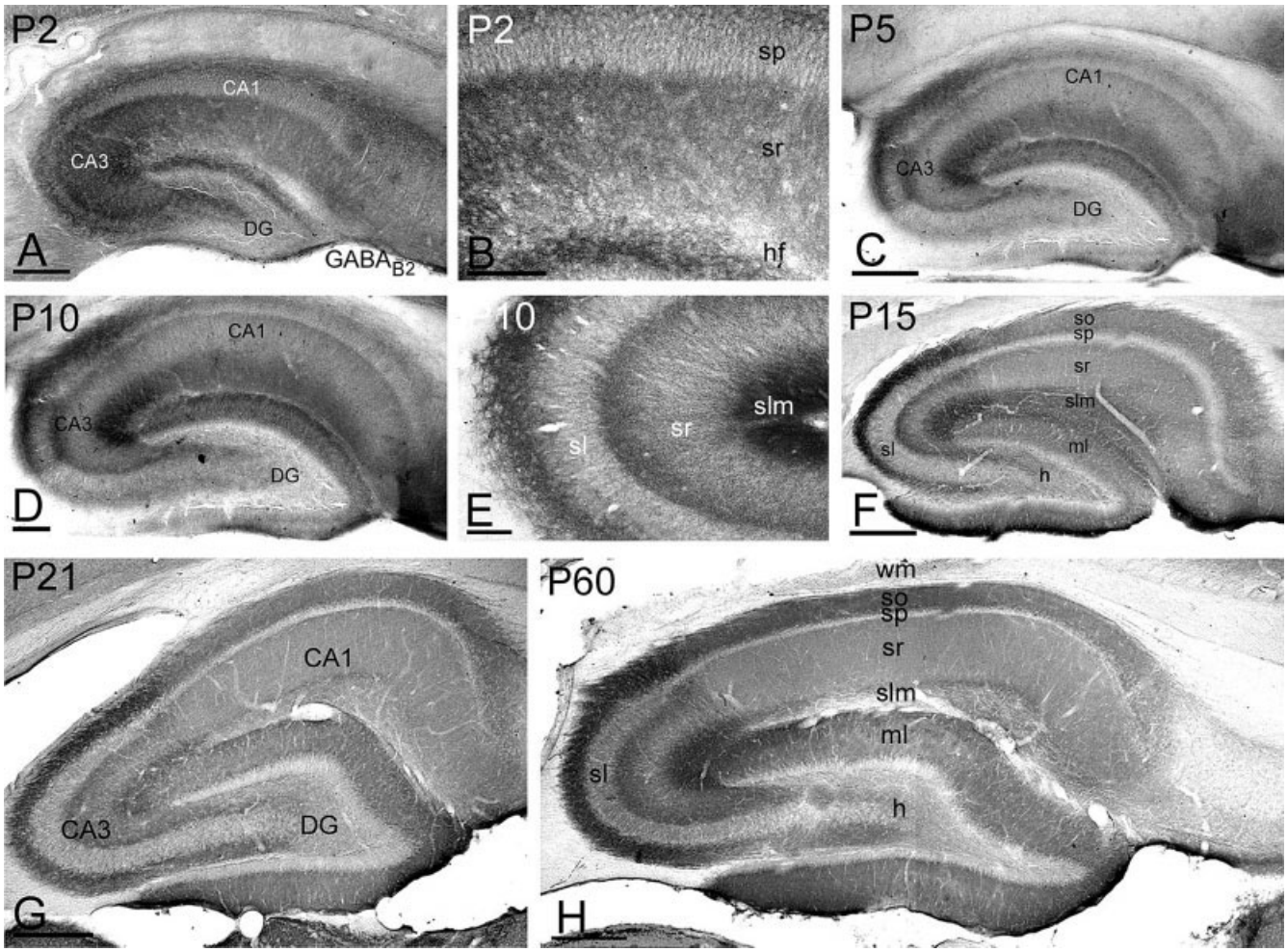

FIGURE 3. Immunoreactivity for γ-aminobutyric acid (GABA)$_{B2}$ in the hippocampus during postnatal development using a pre-embedding immunoperoxidase method at P2, P5, P10, P15, P21, and P60. A,B: At P2, all the hippocampal areas were labeled for GABA$_{B2}$. C–E: At P5-P10, immunreactivity for GABA$_{B2}$ in the strata oriens and radiatum of the CA1 region decreased relative to other regions. F–H: From P15 to adulthood, the expression of the receptor in the hippocampal formation was very similar. Thus, immunoreactivity was strongest in the strata oriens, radiatum, and lacunosum-moleculare of the CA3 region, in the stratum lacunosum-moleculare of the CA1 region and in the molecular layer of the dentate gyrus. The stratum lucidum and the hilus were only weakly labeled. Neither immunopositive cells bodies of interneurons nor glial cells were found at any age. Scale bars = 100 μm in A,C; 50 μm in B,D,E; 300 μm in F,G,H.

Embryonic Hippocampal Cells Express GABA$_B$ Receptors

In the present study, we demonstrated the expression of GABA$_{B1a/b}$ and GABA$_{B2}$ in most marginal zone cells from early stages in the embryonic hippocampus. The expression of functional GABA$_B$ receptors by embryonic neurons was first reported in the hypothalamus (Obrietan and van den Pol, 1999). More recently, GABA$_B$ receptors have been shown to be involved in mechanisms of migrating embryonic cortical cells, both in radial (Behar et al., 2000, 2001) and in tangential migration (López-Bendito et al., 2003). Furthermore, migrating neocortical cells exhibited sensitive responses to GABA$_B$ receptor antagonists, indicating that they express functional receptors (Behar et al., 2001; López-Bendito et al., 2003). We have previously shown that the two subunits colocalize in the same embryonic cortical cells (López-Bendito et al., 2002), a prerequisite to form functional GABA$_B$ receptors. In the hippocampus, the similar expression pattern of GABA$_{B1a/b}$ and GABA$_{B2}$ suggests that GABA$_B$ receptors may also be functionally active during prenatal development.

The presence of GABA$_B$ receptors on embryonic hippocampal cells raises the question of their functional significance. It has been suggested that GABA$_B$ receptors play an inhibitory role in the regulation of the cytosolic Ca^{2+} transients elicited by GABA$_A$ or glutamate receptor activation in hypothalamic neurons (Obrietan and van den Pol, 1998, 1999). By modulating intracellular Ca^{2+} in embryonic hippocampal cells, GABA$_B$ receptors could play a role in the regulation of processes essential for neuronal development.

Postnatal Developmental Distribution of GABA$_B$ Receptors

At the light microscopic level, GABA$_{B1a/b}$ and GABA$_{B2}$ were widely distributed in the postnatal hippocampus showing a strong

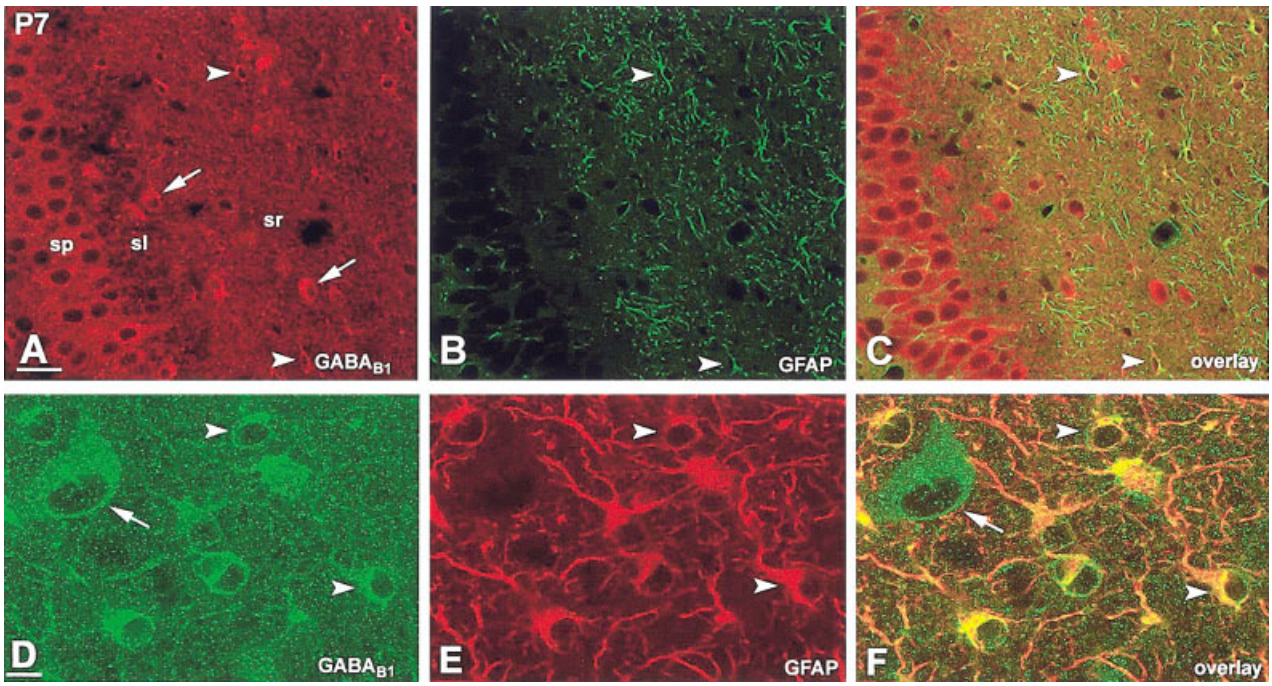

FIGURE 4. Characterization of glial cells expressing γ-aminobutyric acid $(GABA)_{B1a/b}$ during postnatal development. A–F: Immunofluorescence for $GABA_{B1a/b}$ (red) and GFAP (green) in the hippocampus at P7 in the CA3 (A–C) and CA1 region (D–F). Abundant small size cells (arrowheads) and processes immunoreactive for GFAP in all subfields of the hippocampal formation colocalized with the receptor, demonstrating that $GABA_{B1a/b}$ was expressed in glial cells. Large size cells, putative neurons, immunoreactive for $GABA_{B1}$ that did not express GFAP (arrows) were also observed. Scale bars = 100 μm in A–C. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

overlap in distribution. Our labeling pattern is consistent with earlier in situ hybridization (Kaupmann et al., 1998a; Bischoff et al., 1999; Durkin et al., 1999; Lu et al., 1999; Liang et al., 2000), autoradiographic (Swann et al., 1989; Chu et al., 1990; Turgeon and Albin, 1994; Bischoff et al., 1999) and light microscopic studies (Fritschy et al., 1999; Margeta-Mitrovic et al., 1999; Charles et al., 2001). Intensity of immunoreactivity for $GABA_{B1a/b}$ and $GABA_{B2}$ was similar in most hippocampal subfields, with the exception of the stratum pyramidale. One possibility is that very little $GABA_{B2}$ mRNA is translocated to that particular region. Nevertheless, we observed a convincing overlap between the distribution of the $GABA_{B1a/b}$ and $GABA_{B2}$ immunoreactivity in the dendritic fields of principal cells throughout the postnatal hippocampus.

Apart from the pyramidal cell staining, strongly labeled somata of GABAergic cells were scattered all over the hippocampus, consistent with previous morphological studies (Fritschy et al., 1999; Margeta-Mitrovic et al., 1999). Their distinct morphology and location in the hippocampal formation suggest that different populations of interneurons contain $GABA_B$ receptors, (Sloviter et al., 1999) although, remarkably, they showed immunoreactivity for $GABA_{B1a/b}$, but not for $GABA_{B2}$. However, ultrastructural studies demonstrated that both receptor subtypes are present in the plasma membrane. Immunoelectron microscopy further demonstrated that this strong somatic labeling was due to the abundance of receptors in the endoplasmic reticulum. The $GABA_{B2}$ subunit has been shown to be essential for the translocation of $GABA_{B1a/b}$ to the plasma membrane and for the formation of fully functional receptors (Jones et al., 1998; Kaupmann et al., 1998a; White et al., 1998; Kuner et al., 1999). Thus, the lack of the $GABA_{B2}$ subunit in the endoplasmic reticulum in these cells may be responsible for the accumulation of this possible nonfunctional pool of $GABA_{B1a/b}$ receptors.

In addition to neurons, glial cells in the hippocampal formation showed $GABA_{B1a/b}$ immunoreactivity during postnatal development. This result is in agreement with previous findings of $GABA_B$ receptor-mediated effects in glial cells throughout the brain (Hosli and Hosli, 1990). In contrast, no immunostaining for $GABA_{B2}$ was observed in glial cells (Clark et al., 2000). The expression of $GABA_{B1a/b}$ in glial cells, however, appears to be restricted to developmental stages, as we did not detect significant levels in the adult hippocampus (Kulik et al., 2003). These results are consistent with in situ hybridization studies in which no detectable levels of $GABA_{B1a/b}$ were found in the mature hippocampus (Kaupmann et al., 1997; Liang et al., 2000).

Subcellular Localization of $GABA_B$ Receptors

At the ultrastructural level, immunoreactivity for $GABA_B$ receptors revealed that the strong labeling detected in the postnatal hippocampal formation is mainly attributed to the presence of receptors in pyramidal and nonpyramidal cells. This finding is in agreement with in situ hybridization and immunohistochemical studies demonstrating the abundance of $GABA_{B1a/b}$ and $GABA_{B2}$ in hippocampal pyramidal and nonpyramidal cells in adulthood (Kaupmann et al., 1998a; Bischoff

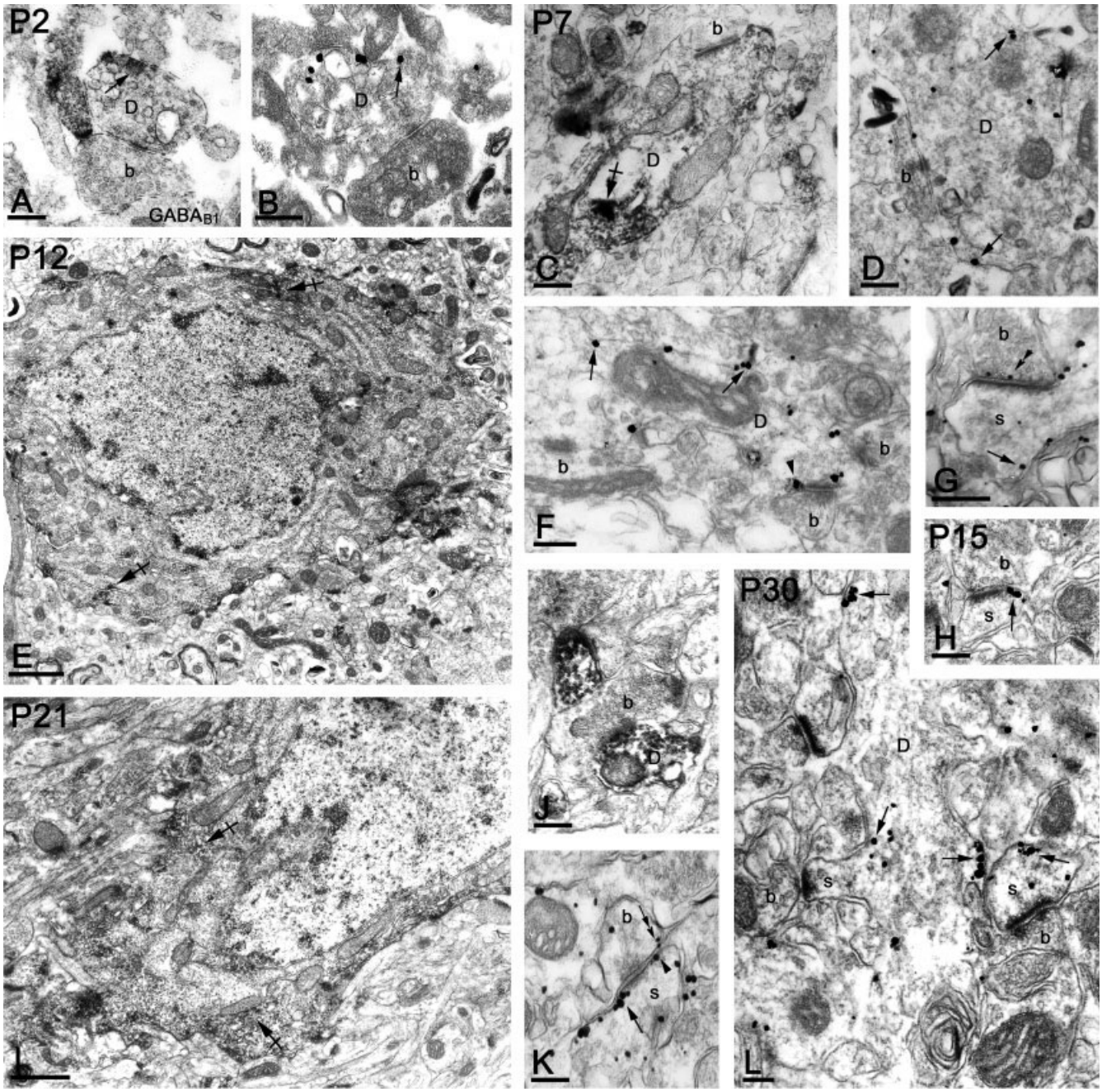

FIGURE 5. Electron micrographs showing immunoreactivity for γ-aminobutyric acid (GABA)$_{B1a/b}$ in the hippocampus during postnatal development in the CA1 strata oriens (A,B) and radiatum (C–G), as well as in the CA3 strata oriens (I–K) and radiatum (H,L), using pre-embedding immunoperoxidase and immunogold methods. A–D: At P2 and P7, GABA$_{B1a/b}$ immunolabeling concentrated at the extrasynaptic plasma membrane of dendrites (D) of pyramidal cells (arrows) postsynaptic to axon terminals (b) and also associated with intracellular membranes (crossed arrow). E–L: From P12 to P30, peroxidase reaction end-product was mainly associated with the endoplasmic reticulum (crossed arrows) and with dendritic shafts (D) of pyramidal cells and interneurons (J). Immunoparticles were localized to the extrasynaptic plasma membrane of dendritic spines (s) of pyramidal cells (arrows in G,H,K) and dendritic shafts (D) of pyramidal cells (arrows in K) and interneurons (arrows in F). Immunoparticles also localized at the edge of the synaptic specialization (arrowheads). Presynaptically, immunoparticles were present along the presynaptic active zone (double arrowheads) and the extrasynaptic plasma membrane of axon terminals (b, double arrows) establishing asymmetrical and symmetrical synapses. Scale bars = 0.5 μm in A–D,F–H,J–L; 2 μm in E,I.

et al., 1999; Liang et al., 2000; Kulik et al., 2003). Previous ultrastructural studies have shown that GABA$_B$ receptors are localized both post- and presynaptically in several brain regions (Kaupmann et al., 1998a; Fritschy et al., 1999; Gonchar et al., 2001; Kulik et al., 2002; López-Bendito et al., 2002). At their postsynaptic localization, GABA$_B$ receptors were found at extrasynaptic sites, at the edge of synapses, and occasionally over the synaptic membrane specialization. In the hippocampus, we

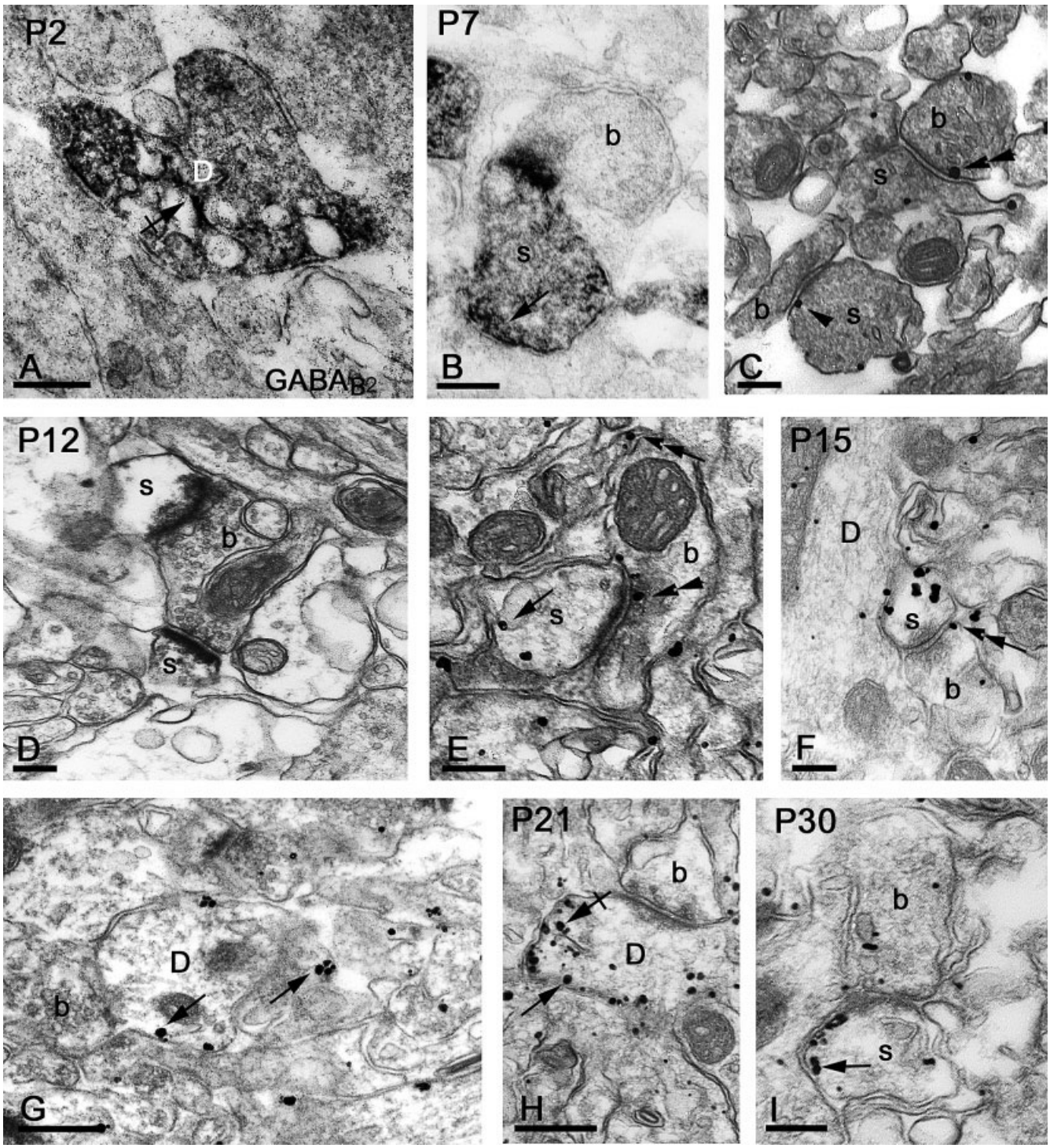

FIGURE 6. Electron micrographs showing immunoreactivity for γ-aminobutyric acid $(GABA)_{B2}$ in the hippocampus during postnatal development in the CA1 strata oriens (A, G) and radiatum (B,C), as well as in the CA3 strata oriens (D,E) and radiatum (F,H,I) using pre-embedding immunoperoxidase and immunogold methods. A–C: At P2 and P7, Peroxidase reaction end-product was found in dendritic spines (s) and dendritic shafts (D) of pyramidal cells, establishing synaptic contacts with axon terminals (b) and also associated with intracellular membranes (crossed arrow). Immunoparticles were localized postsynaptically along the plasma membrane of dendritic spines (s, arrowhead) and presynaptically along the plasma membrane of axon terminals (b, double arrowhead). D–I: From P12 to P30, immunoperoxidase for $GABA_{B2}$ was mainly detected in dendritic spines (s) of pyramidal cells in close apposition to the plasma membrane (arrow in B). Most immunogold particles were detected in the extrasynaptic plasma membrane of dendritic spines of pyramidal cells (arrows in E,F,I) and dendritic shafts of interneurons (arrows in G,H). Immunoparticles also associated to intracellular membranes (crossed arrow in H). At the presynaptic level, immunoparticles were found in the presynaptic active zone (double arrowheads) and the extrasynaptic plasma membrane of axon terminals (b, double arrows) establishing asymmetrical and symmetrical synapses. Scale bars = 0.5 μm in A,B,D,G; 0.2 μm in C,E,F,H; 0.1 μm in I.

have shown that $GABA_B$ receptors were localized pre- and postsynaptic to both glutamatergic and GABAergic synapses during postnatal development, and the same distribution has been observed in the adult (Kulik et al., 2003). Interestingly, at their postsynaptic location, $GABA_B$ receptors were observed in dendritic spines of pyramidal cells, which also express high levels of mGluR5, particularly at a perisynaptic location (Luján et al., 1996, 1997). Overall, the ubiquitous expression of $GABA_{B1a/b}$ and $GABA_{B2}$ in developing hippocampal pyramidal and nonpyramidal neurons suggests the involvement of $GABA_B$ receptors in many intracortical circuits.

Several studies have suggested that, in some locations, $GABA_{B1a}$ was located presynaptically, whereas $GABA_{B1b}$ was located postsynaptically (Bettler et al., 1998; Kaupmann et al., 1998b; but see Benke et al., 1999). However, we were unable to differentiate the detailed subcellular localization of the two subunits during development, which requires further immunoelectron microscopic studies using isoform-specific antibodies. Whatever the specific localization of the two subunits may be, it has been demonstrated that physiological responses following activation of $GABA_B$ receptors require their coassembly with $GABA_{B2}$ (Bowery et al., 2003). The similar subcellular localization of $GABA_{B1a/b}$ and $GABA_{B2}$ that we have observed on neuronal elements of the hippocampal formation further supports that membrane-bound $GABA_B$ receptors form heteromeric assemblies, and that subsets of excitatory and inhibitory synapses may express functional $GABA_B$ receptors.

$GABA_B$ Receptors in Presynaptic Axon Terminals

The excitability of pyramidal cells is regulated by a diverse population of inhibitory interneurons located throughout the hippocampal formation (Freund and Buzsaki, 1996). Interneurons located in the hippocampus form synaptic contacts with specific synaptic regions of the principal cells they innervate. In the present study, we directly demonstrated that a subpopulation of symmetrical, mostly GABAergic, synapses on somata and dendrites of pyramidal cells contain presynaptic $GABA_B$ receptors during postnatal development, but we have not identified the source of the terminals. This result is in line with functional studies showing that $GABA_B$ receptor-mediated inhibition acts on GABAergic terminals (Deisz and Prince, 1989; Davies et al., 1990). In these inhibitory synapses, $GABA_B$ receptors may function as autoreceptors suppressing GABA release by inhibition of L-, P-, and N-type Ca^{2+} channels (Dutar and Nicoll, 1988; Misgeld et al., 1995).

Although activation of inhibitory synapses produces a slow hyperpolarization of pyramidal cells (Shao and Burkhalter, 1999), some of them lacked $GABA_B$ receptor-mediated responses (Funahashi and Stewart, 1998), suggesting that different types of interneurons operate through fast $GABA_A$ and slow $GABA_B$ receptor inhibition. Thus, a differential modulation of interneurons may provide mechanisms to regulate the function of developing principal cells.

The glutamatergic terminals innervating dendritic spines of pyramidal cells and shafts of interneurons express a variable level of presynaptic mGluR7a (Shigemoto et al., 1996). Here, similar to our recent description of the developing neocortex (López-Bendito et al., 2002), we also observed expression of $GABA_{B1a/b}$ and $GABA_{B2}$ in presynaptic membranes of asymmetrical, mostly glutamatergic, synapses in the developing hippocampus. This finding is consistent with previous studies that showed heteroreceptor-mediated presynaptic depression of excitatory responses by $GABA_B$ receptor agonist in the hippocampus (Dutar and Nicoll, 1988). Thus, activation of presynaptic $GABA_B$ receptors on excitatory nerve terminals may suppress glutamate release from hippocampal axon terminals.

Disruption of the $GABA_{B1}$ gene leads to a number of behavioral, biochemical and functional changes in mice (Prosser et al., 2001; Schuler et al., 2001), but the circuits that are disrupted have not been identified yet. The presence of the receptors in axon terminals, which release glutamate and probably not GABA, raises the question of the identity of the agonist for $GABA_B$ receptors. One possible scenario is that heteroreceptors are activated by GABA spilling over from neighboring GABAergic synapses (Scanziani, 2000). Furthermore, the activation of $GABA_B$ receptors by nonsynaptically released GABA or the release of an agonist, possibly taurine, for presynaptic $GABA_B$ receptors from the GABAergic terminal cannot be excluded. Finally, high concentrations of both GABA and glutamate have been reported in the terminals of hippocampal granule cells (Sloviter et al., 1996), which appear to be released after granule cell activation (Gutiérrez, 2000,2002; Walker et al., 2001). Regardless of the mechanism of activation of $GABA_B$ receptors, the presence of $GABA_{B1a/b}$ and $GABA_{B2}$ at excitatory synapses suggests that they are likely to be involved in developmental physiological events that take place in the hippocampus.

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