Differential Distribution of Group I Metabotropic Glutamate Receptors during Rat Cortical Development

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Neurons in the rat cerebral cortex are enriched in group I metabotropic glutamate receptor (mGluR) subtypes and respond to their activation during development. To understand better the mechanisms by which mGluR1 and mGluR5 mediate these effects, the goal of this study was to elucidate the expression pattern and to determine the cellular and the precise subcellular localization of these two receptor subtypes in the rat neocortex and hippocampus during late prenatal and postnatal development. At the light microscopic level, mGluR1α and mGluR5 were first detected in the cerebral cortex with different expression levels at embryonic day E18. Thus, mGluR5 had a moderate expression, whereas mGluR1α was detected as a diffuse and weak labeling. mGluR5 was localized in some Cajal–Retzius cells as well as in other cell types, such as pioneer neurons of the marginal zone. During postnatal development, the distribution of the receptors dramatically changed. From P0 to around P10, mGluR1α was localized in identified, transient Cajal–Retzius cells of neocortex and hippocampus, until these cells disappear. In addition, a population of interneurons localized the receptor from the second/third postnatal week. In contrast, mGluR5 was localized mainly in pyramidal cells and in some interneurons, with a neuropilar staining throughout the cerebral cortex. At the electron microscopic level, the immunoreactivity for both group I mGluR subtypes was expressed postsynaptically. Using immunogold methods, mGluR1α and mGluR5 immunoreactivities were found throughout postnatal development at the edge of postsynaptic specialization of asymmetrical synapses. These results show that the two group I mGluRs have a differential expression pattern in neocortex and hippocampus that may suggest roles for the receptors in the early processing of cortical information and in the control of cortical developmental events.

Introduction

Glutamate is the main excitatory neurotransmitter in the cerebral cortex (Storm-Mathisen and Ottersen, 1988; Tsumoto, 1990; Ottersen, 1991; McCormick, 1992). Its involvement in numerous physiological functions during development and in adulthood is now quite well established (Nakanishi *et al.*, 1994). The effects of glutamate in the cerebral cortex are mediated by activation of ionotropic and metabotropic receptors (Hollmann and Heinemann, 1994; Nakanishi, 1994; Pin and Duvoisin, 1995; Conn and Pin, 1997). Ionotropic glutamate receptors are ligand-gated cation channels that mediate fast excitatory neurotransmission, whereas metabotropic glutamate receptors (mGluRs) are coupled to intracellular signal transduction via G-proteins and mediate slower responses (Baskys, 1992; Nakanishi, 1992, 1994; Pin and Duvoisin, 1995). To date, eight different subtypes of mGluRs have been identified (mGluR1–mGluR8), which have been classified into three groups based on their sequence homology, transduction mechanisms and pharmacological profiles (Nakanishi, 1994; Pin and Duvoisin, 1995; Conn and Pin, 1997). Group I, the subject of the present study, includes mGluR1 and mGluR5, which exist in a number of alternatively spliced forms (mGluR1α, β, c, d and mGluR5a, b). Activation of group I mGluRs lead to increases in phosphoinositide (PI) hydrolysis, resulting in formation of diacylglycerol and IP_3 and mobilization of Ca^{2+} from intracellular stores.

Group I mGluRs are important in the genesis of synaptic plasticity, such as the induction of long-term potentiation and long-term depression (Aiba *et al.*, 1994; Lu *et al.*, 1997). These receptors modulate neuronal excitability and synaptic transmission through regulation of ion channels and ionotropic receptors (Pin and Duvoisin, 1995). Light microscopic immunocytochemistry and *in situ* hybridization studies have shown that group I mGluRs (mGluR1, mGluR5) are particularly abundant in the cerebral cortex of adult animals (Shigemoto *et al.*, 1992; Romano *et al.*, 1995; Luján *et al.*, 1996). Electron microscopic studies revealed the highest density of mGluR1 and mGluR5 located outside the synaptic membrane specialization (Baude *et al.*, 1993; Nusser *et al.*, 1994; Luján *et al.*, 1996, 1997) and they appeared to be restricted to postsynaptic elements (Shigemoto *et al.*, 1997). Via group I mGluRs, glutamate produces slow depolarization in cortical neurons and shifts the firing mode from rhythmic bursting to single spikes, likely resulting from the inhibition of K^+ channels (Pin and Duvoisin, 1995). Group I mGluRs are also especially important for the regulation of pyramidal cell excitability in the hippocampus, including depolarization and increased cell firing (Pin and Duvoisin, 1995).

Messenger RNA transcripts and receptor proteins for mGluRs are expressed in the developing rat cerebral cortex (Catania *et al.*, 1994; Furuta and Martin, 1999; Muñoz *et al.*, 1999). Several lines of evidence suggest that group I activation might play an important role in neuronal differentiation and synaptogenesis during cortical development. First, differentiation of cortical laminae during neurogenesis is accompanied by changes in mGluR expression (Furuta and Martin, 1999; Muñoz *et al.*, 1999). Second, neuronal calcium oscillations, that are thought to be of importance in signal transduction and the regulation of gene expression, are produced by endogenous activation of mGluRs (Flint *et al.*, 1999). Third, phosphoinositide (PI) hydrolysis induced by activation of mGluRs is dramatically enhanced in developing compared to adult brain (Sladeczek *et al.*, 1985; Nicoletti *et al.*, 1986; Schoepp and Hillman, 1990; Sortino *et al.*, 1991; Romano *et al.*, 1995, 1996; Casabona *et al.*, 1997). Fourth, the use of specific group I mGluR agonists evoked $[Ca^{2+}]_i$ increases, indicating functional mGluRs in layer I and demonstrating the presence of mGluR1α in Cajal–Retzius cells in mouse (Martínez-Galán *et al.*, 2001).

Information regarding distribution of mGluRs in the cerebral cortex is important in helping to determine the roles that the different receptor subtypes may play in pre- and postnatal development. In addition, the localization of receptors to identified cells and synapses may help allocate particular subtypes to different functional roles. However, the expression pattern and the discrete cellular and subcellular localization of group I

mGluR proteins during corticogenesis are not fully identified. To understand better the mechanisms by which mGluRs mediate physiological effects in developing cortical neurons, the goal of the present study is to characterize the expression pattern and the cellular and the precise subcellular localization of mGluR1α and mGluR5 in the rat neocortex and hippocampus during pre- and postnatal development. A preliminary report of this work has appeared in abstract form (López-Bendito *et al*., 2000).

Materials and Methods

Preparation of Animals and Tissue

The present study was carried out on the brains of a total of 47 Wistar rats, ranging in age from embryonic day 14 (E14) to adulthood. Animals were grouped as follows: embryonic day 14 = E14 (n = 3), E16 (n = 3), E18 (n = 6); day of birth = P0 (n = 4); postnatal day 2 = P2 (n = 5), P5 (n = 4), P7 (n = 5), P10 (n = 5), P15 (n = 4), P21 (n = 4) and P60 (n = 4). The care and handling of the animals prior to and during the experimental procedures followed European Union regulations, and were approved by the Animal Care and Use Committees of the authors' institutions. All efforts were made to reduce the number of animals used and minimize animal suffering.

Antibodies and controls

Affinity-purified polyclonal antibodies against mGluR1α and mGluR5 were raised in rabbits. The characteristics and specificity of these antibodies have been described elsewhere (Shigemoto *et al*., 1993; Jaarsma *et al*., 1998). A monoclonal anti-reelin antibody, a gift of Dr A.M. Goffinet, was also used (de Bergeyck *et al*., 1998), as well as a monoclonal anti-Calbindin antibody (Chemicon, Temecula, CA).

To test method specificity in the procedures for light and electron microscopy, the primary antibody was omitted or replaced by 5% (v/v) normal serum of the species of the primary antibody. Under these conditions, no selective labeling was observed. Staining patterns were also compared to those obtained by calretinin and calbindin (Chemicon, Temecula, CA); only the antibodies to mGluR1α and mGluR5 consistently labeled the plasma membrane of cells. When double labeling 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 of secondary antibodies. Other sections were incubated with two primary antibodies and one secondary antibody, followed by the full sequence of signal detection. No cross-labeling was detected that would influence the results.

Immunocytochemistry for Metabotropic Glutamate Receptors

Light Microscope Procedures

Foetuses were collected by Caesarean section after anaesthesia of the dam with an i.p. injection of a Rompun/Imalgene mixture (0.1 ml/kg body wt). Animals were deeply anaesthetized by hypothermia (E14–P5) or by i.p. injection of Rompun–Imalgene 1:1 (0.1 ml/kg body wt) and then the hearts were surgically exposed for perfusion fixation. Perfusion was carried out as similar as described earlier (López-Bendito *et al*., 2001). After perfusion, tissue blocks containing the cerebral cortex and hippocampus were dissected and washed thoroughly in 0.1 M phosphate buffer (PB) for several hours. 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 h. Sections were then incubated for 48 h with affinity-purified polyclonal antibodies against mGluR1α or mGluR5 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 h in biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted 1:50 in TBS containing 1% NGS. They were then transferred into avidin–biotin–peroxidase complex (ABC kit, Vector Laboratories) diluted 1:100 for 2 h at room temperature. 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 prior to observation in a Leica DMRS photomicroscope equipped with differential interference contrast optics.

Alternate sections were used for immunofluorescence experiments using a laser scanning confocal microscope. Primary antibodies used in combination with polyclonal antibodies to group I metabotropic receptors were monoclonal anti-reelin antibody G10 diluted 1:1000, and a 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 2 h 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).

Each labeling combination was analyzed in sections from two to four prenatal or postnatal animals. Co-localization of mGluR1α or mGluR5 with the neurochemical markers reelin and calbindin was assessed by confocal laser microscopy (Leica TLSCM). For each marker, a semi-quantitative estimation of the proportion of double labeled neurons was performed by analyzing samples of 100–130 cells, collected in eight to ten coronal sections through the prenatal or postnatal neocortex and hippocampus. Images were stored and analyzed using appropriate hardware and 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 Microscope Procedures

Animals were anaesthetized as described above and perfused as described earlier (Luján *et al*., 1996; López-Bendito *et al*., 2001). After perfusion, tissue blocks containing the cerebral cortex and hippocampus were dissected and washed thoroughly in 0.1 M PB for several hours. Coronal 60 μm sections were cut with a Vibratome and collected in 0.1 M PB.

Pre-embedding Immunoperoxidase Method. Immunocytochemical reactions were carried out as described earlier (Luján *et al*., 1996; López-Bendito *et al*., 2001). Free-floating sections were incubated in 10% NGS diluted in TBS for 1 h. Sections were then incubated for 48 h in an affinity-purified polyclonal antibody anti-mGluR1α or anti-mGluR5 at a final protein concentration of 1–2 μg/ml diluted in TBS containing 1% NGS. After washes in TBS, the sections were incubated for 2 h in biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted 1:50 in TBS containing 1% NGS. They were then transferred into avidin–biotin–peroxidase complex (ABC kit, Vector Laboratories) diluted 1:100 for 2 h at room temperature. Peroxidase enzyme activity was revealed using DAB (0.05% in TB, pH 7.4) as chromogen and 0.01% H_2O_2 as substrate.

Pre-embedding Immunogold Method. 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 for 3 h in goat anti-rabbit IgG coupled to 1.4 nm gold (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 in PBS for 10 min. 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-labeled sections were processed for electron microscopy. This included treatment with OsO_4 (1% in 0.1 M PB), block-staining with uranyl acetate, dehydration in graded series of ethanol and flat-embedding on glass slides in Durcupan (Fluka) resin.

Results

Distribution of mGluR1α and mGluR5 at the Light Microscopic Level

Prenatal Development

The expression of group I mGluRs was studied at prenatal ages E14, E16 and E18. At E14 and E16, we did not detect expression for group I mGluRs. Significant levels of immunoreactivity for

mGluR1α and mGluR5 were only detected in the telencephalon on E18 (Figs 1 and 2). At that age, the two receptors showed a differential pattern of distribution in the neocortex and hippocampus during development.

mGluR1α. The expression of mGluR1α was low at E18 in the developing neocortex and hippocampus (Fig. 1*A*,*B*). In both regions, the labeling was weak and diffuse in the cortical plate and marginal zone and in the cortical subplate (Fig. 1*A*,*B*). No immunopositive cell bodies could be detected at that age (Fig. 1*A*,*B*). However, the expression level was very high in other regions such as the striatum, where labeling was mainly restricted to cell bodies and dendrites (Fig. 1*A*).

mGluR5. In the marginal zone, subplate and cortical plate of the neocortex, immunolabeling for mGluR5 was intense and diffuse at E18 (Fig. 2*A–C*). Immunolabeling was also intense and diffuse in the claustrum primordium, which is ventral to and continuous with the subplate (Fig. 2*A*). Cell bodies located in the marginal zone, some of them with the typical horizontal orientation of Cajal–Retzius cells, and in the subplate were also labeled (Fig. 2*A*,*C*). In the hippocampus, mGluR5 had a moderate expression level (Fig. 2*A*,*B*) and immunopositive neurons could be also detected.

Postnatal Development

An intense expression of mGluR1α and mGluR5 was already found in the cortical areas from the day of birth (P0) and progressively increased until P21, when the adult levels were reached.

mGluR1α in the Neocortex during Postnatal Development. During the first week of postnatal development, immunoreactivity for mGluR1α was mainly detected in cortical layer I neurons (Fig. 3*A–C*). These labeled neurons resembled Cajal–Retzius cells (Fig. 3*B*), having the typical fusiform morphology and horizontal orientation and being located at a distance of ~20 μm from the pial surface (Meyer *et al.*, 1998). By P10, the number of cortical layer I immunoreactive mGluR1α neurons decreased (Fig. 3*C*) and the expression could not be detected shortly after that age. In cortical layers II–VI, mGluR1α immunoreactivity was weakly detected in interneurons (data not shown). This distribution of the receptor in interneurons was more prominent after the third postnatal week (Fig. 3*D–F*), when the adult expression was reached. Interneurons expressing mGluR1α throughout the neocortex had a bipolar or multipolar morphology (Fig. 3*F*).

mGluR1α in the Hippocampus during Postnatal Development. In the hippocampus, mGluR1α immunoreactivity was detected in neurons displaying the morphology of Cajal–Retzius cells (Drakew *et al.*, 1998) located close to the hippocampal fissure, in the stratum lacunosum-moleculare, and in the dentate outer molecular layer (Fig. 5*B*,*C*,*F*,*I*,*J*). MGluR1α immunoreactivity in these horizontal cells was detected in the somata and dendrites beneath the plasma membrane (Fig. 5*J*). After the

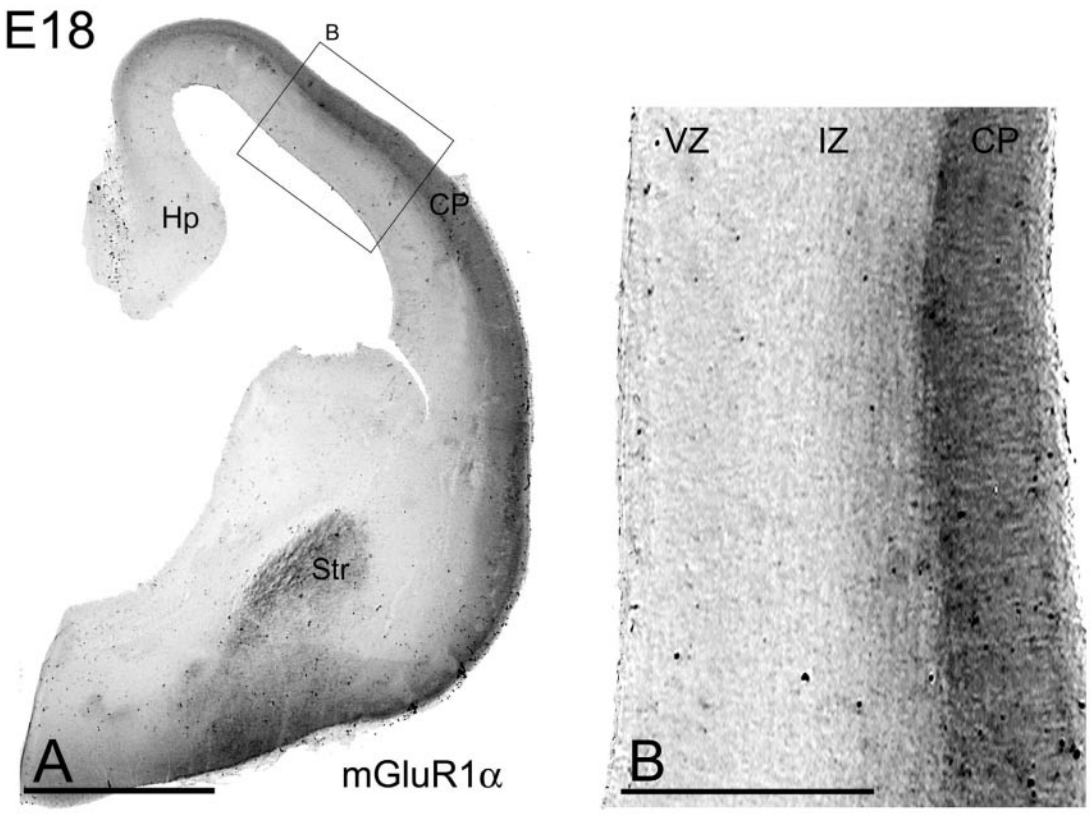

Figure 1. Immunoreactivity for mGluR1α at E18 using a pre-embedding immunoperoxidase method. (*A*) Mid-caudal coronal section of the telencephalon showing the expression and distribution of mGluR1α. The receptor was mainly localized in the cortical plate (CP) and in the striatum (Str). (*B*) Higher magnification of a cortical area labeled for the receptor. Note that only a diffuse immunolabeling was found along the CP.Scale bars: 1 mm (*A*); 200 μm (*B*).

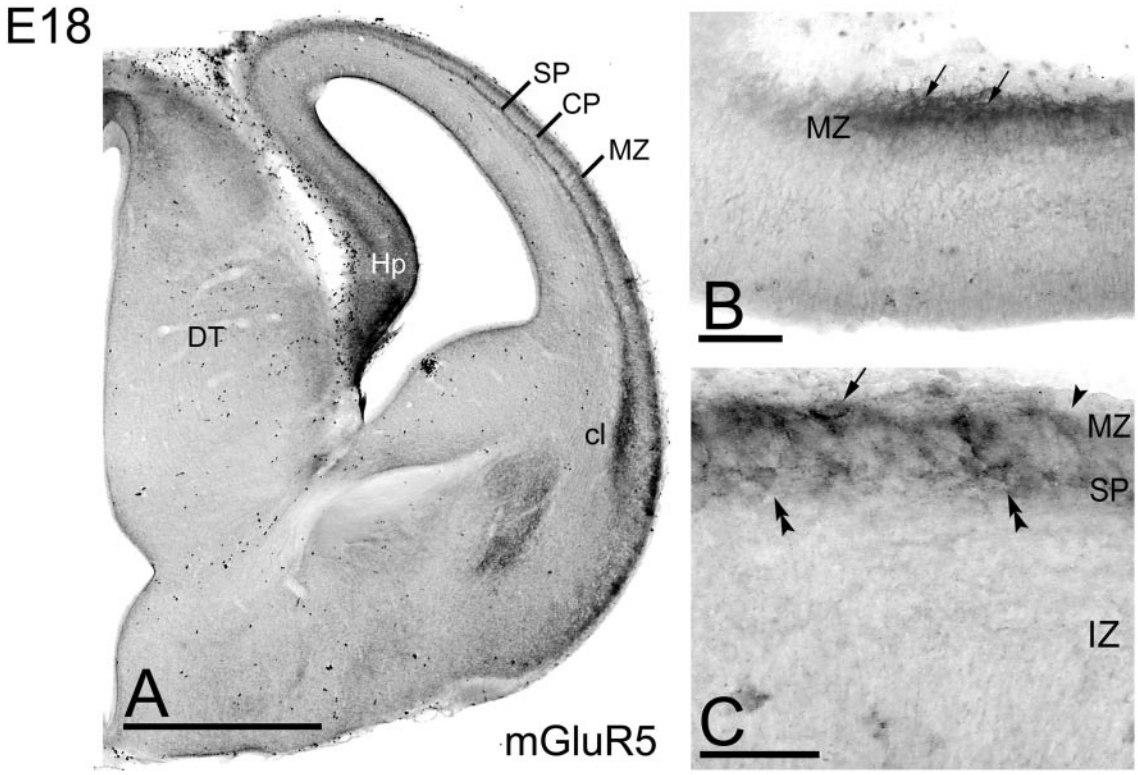

Figure 2. Immunoreactivity for mGluR5 at E18 using a pre-embedding immunoperoxidase method (*A–C*). (*A*) Mid-caudal coronal section labeled for mGluR5. Immunoreactivity was found in the marginal zone (MZ) and subplate (SP) of the cerebral cortex, in the claustrum (Cl) and in the hippocampus (Hp). (*B,C*) High magnification views of the hippocampus and cortex immunopositive for mGluR5. In the MZ of both structures we found a strong somatic labeling in cells located more superficially in this layer (arrows). In the cortex we observed another cell type immunoreactive for mGluR5, but located deeper in this layer (arrowhead). Note also the presence of immunoreactive cells in the SP (double arrowheads). Scale bars: 1 mm (*A*); 100 μm (*D*); 50 μm (*E*).

second and third postnatal week, mGluR1α was distributed in neurons resembling the somatostatin-containing GABAergic neurons of the CA1 alveus/oriens and in interneurons of the CA3 region and hilus of the dentate gyrus (Fig. 5*M*), as previously described (Baude *et al.*, 1993).

mGluR5 in the Neocortex during Postnatal Development. At P0–P2, layer I was strongly labeled for mGluR5 but, in contrast to E18, immunolabeled cell bodies of Cajal–Retzius cells were no longer detected (Fig. 4*A*). During the first postnatal week, neurons of layer II–III did not show mGluR5 immunoreactivity; only apical dendrites of pyramidal cells from deeper layers were labeled in that cortical layer (Fig. 4*A*). MGluR5 immunoreactivity in the neocortex was diffusely distributed in cortical layers IV–VI (Fig. 4*A*). However, the still undifferentiated upper cortical plate was not immunoreactive at those ages (Fig. 4*A*).

At P7–P10, the immunoreactivity for mGluR5 was very intense in the neuropil of layer IV barrel hollows, excluding the barrel septa (Fig. 4*B*,*C*). This neuropilar staining of layers I–IV was still observed during the third postnatal week (Fig. 4*D*). Around P21–P30, all cortical layers reached the same level as labeling (Fig. 4*E*). MGluR5 immunolabeling was then exclusively observed in the neuropil surrounding cell bodies of pyramidal and non-pyramidal cells (Fig. 4*E*). We did not detect axon terminal or glial labeling at any age.

mGluR5 in the Hippocampus during Postnatal Development. In the hippocampus, a high mGluR5 expression was already detected at P0–P2 (Fig. 5*D*). From the first postnatal week, we observed a progressive increase in immunolabeling (Fig. 5*D*,*G*,*K*), similar to what was detected in neocortex. Within the hippocampal CA1 and CA3 areas, the stratum oriens showed the strongest mGluR5 immunoreactivity (Fig. 5*G*,*K*). The distal regions of the stratum radiatum showed weaker expression than the proximal regions and the stratum lacunosum-moleculare (Fig. 5*G*,*K*). The stratum pyramidale was devoid of staining at any postnatal age (Fig. 5*D*,*G*,*K*). At P15–P21, adult levels of mGluR5 expression were reached (Fig. 5*N*). Thus, as previously described (Luján *et al.*, 1996), a strong mGluR5 immunoreactivity was observed in the strata oriens and radiatum (Fig. 5*N*). The stratum lacunosum-moleculare of the CA1 and CA3 regions and the dentate gyrus were weakly labeled. The stratum lucidum of the CA3 region was almost devoid of any mGluR5 labeling.

Characterization of Neurons Expressing Group I mGluRs during Perinatal Development

In order to investigate whether the neurons expressing group I mGluRs in cortical layer I and in the hippocampal marginal zones were Cajal–Retzius cells, we carried out a double-labeling experiments using confocal microscopy for the mGluRs and for reelin, an extracellular matrix glycoprotein that is synthesized and secreted by Cajal–Retzius cells (D'Arcangelo *et al.*, 1995, 1997; Ogawa *et al.*, 1995). This experiment was carried out because reelin expression, and not just cell morphology, is the essential trait of Cajal–Retzius cells (Meyer *et al.*, 1998, 1999). Additionally, we carried out double-labeling experiments for mGluR5 and calbindin. The calcium-binding protein calbindin is

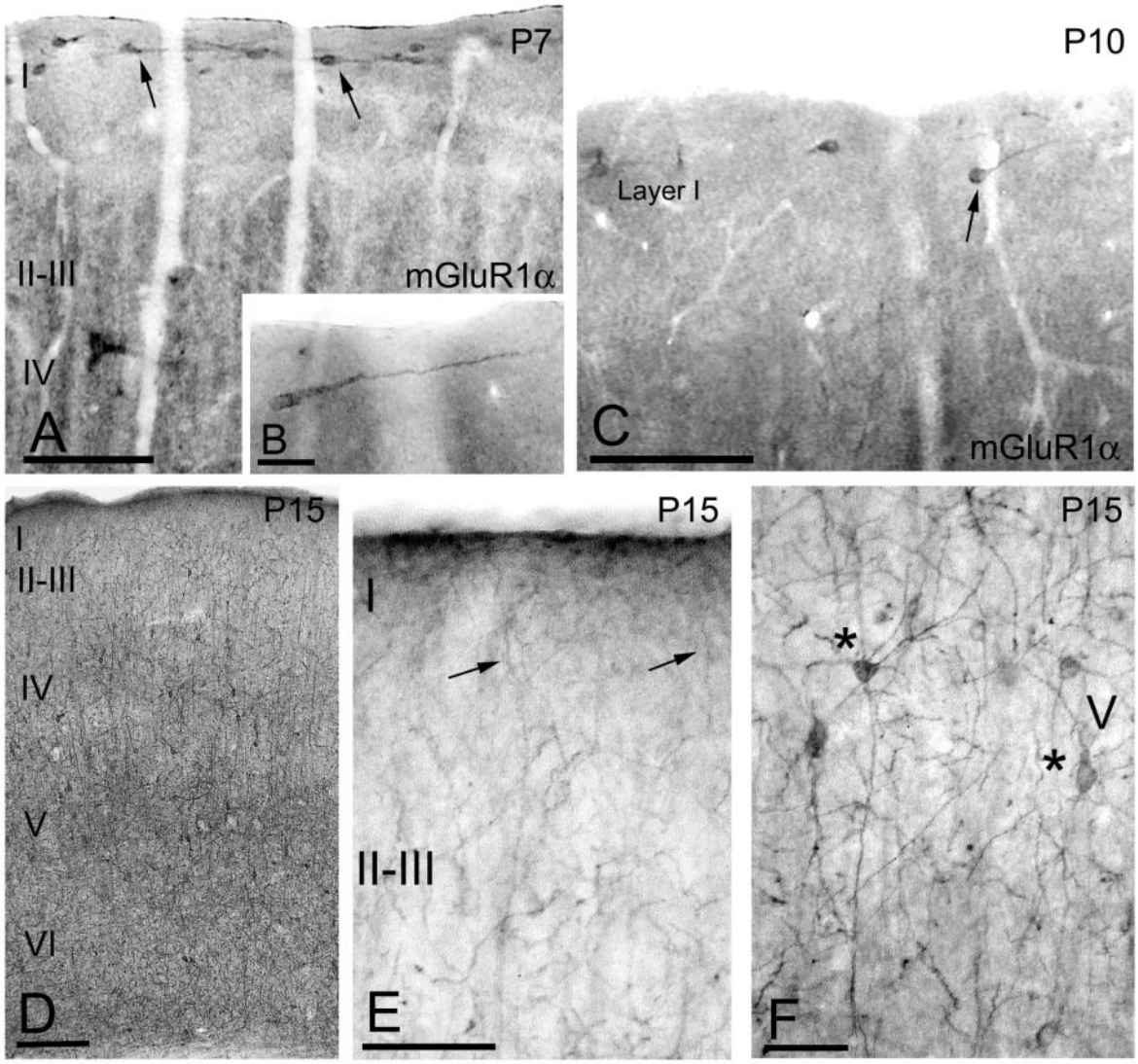

Figure 3. Immunoreactivity for mGluR1α in the neocortex during postnatal development using a pre-embedding immunoperoxidase method (*A–F*). (*A*) Coronal section of a P7 rat brain showing the expression for mGluR1α in the supragranular layers of the cerebral cortex. The receptor was highly expressed in cells located horizontally in layer I with processes running parallel to the pial surface (arrows). (*B*) High magnification of a mGluR1α positive layer I cell with Cajal–Retzius-like morphology. (*C*) At P10, the number of immunolabeled cells in layer I (arrow) decreased. (*D–F*) Later on in development (P15), mGluR1α expression was localized in interneurons along all the cortical layers. We did not observe any cells showing the morphology of Cajal–Retzius cells in layer I at this age. Immunoreactive interneurons were found throughout all layers (asterisks), some of them with their dendrites running up to layer I (arrows), crossing the still undifferentiated upper part of the cortical plate. Scale bars: 100 μm (*A*,*C–E*); 25 μm (*B*); 50 μm (*F*).

a marker of marginal zone pioneer neurons in the rat (Meyer *et al*., 1998, 1999; Soria and Fairén, 2000).

*mGluR1*α

During prenatal development, we did not find co-localization for mGluR1α and reelin in either the neocortex or hippocampus. However, during postnatal development, there was an estimated co-localization of ~98% for mGluR1α- and reelin-immunoreactivity in both cortical layer I (Fig. 6*G–I*) and hippocampal marginal zones (Fig. 4*J–L*). This demonstrated that the neurons expressing mGluR1α were Cajal–Retzius cells.

During prenatal development, we could not detect co-localization for mGluR1α and calbindin either in the neocortex or hippocampus.

mGluR5

During prenatal development, we found that ~25% of cells expressing reelin also expressed mGluR5 (Fig. 6*A–C*), indicating that a subpopulation of mGluR5-immunoreactive neurons in the cortical and hippocampal marginal zones were Cajal–Retzius cells. However, during postnatal development, we could not detect any co-localization of reelin and mGluR5. In addition, ~45% of mGluR5-expressing neurons also expressed calbindin (Fig. 6*D–F*). This, together with the rounded forms of these cells, suggested they could be pioneer neurons of the marginal zone.

*Distribution of mGluR1*α *and mGluR5 at the Electron Microscopic Level*

In order to investigate the precise subcellular localization of group I mGluRs in neocortex and hippocampus during postnatal development, we carried out high-resolution immunohistochemical studies using pre-embedding immunoperoxidase and immunogold techniques. Overall, the pattern of distribution of

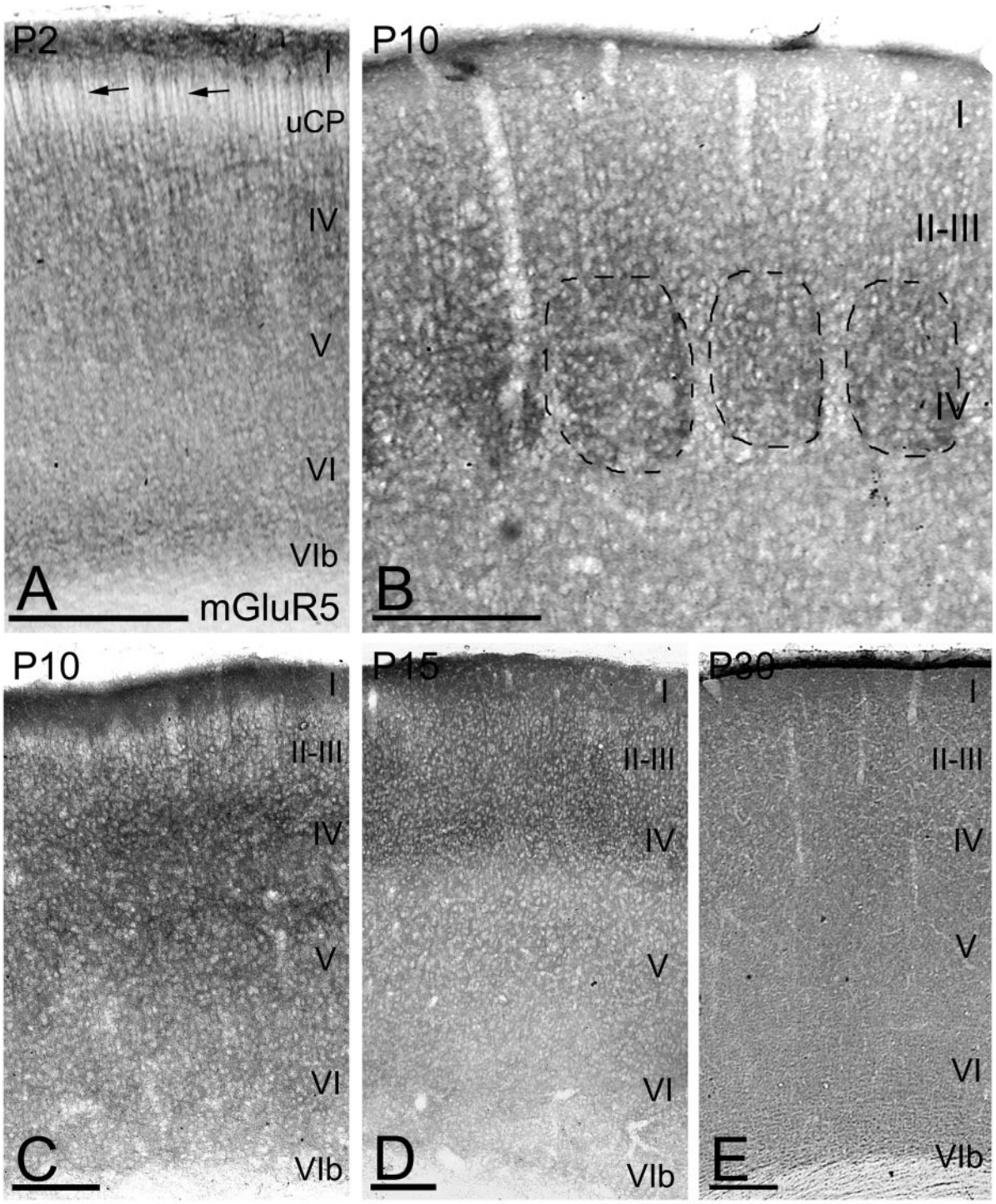

Figure 4. Immunoreactivity for mGluR5 in the neocortex during postnatal development using a pre-embedding immunoperoxidase method (*A–E*). (*A*) Immunolabeling for mGluR5 along the cerebral cortex at P2. The receptor was localized in the neuropil of all cortical layers, but was stronger in layers I and IV. At this age, we observed numerous apical dendrites of pyramidal cells immunoreactive for mGluR5 (arrows). (*B*,*C*) During the second week of postnatal development, mGluR5 was mainly localized in the neuropil of all cortical layers. Intense immunolabeling for the receptor was observed in the layer IV barrel cortex. (*D*) During the third week of postnatal development, mGluR5 was mainly localized in all cortical layers, with a strong labeling in layers I-IV. (*E*) From the fourth postnatal week to adulthood we observed a strong neuropilar staining in all cortical layers. Scale bars: 100 μm (*A*,*B*); 200 μm (*C–E*).

the immunoreactivity for group I mGluRs was consistent with the light microscopic labeling.

mGluR1α

During the first postnatal week, all immunolabeled profiles in cortical layer I and hippocampus were identified as somata or dendrites of Cajal–Retzius cells, as expected on the basis of the light microscopic data. Together with the results of the co-localization experiments (see above), such identification was based on their subpial position in the marginal zones, on their elongated shape and on the presence of abundant rough endoplasmic reticulum in their cytoplasm (Edmunds and Parnavelas,

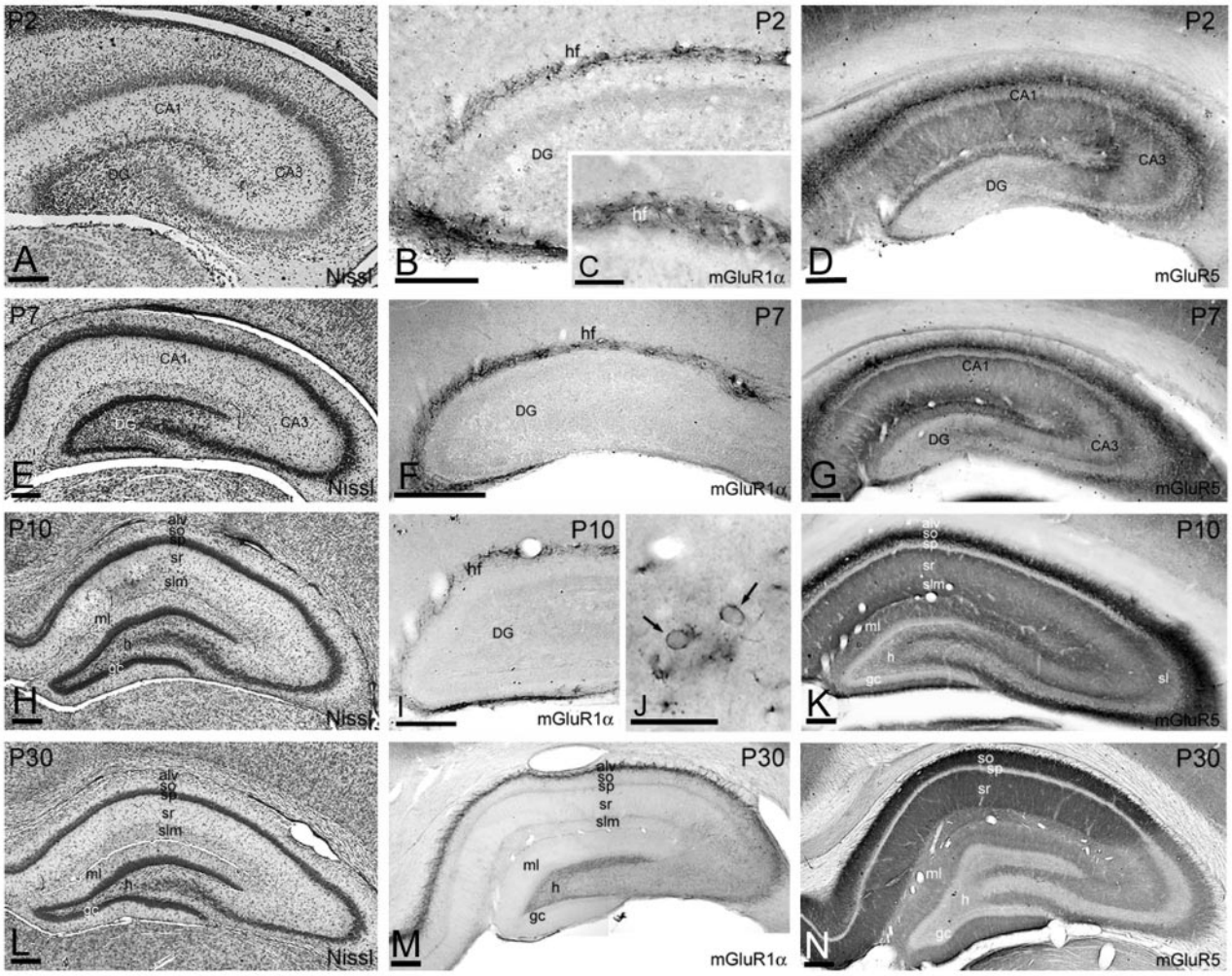

Figure 5. Expression of group I mGluRs during hippocampal development using a pre-embedding immunoperoxidase method. (*A*,*E*,*H*,*L*) Nissl staining of P2, P7, P10 and P30 hippocampi, respectively. (*B*,*C*,*F*) At P2 and P7, mGluR1α immunoreactive cells were observed at both sides of the hippocampal fissure (hf), in the outer molecular layer of the fascia dentata and the stratum lacunosum-moleculare of the hippocampus. Those cells had an elongate morphology resembling to Cajal–Retzius cells. (*I*,*J*) At P10, the number of immunolabeled cells for mGluR1α in the hf decreased. Note the location of the receptor in the plasma membrane (arrows). (*M*) From P15 to adulthood, mGluR1α immunoreactivity was only detected in the alveus, some scattered interneurons in the strata oriens and radiatum of the CA1 and CA3 regions, and in the hilus (h) of the dentate gyrus (de). (*D*,*G*,*K*,*N*) Coronal section of the hippocampus of P2 (*G*), P7 (*H*), P10 (*I*) and P30 (*J*) rat brains. Immunoreactivity for mGluR5 increased during postnatal development. During the first and second postnatal weeks (*D*,*G*,*K*), mGluR5 immunoreactivity was strongest in the stratum oriens of the CA1 and CA3 region. At P30–P60 (*N*), immunoreactivity was very strong in the strata oriens and radiatum of the CA1 region. The alveus, stratum lacunosum-moleculare of CA1, strata oriens and radiatum of the CA3 regions and the molecular layer (ml) of the dentate gyrus were labeled less intensely, with the stratum lucidum (sl) and the dentate hilus (h) being weakly labeled. gc, granule cell layer. Scale bars: 200 μm (*A*,*B*,*D*–*I*,*K*–*N*); 50 μm (*C*); 30 μm (*J*).

1982; Derer and Derer, 1990). The dense reaction product was mainly localized in the plasma membrane of somata and dendrites (Fig. 7*A*,*B*). In somata, immunoperoxidase reaction product was also associated with rough endoplasmic reticulum and Golgi apparatus (Fig. 7*A*). The dendritic spines of these neurons showed mGluR1α expression establishing asymmetrical synaptic contacts with axons terminals (Fig. 7*C*). Immunogold particles were mainly localized on plasma membranes and were less frequently detected in the cytoplasm (Fig. 7*D*). In all cases, immunogold particles were located in perisynaptic sites or associated with non-synaptic plasma membranes (Fig. 7*E*,*F*). Labeled synapses were more frequently found in P7–P10 animals as compared to P0–P2 animals. Pre- synaptic terminals were irregular in shape (e.g. Fig. 7*C*,*E*,*F*) and were never immunolabeled for mGluR1α. They mostly formed synaptic contact on dendritic shafts (Fig. 7*E*) and dendritic spines (Fig. 7*C*,*F*).

Once Cajal–Retzius cells disappeared from layer I at around P12, mGluR1α immunoreactivity was mainly found in interneurons throughout the neocortex. Immunoperoxidase reaction end-product was associated with rough endoplasmic reticulum; however, the end-product was mainly distributed on extrasynaptic plasma membranes (Fig. 8*A*) or displayed a close relationship with synapses (Fig. 8*B*). The proximal (Fig. 8*C*,*D*) and distal (Fig. 8*E*) dendritic shafts of interneurons were also mGluR1α immunoreactive.

mGluR5

Immunoreactivity for mGluR5 was confined to postsynaptic elements, including dendrites and dendritic spines (Fig. 9) at any postnatal age. These findings were consistent with those found using light microscopy. Cell bodies of pyramidal cells or interneurons were mostly devoid of mGluR5 immunoreactivity throughout the cerebral cortex. In cortical layer I, no Cajal–Retzius cell bodies were labeled and only some pyramidal

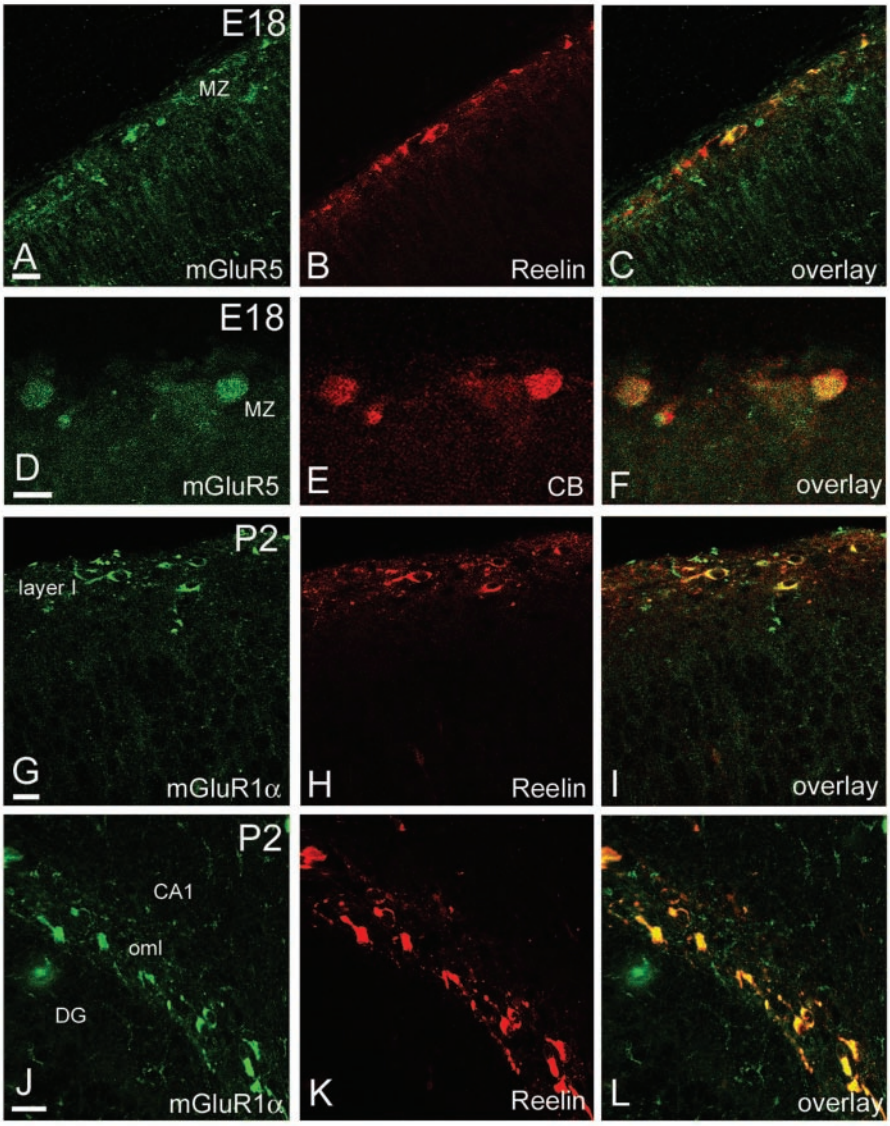

Figure 6. Characterization of the cell population expressing group I mGluRs during pre- and postnatal development. Double-labeling for mGluR1α or mGluR5 and different neuronal markers (calbindin and reelin). (*A–C*) Immunofluorescence for mGluR5 (green) and reelin (red) in the cerebral cortex at E18. Approximately 25 % of the reelin-positive cells in MZ co-localized the receptor, demonstrating that mGluR1α was expressed in Cajal–Retzius cells. The co-localization is shown in (*C*). (*D–F*) High magnification of MZ region labeled with mGluR5 (green) and Calbindin (red). Around 45.% of the calbindin-positive cells in the MZ co-localized mGluR5, as shown in (*F*). (*G–L*) Double immunofluorescence for mGluR1α (green) and reelin (red) in layer I of the cortex (*G–I*) and in the hippocampus (*J–L*) at P2. Virtually all neurons immunopositive for reelin co-expressed mGluR1α in both regions, as shown in (*I*) and (*L*), respectively. hf, hippocampal fissure; DG, dentate gyrus. Scale bars: 20 μm.

cell dendrites and dendritic spines at P3 were mGluR5 immunoreactive (Fig. 9*A*,*B*). In these pyramidal cells, immunogold particles were located in perisynaptic sites or associated with non-synaptic membranes (Fig. 9*C*). In deeper cortical layers, as illustrated at P10, the peroxidase deposit was also distributed in pyramidal cell and interneuron dendritic shafts (Fig. 9*D*), as well as in pyramidal cell dendritic spines (Fig. 9*E*) establishing asymmetrical synapses with axon terminals. Immunogold particles were associated with plasma membranes. In dendritic spines, immunogold particles were concentrated at perisynaptic membrane sites adjacent to postsynaptic densities of putative glutamatergic terminals and at extrasynaptic sites (Fig. 9*F*,*G*).

Discussion

This study provides the first description of the precise cellular and subcellular localization of group I mGluRs in the rat neocortex and hippocampus during pre- and postnatal development. These receptors undergo subtype-specific regulation of their expression and localization during neocortical and hippocampal development, showing evidence for a dynamic and differential pattern of distribution during corticogenesis.

mGluR1α immunoreactivity showed a progressive increase in intensity in both neocortex and hippocampus as development proceeded. The results reported here tend to follow the same sequence of developmental variations in the levels of mGluR1 expression (Shigemoto *et al.*, 1992; Catania *et al.*, 1994). It has also been demonstrated that cortical mGluR5 expression increases perinatally, peaks around the second postnatal week and decreases thereafter (Catania *et al.*, 1994; Romano *et al.*, 1996). These changes in expression are associated with a decline in mGluR5a mRNA expression and an increase in mGluR5b mRNA levels, which predominates in adults (Minakami *et al.*, 1995; Romano *et al.*, 1996). In the cortex, we did not find a clear

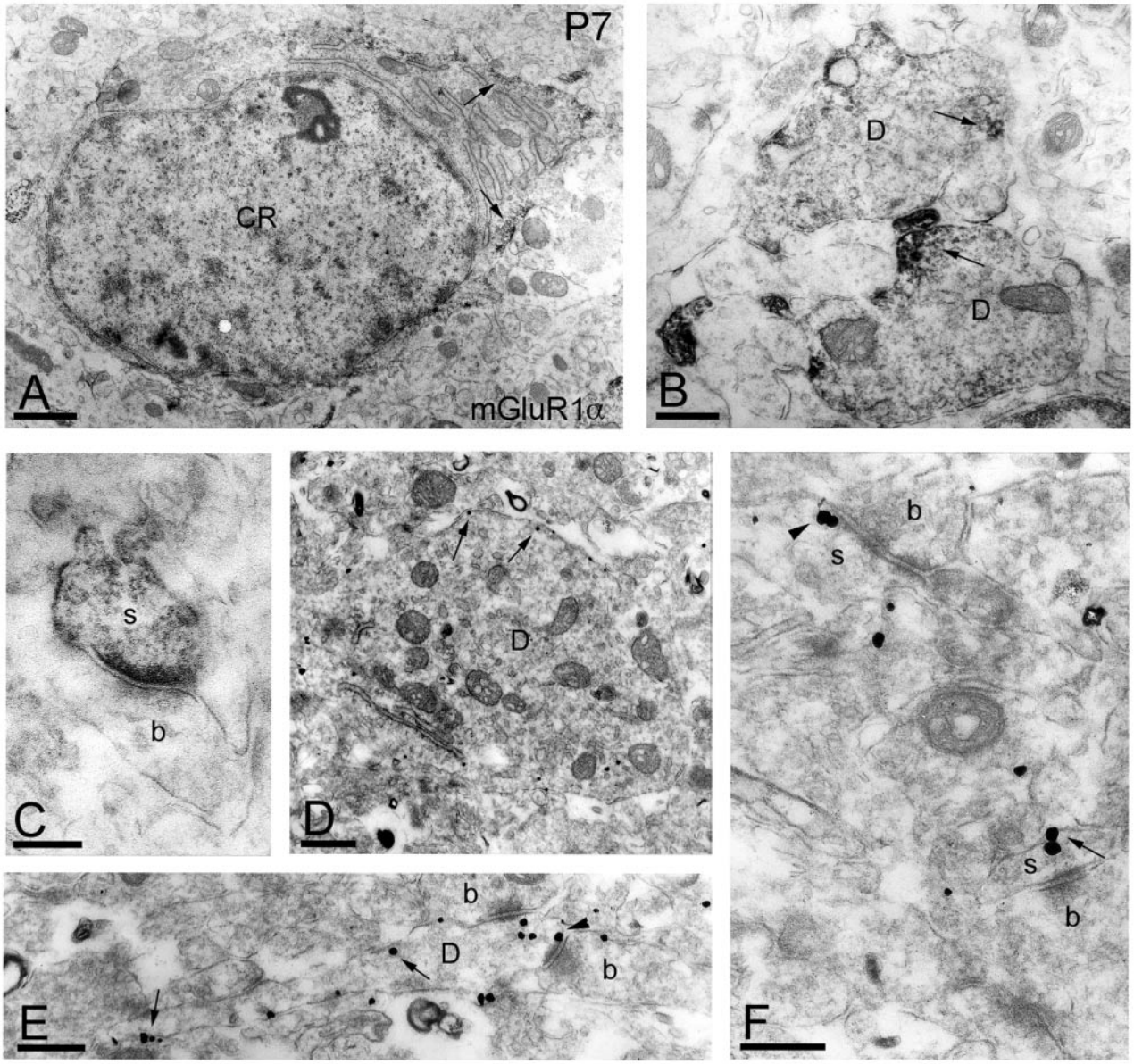

Figure 7. Immunoreactivity for mGluR1α in layer I of the cortex and hippocampus at P7 as demonstrated by pre-embedding immunoperoxidase and immunogold reactions — (*A*,*B*) cortex, (*C–F*) hippocampus — showing the subcellular localization of the receptor in Cajal–Retzius cells. The neuron contained a rounded large nucleus and abundant stacks of endoplasmic reticulum, identifying it as a Cajal–Retzius (CR) cell. (*A–C*) the peroxidase reaction end-product was detected in somata, in dendrites (D) and in dendritic spines (s), mainly in association with the plasma membrane — arrows in (*A*,*B*). Many of the immunoreactive spines were postsynaptic to axon terminals (b), forming asymmetrical synapses. (*D–F*) Immunoreactive dendrites and spines revealed by an immunogold method. Immunoparticles were associated with the internal surface of the plasma membrane (arrows) of dendrites (D) and spines (s) at extrasynaptic sites. Gold particles were often present at the edge (arrowheads) of the postsynaptic membrane specialization of asymmetrical synapses between dendrites (D) or spines (s) of Cajal–Retzius cells and axon terminals (b). Scale bars: 10 μm (*A*); 0.2 μm (*C*,*F*); 0.3 μm (*B*,*E*); 0.5 μm (*D*).

drop in mGluR5 immunoreactivity, but we found no major increase comparable to that revealed for mGluR1α; this is similar to what has been described in thalamus (Liu *et al*., 1998).

Interestingly, mGluR5 immunolabeling showed an important reorganization of cortical pyramidal cells. Thus, during the first postnatal week, mGluR5 was preferentially located in pyramidal cell dendrites terminating in layer I. As development proceeded, the receptor became more widely distributed on the surface of the pyramidal cells. The mGluR5 became localized on dendritic spines and associated with synapses. A similar redistribution of group I mGluRs during development of corticothalamic function has also been described in the thalamus (Liu *et al*., 1998).

Group I mGluRs are Transiently Expressed in the Marginal Zone/Layer I

Group I mGluRs expression in the marginal zone (future layer I) changed during development and peaked perinatally. This developmental pattern suggests that mGluR1α and mGluR5 may function in cortical morphogenesis.

During early corticogenesis, the insertion of the cortical plate within the preplate splits this primitive layer into the marginal zone and the subplate (Marín-Padilla, 1971; Allendoerfer and Shatz, 1994). The marginal zone contains a population of transient neurons – the Cajal–Retzius cells (Meyer *et al*., 1999; Mienville, 1999) – that govern the laminar positioning of cor-

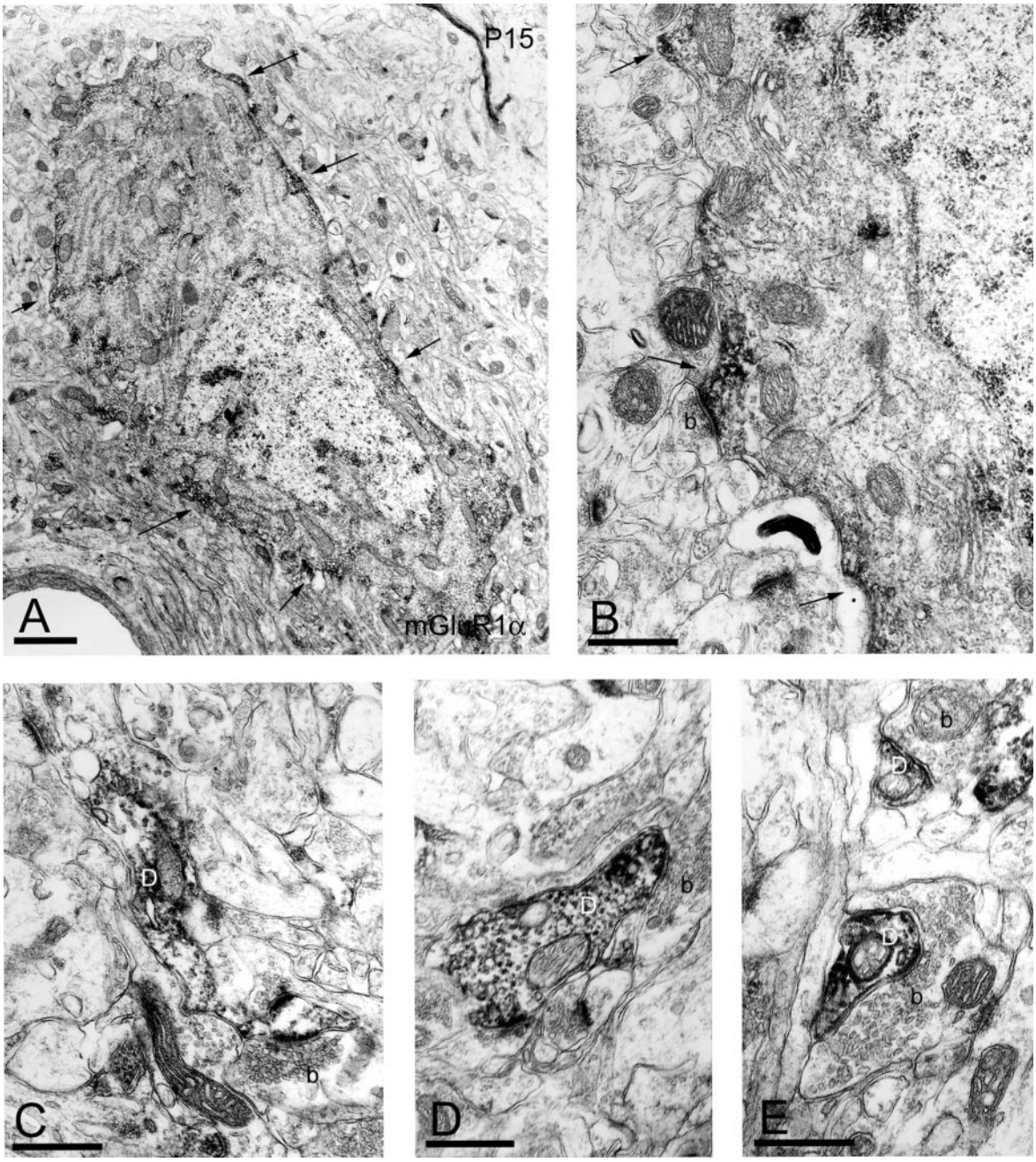

Figure 8. Electron micrographs of layer V of the cortex showing immunoreactivity for mGluR1α in interneurons at P15 as demonstrated by a pre-embedding immunoperoxidase method. (*A*,*B*) Aggregates of immunoperoxidase reaction product were mainly associated with the somatic plasma membrane (arrows) and also with the cytoplasmic face of membranes of the endoplasmic reticulum. The soma received abundant synaptic contacts with axon terminals (b) and immunolabeling for the receptor could be seen very close to these synapses (arrow in figure *B*). The proximal (D in *C* and *D*) and distal dendrites (D in *E*) of these interneurons were immunolabeled for mGluR1α and often surrounded by axon terminals (b), forming symmetrical or asymmetrical synapses. Scale bars: 1 μm (*A*); 0.5 μm (*C*–*E*).

tical plate neurons according to an inside-out gradient of radial migration (Angevine and Sidman, 1961; Rakic, 1974). Cajal–Retzius cells exert this organizational function by synthesizing and secrete reelin, an extracellular matrix glycoprotein (D'Arcangelo *et al.*, 1995; Ogawa *et al.*, 1995). In reelin-deficient reeler mutant mice, neocortical and hippocampal neuronal migration is severely altered (Rakic and Caviness, 1995; Curran and D'Arcangelo, 1998; Frotscher, 1998). As the cortex develops, the marginal zone becomes layer I. The Cajal–Retzius cells disappear either by cell death (Derer and Derer, 1990; Spreafico *et al.*, 1995) or by differentiation into interneurons (Edmunds and Parnavelas, 1982; Parnavelas and Edmunds, 1983). As in the neocortical marginal zone, the marginal zones of the hippocampus and fascia dentata, the stratum lacunosum-moleculare and the dentate outer molecular layer, also contain Cajal–Retzius cells (D'Arcangelo *et al.*, 1995; Del Rio *et al.*, 1997; Alcántara *et al.*, 1998; Drakew *et al.*, 1998). Cajal–Retzius cells express diverse types of ionotropic neurotransmitter receptors including NMDA, AMPA and $GABA_A$ receptors (Schwartz *et al.*, 1998; Mienville, 1999; Mienville and Pesold, 1999). We have recently demonstrated that mice also express functional metabotropic neurotransmitter receptors that include

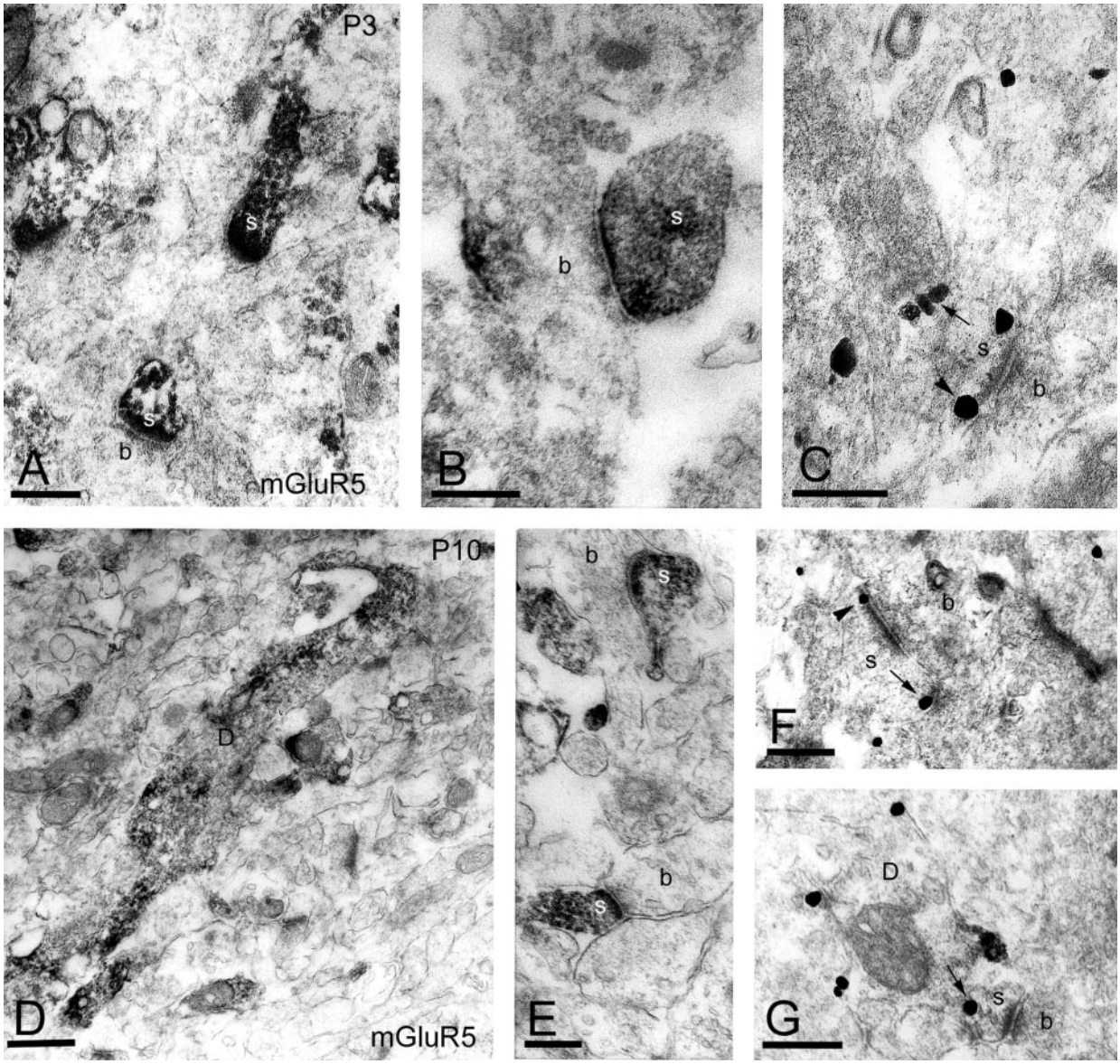

Figure 9. Immunoreactivity for mGluR5 in the cortex during postnatal development as demonstrated by pre-embedding immunoperoxidase and immunogold reactions. (*A–C*) Subcellular localization of mGluR5 in layer I at P3 showing intense immunoreactivity for the receptor in dendritic spines (s) of pyramidal cells. Many of these dendritic spines formed asymmetrical synapses with axon terminals (b). Immunoparticles were associated with the extrasynaptic plasma membrane (arrows) of the spines and often were localized at the edge of the synaptic specialization (arrowhead). (*D*, *E*) At P10, dendritic shafts (D) of interneurons (illustrated from layer II–III) and pyramidal cells were also immunoreactive for mGluR5 throughout the cortex and hippocampus. Immunoreactive dendritic spines (s) belonging to pyramidal cells of layer V formed asymmetrical synapses with axon terminals (b). (*F*, *G*) Immunoparticles were also associated with the extrasynaptic plasma membrane (arrows) of dendrites (D) and the dendritic spines (s) and also are localized at the edge of the synaptic specialization (arrowhead). Scale bars: 0.3 μm (*A–C*, *E–G*); 0.5 μm (*D*).

mGluR1α (Martínez-Galán *et al.*, 2001) and $GABA_B$ receptors (López-Bendito *et al.*, 2002). Here, we have shown that virtually all Cajal–Retzius cells in the postnatal rat expressed mGluR1α postnatally, whereas mGluR5 was not detected in these cells during postnatal development. Furthermore, mGluR1α in Cajal–Retzius cells is present on extrasynaptic and perisynaptic plasma membrane sites, similar to the localization of mGluR1α in other cell types in adulthood or during development (Luján *et al.*, 1996; Liu *et al.*, 1998; Alvarez *et al.*, 2000; López-Bendito *et al.*, 2001). Interestingly, during late prenatal development, a subpopulation of Cajal–Retzius cells does express mGluR5, whereas we were not able to detect mGluR1α before birth in either the neocortex or hippocampus. The non-overlapping temporal mGluR5 and mGluR1α expression in Cajal–Retzius cells during prenatal (mGluR5) and postnatal (mGluR1α) development suggests a switch in the expression of these group I mGluRs in a small population of these cells.

The spatio-temporal expression of group I mGluRs reported here in the marginal zone/layer 1 may be a crucial mechanism in evoking different Ca^{2+} dynamics, which is thought to play a role in the regulation of gene expression and control of developmental events. Glutamate activation of mGluR1 and mGluR5 evokes non-oscillatory and oscillatory intracellular Ca^{2+} signals, respectively (Kawabata *et al.*, 1996). Recent studies have found that developing cortical neurons exhibit calcium oscillations in response to direct activation of mGluR5 (Flint *et al.*, 1999). These data are consistent with our previous results, in which we demonstrated that in mouse, intracellular Ca^{2+} level increases in

layer I cells were non-oscillatory, also showing that Cajal–Retzius cells respond to group I mGluR agonists (Martínez-Galán *et al.*, 2001).

Activation of group I mGluRs induces phosphoinositide hydrolysis, with subsequent formation of diacylglycerol and inositol-1,4,5-triphosphate, activation of protein kinase C and mobilization of Ca^{2+} from intracellular stores (Nakanishi *et al.*, 1994; Pin and Duvoisin, 1995; Fagni *et al.*, 2000). Alterations in intracellular Ca^{2+} are involved in many aspects of development, plasticity and neurotoxicologic processes. Thus, it is possible that cortical mGluR5 activation may result in elevation of internal Ca^{2+} levels, which may be associated with dendritic growth. This is supported by the fact that PI hydrolysis associated with group I mGluRs activation is enhanced during early postnatal development (Sladeczek *et al.*, 1985; Nicoletti *et al.*, 1986; Schoepp and Hillman, 1990; Sortino *et al.*, 1991; Romano *et al.*, 1996).

In general, the data presented here indicate that the group-I-mediated intracellular signaling mechanism operating through PI hydrolysis and mobilization of intracellular Ca^{2+} is developmentally regulated in the cerebral cortex. This mechanism may be important in selecting and stabilizing afferents in a layer-dependent manner and it may be supported by the finding that mGluR1 is involved in the regression of supernumerary climbing fibre synapses in the cerebellum (Kano *et al.*, 1997; López-Bendito *et al.*, 2001). The enrichment of mGluR5 in neuropil of different cortical layers in the developing neocortex and hippocampus suggests an involvement of the receptor in morphogenesis. Although there is an absence of CNS structural abnormalities in mice deficient in mGluR5, these animals show abnormal synaptic function (Lu *et al.*, 1997).

Differential Localization of Group I mGluRs during Development

The expression and cellular localization of mGluR1α and mGluR5 changed during pre- and postnatal development. Both receptors were found in neuropil and in neocortical and hippocampal neuronal somata; however, the level of receptor expression depended on the developmental stage studied. Thus, during late prenatal development, mGluR1α was mainly found in neuropil, while mGluR5 was found in somata. In contrast, during postnatal development mGluR1α was mainly found in neuronal somata, whereas mGluR5 was localized in neuropil. In the developing and adult neocortex and hippocampus, mGluR1α appeared to be expressed solely in non-pyramidal neurons, whereas mGluR5 was expressed by both pyramidal and non-pyramidal neurons. These results are consistent with previous adult studies (Baude *et al.*, 1993; Luján *et al.*, 1996; Petralia *et al.*, 1997; Cauli *et al.*, 2000; Stinehelfer *et al.*, 2000). Virtually all mGluR1α-immunoreactive neurons in adult neocortex and hippocampus were GABAergic interneurons (Baude *et al.*, 1993; Cauli *et al.*, 2000; Stinehelfer *et al.*, 2000). In neocortex, mGluR1α was expressed by specific subclasses of GABAergic neurons, mainly somatostatin-, calretinin-, calbindin- and VIP-positive neurons (Cauli *et al.*, 2000; Stinehelfer *et al.*, 2000). However, in the hippocampus mGluR1α was mainly expressed in somatostatin- (Baude *et al.*, 1993) and in VIP-positive interneurons (Ferraguti *et al.*, 2001). The morphology and distribution of mGluR1α-immunoreactive interneurons found in the present study seems to indicate that such is also the case during development, though additional double-labeling experiments using different interneuron markers are needed to confirm that.

Previous studies have shown that mGluR1α and mGluR5 are mainly localized postsynaptically in several brain regions, including the cerebral cortex (Martin *et al.*, 1992; Shigemoto *et al.*, 1992, 1993; Baude *et al.*, 1993; Fotushi *et al.*, 1993; Grandes *et al.*, 1994; Kharazia *et al.*, 1995; Romano *et al.*, 1995; van den Pol *et al.*, 1995; Godwin *et al.*, 1996; Luján *et al.*, 1996, 1997; Vidyanszky *et al.*, 1996; Negyessy *et al.*, 1997; Liu *et al.*, 1998; Muñoz *et al.*, 1999; Alvarez *et al.*, 2000; Martínez-Galán *et al.*, 2001). This pattern of group I mGluR expression is consistent with the developmental pattern described in this study. However, our data differ from a recent developmental study in the somatosensory cortex that showed a strong mGluR1α neuropilar staining in all cortical layers (Muñoz *et al.*, 1999). This discrepancy may be explained by the use of different antibodies against distinct sequences of the receptor; which would lead to different patterns of labeling.

In addition to neuronal labeling, mGluR5 has also been localized in astrocytic cell bodies in the cerebral cortex (Romano *et al.*, 1995; Muñoz *et al.*, 1999), thalamus (Liu *et al.*, 1998) and hypothalamus (van den Pol *et al.*, 1995), as well as in axon terminals (Romano *et al.*, 1995; van den Pol *et al.*, 1995). In this study, mGluR5 immunoreactivity was not detected in glial processes at any developmental age, which is consistent with previous studies that used the same antiserum (Shigemoto *et al.*, 1993; Luján *et al.*, 1996, 1997). Furthermore, we found no evidence for the presence of presynaptic mGluR5 in the cortex (Shigemoto *et al.*, 1997) during development.

The current view of the subcellular distribution of group I mGluRs in the CNS is mainly based on data obtained in the adult cerebral cortex and cerebellum (Nusser *et al.*, 1994; Luján *et al.*, 1996, 1997). Those studies showed that mGluR1α and mGluR5 were found at the edges of asymmetrical postsynaptic specialization, which has also been recently confirmed in other brain regions and in several species (Hanson and Smith, 1999; Alvarez *et al.*, 2000; Hubert and Smith, 2001). Our findings confirm these results in the cerebral cortex during development. Immunogold particles for mGluR1α and mGluR5 were frequently found perisynaptically at putative glutamatergic synapses and also localized extrasynaptically along the plasma membrane. The receptors were not significantly associated with GABAergic synapses. This localization pattern is consistent with recent developmental studies in the cerebellum and thalamus using comparable methods (Liu *et al.*, 1998; Petralia *et al.*, 1998; López-Bendito *et al.*, 2002). However, group I mGluRs have recently been localized in the postsynaptic membrane specialization of GABAergic synapses established by striatal terminals (Hanson and Smith, 1999; Hubert and Smith, 2001). The lack of mGluR immunoreactivity in the main body of asymmetric synapses might arise from inability of the antibody to reach the epitope. The consistency of the perisynaptic localization in asymmetric synapses using different antibodies, techniques, species and developmental stages suggests that is unlikely to be the case, but rather represents a genuine phenomenon in the CNS for group I mGluRs. This perisynaptic localization described throughout development may account for the fact that slow group I mGluRs are activated only after high-frequency stimulation (Charpak and Gahwiler, 1991; McCormick and von Krosigk, 1992; Golshani *et al.*, 1998).

It is clear that mGluR1α and mGluR5 expression is differentially regulated. These regulatory systems control the differential distribution of the receptor subtypes on the plasma membrane of cortical neurons. The differential expression is consistent with recent electrophysiological studies demonstrating that mGluR1 and mGluR5 differentially regulate CA1 pyramidal cell function in the hippocampus (Mannaioni *et al.*, 2001). Together,

these studies suggest that a differential distribution of group I mGluRs in a cortical neuronal population correlate with distinct functions. Additional studies are needed to characterize the functional roles of the two group I mGluRs that will correlate with the anatomical observations reported here.

Notes

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