Histol Histopathol (2025) 40: 1967-1984

Histology and Histopathology
From Cell Biology to Tissue Engineering

ORIGINAL ARTICLE Open Access

Developmental regulation of $GABA_B$ receptors and downstream molecules in the mouse brain

Carolina Aguado[1,2], Ana Fajardo-Serrano[1], Rocío Alfaro-Ruiz[1,2,3], María Llanos Martínez-Poyato[1], Ana Esther Moreno-Martínez[1,2], Sebastián García-Madrona[1,4], Alberto Roldán-Sastre[1], Pablo Alonso-Gómez[1], Miriam Fernández[1,2], Ricardo Puertas-Avendaño[1], Ryuichi Shigemoto[5], Kirill A. Martemyanov[6] and Rafael Luján[1,2]

[1]*Synaptic Structure Laboratory*, Instituto de Biomedicina de la UCLM (IB-UCLM), Departamento de Ciencias Médicas, Facultad de Medicina, Universidad Castilla-La Mancha, Campus Biosanitario, [2]Laboratorio de Estructura Sináptica, Instituto de Investigación Sanitaria de Castilla-La Mancha (IDISCAM), Albacete, [3]Universidad Europea de Valencia, Valencia, [4]Instituto Ramón y Cajal de Investigación Sanitaria, Hospital Universitario Ramón Y Cajal, Madrid, Spain, [5]Institute of Science and Technology (IST Austria), Klosterneuburg, Austria and [6]Department of Neuroscience, The Scripps Research Institute, Jupiter, FL, USA

Summary. Metabotropic GABA ($GABA_B$) receptors have modulatory functions on neuronal excitability and neurotransmitter release. To fulfil these functions, $GABA_B$ receptors form macromolecular signaling complexes with G proteins, effectors, and other associated proteins. Here we investigated the postnatal development of $GABA_B$ receptors ($GABA_{B1}$ and $GABA_{B2}$ subunits) in mouse brain, focusing on potential similarities in the spatial and temporal expression pattern of their associated proteins $Ca_V2.1$, $G\alpha_o$, Gβ5, and RGS7, using histoblots, immunofluorescence, and immunoelectron microscopic techniques. At all ages analyzed, histoblot showed that the six proteins were widely expressed in the brain, with mostly an overlapping pattern throughout postnatal development. In the hippocampus, immunoelectron microscopy and quantitative analysis of immunoparticles for $GABA_{B1}$, $GABA_{B2}$, $G\alpha_o$, Gβ5, and RGS7 revealed their progressive enrichment around excitatory synapses on dendritic spines of CA1 pyramidal cells toward P15. At presynaptic sites, $GABA_B$ receptors colocalize with $Ca_V2.1$, $G\alpha_o$, Gβ5, and RGS7 in the active zone and extrasynaptic membranes of axon terminals, establishing synapses on dendritic spines of CA1 pyramidal cells. In the cerebellum, double immunofluorescence at P7 and P10 revealed the colocalization of $GABA_{B1}$ and $Ca_V2.1$ in the whole dendritic tree of developing Purkinje cells. Immunoelectron microscopy at P15 showed that $GABA_{B1}$, $GABA_{B2}$, $Ca_V2.1$, $G\alpha_o$, Gβ5, and RGS7 are distributed along the dendritic surface of Purkinje cells, enriched close to excitatory synapses in spines. Altogether, these data suggest that macromolecular complexes composed of $GABA_{B1}$ /$GABA_{B2}$/$Ca_V2.1$/$G\alpha_o$/Gβ5/RGS7 are pre-assembled during key stages of postnatal development in hippocampal and cerebellar neurons.

Key words: Postnatal development, Hippocampus, Cerebellum, $GABA_B$ receptors, G proteins, Calcium channels, Histoblot, Electron microscopy

Corresponding Author: Dr. Carolina Aguado and Dr. Rafael Luján, *Synaptic Structure Laboratory*, Instituto de Biomedicina de la UCLM (IB-UCLM), Departamento de Ciencias Médicas, Facultad de Medicina, Universidad Castilla-La Mancha, Campus Biosanitario, C/ Almansa 14, 02008 Albacete, Spain. e-mail: Carolina.Aguado@uclm.es and Rafael.Lujan@uclm.es

www.hh.um.es. DOI: 10.14670/HH-18-970

Introduction

The environment around neurons is so dynamic that they have developed mechanisms to transport extracellular signals into the cell, thus allowing their adaptation to the changing environment. This process, known as signal transduction, involves the activation of receptors mostly located on the neuronal surface. G protein-coupled receptors (GPCRs) are one of the largest and most diverse families of receptors (Rosenbaum et al., 2009), which respond to neurotransmitters in the brain. GPCRs catalyze GDP/GTP exchange on the heterotrimeric G protein, leading to dissociation of Gα-GTP and Gβγ subunits and their subsequent modulation of enzymes or ion channels (Smrcka, 2008). Signaling is stopped by regulator of G-protein signaling (RGS) proteins, which mediate the hydrolysis of GTP by Gα, resulting in re-association of Gα and Gβγ (Dong et al., 1997).

The GPCR that mediates the slow responses to synaptically released γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the brain, is the

$GABA_B$ receptor (Bettler et al., 2004). $GABA_B$ receptors have modulatory functions on neuronal excitability and neurotransmitter release, providing a crucial component of inhibitory neurotransmission in the brain via coupling to $G\alpha_{i/o}$ type G proteins (Bettler et al., 2004). $GABA_B$ receptors mediate their inhibitory effects by activating several signaling pathways that include the direct modulation of ion channels by Gβγ subunits. In postsynaptic compartments, Gβγ binds and opens G-protein-gated inwardly rectifying K^+ (GIRK/Kir3) channels, producing slow IPSCs and hyperpolarizing neurons (Lüscher and Slesinger, 2010). At presynaptic sites, Gβγ binds and inhibits Ca_V2 voltage-gated channels (N and P/Q types), suppressing neurotransmitter release (Zamponi and Currie, 2013). Dysfunction of this $GABA_B$ signaling through GIRK and Ca_V2 channels contributes to a variety of neurological and psychiatric disorders, including epilepsy, depression, addiction, cognitive impairment, and anxiety (Fritzius and Bettler, 2020; Vlachou, 2022).

The $GABA_B$ receptor functions as an obligatory heterodimer, consisting of the $GABA_{B1}$ and $GABA_{B2}$ subunits, whereby the $GABA_{B1}$ subunit is necessary for agonist activation and the $GABA_{B2}$ subunit for surface trafficking and G-protein coupling (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999). Many studies using autoradiography, light microscopic immunocytochemistry, and *in situ* hybridization showed that $GABA_B$ receptors are widely distributed throughout the adult and developing brain (Chu et al., 1990; Turgeon and Albin, 1994; Kaupmann et al., 1997; Bischoff et al., 1999; Margeta-Mitrovic et al., 1999; Fritschy et al., 2004). Ultrastructural analysis confirmed that $GABA_B$ receptors are present in excitatory and inhibitory synapses at both postsynaptic and presynaptic locations (Kulik et al., 2002, 2003). During development, $GABA_B$ receptors were localized to the extrasynaptic and perisynaptic plasma membrane in dendrites and spines of CA1 pyramidal cells and Purkinje cells (López-Bendito et al., 2004; Luján and Shigemoto, 2006).

To fulfill their physiological roles with high efficiency in mature neurons, $GABA_B$ receptors are frequently identified as components of GPCR-effector macromolecular membrane assembly (GEMMA), which is defined as a pre-assembled signaling complex composed of combinations of GPCRs, G proteins, effectors, and other associated transmembrane proteins along the plasma membrane (Ferré et al., 2022). We have previously shown that $GABA_B$ receptors form macromolecular complexes with GIRK channels, Ca_V2 channels, and RGS7/Gβ5 proteins in the adult cerebellum (Aguado et al., 2016a; Luján et al., 2018) and hippocampus (Fajardo-Serrano et al., 2013; Ostrovskaya et al., 2014, 2018). The question of when and in what neuronal compartments these $GABA_B$ pre-assembled signaling complexes are formed remains unsettled. The answer to this question will provide key information to understand the contribution of $GABA_B$ receptors and their associated proteins to developmental processes and brain functions. Here, to understand how the expression of these proteins is regulated during postnatal development and becomes organized along the neuronal surface, we used histoblotting, immunofluorescence, and immunoelectron microscopy approaches. Our findings are in line with the view that $GABA_{B1}$, $GABA_{B2}$, $Ca_V2.1$, $G\alpha_o$, Gβ5, and RGS7 show extensive co-localization within the same microdomains, beginning at early stages of development, and suggest the formation of signaling complexes involved in developmental processes.

Materials and methods

Animals

OF-1 mice, from the day of birth [postnatal day (P)0] to adulthood (obtained from the Animal House Facility, School of Medicine, University of Castilla-La Mancha), were used in this study for histoblots, immunofluorescence, and pre-embedding immunogold analyses. The care and handling of animals prior to and during the experimental procedures were in accordance with Spanish (RD 1201/2005) and European Union (86/609/EC) regulations, and the protocols were approved by the University's Animal Care and Use Committee. For each developmental stage, the animals used were from different litters and were grouped as follows: P0, P5, P10, P15, P21, and P60, n=3 animals per group for histoblots; P7 and P12, n=3 animals for immunofluorescence; and P10 and P15, n=3 animals for pre-embedding immunoelectron microscopy.

For histoblotting, animals were deeply anaesthetized by hypothermia (P0-P5) or by intraperitoneal injection of ketamine/xylazine 1:1 (0.1 mL/kg b.w.), and the brains were quickly frozen in liquid nitrogen. For immunofluorescence, animals were anaesthetized by intraperitoneal injection of ketamine/xylazine 1:1 (0.1 mL/kg b.w.) and transcardially perfused with ice-cold fixative containing 4% paraformaldehyde and 15% (v/v) saturated picric acid made up in 0.1 M phosphate buffer (PB, pH 7.4). For pre-embedding immunogold, animals were anaesthetized by intraperitoneal injection of ketamine/xylazine 1:1 (0.1 mL/kg b.w.) and transcardially perfused with ice-cold fixative containing 4% paraformaldehyde, with 0.05% glutaraldehyde and 15% (v/v) saturated picric acid made up in 0.1 M phosphate buffer (PB, pH 7.4). After perfusion, brains were removed and immersed in the same fixative for 2 hours or overnight at 4°C. Tissue blocks were washed thoroughly in 0.1 M PB. Coronal 60 µm-thick sections were cut on a Vibratome (Leica V1000).

Antibodies and chemicals

The following primary antibodies were used: rabbit anti-$GABA_{B1}$ (B17, aa. 901-960 of rat $GABA_{B1}$); affinity-purified guinea pig anti-$GABA_{B2}$ polyclonal

(AB2255; Chemicon, Temecula, CA, USA); affinity-purified guinea pig anti-$Ca_V2.1$ (α-1A) polyclonal (GP-Af810; aa. 361-400 of mouse $Ca_V2.1$; Frontier Institute Co., Japan); affinity-purified rabbit anti-$Ca_V2.1$ (Cat. No. 152 203; aa. 1921 to 2212 from rat $Ca_V2.1$, P54282; Synaptic Systems, Gottingen, Germany); affinity-purified rabbit anti-$G\alpha_o$ polyclonal (ref#ab154001; Recombinant fragment corresponding to Human GNAO1 aa 104-338; Abcam, Cambridge, UK); and affinity-purified rabbit anti-RGS7 and rabbit anti-Gβ5 (generous gifts from Dr. William Simonds, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health). The antibody (B17) raised against the intracellular amino acid residues is specific to both 1a and 1b splice variants of the $GABA_{B1}$ subunit ($GABA_{B1a/b}$ antibody). The characteristics and specificity of the anti-$GABA_{B1}$ and anti-$GABA_{B2}$ antibodies (Kulik et al., 2002; Luján and Shigemoto, 2006; Vigot et al., 2006), the guinea pig anti-$Ca_V2.1$ antibody (Indriati et al., 2013), the rabbit anti-$Ca_V2.1$ antibody (Rebola et al., 2019), the rabbit anti-$G\alpha_o$ antibody (Roldán-Sastre et al., 2021), and the anti-RGS7 and rabbit anti-Gβ5 antibodies (Cao et al., 2008; Xie et al., 2012) have been described previously.

The secondary antibodies used were as follows: alkaline phosphatase (AP)-goat anti-rabbit IgG (H+L) (1:5000; Sigma-Aldrich, St. Louis, MO, USA); Alexa®-488-goat anti-guinea pig IgG (H+L) and Alexa®-594-goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch Europe Ltd., Ely CB7 4EX, UK); and goat anti-rabbit and anti-guinea pig IgG coupled to 1.4 nm gold (1:100; Nanoprobes Inc., Stony Brook, NY, USA).

Histoblotting

The regional distribution of $GABA_{B1}$, $GABA_{B2}$, $Ca_V2.1$, $G\alpha_o$, Gβ5, and RGS7 was analyzed in developing mouse brains using the histoblot technique (Aguado et al., 2023). Briefly, horizontal cryostat sections (10 µm) from mouse brains were transferred to nitrocellulose membranes moistened with 48 mM Tris-base, 39 mM glycine, 2% (w/v) sodium dodecyl sulphate and 20% (v/v) methanol for 15 min at room temperature (~20ºC). After blocking in 5% (w/v) non-fat dry milk in phosphate-buffered saline, nitrocellulose membranes were treated with DNase I (5 U/mL), washed and incubated in 2% (w/v) sodium dodecyl sulphate and 100 mm β-mercaptoethanol in 100 mM Tris-HCl (pH 7.0) for 60 min at 45ºC to remove adhering tissue residues. After extensive washing, the blots were reacted with affinity-purified anti-$GABA_{B1}$, anti-$GABA_{B2}$, anti-$Ca_V2.1$, anti-$G\alpha_o$, anti-Gβ5, or anti-RGS7 antibodies (0.5 mg/mL) in blocking solution overnight at 4ºC. The bound primary antibodies were detected with alkaline phosphatase-conjugated anti-rabbit IgG secondary antibodies. A series of primary and secondary antibody dilutions and incubation times were used to optimize the experimental conditions for the linear sensitivity range of all reactions and to confirm that all labeling was below saturation levels. To compare the expression levels of each protein during development, all nitrocellulose membranes were processed in parallel, and the same incubation time for each reagent was used for all antibodies at all ages.

Digital images were acquired by scanning the nitrocellulose membranes using a desktop scanner (HP Scanjet 8300). Image analysis and processing were performed using Adobe Photoshop software (Adobe Systems, San Jose, CA, USA) as described previously (Martín-Belmonte et al., 2020). All the images were processed in the same way to allow comparison of the intensity of grayscale images at different postnatal ages and brain regions. The pixel intensity of immunoreactivity was measured using open circular cursors with a diameter of 0.10 mm. The cursors were placed at different brain regions identified based on the adjacent cresyl violet-stained sections. The average of eight background determinations carried out near the brain protein-containing areas of the immunostained nitrocellulose membranes was subtracted from the average pixel intensities measured within brain regions. Following background corrections, the average pixel intensity for the whole region from one animal was counted as one 'n'. Data were analyzed and plotted using the software Analysis (Soft Imaging Systems, Munster, Germany). Differences during development between the brain regions were assessed using a one-way analysis of variance (ANOVA), and further compared with the Bonferroni *post-hoc* test, at a minimum confidence level of $p<0.05$.

Immunohistochemistry for confocal microscopy

Free-floating sections were first blocked in 10% Normal Goat Serum (NGS) made up in Tris-buffered saline (TBS) for 1h before incubation in a mixture of primary antibodies for $Ca_V2.1$ (Frontier Institute Co., Japan) and $GABA_{B1}$ (B17) in TBS containing 3% NGS at 4ºC overnight. After several washes in TBS, the sections were incubated in a mixture of secondary antibodies (anti-guinea pig Alexa®-488 for $Ca_V2.1$ and anti-rabbit Alexa Fluor®-594 for $GABA_{B1}$) made up in TBS for 2h at room temperature. After further washes in TBS, sections were mounted on gelatine-coated slides and coverslipped with fluorescence mounting medium (Vectashield, Vector Lab.). Immunofluorescence labeling was examined using a confocal laser-scanning microscope (Zeiss LSM 510-Meta, Jena, Germany). Separate color channels were acquired sequentially to avoid crosstalk between fluorochromes.

Immunohistochemistry for electron microscopy

Immunohistochemical reactions for electron microscopy were carried out using the pre-embedding immunogold method described previously (Luján et al., 1996). Briefly, free-floating sections were incubated in

10% (v/v) NGS diluted in TBS. Sections were then incubated in anti-$GABA_{B1}$, anti-$GABA_{B2}$, anti-$Ca_V2.1$, anti-$G\alpha_o$, anti-Gβ5, or anti-RGS7 antibodies [3-5 μg/mL diluted in TBS containing 1% (v/v) NGS], followed by incubation in goat anti-rabbit IgG, anti-guinea pig IgG or anti-mouse IgG coupled to 1.4 nm gold (Nanoprobes Inc., Stony Brook, NY, USA). Sections were postfixed in 1% (v/v) glutaraldehyde and washed in double-distilled water, followed by silver enhancement of the gold particles with an HQ Silver kit (Nanoprobes Inc.). Sections were then treated with osmium tetroxide (1% in 0.1 M phosphate buffer), block-stained with uranyl acetate, dehydrated in a graded series of ethanol, and flat-embedded on glass slides in Durcupan (Fluka) resin. Regions of interest were cut at 70-90 nm on an ultramicrotome (Reichert Ultracut E, Leica, Austria) and collected on single-slot pioloform-coated copper grids. Staining was performed on drops of 1% aqueous uranyl acetate, followed by Reynolds's lead citrate. Ultrastructural analyses were performed in a JEOL-1400Flash electron microscope equipped with a digital high-sensitivity sCMOS camera.

Quantification of protein immunoreactivities

To determine the relative abundance of $GABA_B$ receptors and downstream molecules ($Ca_V2.1$, $G\alpha_o$, Gβ5, and RGS7) in dendritic spines of CA1 pyramidal cells or Purkinje cells during postnatal development, and their association with excitatory synapses, immuno-particles identified in each reference area and present in dendritic spines were counted. The procedure was similar to that used previously (Luján and Shigemoto, 2006). Briefly, for each of the three adult animals, three samples of tissue were obtained for the preparation of embedding blocks. To minimize false negatives, electron microscopic ultrathin serial sections were cut close to the surface of each block, as immunoreactivity decreased with depth. We estimated the quality of immunolabeling by always selecting areas with optimal gold labeling at approximately the same distance from the cutting surface. Randomly selected areas were then captured at a final magnification of 30,000 X. Developing excitatory synapses were identified by the presence of: (i) a postsynaptic density (PSD), (ii) synaptic vesicles at the presynaptic terminal, and (iii) opposing membranes between the pre- and post-synaptic terminals. Quantification of immunolabeling was performed in two different ways:

Postsynaptic distribution of proteins relative to glutamate release sites. We determined the relative abundance of $GABA_{B1}$, $G\alpha_o$, Gβ5, and RGS7 in dendritic spines of CA1 pyramidal cells at P10 and P15 in the *stratum radiatum*, as well as $GABA_{B1}$, $GABA_{B2}$, $Ca_V2.1$, and $G\alpha_o$ in dendritic spines of Purkinje cells at P15 in the molecular layer. We counted the immuno-particles and then measured the length of the dendritic spine membrane from the edge of the synaptic junction. The position of the center of each immunoparticle attached to the plasma membrane of the dendritic spine as a function of distance from the edge of the postsynaptic density was measured using a digitizing tablet and appropriate software (ImageJ). Finally, to obtain a normalized value of the relative abundance of the proteins along the dendritic spines, the number of gold particles was expressed as relative frequency in bins corresponding to 60-nm membrane segments of the spine membrane.

Presynaptic distribution of proteins relative to glutamate release sites. To establish the relative frequency of $GABA_{B1}$, $GABA_{B2}$, $Ca_V2.1$, $G\alpha_o$, Gβ5, and RGS7 in axon terminals, immunoparticles identified in this compartment were counted at P15 in the *stratum radiatum*. The perimeter of each axon terminal was measured in reference areas totaling ~1,900 μm^2 for each protein. All axon terminals establishing excitatory synapses with dendritic spines of CA1 pyramidal cells were counted and assessed from single ultrathin sections. The lengths of the active zone and the extrasynaptic membrane surrounding the active zone were measured using a digitizing tablet and appropriate software (Image J). The distances of the immunoparticles (n=142 for $GABA_{B1}$; n=151 for $GABA_{B2}$; n=173 for $Ca_V2.1$; n=138 for $G\alpha_o$; n=112 for Gβ5; and n=121 for RGS7) from the edge of the active zone and the center of the immunoparticles were measured. To obtain a normalized value of the relative frequency of the six proteins along the axon terminal, the number of gold particles was expressed as relative frequency in bins corresponding to 60 nm membrane segments of the plasma membrane. The data expressed in this way show the change in density of the four proteins as a function of distance along the axon terminal.

Controls

To test method specificity in the procedures for histoblots and electron microscopy, the primary antibodies were either omitted or replaced with 5% (v/v) normal serum of the species of the primary antibody. Under these conditions, no selective labeling was observed.

Data analysis

To avoid observer bias, we performed blinded experiments for histoblots and immunohistochemistry prior to data analysis. Statistical analyses were performed using GraphPad Prism (San Diego, CA, USA), and data were presented as mean ± SEM unless indicated otherwise. Statistical significance was defined as $p<0.05$. The statistical evaluation of the histoblots was performed using one-way analysis of variance (ANOVA), and further compared with the Bonferroni *post-hoc* test. To compute SEM error bars, three blots were measured from each animal.

Results

Brain expression of $GABA_B$ receptors in postnatal development

We used the histoblot technique to determine the regional distribution and expression levels of the $GABA_{B1}$ and $GABA_{B2}$ subunits of $GABA_B$ receptors in the brain during postnatal development and adulthood. In adult brain (P60), the overall $GABA_{B1}$ and $GABA_{B2}$ expression profiles were very similar, showing mostly overlapping labeling patterns (Fig. 1A-C). Labeling for $GABA_{B1}$ and $GABA_{B2}$ was widely distributed throughout the brain, with strongest immunoreactivities in the cerebellum and thalamus, moderate labeling in the hippocampus, cortex and septum and weak labeling in the caudate putamen and midbrain nuclei (Fig. 1A-C).

During postnatal development, both $GABA_{B1}$ and $GABA_{B2}$ proteins were expressed from the day of birth (P0), also showing overlapping labeling patterns and displaying differences in a region-dependent manner (Fig. 1A). The hippocampus and cerebellum were further examined in a subregion- and layer-specific manner. Densitometric measurements performed in the hippocampus demonstrated that at all developmental ages, immunoreactivity for $GABA_{B1}$ and $GABA_{B2}$ was higher within the CA3 field compared to the CA1 field and the dentate gyrus (Fig. 1B,C). Furthermore, in all sub-regions of the hippocampus, weak $GABA_{B1}$ and $GABA_{B2}$ expression was detected at P0 and P5, which increased from P10 to P21 to reach a peak at P60 (Fig. 1A-C). In the cerebellum, weak $GABA_{B1}$ and $GABA_{B2}$ expression was observed in the molecular and granule cell layers at P0 and P5, which increased steadily in the molecular layer from P10 to P60, although in the granule cell layer it increased from P10 to P15 and then decreased until adulthood (Fig. 1A-C).

Other brain regions analyzed were the thalamus and the caudate putamen. In the thalamus, weak $GABA_{B1}$ and $GABA_{B2}$ expression was found at P0 and P5 and increased steadily from P10 to adulthood (Fig. 1B,C). In the caudate putamen, weak $GABA_{B1}$ and $GABA_{B2}$ expression was found at P0 and P5, which increased from P10 to P15, and then decreased until adulthood (Fig. 1B,C).

Brain expression of $Ca_V2.1$ channels in postnatal development

To determine the regional and developmental expression of P/Q-type channels in the mouse brain, we used $Ca_V2.1$ subunit-specific antibodies in conventional histoblotting. In the adult brain, very strong $Ca_V2.1$ immunoreactivity was observed in the cerebellum, with moderate labeling in the hippocampus, thalamus, septum, caudate putamen, and cortex (Fig. 2A,B). In the hippocampus, immunoreactivity for $Ca_V2.1$ was strong in the *strata oriens* and *radiatum* of the CA1 and CA3 fields, the *stratum lucidum* of the CA3 field, and the molecular layer of the dentate gyrus (Fig. 2A,B). Moderate labeling was observed in the *stratum lacunosum-moleculare* of CA1 and CA3 fields and the hilus of the dentate gyrus (Fig. 2A,B). In the cerebellum, immunoreactivity for $Ca_V2.1$ was significantly stronger in the molecular layer than in the granule cell layer, and very weak in the white matter (Fig. 2A,B).

During postnatal development, $Ca_V2.1$ was expressed from P0, showing differences in a region-dependent manner (Fig. 2A,B). At all developmental ages, immunoreactivity for $Ca_V2.1$ was strong in the CA1 and CA3 fields and the molecular layer of the dentate gyrus (Fig. 2A,B). In these sub-regions of the hippocampus, weak $Ca_V2.1$ expression was detected at P0 and increased progressively to reach a peak at P60 (Fig. 2A,B). In the cerebellum, weak $Ca_V2.1$ expression was observed in the molecular and granule cell layers at P0 and increased steadily to P60 (Fig. 2A,B). A similar developmental expression pattern was found in the thalamus, septum, and caudate putamen (Fig. 2A,B).

Brain expression of $G\alpha_o$ in postnatal development

We next studied the regional and developmental distribution of $G\alpha_o$ to analyze its extent of overlap with $GABA_B$ receptors. The brain expression of $G\alpha_o$ revealed region-specific differences, with the strongest immunoreactivities in the hippocampus, septum, caudate putamen, cortex, and cerebellum, and moderate labeling in the thalamus and midbrain nuclei (Fig. 3A,B). In the adult brain (P60), expression of $G\alpha_o$ in the hippocampus was strong in the *strata oriens* and *radiatum* of the CA1 and CA3 fields, the molecular layer of the dentate gyrus, and the *stratum lucidum* of the CA3 field (Fig. 3A,B). However, weaker immunoreactivity was evident in the *stratum lacunosum-moleculare* of CA1 and CA3 fields, and in the hilus of the dentate gyrus (Fig. 3A,B). In the cerebellum, immunostaining for $G\alpha_o$ was significantly stronger in the molecular layer than in the granule cell layer, in which moderate to weak labeling was found (Fig. 3A,B). The white matter consistently showed very weak $G\alpha_o$ labeling (Fig. 3A,B).

During postnatal development, $G\alpha_o$ was expressed from the day of birth (P0), also showing overlapping labeling patterns and displaying differences in a region-dependent manner (Fig. 3A,B). At all developmental ages, immunoreactivity for $G\alpha_o$ was higher within the CA3 field compared to the CA1 field and the dentate gyrus (Fig. 3A,B). Furthermore, in all those sub-regions of the hippocampus, weak $G\alpha_o$ expression was detected at P0, which increased from P5 to P10 to reach a peak at P15 and then decreased to P21 and P60 (Fig. 3A,B). In the cerebellum, weak $G\alpha_o$ expression was observed in the molecular layer at P5, and undetectable at P0, increased steadily in the molecular layer from P10 to P15, and then decreased until adulthood (Fig. 3A,B).

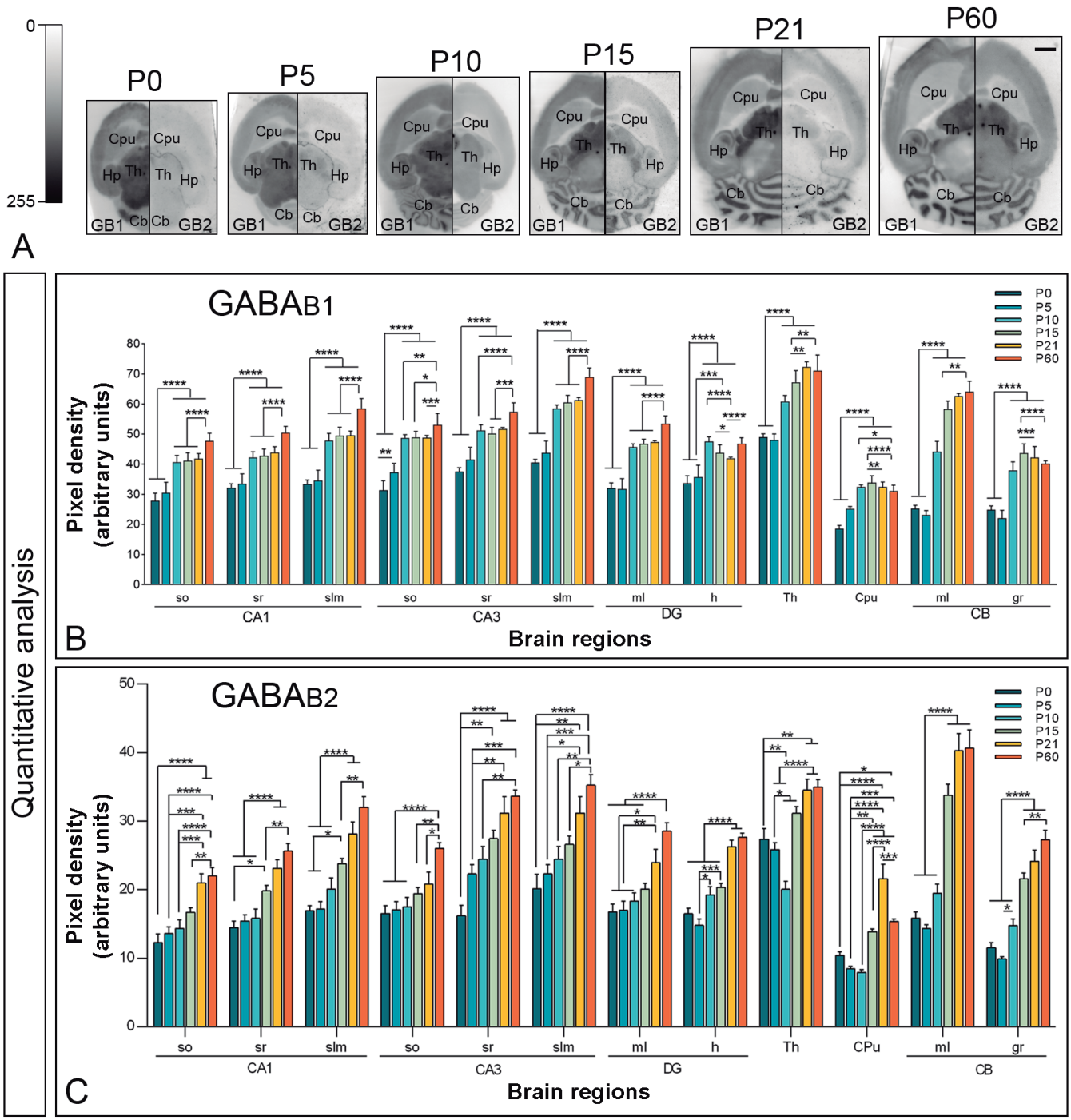

Fig. 1. Regional distribution of GABA$_B$ receptors in the developing mouse brain. **A.** Distribution of GABA$_B$ proteins was visualized on histoblots of horizontal brain sections at various stages of postnatal development using subunit-specific affinity-purified anti-GABA$_{B1}$ and anti-GABA$_{B2}$ antibodies. The two GABA$_B$ receptor subunits showed broad and overlapping distribution patterns in the developing and adult brain. Strong immunoreactivity for GABA$_{B1}$ (GB1) and GABA$_{B2}$ (GB2) was detected in the cerebellum (Cb) and thalamus (Th), moderate labeling in the hippocampus (Hp), cortex (Cx), and septum (Sp), and weak labeling in the caudate putamen (CPu). **B-C.** The histoblots were scanned and densitometric measurements from five independent experiments were averaged to compare the protein densities for each developmental time point. Overall, GABA$_{B1}$ and GABA$_{B2}$ expression were low at P0 and P5 and increased from P10 to adulthood. Error bars indicate SEM; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Scale bar: 0.4 cm.

Brain expression of RGS7 and Gβ5 in postnatal development

Next, we analyzed the regional and developmental expression of RGS7 and Gβ5 in the mouse brain and their extent of overlap with $GABA_B$ receptors and other associated proteins. In the adult brain (P60), the overall expression of RGS7 and Gβ5 proteins showed very similar expression patterns (Fig. 4A). They were widely distributed in the brain, with strong immunoreactivities in the neocortex, cerebellum, hippocampus, thalamus, and caudate putamen, with moderate labeling in the midbrain nuclei (Fig. 4A-C). In the hippocampus, immunoreactivity for RGS7 and Gβ5 was very strong in the *strata oriens* and *radiatum* of the CA1 and CA3 fields and the *stratum lucidum* of CA3, moderate in the *stratum lacunosum-moleculare* of CA3, and weak immunoreactivity was evident in the *stratum lacunosum-moleculare* of CA1 (Fig. 4A-C). In the cerebellum, immunoreactivity for RGS7 and Gβ5 was significantly stronger in the molecular layer than the granule cell layer, in which moderate to weak labeling was consistently detected, and very weak in the white matter (Fig. 4A-C).

The RGS7 and Gβ5 proteins were expressed in the developing brain from the day of birth (P0), showing virtually the same distribution patterns during postnatal development in all regions analyzed (Fig. 4A-C). In the hippocampus, weak RGS7 expression was detected at P0 in all subfields analyzed, increased significantly at P5 to reach similar intensity levels until P60, with no significant changes from P5 to P60 (Fig. 4A-C). Labeling for Gβ5 in the hippocampus was very weak at P0 and more detectable at P5, increased progressively to reach a peak at P21-P60 (Fig. 4A-C). In the cerebellum, the expression of RGS7 and Gβ5 was low in the

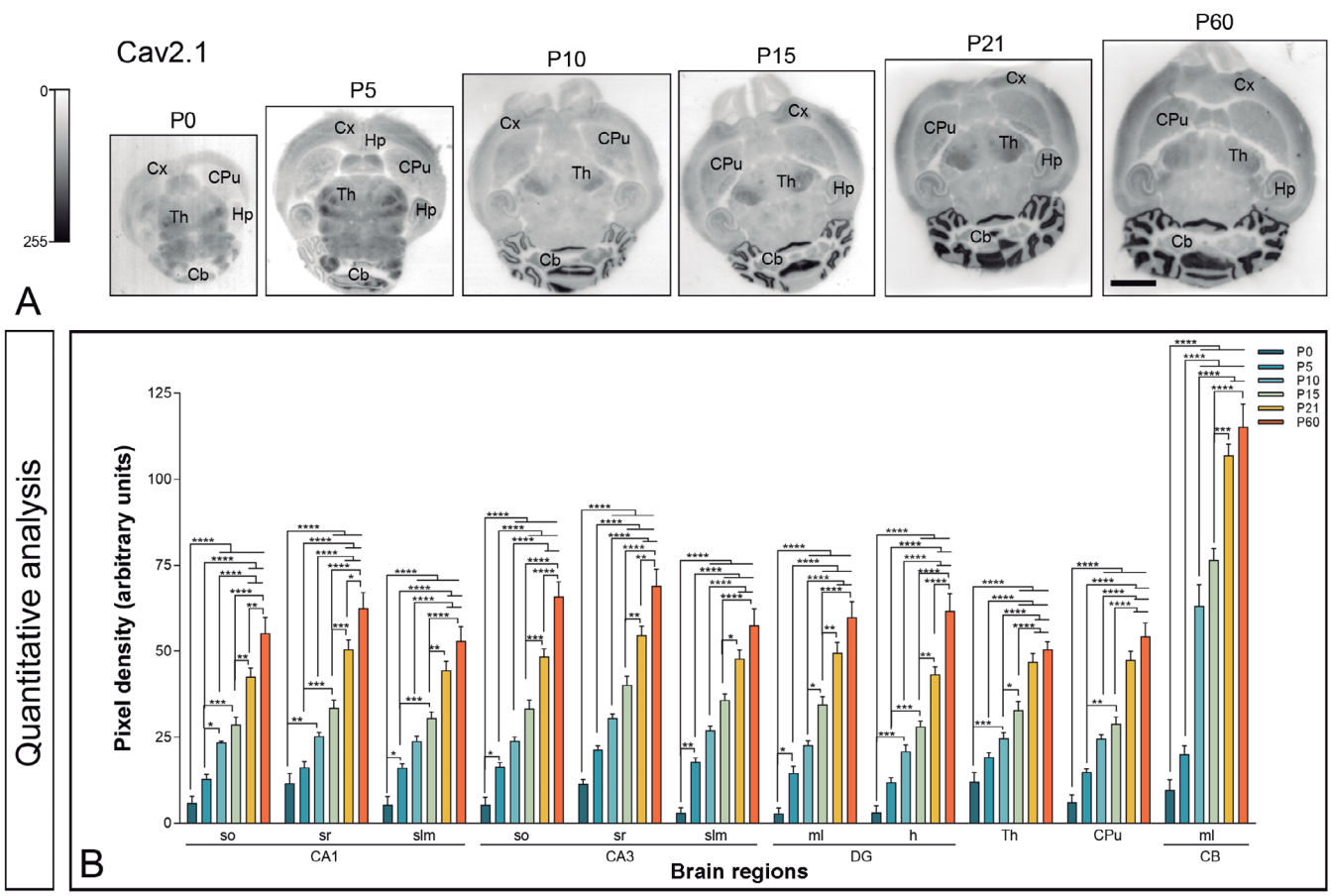

Fig. 2. Regional distribution of $Ca_V2.1$ channels in the developing mouse brain. **A.** $Ca_V2.1$ protein distribution was visualized on histoblots of horizontal brain sections at different stages of postnatal development using an affinity-purified anti-$Ca_V2.1$ antibody. $Ca_V2.1$ showed broad distribution patterns in the developing and adult brain. $Ca_V2.1$ was expressed in the brain since the day of birth (P0), and at all stages the strongest expression was observed in the cerebellum (Cb), with moderate labeling in the hippocampus (Hp), thalamus (Th), septum (Sp), caudate putamen (CPu), and cortex (Cx). **B.** The histoblots were scanned and densitometric measurements from four independent experiments were averaged to compare the protein densities for each developmental time point. In all brain regions analyzed, as well as in all layers or subfields from each region, $Ca_V2.1$ expression increased from its lowest expression at P0 to a peak at P60. Error bars indicate SEM; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Scale bar: 0.5 cm.

molecular layer at P5, increased at P10, and then decreased from P15 to adulthood (Fig. 4A,B). A similar developmental expression pattern was found in the thalamus and caudate putamen (Fig. 4A-C).

Close spatial association of downstream molecules in the hippocampus during postnatal development

We have previously demonstrated that $GABA_B$ receptors show overlapping subcellular localization and form macromolecular complexes with GIRK and Ca_V2 channels, RGS7, and Gβ5 proteins in the adult hippocampus, both postsynaptically and presynaptically (Fajardo-Serrano et al., 2013; Ostrovskaya et al., 2014, 2018). This prompted us to investigate the spatial relation of $GABA_B$ receptors with different signaling proteins in the hippocampus during postnatal development to identify when and where macromolecular complexes are formed. For such purpose, we used a pre-embedding immunogold approach and quantitative analysis in the *stratum radiatum* of the CA1 field at two key postnatal ages: P10 and P15 (Fig. 5). At these two ages, immunoreactivity for $GABA_{B1}$, $G\alpha_o$, Gβ5, and RGS7 was primarily found along the extrasynaptic plasma membrane of dendritic spines and shafts of CA1 pyramidal cells, and less frequently associated with the

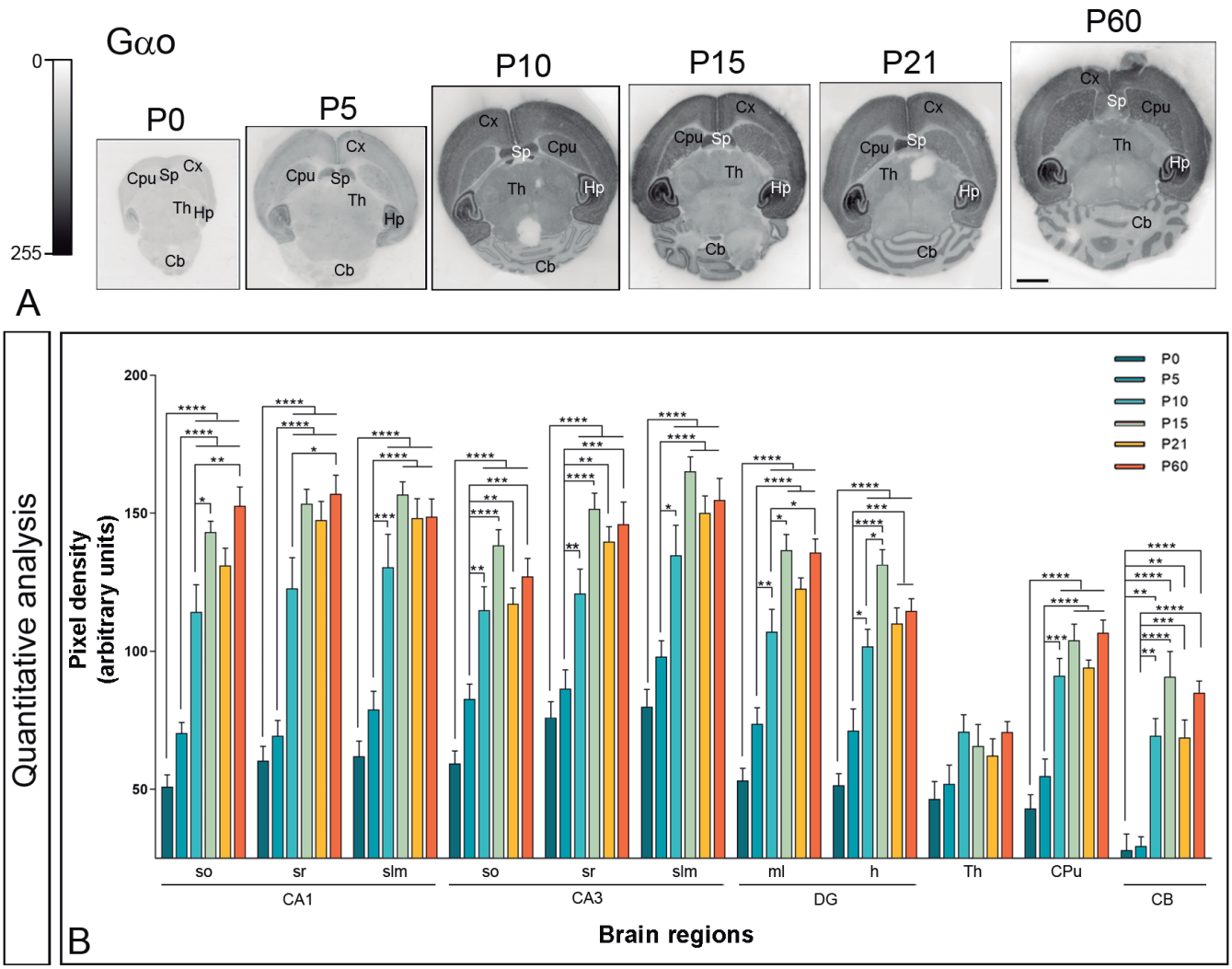

Fig. 3. Regional distribution of the $G\alpha_o$ protein in the developing mouse brain. **A.** Distribution of $G\alpha_o$ protein was visualized on histoblots of horizontal brain sections at various stages of postnatal development using subunit-specific affinity-purified anti-$G\alpha_o$ antibody. The $G\alpha_o$ protein expression showed marked region-specific differences in the developing and adult brain, with the strongest immunoreactivity in the hippocampus (Hp), followed by septum (Sp) cortex (Cx), caudate putamen (CPu), and cerebellum (Cb), and moderate expression in the thalamus (Th). **B.** The histoblots were scanned and densitometric measurements from five independent experiments were averaged to compare the protein densities for each developmental time point. Overall, $G\alpha_o$ expression was low at P0, increased progressively reaching a peak at P15, and then decreased to adulthood. Error bars indicate SEM; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Scale bar: 0.4 cm.

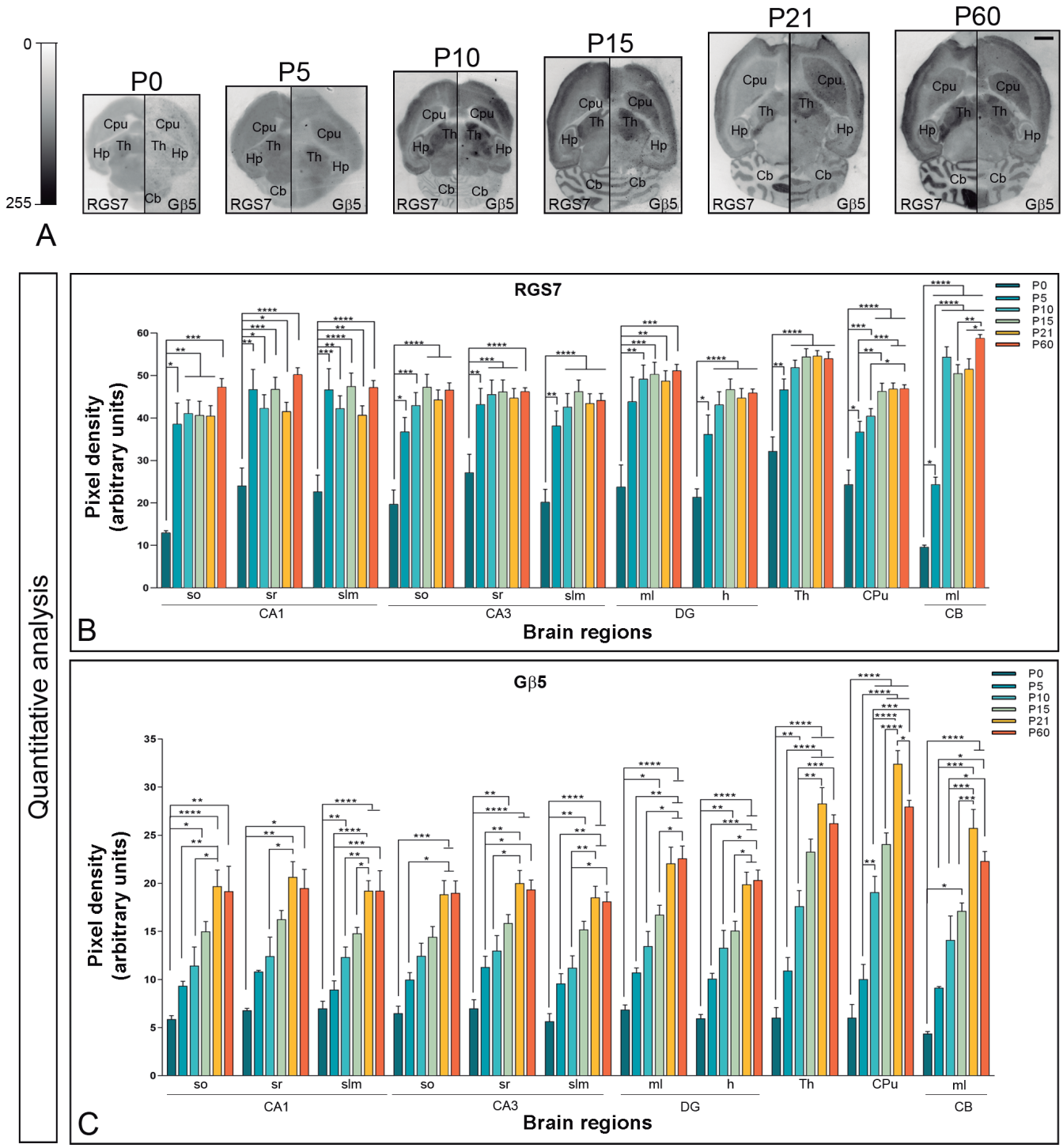

Fig. 4. Regional distribution of Gβ5 and RGS7 proteins in the developing mouse brain. **A.** Distribution of Gβ5 and RGS7 proteins was visualized on histoblots of horizontal brain sections at various stages of postnatal development. Gβ5 and RGS7 showed broad and overlapping distribution patterns in the developing and adult brain. Strong immunoreactivity for Gβ5 and RGS7 was detected in the thalamus (Th), followed by the caudate putamen (CPu), molecular layer of the cerebellum (Cb), hippocampus (Hp), cortex (Cx), and septum (Sp). **B-C.** The histoblots were scanned and densitometric measurements from five independent experiments were averaged to compare the protein densities for each developmental time point. Overall, Gβ5 and RGS7 expression were low at P0 and increased progressively to adulthood. Error bars indicate SEM; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Scale bar: 0.4 cm.

endoplasmic reticulum cisterna of dendritic shafts and the spine apparatus (Fig. 5A-D,I-L).

To compare the distribution of $GABA_B$ receptors with that of $G\alpha_o$, Gβ5, and RGS7 on spines of pyramidal cells in relation to glutamatergic synapses, the distances of the immunoparticles from the edge of asymmetrical synaptic specializations were measured at P10 (Fig. 5E-H) and P15 (Fig. 5M-P). At P10, around 50% of the particles for $GABA_{B1}$, $G\alpha_o$, Gβ5, and RGS7 were found between 0 and 300 nm from the synapses (Fig. 5E-H). At P15, the distribution of the four proteins showed a peak between 0 and 300 nm from the synapses (68% of the $GABA_{B1}$ particles, 64% of the $G\alpha_o$ particles, 71% of the Gβ5 particles, and 69% of the RGS7 particles) (Fig. 5M-P). Therefore, this approach revealed that on dendritic spines, the four proteins have the same

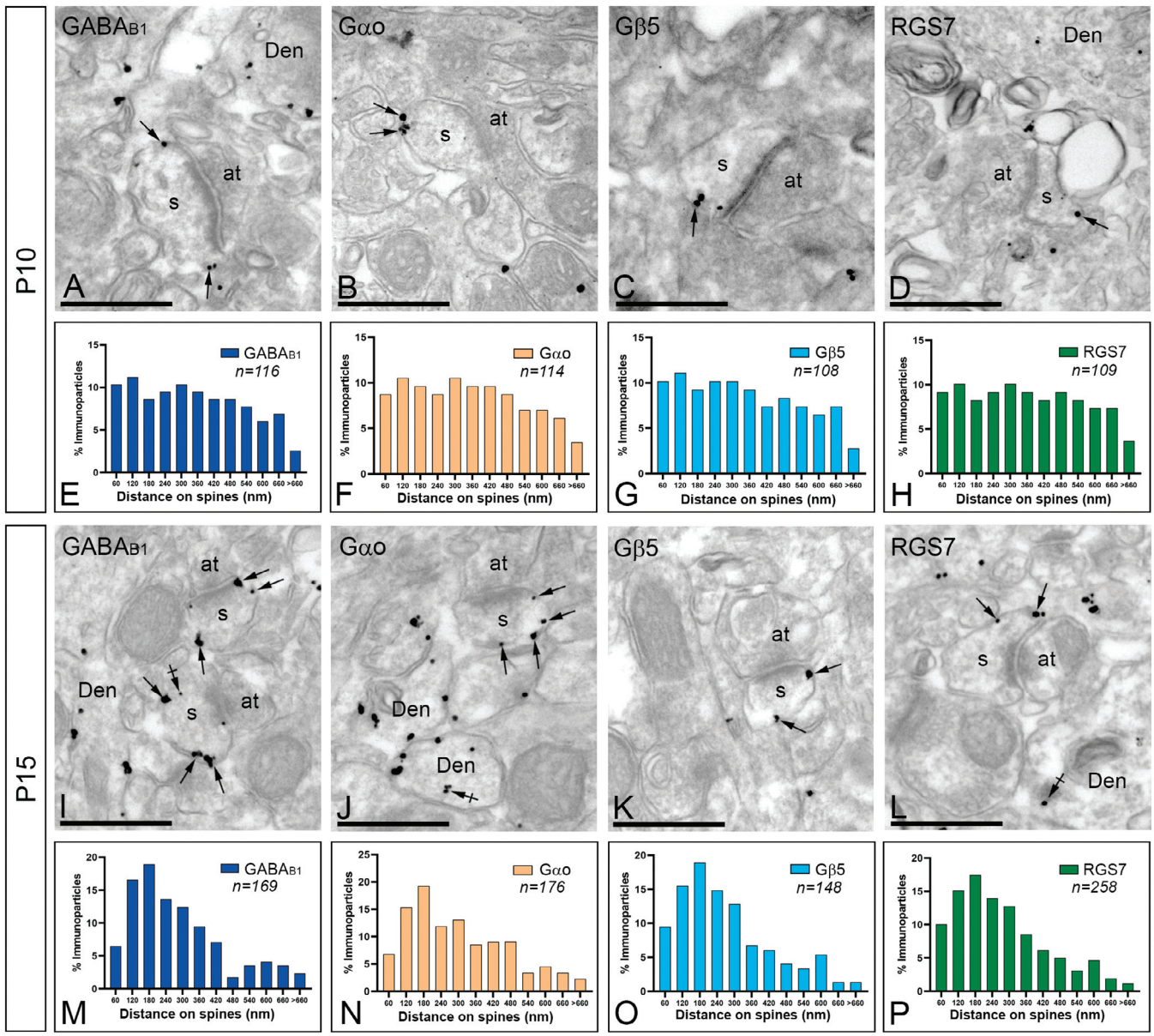

Fig. 5. Subcellular localization of postsynaptic $GABA_B$ receptors with downstream molecules during postnatal development in the hippocampus. Electron micrographs of the *stratum radiatum* of the hippocampal CA1 field showing immunoparticles for $GABA_{B1}$, $G\alpha_o$, Gβ5, and RGS7, as detected using a pre-embedding immunogold method. **A-P.** At P10 and P15, immunoparticles for $GABA_{B1}$, $G\alpha_o$, Gβ5, and RGS7 were localized to the extrasynaptic plasma membrane (arrows) of dendritic shafts (Den) and dendritic spines (s) of pyramidal cells contacted by terminals (at), as well as at intracellular sites (crossed arrows). Dendritic compartmentalization of $GABA_{B1}$, $G\alpha_o$, Gβ5, and RGS7 in CA1 pyramidal cells was assessed using quantitative analyses. Distances of immunogold particles were measured from the closest edge of the PSD along the surface of dendritic spines. Values were allocated to 60-nm-wide bins and expressed as relative frequencies. The histogram shows an enrichment of all four proteins in the proximity of asymmetrical synapses on dendritic spines during postnatal development that is larger at P15. Scale bar: 500 nm.

distribution patterns around asymmetrical synapses in the hippocampus, where they accumulate progressively close to excitatory synapses during postnatal development.

We next investigated the spatial relationship of $GABA_B$ receptors with that of $Ca_V2.1$, $G\alpha_o$, Gβ5, and RGS7 on excitatory axon terminals, presumably from CA3 pyramidal cells, in the *stratum radiatum* at P15 (Fig. 6). Immunoparticles for $GABA_{B1}$, $GABA_{B2}$, $Ca_V2.1$, $G\alpha_o$, Gβ5, and RGS7 were observed both in the active zone and extrasynaptic plasma membrane of axon terminals (Fig. 6A-F). The quantitative analysis on the distribution of the six proteins at presynaptic sites showed the overlapping distribution within the active zone and the surrounding extrasynaptic plasma membrane of the axon terminals (Fig. 6A-F).

Close spatial association of downstream molecules in the cerebellum during postnatal development

Another brain region where $GABA_B$ receptors show overlapping subcellular localization and form macromolecular complexes with different associated proteins is the cerebellum (Ciruela et al., 2010; Aguado et al., 2016b; Luján et al., 2018). To provide insights into the spatial relation of $GABA_B$ receptors with different signaling proteins during postnatal cerebellar development, we used immunofluorescence and immunoelectron microscopy combined with quantitative approaches (Fig. 7). Double-labeling immunofluorescence experiments were carried out to colocalize $Ca_V2.1$ and $GABA_{B1}$ at P7 and P12 (Fig. 7A,B) At these two postnatal ages, strong immunoreactivity for $Ca_V2.1$ and $GABA_{B1}$ was found in Purkinje cell somata and their developing dendrites in the molecular layer, showing mostly overlapping distributions (Fig. 7A,B). Compared with the intense immunoreactivity in Purkinje cells, weak labeling was seen in the internal and external granular layer for $GABA_{B1}$ (Fig. 7A2,B2) and $Ca_V2.1$ (Fig. 7A1,B1).

To establish the precise subcellular localization of $GABA_B$ receptors, $Ca_V2.1$, and $G\alpha_o$ during postnatal

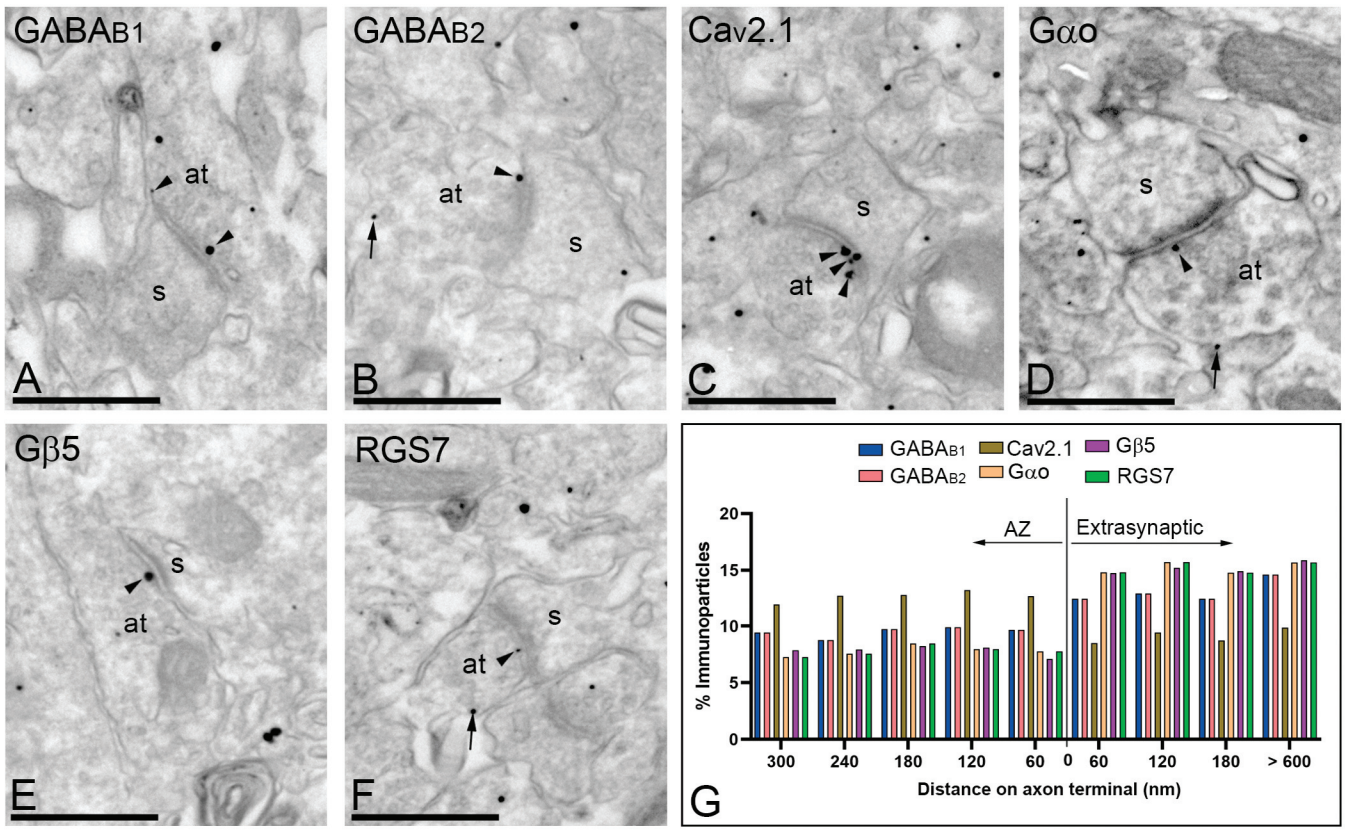

Fig. 6. Subcellular localization of presynaptic $GABA_B$ receptors with downstream molecules during postnatal development in the hippocampus. Electron micrographs of the *stratum radiatum* of the hippocampal CA1 field showing immunoparticles for $GABA_{B1}$, $GABA_{B2}$, $Ca_V2.1$, $G\alpha_o$, Gβ5, and RGS7 at presynaptic sites, as detected using a pre-embedding immunogold method at P15. **A-F.** Immunoparticles for the six proteins were localized to the extrasynaptic plasma membrane (arrows) and to the active zone (arrowheads) of axon terminals (at), establishing asymmetrical synapses with spines (s). **G.** Distances of immunogold particles (n=142 for $GABA_{B1}$; n=151 for $GABA_{B2}$; n=173 for $Ca_V2.1$; n=138 for $G\alpha_o$; n=112 for Gβ5; n=121 for RGS7) were measured from the edge of the active zone (AZ) along the extrasynaptic membrane surrounding the AZ. Values were allocated to 60-nm-wide bins and expressed as relative frequencies. Histogram showing the spatial distribution of immunoparticles for the six proteins on excitatory axon terminals. These data show that $GABA_{B1}$, $GABA_{B2}$, $Ca_V2.1$, $G\alpha_o$, Gβ5, and RGS7 are present within the same nanodomains of the axon terminals, suggesting their possible association during postnatal development. Scale bar: 500 nm.

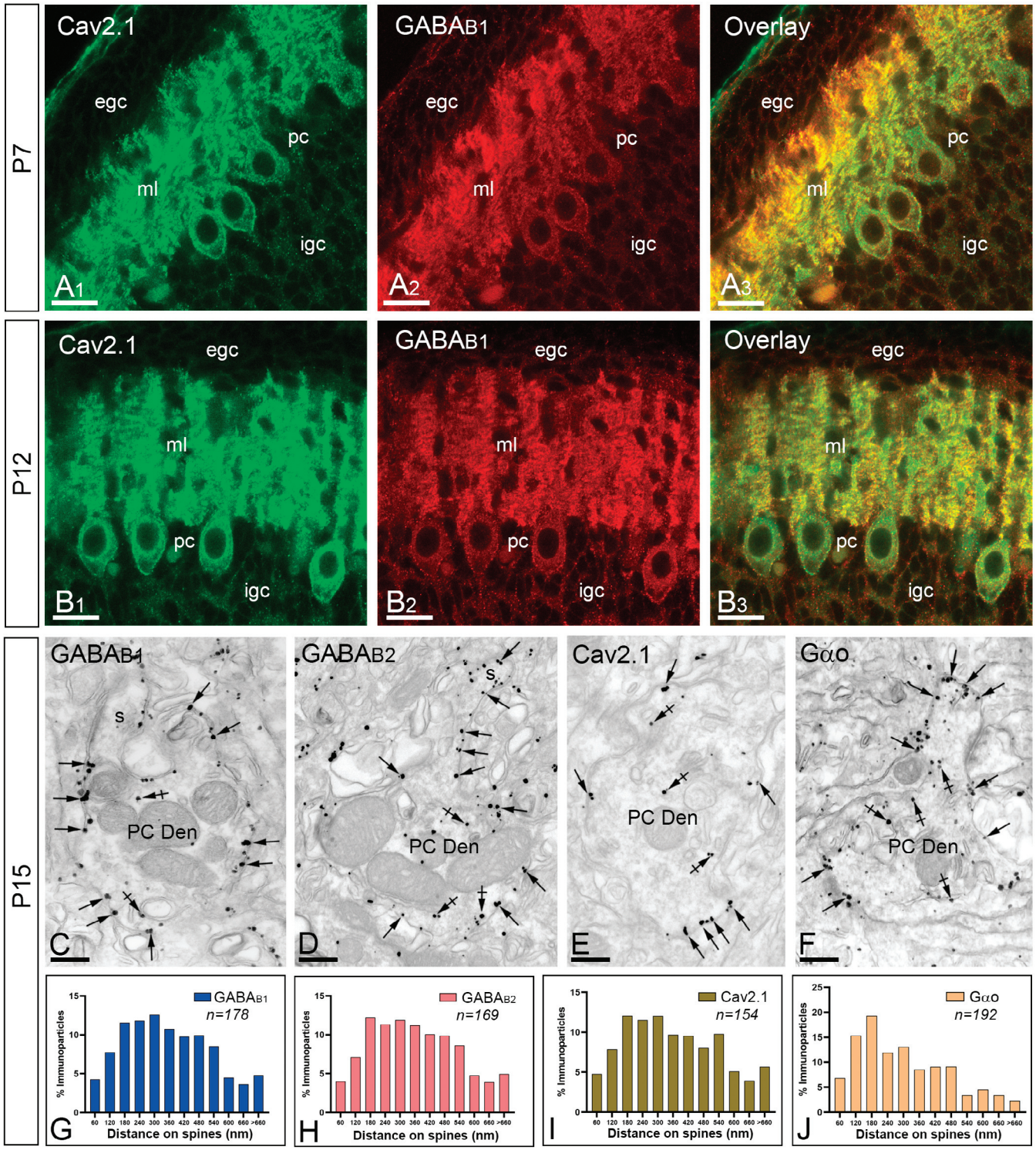

Fig. 7. Association of GABA$_B$ receptors with downstream molecules during postnatal development in the cerebellum. **A, B.** Co-localization of Ca$_V$2.1 (green) and GABA$_{B1}$ (red) in the cerebellum at P7 and P12, as demonstrated using double immunofluorescence. Double labeling between Ca$_V$2.1 and GABA$_{B1}$, as shown in color-separated images and in the corresponding overlay. Strong immunoreactivity for Ca$_V$2.1 and GABA$_{B1}$ was found in Purkinje cell (pc) somata and their developing dendrites in the molecular layer (ml), showing overlapping distribution patterns. **C-F.** Electron micrographs of the cerebellar cortex showing immunoreactivity for GABA$_{B1}$, GABA$_{B2}$, Ca$_V$2.1, and Gα$_o$, as revealed by a pre-embedding immunogold method at P15. Immunoparticles for the four proteins were mainly located at the extrasynaptic plasma membranes (arrows) of dendritic shafts (PC Den) and spines (s) of Purkinje cells, as well as at intracellular sites (crossed arrows). **G-J.** Distances of immunogold particles were measured from the closest edge of the PSD along the surface of dendritic spines. Values were allocated to 60-nm-wide bins and expressed as relative frequencies. The histogram shows an enrichment of GABA$_{B1}$, GABA$_{B2}$, Ca$_V$2.1, and Gα$_o$, which are present in the same nanodomain of Purkinje cells to form macromolecular complexes at P15. egc, external granular layer; igc, internal granular layer. Scale bar: 500 nm.

development of Purkinje cells, we carried out electron microscopic investigations using the pre-embedding immunogold technique at P15 (Fig. 7C). At this age, immunoreactivity for $GABA_{B1}$ and $GABA_{B2}$ was characterized by a progressive accumulation of immunoparticles on the extrasynaptic plasma membrane of Purkinje cell dendritic shafts and spines, establishing synapses with parallel fibers (Fig. 7C,D). Similarly, immunoreactivity for $Ca_V2.1$ and $G\alpha_o$ was distributed along the extrasynaptic plasma membrane of dendritic shafts and spines of Purkinje cells (Fig. 7E,F). We next determined the spatial relationship of $GABA_{B1}$, $GABA_{B2}$, $Ca_V2.1$, and $G\alpha_o$ on dendritic spines relative to glutamate release sites (Fig. 7G-J), to gain further insight into their subcellular compartmentalization. The data revealed that along the extrasynaptic membrane of dendritic spines of Purkinje cells, the four proteins showed the same distribution pattern relative to parallel fiber synapses (Fig. 7G-J). Around 50% of the particles for $GABA_{B1}$, $GABA_{B2}$, $Ca_V2.1$, and $G\alpha_o$ were found between 0 and 300 nm from the synapses (Fig. 7G-J). This data shows that the four proteins are present in the same nanodomain of Purkinje cells, supporting their close spatial relationship to form macromolecular complexes at P15.

Discussion

In the present study, we have investigated the spatiotemporal expression of $GABA_B$ receptors and different downstream molecules that are part of the macromolecular complex or signalosome in the mouse brain during postnatal development, with the general aim of determining when and where such an association might take place. Using histoblots, we have shown that the $GABA_{B1}$ and $GABA_{B2}$ subunits of $GABA_B$ receptors are widely distributed from early stages in the postnatal developing brain, and that $Ca_V2.1$, $G\alpha_o$, Gβ5, and RGS7 exhibit temporally and regionally overlapping distribution patterns. Using quantitative immunoelectron microscopy in the developing hippocampus, we revealed the close spatial association of $GABA_{B1}$, $G\alpha_o$, Gβ5, and RGS7 with excitatory synapses at postsynaptic sites, and $GABA_{B1}$, $GABA_{B2}$, $Ca_V2.1$, $G\alpha_o$, Gβ5, and RGS7 at presynaptic sites. Using confocal and quantitative immunoelectron microscopy in the developing cerebellum, we showed that $GABA_{B1}$ and $Ca_V2.1$ exhibit overlapping distribution in Purkinje cells from early stages of development, as well as tight co-distribution of $GABA_{B1}$, $GABA_{B2}$, $Ca_V2.1$, and $G\alpha_o$ in dendrites and spines. The analogous subcellular and developmental distribution of proteins ($Ca_V2.1$, $G\alpha_o$, Gβ5, and RGS7) with that of $GABA_{B1}$ and $GABA_{B2}$ in association with the establishment of glutamatergic synapses supports the idea that the formation of the $GABA_B$ receptor signalosome take place at early stages and favors their possible involvement in regulating hippocampal and cerebellar processes during postnatal development.

Methodological considerations

Although the regulation and changes in expression of some receptors and ion channels have been previously analyzed in the developing brain (Martin et al., 1998; Lopez-Tellez et al., 2004; Ballesteros-Merino et al., 2012, 2014; Martínez-Hernández et al., 2013), these studies were performed using western blots on native tissue prepared from the whole brain, the hippocampus, or the cerebellum, thus not allowing any detailed examination of the different layers or subfields of the given brain area. The histoblot technique became a reliable and fast alternative for western blots and for conventional immunohistochemistry (Aguado et al., 2023). The characteristics of the technique result in an appropriate spatiotemporal pattern in which proteins are arranged within a brain section and affords accurate quantitative analyses of the different brain regions, allowing its successful application to determine the developmental regulation of different proteins in the mouse brain (Tönnes et al., 1999; Pickard et al., 2000; Fernández-Alacid et al., 2011; Ferrándiz-Huertas et al., 2012; Aguado et al., 2016a;). Here, we have extended those studies to establish the spatiotemporal distribution and expression of $GABA_{B1}$, $GABA_{B2}$, $Ca_V2.1$, $G\alpha_o$, Gβ5, and RGS7 in brain regions present on tissue sections and all subdivision layers of the hippocampus and cerebellum during postnatal development from the day of birth.

At the subcellular level, it was not possible to provide a direct demonstration of the colocalization between $GABA_{B1}$, $GABA_{B2}$, $Ca_V2.1$, $G\alpha_o$, Gβ5, and RGS7 with the antibodies available, which were mostly raised in rabbits. It was, nevertheless, evident that these proteins were expressed, at times, by the same neuron population and the same neuronal compartment. Using quantitative analysis in spines of developing CA1 pyramidal and Purkinje cells, as well as in axon terminals establishing synapses with CA1 pyramidal cells, we here show that the subcellular localization of $GABA_B$ receptors remains indistinguishable from the associated proteins in those neuronal compartments during critical periods of synaptogenesis. This indicates that they are located within the same nanodomain, which favors their molecular association during postnatal development. We have previously shown that macromolecular complexes formed by $GABA_B$/GIRK/Gβ5/RGS7 exist and that these four proteins share the same co-localization in mature CA1 pyramidal cells (Fajardo-Serrano et al., 2013).

Similar developmental expression profile of $GABA_B$ receptors and downstream molecules

The neurotransmitter GABA and the maturation of the inhibitory system play critical roles in correct brain functioning (Tang et al., 2021). $GABA_B$ receptors are expressed at early stages of development, well before the formation of synaptic circuits in the brain (López-

Bendito et al., 2004). Although data obtained from $GABA_{B1}$ KO (knockout) mice indicated that $GABA_B$ receptors do not seem to be essential for normal brain development (Schuler et al., 2001), they likely play some roles in mediating both synaptic and non-synaptic signaling during neonatal development. In the present study, we found widespread expression of the $GABA_{B1}$ and $GABA_{B2}$ subunits in the postnatal brain, with the most intense labeling in the molecular layer of the cerebellum, the thalamus, and some layers of the hippocampus throughout development. Our findings are consistent with *in situ* hybridization (Kaupmann et al., 1998; Bischoff et al., 1999; Durkin et al., 1999) and light microscopic studies (Fritschy et al., 1999; López-Bendito et al., 2004; Luján and Shigemoto, 2006), reinforcing the idea that $GABA_{B1}$ and $GABA_{B2}$ subunits exhibit regionally and temporally overlapping distribution patterns. The increase in $GABA_{B1}$ and $GABA_{B2}$ expression seen between P10 and P21 in most brain regions parallels the formation and maturation of synapses, especially in the hippocampus and cerebellum. This suggests that synaptic activity might regulate $GABA_B$ receptors during postnatal development.

Most of the physiological functions of GPCRs are mediated through their coupling to heterotrimeric G proteins, composed of α, β, and γ subunits. To date, there are 16 Gα, 5 Gβ, and 12 Gγ genes in the human and mouse genomes (Syrovatkina et al., 2016). Based on the sequence and functional similarities, Gα proteins are grouped into four main families ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$) that differ in effector recognition (Syrovatkina et al., 2016). $GABA_B$ receptors act via $G\alpha_{i/o}$ signaling, which is blocked by pertussis toxin to inhibit receptor interaction (Bettler et al., 2004). Among Gβ subunits, Gβ5 is unique because it shares fewer similarities with the remaining subunits, is mainly found in the brain, and can heterodimerize with members of the R7-RGS subfamily (Syrovatkina et al., 2016). Gβ5/R7-RGS complexes selectively deactivate the $G\alpha_{i/o}$ subunits that mediate the actions of GPCRs (Posner et al., 1999), including $GABA_B$ receptors. Here, we show that $G\alpha_o$, Gβ5, and RGS7 exhibit regionally overlapping distributions during postnatal development. However, $G\alpha_o$ is more widely distributed throughout the brain, likely because of its functional association with many different GPCRs (Wettschureck and Offermanns, 2005). The comparison of their labeling patterns with those of $GABA_{B1}$ and $GABA_{B2}$ indicated that they largely overlap throughout the adult and developing brain, suggesting that the downstream molecules involved in the activation and deactivation of the G protein-mediated signaling system might associate with their parent GPCR since early stages of postnatal development. The only brain region where labelling patterns are not virtually the same is the thalamus, which shows lower levels of $G\alpha_o$ protein compared to all other proteins throughout development, suggesting that a less efficient functional coupling.

Ca_V2 channels can be modulated by $GABA_B$ receptors (Padgett and Slesinger, 2010) in a region- and neuronal compartment-dependent manner (Luján et al., 2018; Martín-Belmonte et al., 2024). $GABA_B$ receptors couple to $Ca_V2.1$ channels at presynaptic sites in the hippocampus (Takahashi et al., 1998) and at postsynaptic sites in the cerebellum (Luján et al., 2018). During development, Ca^{2+} and Cav channels are involved in several functions, including neurogenesis, neurite outgrowth, synapse formation and elimination, cell differentiation, and neuronal death (Rosenberg and Spitzer, 2011). Our findings show that the $Ca_V2.1$ protein was widely expressed throughout the brain during postnatal development, consistent with previous *in situ* hybridization (Ludwig et al., 1997). At the protein level, the intensity of immunolabeling for $Ca_V2.1$ varied markedly between specific brain regions and showed its most intense labeling in the molecular layer of the cerebellum, the thalamus, and the hippo-campus throughout development. This developmental and regional expression pattern was similar to that described for $GABA_{B1}$ and $GABA_{B2}$ subunits, suggesting that activation of $GABA_B$ receptors modulates $Ca_V2.1$ channels from early stages of postnatal development.

Subcellular localization of $GABA_B$ receptors and downstream molecules during development

Heterodimerization of the $GABA_{B1}$ and $GABA_{B2}$ subunits to form $GABA_B$ receptors *in vivo* was demonstrated in $GABA_{B1}$ KO mice, showing the absence of functional $GABA_B$-mediated responses (Schuler et al., 2001). Consistent with this data, our findings reveal that $GABA_{B1}$ and $GABA_{B2}$ show a very similar developmental profile in the brain and indistinguishable subcellular localization in both the hippocampus and cerebellum. This data suggests that $GABA_{B1}$ and $GABA_{B2}$ heterodimers, and therefore functional $GABA_B$ receptors, can be formed from early stages of brain development, consistent with previous findings (Fritschy et al., 2004). However, the existence of a small fraction of $GABA_{B1}$ and/or $GABA_{B2}$ that are not part of dimeric $GABA_B$ receptors cannot be ruled out (Fritschy et al., 2004).

Besides heterodimerization, oligomerization of $GABA_B$ receptors has also been described in the brain (Schwenk et al., 2016). There is now compelling evidence that $GABA_B$ receptors are frequently identified as components of GEMMAs (Ferré et al., 2022) and show specificity for subcellular compartments in the hippocampus and cerebellum (Luján et al., 2018; Martín-Belmonte et al., 2024). Considering the role played by these GEMMAs in neuronal function, the crucial question arises of when GEMMA organization is taking place. To explore the microarchitecture of proteins in specialized neuronal compartments during postnatal development, quantitative immunoelectron microscopy was used. Our data show that $GABA_B$ receptors and their downstream signaling partners, such as $G\alpha_o$, Gβ5, and RGS7, are present in the nanodomain

in dendritic spines of CA1 pyramidal cells at both P10 and P15. In the cerebellum, $GABA_B$ receptors are extensively colocalized with $Ca_V2.1$ in Purkinje cells at P7 and P12, a period of great developmental activity in these cells with considerable outgrowth and differentiation of the dendritic arborization and parallel fiber synapse formation (Altman and Bayer, 1997). In addition, immunoparticles for $GABA_B$ receptors, $G\alpha_o$ and $Ca_V2.1$ were detected along the extrasynaptic plasma membrane of dendritic shafts of Purkinje cells and were more abundant around excitatory synapses in the same nanodomain in dendritic spines at P15, when final maturation of excitatory synapses is reached (Altman and Bayer, 1997). Given these data, it seems reasonable to suggest that $G\alpha_o$ and $Ca_V2.1$ mediate the dominant component of $GABA_B$-mediated postsynaptic inhibition during critical periods of development. Consistent with this data, a quantitative proteomic study suggested that $GABA_B$ receptors strongly interact with $Ca_V2.1$ channels in the brain (Müller et al., 2010). Furthermore, using co-immunoprecipitation and immunoelectron microscopy techniques, we previously showed that $GABA_B$ receptors form complexes with $Ca_V2.1$ channels in the adult cerebellum, where they co-cluster within a nanometer scale (Luján et al., 2018). Therefore, our data suggest that macromolecular complexes of $GABA_B$ receptors with downstream molecules are assembled at postsynaptic compartments during postnatal development in hippocampal and cerebellar neurons.

Presynaptically, $GABA_B$ receptors are predominantly localized at glutamatergic axon terminals to mediate presynaptic inhibition of glutamate release (Vigot et al., 2006), through inhibition of voltage-gated P/Q- and N-type Ca^{2+} channels. Thus, activation of $GABA_B$ receptors at these axon terminals induces inhibition of synaptic vesicle release through suppression of Ca^{2+} flux (Laviv et al., 2010). To fulfill this presynaptic inhibition at hippocampal synapses, $GABA_B$ receptors form macromolecular complexes with different proteins, regardless of synaptic activity and agonist stimulation (Laviv et al., 2011). Consistent with these findings, our data showed the colocalization and close spatial association of $GABA_{B1}$, $GABA_{B2}$, $Ca_V2.1$, $G\alpha_o$, Gβ5, and RGS7 in the active zone and extrasynaptic plasma membrane of axon terminals, establishing excitatory synapses with dendritic spines of CA1 pyramidal cells. The $Ca_V2.1$ channel, however, was mostly distributed in the active zone of axon terminals during development, largely confirming previous ultrastructural findings in the adult brain (Althof et al., 2015; Martín-Belmonte et al., 2024). Overall, our data suggest that precoupling of $GABA_B$ receptors, G proteins, RGS proteins, and $Ca_V2.1$ channels in a signaling nanodomain is required for proper regulation of neurotransmitter release at developing hippocampal synapses.

In summary, heterodimerization of $GABA_{B1}$ and $GABA_{B2}$ is essential for $GABA_B$ receptor function, and their oligomerization with downstream molecules is important to fulfill different functions, assuring the specificity and efficacy of signal transduction. The comparison of labeling patterns for $GABA_{B1}$ and $GABA_{B2}$ with those for $G\alpha_o$, $Ca_V2.1$, Gβ5, and RGS7 at different stages of development indicated that they largely overlap throughout the adult and developing brain. Although the exact compositions and molecular arrangements of most signaling complexes remain to be elucidated, not to mention the mechanisms by which they are assembled and disassembled, our findings show that $GABA_B$ receptors and downstream molecules ($Ca_V2.1$, $G\alpha_o$, Gβ5, and RGS7) cohabit the same microdomain of dendritic spines and axon terminals in the hippocampus and cerebellum from early stages of postnatal development. Therefore, our data suggest that compartmentalization of $GABA_B$ receptors, $G\alpha_o$, Gβ5, RGS7, with or without $Ca_V2.1$ channels (depending on the type of neuron), may constitute a general mechanism underlying postsynaptic and presynaptic signaling throughout postnatal development.

Acknowledgements. We thank Ms. Diane Latawiec for the English revision of the manuscript. Funding sources were the Spanish Ministerio de Economía y Competitividad, and Junta de Comunidades de Castilla-La Mancha (Spain).

Funding. Grants PID2021-125875OB-I00 funded by MCIN/AEI/10.13039/501100011033 and "ERDF A way of making Europe" to RL. This work was also supported by Castilla-La Mancha Regional Government and the European Regional Development Fund (SBPLY/21/180501/000064) and the Universidad de Castilla-La Mancha (2023-GRIN-34187) to RL.

Availability of data and material. All data used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' Contributions. All authors had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. CA and RL designed the project and performed immunoelectron microscopy analysis; AFS performed early histoblot and electron microscopy for $GABA_B$ receptors, Gβ5, and RGS7 proteins; SGM performed early histoblot for $Ca_V2.1$; ARS performed early histoblot for $G\alpha_o$; RAR, AMB, CA, and AEMM performed histoblot analysis; CA, AFS, AMB, RAR, and RL analyzed data; RS provided reagents and feedback on the manuscript; KAM provided reagents and feedback on the manuscript; RL wrote the paper. All authors read and approved the final manuscript.

Ethical Approval and Consent to Participate. All animal experimental procedures were performed in accordance with Spanish (RD 53/2013) and European Union regulations (2010/63/UE), and the protocols were approved by the local Animal Care and Use Committee.

Consent for publication. All co-authors of the present manuscript can certify that it has not been submitted to more than one journal for simultaneous consideration and that the manuscript has not been published previously (partly or in full). The authors can also certify that the main study is not split up into several parts to increase the quantity of submissions, that none of the data presented here have been fabricated or manipulated, and that we present our own data/text/theories/ideas. All authors and authorities have explicitly provided their consent to submit the present manuscript, and in general, we all agree with the ethical responsibilities of authors of the journal.

Finally, all authors give consent for publication in *Histology and Histopathology*.

Competing interests. The authors of this manuscript declare that they have no competing interests.

References

Aguado C., García-Madrona S., Gil-Minguez M. and Luján R. (2016a). Ontogenic changes and differential localization of T-type Ca^{2+} channel subunits Cav3.1 and Cav3.2 in mouse hippocampus and cerebellum. Front. Neuroanat. 10, 83.

Aguado C., Orlandi C., Fajardo-Serrano A., Gil-Minguez M., Martemyanov K.A. and Luján R. (2016b). Cellular and subcellular localization of the RGS7/Gβ5/R7BP complex in the cerebellar cortex. Front. Neuroanat. 10, 114.

Aguado C., Martín-Belmonte A., Alfaro-Ruiz R., Martínez-Moreno A. E. and Luján R. (2023). Histoblot: A sensitive method to quantify the expression of proteins in normal and pathological conditions. Histol Histopathol. 38, 725-737.

Althof D., Baehrens D., Watanabe M., Suzuki N., Fakler B. and Kulik Á. (2015). Inhibitory and excitatory axon terminals share a common nano-architecture of their $Ca_V2.1$ (P/Q-type) Ca^{2+} channels. Front. Cell. Neurosci. 9, 315.

Altman J. and Bayer S. (1997). Development of the Cerebellar System: in Relation to its Evolution, Structure, and Functions. 1st Ed. CRC-Press. 783 p.

Ballesteros-Merino C., Lin M., Wu W.W., Ferrandiz-Huertas C., Cabañero M.J., Watanabe M., Fukazawa Y., Shigemoto R., Maylie J., Adelman J.P. and Luján R. (2012). Developmental profile of SK2 channel expression and function in CA1 neurons. Hippocampus 22, 1467-1480.

Ballesteros-Merino C., Watanabe M., Shigemoto R., Fukazawa Y., Adelman J.P. and Luján R. (2014). Differential subcellular localization of SK3-containing channels in the hippocampus. Eur. J. Neurosci. 39, 883-892.

Bettler B., Kaupmann K., Mosbacher J. and Gassmann M. (2004). Molecular structure and physiological functions of $GABA_B$ receptors. Physiol. Rev. 84, 835-867.

Bischoff S., Leonhard S., Reymann N., Schuler V., Shigemoto R., Kaupmann K. and Bettler B. (1999). Spatial distribution of $GABA_BR1$ receptor mRNA and binding sites in the rat brain. J. Comp. Neurol. 412, 1-16.

Cao Y., Song H., Okawa H., Sampath A.P., Sokolov M. and Martemyanov K.A. (2008). Targeting of RGS7/Gβ5 to the dendritic tips of ON-bipolar cells is independent of its association with membrane anchor R7BP. J. Neurosci. 28, 10443-10449.

Chu D.C.M., Albin R.L., Young A.B. and Penney J.B. (1990). Distribution and kinetics of $GABA_B$ binding sites in rat central nervous system: A quantitative autoradiographic study. Neuroscience 34, 341-357.

Ciruela F., Fernández-Dueñas V., Sahlholm K., Fernández-Alacid L., Nicolau J.C., Watanabe M. and Luján R. (2010). Evidence for oligomerization between $GABA_B$ receptors and GIRK channels containing the GIRK1 and GIRK3 subunits. Eur. J. Neurosci. 32, 1265-77.

Dong H., O'Brien R.J., Fung E.T., Lanahan A.A., Worley P.F. and Huganir R.L. (1997). GRIP: A synaptic PDZ domain-containing protein that interacts with AMPA receptors. Nature 386, 279-284.

Durkin M.M., Gunwaldsen C.A., Borowsky B., Jones K.A. and Branchek T.A. (1999). An *in situ* hybridization study of the distribution of the $GABA_{B2}$ protein mRNA in the rat CNS. Brain Res. Mol. Brain Res. 71, 185-200.

Fajardo-Serrano A., Wydeven N., Young D., Watanabe M., Shigemoto R., Martemyanov K.A., Wickman K. and Luján R. (2013). Association of Rgs7/Gβ5 complexes with girk channels and $GABA_B$ receptors in hippocampal CA1 pyramidal neurons. Hippocampus 23, 1231-1245.

Fernández-Alacid L., Watanabe M., Molnár E., Wickman K. and Luján R. (2011). Developmental regulation of G protein-gated inwardly-rectifying K^+ (GIRK/Kir3) channel subunits in the brain. Eur. J. Neurosci. 34, 1724-1736.

Ferrándiz-Huertas C., Gil-Mínguez M. and Luján R. (2012). Regional expression and subcellular localization of the voltage-gated calcium channel β subunits in the developing mouse brain. J. Neurochem. 122, 1095-1107.

Ferré S., Ciruela F., Dessauer C. W., González-Maeso J., Hébert T.E., Jockers R., Logothetis D.E. and Pardo L. (2022). G protein-coupled receptor-effector macromolecular membrane assemblies (GEMMAs). Pharmacol. Ther. 231, 107977.

Fritschy J., Meskenaite V., Weinmann O., Honer M., Benke D. and Mohler H. (1999). $GABA_B$ receptor splice variants GB1a and GB1b in rat brain: Developmental regulation, cellular distribution and extrasynaptic localization. Eur. J. Neurosci. 11, 761-768.

Fritschy J.M., Sidler C., Parpan F., Gassmann M., Kaupmann K., Bettler B. and Benke D. (2004). Independent maturation of the $GABA_B$ receptor subunits $GABA_{B1}$ and $GABA_{B2}$ during postnatal development in rodent brain. J. Comp. Neurol. 477, 235-252.

Fritzius T. and Bettler B. (2020). The organizing principle of $GABA_B$ receptor complexes: Physiological and pharmacological implications. Basic Clin. Pharmacol. Toxicol. 126, 25-34.

Indriati D. W., Kamasawa N., Matsui K., Meredith A. L., Watanabe M. and Shigemoto R. (2013). Quantitative localization of $Ca_V2.1$ (P/Q-Type) voltage-dependent calcium channels in Purkinje cells: Somatodendritic gradient and distinct somatic Coclustering with calcium-activated potassium channels. J. Neurosci. 33, 3668-3678.

Jones K.A., Borowsky B., Tamm J.A., Craig D.A., Durkin M.M., Dai M., Yao W.J., Johnson M., Gunwaldsen C., Huang L.Y., Tang C., Shen Q., Salon J.A., Morse K., Laz T., Smith K.E., Nagarathnam D., Noble S.A., Branchek T.A. and Gerald C. (1998). $GABA_B$ receptors function as a heteromeric assembly of the subunits $GABA_BR1$ and $GABA_BR2$. Nature 396, 674-679.

Kaupmann K., Huggel K., Heid J., Flor P.J., Bischoff S., Mickel S.J., McMaster G., Angst C., Bittiger H., Froestl W. and Bettler B. (1997). Expression cloning of $GABA_B$ receptors uncovers similarity to metabotropic glutamate receptors. Nature 386, 239-246.

Kaupmann K., Malitschek B., Heid J., Froestl W., Beck P., Mosbacher J., Bischoff S., Kulik A., Shigemoto R., Karschin A. and Bettler B. (1998). $GABA_B$-receptor subtypes assemble into functional heteromeric complexes. Nature 60, 683-687.

Kulik Á., Nakadate K., Nyíri G., Notomi T., Malitschek B., Bettler B. and Shigemoto R. (2002). Distinct localization of $GABA_B$ receptors relative to synaptic sites in the rat cerebellum and ventrobasal thalamus. Eur. J. Neurosci. 15, 291-307.

Kulik Á., Vida I., Luján R., Haas C. A., López-Bendito G., Shigemoto R. and Frotscher M. (2003). Subcellular localization of metabotropic $GABA_B$ receptor subunits $GABA_{B1a/b}$ and $GABA_{B2}$ in the rat hippocampus. J. Neurosci. 23, 11026-11035.

Kuner R., Kohn G., Grunewald S., Eisenhardt G., Bach A. and Kornau H. C. (1999). Role of heteromer formation in $GABA_B$ receptor function. Science 283, 74-77.

Laviv T., Riven I., Dolev I., Vertkin I., Balana B., Slesinger P.A. and Slutsky I. (2010). Basal GABA regulates $GABA_BR$ conformation and release probability at single hippocampal synapses. Neuron 67, 253-267.

Laviv T., Vertkin I., Berdichevsky Y., Fogel H., Riven I., Bettler B., Slesinger P.A. and Slutsky I. (2011). Compartmentalization of the $GABA_B$ receptor signaling complex is required for presynaptic inhibition at hippocampal synapses. J. Neurosci. 31, 12523-12532.

López-Bendito G., Shigemoto R., Kulik A., Vida I. and Lujan R. (2004). Distribution of metabotropic GABA receptor subunits $GABA_{B1a/b}$ and $GABA_{B2}$ in the rat hippocampus during prenatal and postnatal development. Hippocampus 848, 836-848.

Lopez-Tellez J.F., Vela J., Del Rio J.C., Ramos B., Baglietto-Vargas D., Santa-Maria C., Ruano D., Gutierrez A. and Vitorica J. (2004). Postnatal development of the α1 containing $GABA_A$ receptor subunit in rat hippocampus. Dev. Brain Res. 148, 129-141.

Ludwig A., Flockerzi V. and Hofmann F. (1997). Regional expression and cellular localization of the and β subunit of high voltage-activated calcium channels in rat brain. J. Neurosci. 17, 1339-1349.

Luján R. and Shigemoto R. (2006). Localization of metabotropic GABA receptor subunits $GABA_{B1}$ and $GABA_{B2}$ relative to synaptic sites in the rat developing cerebellum. Eur. J. Neurosci. 23, 1479-1490.

Luján R., Nusser Z., Roberts J.D.B., Shigemoto R. and Somogyi P. (1996). Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the rat hippocampus. Eur. J. Neurosci. 8, 1488-1500.

Luján R., Aguado C., Ciruela F., Cózar J., Kleindienst D., de la Ossa L., Bettler B., Wickman K., Watanabe M., Shigemoto R. and Fukazawa Y. (2018). Differential association of $GABA_B$ receptors with their effector ion channels in Purkinje cells. Brain Struct. Funct. 223, 1565-1587.

Lüscher C. and Slesinger P.A. (2010). Emerging roles for G protein-gated inwardly rectifying potassium (GIRK) channels in health and disease. Nat. Rev. Neurosci. 11, 301-315.

Margeta-mitrovic M., Mitrovic I., Riley R.C., Jan L.Y. and Basbaum A.I. (1999). Immunohistochemical localization of $GABA_B$ receptors in the rat central nervous system. J. Comp. Neurol. 321, 299-321.

Martin L.J., Furuta A. and Blackstone C.D. (1998). AMPA receptor protein in developing rat brain: Glutamate receptor-1 expression and localization change at regional, cellular, and subcellular levels with maturation. Neuroscience 83, 917-928.

Martín-Belmonte A., Aguado C., Alfaro-Ruíz R., Itakura M., Moreno-Martínez A. E., de la Ossa L., Molnár E., Fukazawa Y. and Luján R. (2020). Age-dependent shift of AMPA receptors from synapses to intracellular compartments in Alzheimer's disease: Immunocytochemical analysis of the CA1 hippocampal region in APP/PS1 transgenic mouse model. Front. Aging Neurosci. 12, 577996.

Martín-Belmonte A., Aguado C., Alfaro-Ruiz R., Kulik A., de la Ossa L., Moreno-Martínez A. E., Alberquilla S., García-Carracedo L., Fernández M., Fajardo-Serrano A., Aso E., Shigemoto R., Martín E.D., Fukazawa Y., Ciruela F. and Luján R. (2024). Nano-architecture of $Ca_V2.1$ channels and $GABA_B$ receptors in the mouse hippocampus: Impact of APP/PS1 pathology. Brain Pathol. 35, e13279.

Martínez-Hernández J., Ballesteros-Merino C., Fernández-Alacid L., Nicolau J.C., Aguado C. and Luján R. (2013). Polarised localisation of the voltage-gated sodium channel $Na_V1.2$ in cerebellar granule cells. Cerebellum 12, 16-26.

Müller C.S., Haupt A., Bildl W., Schindler J., Knaus H.G., Meissner M., Rammner B., Striessnig J., Flockerzi V., Fakler B. and Schulte U. (2010). Quantitative proteomics of the Ca_V2 channel nanoenvironments in the mammalian brain. Proc. Natl. Acad. Sci. USA 107, 14950-14957.

Ostrovskaya O., Xie K., Masuho I., Fajardo-Serrano A., Lujan R., Wickman K. and Martemyanov K.A. (2014). RGS7/Gβ5/R7BP complex regulates synaptic plasticity and memory by modulating hippocampal $GABA_BR$-GIRK signaling. Elife 3, e02053.

Ostrovskaya O.I., Orlandi C., Fajardo-Serrano A., Young S.M., Lujan R. and Martemyanov K.A. (2018). Inhibitory signaling to ion channels in hippocampal neurons is differentially regulated by alternative macromolecular complexes of RGS7. J. Neurosci. 38, 10002-10015.

Padgett C.L. and Slesinger P.A. (2010). $GABA_B$ receptor coupling to G-proteins and ion channels. Adv. Pharmacol. 58, 123-147.

Pickard L., Noel J., Henley J.M., Collingridge G.L. and Molnar E. (2000). Developmental changes in synaptic AMPA and NMDA receptor distribution and AMPA receptor subunit composition in living hippocampal neurons. J. Neurosci. 20, 7922-7931.

Posner B.A., Gilman A.G. and Harris B.A. (1999). Regulators of G protein signaling 6 and 7. Purification of complexes with Gβ5 and assessment of their effects on G protein-mediated signaling pathways. J. Biol. Chem. 274, 31087-31093.

Rebola N., Reva M., Kirizs T., Szoboszlay M., Lőrincz A., Moneron G., Nusser Z. and DiGregorio D.A. (2019). Distinct nanoscale calcium channel and synaptic vesicle topographies contribute to the diversity of synaptic function. Neuron 104, 693-710.e9.

Roldán-Sastre A., Aguado C., Martín-Belmonte A., Alfaro-Ruiz R., Moreno-Martínez A.E. and Luján R. (2021). Cellular diversity and differential subcellular localization of the G-Protein $Gα_o$ subunit in the mouse cerebellum. Front. Neuroanat. 15, 686279.

Rosenbaum D.M., Rasmussen S.G.F. and Kobilka B.K. (2009). The structure and function of G-protein-coupled receptors. Nature 459, 356-363.

Rosenberg S.S. and Spitzer N.C. (2011). Calcium signaling in neuronal development. Cold Spring Harb. Perspect. Biol. 3, 1-13.

Schuler V., Lüscher C., Blanchet C., Klix N., Sansig G., Klebs K., Schmutz M., Heid J., Gentry C., Urban L., Fox A., Spooren W., Jaton A.L., Vigouret J.M., Pozza M., Kelly P.H., Mosbacher J., Froestl W., Käslin E., Korn R., Bischoff S., Kaupmann K., Van Der Putten H. and Bettler B. (2001). Epilepsy, hyperalgesia, impaired memory, and loss of pre- and postsynaptic $GABA_B$ responses in mice lacking $GABA_{B1}$. Neuron 31, 47-58.

Schwenk J., Pérez-Garci E., Schneider A., Kollewe A., Gauthier-Kemper A., Fritzius T., Raveh A., Dinamarca M.C., Hanuschkin A., Bildl W., Klingauf J., Gassmann M., Schulte U., Bettler B. and Fakler B. (2016). Modular composition and dynamics of native $GABA_B$ receptors identified by high-resolution proteomics. Nat. Neurosci. 19, 233-242.

Smrcka A.V. (2008). G protein βγ subunits: Central mediators of G protein-coupled receptor signaling. Cell. Mol. Life Sci. 65, 2191-2214.

Syrovatkina V., Alegre K.O., Dey R. and Huang X.Y. (2016). Regulation, signaling, and physiological functions of G-Proteins. J. Mol. Biol. 428, 3850-3868.

Takahashi T., Kajikawa Y. and Tsujimoto T. (1998). G-protein-coupled

modulation of presynaptic calcium currents and transmitter release by a $GABA_B$ receptor. J. Neurosci. 18, 3138-3146.

Tang X., Jaenisch R. and Sur M. (2021). The role of GABAergic signalling in neurodevelopmental disorders. Nat. Rev. Neurosci. 22, 290-307.

Tönnes J., Stierli B., Cerletti C., Behrmann T., Molnár E. and Streit P. (1999). Regional distribution and developmental changes of GluR1-flop protein revealed by monoclonal antibody in rat brain. J. Neurochem. 73, 2195-2205.

Turgeon S.M. and Albin R.L. (1994). Postnatal ontogeny of $GABA_B$ binding in rat brain. Neuroscience 62, 601-613.

Vigot R., Barbieri S., Bräuner-Osborne H., Turecek R., Shigemoto R., Zhang Y., Luján R., Jacobson L.H., Biermann B., Fritschy J., Vacher C.-M., Müller M., Sansig G., Guetg N., Cryan J.F., Kaupmann K., Gassmann M., Oertner T.G. and Bettler B. (2006). Differential compartmentalization and distinct functions of $GABA_B$ receptor variants. Neuron 50, 589-601.

Vlachou S. (2022). $GABA_B$ receptors and cognitive processing in health and disease. Curr. Top. Behav. Neurosci. 52, 291-329.

Wettschureck N. and Offermanns S. (2005). Mammalian G proteins and their cell type specific functions. Physiol. Rev. 85, 1159-1204.

White J.H., Wise A., Main M.J., Green A., Fraser N.J., Disney G.H., Barnes A.A., Emson P., Foord S.M. and Marshall F.H. (1998). Heterodimerization is required for the formation of a functional $GABA_B$ receptor. Nature 396, 679-682.

Xie K., Ge S., Collins V.E., Haynes C.L., Renner K.J., Meisel R.L., Lujan R. and Martemyanov K.A. (2012). Gβ5-RGS complexes are gatekeepers of hyperactivity involved in control of multiple neurotransmitter systems. Psychopharmacology 219, 823-834.

Zamponi G.W. and Currie K.P.M. (2013). Regulation of Ca_V2 calcium channels by G protein coupled receptors. Biochim. Biophys. Acta 1828, 1629-1643.

Accepted July 18, 2025