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# GABA $\rho$ subunits confer a bicuculline-insensitive component to GFAP<sup>+</sup> cells of cerebellum

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**GABA-A receptors mediating synaptic or extrasynaptic transmission are molecularly and functionally distinct, and glial cells are known to express a plethora of GABA-A subunits. Here we demonstrate that GFAP<sup>+</sup> cells of the granular layer of cerebellum express GABA $\rho$  subunits during early postnatal development, thereby conferring peculiar pharmacologic characteristics to GABA responses. Electron microscopy revealed the presence of GABA $\rho$  in the plasma membrane of GFAP<sup>+</sup> cells. In contrast, expression in the adult was restricted to Purkinje neurons and a subset of ependymal cells. Electrophysiological studies *in vitro* revealed that astrocytes express functional receptors with an EC<sub>50</sub> of 52.2 ± 11.8 μM for GABA. The evoked currents were inhibited by bicuculline (100 μM) and TPMPA (IC<sub>50</sub>, 5.9 ± 0.6 μM), indicating the presence of a GABA $\rho$  component. Coimmunoprecipitation demonstrated protein–protein interactions between GABA $\rho$ 1 and GABA $\alpha$ 1, and double immunofluorescence showed that these subunits colocalize in the plasma membrane. Three populations of GABA-A receptors in astrocytes were identified: classic GABA-A, bicuculline-insensitive GABA $\rho$ , and GABA-A–GABA $\rho$  hybrids. Clusters of GABA-A receptors were distributed in the perinuclear space and along the processes of GFAP<sup>+</sup> cells. Time-lapse microscopy showed GABA $\rho$ 2-GFP accumulation in clusters located in the soma and along the processes. The clusters were relatively immobile, with mean displacement of 9.4 ± 0.9 μm and a net distance traveled of 1–2 μm, owing mainly to directional movement or simple diffusion. Modulation of GABA $\rho$  dynamics may be a novel mechanism of extrasynaptic transmission regulating GABAergic control of GFAP<sup>+</sup> cells during early postnatal development.**

astrocytes | cerebellum | GABA-A receptor | GABA $\rho$  receptor | protein trafficking

The role of GABAergic signaling is fundamental in the cerebellum, not only for influencing cell differentiation and neurotransmitter specification during early postnatal development, but also for controlling precise movements in the adult life (1–3). The expression of ionotropic GABA-A receptors with high affinity for the neurotransmitter is now well recognized, although the source of GABA involved in this process is controversial (4–6).

GABA-A receptors mediating synaptic (phasic) or extrasynaptic (tonic) transmission are molecularly and functionally distinct. In contrast to neurons, the depolarizing effect of astrocytic GABA-A receptors persists through postnatal development, although the response may attenuate with age (7). In cerebellar astrocytes, the array of GABA-A subunits is heterogeneous, and modulation by benzodiazepines is different from that by neurons (8). Indeed, GABA responses of Bergmann cells and ependymal glial cells (EGCs) are insensitive to benzodiazepines or pentobarbital, owing to the assembly of receptors that include GABA $\delta$  or GABA $\rho$  subunits (9, 10).

GABA $\rho$  subunits are part of the ionotropic GABA-A receptor family, which includes 19 identified genes that code for the same number of known proteins:  $\alpha$ 1– $\alpha$ 6,  $\beta$ 1– $\beta$ 3,  $\gamma$ 1– $\gamma$ 3,  $\delta$ ,  $\epsilon$ ,  $\Theta$ ,  $\pi$ , and  $\rho$ 1– $\rho$ 3 (11). GABA-A receptors are pentameric heterocomplexes composed of a combination of subunits, most commonly the

$\alpha$ 1 $\beta$ 2 $\gamma$ 2 combination, that gate a Cl<sup>−</sup> channel on activation (11, 12). Other arrays may include  $\delta$ ,  $\rho$ , or  $\epsilon$  subunits, which confer distinctive functional and pharmacologic properties. GABA $\rho$  subunits can combine in homopentameric arrangements that form receptors with high affinity for the neurotransmitter (GABA EC<sub>50</sub>, 1–5 μM) and a low rate of desensitization, making them suitable for tonic transmission (11, 13, 14). GABA $\rho$  subunits are known to be expressed in the retina, where their presence in bipolar neurons controls the glutamatergic output (15, 16); they are present in other areas of the central nervous system as well, including striatum, hippocampus, and cerebellum, but their function there is not fully understood (17–19).

The role of GABA $\rho$  in neuronal tonic (extrasynaptic) and phasic (synaptic) transmission has been demonstrated in the Purkinje neurons of the cerebellum (20); however, GABA $\rho$  subunits are also expressed in a large fraction of EGCs, specialized, ciliated GFAP<sup>+</sup> cells that permit the flow of cerebrospinal fluid circulating in the fourth ventricle (10, 21). GABA-ionic currents in these cells are insensitive to pentobarbital and partially blocked by the GABA-A antagonist bicuculline as well as by (1,2,5,6-Tetrahydropyridin-4-yl)methylphosphonic acid (TPMPA), the first synthesized GABA $\rho$  antagonist (22). GABA-A receptors that include GABA $\rho$  subunits are also expressed in approximately 30% of the GFAP<sup>+</sup> cells present in the striatum (19). Thus, it seems that cells of glial origin, such as EGCs and astrocytes, present a diverse array of ionotropic GABA receptors that include GABA $\rho$  subunits and whose functional role remains unidentified.

In the course of recent work on assessing the presence of GABA $\rho$  subunits during early postnatal development of cerebellar

## Significance

**Early postnatal development of the cerebellum involves a number of events that require signaling via the neurotransmitter GABA, which acts on specific receptors anchored in the plasma membrane. GABAergic transmission regulates the proliferation and migration of neuronal precursors of astrocytic lineage. Glial cells are known to express GABA-A receptors that include GABA $\rho$  subunits, but their expression pattern, functional properties, and trafficking dynamics remain unknown. This study found that a large number of glial cells express GABA $\rho$  in the cerebellum. Functional properties and intracellular trafficking of GABA-A receptors in glial cells grown *in vitro* suggest a different mechanism of GABAergic control of extrasynaptic transmission.**

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EGCs, we corroborated their presence in this area but, unexpectedly, found that they are also widely distributed in a large proportion of GFAP<sup>+</sup> cells of the granular layer (GL) that appear to be astrocytes. In this paper we report in detail the expression pattern of GABA $\rho$  subunits in GFAP<sup>+</sup> cells of the cerebellum, the functional characterization of GABA responses of cerebellar astrocytes grown in vitro, and the participation of a GABA $\rho$  component in these responses. In addition, we provide evidence of the intracellular trafficking of GABA $\rho$  in astrocytes grown in vitro, and speculate about the possible synthesis of proteins in the processes of these cells.

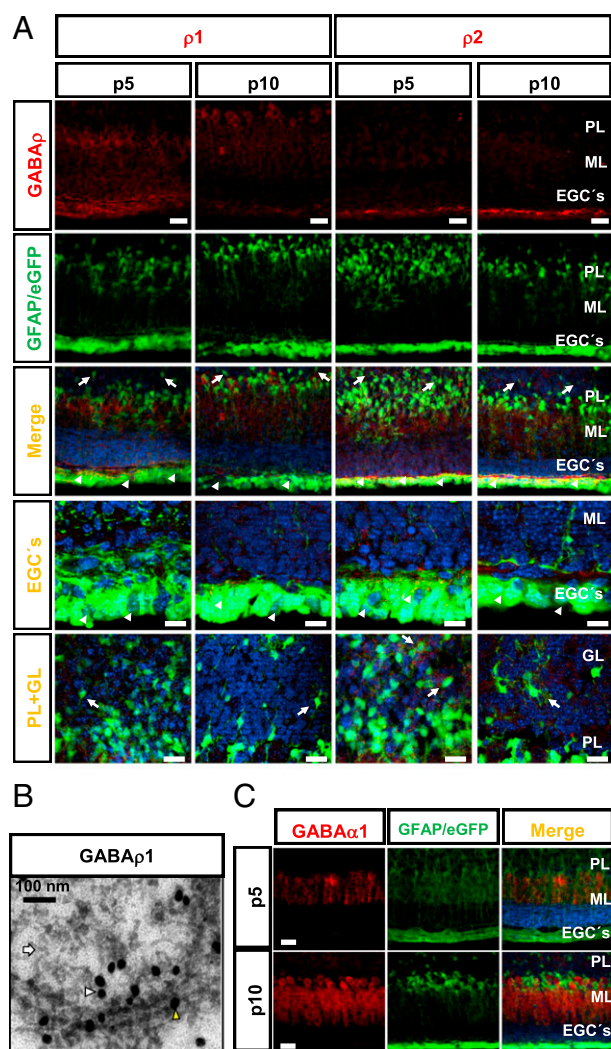
## Results

**GABA-A Receptors in Cerebellar GFAP<sup>+</sup> Cells.** Previous findings indicated that GABA $\rho$  subunits are expressed in EGCs of the cerebellum, in the zone facing the fourth ventricle (10) (Fig. S1A), which encouraged us to analyze the expression of these subunits during earlier stages of development in more depth. RT-PCR revealed GABA $\rho$ 1, GABA $\rho$ 2, and GABA- $\alpha$ 1 expression at postnatal day (P) 5, P10, and P30 (Fig. S1B). Unexpectedly, immunofluorescence revealed the presence of GABA $\rho$ 1 and GABA $\rho$ 2 in most, but not all, GFAP<sup>+</sup> cells of the subventricular zone (Fig. 1A, rows three and four, arrowheads) and GL (Fig. 1A, rows three and five, arrows). The proportion of GFAP<sup>+</sup> cells expressing GABA $\rho$ 1 was larger at P5 than at P10, in contrast to the expression of GABA $\rho$ 2, which was more abundant at P10 (Fig. S2 A and B, columns one and two). Nevertheless, expression of GABA $\rho$  subunits decreased during the development in GFAP<sup>+</sup> cells, such that in the adult it was limited to a few end feet of Bergmann glial cells and EGCs (Fig. S2 A and B, column three).

At P5 and P10, between 20% and 30% of GFAP<sup>+</sup> cells of the GL were labeled with selective GABA $\rho$ 1 and GABA $\rho$ 2 antibodies (Fig. S3C). At P5, the GABA $\rho$ 1 and GABA $\rho$ 2 signals were located in soma and processes of GFAP cells, whereas at P10, the label was redistributed to the periphery of these cells (Fig. S3 A and B). We used an immunogold assay and transmission electron microscopy to gain insight into the location of this receptor. Evaluation of several images each from three independent experiments revealed the presence of GABA $\rho$ 1 at the plasma membrane of cells distinguished by their internal filamentous structures, criteria that unequivocally distinguish astrocytes under electron microscopy (23). These cells were distributed in the GL, and the label was observed along many processes and was associated with intracellular filaments; an example of these cells is shown in Fig. 1B.

Expression of GABA $\alpha$ 1 is crucial for proper GABAergic neurotransmission in the cerebellum (24), and this subunit is known to be present mainly in the adult neurons with scattered expression in GFAP<sup>+</sup> cells (10). At P5 and P10, immunolabeling for GABA $\alpha$ 1 was present mainly in non-GFAP<sup>+</sup> cells, although Bergmann glial processes in the close vicinity of Purkinje cells exhibited limited immunolabeling at P5 (Fig. 1C, upper row), but not at P10 (Fig. 1C, lower row).

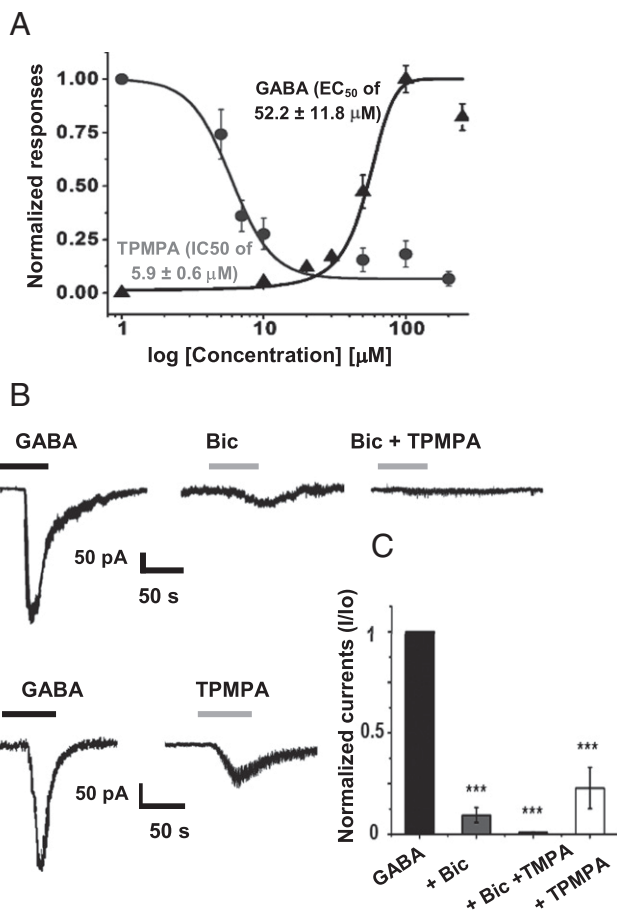
**Cerebellar Ionotropic GABA Receptors Include a GABA $\rho$  Component.** After identifying in situ GABA-A receptors that include a GABA $\rho$  component, we cultured astrocytes to study the functional and pharmacologic characteristics of the receptor and to assess under more controlled conditions the intracellular trafficking of the receptor. Cerebellar cells obtained from P5 mice and grown in vitro for 5 d were 91  $\pm$  7.5% GFAP<sup>+</sup> (mean  $\pm$  SE). The cells were divided into three populations according to morphology: flat cells (50  $\pm$  4.9%), flat polygonal cells (45  $\pm$  3.7%), and star-like cells (5  $\pm$  1.4%) (Fig. S4A). GABA currents from these GFAP<sup>+</sup> cells exhibited two components, one component that inactivates promptly ( $\tau_{\text{inac}} = 3.6 \pm 0.5$  s) and a slowly inactivating component ( $\tau_{\text{inac}} = 48.6 \pm 16.4$  s). GABA EC<sub>50</sub> was



**Fig. 1.** Expression of GABA-A receptor subunits in cerebellar GFAP<sup>+</sup> cells. (A) GABA $\rho$ 1 or GABA $\rho$ 2 (first row) at P5 and P10 in GFAP-eGFP transgenic mice (second row). The merged image shows that GFAP<sup>+</sup> cells (green) of the subventricular zone (arrowheads) and GL (arrows) express GABA $\rho$ 1 and GABA $\rho$ 2 (red). The last two columns show maximizations of EGCs and GFAP<sup>+</sup> cells of the GL expressing GABA $\rho$ . (Scale bars: 50  $\mu$ m.) (B) Ultrastructural location of GABA $\rho$ 1 in glial cells of the GL of cerebellum. The yellow arrowhead points to a gold particle labeling GABA $\rho$ 1 in the plasma membrane; the white arrowhead, to a GABA $\rho$ 1 label in submembranous structures. Astrocytes were identified by the presence of filamentous structures (white arrows). (Scale bar: 100 nm.) (C) Expression of GABA $\alpha$ 1 (red) at P5, but not at P10, is located in Bergmann glial processes (green).  $n = 3$ . ML, molecular layer; PL, Purkinje layer. (Scale bars: 50  $\mu$ m; 20  $\mu$ m for maximizations.)

52.2  $\pm$  11.8  $\mu$ M (Fig. 2A and Fig. S5A), and the GABA currents were partially blocked by 100  $\mu$ M bicuculline, which inhibited the GABA responses by 90.5  $\pm$  3.7% (Fig. 2B); these responses were totally abolished by a combination of 100  $\mu$ M bicuculline and 10  $\mu$ M TPMPA (Fig. 3B). Consecutive applications of 50  $\mu$ M GABA consistently reduced the responses; however, a residual nondesensitizing component remained in all of the cells recorded after five applications (Fig. S5B). This component could be attributed to the expression of GABA $\rho$  subunits. The desensitizing component reappeared after 28 min of wash perfusion (Fig. S5C). These results indicate that GABA-A receptors from astrocytes are functional and have a GABA $\rho$  component.

Application of 10  $\mu$ M TPMPA inhibited the GABA response by 81.1  $\pm$  5.9% (Fig. 3B), further supporting the presence of



**Fig. 2.** GABA responses of cerebellar astrocytes in culture. (A) GABA and TPMPA dose–response curves ( $n = 6$ ). (B) GABA responses (from left to right): Bicuculline  $100 \mu\text{M}$  inhibited the GABA response ( $90.5 \pm 3.7\%$ ), bicuculline  $100 \mu\text{M}$  plus TPMPA  $10 \mu\text{M}$  blocked 100% of the GABA response ( $n = 6$ ), and TPMPA  $10 \mu\text{M}$  inhibited the GABA response ( $81.1 \pm 5.9\%$ ) ( $n = 6$ ). (C) Dose–response relationship for GABA and TPMPA blockage. Quantitation of inhibition of GABA responses by bicuculline and GABA was significant.  $***P < 0.001$ , one-way ANOVA.

heterogeneous populations of ionotropic GABA receptors: “classic” GABA-A, a GABA $\rho$  component, and heteromeric GABA-A–GABA $\rho$  receptors. This hypothesis is partially supported by the unexpected sensitivity of the ionotropic GABA receptors to TPMPA ( $\text{IC}_{50}$ ,  $5.9 \pm 0.07 \mu\text{M}$ ) (Fig. 2A and Fig. S5C), which is close to that of homomeric GABA $\rho$  receptors ( $1\text{--}5 \mu\text{M}$ ) and far lower than that of classic GABA-A receptors ( $500 \mu\text{M}$ ) (11).

To test whether the peculiar responses to GABA found in astrocytes correspond to the expression of multiple heterogeneous GABA-A subunits, we confirmed the expression of GABA-A ( $\alpha 1$  and  $\gamma 2$ ), GABA $\rho 1$ , and GABA $\rho 2$  by RT-PCR and Western blot analysis (Fig. 3A and B). In addition, immunolabeling revealed the presence of GABA $\alpha 1$  in  $78 \pm 2.5\%$  of GFAP $^+$  cells, GABA $\gamma 2$  in  $93 \pm 1.2\%$ , GABA $\rho 1$  in  $60.0 \pm 6.8\%$ , and GABA $\rho 2$  in  $70.0 \pm 4.9\%$ . The receptor subunits were seen to accumulate in the soma and to have a punctate distribution along the processes (Fig. 3C). Clustering and distribution of GABA $\alpha 1$ , GABA $\rho 1$ , and GABA $\rho 2$  were similar in the three GFAP $^+$  cell morphologies, whereas GABA $\gamma 2$  was more likely to accumulate in fewer clusters in the plasma membrane around the nucleus (Fig. S4B and C).

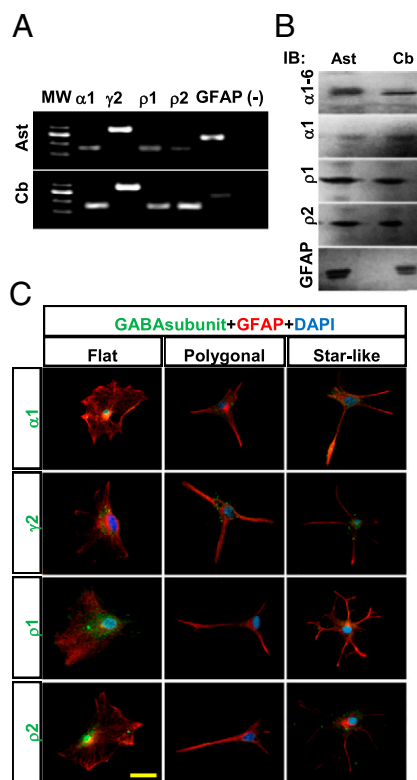
**GABA $\rho 1$  Associates with GABA $\alpha 1$ .** Although the existence of heteromeric GABA-A–GABA $\rho$  receptors is indicated by their current kinetics, hybrid pharmacologic properties, and similar

distribution in astrocytes in culture, we still needed to demonstrate their physical interaction. For this, we used an antibody selective for GABA $\rho 1$  to assess the potential interactions with GABA $\alpha$  and GABA $\rho 1$  by coimmunoprecipitation (Fig. 4A). The results indicate that in membranes of GFAP $^+$  cells, an anti-GABA $\rho 1$  pulls down at least one GABA-A $\alpha$  subunit, given that anti-GABA $\alpha 1\text{--}6$  and anti-GABA $\alpha 1$  were detected in the immunoprecipitate. In addition, GABA $\rho 2$  was recognized by a selective antibody, suggesting that the GABA $\rho 1$ –GABA $\rho 2$  interaction that occurs in the retina (25, 26). These interactions were demonstrated in membranes isolated from cerebellum as well (Fig. 4A).

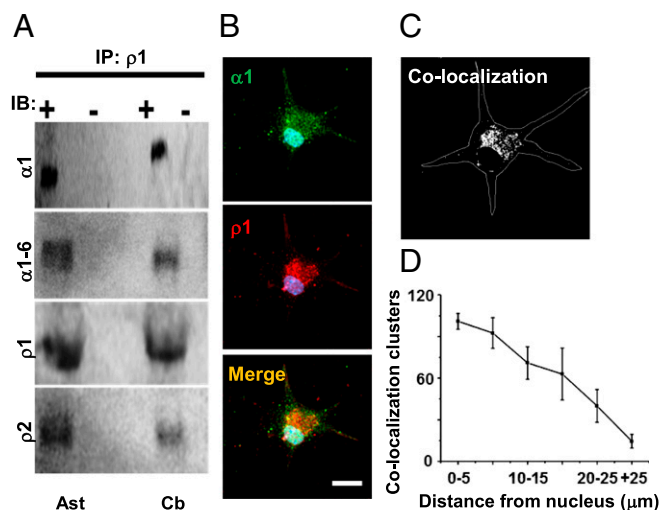
As shown in Fig. 3, most of the immunolabeling for GABA $\rho 1$  and GABA-A $\alpha 1$  in cultured astrocytes was distributed similarly. To determine the spatial colocalization of these subunits in astrocytes, we used double immunofluorescence analysis of astrocytes in culture. Fig. 4B and C shows a sample astrocyte in which we used antibodies that recognize the extracellular domain of GABA $\rho 1$  or GABA-A $\alpha 1$ . As indicated, these subunits were detected in the area surrounding the soma ( $101 \pm 5.7$  clusters;  $n = 20$ ) and, to a lesser extent, in distal processes ( $14.5 \pm 5$  clusters;  $n = 20$ ) (Fig. 4D).

The foregoing evidence supports the conclusion that cerebellar astrocytes in culture express functional GABA-A receptors, and that some of these receptors are composed of GABA $\rho$  subunits that interact with classic GABA-A subunits, such as GABA $\alpha 1$  and GABA $\rho 2$ .

**Distribution and Trafficking of GABA $\rho 2$ .** It was previously shown that in astrocytes grown in vitro, GABA $\rho 2$  is located in soma and



**Fig. 3.** GABA-A receptors in cerebellar astrocytes in culture and in cerebellum. (A) Expression of mRNA for GABA $\alpha 1$ , GABA $\gamma 2$ , GABA $\rho 1$ , GABA $\rho 2$ , and GFAP in cerebellar astrocytes in culture (Ast) and in whole cerebellum (Cb). (B) Western blot analysis for GABA $\alpha 1\text{--}6$ , GABA $\alpha 1$ , GABA $\rho 1$ , GABA $\rho 2$ , and GFAP in extracted membranes from astrocytes (Ast) and whole cerebellum (Cb). (C) Distribution of GABA $\alpha 1$ , GABA $\gamma 2$ , GABA $\rho 1$ , and GABA $\rho 2$  subunits (green) in GFAP $^+$  cells (red) with different morphologies. Nuclei were counterstained with DAPI (blue).  $n = 3$ . (Scale bar:  $20 \mu\text{m}$ .)



**Fig. 4.** Protein–protein interaction between GABA $\rho$ 1 and GABA $\alpha$  subunits. (A) Coimmunoprecipitation using antibody against GABA $\rho$ 1 showing interactions among GABA $\alpha_{1-\epsilon}$ -GABA $\rho$ 1; GABA $\alpha_1$ -GABA $\rho$ 1, and GABA $\rho$ 1-GABA $\rho$ 2 subunits in cerebellar astrocytes (Ast) in culture and in adult cerebellum (Cb). Membrane proteins without anti-GABA $\rho$ 1 were negative. (B) Immunolocalization of GABA-A $\alpha_1$  (green) and GABA $\rho$ 1 (red). Merged yellow clusters indicate colocalization of GABA $\alpha_1$  and GABA $\rho$ 1. (C) Fluorescence from clusters colabeled for GABA-A $\alpha_1$  and GABA $\rho$ 1 converged mainly at the plasma membrane around the soma, and to a lesser extent in the distal processes of GFAP $^+$  cells in culture (450 clusters from five cells). (D) Quantification of colabeled clusters.

proximal processes (27). In the present study, we reexamined the distribution of this receptor subunit in astrocytes with all three morphologies and corroborated those findings. In 40 cells, the chimeric receptors were located mainly in the soma ( $42 \pm 4.9$  clusters) and to some extent in distal processes ( $8 \pm 2$  clusters). The previous study also found that GABA $\rho$ 2 clusters remain stationary for long periods, which does not explain their presence in terminal processes of astrocytes either in culture or in situ. Thus, we examined the trajectories of GABA $\rho$ 2 tagged with GFP expressed by adenoviral transduction (Fig. S6).

Receptor clusters displayed a mean cumulative displacement of  $9.4 \pm 0.9 \mu\text{m}$  over 30 min, but remained practically stationary, with a net distance traveled of only  $1.5 \pm 0.23 \mu\text{m}$  (Fig. 5B). No significant differences were observed among cells with different morphologies. A plot of the first 10 points of mean squared displacement (MSD) revealed slow and limited, directed movement by polygonal and flat astrocytes, as illustrated by the exponential fitting of the MSD curves (Fig. 5C). In contrast, MSD curves in star-like astrocytes exhibited a strong linear correlation, suggesting that GABA $\rho$ 2 is transported by means of free diffusion (Fig. 5C). Thus, it appears that transport of GABA $\rho$ 2 is not consistent with classic vesicular traffic and may be explained by local synthesis of proteins that gives rise to clustered receptors in the astrocyte processes. Alternatively, the size of the vesicles that transport the receptor might be smaller than the resolution limit of the epifluorescence microscope ( $\sim 200$  nm in the lateral direction and  $\sim 600$  nm in the axial direction) (28).

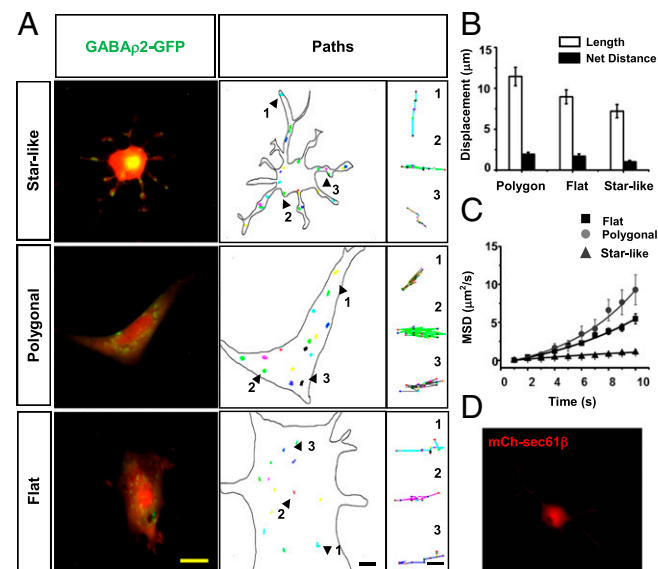
To support the first view, we labeled the endoplasmic reticulum (ER) of astrocytes by transiently expressing the protein Sec61 $\beta$  tagged with mCherry. Sec61 $\beta$  is a major component of the translocation apparatus for proteins in the ER (29). Fluorescence was consistently detected around the nucleus of astrocytes in culture, but—important for test of our suggestion—many fluorescent clusters were observed in the processes of astrocytes (Fig. 5D), indicating the presence of ER, the cell compartment containing the ribosomes on which protein synthesis occurs.

## Discussion

The periventricular layer of the roof of the fourth ventricle is formed by ependymal cells of glial origin. These cells generate GABA currents on exogenous administration of GABA owing to the presence of an array of GABA-A subunits that include GABA $\rho$ . GABA responses are partially blocked by TPMPA or bicuculline and totally abolished by a combination of the two compounds (10). Here we present evidence that during postnatal development of the cerebellum, GABA $\rho$  subunits are widely expressed in EGCs and even in GFAP $^+$  cells of the GL and subventricular zone. Expression of GABA $\rho$  subunits is down-regulated in young mice, but limited to Purkinje neurons and a small fraction of GFAP $^+$  cells in adults.

The developmental pattern of GABA $\rho$  subunit localization partially agrees with earlier reports suggesting expression of GABA $\rho$ 1 and GABA $\rho$ 2 at low levels in rat Purkinje and Golgi neurons during early postnatal development (30, 31). Previous studies have unequivocally demonstrated the presence of other GABA-A subunits ( $\alpha_x$ ,  $\beta_x$ ,  $\gamma_x$ ) in immature neurons of the cerebellum, and the expression of this receptor is a fundamental element in proper formation of the synaptic circuitry (32–34). Glial cells are also part of the tripartite synapse, and GABA-A subunits have been identified in Bergmann glia ( $\alpha_2$ ,  $\gamma_1$ ,  $\delta$ ) and EGCs ( $\alpha_1$ ,  $\rho_1$ ) (9, 10, 35), where they might play a key role as extrasynaptic sensors of GABAergic tone, similar to what occurs in astrocyte-like GFAP $^+$  progenitors (36). GABA-A receptors found in cerebellar astrocytes include a bicuculline-resistant nondesensitizing component ( $\tau = 48.6 \pm 16.4$  s), the result of the presence of GABA $\rho$  subunits, which are functionally suitable to control tonic transmission.

The detection of GABA $\rho$  subunits in glia (Fig. 1 and Figs. S1 and S2) should be attributed to the accurate identification of



**Fig. 5.** Distribution and trafficking of GABA $\rho$ 2-GFP. (A) Left to right columns: Fluorescence emitted by GABA $\rho$ 2-GFP (green) and soluble mCherry (red). GABA $\rho$ 2-GFP accumulates in clusters located in the soma and along the processes. Arrowheads and numbers indicate samples of isolated tracked paths of GABA $\rho$ 2-GFP. (Scale bars: 25, 5, and 1  $\mu\text{m}$ , respectively.) (B) Displacement length and net distance traveled of GABA $\rho$ 2-GFP did not differ significantly among astrocytes with different phenotypes. (C) MSD curves of GABA $\rho$ 2-GFP fluorescent clusters. Exponential correlation indicates directional movements in soma and processes of flat and polygonal cells, and linear correlation is evidence of simple diffusion in star-like cells (510 clusters from 40 cells). (D) Distribution of mCh-Sec61 $\beta$  in soma and distal processes. (Scale bar: 20  $\mu\text{m}$ .)

GFAP<sup>+</sup> cells of the transgenic mice used for immunofluorescence (37), a valuable tool that allowed differentiation of the labeled glial cells from other cell lineages. Although a role for the GABA<sub>A</sub> subunits expressed in GFAP<sup>+</sup> cells is not yet evident, these subunits reportedly participate in radial neuronal migration during brain cortex development (38). Immature neurons of the intermediate zone express a combination of classical GABA<sub>A</sub> subunits and GABA<sub>A</sub>, and it has been inferred that activation of the GABA<sub>A</sub> component provides a precise signal for the immature neurons to migrate toward the external zone of the cortex (38). GABA<sub>A</sub> may have a similar function in the developing cerebellum, considering that immature GABAergic neurons are known to transiently express GFAP during proliferation, and the possibility exists that the population of cells that we have identified as glia correspond to multipotent cells that will give rise to both interneurons and astrocytes (39).

We also examined the location of GABA<sub>A</sub>1 in GFAP<sup>+</sup> cells by immunogold assay and transmission electron microscopy, which indicated the presence of the receptors in the plasma membrane of glial cell processes. This position would render those cells with a potent detector for sensing environmental GABA, which is known to modulate important developmental events in the cerebellum and in the nervous system in general (40, 41).

Trafficking of GABA<sub>A</sub> receptors in neurons grown in vitro has been widely studied (42, 43). For example, receptor lateral diffusion from perisynaptic loci is known to be relevant for replenishing the receptor pool at inhibitory synapses; this mechanism is subtly dependent on the  $\alpha$  subunit that forms the pentameric heterocomplex and seems to be a mechanism of finely controlling GABAergic signaling (44, 45). In contrast, the role and trafficking of ionotropic GABA receptors in glia are not clearly understood. It is evident that hybrid GABA<sub>A</sub>-GABA<sub>A</sub> complexes are present in vitro and in vivo, and that the positions of the subunits partially converge in the soma of GFAP<sup>+</sup> cells in culture. Their dynamic insertion into the plasma membrane was not determined in the present study, but antibodies that detect an extracellular domain of the receptor reveal the presence of GABA<sub>A</sub>2 in the plasma membrane of soma and processes. In contrast, our analyses of the intracellular trajectories of GABA<sub>A</sub>2 tagged with GFP suggest that the mobility of this subunit is limited, and that differences exist among astrocyte phenotypes. In flat and polygonal cells the receptor movements are slow and directed, whereas in star-shaped cells, the receptors move by simple diffusion, similar to the trafficking of the cannabinoid receptor 1 in flat astrocytes grown in vitro (46).

The foregoing results suggest that temporal regulation of the expression of GABA<sub>A</sub> subunits could contribute to the precise regulation of glial development in the cerebellum. The presence of GABA<sub>A</sub> subunits either independently or in combination with other GABA<sub>A</sub> subunits confers even more complexity to GABAergic signaling during early postnatal life. Although GABA<sub>A</sub> receptors are known to reside in GFAP<sup>+</sup> cell processes, how they are delivered to the plasma membrane remains undetermined. Possibilities include transport of the receptors in vesicles smaller than the resolution limit and local synthesis of the receptors in the distal processes. Localization of ribosomal complexes and RNA in distal processes of astrocytes would support the latter possibility, which also seems likely because ER was detected in these processes in this and previous studies (47, 48).

## Methods

**Tissue Processing, Immunofluorescence, and Immunogold Assays.** All experiments were conducted in accordance with the guidelines of the National Institutes of Health's Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the Universidad Nacional Autónoma de México. For the cerebellum slice preparation, male GFAP-eGFP transgenic mice (37) at P5, P10, and P30 were processed as described previously (18). Astrocytes were grown in vitro (49),

and protein extracts and membrane preparations were obtained as reported previously (50). Antibodies were used for Western blot analysis and to locate the GABA<sub>A</sub> receptors in cerebellum and in cultured cerebellar astrocytes (51). The antibodies used in this study are listed in Table S1.

Double immunofluorescence was performed to determine GABA<sub>A</sub> subunit expression in GFAP<sup>+</sup> cells. The cells were counterstained with DAPI and then dehydrated and mounted in Vectashield (Vector Laboratories). Images were obtained using a Zeiss LSM510 Meta confocal microscope, with wavelengths of 561 nm for excitation of Alexa Fluor 594, 488 nm for excitation of Alexa Fluor 488 and eGFP, and 750 nm for excitation of DAPI. For image quantitative analysis, the z-stack images were processed in ImageJ analysis software (Colocalization and Analyzed Particles plug-ins).

Immunogold assays were performed as described previously (19) in P10 CD1 male mice. The primary antibody was 1:50 rabbit IgG anti-GABA<sub>A</sub>1 (Santa Cruz Biotechnology), and the secondary antibody was 1:80, 25 nm colloidal gold-conjugated, goat anti-rabbit IgG (25116; Electron Microscopy Sciences). The samples were observed with a JEOL JEM-1010 transmission electron microscope at 80 kV.

**RT-PCR.** RNA was isolated from P5, P10, and P30 cerebellum and from cerebellar astrocytes in culture (5 d in vitro) using TRIzol reagent (Invitrogen), following the manufacturer's instructions. Gene-specific primers designed for the GABA subunits GABA $\alpha$ 1, GABA $\gamma$ 2, GABA<sub>A</sub>1, GABA<sub>A</sub>2, and GFAP are listed in Table S2.

**Electrophysiological Recordings.** Electrophysiological recordings of astrocytes in culture were described previously (49). Once the glial identity was verified, the membrane potential was held at  $-60$  mV, and GABA was applied for 30 s. Concentration-response and desensitization curves were determined using application intervals of 7 min. Antagonism of responses elicited by 50  $\mu$ M GABA was studied by preincubating and coapplying the GABA<sub>A</sub> receptor antagonist bicuculline (100  $\mu$ M) or the GABA<sub>A</sub>-selective antagonist TPMPA (10  $\mu$ M). Finally, dose-response curves for TPMPA were constructed to determine the potency of the drug. Stock solutions of GABA, bicuculline (Sigma-Aldrich), and TPMPA (TOCRIS) were prepared according to the manufacturer's instructions and then diluted in extracellular bath solution.

**Coimmunoprecipitation.** Assays were performed on membranes isolated from either P10 cerebella or astrocytes in culture (5 d in vitro) using a goat primary anti-GABA<sub>A</sub>1 antibody, following the basic protocol reported earlier (20). G-Sepharose was added to the mix for 4 h at 4 °C. To remove the resin, samples were centrifuged at 9,500  $\times$  g for 10 min at 4 °C, the supernatant was removed, and the pellet was washed and centrifuged three times at 9,500  $\times$  g for 5 min. Then 15  $\mu$ L of loading buffer was added, and the samples were denatured by heating for 5 min at 95 °C. Before PAGE, the samples were centrifuged at 9,500  $\times$  g for 3 min and electroblotted onto PVDF membranes; proteins were detected as described previously (20).

**Expression of Fluorescent Proteins in GFAP<sup>+</sup> Cells in Culture and Analyses of Receptor Trajectories.** Trafficking of GABA<sub>A</sub>2 was assessed using AdGABA<sub>A</sub>2-GFP (27), and cell morphology was highlighted using AdmCherry (6.4  $\times$  10<sup>6</sup> pfu) (Vector Biolabs). Fluorescent clusters were tracked under an inverted epifluorescence microscope (Olympus CKX41, objective: LCAch 40 $\times$ /0.55 Ph), and images were obtained at 0.017 Hz at room temperature. The distribution and dynamics of fluorescent particles were analyzed with ImageJ (Analyze Particles and MTrackJ plug-ins), and the motility of fluorescent clusters and MSD were determined and plotted in Origin 8 Pro (46).

For localizing ER in astrocytes in culture, the plasmid mChe-Sec61 $\beta$  (49155; Addgene) (52) was transfected using Lipofectamine PLUS reagent (Life Technologies), and the distribution of the fluorescent protein was observed 48–72 h later (53).

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