Amino Acids (2004) 26: 77–84
DOI 10.1007/s00726-003-0006-z

Amino Acids
Printed in Austria

Immunocytochemical study by two photon fluorescence microscopy of the distribution of $GABA_A$ receptor subunits in rat cerebellar granule cells in culture

F. Merlo [1], **R. Balduzzi** [1], **A. Cupello** [2], and **M. Robello** [1]

[1] INFM, Dipartimento di Fisica, Università di Genova, Genova, Italy
[2] Istituto di Bioimmagini e Fisiologia Molecolare, CNR, Sezione di Genova, Genova, Italy

Received January 15, 2003
Accepted March 3, 2003
Published online May 28, 2003; © Springer-Verlag 2003

Summary. An immunocytochemical investigation of the expression of α_1, α_6, $\beta_{2/3}$, γ_2 and δ subunits was performed on rat cerebellum granule cells in culture by the two photon microscopy technique.

The first four subunits appear to be expressed abundantly in these cells, whereas the δ one seems to be expressed at a lower level. Another major difference in the distribution of these subunits is that whereas α_6, $\beta_{2/3}$ and γ_2 appear only on plasma membranes α_1 and δ are present mainly in the cell bodies cytoplasm. Still another difference was found in that the presence of γ_2 on neurites is "polarized", preferentially labelling neurites with the appearance of dendrites. The subunits α_6 and $\beta_{2/3}$ appear to label all types of neurites, with $\beta_{2/3}$ being by far the most heavily expressed subunit type. A final distinct characteristic is that α_6 and, even more, γ_2 appear to accumulate in the cytoplasmic domains immediately below the cone of emergence of neurites. This suggests a conspicuous transport of such subunits from the site of synthesis in the cell body to the site of final expression in the neurites (dendrites and axon terminals).

Keywords: Fluorescence microscopy – Immunocytochemistry – $GABA_A$ receptors – Subunits – Cerebellar granules – *In vitro*

Introduction

The cerebellar granule layer has a pivotal role in receiving and elaborating the afferent inputs to the cerebellum. The granule cells are excited by the mossy fiber inputs to their dendrites at the glomerular complexes and the excitation of their dendrites is controlled by GABA-ergic inhibition by Golgi cells (Kandel et al., 2000). The inhibitory GABA-ergic control of the granule cells activity is powerful and involves two types of $GABA_A$ inhibitions, a phasic one by means of the GABA-ergic synaptic input to the granule cells dendrites and a tonic one involving extrasynaptic $GABA_A$ receptors (Brickley et al., 1996; Nusser et al., 1998; Cupello et al., 2000). These two types of GABA mediated inhibitions are due to different types of $GABA_A$ receptors with different subunit compositions and therefore with different physiological and pharmacological characteristics (Nusser et al., 1998; Cupello et al., 2000; Robello et al., 1999). The endeavour to characterize the subunit composition of the different populations of $GABA_A$ receptors of cerebellar granules is thus of particular interest. Both *in situ* hybridization studies of messenger RNA's (Laurie et al., 1992) and more recent immunocytochemical investigations of the subunits (Pirker et al., 2000) have indicated α_1, α_6, $\beta_{2/3}$, γ_2 and δ as the main $GABA_A$ receptors subunits expressed in the cerebellar granules of the adult rat. Referring to the neonatal rat granule cells grown in culture, although many studies have been performed on the expression of the subunits' mRNA's (Bovolin et al., 1992a; Bovolin et al., 1992b; Beattie et al., 1993; Mathews et al., 1994; Santi et al., 1994), relatively few papers have dealt with the immunocytochemical localization of the $GABA_A$ receptors subunits of the granule cells (Caruncho et al., 1995; Gao et al., 1995).

In this report we present our results of an immunocytochemical study about the subcellular distribution in cerebellar granules in culture of the $GABA_A$ receptors subunits which have been described as the more expressed in this cell type (α_1, α_6, $\beta_{2/3}$, γ_2 and δ). This study has been performed with the sensitive technique of two photons fluorescence microscopy.

Materials and methods

Antibodies

Affinity purified polyclonal antisera from rabbit against the $GABA_A$ receptor α_1 and α_6 subunits were purchased from Chemicon International, Temecula, CA. According to the manufacturer, they were raised, respectively, against peptide residues 1–16 of rat or mouse α_1 subunit and against a synthetic peptide corresponding to amino acids 1–16 of bovine $GABA_A$ receptor α_6 subunit. From Chemicon International was also the rabbit anti-γ_2 subunit polyclonal antibody. This was against a synthetic peptide from the N-terminus of rat $GABA_A$ receptor γ_2 subunit.

The antibody against the $\beta_{2/3}$ subunit was the mouse monoclonal antibody bd-17, purchased from Boehringer Mannheim, Germany. Finally, we used an affinity purified goat polyclonal antibody raised against a peptide mapping at the carboxy terminus of human $GABA_A$ receptor δ subunit. This antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

Alexa Fluor 488 goat anti-rabbit IgG (H + L), Oregon Green™ 488 goat anti mouse IgG and Alexa Fluor® 350 donkey anti-goat IgG labelled antibodies were obtained from Molecular Probes, Inc., Leiden, The Netherlands.

Granule cells cultures

Granule cells were prepared from cerebella of 7 days old Sprague-Dawley rats following the procedure of Levi et al. (1984). In brief, the tissue was minced and then suspended in trypsin (0.25 mg/ml, Type III Sigma) for 15 min at 37°C in a shaking water bath and then in deoxyribonuclease and trypsin inhibitor. Finally, the tissue was dispersed by gently drawing it into a fire-polished Pasteur pipette. Cells were resuspended in basal Eagle's medium with Earle's salts supplemented with 10% fetal calf serum (Gibco Bio-Cult, UK), 25 mM KCl, 2 mM glutamine and 100 μg/ml gentamicine and plated on poly-L-Lysine coated glass cover slips placed in 10 mm sterilized glass dishes at a density of 1×10^6 per dish and kept at 37°C in a humified 95% air-5% CO_2 atmosphere.

Immunolabelling

Seven days after having been put in culture, the cells were washed three times for 5 min each in PBS buffer (0.01 M, pH 7.4), fixed in 3.7% paraformaldehyde in the same buffer for 5 min at room temperature and washed again three times for 5 min each with PBS. Cells were permeabilized with 0.1% Triton X-100 in PBS + BSA (Bovin Serum Albumine) for 30 min and incubated overnight at 4°C with primary antibody:

- Polyclonal rabbit antibody against α_1 subunits (amino acids 1–16) of the rat $GABA_A$ receptor, concentration 1.5 μg/ml in 1% BSA in PBS;
- Polyclonal rabbit antibody against α_6 subunits (amino acids 1–16) of the $GABA_A$ receptor, concentration 1 μg/ml in 1% BSA in PBS;
- Rabbit anti-rat $GABA_A$ receptor γ_2 subunits (N-terminus) polyclonal antibody, concentration 1.3 μg/mL in 1% BSA in PBS;
- Mouse monoclonal antibody against $\beta_{2/3}$ subunits of $GABA_A$ receptor (clone bd 17), concentration 1.5 μg/ml in 1% BSA in PBS;
- Goat polyclonal antibody raised against the carboxy terminus of δ subunit of rat $GABA_A$ receptor, concentration 2 μg/ml in 1% BSA in PBS;

Cells were washed with 1% BSA in PBS three times for 10 min each and incubated for 1 h at 37°C with secondary antibodies, conjugated with dyes:

- Alexa Fluor 488 goat anti-rabbit IgG (H + L) (against α_1, α_6 and γ_2 subunits), concentration 2.5 μg/ml;
- Alexa Fluor® 350 Donkey anti-goat IgG antibody (against δ subunits), concentration 7.5 μg/ml;
- Oregon Green™ 488 goat anti-mouse IgG antibody (against $\beta_{2/3}$ subunits), concentration 10 μg/ml.

Cells were washed three times in PBS buffer for 10 min and mounted in glycerol/buffer (70/30). To exclude autofluorescence control cells were incubated with only primary or secondary antibodies.

Image acquisition

The granule cells were imaged using a two-photon laser scanning microscope Nikon PCM 2000 (Nikon Instuments, Florence, Italy) described in details elsewere (Diaspro et al., 2000). Alexa Fluor 488 and Oregon Green™ 488 were two-photon excited at 750 nm, by a Titanium:Sapphire infrared pulsed laser (Tsunami 3960, Spectra Physics, Mountain View, CA, USA); fluorescence was collected with a selective emission filter centred on 535 nm with a bandwith of 50 nm; Alexa Fluor® 350 was two photon excited at 700 nm and the fluorescence collected with a selective emission filter centred on 460 nm with a bandwidth of 50 nm. We used an oil immersion 100X objective; EZ 2000 (Coord, Amsterdam, NL) software was used for acquisition, storage and visualization.

For each sample, we divided the cell (thickness 10 μm) into 15 optical sections. For each subunit experiment we used at least 15 cells.

Results

First we observed that untreated cells and cells used as controls did not show fluorescence. Then we considered the distribution of fluorescence in granule cells marked with antibodies against the different subunits of $GABA_A$ receptor. Of the different antibodies used by us, the most thoroughly tested with the respective subunits are the monoclonal one against the $\beta_{2/3}$ subunits (bd 17) and the polyclonal one against α_1 subunit. However, also the other ones presented very specific reaction distributions (see results below), this appears to exclude unspecific cross-reactions.

In Fig. 1 we report two photon optical sections of a granule cell labelled with antibodies against α_1 subunit of $GABA_A$ receptor. The fluorescence is distributed homogeneously in the whole cell body and the signal has practically the same intensity in every section of the cell body (distance of 0.5 μm one from each other). This subunit appears more in the cytoplasm than on the plasma membrane. In other words, the conspicuous presence in the cytoplasm hides an accumulation on the plasma membrane. The neurites result partly marked, but there the fluorescence shows a lower intensity in comparison with the cell body.

Figure 2 shows the typical distribution of α_6 subunit of $GABA_A$ receptor in different focal planes (distance of 0.5 μm) of a granule cell. One can notice that α_6 subunit is localized mainly on plasma membranes of the cell body and of neurites, while fluorescence is very low in the cytoplasm. Processes are always intensely marked and their fluorescence is comparable to that of the cell body. In some cases the labelling is more concentrated at one of the cell poles and on the plasma membrane of the process

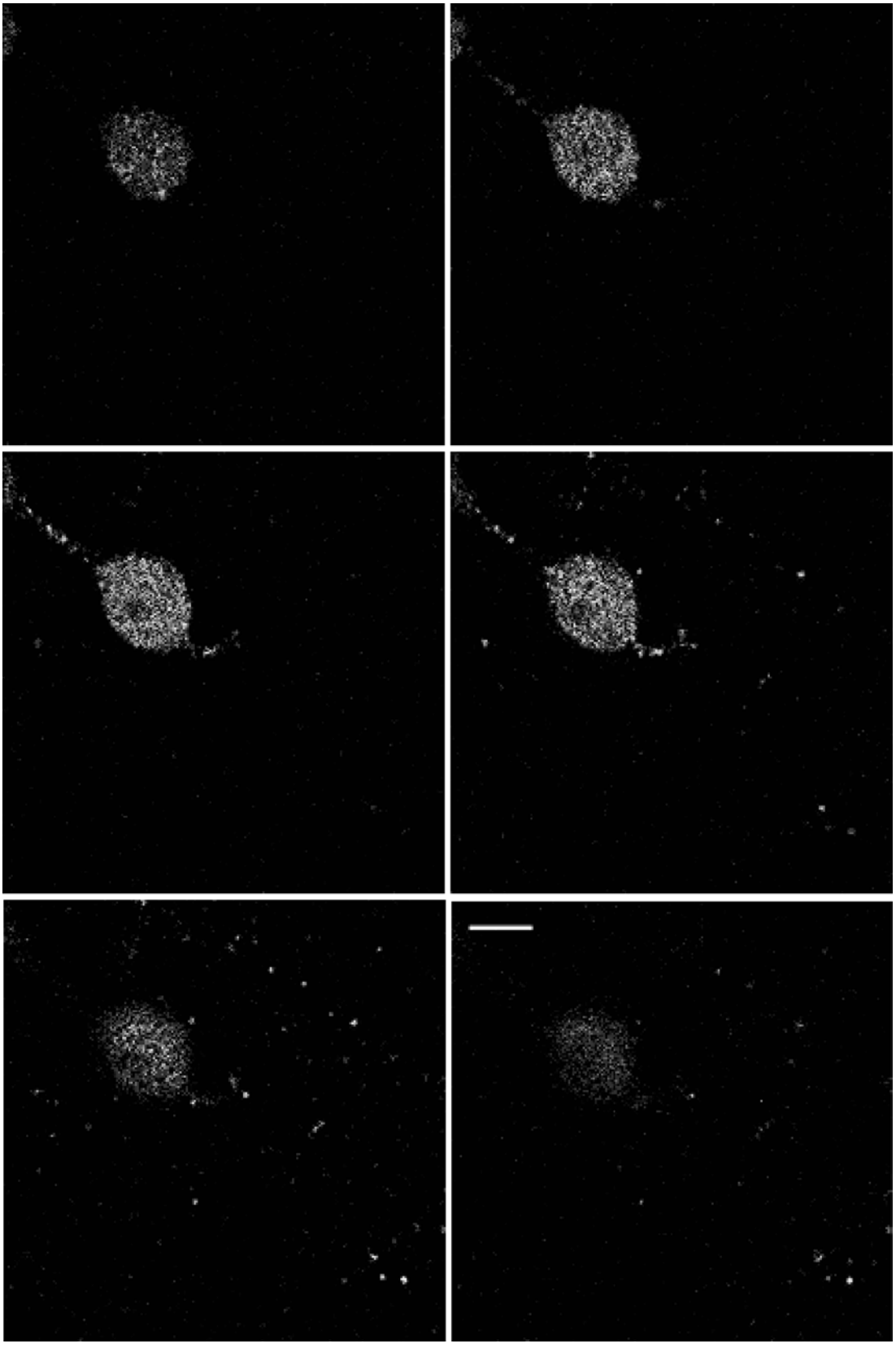

Fig. 1. Two photons optical sections showing $GABA_A$ receptor α_1 subunit distribution in a cerebellar granule cell from a 7 days old rat and kept 7 days *in vitro*. The various images represent six different focal planes at the distance of 0.5 μm one from the other. The bar represents a length of 5 μm

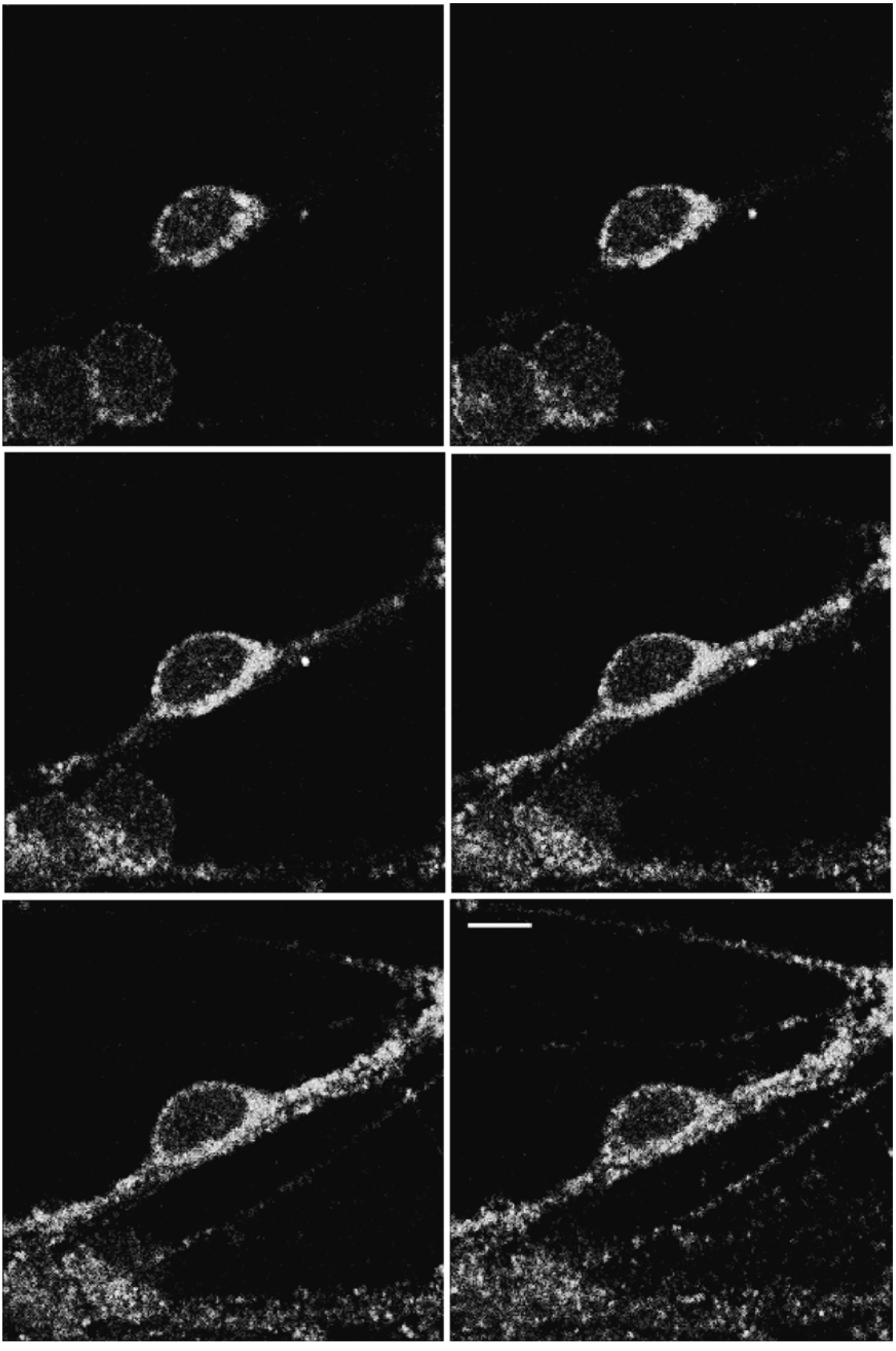

Fig. 2. Optical sections of a rat cerebellum granule cell marked with antibodies against the α_6 subunit of $GABA_A$ receptor. Bar $= 5\,\mu$m

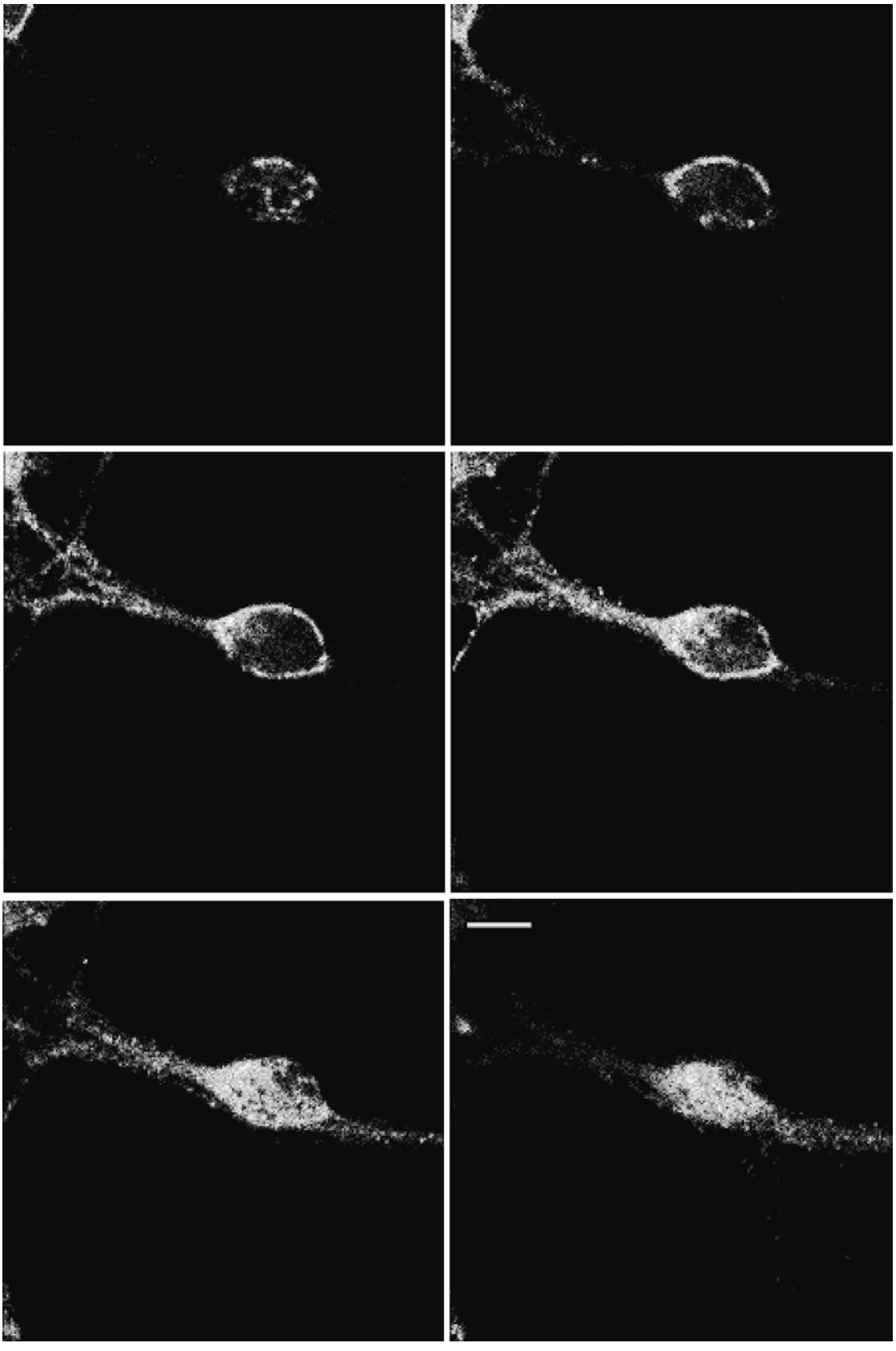

Fig. 3. Optical sections of a rat cerebellum granule cell marked with antibodies against the γ_2 subunit of $GABA_A$ receptor. Bar $= 5\,\mu$m

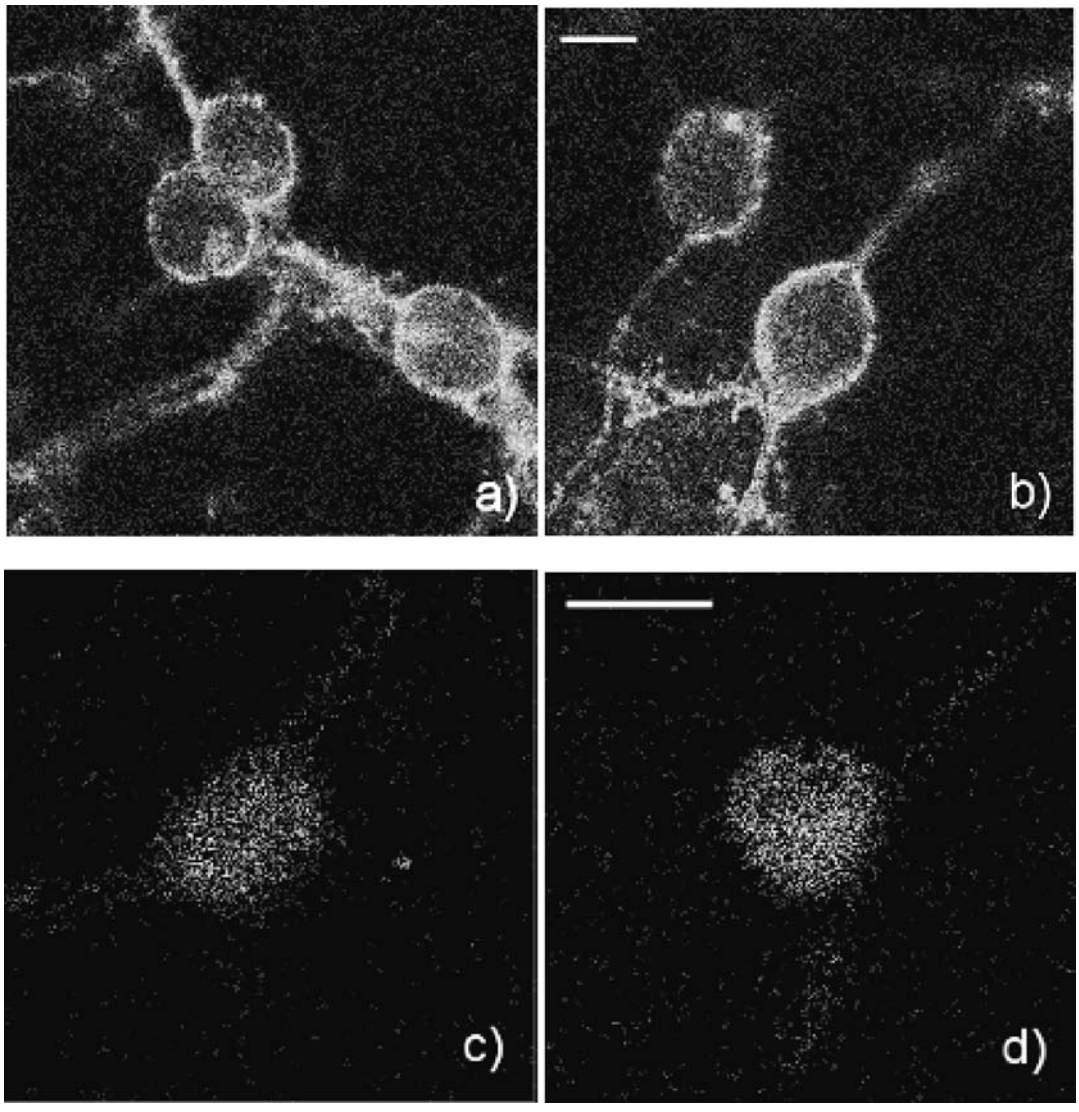

Fig. 4. Middle planes of various granule cells marked with antibodies against the $\beta_{2/3}$ subunits (case **a** and **b**) and of a granule cell labelled for the δ subunit (case **c** and **d**) of $GABA_A$ receptor. Here too, the bar represents 5 μm

that emerges from this pole. Patches of immunoreactivity are present both on cell bodies and neurites plasma membranes.

As for the α_6 subunit, also the γ_2 subunit results distributed on cell body and processes and it presents a distinct accumulation on the plasma membranes (see Fig. 3). Here too, part of the immunoreactivity appears to be present in patches along the plasma membranes. In addition, there is a fluorescence accumulation in a cell region just below the cone of emergence of granule cell dendrites. The dendritic nature of the labelled neurites is suggested by their shape, which is very much reminiscent of the appearance of the granule cells dendritic tree *in situ*.

Figure 4a and b represent single sections of the middle planes of cerebellar granule cells marked for the $\beta_{2/3}$ subunit. These images show that this subunit is almost exclusively localized on cell body and neurites plasma membrane. The fluorescence shows clusters of high intensity. Fig. 4c and d instead show middle focal planes of a typical granule cell labelled with antibodies against the δ subunit. Here fluorescence is quite low. This may indicate that the δ subunit is not expressed in high concentration in granule cells at the developmental stage studied by us. The subcellular distribution of this subunit shows a localization of fluorescence on the cell body rather than on processes. In turn, the labelling of the cell body

is cytoplasmatic (endoplasmic reticulum) rather than on the plasma membrane.

Discussion

The subunits we studied in the present work are those which are more represented in the adult rat and mouse cerebellum granule layer both in terms of mRNA (Laurie et al., 1992; Wu et al., 1995) and subunits proper (Pirker et al., 2000; Gao et al., 1995). An important issue in the characterization of $GABA_A$ receptor mediated inhibition of cerebellar granules is to differentiate the receptor populations which respectively mediate the phasic and tonic form of inhibition of these cells (Brickley et al., 1996). The first one involves GABAergic synaptic contacts whereas the second one is mediated by extrasynaptic $GABA_A$ receptors such as those on granule cells bodies (Nusser et al., 1998; Cupello et al., 2000). Immunocytochemical studies on cerebellar slices at the electron microscope level showed that the α_6 (Baude et al., 1992), the α_1 and $\beta_{2/3}$ (Nusser et al., 1995) subunits are enriched at dendritic postsynaptic membranes. Colocalization of α_1 and α_6 at inhibitory synapses was later described (Nusser et al., 1996). On the contrary, the δ subunit was found to be present only in extrasynaptic receptors (Nusser et al., 1998). The γ_2 subunit is instead concentrated in synaptic $GABA_A$ receptors (Nusser et al., 1998).

Previous immunocytochemical studies of $GABA_A$ receptor subunits in cerebellar granules in culture involving the very same subunits we studied here were performed by two different groups and with different techniques (Caruncho et al., 1995; Gao et al., 1995). Our study confirms the presence of α_1, α_6, $\beta_{2/3}$, γ_2 and δ in cerebellar granules obtained from 7 days old rats and kept 7 days *in vitro*. Other features which confirm the results of the previous studies are the relatively lower expression of the δ subunit and the presence of patches of immunoreactivity (see α_6, $\beta_{2/3}$ and γ_2) on the plasma membrane of both cell bodies and neurites. However, there are two distinct differences with the previous results. First, the α_1 subunit immunoreactivity is diffuse in the cell body cytoplasm so that it is difficult to distinguish the labelling of the cell body plasma membrane. This result was not apparent in the work by Gao and Fritschy (1995) on cerebellar granules in culture nor in other immunocytochemical studies in cerebellar slices from adult animals (Baude et al., 1992; Fritschy et al., 1994). However, in an early study Meinecke et al. (1989), using the antibody E9, directed against the α subunit of the GABA/benzodiazepine receptor, found immunoreactivity in the perikaryal cytoplasm of rat cerebellum granule cells. This antibody most probably recognizes the α_1 subunit (Sweetnam et al., 1987). In any case, our results suggest a strong presence of the α_1 subunit in receptors in the process of being assembled in the endoplasmic reticulum (Barnes et al., 2000). A similar distribution we also found for the δ subunit.

Secondly, we found, more markedly for the γ_2 but also for the α_6 subunit, an accumulation in cytoplasmic domains just below the site of emergence of neurites. This suggests that these subunits are synthesized in the cell body but are then transported to a final membrane destination in neurites. The distribution of the γ_2 subunit is ''polar'', the immunoreactivity being on the neurite of only one side of the cells (see Fig. 3) as opposite to the one of α_6 which is on neurites on both sides of the cell body (see Fig. 2). This circumstance and the very appearance of the neurites involved (Fig. 3) suggest that the γ_2 subunit is transported to a dendritic site. These sites most probably correspond to what *in situ* would be postsynaptic sites. The fact that the α_6 subunit does not show such a polarity albeit being involved in synaptic receptors *in situ* (Baude et al., 1992) may be explained recalling that benzodiazepine-insensitive $GABA_A$ receptors are expressed on parallel fiber terminals too (Schmid et al., 1996). These receptors may well contain the α_6 subunit (Raiteri et al., 2001), which actually renders them insensitive to benzodiazepine agonists (Luddens et al., 1990). Thus, it is not surprising to find α_6 subunits expressed also in granule cells neurites (axons) which are the *in vitro* cell structures which correspond to the *in situ* parallel fibers of the cerebellar cortex.

References

Barnes EM (2000) Intracellular trafficking of $GABA_A$ receptors. Life Sci 66: 1063–1070

Baude A, Sequier J-M, McKernan RM, Olivier KR, Somogyi P (1992) Differential subcellular distribution of the α_6 subunit versus the α_1 and $\beta_{2/3}$ subunits of the $GABA_A$/Benzodiazepine receptor complex in granule cells of the cerebellar cortex. Neuroscience 51: 739–748

Beattie CE, Siegel RE (1993) Developmental cues modulate $GABA_A$ receptor subunit mRNA expression in cultured cerebellar granule neurons. J Neurosci 13: 1784–1792

Bovolin P, Santi MR, Memo M, Costa E, Grayson DR (1992a) Distinct developmental patterns of expression of rat alpha 1, alpha 5, gamma 2S, and gamma 2L gamma-aminobutyric $acid_A$ receptor subunit mRNAs *in vivo* and *in vitro*. J Neurochem 59: 62–72

Bovolin P, Santi MR, Puia G, Costa E, Grayson DR (1992b) Expression patterns of gamma-aminobutyric acid type A receptor subunit mRNAs in primary cultures of granule neurons and astrocytes from neonatal rat cerebella. Proc Natl Acad Sci USA 89: 9344–9348

Brickley SG, Cull-Candy SG, Farrant M (1996) Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of $GABA_A$ receptors. J Physiol 497: 753–759

Caruncho HJ, Puia G, Mohler H, Costa E (1995) The density and distribution of six $GABA_A$ receptor subunits in primary cultures of rat cerebellar granule cells. Neuroscience 67: 583–593

Cupello A, Robello M (2000) $GABA_A$ receptor modulation in rat cerebellum granule cells. Receptors Channels 7: 151–171

Diaspro A, Robello M (2000) Two photons excitation of fluorescence for three dimensional optical imaging of biological structures. J Photochem Photobiol 55: 1–8

Fritschy J-M, Paysan J, Enna A, Mohler H (1994) Switch in the expression of rat $GABA_A$-receptor subtypes during postnatal development: an immunohistochemical study. J Neurosci 14: 5302–5324

Gao B, Fritschy J-M (1995) Cerebellar granule cells *in vitro* recapitulate the *in vivo* pattern of $GABA_A$-receptor subunit expression. Dev Brain Res 88: 1–16

Kandel E, Schwartz JH, Jessel TM (2000) Principles of neural sciences, Chp 42. Mc Graw Hill, New York

Laurie DJ, Seeburg PH, Wisden W (1992) The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. II. Olfactory bulb and cerebellum. J Neurosci 12: 1063–1076

Levi G, Aloisi F, Ciotti MT, Gallo V (1984) Autoradiographic localization and depolarization induced release of amino acids in differentiating granule cells in culture. Brain Res 290: 77–86

Luddens H, Pritchett DB, Kohler M, Killisch I, Keinanen K, Monyer H, Sprengel R, Seeburg PH (1990) Cerebellar $GABA_A$ receptor selective for a behavioural alcohol antagonist. Nature 346: 648–651

Mathews GC, Bolos-Sy AM, Holland KD, Isenberg KE, Covey DF, Ferrendelli JA, Rothmann SM (1994) Developmental alteration in $GABA_A$ receptor structure and physiological properties in cultured cerebellar granule neurons. Neuron 13: 149–158

Meinecke DL, Tallman J, Rakic P (1989) GABA/benzodiazepine receptor-like immunoreactivity in rat and monkey cerebellum. Brain Res 493: 303–319

Nusser Z, Roberts JDB, Baude A, Richards JG, Somogyi P (1995) Relative densities of synaptic and extrasynaptic $GABA_A$ receptors on cerebellar granule cells as determined by a quantitative immunogold method. J Neurosci 15: 2948–2960

Nusser Z, Sieghart W, Somogyi P (1998) Segregation of different $GABA_A$ receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. J Neurosci 18: 1693–1703

Nusser Z, Sieghart W, Stephenson FA, Somogyi P (1996) The α_6 subunit of the $GABA_A$ receptor is concentrated in both inhibitory and excitatory synapses on cerebellar granule cells. J Neurosci 16: 103–114

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G (2000) GABA(A) receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 101: 815–850

Raiteri L, Schmid G, Prestipino S, Raiteri M, Bonanno G (2001) Activation of alpha 6 $GABA_A$ receptors on depolarized cerebellar parallel fibers elicits glutamate release through anion channels. Neuropharmacology 41: 943–951

Robello M, Amico C, Cupello A (1999) Evidence of two populations of $GABA_A$ receptors in cerebellar granule cells in culture: different desensitization kinetics, pharmacology, serine/threonine kinase sensitivity, and localization. Biochem Biophys Res Commun 266: 603–608

Santi MR, Vicini S, Eldadah B, Neale JH (1994) Analysis by polymerase chain reaction of alpha 1 and alpha 6 $GABA_A$ receptor subunit mRNAs in individual cerebellar neurons after whole-cell recordings. J Neurochem 63: 2357–2360

Schmid G, Bonanno G, Raiteri M (1996) Functional evidence for two native $GABA_A$ receptor subtypes in adult rat hippocampus and cerebellum. Neuroscience 73: 697–704

Sweetnam P, Nestler E, Gallombardo P, Brown S, Duman R, Stefon Bracha H, Tallman J (1987) Comparison of the molecular structure of GABA/benzodiazepine receptors purified from rat and human cerebellum. Mol Brain Res 2: 223–233

Wu C-H, Frostholm A, De Blas AL, Rotter A (1995) Differential expression of $GABA_A$/Benzodiazepine receptor subunit mRNAs and ligand binding sites in mouse cerebellar neurons following *in vivo* ethanol administration: an autoradiographic analysis. J Neurochem 65: 1229–1239

Authors' address: Prof. Aroldo Cupello, Istituto di Bioimmagini e Fisiologia Molecolare, CNR, Sezione di Genova, Via De Toni 5, I-16132 Genova, Italy,

Fax: 39-010354180, E-mail: dcupel@neurologia. unige.it