

MORPHOLOGICAL AND NEUROCHEMICAL DIFFERENTIATION OF LARGE GRANULAR LAYER INTERNEURONS IN THE ADULT RAT CEREBELLUM

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Abstract—The granular layer of the cerebellar cortex consists of densely packed neuronal cells, classified into granule cells and large interneurons. In this study, we provide a comparative survey of large granular layer interneurons in the adult rat cerebellum based on both morphological and neurochemical criteria. To this end, double immunofluorescence histochemistry was performed by combining antibodies against the cytoplasmic antigen Rat-303, calretinin, the metabotropic glutamate receptor mGluR2 and somatostatin. Based on Rat-303/calretinin double immunohistochemistry, three distinct populations of large granular layer interneurons could be discerned: cells immunopositive for Rat-303, calretinin or both. Rat-303 or calretinin single-labeled cells represented Golgi cells and unipolar brush cells, respectively. Rat-303/calretinin double-labeled cells located just underneath the Purkinje cell layer represented Lugaro cells. Morphometrical analysis distinguished two populations of Rat-303-positive Golgi cells according to their location: vermis versus hemisphere. Immunostaining for the metabotropic glutamate receptor mGluR2 combined with Rat-303 or calretinin revealed that the majority of Golgi cells (about 90%) appeared to be mGluR2 positive. Lugaro cells were mGluR2 negative. In addition, a limited population of large polymorphous interneurons in the depth of the granular layer with morphological features resembling Golgi cells also displayed Rat-303/calretinin immunoreactivity and were mGluR2 negative. Double immunohistochemistry for Rat-303 and somatostatin revealed three populations of labeled cells in the depth of the granular layer. Besides double-labeled Golgi cells, Rat-303 or somatostatin single-labeled cells were present. Based on mGluR2/somatostatin and calretinin/somatostatin double immunostainings, Rat-303 single-labeled cells were found to correspond to Rat-303/calretinin-positive, mGluR2-negative Golgi-like cells, while the identity of somatostatin single-labeled cells remained unclear.

The data presented in this article elaborate previous reports on the morphological and neurochemical differentiation of large interneurons in the rat cerebellar granular layer. In addition, they indicate that the current classification of these cells into Golgi cells, Lugaro cells and unipolar brush cells does not describe the observed neurochemical heterogeneity. © 2001 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: Golgi cell, Lugaro cell, unipolar brush cell, immunohistochemistry, Rat-303, metabotropic glutamate receptor mGluR2.

Understanding how information is processed by the cerebellar cortex implies a thorough knowledge of cerebellar circuitry. This requires an accurate description of the neuronal cell types involved (for reviews, see Refs 43 and 59). The granular layer of the cerebellar cortex consists of densely packed neuronal cells. They are classified into two major morphologically and functionally distinct neuronal types: small granule cells and large interneurons.^{8,20} Characterization and differentiation of the large granular layer interneurons started at the end of the 19th century with the classical Golgi impregnations of Golgi¹⁵ and Lugaro.²⁹ The availability of specific antibodies nowadays offers an alternative method for the visualization of neurons and allows us to reinvestigate

the differentiation of large granular layer interneurons, currently classified into Golgi cells,^{8,63} Lugaro cells^{9,27} and unipolar brush cells (UBCs).^{34,35} In this regard, four immunohistochemical markers deserve further attention.

The first marker, called Rat-303, represents an unidentified cytoplasmic antigen. An antibody against Rat-303 was produced by immunizing Balb/c mice with homogenized rat spinal cord.¹⁸ Immunohistochemical studies using the Rat-303 antibody on the cerebellum of rats and cats revealed large cells located in the granular layer of the cerebellar cortex. Based on the position, shape and size of immunopositive neurons, it was concluded that the Rat-303 antibody selectively stains Golgi cells.^{18,51}

A second marker is calretinin (CRT), an intracellular calcium-binding protein closely related to calbindin (a calcium-binding protein abundantly present in cerebellar Purkinje cells).^{28,49} CRT immunoreactivity (IR) has been reported, amongst others, in the cerebellum of rats and humans.^{3,9,14,35,47,48} Within the cerebellar granular layer, CRT-IR was observed in two types of large interneurons:

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Abbreviations: CRT, calretinin; FITC, fluorescein isothiocyanate; Ig, immunoglobulin; IR, immunoreactivity; mGluR, metabotropic glutamate receptor; PBS, phosphate-buffered saline; SOM, somatostatin; UBC, unipolar brush cell.

Table 1. Characteristics of antibodies used

Antigen	Source	Dilution	Species	Reference
Rat-303	Hockfield	1:5	Mouse	18
Calretinin	SWant	1:2500	Rabbit	
mGluR2	Shigemoto	1:500	Mouse	37
Somatostatin	Euro-Diagnostica	1:50	Rabbit	
Somatostatin	Buchan	1:500	Mouse	5

Lugaro cells^{9,48} and UBCs.^{3,14,35,47} Recently, calretinin was demonstrated to play a major role in cerebellar physiology.⁵²

The neuropeptide somatostatin (SOM) represents a third marker of interest. In the CNS of adult rats, a widespread occurrence of SOM-positive nerve cell bodies has been reported.^{2,4,13,23} The cerebellum, however, contained only few SOM-positive cell bodies.²³ Positively labeled cell bodies within the cerebellar granular layer were identified as Golgi cells.^{19,23,58} Despite the low level of cerebellar SOM-IR, the cerebellum may be activated during SOM-induced behavior.²⁴

The fourth marker is the metabotropic glutamate receptor mGluR2. Within the cerebellar cortex, mGluR2 is expressed in Golgi cells^{37–40} and to some degree in UBCs.^{21,39} Based on the presence of mGluR2 in Golgi cells, transgenic mice were generated in which elimination of Golgi cells using the immunotoxin-mediated cell targeting technique caused severe motor disorders.⁶³

The aim of the present study was to differentiate large granular layer interneurons in the adult rat cerebellum based on morphological and neurochemical criteria. Therefore, we have performed double immunostainings with antibodies raised against the above-mentioned markers (Rat-303, CRT, mGluR2 and SOM).

EXPERIMENTAL PROCEDURES

Animals

Three adult male and three adult female Wistar rats, weighing 235–325 g (Iffa-Credo, Brussels, Belgium), were used in this study. All rats were kept in acrylic cages with wood shavings in a climatized room (12-h/12-h light–dark cycle; 22 ± 3°C), with food pellets and water available *ad libitum*. National and international principles of laboratory animal care were followed, and the experiments were approved by the local ethics committee of the University of Antwerp.

Tissue processing

Animals were killed with an overdose of sodium pentobarbital (Nembutal; i.p.) and transcardially perfused with heparinized physiological saline, followed by a fixative consisting of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After 30 min perfusion, the cerebellum was dissected out and immersed in the same fixative for another 2 h. Following several washes with 10 mM phosphate-buffered saline (PBS; pH 7.4), the cerebellum was divided into vermis and hemispheres. Tissue parts were immersed in PBS containing 30% sucrose for 24 h, frozen in Tissue Tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands) on a cryostat chuck and cut into 14- to 16- μ m-thick sagittal sections on a Microm cryostat (Walldorf, Germany). Cryosections were mounted onto gelatin- or poly-L-lysine-coated glasses and immediately processed for immunohistochemistry.

Immunohistochemistry

After rinsing in PBS and preincubation for 30 min with the same solution as used for the dilution of primary and secondary antibodies (10% normal goat serum, 0.1% bovine serum albumin, 0.05% thimerosal, 0.01% NaN₃ and 1% Triton X-100 in PBS) to block non-specific binding of the antibodies, double immunofluorescence histochemistry was performed. The antibodies used in this study are summarized in Table 1: the mouse monoclonal Rat-303 antibody (1:5; a generous gift from Prof. S. Hockfield), a rabbit polyclonal anti-CRT antibody (1:2500; SWant, Bellinzona, Switzerland), a mouse monoclonal anti-mGluR2 antibody (1:500; MAb mG2Na-5³⁷), a rabbit polyclonal anti-SOM antibody (1:50; Euro-Diagnostica, Arnhem, The Netherlands) and a mouse monoclonal anti-SOM antibody (1:500; S10,⁵ a generous gift from Prof. A. M. J. Buchan). All incubations were carried out at room temperature in a humid atmosphere. Cryosections were incubated overnight for the following combinations: (1) Rat-303 and CRT; (2) mGluR2 and CRT; (3) Rat-303 and SOM; (4) CRT and SOM; and (5) mGluR2 and SOM. Sections were then rinsed in PBS and incubated for 2 h with the appropriate secondary antibodies: for combinations (1), (2) and (4), a Cy3-conjugated goat anti-mouse immunoglobulin (Ig; 1:200) and a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit Ig (1:100); for combinations (3) and (4), an FITC-conjugated goat anti-mouse Ig (1:100) and a Cy3-conjugated goat anti-rabbit Ig (1:200). Secondary antibodies were obtained from Jackson Laboratories (West Grove, PA, USA). Sections were thoroughly washed with PBS, to remove unreacted secondary antibodies, and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA).

In order to perform double immunostainings for (1) Rat-303 and mGluR2, and (2) CRT and SOM with antibodies raised in the same host species, the indirect tyramide signal amplification method^{33,54,62} (NEN Life Science Products, Boston, MA, USA) was used. Cryosections were air-dried, rinsed in PBS, preincubated for 10 min with 3% H₂O₂ (in 50% methanol) to block endogenous peroxidase activity, rinsed again in PBS and preincubated for 30 min with the same solution as before to block non-specific binding of antibodies. Subsequently, sections were incubated overnight with (1) the mGluR2 antibody (1:10,000) or (2) the CRT antibody (1:50,000). The following day, the mGluR2 or CRT signal was revealed. Between subsequent steps, sections were washed with 0.05% Tween 20 in PBS. Amplification and visualization of the mGluR2 signal was achieved by incubation of sections with biotinylated sheep anti-mouse Ig (1:500; 1 h; Amersham, Buckinghamshire, UK), followed by horseradish peroxidase-conjugated ExtrAvidin (1:500; 1 h; Sigma, Bornem, Belgium), biotinylated tyramide (1:50; 7 min; NEN Life Science Products) and FITC-conjugated streptavidin (1:200; 10 min; Amersham). After rinsing in PBS, sections were incubated overnight with the Rat-303 antibody (1:50). Amplification and visualization of the CRT signal was achieved by subsequent incubation of sections with biotinylated goat anti-rabbit Ig (1:500; 1 h; DAKO, Glostrup, Denmark), horseradish peroxidase-conjugated ExtrAvidin (1:500; 1 h), biotinylated tyramide (1:50; 7 min) and FITC-conjugated streptavidin (1:200; 10 min). After rinsing in PBS, sections were incubated overnight with the rabbit polyclonal anti-SOM antibody (1:50). Finally, sections were washed with PBS and incubated for 2 h with a Cy3-conjugated goat anti-mouse Ig or a Cy3-conjugated goat anti-rabbit Ig (1:200; Jackson Laboratories), respectively, to reveal the Rat-303 or SOM signal, followed by a thorough wash with PBS and mounting in Vectashield. All incubations were performed at room temperature in a humid atmosphere.

To test method specificity, cerebellar sections were incubated as described above, but the primary antibodies were omitted. This resulted in a complete absence of immunostaining.

Microscopic analysis

Fluorescent signals were imaged by an epifluorescence microscope (Zeiss Axiophot), equipped with filter combinations for separate or simultaneous visualization of FITC and Cy3, or by a confocal laser scanning microscope (Zeiss LSM 410) and the

attached image reconstruction facilities (Imaris 2.7 software, Fairfield Imaging) on a Silicon Graphics Indigo 2 workstation. In the latter case, an argon laser (488 nm) and a helium–neon laser (543 nm) in combination with the appropriate dichroic mirrors and emission filters were used for the excitation and observation of FITC and Cy3, respectively.

Morphometrical analysis

Quantitative morphometrical analysis was performed with the aid of an Olympus BX-50 epifluorescence microscope equipped with a 3CCD camera (DXC9100P, Sony) and a computerized stage system (Märzhäuser motorized scanning table supported by MultiControl 2000), coupled to a personal computer with image processing software (analysis 3.0, Soft Imaging System, Münster, Germany). The size of neurochemically defined cell bodies within specified cerebellar regions, based on the atlas of Paxinos and Watson,⁴⁴ was estimated by measuring their cell body cross-section area in vermal and hemispherical cryosections from both male and female rats. Because labeled cell bodies displayed a variety of shapes (e.g. round, polygonal, fusiform), we have measured cell body cross-section area instead of diameter as it generates, in our opinion, a better estimation of the actual size. Although this method does not provide the actual cell body size, it allows comparison of the different neurochemical populations. The morphometrical analysis was performed semi-automatically. After focusing, cells were presented on the monitor. Only cells which clearly displayed a nucleus were included. Subsequently, the outline of cell bodies was drawn with the aid of a mouse. Based on this perimeter, cell body cross-section area was computed by the analysis image processing software. The data were compiled and subjected to a three-way ANOVA test (proc. mixed SAS V6.12). Cell size was regarded as dependent variable and cell type (Golgi cell, Lugaro cell, UBC), region (vermis, hemisphere), individual (different rats) and all interactions were entered as independent variables. A probability of $P < 0.05$ was set as the level of significance in all analyses.

RESULTS

Rat-303 and calretinin double immunostaining

In the granular layer of cerebellar sections stained for Rat-303 and CRT, three distinct populations of neuronal cells could be discerned: cells immunopositive for Rat-303, CRT or both. As demonstrated in Fig. 1A, Rat-303- or CRT-immunostained neuronal cells were scattered throughout the granular layer.

Rat-303 single staining was observed in large (up to 20 μm in diameter) round or polygonal cell bodies with radiating dendritic arborizations (Fig. 2A). They represented Golgi cells, which are the most abundant large interneurons throughout the granular layer. Golgi cell somata displayed intensely labeled intracellular spots (Fig. 2A), a typical feature of Rat-303 staining.⁵¹ Visualization of the dendritic arborizations was usually rather limited. Inherent to the experimental procedure, only proximal dendrites were clearly delineated (Fig. 2A). Although Rat-303-positive Golgi cells exhibited a similar shape, they showed a marked variation in size compared to other labeled cells (Fig. 2B). In an attempt to quantify this apparent large variability in size, we estimated Golgi cell body size by measuring their cell body cross-section area in both vermal and hemispherical sections from three different animals. A total of 736 cells was measured, with a mean \pm S.D. of 123 ± 24 cells in each tissue sample. Golgi cell body cross-section

area ranged from 40 to 400 μm^2 and seemed unevenly distributed. Statistical analysis, however, revealed this distribution to be caused by a significant interaction between the cerebellar region (vermis versus hemisphere) and the measured value (cell body cross-section area). Golgi cells were thus found to differ according to their location: mean cell body cross-section area \pm S.D. was $147 \pm 57 \mu\text{m}^2$ in the hemispheres versus $165 \pm 65 \mu\text{m}^2$ in the vermis (Table 2). Apart from location, Golgi cells displayed no further statistical variability.

CRT single staining, however, was seen in a medium-sized neuronal cell type, intermediate in size between granule cells and Golgi cells, which gave rise to a single paint brush-like dendritic tree (Fig. 2C). This cell type, the UBC, although predominantly present in the vestibulo-cerebellum, was found in all lobules of the cerebellar cortex. Besides their inhomogeneous distribution, UBCs exhibited no apparent variability in size or shape.

Thirdly, a widespread population of large granular layer interneurons displayed both Rat-303- and CRT-IR. Rat-303/CRT-immunopositive cell bodies were usually fusiform and located just underneath the Purkinje cell layer (Fig. 2D). They emitted, emerging from opposite poles, two thick dendrites in the sagittal plane, parallel to the Purkinje cell layer (Fig. 2D). Based on their morphology, these cells were identified as Lugaro cells. Hence, using neurochemical markers, Lugaro cells (arrowhead) could be clearly distinguished from Golgi cells (arrow) and UBCs (asterisks) (Fig. 1B). Rat-303 staining of Lugaro cells, although slightly less pronounced than in Golgi cells, again revealed the presence of intensely labeled intracellular spots (Fig. 1B). CRT immunostaining of Lugaro cells appeared somewhat less bright than CRT staining of UBCs (Fig. 1B). Although Lugaro cells were equally present in all lobules, they showed a peculiar anatomical inhomogeneity. Lugaro cells located in the buried, concave parts of the folia exhibited a tendency to cluster (Fig. 1C, arrowheads). Such clustering was less obvious in other parts of the folium. Apart from Lugaro cells, another population of large Rat-303/calretinin-positive interneurons could be observed, although in much lower numbers. These cells were located in the depth of the granular layer and displayed a variety of shapes, often appearing quite similar to Golgi cells (Fig. 1D, open arrowhead).

Rat-303 and mGluR2 double immunostaining

Double immunohistochemistry for Rat-303 and mGluR2 revealed two populations of Rat-303-positive neuronal cells. Besides Rat-303/mGluR2 double-labeled cells (arrow), a population of Rat-303-positive, mGluR2-negative cells (arrowhead) was present (Fig. 3). While the population of Rat-303/mGluR2-positive cells clearly displayed Golgi cell morphology, the population of Rat-303-positive, mGluR2-negative cells showed a variety of shapes. This was not surprising since Rat-303 staining, as described above, did not label exclusively Golgi cells. In addition, Neki *et al.*³⁸ demonstrated that only 90% of cerebellar Golgi cells exhibited mGluR2-IR. In order to

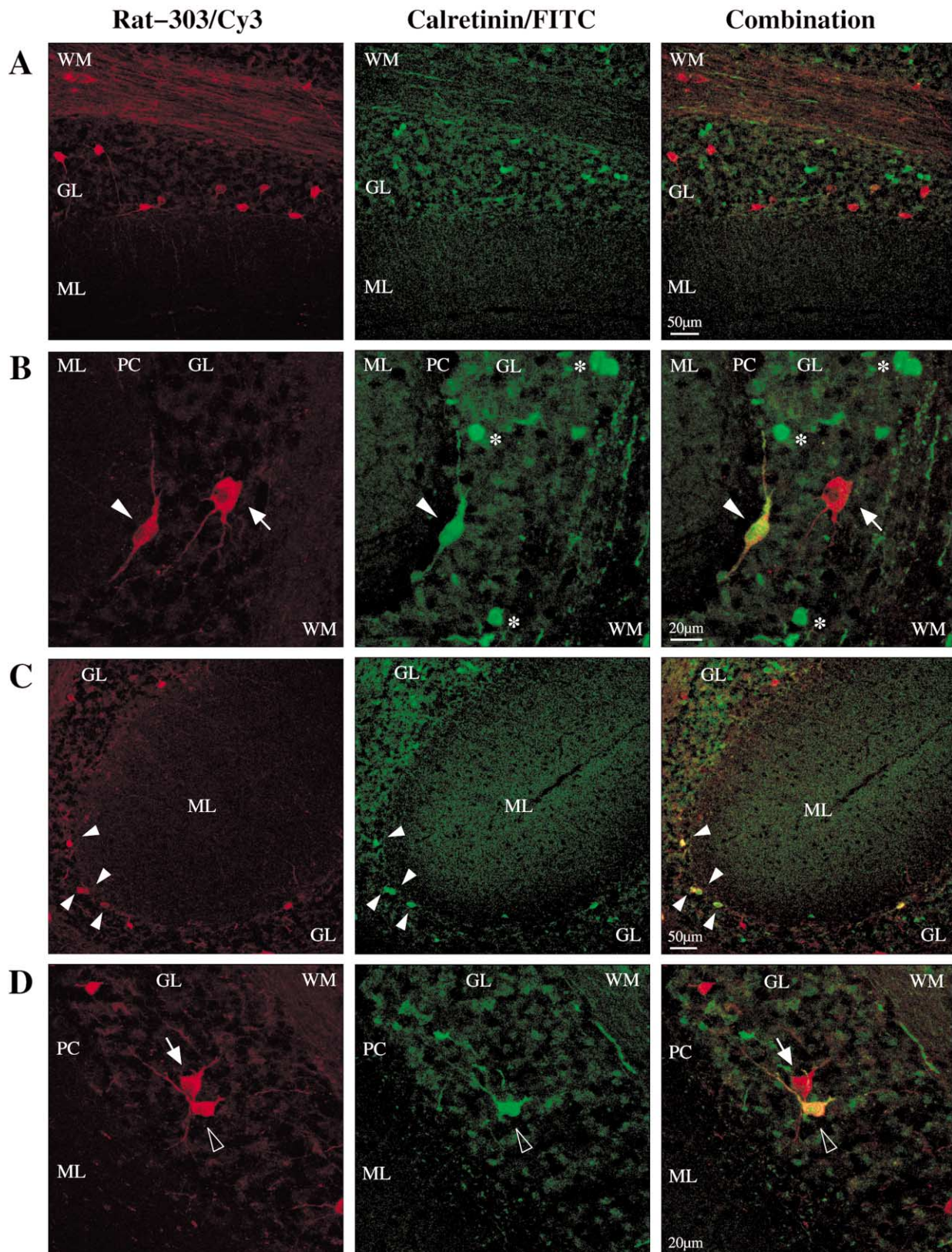


Fig. 1. Confocal microscopic images from adult rat cerebellar cryosections immunostained for Rat-303 (red Cy3 fluorescence) and calretinin (green FITC fluorescence). (A) Low-magnification overview of the cerebellar cortex showing two distinct neuronal populations, immunoreactive for either Rat-303 or CRT, within the granular layer. (B) High-magnification overview of the granular layer displaying three different types of large interneurons: Rat-303-positive Golgi cells (arrow), Rat-303/CRT-positive Lugaro cells (arrowhead) and CRT-positive UBCs (asterisks). (C) Cluster of Rat-303/CRT-positive Lugaro cells (indicated by arrowheads) located just underneath the Purkinje cell layer in the buried, concave part of a cerebellar folium. (D) Rat-303-positive Golgi cell (arrow) and Rat-303/CRT-positive large interneuron (open arrowhead) with quite similar morphologies, lying closely together in the middle of the granular layer. GL, granular layer; ML, molecular layer; PC, Purkinje cell layer; WM, white matter.

Table 2. Cell body cross-section area frequency distribution of Rat-303-positive, calretinin-negative Golgi cells

Cell body cross-section area (μm^2)	Rat-303-positive Golgi cells in hemisphere		Rat-303-positive Golgi cells in vermis	
	$n = 372$	Per cent of total	$n = 364$	Per cent of total
40–54	9	2.42	5	1.37
55–69	14	3.76	6	1.65
70–84	22	5.91	24	6.59
85–99	53	14.25	39	10.71
100–114	27	7.26	16	4.4
115–129	39	10.48	33	9.07
130–144	24	6.45	32	8.79
145–159	38	10.21	24	6.59
160–174	30	8.06	30	8.24
175–189	34	9.14	26	7.14
190–204	25	6.72	28	7.69
205–219	16	4.3	25	6.87
220–234	17	4.57	27	7.42
235–249	6	1.61	11	3.02
250–264	9	2.42	14	3.85
265–279	3	0.81	10	2.75
280–294	3	0.81	3	0.82
295–309	0	0	2	0.55
310–324	0	0	5	1.37
325–339	0	0	1	0.27
340–354	1	0.27	0	0
355–369	2	0.54	1	0.27
370–384	0	0	1	0.27
385–399	0	0	0	0
400–414	0	0	1	0.27
Average	$147 \pm 57 \mu\text{m}^2$		$165 \pm 65 \mu\text{m}^2$	

Statistical analysis showed a significant difference in Golgi cell body cross-section area between sections obtained from the hemisphere (mean Golgi cell body cross-section area \pm S.D.: $147 \pm 57 \mu\text{m}^2$) or from the vermis (mean area \pm S.D.: $165 \pm 65 \mu\text{m}^2$).

address the identity of these Rat-303-positive, mGluR2-negative cells, we conducted mGluR2/CRT double immunohistochemistry.

mGluR2 and calretinin double immunostaining

As demonstrated in Fig. 4A, mGluR2-immunoreactive Golgi cells (arrow) were CRT negative. CRT-immunopositive Lugaro cells, in contrast, were mGluR2 negative (Fig. 4B, arrowhead). Therefore, part of the above-described population of Rat-303-positive, mGluR2-negative cells most likely represented Lugaro cells. In addition, the population of large polymorphous CRT-immunoreactive cells in the depth of the granular layer appeared to be mGluR2 negative as well (Fig. 4C, open arrowhead; also see Fig. 1D). Consequently, these cells may also contribute to the population of Rat-303-positive, mGluR2-negative cells.

To determine the ratio of these contributions, a quantitative analysis was performed. First, the ratio of Golgi cells, Lugaro cells and deep CRT-positive cells was determined on sections stained for Rat-303 and CRT. Sections were obtained from two different rats and a total of 1041 cells was counted ($n = 610$ and 431 , respectively). Golgi cells represented $75.9 \pm 3.9\%$ of Rat-303 labeled cells, whereas Lugaro cells and deep CRT-positive cells accounted for $20.7 \pm 4.3\%$ and $3.4 \pm 0.4\%$,

respectively (all data are given as mean \pm S.D.). Second, the ratio of Rat-303-positive, mGluR2-negative versus Rat-303/mGluR2-positive cells was determined. In sections stained for Rat-303 and mGluR2, a total of 916 cells was counted. Sections were obtained from two different rats ($n = 482$ and 434 , respectively); $68.7 \pm 2.0\%$ of the cells were double stained, while $31.3 \pm 2.0\%$ were mGluR2 negative. Consequently, besides Lugaro cells (about 20.7% of the 31.3%) and deep CRT-positive cells (about 3.4% of the 31.3%), CRT-negative Golgi cells may also contribute to the population of Rat-303-positive, mGluR2-negative cells (about 7.2% of the 31.3%).

In addition to Golgi cells, CRT-positive UBCs displayed mGluR2-IR as well. However, the mGluR2 immunostaining of UBCs appeared very faintly and allowed no further quantification.

Rat-303 and somatostatin double immunostaining

Sections stained for Rat-303 and SOM revealed many large-sized double-labeled neuronal cells within the granular layer (Fig. 5A). Rat-303/SOM-immunopositive cells appeared as large round or polygonal cell bodies with radiating dendritic arborizations and were consequently considered to be Golgi cells. Besides Rat-303/SOM double-labeled Golgi cells (arrow), a population of

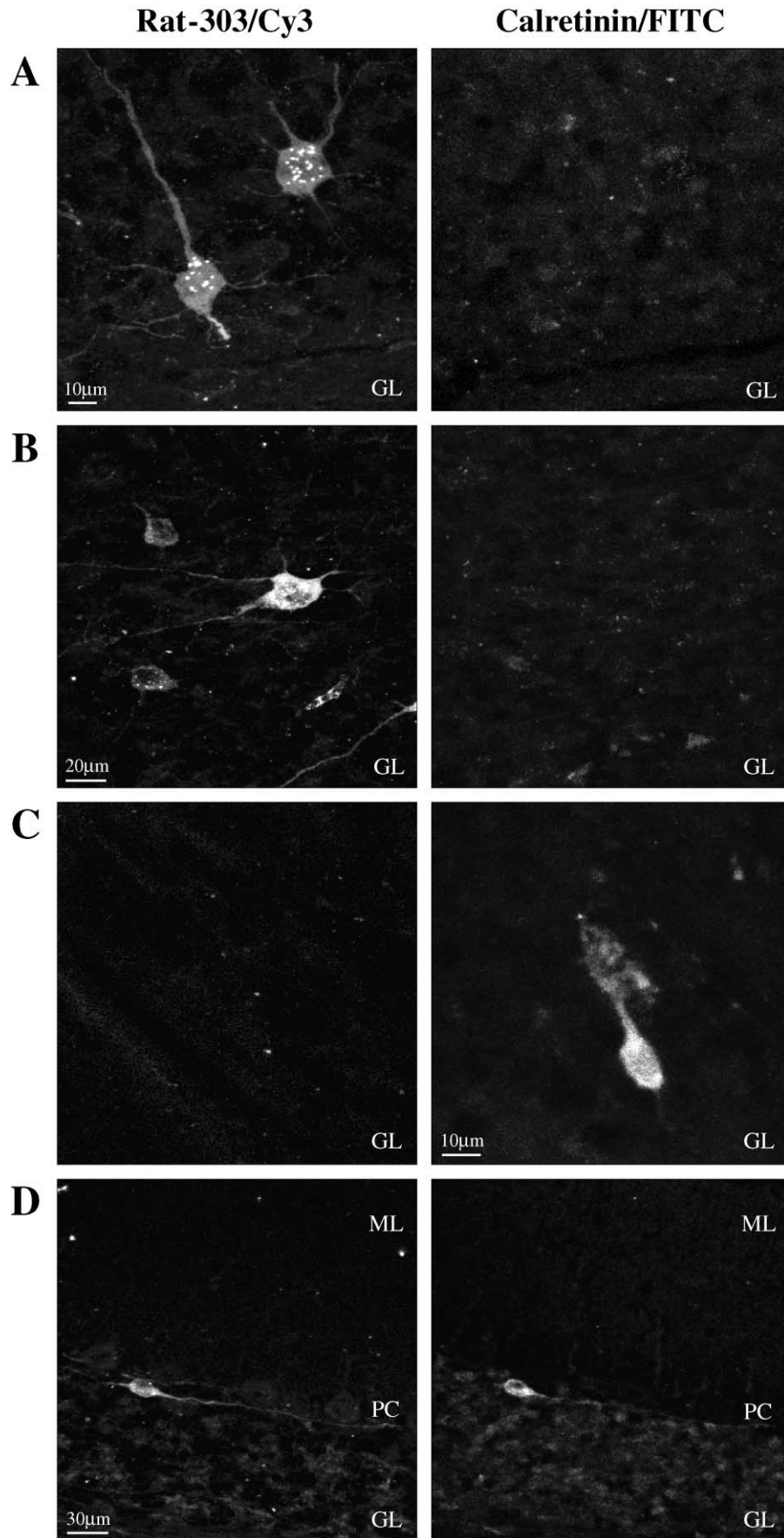


Fig. 2.

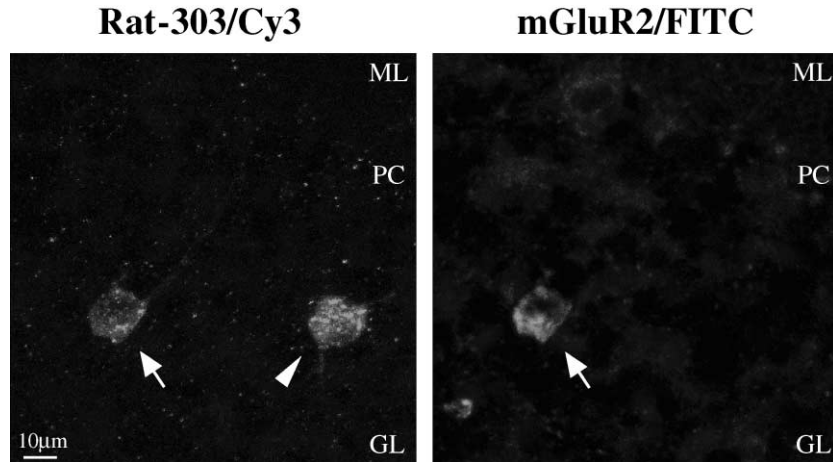


Fig. 3. Confocal microscopic images from adult rat cerebellar cryosections immunostained for Rat-303 (visualized with Cy3) and mGluR2 (visualized with FITC). Two types of Rat-303-positive neuronal cells can be observed: a Rat-303/mGluR2 double-labeled Golgi cell (arrow) and a Rat-303 single-labeled large interneuron (arrowhead). GL, granular layer; ML, molecular layer; PC, Purkinje cell layer.

Rat-303 single-labeled cells was present in the depth of the granular layer (Fig. 5B, open arrowhead). In addition, a population of neuronal cells appeared to be only SOM immunoreactive (Fig. 5C, open arrow). SOM single-labeled cells tended to have a smaller soma size. Morphometrical analysis on both vermal and hemispherical cryosections from three different animals ($n = 281$) revealed that their cell body cross-section area ranged from 45 to 130 μm^2 , with a mean \pm S.D. of $88 \pm 14 \mu\text{m}^2$. Statistical analysis showed no significant differences between vermis and hemispheres. Morphometrical analysis of CRT-positive UBCs on Rat-303/CRT-stained sections ($n = 151$) showed similar results. UBC cell body cross-section area ranged from 35 to 130 μm^2 , with a mean of $85 \pm 13 \mu\text{m}^2$. Again, no significant differences were found between the distinct cerebellar regions. (The mean \pm S.D. number of cells measured in each sample for both analyses was 48 ± 4 .) In order to determine whether small Rat-303-negative, SOM-positive cells and CRT-immunoreactive UBCs represented the same neuronal population, CRT/SOM double immunohistochemistry was performed.

Calretinin and somatostatin double immunostaining

Double immunohistochemistry for CRT and SOM was performed by means of the indirect tyramide signal amplification method. No CRT/SOM double-stained UBCs were found. This was particularly evident in the vestibulocerebellum. Although UBCs are prominently present in this part of the cerebellum, no difference in

SOM labeling was observed compared to other cerebellar regions. To ensure that the amplification method did not generate false negative results caused by the deposition of reaction products, CRT/SOM double immunostaining was also performed using the S10 SOM antibody. Again, CRT-positive UBCs were SOM negative (Fig. 6A, asterisk). Lugaro cells (Fig. 6A, arrowhead) and deep CRT-positive cells (Fig. 6B, open arrowhead) were also SOM negative. CRT-negative Golgi cells, in contrast, appeared to be SOM positive (Fig. 6A, B, arrow).

mGluR2 and somatostatin double immunostaining

Sections stained for mGluR2 and SOM revealed that all mGluR2-positive Golgi cells (arrow) were also SOM positive (Fig. 7A, B). Besides mGluR2/SOM-positive Golgi cells, a population of large SOM-positive, mGluR2-negative cells was present (Fig. 7A, arrowhead). As these cells exhibited Golgi-like morphology and only Golgi cells were SOM positive (see Fig. 6), they were identified as mGluR2-negative Golgi cells.

mGluR2/SOM double immunohistochemistry again revealed (also see Fig. 5C) the presence of small SOM single-labeled cells (Fig. 7B, open arrow). These cells were thus mGluR2 negative. To estimate the number of small SOM single-labeled cells, they were compared to the population of Rat-303 single-labeled cells in Rat-303/SOM stained sections from two different rats. A total of 111 single-labeled cells was counted, with a mean \pm S.D. of 37 ± 4 cells in each tissue sample; $64 \pm 5\%$ were SOM single labeled, whereas $36 \pm 5\%$ were Rat-303 single labeled.

Fig. 2. Confocal microscopic images from adult rat cerebellar cryosections immunostained for Rat-303 (visualized with Cy3) and calretinin (visualized with FITC). (A) High magnification of two Rat-303-positive cells. They display the classical features of Golgi cells: large round or polygonal cell bodies with radiating dendritic arborizations, extending in all directions. Note the intensely labeled intracellular spots. (B) Rat-303-positive Golgi cells of different sizes. (C) High magnification of a CRT-positive UBC with characteristic paint brush-like dendritic tree. (D) Rat-303- and CRT-positive Lugaro cell located just underneath the Purkinje cell layer. The spindle-shaped soma emits from opposite poles two thick dendrites in the sagittal plane, parallel to the Purkinje cell layer. GL, granular layer; ML, molecular layer; PC, Purkinje cell layer.

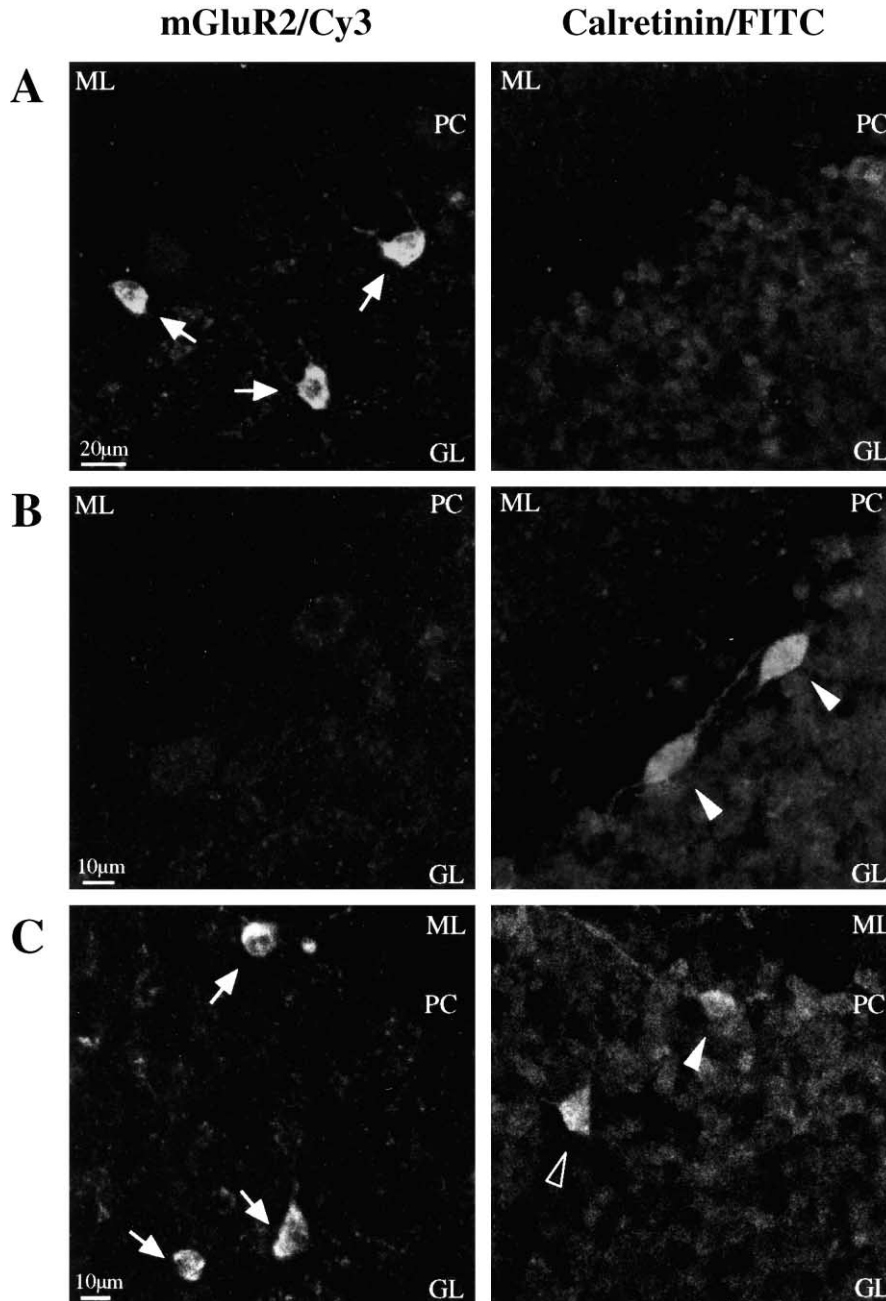


Fig. 4. Confocal microscopic images from adult rat cerebellar cryosections immunostained for mGluR2 (visualized with Cy3) and calretinin (visualized with FITC). (A) mGluR2-positive Golgi cells (arrows) are CRT negative. (B) CRT-positive Lugaro cells (arrowheads) are mGluR2 negative. (C) Large CRT-positive interneurons located in the depth of the granular layer (open arrowhead) are mGluR2 negative. This cell type resembles the neurochemical features of a Lugaro cell (arrowhead), but exhibits Golgi-like morphology. Golgi cells are indicated by arrows. GL, granular layer; ML, molecular layer; PC, Purkinje cell layer.

DISCUSSION

The granular layer of the cerebellar cortex can be considered as an input layer in which mossy fiber afferent input is preprocessed before transmission onto Purkinje cells.^{6,12,20,31,34,60,61} It is composed of two major morphologically and functionally distinct neuronal cell types: granule cells and large interneurons. Granule cells, the most common cerebellar interneurons, are responsible for the transduction of mossy fiber afferent input onto

Purkinje cells.²⁰ Large interneurons modulate this signal transduction directly by inhibiting granule cells (Golgi cells^{6,12}), or indirectly by acting on Golgi cells (Lugaro cells⁹) or mossy fiber input (UBCs^{10,34}). In line with previous reports on rat cerebellar Purkinje cell layer²⁶ and molecular layer interneurons,⁵⁶ this study provides a comparative survey of large interneurons in the rat cerebellar granular layer. Contrary to the above-mentioned reports, both morphological and neurochemical characteristics are included. Table 3 summarizes the

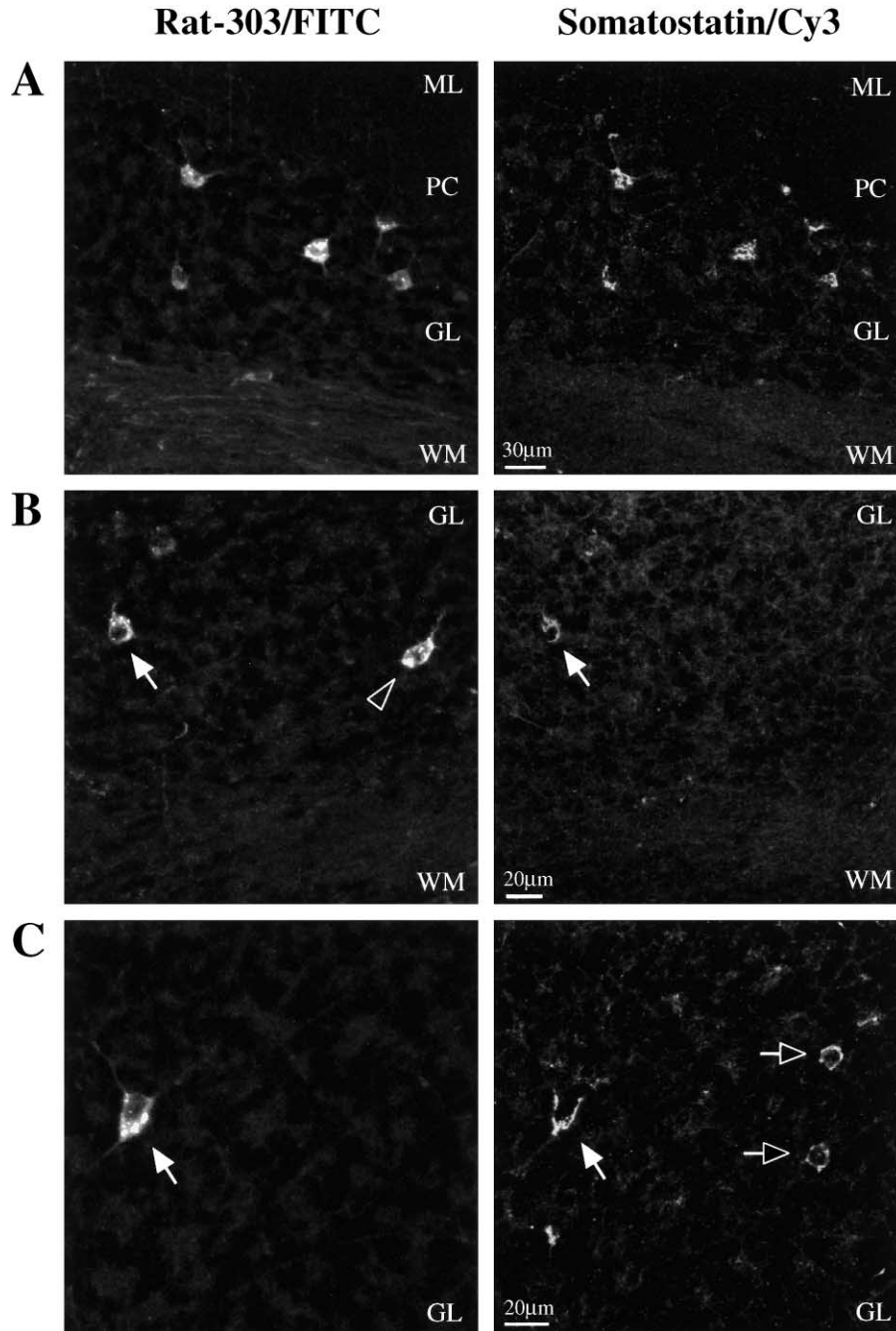


Fig. 5. Confocal microscopic images from adult rat cerebellar cryosections immunostained for Rat-303 (visualized with FITC) and somatostatin (visualized with Cy3). (A) High-magnification overview of the cerebellar cortex showing five large Rat-303/SOM-positive polygonal cell bodies with radiating dendritic arborizations in the depth of the granular layer, characteristic of Golgi cells. (B, C) Besides double-labeled Golgi cells (arrows), Rat-303 (B, open arrowhead) or SOM (C, open arrow) single-labeled neuronal cells are also present in the depth of the granular layer. GL, granular layer; ML, molecular layer; PC, Purkinje cell layer; WM, white matter.

neurochemical coding and reports the relative number of distinct populations of large interneurons. The population of Golgi cells is set as reference (= 100).

Rat-303 and calretinin double immunostaining

Based on Rat-303 and CRT double immunofluorescence histochemistry, three distinct populations of large granular layer interneurons can be observed: cells

immunopositive for Rat-303, CRT or both. Rat-303-immunoreactive neuronal cell bodies display intensely labeled intracellular spots, as reported previously in the cat.⁵¹ The presence of these spots therefore demonstrates Rat-303 antibody specificity in rat cerebellar tissue. Unlike the situation in the cat, Rat-303 staining is not only observed in Golgi cells, but also in neurons with Lugaro-like morphology. Hence, the Rat-303 antibody may exclusively label Golgi cells in the cat,⁵¹ but in

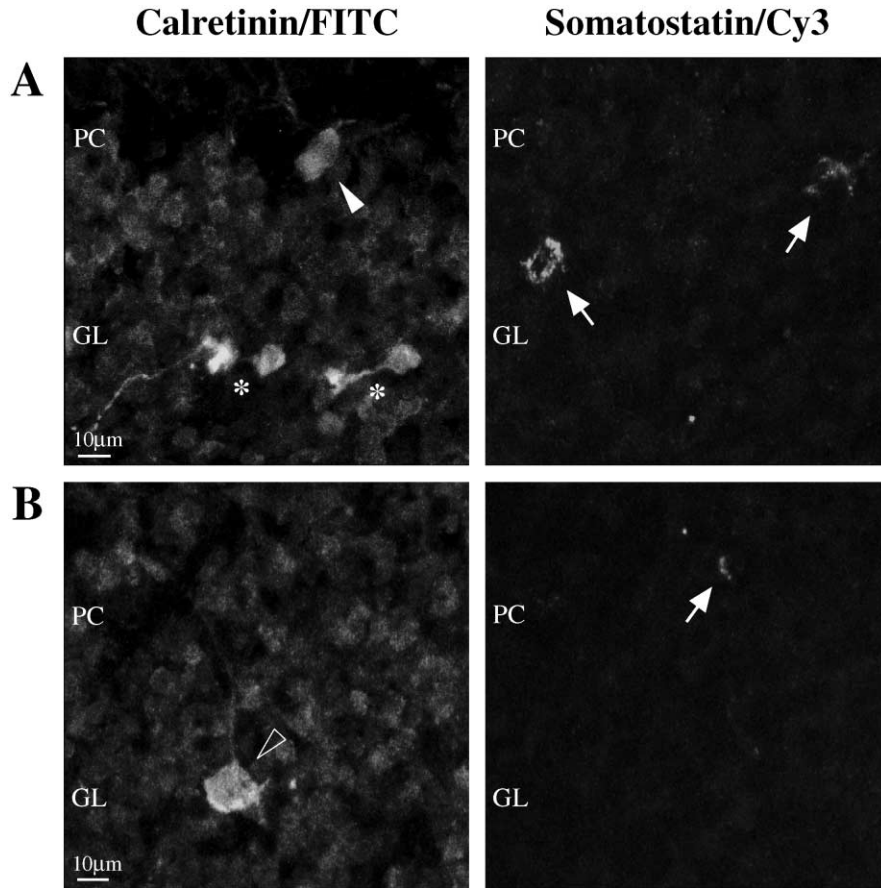


Fig. 6. Confocal microscopic images from adult rat cerebellar cryosections immunostained for calretinin (visualized with FITC) and somatostatin (visualized with Cy3). (A) High-magnification overview of the cerebellar granular layer. CRT-positive UBCs (asterisks) and Lugo cells (arrowhead) are SOM negative. Golgi cells, however, are SOM positive (arrows) and CRT negative. (B) Besides UBCs and Lugo cells, deep CRT-positive Golgi-like cells (open arrowhead) are also SOM negative. The depicted SOM-positive Golgi cell (arrow) is only partially present in the section. GL, granular layer; PC, Purkinje cell layer.

the rat the antibody recognizes both Golgi cells and Lugo cells (see also Ref. 7). This does not imply that the Rat-303 antibody cannot be used for the identification of large granular layer interneurons in the rat cerebellum. When combined with CRT, a useful marker for Lugo cells and UBCs as also demonstrated in rat cerebellar cell cultures,³² the Rat-303 antibody proves to be effective in differentiating Rat-303-positive Golgi cells, CRT-positive UBCs and Rat-303/CRT-positive Lugo cells. Besides Lugo cells, another population of large Rat-303/CRT-positive interneurons is present. They are located in the depth of the granular layer and often display Golgi-like morphology. As shown in Table 3, Lugo cells are found to be six times as numerous as these deep CRT-positive cells (relative numbers 27 and 4.5, respectively). One interpretation of this result, as mentioned by Dino *et al.*,¹¹ is that a subpopulation of Golgi cells is CRT immunopositive. However, it may also point towards the existence of “deep” Lugo cells, as already suggested in the cat using the surface-associated Cat-301 proteoglycan as a Lugo cell-specific marker.⁵¹ Finally, Rat-303/CRT double immunolabeling reveals a peculiar morphological feature of rat cerebellar Golgi cells. In line with earlier reports indicating a morphological heterogeneity within the Golgi cell

population,^{20,43,46} Rat-303-positive Golgi cells could be differentiated according to their location: vermis versus hemisphere.

mGluR2 immunohistochemistry

Rat-303 and mGluR2 double immunohistochemistry reveals two populations of Rat-303-labeled cells. Besides Rat-303/mGluR2 double-labeled Golgi cells, Rat-303 single-labeled cells are present. They represent $68.7 \pm 2.0\%$ and $31.3 \pm 2.0\%$ of Rat-303-positive cells, respectively. In addition, mGluR2 and CRT double immunostaining demonstrates that Lugo cells are mGluR2 negative. Also, the population of large CRT-positive interneurons located in the depth of the granular layer is mGluR2 negative. The absence of mGluR2-IR in the latter population provides another argument in favor of their identity as “deep” Lugo cells. Furthermore, Neki *et al.*³⁸ reported that about 10% of cerebellar Golgi cells display no mGluR2-IR. Indeed, the above-mentioned population of Rat-303-positive, mGluR2-negative cells seems to be composed of Lugo cells, deep CRT-positive cells and CRT-negative Golgi cells. Quantitative analysis reveals their relative contributions as about 20.7%, 3.4% and 7.2%, respectively. In conclusion, as

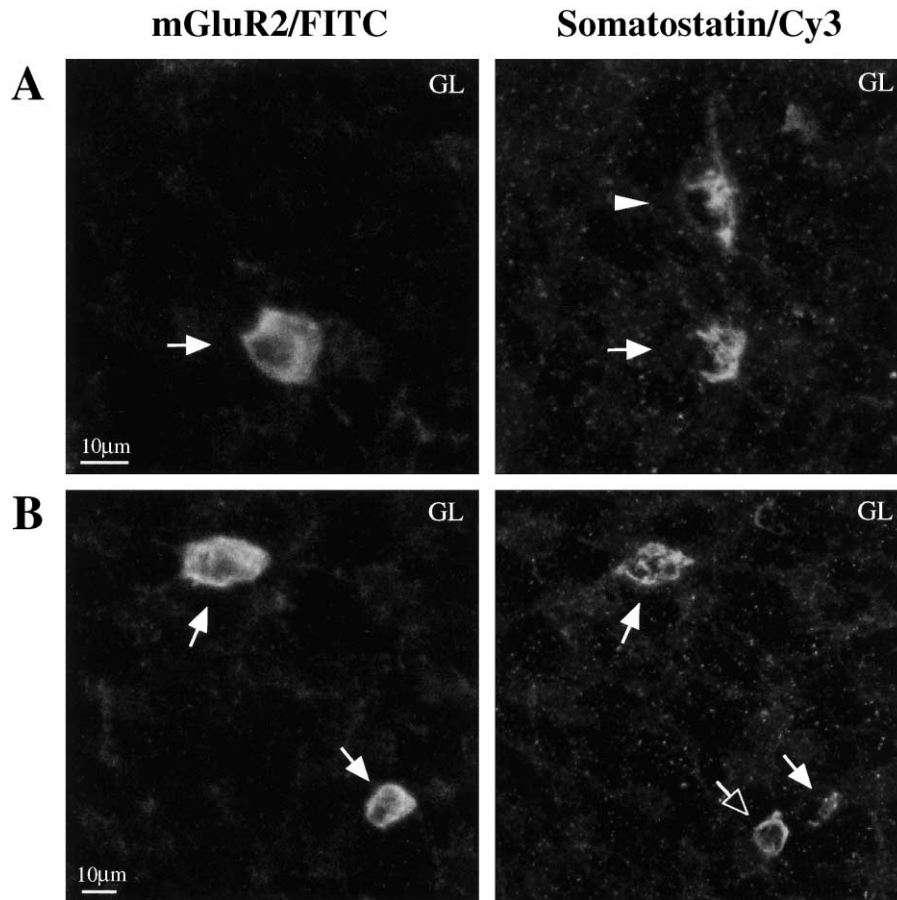


Fig. 7. Confocal microscopic images from adult rat cerebellar cryosections immunostained for mGluR2 (visualized with FITC) and somatostatin (visualized with Cy3). (A) Besides mGluR2/SOM double-labeled Golgi cells (arrow), SOM single-labeled Golgi cells (arrowhead) are present. (B) In addition, a population of small SOM single-labeled cells (open arrow) can be seen. They exhibit no apparent morphological characteristics. Compare their size to mGluR2/SOM double-labeled Golgi cells (arrows), which display, as mentioned before, a marked variation in size. GL, granular layer.

Golgi cells represent 75.9% of Rat-303-positive interneurons, about 90.5% of them (68.7% of the 75.9%) are mGluR2 immunoreactive (also see Table 3). Of course, if deep CRT-positive cells represent Golgi cells as well, this fraction will be somewhat smaller.

Whether mGluR2-negative Golgi cells express mGluR5, as described by Neki *et al.*,³⁸ is not completely clear. Abe *et al.*¹ described the presence of mGluR5 mRNA in a small population of Golgi cells. Based on

an immunohistochemical study with the use of a polyclonal mGluR5 antibody,⁵³ Neki *et al.*³⁸ reported that about 10% of Golgi cells showed mGluR5-IR. This result was confirmed by Negyessy *et al.*,³⁶ using their own mGluR5 antiserum. However, Negyessy *et al.*³⁶ also found Lugaro cells to be mGluR5 immunopositive. Romano *et al.*,⁵⁰ in contrast, were unable to demonstrate mGluR5-IR of Golgi cells with the use of their m5.12 antibody. Also, Dieudonné⁸ questioned the mGluR5-IR

Table 3. Overview of large granular layer interneurons in the adult rat cerebellum based on morphological and neurochemical properties

	Rat-303	Calretinin	mGluR2	Somatostatin	Number†
Golgi cell	+	–	+*/–**	+	100 (90/10)
Lugaro cell	+	+	–	–	27
Unipolar brush cell	–	+	(+)§	–	Variable‡
Deep calretinin-positive cell	+	+	–	–	4.5
Small somatostatin single-labeled cell	–	–	–	+	8

The symbols + and – indicate “immunopositive” and “immunonegative”, respectively. *Ninety per cent of cells are immunopositive for the marker.

**Ten per cent of cells are immunonegative for the marker.

†The relative number is given. The population of Golgi cells is set as reference (= 100).

‡Unipolar brush cells are inhomogeneously distributed. Their number varies substantially among different lobules.

§The observed mGluR2 immunoreactivity in unipolar brush cells is weak.

of cerebellar Golgi cells. These inconsistent findings made us look for an additional mGluR5 antibody to investigate this issue. Unfortunately, our results (with the mGluR5 antiserum from Upstate Biotechnology) yielded an extensive, non-specific immunostaining of most cerebellar structures (data not shown). Consequently, while mGluR2-IR of Golgi cells is prominent,^{37–39,45} mGluR5 staining appears to be highly dependant on the specificity of the antibodies used.

Somatostatin immunohistochemistry

Besides Rat-303, CRT and mGluR2, SOM is used as a neurochemical marker. Rat-303/SOM double immunostaining reveals many large-sized double-labeled neuronal cells within the granular layer. Based on their morphology and in agreement with previous results,^{18,23,58} Rat-303/SOM-positive cells are identified as Golgi cells. In addition, two populations of neuronal cells, immunoreactive for either Rat-303 or SOM, are also present in the depth of the granular layer. Rat-303-negative, SOM-positive cells are almost twice as numerous as Rat-303-positive, SOM-negative cells (relative numbers 8 and 4.5, respectively; Table 3). As SOM-IR is most apparent in cell bodies but less clear in cell processes^{22,23} (also see Fig. 5C), no morphological identification of the population of SOM single-labeled cells can be performed. They appear to be of the same size as CRT-positive UBCs, but do not correspond with these cells (see Fig. 6A). Also, mGluR2/SOM double immunohistochemistry reveals no further information, as these cells exhibit no mGluR2-IR. Therefore, the identity of SOM single-labeled cells remains unclear. Rat-303 single-labeled cells, however, seem to correspond to deep CRT-positive cells. Indeed, mGluR2/SOM and CRT/SOM double immunohistochemistry reveals SOM-IR in both mGluR2-positive and mGluR2-negative Golgi cells, but not in deep CRT-positive cells. Consequently, deep CRT-positive cells exhibit CRT- and Rat-303-IR but are mGluR2 and SOM immunonegative (also see Table 3).

Whether deep CRT-positive cells represent “deep” Lugaro cells or point towards a neurochemical heterogeneity within the population of Golgi cells is unclear. Neurochemical differences amongst Golgi cells, however, have been reported before, not only in the expression of metabotropic glutamate receptors,³⁸ but also in the expression^{41,42} and uptake⁶⁴ of glycine. Indeed, the

observations of [³H]glycine uptake were consistent with the view that there were two biochemically separate populations of Golgi neurons, one transporting glycine, the other GABA.⁶⁴ However, based on immunohistochemistry for glycine and GABA, it was subsequently concluded that a subpopulation of Golgi cells contained both glycine and GABA.^{41,42} More recently, the finding of Golgi cell labeling for the glycine transporter GLYT2 supports a transmitter role for glycine in these neurons.³⁰

CONCLUSION

Taken together, these results indicate that the current classification of large granular layer interneurons into Golgi cells, Lugaro cells and UBCs does not describe the observed neurochemical heterogeneity adequately. However, they do not exclude that the populations of deep CRT-positive cells or small SOM single-labeled cells may represent a (sub)population of Lugaro cells, Golgi cells or UBCs. Indeed, the finding that a given molecule is differentially expressed within a neuronal cell population does not necessarily mean that they are not the same cell type. In order to address the question whether a given molecule is expressed by a subpopulation of the same cell type or really defines a different cell population, further research is required. Still, as demonstrated in the hippocampus,^{16,17,25,55,57} the use of neurochemical markers provides a useful tool for the specification of a particular (sub)population of interneurons. Moreover, neurochemical differences may accompany functional differences. Gulyas *et al.*¹⁶ demonstrated that hippocampal interneurons containing CRT only innervate interneurons, whereas interneurons that do not express CRT innervate principal cells. Whether the observed neurochemical heterogeneity in the cerebellum points towards the existence of different functional populations of large granular layer interneurons beyond the current classification remains to be elucidated.

Acknowledgements—We would like to thank Prof. Dr S. Hockfield (Yale University School of Medicine, USA) and Prof. Dr A. M. J. Buchan (University of British Columbia, Canada) for providing Rat-303 and S10 SOM antibody, respectively, and Dr F. Adriaensen for help with the statistical analysis. This work was supported by an IWT fellowship from the Flemish government (SB 981250) to F.J.G., a concerted research project (99/2/33) of the University of Antwerp, FWO project G.0401.00 and HFSP0 grant RG0091/1999.

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(Accepted 30 January 2001)