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EXTENDING THE CEREBELLAR LUGARO CELL CLASS

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Abstract—We describe here, in Golgi-impregnated rat cerebellar cortex, a new group of large granular layer neurons. These cells have a globular soma located at variable depths in the granular layer, and three to four long radiating dendrites coursing through the three layers of the cortex. The axon projects more or less directly into the molecular layer, where it expands in a local plexus of oblique and tortuous thick collaterals ascending through the major part of the layer. Interestingly, the axons of several of these cells give off a collateral that courses for a long distance in the transverse direction, just above the Purkinje cell somata, parallel to the parallel fibers.

While the granular layer location and the polymorphous somato-dendritic pattern of these cells is reminiscent of that of Golgi cells, their axonal pattern is clearly of the same type as that of another large granular layer interneuron, the Lugaro cell. Moreover, double anti-calretinin and anti-calbindin immunolabellings show that Lugaro cells as well as some globular somata dispersed in the granular layer are both calretinin-positive and in close apposition with numerous calbindin-positive varicosities of Purkinje cell axon recurrent collaterals. These latter are known from previous ultrastructural studies to be pre-synaptic to Lugaro cells.

The common granular layer location and calretinin labelling, the striking similarity in axonal projection pattern, and the important common recurrent afferentation by Purkinje cell axons strongly argue in favor of the classification of these globular interneurons as a subgroup of a widened Lugaro cell type.

Key words: cerebellum, Golgi technique, interneuron, molecular layer axonal projection, calretinin.

Morphological classification of neurons, historically based on Golgi-impregnated material, rely on three complementary characteristics: shape and location of the soma, distribution of the dendritic arborization, and projections of the axonal plexus. As each of these basic features is subject to a degree of variability, it is absolutely necessary that, for a given cell, all three criteria be ascertained so it can be reliably classified in a particular neuronal population, thus indicating a similarity of input and output connections for each member of the neuronal class and pointing towards a comparable functional role in the neuronal network. More recently, the advent of immunocytochemistry has added a degree of complexity to pre-existing classifications, as this technique often discloses a molecular heterogeneity within a well-established, morphologically homogeneous, neuronal type.

In contrast with textbook simplifications, neuronal cell classes of the mammalian cerebellar cortex appear to be more numerous than previously thought, and the complexity of this structure does not yet seem to be entirely unravelled. This is particularly true for the large neurons of the granular layer, the classification of which now includes three distinct types of interneurons: the unipolar brush cell, the Golgi cell, and the Lugaro cell.

The unipolar brush cell has only recently been described with tritiated thymidine, immunocytochemical, and Golgi techniques (Altman and Bayer, 1977; Hockfield, 1987; Rogers, 1989; Munoz, 1990; Mugnaini and Floris, 1994; Berthié and Axelrad, 1994; Diño et al., 1999). It has a rounded soma with a very specific single thick dendrite ending in a brush-like formation, and a contorted, branched, axon confined within the granular layer, presenting rosette-like enlargements. The rosettes contact granule cell dendritic digits and brushes of other unipolar brush cells (Diño et al., 2000). These neurons appear to constitute a rather homogeneous neuronal type, by both morphological and neurochemical criteria, but, in contrast with the two other large granular interneurons, ubiquitously distributed in all the folia of the cerebellar cortex, they are mainly found in the vestibulocerebellum.

Described since the advent of silver impregnation studies of the brain, the Golgi cells (Golgi, 1874; Ramón y Cajal, 1911) have a rounded or polygonal soma, scattered throughout the granular layer, with radiating dendrites often reaching the upper molecular layer, and a very distinctive thin and profuse axonal plexus, exclusively spanning the granular layer. However, based on morphological or immunocytochemical features, the heterogeneity of this cell class has frequently been advocated. Anatomical subtypes have been described on the basis of somato-dendritic size and location inside the granular layer, as well as on the spatial extent of the axonal arbor (Ramón y Cajal, 1911; O'Leary et al., 1968; Mugnaini, 1972; Palay and Chan-Palay, 1974; Braak and Braak, 1983). Different immunoreactive subpopulations have also been shown to exist, in relation

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Abbreviations: PC, Purkinje cell.

with either different neurotransmitters (Ottersen et al., 1987, 1988; Illing, 1990; Ikeda et al., 1991), neuropeptides (Schulman et al., 1981; Johansson et al., 1984; Geurts et al., 2001), or a differential expression of metabotropic glutamate receptors (Neki et al., 1996; Négyessy et al., 1997; Geurts et al., 2001). The significance of this heterogeneity within the Golgi cell population remains problematic, as neurochemical subtypes do not seem to correspond to anatomically defined subtypes. Moreover, identification of immunolabelled neurons is in some instances subject to caution, since many neurochemical markers are often restricted to soma and proximal dendrites and do not label the axonal arborization, which is an essential feature for the secure assignment of a given neuron to the Golgi cell class.

The intermediate cell of Lugaro (Golgi, 1874; Lugaro, 1894; Ramón y Cajal, 1911; Fox, 1959; Palay and Chan-Palay, 1974; Sahin and Hockfield, 1990), a fusiform neuron with long horizontal dendrites lying just beneath the Purkinje cell (PC) layer, was until recently rarely listed as a component of the cortico-cerebellar network, due to the uncertainty about its axonal projections and absence of physiological recordings. Recent studies have confirmed that the Lugaro cell is a distinct cortico-cerebellar interneuron with unique somato-dendritic, axonal, and physiological features (Lainé and Axelrad, 1996, 1998a; Dieudonné and Dumoulin, 2000). In particular, our group (Lainé and Axelrad, 1996, 1998a) demonstrated that, in spite of an important variability in the trajectory of the axon, Lugaro cells always project inside the molecular layer, where they form inhibitory synapses exclusively upon interneurons, basket cells, stellate cells and putative Golgi cells, but never on Purkinje cells. We also confirmed and extended previous descriptions of long axonal collaterals coursing in the mediolateral direction, parallel to the parallel fibers (Lugaro, 1894; Fox, 1959). Data from patch-clamp recordings of individual Lugaro or Golgi cells in rat cerebellar slices also point towards such connections (Dieudonné and Dumoulin, 2000).

The present report describes a group of large granular layer interneurons with distinctive morphological characteristics. All these cells have somato-dendritic features strongly resembling that of Golgi cells, but an axon strikingly similar to that of Lugaro cells. These neurons thus set a problem of classification and raise the question of the extent of morphological variability within a given neuronal class.

EXPERIMENTAL PROCEDURES

In accordance with the European Communities Directive 86/609/EEC, the strict minimum of experimental animals were used and all efforts were made to avoid any suffering during the perfusion protocol.

Golgi stainings

Young albino male rats (C.E.R.J., Le Genest-St-Isle, France) were deeply anesthetized and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. The cerebellum was processed according to the rapid Golgi method (for details see Lainé and Axelrad, 1994), followed by a nitrocellulose embedding. It was then cut into 100-µm parasagittal serial sections, mounted between two coverslips to allow obverse–reverse analysis of the impregnated cells. Observations at high magnification with Nomarski optics, camera lucida drawings, and photomicrographs were performed with a Zeiss Axioplan microscope.

Immunocytochemistry

Adult albino rats ($n = 2$) were first perfused with saline, followed by 8% paraformaldehyde. Single peroxidase and double fluorescent immunolabellings were performed on 40-µm-thick, vibratome-cut, free-floating parasagittal sections of the cerebellar vermis.

Single-labelled sections were first incubated overnight in a polyclonal rabbit anti-calretinin antibody (1/5000, SWant, Bellinzona, Switzerland). After incubation (2 h) in a secondary biotinylated anti-rabbit antibody (1/200, Vector Laboratories, Burlingame, CA, USA), the immunoreactivity was revealed by an avidin–biotin complex reaction (Vectastain Elite, Vector Laboratories), using 0.005% diaminobenzidine as the chromogen.

Double-labelled sections were first simultaneously incubated in the anti-calretinin (1/1000) and a monoclonal mouse anti-calbindin (1/1000, Sigma, St. Louis, MO, USA) antibodies, then in a biotinylated horse anti-mouse antibody (1/200, Vector Laboratories; 2 h), and finally, for 2 h, in a mix of FITC anti-rabbit secondary antibody (1/200, Silenus Laboratories, Victoria, Australia) and streptavidin–biotin–Texas Red (1/200, Gibco BRL, Invitrogen, Carlsbad, CA, USA). Control sections, processed with omission of the primary antibodies, did not show any specific staining. Immunoperoxidase sections were observed and photographed on a Leica DMRB microscope. Fluorescently labelled sections were observed under a laser-scanning confocal microscope (Leica TCS 400), and images were generated with Adobe Photoshop 5.0.

Measurements and 3D reconstructions

The depth location of the Golgi or immunocytochemical stained neuronal somata was measured directly with an oculomicrometer at the ×100 objective magnification. The area of the largest cross-section of neuronal somata was manually calculated from camera lucida drawings (final magnification ×1280).

3D computer reconstructions of Golgi-impregnated neurons were done with a fil-de-fer program written in the laboratory by Dr A. Crivat, as detailed in Lainé and Axelrad (1994).

RESULTS

The description we provide here is based on a series of 15 Golgi-stained neurons with an impregnated axonal arborization. These were selected by a thorough scanning of serial parasagittal sections of the vermises of a collection of young rat cerebella aged 12–30 post-natal days. Cells with an axon impregnated beyond the initial segment were only found in eight rats aged 16–24 days. This could be due to the development of a myelin sheath around these axons in older animals, thus leading to a more difficult impregnation by the Golgi method. Considering the global morphology of their somata (see Table 1), these neurons will, provisionally, be termed ‘globular cells’.

Location and somato-dendritic features

The folial distribution of globular cells as well as nu-

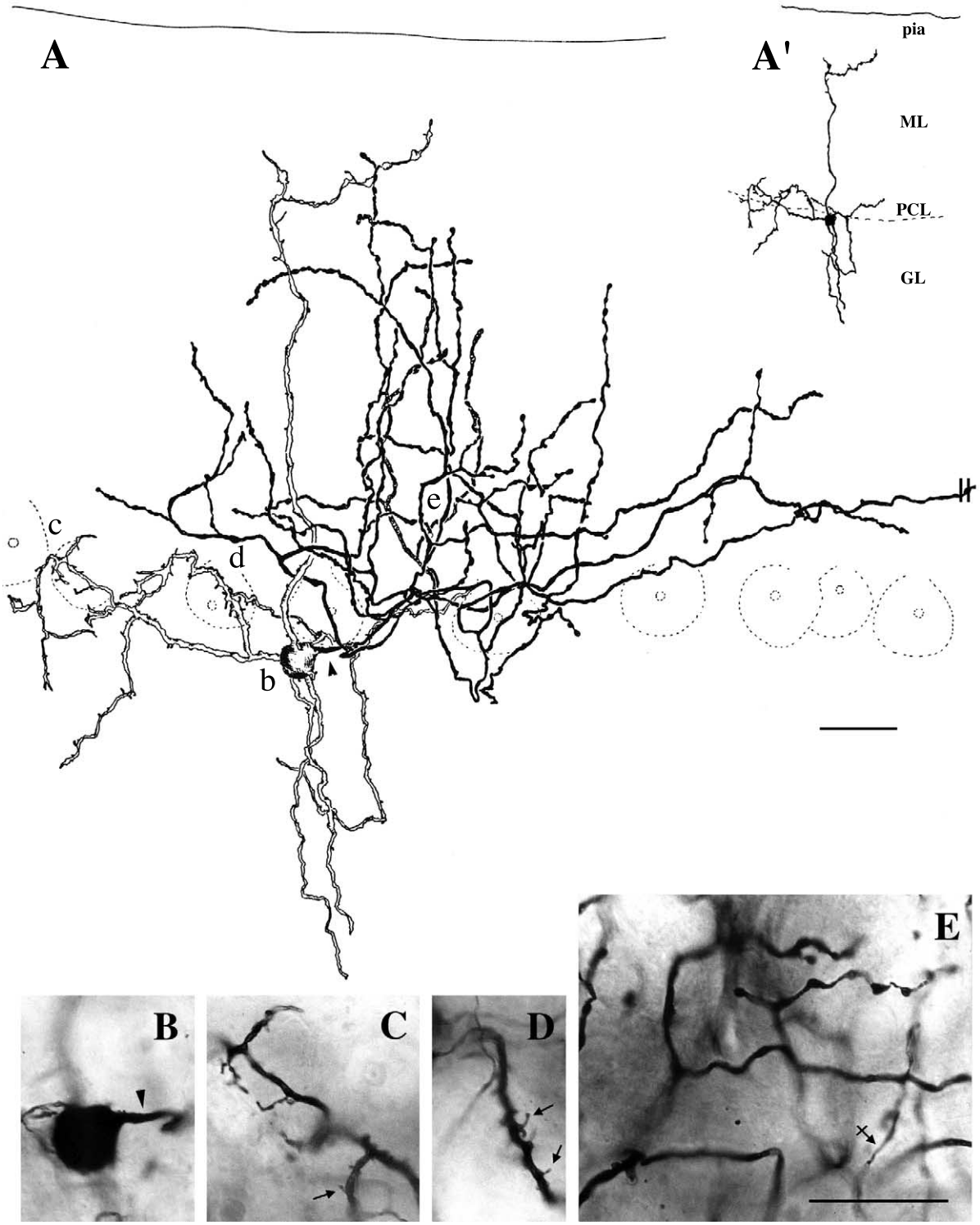

Fig. 1. Camera lucida drawing and photomicrographs of a globular neuron (rapid Golgi impregnation). (A, A′) The spherical cell body is located at the upper border of the granular layer. The dendritic arborization, outlined in A′, displays a radiating pattern with dendrites emerging from the soma and spreading at right angles through the three layers of the cerebellar cortex. Note that if the axon were not impregnated, such a neuron could be confused with a Golgi cell. The axon emerges with a thick initial segment and takes an ascending course. It branches profusely at the level of the supraganglionic plexus and ascends through the inferior two thirds of the molecular layer. A horizontal branch extends parasagittally for a long distance in the low molecular layer. It ends about 70 µm farther than the parallel bars. (B–E) Photomicrographs of some details of the cell (b–e in the camera lucida drawing). (B) The spherical cell body with the thick initial segment of the axon. (C, D) Two dendritic branches, coursing in the PC layer, display numerous peduncular spines (arrows). (E) Beaded collaterals of the axon in the molecular layer. The diameter of these branches is far thicker than that of the nearby ascending axon of a granular cell (crossed arrow). Parasagittal sections of 21-day-old rat cerebellar vermis, folium III. Scale bars = 25 µm (bar in E is for B–E). In this and subsequent figures: dotted line indicates the inferior border of PC layer and arrowhead points to the axonal initial segment. Pia = pia mater, PCL = PC layer, GL = granular layer, ML = molecular layer.

merical data concerning characteristics of their somata and number of dendrites are detailed in Table 1. These cells are found scattered in the different folia of the vermis, thus appearing to be distributed throughout the cerebellar cortex. It must nevertheless be noted that about half of the neurons in our series are located in the vestibulo-cerebellum, but, due to the small size of our sample, this observation may not be generalized for the entire population of globular neurons.

The soma nearly always (14/15) lies in the upper third of the granular layer (Figs. 1, 2 and 4) or inside the PC layer. In all globular cells the soma has a multipolar morphology, with radiating dendrites, a pattern highly reminiscent of that of Golgi cells. The pericaryon is more or less rounded, most often globular, and has a mean great axis of 14.5 μm and a mean somatic area of 129 μm^2. Nevertheless, some somata are more or less triangular or polyedric. The outstanding majority of our neurons (12/15) have no more than three to four dendritic trunks (see Table 1) and, frequently, the neurites draw a cross-like figure: three thick dendrites extend at right angles from the three cardinal points of the soma, whilst the axon emerges at the fourth (Figs. 1, 2 and 4). The dendritic arbor appears to have no stereotyped spatial spread. One or two dendrites are always seen ramifying throughout the molecular layer, but the others may extend in the PC or/and granular layers. In the cells of Figs. 1 and 4, the dendritic arborization spans the three layers, whilst, in the cell of Fig. 2, no dendrite is seen to extend inside the granular layer. As a rule, the dendrites are rather poorly ramified, and their shafts are moderately covered with spines. These can be either pedunculated, with a thin neck and a globulous ending, most frequently on the molecular layer dendrites (Figs. 1D and 2C), or sessile and bulbous (Fig. 1C). In contrast with Golgi cells, the dendritic tree of these neurons has a more restricted spatial spread in the mediolateral direction, frequently less than 100 μm, a feature that could be used to differentiate both cell types.

The axon

The axon usually originates with a rather thick initial segment, directly from the pericaryon (13/15) or from a proximal dendrite. The axonal stem can either directly ascend into the molecular layer where it ramifies (Figs. 1, 4), or it may first engage in a descending course (Fig. 2), giving off several thick collaterals which, after making a hairpin turn, re-ascend towards the molecular layer in which they branch. Before reaching the molecular layer, these collaterals usually give off short beaded fibers ending inside the granular layer (Figs. 1, 2).

The axonal arborization in the molecular layer is quite intricate with rather profuse ramified branches. These fibers have a more or less homogeneous diameter, in the order of 1 μm, and exhibit numerous beaded swellings. They are mainly distributed in the inferior two thirds of the layer (Figs. 1 and 2) but, in some cases, may span the entire height of the molecular layer, reaching the pia mater (Fig. 4).

Most interestingly, five of these cells exhibit an axonal collateral which runs for long distances in the supraganglionic plexus, just above the apexes of PC somata. These collaterals follow a transverse direction, parallel to the great axis of the folium, i.e. exactly in the same direction as the parallel fibers. One such collateral, which has been followed and computer-reconstructed through five 100-μm-thick parasagittal serial sections, is shown in Fig. 3B, C. It has a much larger diameter than adjacent impregnated parallel fibers and emits, from time to time, rather short, and sometimes branching, beaded fibers towards the PC or low molecular layers. Such a collateral is identical to similar fibers found in Lugaro cell axons (Lugaro, 1894; Fox, 1959; Lainé and Axelrad, 1996). Moreover, as in the case of Lugaro cells, this type of long collateral does not seem present in all the globular neurons of our series. This may be due to an arrest of silver impregnation, or to the difficulty to spot and individualize such fibers in very richly stained Golgi

Table 1. Distribution and somato-dendritic characteristics of the 15 Golgi-impregnated globular cells

Folium	Location[a]	Shape	Great axis (GA) (μm) $m = 14.54$ $\sigma = 2.54$	Small axis (SA) (μm) $m = 11.06$ $\sigma = 2.22$	GA/SA $m = 1.39$ $\sigma = 0.54$	Somatic area (μm^2) $m = 129.04$ $\sigma = 30.94$	Number of dendritic trunks $m = 3.66$ $\sigma = 0.82$
I	98%	spherical	12.7	11.2	1.05	125.39	3
II	100%	ovoid	14.4	9.7	1.48	111.32	5
II	100%	spherical	14.4	12.7	1.13	130.71	5
IIIb	95%	spherical	11.5	10.8	1.06	90.33	4
IV	100%	elliptical	19.2	6.8	2.82	118.69	4
IV	100%	spherical	14.4	13	1.11	137.46	4
IV	85%	triangular	11.7	11.3	1.04	96.09	3
VIa	83%	elliptical	14.1	10.9	1.29	135.50	3
IXc	100%	spherical	11.9	10.2	1.17	90.49	3
IXc	82%	triangular	14.4	12.7	1.13	179.55	5
X	97%	spherical	17.8	14.4	1.24	182.42	3
X	65%	polyedric	19.7	7.9	2.49	141.97	4
X	97%	spherical	12.7	10.2	1.25	99.11	3
X	90%	ovoid	14.4	9.3	1.55	96.95	3
X	83%	spherical	14.8	14.8	1.00	166.63	3

[a]Distance of the center of the globular cell soma from the border of the white matter expressed as a percentage of the total height of the granular layer.

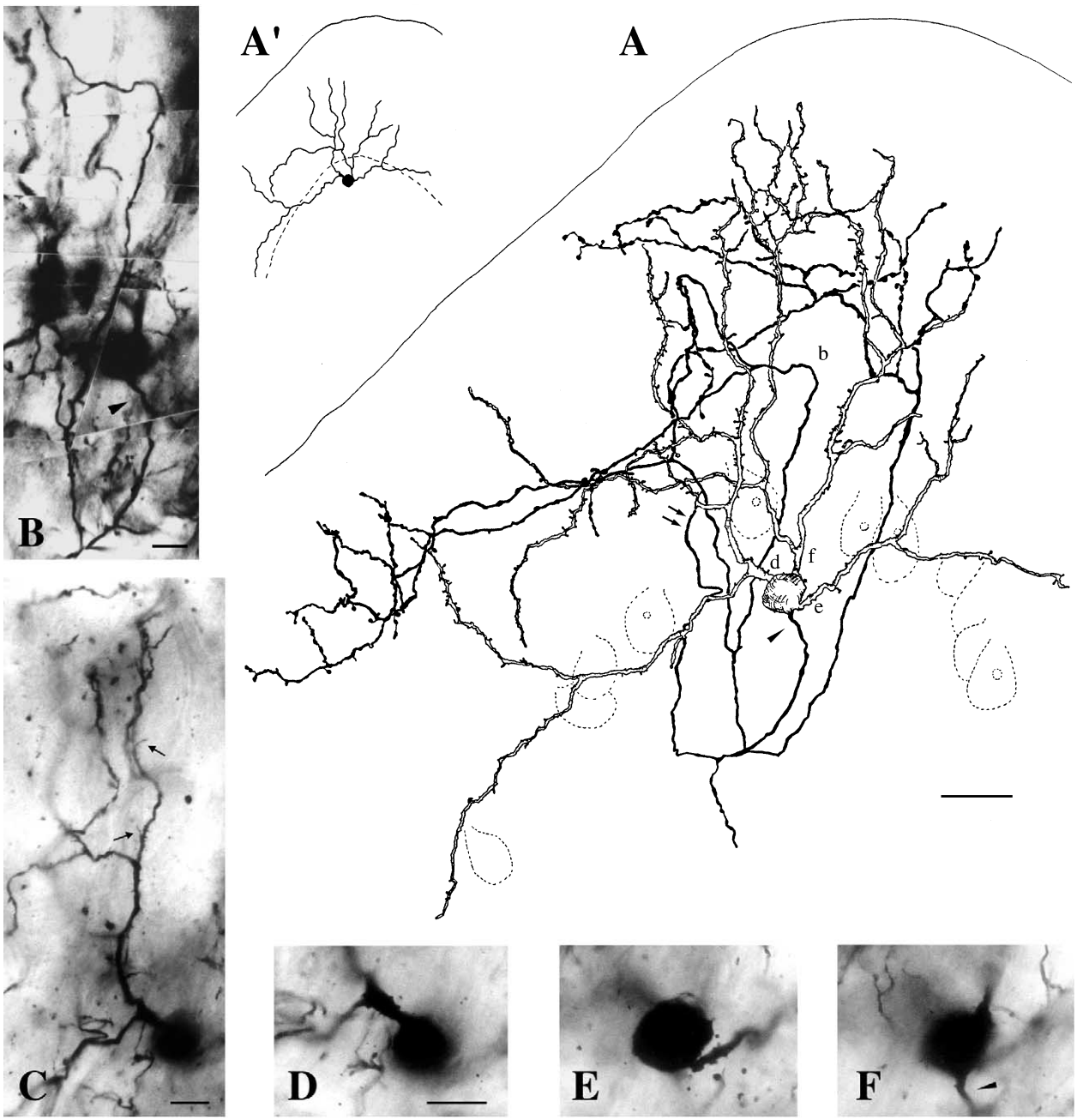

Fig. 2. (A, A′) Camera lucida drawings of a globular cell reconstructed from six parasagittal 100-µm-thick serial sections. The soma and dendrites are restricted to a single section, while the axon extends through all the six sections. The spherical cell body is located in the upper granular layer. The radiating pattern of dendrites appears clearly in the somato-dendritic outline of inset A′. The axon first descends obliquely into the middle granular layer and gives off three collaterals, which directly ascend into the molecular layer where they ramify just above the parent cell. One of these collaterals ascends up to the apexes of PC somata, and can then be followed through five successive sections (see Fig. 3). For the sake of clarity, only the beginning of its trajectory is figured here (double arrows). (B) Photomontage showing the continuity of the primary shaft of the axon with the ascending trajectory of one of its molecular layer collaterals (b in A). (C) Photomicrograph of a dendrite ascending vertically throughout the molecular layer, studded with thin long-necked spines (arrows). (D–F) High magnification photomicrographs, at different focal depths, showing the four neuritic trunks which emerge from the four cardinal directions of the spherical cell body. 18-day-old rat vermis, folium X. Scale bar = 25 µm for A, 10 µm for B–D. Bar in D is for D, E.

sections. Consequently, we cannot at this stage ascertain that these long transverse collaterals are systematically present in this type of neuron.

We used 3D computer reconstructions to analyze the spatial spread of the axonal arborization and, specifically, its longitudinal extension. Fig. 3 shows reconstructions of the axonal plexus of the globular cell of Fig. 2 in the three orthogonal planes. In Fig. 3B, C, the axon is displayed in the transverse and horizontal planes, respectively. It shows two clearly distinct domains. A tangled plexus spreads throughout the molecular layer just above the somato-dendritic field of the cell of origin, this latter

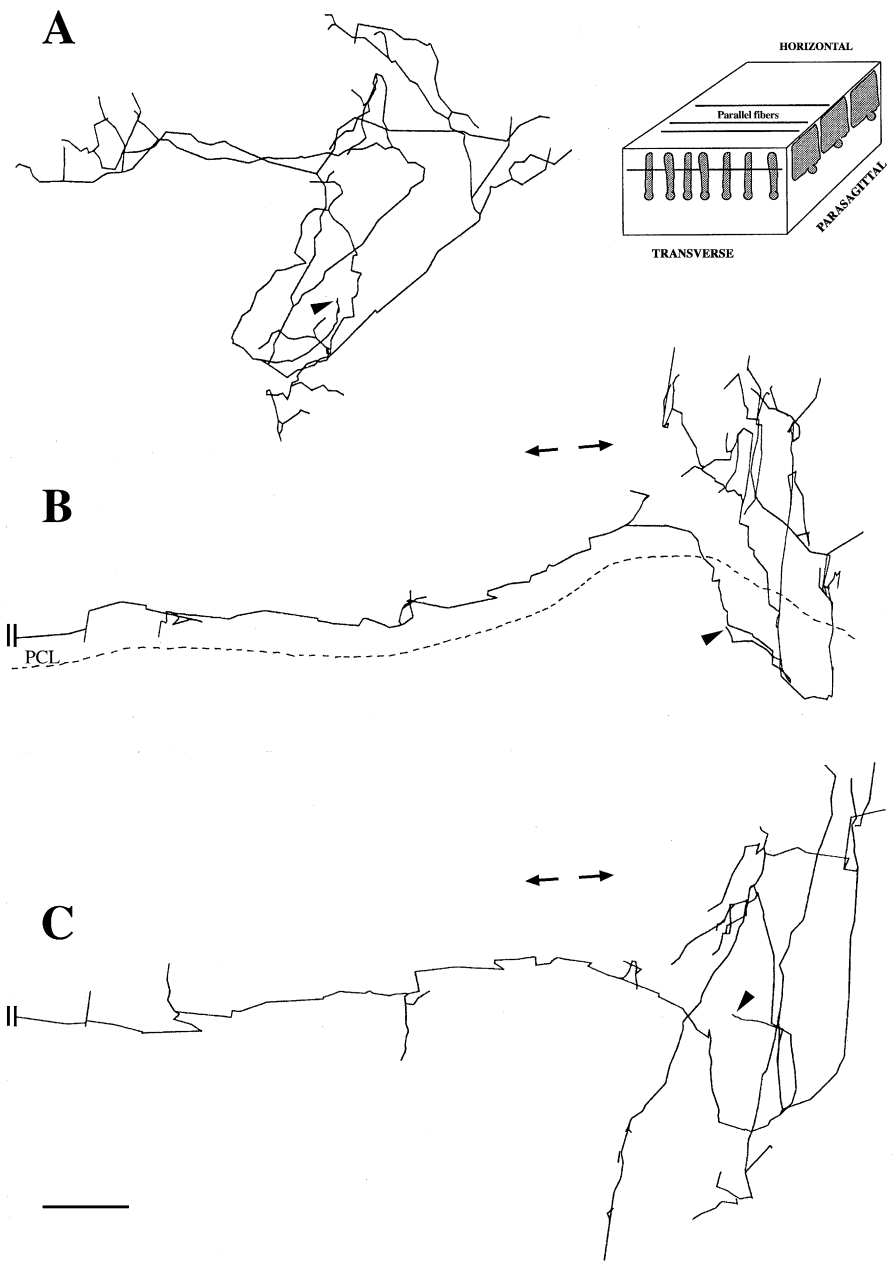

Fig. 3. 3D computer reconstructions of the entire axonal arbor of the globular cell of Fig. 2. The inset at the upper right schematizes the three orthogonal planes of visualization. (A) View of the reconstructed axon in the parasagittal plane, as originally drawn. (B) A 90° rotational view in the transverse plane. The axonal arborization displays two clearly distinct domains: an intricate local plexus spreading in the molecular layer, just above the parent cell, and a long-projecting collateral. This latter courses in the lower molecular layer in the same mediolateral direction as the parallel fibers (orientation indicated by paired opposite arrows) just above the PC somata, and gives off sparse short collaterals at regular intervals. Its 3D length, measured from the axon hillock, is 685 µm, but it is certainly longer as its continuation is obscured by crystalline artifacts (parallel bars). (C) View in the horizontal plane. The local plexus displays a general elliptical envelope, with a parasagittal great axis and a transverse short axis. In this plane, the distal projection also appears to run exactly in the same direction as parallel fibers, demonstrating that both fiber types have strictly parallel courses. Scale bar = 50 µm.

being indicated by the location of the axonal initial segment (arrowhead). This arborization seems devoid of any evident geometrical regularity, and when seen in the horizontal plane (Fig. 3C) appears confined to an elliptical envelope with a longer parasagittal axis and a shorter latero-lateral span. A branch leaves this local plexus and takes a latero-lateral direction just above the PC layer with some sparse collaterals along its course. It is quite long, with a 3D length exceeding 685 µm from axon hillock to tip (as calculated from the computer reconstruction). Seen in both transverse and horizontal planes its direction is exactly the same as that of the parallel fibers (indicated by paired opposite arrows). A reconstruction in the transverse plane of the axon of

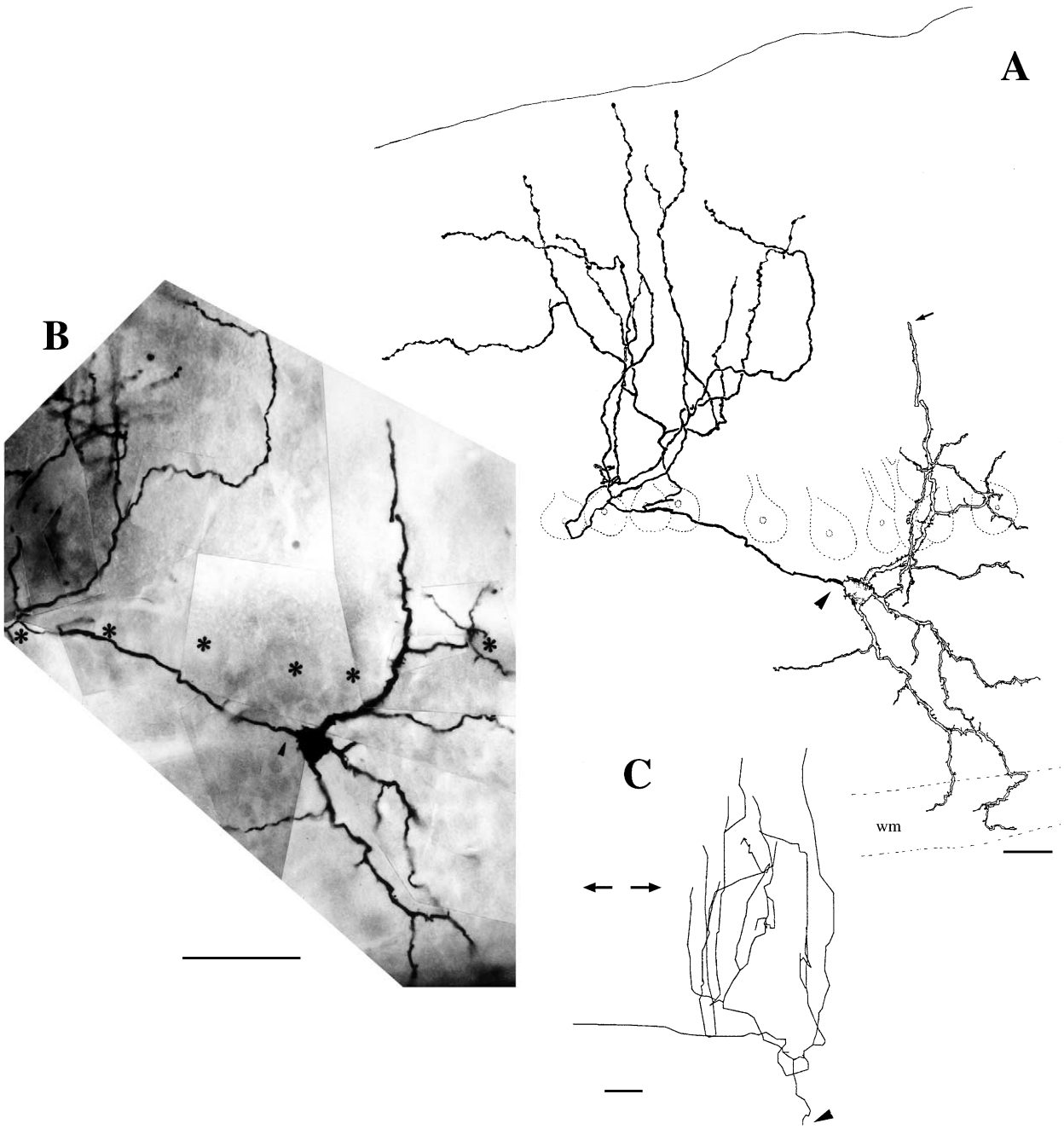

Fig. 4. (A) Camera lucida drawing of another globular cell, reconstructed from three parasagittal sections. The ovoid soma is located in the outer third of the granular layer, at a distance below the PC somata. Three dendrites emerge at right angles, the axon emerging from the fourth cardinal point. The dendrites span mainly the PC and granular layers, even reaching the white matter (wm). The vertical dendrite in the molecular layer is probably incompletely stained, considering its blunt termination (arrow). The axon has an oblique but direct course to the PC layer where it gives off a series of intricate collaterals ascending through nearly the whole thickness of the molecular layer. The present axonal arborization is less dense than is the case for the cells in Figs. 1, 2, certainly due to an incomplete impregnation. (B) Photomontage of the same cell as in A, evidencing the axonal projection into the molecular layer. Indeed, the soma location and the dendritic pattern look quite similar to those of a Golgi cell, but the axon, instead of ramifying profusely in the granular layer as is the case for Golgi cells, ascends directly into the PC layer (asterisks indicate PC somata). (C) 3D computer reconstruction of the axon, rotated in a transverse plane, showing the same general organization as in Fig. 3: a local plexus just above the parent cell, and a longitudinal collateral which courses distally just above the PC somata, in the same direction as the parallel fibers (paired opposite arrows). 23-day-old rat vermis, folium VI a. Scale bars = 25 μm.

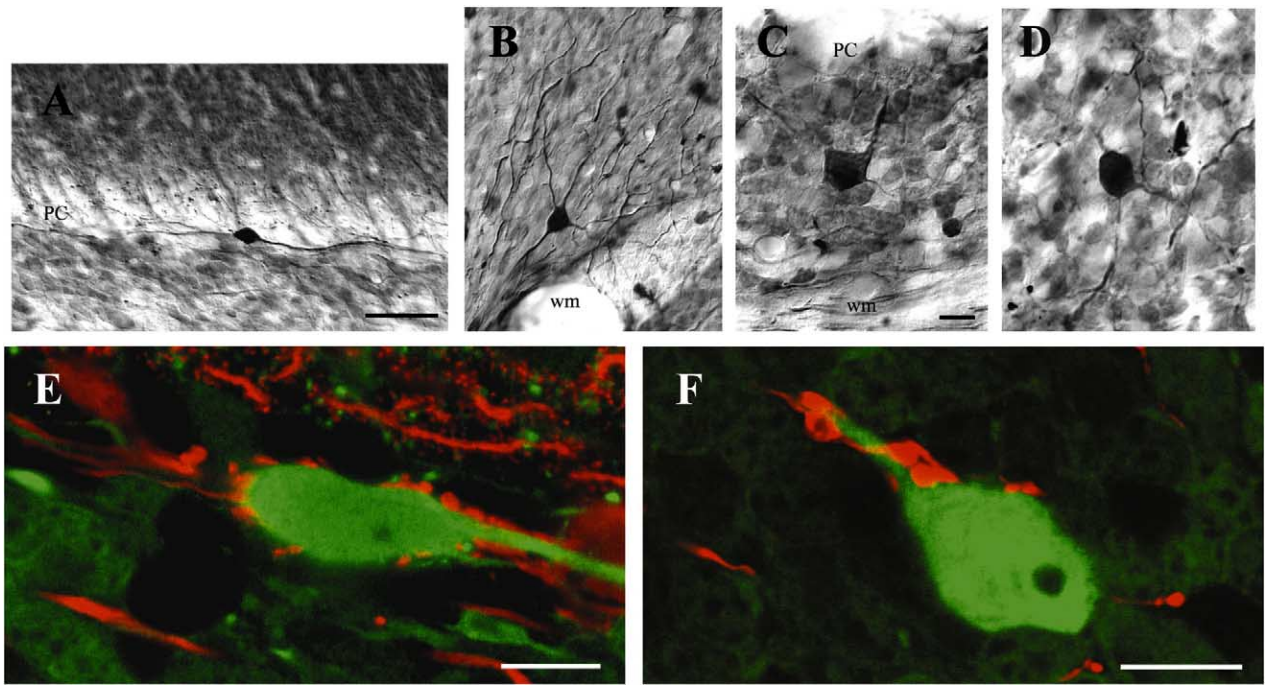

Fig. 5. Immunocytochemical labelling shows that Lugaro and globular neurons are both calretinin-positive and contacted by recurrent collaterals of Purkinje cell axons. (A–D) Anti-calretinin immunoperoxidase labellings. (A) A typical Lugaro cell displaying a fusiform soma and long bipolar dendrites, located immediately below the layer of calretinin negative PC somata (PC). (B–D) Three calretinin-positive globular cells. In B, the soma, located near the white matter (wm), gives rise to four radiating neurites. The polygonal cell body in C and the ovoid soma in D are located in the mid-granular layer. Note that when, as is the case for these two neurons, only soma and proximal dendrites are labelled, they appear quite similar to and can be confused with Golgi cells (see also Fig. 1A, A′). (E, F) High magnification confocal views of double calretinin (FITC) and calbindin (Texas Red) immunolabellings. (E) A calretinin-labelled Lugaro cell within the PC layer. Its soma and a proximal dendrite appear nearly completely outlined by the calbindin-labelled varicosities of PC axon recurrent collaterals. (F) A calretinin-positive globular neuron in the depth of granular layer also displays close appositions of PC recurrent collaterals as large varicosities linked by thin axonal threads. Scale bars = 50 μm in A, B and 10 μm in C–F. Bar in A is for A, B, and bar in C is for C, D. Adult Wistar rats.

another globular cell, illustrated in Fig. 4, shows a similar pattern (Fig. 4C).

The axonal arborization of these neurons thus frequently, if not systematically, comprises two types of projections in the molecular layer: a profuse proximal projection, spreading in the same parasagittal zone as the soma and dendrites, and a poorly branched transverse projection, extending longitudinally in the direction of the folial axis, coursing in the lower molecular layer for several hundred microns, and giving few short horizontal collaterals distant from the parent cell.

Immunocytochemical analysis of globular and Lugaro neurons

The great similarity between the axonal projections of these Golgi-impregnated globular cells and those of Lugaro cells suggests a common efferent connectivity and, possibly, identical functional roles. We then sought other morphological features that could be common to these two kinds of neurons. Rat Lugaro cells are characterized by both a high cytoplasmic content of the calcium-binding protein, calretinin (Rogers, 1989; Arai et al., 1991; Floris et al., 1994) and a heavy pre-synaptic afferentation by PC axon recurrent collaterals (Palay and Chan-Palay, 1974; Lainé and Axelrad, 1998b). The PCs contain another type of calcium-binding protein marker, calbindin (Baimbridge and Miller, 1982). We therefore used anti-calretinin and anti-calbindin immunolabellings to analyze if globular cells presented the two same above-mentioned features as Lugaro neurons.

In the rat cerebellar cortex, anti-calretinin labelling produces a faint staining of granule cells' bodies and of their axons, the parallel fibers. In contrast, two large granular layer cell types are strongly labelled: the unipolar brush cells, clearly recognizable due to their unique and thick dendrite ending in a brush-like formation, and the Lugaro cells, located at the interface between the granular cell layer and the PC layer and readily identified by their fusiform soma and elongated dendrites (Fig. 5A, E). The Lugaro cell axons are also clearly labelled, and appear as intricate and beaded oblique fiber plexuses in the molecular layer. Their morphological features correspond to the axonal pattern described with Golgi stains (Lainé and Axelrad, 1996). A third type of neuron is also found to be calretinin-positive. These cells are few in number, have a globular, triangular or polygonal soma with generally three to four radiating dendrites, and are located in all the different folia, with about one third in the vestibulo-cerebellum. They lie at varying depths in

the granular layer (Fig. 5B–D, F). Of 28 cells found on a single vermal section, 75% were located in the PC layer or upper third of the granular layer, 21.4% in the middle third of the granular layer and 3.6% in its lower third. On some occasions their dendrites appear stained and can be seen radiating through the granular layer (Fig. 5B), giving these globular neurons a Golgi cell-like somato-dendritic pattern.

The somato-dendritic morphology and distribution of these calretinin-positive globular neurons thus appear to fit well the features of the Golgi-impregnated globular cells, as described above. We calculated the relative densities of Lugaro and globular neurons in calretinin-immunostained sections, and found a ratio of about one calretinin-positive globular cell for 7.7 Lugaro cells ($n = 763$ neurons). However, the number of the calretinin-positive globular neurons is likely underestimated. Indeed, to ensure an unambiguous classification and no overlap with partially cut unipolar brush cells or bipolar Lugaro neurons, only globular neurons with clearly visible somata and dendritic emergences were taken into account.

As noted above, the anti-calbindin antibody is a specific marker of PCs (Baimbridge and Miller, 1982). It labels, intensely and exclusively, all parts of these neurons: soma, dendritic trunks and spiny dendrioles, as well as the entire axon. This includes the recurrent collaterals and their synaptic varicosities, mainly located in the rat within and just beneath the PC layer. In cerebellar sections double-immunostained with anti-calretinin and anti-calbindin antibodies, Lugaro cells clearly appear as one of the main targets of PC axon recurrent collaterals: their calretinin-labelled soma and bipolar dendrites are densely covered with calbindin-positive axonal varicosities (Fig. 5E). Quite interestingly, the soma and dendrites of most of the calretinin-positive globular neurons, located in the depth of the granular layer, are also found to be contacted by numerous calbindin-positive varicosities. This is illustrated, at high magnification, in the confocal micrograph of Fig. 5F, where the PC axon recurrent collaterals appear as large calbindin-positive varicose endings linked by a thin axonal thread, and are seen to be closely apposed to a calretinin-positive globular neuronal soma and its proximal dendrite, located in the mid-granular layer. It must nevertheless be noticed that a small number of both calretinin-positive globular neurons and Lugaro cells appear devoid of any apposition by calbindin-labelled varicosities.

DISCUSSION

We present here a previously undescribed group of large interneurons located in the granular layer of the rat cerebellar cortex. We propose that these globular cells be considered as a subpopulation of an extended Lugaro cell class, with whom they share a unique pattern of axonal projection, a molecular specification by calretinin and an important afferentation by recurrent collaterals of PC axons.

Specificity of globular cells

Globular cells have distinctive morphological characteristics that clearly differentiate them from two of the four other types of large neurons present in the vicinity.

They can easily be differentiated from candelabrum cells (Lainé and Axelrad, 1994), whose soma generally lies inside the PC layer and who exhibit an asymmetrical dendritic pattern (short basal granular layer dendrites and long apical molecular layer ones) and a number of parallel axonal branches ascending vertically in the molecular layer not far from the parent cell.

No morphological similarity can be found between the globular cells and the monodendritic unipolar brush cells, whose axons course tortuously in the granular layer with specific rosette-like enlargements (Mugnaini and Floris, 1994; Berthié and Axelrad, 1994).

In contrast, on the pure ground of their somato-dendritic appearance and location, globular cells cannot be easily distinguished from Golgi cells. Although their dendritic expansion seems more confined in the latero-lateral direction and the number of their dendrites is less than is generally the case for Golgi neurons, the general appearance of globular cells in silver-impregnated material, when their axon is not impregnated, bears a resemblance with these latter neurons (see Figs. 1A′, 2A′ and the somato-dendritic aspect of the cell in Fig. 3A). This possible confusion is even more true in many immunocytochemical studies, where not only the axon is seldom labelled but often only the soma and the proximal part of the dendritic tree are apparent. Such ambiguity in assigning these neurons to a particular cell class on the sole basis of somato-dendritic features again stresses the importance of visualizing the axonal trajectory. Indeed, the respective axonal projections of Golgi cells and globular cells exhibit clear-cut differences. On the one hand, Golgi cells have a thin varicose and very richly ramified plexus expanding exclusively in the granular layer, whilst, on the other hand, globular cells have thick beaded fibers forming a less dense plexus in the molecular layer, sometimes spanning the entire thickness of the layer. This is why the sample of 15 globular cells on which the present study is based comprises neurons chosen with a sufficiently complete impregnation of dendrites and axons to securely classify them as a homogeneous group, different from Golgi cells.

Similarities of the axonal arborizations of Lugaro and globular neurons

In contrast with the three previous large cerebellar interneurons, morphological differences between globular and Lugaro cells present a problem of classification. In their typical appearance, Lugaro cells have a fusiform soma from which emerge long and poorly ramified horizontal and bipolar dendrites, parasagittally elongated under the PCs somata. This somato-dendritic outline differs from that of the radiating globular multipolar cells described above. However, both neurons are the only large granular layer cells whose axon projects into the molecular layer and, obviously, their axonal arboriza-

tions display common and very specific characteristics. (1) Both axons appear to be very rarely stained with Golgi methods, except in relatively young animals, suggesting they may be at least partly endowed with a myelin sheath, still incomplete at the young ages we have studied. (2) Both of them ascend to the molecular layer, either directly or indirectly, after a hairpin turn of their primary branches. (3) Both break in a complex arborization of thick, obliquely oriented and tortuous beaded branches throughout the overlying molecular layer. (4) Most important, the organization of the two projection patterns in transverse and horizontal planes is remarkably similar, as evidenced by 3D computer reconstructions (Figs. 3, 4 in the present paper and, for Lugaro cells, figure 15 in Lainé and Axelrad, 1996). They are both characterized by a local dense plexus ramifying in the parasagittal plane above or near the parent cell, frequently associated with a remarkable type of collateral, extending for quite a long distance in the latero-lateral direction, exactly parallel to the parallel fibers.

This dual axonal pattern is unique in the cerebellar cortex, and very likely points towards common characteristics of post-synaptic targets for both Lugaro and globular neurons. Recent data suggest that the specificity of the dual axonal pattern of Lugaro cells may be in relation with two distinct types of synaptic targets. On the one hand, as shown by a Golgi gold-toning and immunocytochemical electron microscopic study (Lainé and Axelrad, 1998a), the parasagittally spread proximal axonal plexus engages GABAergic symmetrical synapses with basket and stellate interneurons of the molecular layer and likely also with Golgi cell dendrites, but never synapse with PC dendrites. On the other hand, a patch-clamp electrophysiological and immunocytochemical report (Dieudonné and Dumoulin, 2000) has indirectly shown that a serotonin-induced excitation of Lugaro cells could account for the serotonin-driven inhibitory activity recorded from Golgi cells. The authors suggest that myelinated, calretinin-positive, transverse, axonal branches located just above PC somata could correspond to the Lugaro cell long transverse axonal branches previously described in Golgi studies (Lugaro, 1894; Fox, 1959; Lainé and Axelrad, 1996), and that these fibers could be pre-synaptic to Golgi cells dendrites.

From both these studies, it can be argued that the Lugaro cell class thus represents a specific population of inhibitory interneurons in the cerebellum, dedicated to the regulatory control of the other inhibitory interneurons in distinct parasagittal zones. Another interesting specificity of the Lugaro cells appears to be a mixed GABAergic and glycinergic mode of inhibitory action (Ottersen et al., 1987, 1988; Dumoulin et al., 2001).

Neurochemical and input similarities between Lugaro and globular cells

The striking morphological features shared by Golgi-impregnated Lugaro cell and globular neuron axonal projections may be in keeping with other similarities disclosed by several previous reports and by the present immunocytochemical study. Immunocytochemical reports using Cat 301 (Sahin and Hockfield, 1990) and anti-calretinin (Arai et al., 1991; Floris et al., 1994) antibodies point to the existence of some 'Golgi cell-like neurons' with specific molecular characteristics differentiating them from Golgi cells. Only two types of cells were labelled with the cell-surface Cat 301 antibody in the cat cerebellar cortex: a first group matching the classical description of Lugaro cells' somato-dendritic pattern, and another group of cells of varying soma shapes, located throughout the granular layer, and 'quite similar to Golgi cells', but never labelled by Rat 303 antibody, which appears to be a Golgi cell-specific marker in the cat. In contrast with the cat, Rat 303 antibody appears in the rat to label both Golgi and Lugaro cells (Geurts et al., 2001 and our personal observations). In the rat, calretinin studies also describe some immunopositive 'Golgi cell-like neurons', as well as immunopositive Lugaro and unipolar brush cells, while the bulk of Golgi cells is not calretinin-positive. Therefore, these reports agree to specify a small group of large granular layer neurons with a 'Golgi cell-like' somato-dendritic pattern, and sharing several biochemical specificities with Lugaro cells. A recent report, aiming at differentiating the large cells of the rat granular layer (Geurts et al., 2001), has confirmed that a small group of large polymorphous cells are, together with Lugaro cells, positive for both Rat 303 and calretinin, whilst Golgi cells are only positive for Rat 303, and unipolar brush cells are only calretinin-labelled. The quantitative evaluation obtained by these authors is that of 1 globular neuron (termed by them 'deep calretinin-positive cells') for six Lugaro cells.

In our sections, we noted that the somato-dendritic morphology and distribution of this group of calretinin-labelled Golgi cell-like neurons are the same as those characterizing silver-impregnated globular cells, thus seemingly identifying the same cell type: they are scarce, may be found in all folia, and have a globular or polygonal soma. However, we noticed that these globular neurons are mostly located in the upper part of the granular layer, as is the case for the Golgi-impregnated globular cells. This is in contrast with the indications given by Geurts et al. (2001), who find these 'deep calretinin-positive cells' exclusively in the depth of the granular layer. Noticeably, the ratio of 1 globular cell for 7.7 Lugaro cells that we calculated is of the same order of magnitude as the one found by these latter authors.

An additional and important finding, arguing for a strong propinquity between Lugaro and globular neurons, stems from our double calretinin and calbindin immunolabellings. As specified above, both these groups of neurons demonstrate a massive and specific afferentation of soma and dendrites by the varicosities of calbindin-positive recurrent collaterals of PC axons, an afferentation already demonstrated for the Lugaro cells at the ultrastructural level (Palay and Chan-Palay, 1974; Lainé and Axelrad, 1998b). Interestingly, it can be noted that Ramón y Cajal also illustrated, a century ago, the existence of some cells in the bulk of the granular layer that were completely surrounded by recurrent collaterals of Purkinje cell axons (Ramón y Cajal, 1911: figure 37,

p. 52). This would then imply that all these cells, Lugaro and globular, integrate the feedback from numerous Purkinje cells, the only output of the entire cerebellar network, allowing them, in turn, to regulate the entire activity of the inhibitory interneurons of one or a number of zones, more or less distant from the parent cell.

A widened Lugaro cell type

The morphological, biochemical, and input specificities of globular cells, described and discussed above, argue in favor of them being classified as a subgroup of an enlarged Lugaro cell type rather than as a totally new cerebellar cell class. Indeed, both neurons appear to share three decisive features: (1) their axonal projection pattern – of a unique type in the cerebellar cortex – likely implying common post-synaptic targets; (2) a cytoplasmic high content of the calcium buffer calretinin; and (3) an important and specific afferentation by the recurrent collaterals of PC axons. This classification appears straightforward but for the somato-dendritic disparity. The observed variability in the shape of the dendritic arbor must, however, be put in perspective with the existence of Lugaro cells that diverge more or less from their standard fusiform and bipolar appearance (see figures 10A, 13, 14 in Lainé and Axelrad, 1996). The existence of this graded change in the spatial extent and organization of the dendritic tree suggests that the variability in the morphology of the dendritic pole of these neurons is greater than generally estimated.

The dendritic dissimilarity of Lugaro and globular cells most certainly indicates a change in the balance of excitatory and inhibitory inputs impinging on each neuron. As has been shown by previous ultrastructural studies, Lugaro cells receive two types of inputs, an inhibitory one from recurrent collaterals of PC axons and an excitatory one from granule cell axons (Palay and Chan-Palay, 1974). Our own ultrastructural data confirm that the soma and proximal dendrites of Lugaro cells are mainly synaptically contacted by the inhibitory recurrent collaterals of Purkinje cell axons and by some granule cell axons, but also, very interestingly, by another excitatory type of large synapse (Lainé and Axelrad, 1998b, and in preparation). From our present results with double anti-calbindin/anti-calretinin immunostaining, it appears that globular neurons' soma and proximal dendrites receive the same heavy inhibitory recurrent afferentation from PCs as Lugaro cells. The main difference in the modulatory balance would then come from the sampling of the excitatory contacts of granule cell axons. Indeed, dendrites from globular cells, which ascend vertically through the entire molecular layer, are post-synaptic to the densely packed parallel fibers, whilst the horizontal dendrites of the fusiform Lugaro cells, which extend in the granular layer, receive inputs from the more scarce vertically ascending part of granule cells' axons. It can then be hypothesized from these morphological data that globular cells are more effectively driven by granule cells' activity than Lugaro cells.

Phylogenetic studies will be necessary to specify the presence and relative proportions of this globular subtype of Lugaro cells in different species. This could establish if our observations, made in a single species, can be understood in the frame of an evolutionary process leading to the differentiation of two different cell types from a former common population.

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