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ORIGINAL PAPER

Secretagogin Immunoreactivity Reveals Lugaro Cells in the Pigeon Cerebellum

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Abstract

Lugaro cells are inhibitory interneurons found in the upper granular layer of the cerebellar cortex, just below or within the Purkinje cell layer. They are characterized by (1) a fusiform soma oriented in the parasagittal plane, (2) two pairs of dendrites emanating from opposite ends of the soma, (3) innervation from Purkinje cell collaterals, and (4) an axon that projects into the molecular layer akin to granular cell parallel fibers. Lugaro cells have been described in mammals, but not in other vertebrate classes, save one report in teleost fish. Here, we propose the existence of Lugaro cells in the avian cerebellum based on the morphological characteristics and connectivity described above. Immunohistochemical staining against the calcium binding protein secretagogin (SCGN) revealed Lugaro-like cells in the pigeon cerebellum. Some SCGN-labeled cells exhibit fusiform somata and dendrites parallel to the Purkinje cell layer in the parasagittal plane, as well as long axons that project into the molecular layer and travel alongside parallel fibers in the coronal plane. While mammalian Lugaro cells are known to express calretinin, the SCGN-labeled cells in the pigeon do not. SCGN-labeled cells also express glutamic acid decarboxylase, confirming their inhibitory function. Calbindin labeling revealed Purkinje cell terminals surrounding the SCGN-expressing cells. Our results suggest that Lugaro cells are more widespread among vertebrates than previously thought and may be a characteristic of the cerebellum of all vertebrates.

Keywords Lugaro cells · Secretagogin · Immunohistochemistry · Cerebellum · Birds · Cerebellar circuits · Evolution

Introduction

The mammalian cerebellar cortex consists of numerous cell types including Purkinje cells (PCs), basket and stellate cells in the molecular cell layer (mcl), and granule cells in the granular cell layer (gcl) (Fig. 1). Also located in the gcl are large inhibitory interneurons including Lugaro and Golgi cells. While both Golgi [1] and Ramon y Cajal [2] grouped Lugaro and Golgi cells together, Lugaro [3] distinguished smaller fusiform cells located right below the Purkinje cell layer (pcl) in the cat cerebellum as a distinct class from larger globular (Golgi) cells and named the former "intermediate cells". Indeed, Lugaro and Golgi cells differ in several aspects. Lugaro cells are mainly located in the gcl, just below the PCs, or sometimes within the pcl itself, while Golgi cells usually reside deeper in the gcl [4]. Lugaro cells are characterized by their fusiform spindle-like shape and dendrites that emerge from opposite ends of their cell bodies [4]. In terms of their projection pattern, Lugaro cell axons always project into the molecular cell layer (mcl), and travel in the coronal plane alongside parallel fibers that arise from granule cells (Fig. 1) [3–5]. Golgi cells on the other hand exhibit a very thin and profuse axonal plexus that remains within the gcl, whereas their dendrites radiate into the mcl [6]. Nevertheless, recent evidence has blurred the morphological distinction between Golgi and Lugaro cells as the existence of a globular Lugaro cell type has been proposed [6]. In addition to differences in morphology, Lugaro and Golgi cells also differ in the expression of several molecular markers [7]. For example, both globular and fusiform Lugaro cells, but not Golgi cells, are immunoreactive for calretinin [6, 8–10]. Additionally, Golgi cells

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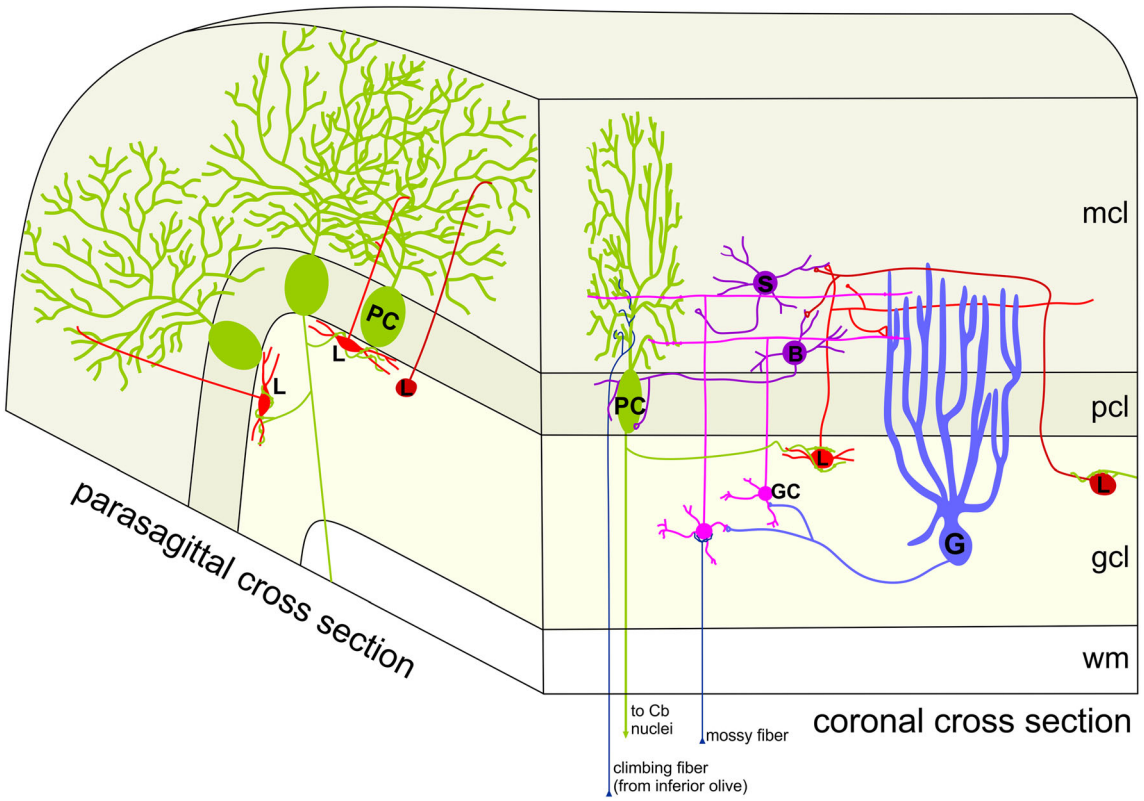

Fig. 1 Simplified cerebellar circuitry. The schematic shows simple cerebellar connectivity and a view of a cerebellar folium in the parasagittal and coronal planes. Climbing fibres (CFs; navy) and mossy fibres (mFs; navy) are the two main inputs into the cerebellum. CFs come from the inferior olive and synapse directly onto the Purkinje cells (PCs; green) while mFs terminate on granule cells (GC; magenta) and Golgi cells (G; blue) in the granular cell layer (gcl). Granule cells project their axons into the molecular cell layer (mcl) and bifurcate to send off parallel fibres that synapse extensively onto the PCs. Lugaro cells (L; red) also project their axons into the mcl, and their axons travel through the coronal plane alongside granule cell parallel fibres to synapse on basket (B) and stellate (S) cells (purple). Basket and stellate cells in turn synapse on PCs, and PC collaterals synapse on Lugaro cells. Lugaro cells also synapse onto Golgi cells. Note both fusiform (bright red) and globular (dark red) types of Lugaro cells. The fusiform somata are only noticeable in parasagittal cross sections. pcl = Purkinje cell layer; wm = white matter

express metabotropic glutamate receptor 2 (mGluR2) and neurogranin while Lugaro cells do not [8, 10].

With respect to circuitry, globular and fusiform Lugaro cells receive inhibitory inputs from PC axon collaterals [11–14], and their axons project into the mcl to innervate stellate cells and basket cells, which in turn inhibit PCs (Fig. 1) [5, 15]. Lugaro cells have also been found to inhibit Golgi cells [16, 17] and Purkinje cells [18]. Several studies in juvenile rats have shown that Lugaro cells are excited by serotonin [18, 19], but this is not exclusive of Lugaro cells, as golgi and globular cells are also excited by serotonin [19]. Lugaro cells have been described in a broad array of mammalian species (e.g., echidna; [20]; rat: [5]; cat: [7]; human: [21]), but evidence for the existence of Lugaro cells in other vertebrates is scarce. Pushchina and Varaksin [22], based only on histochemistry against nitric oxidase, described Lugaro-like cells in the cerebellum of a teleost. In chickens, Rogers [23], based solely on the location near the Purkinje cells layer, suggested that some weakly calretinin positive cells could be either basket or Lugaro cells (we previously found no calretinin positive cells in the molecular layer of the pigeon cerebellum [24]). While this evidence suggests that Lugaro cells could exist in vertebrates other than mammals, solid evidence is needed.

Upon investigating the expression of secretagogin (SCGN), a recently discovered calcium binding protein (reviewed in Alpár et al. [25]), we noted secretagogin-labeling of large cells throughout the gcl in the pigeon cerebellum. In this report, based on cell morphology, local connectivity, and immunohistochemistry, we argue that at least some of the SCGN-labeled cells correspond to Lugaro cells in the pigeon cerebellum.

Materials and Methods

Perfusion and Brain Processing

The methods used adhere to the guidelines established by the Canadian Council on Animal Care and were approved by the Biosciences Animal Care and Use Committee at the University of Alberta. Three rock pigeons (*Coulmba livia*) of either sex, obtained from a local supplier, were anesthetized with an intramuscular injection of a ketamine (65 mg/kg) and

xylazine (8 mg/kg) cocktail, followed by sodium pentobarbital (100 mg/kg). A transcardial perfusion with phosphate-buffered saline (PBS; 0.9% NaCl, 0.1 M phosphate buffer) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4) was then performed. The brains were extracted from the skull and immersed in paraformaldehyde for 3–4 h at 4 °C, then immersed in 30% sucrose in 0.1 M phosphate buffer until they sank (48 h). Brains were subsequently embedded in gelatin, post-fixed in 4% PFA and 30% sucrose in 0.1 phosphate buffer for 5 h, and cryoprotected in 30% sucrose in 0.1 M PBS overnight. The gelatin-embedded brains were then frozen and sliced into 40-μm-thick sections (coronal or sagittal) using a sliding microtome. Sections were collected in four series throughout the extent of the cerebellum. Two rat cerebella (archival tissue) were also processed. One was cut in the parasagittal plane and the other in the coronal plane. Sections were stored in 0.1 M PBS (pH 7.4) between sectioning and immunohistochemistry.

Antigen Retrieval

For the sections that were subject to antigen retrieval, prior to processing the tissue using the immunohistochemical procedures below, well plates filled with 10 mM sodium citrate buffer (pH 8) were placed in a hot water bath (80 °C) for 15 min to allow the buffer to reach 80 °C. Sections were rinsed five times for 5 min in 0.1 M phosphate buffer after which they were placed in 10 mM sodium citrate buffer (pH 8) for 30 min at 80 °C. The well plate containing the tissue was removed from the water bath and left to cool for 15 min with the lid removed. The tissue was rinsed another five times for 5 min in 0.1 M phosphate buffer.

Immunohistochemistry

The immunohistochemistry protocols differed for each antibody (see Table 1), but the general protocol was as outlined here. Tissue was first rinsed with 0.1 M PBS, then blocked with 10% normal donkey serum (Jackson Immunoresearch Laboratories, West Grove, PA) and 0.4% Triton X-100 in PBS for 1 h. Tissue was then incubated in PBS containing 0.1% Triton X-100, 2.5% normal donkey serum, and the appropriate primary antibody concentration (see Table 1) for 24–48 h at 4 °C. In some cases, sections were incubated with two antibodies at the same time. Only antibodies from different hosts were used in conjunction. After primary antibody incubation, sections were rinsed five times in 0.1 M PBS and incubated with animal host appropriate fluorescent secondary antibodies AlexaFluor 594 or 488 (either anti-mouse, -goat, or -rabbit; 1:200, Jackson Immunoresearch Laboratories, West Grove, PA) in PBS, 2.5% normal donkey serum, and 0.4% Triton X-100 for 3 h at room temperature. In some cases, two secondary fluorescent antibodies (one of each color) were used. The tissue was then rinsed five times for 5 min in 0.1 M PBS and mounted onto gelatinized slides for viewing. Although SCGN expression was the focus of this study, several other antibodies were also used (see Table 1). The two antibodies used against SCGN were one raised in rabbit and one raised in goat (see Table 1). Both are polyclonal antibodies raised against a portion of recombinant human SCGN. Specificity of these antibodies against secretagogin has previously been tested and validated in an avian species (chicken) where it recognizes a band of 32 kDa, the same as in mouse [21].

For the tyramide signal amplification (TSA), after primary antibody incubation, tissue was rinsed five times for 5 min, and then incubated with a biotin secondary (1:1000) for 2 h at room temperature. After rinsing five times for 5 min, sections were incubated in avidin-biotin peroxidase complex (ABC; Vectastain Elite ABC Kit, Vector Laboratories Inc., Burlingame, CA, USA; 3.2 μl/ml in PBS-Tx 0.5%/4% NaCl) for 1 h. Sections were again rinsed five times. This was followed by TSA where sections were incubated in 0.0001% biotin-tyramide (IRIS Biotech GmbH, Marktredwitz, Germany; Cat# LS-3500, Lot. 1407008) and 0.003% H_2O_2 in 0.05 M borate

Table 1 List of antibodies used for immunohistochemistry (IH) and the source, concentration, and incubation times used for each

Primary antibody	Source	Host	IH dilution	Time incubated
Secretagogin	R&D Systems Product #AF4878	goat	1:500	48 h
Secretagogin	Atlas Antibodies Product #HPA006641	rabbit	1:5000	72 h
Calretinin	SWant Product #7697	rabbit	1:2000	48 h
Calbindin D-28 k	SWant Product #CB-38a	rabbit	1:2000	48 h
GAD	Millipore Sigma Product #AB1511	rabbit	1:1000	24 h
Neurogranin	Millipore Sigma Product # AB5620	rabbit	1:500	48 h

buffer (pH 8.5) for 1 h. Sections were once again rinsed five times for 5 min. Last, sections were incubated for 1 h with Streptavidin-Alexa594 (Streptavidin Alexa Fluor 594 conjugate, Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:500 in 0.5% Triton X-100 in PBS.

NADPH-d Staining

Because Lugaro and Golgi cells sometimes contain nitric oxide synthase (e.g., in the rabbit cerebellum; Okhotin and Kalinichenko [22]), the tissue was stained for nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) according to the protocol of Fischer and Stell [26]. Sections were mounted on the slides and left to dry for 2–3 days. The slides were washed in 0.1 M Tris buffer (pH 8.0) for 5 min. The NADPH-d staining solution (1 mM NADPH, 18 mM CaCl2, 0.5 nM nitrotetrazolium blue (NTB), and 0.3% Triton-X) was applied directly to the slides. The slides were then covered with a large Petri dish to avoid evaporation of the staining solution during incubation. The different slides were incubated between 20 and 60 min at 37 °C. After the incubation period, the slides were rinsed five times for 5 min with 0.1 M phosphate-buffered saline (pH 7.4).

Microscopy

Sections were viewed with a compound light microscope (Leica DM6B, Concord, ON) equipped with TX2 (red) and L5 (green) fluorescence filters. Images were captured with a DFC7000 T camera using Leica Application Suite X imaging software (Leica Microsystems, Concord, ON). Adobe Photoshop CC (San Jose, CA) was used to adjust contrast and brightness.

Cell Position Measurements

We measured the relative position of all SCGN cells in microphotographs of five coronal sections through the anteroposterior extent of the pigeon cerebellum. The relative position was calculated as the distance of the cells to the white matter divided by the total depth of the granular cells layer. Cells closer than 20 μm from the PC layer were considered as being within it. Measurement included all folia and was in a total of 1653 cells. Measurements were performed in ImageJ.

Results

Secretagogin Labeling in the Cerebellum

As previously mentioned, we visualized SCGN labeling in the cerebellum with the following techniques: (1) classic immunohistochemistry, (2) using amplification with biotin tyramide and streptavidin, and (3) using an antigen retrieval protocol prior to immunohistochemistry. Figure 2 shows SCGN immunoreactivity (SCGN-ir) from each of these protocols in coronal sections through the cerebellum (a-c, e-f, and h-j) and the optic tectum (d, g, and k). Although SCGN-ir was apparent in the optic tectum without amplification or antigen retrieval (Fig. 2d), immunoreactivity in the cerebellum was very faint (Fig. 2a–c). Upon treatment of the tissue with amplification or antigen retrieval, SCGN immunolabeling in the cerebellum became clearly apparent, particularly with the latter (Fig. 2a–c, e–f). Immunoreactivity was apparent in the somata, proximal dendrites, and axons of neurons in the gcl. Most of these cells appeared near the pcl. Generally, in coronal sections, the somata of these neurons appeared round (Fig. 2i). We also noted SCGN-labeled fibers traveling through the mcl in the coronal plane (Fig. 2h). The SCGN antibody raised in a rabbit host is particularly effective at labeling Lugaro cell axons, as shown in Fig. 5, while the SCGN antibody raised in a goat host is not. Finally, labeled mossy fiber rosettes were occasionally apparent (Fig. 3d, e).

To show the proximity of SCGN-ir cells to the pcl, some sections were immunoprocessed for Calbindin-D28k (CB), which is expressed by all PCs. Figures 3 and 4 both show SCGN (red) and CB (green) labeling in sagittal cerebellar sections. SCGN-labeled cells consistently appeared in the gcl both close to the PCs, and deeper within the gcl (Figs. 3a and 4a). Moreover, some of these cells were clearly within the pcl (e.g., Fig. 4b). The somata of the SCGN-ir cells nearest the pcl were smaller, and often fusiform, compared to those deeper in the gcl (e.g., Figs. 3a and 4b, f). We also noted SCGN-ir cells at the border of the gcl and white matter (Fig. 3b). Quantification of the relative position of SCGN-labeled cells in the granular cell layer shows that 60.8% of SCGN-labeled cells were within the PC layer, while 14.7% were in the granular cell layer and 24.5% in the white matter.

In sagittal sections, some of the SCGN-ir cells closest to the pcl resembled the morphology of Lugaro cells described in mammals (Fig. 4b–i). The cell bodies were fusiform in shape, with dendrites emerging from both poles parallel to the pcl (e.g., Fig. 4f). Some of the cells were more globular in shape, but these were situated slightly further away from the pcl than the fusiform cells (e.g., Fig. 4d–i). Often, the SCGN-labeled cells were encapsulated by CB-labeled varicosities (e.g., Fig. 4d, f). Presumably, these arise from PC axon collaterals.

An additional characteristic of mammalian Lugaro cells is the existence of parallel-fiber-like axonal projections through the mcl. When viewing SCGN labeling in coronal sections, we noted similar parallel-fiber-like projections (Fig. 5). The white arrowheads in Fig. 5 highlight a single SCGN immunopositive fiber (arrow heads) coursing into the mcl, making an abrupt turn (empty arrowhead), and traveling in the same plane as parallel fibers of granule cells.

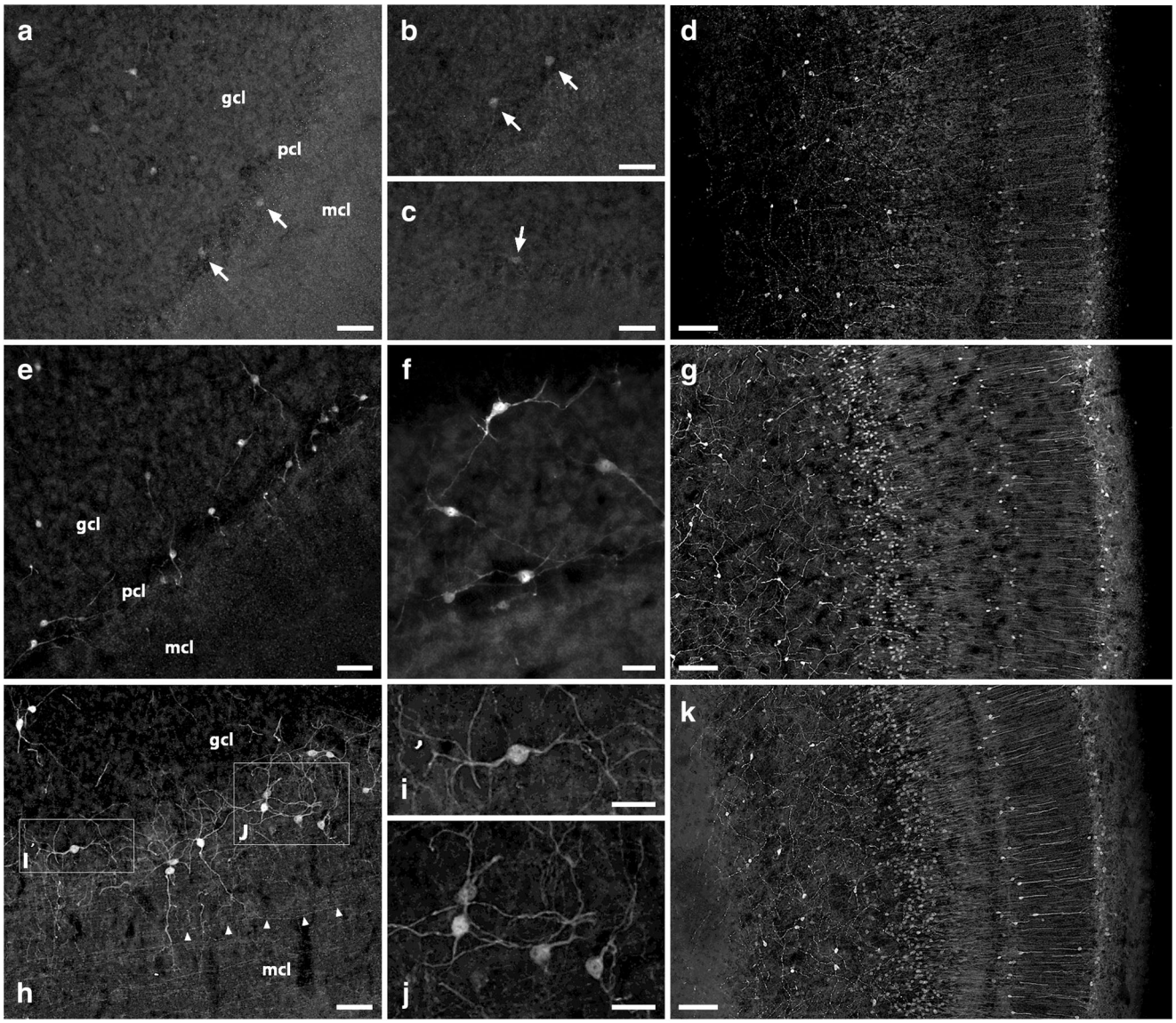

Fig. 2 Secretagogin (SCGN) labeling in the pigeon cerebellum and optic tectum. **a**–**d** SCGN labeling without any enhancement methods for sections cut in the coronal plane. **a**–**c** Labeling in the cerebellum, and **d** shows labeling in the optic tectum for comparison. In the cerebellum, cells in the granular cell layer were very faintly labelled (arrows). With amplification (**e**, **f**) and antigen retrieval (**h**–**j**), these cells showed strong SCGN immunoreactivity. Note SCGN immunoreactive fibers in the mcl in **h**. **g**, **k** SCGN immunoreactivity in the optic tectum with amplification and antigen revival. The SCGN used in these sections was cloned in rabbit. mcl = molecular cell layer; pcl = Purkinje cell layer; gcl = granular cell layer. Scale bars = 25 μm in **f**, **i**, **j**, 50 μm in **a**–**c**, **e**, **h**, 100 μm in **d**, **g**, **k**

As Lugaro cells are inhibitory, we examined the co-expression of SCGN and GAD (Fig. 6). SCGN immunopositive cells in the gcl co-expressed GAD.

Calretinin and SCGN Labeling in Rat v. Pigeon

As apparent Lugaro cells express SCGN in pigeons, we used the same immunohistochemical techniques to see whether SCGN is expressed by Lugaro cells in rats. Figure 7a, b shows a section through the rat cerebellum immunoprocessed for SCGN (red) as well as CB (green) to visualize the PCs. There was no trace whatsoever of SCGN labeling in the cerebellum. However, this was not due to failure of the antibody as SCGN-expressing cells were seen in the central gray of the rat mesencephalon (Fig. 7c). For comparison, pigeon CB and SCGN labeling is shown in Fig. 7d.

Because Lugaro cells in the rat cerebellum express CR [6, 24], we examined whether the apparent Lugaro cells that express SCGN in pigeons also expressed CR. Immunolabeling for CR is shown in the rat cerebellum (Fig. 7e–j). We noted

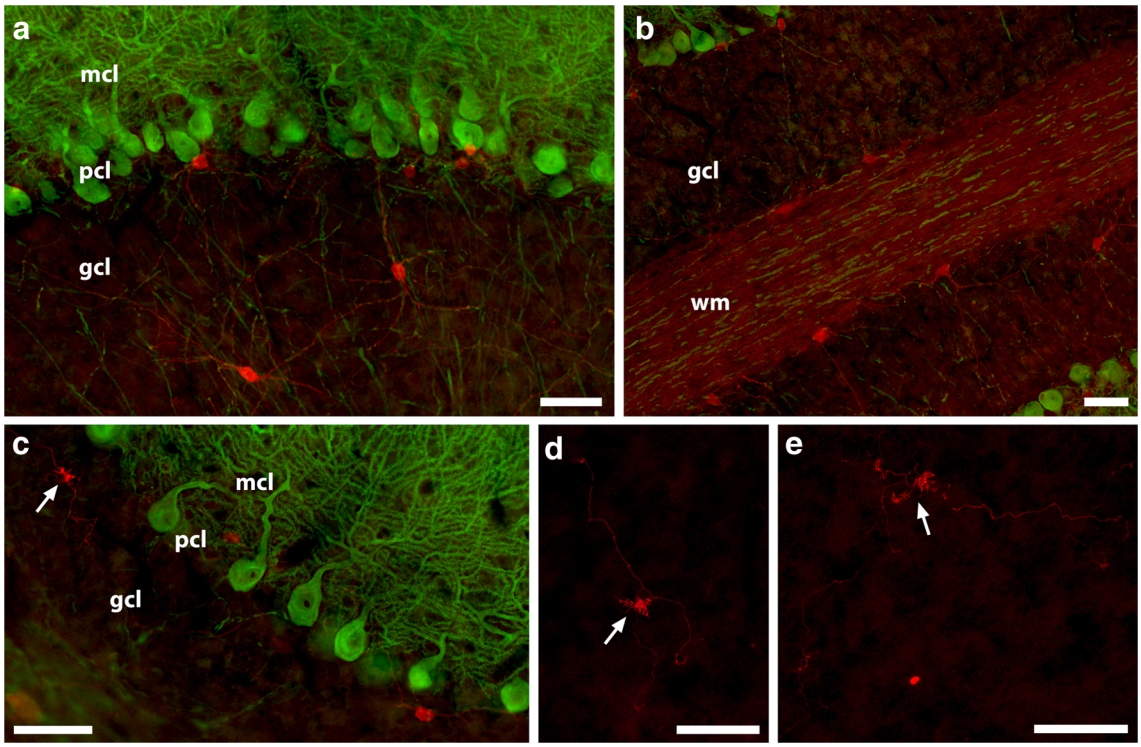

Fig. 3 Secretagogin (SCGN) labeling in the cerebellum. Tissue was immunoprocessed for secretagogin (SCGN; red) and Calbindin-D28k (CB; green). Purkinje cells (PCs) are CB immunopositive. **a** Different types of SCGN-labeled cells: smaller ones closer to the Purkinje cell layer (pcl) and larger ones deeper in the granular cell layer (gcl). PCs are labeled by CB in green. **b** SCGN-labeled cells in the gcl adjacent to the white matter (wm). **c**–**e** Mossy fiber rosettes labeled by SCGN (arrows). mcl = molecular cell layer. Scale bars = 50 μm in **a**–**e**

many fusiform cells in the gcl below the pcl, which were likely Lugaro cells (arrows in Fig. 7f–j). In coronal rat cerebellar sections, we noted CR-immunopositive parallel-like fibers coursing through the mcl (Fig. 7e). In pigeons, the SCGN-immunolabeled cells did not express CR (Fig. 7k–m). Additionally, we attempted to distinguish Golgi from Lugaro cells using immunohistochemistry against neurogranin. In mammals, neurogranin has been shown to label Golgi, but not Lugaro cells [10]. In the pigeon cerebellum, neurogranin labeled Purkinje cells, but did not appear to label the proposed Lugaro cells (data not shown).

NADPH-Diaphorase Staining

NADPH-diaphorase (NADPH-d) selectively stains cells that contain nitric oxide synthase and thus nitric oxide. Lugaro cells in the rabbit cerebellum are known to contain nitric oxide and thus stain for NADPH-d [25], though in humans and cats, they do not. We did not see any NADPH-d-stained cell bodies in the pigeon cerebellum (Fig. 8a), though we noted differential staining of the separate cerebellar layers, which has been previously reported in the pigeon [27]. Some cells in the optic tectum were stained for NADPH-d (Fig. 8b) indicating that the procedure was reliable.

Discussion

Here, we show for the first time the existence of Lugaro cells in the cerebellum of a bird, the rock pigeon. In the cerebellum of this species, Lugaro cells are characterized by the expression of a calcium binding protein, SCGN. This is very distinct from the expression of other calcium binding proteins in the avian cerebellum which are not expressed in large interneurons of the gcl. Whereas parvalbumin is expressed mostly in PCs and mcl interneurons [28], CB is expressed mainly in PCs [29], and CR is expressed in mossy fibers [20]. Noticeably, we could only observe SCGN-ir either after antigen retrieval (Fig. 2)h-j or amplification of the fluorescent signal through a TSA protocol (Fig. 2)e-f suggesting that cells in the cerebellum of the pigeon only weakly express SCGN. This seems to be also the case in chickens [30]. Gáti et al. [21] had previously described expression of SCGN in several regions of the chicken brain but failed to find any in the cerebellum.

Characterization of SCGN-Immunopositive Cells

Large SCGN-immunopositive cells in the gcl of the pigeon cerebellum exhibited a striking similarity to mammalian Lugaro cells in terms of both morphology and circuitry.

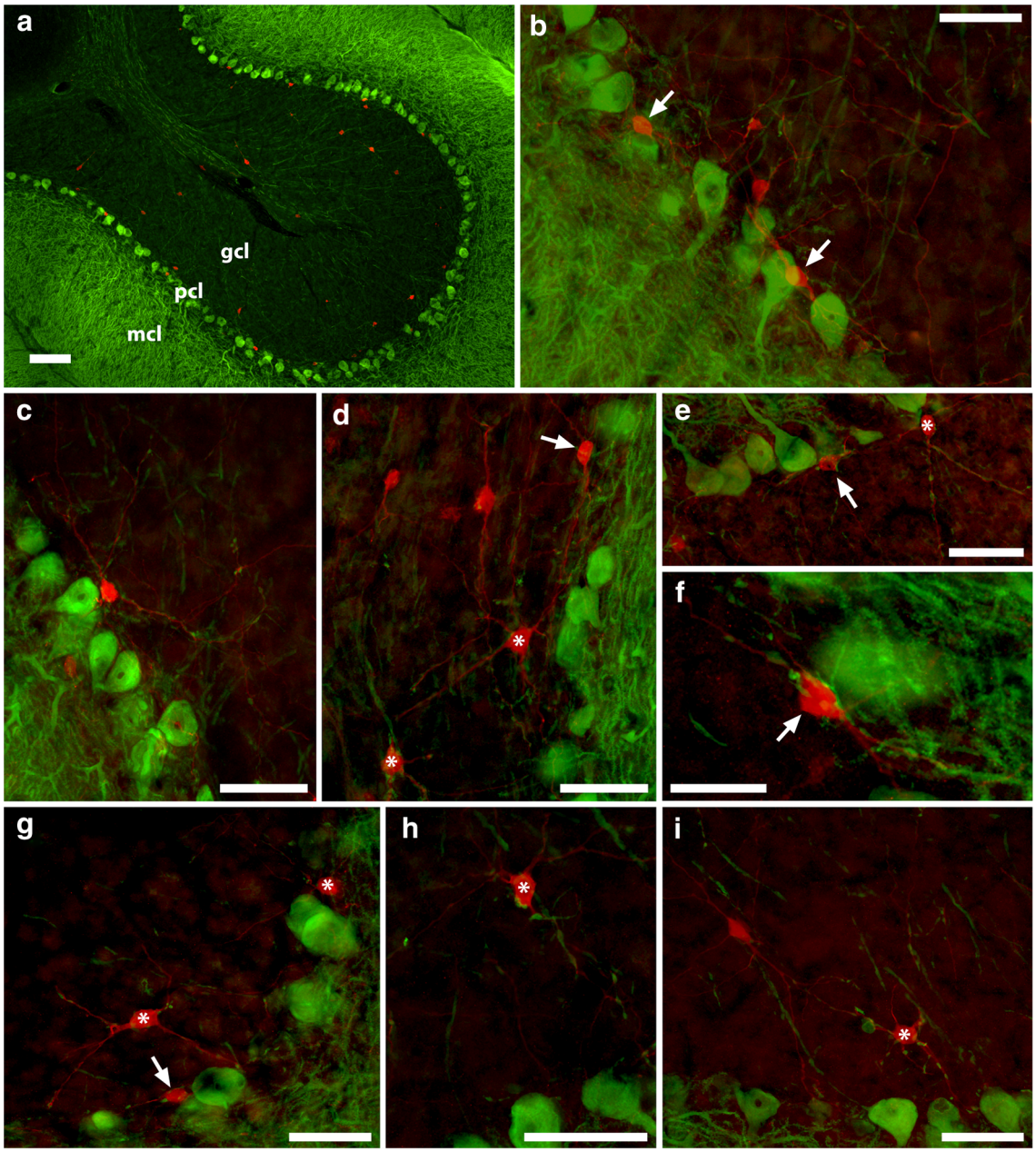

Fig. 4 SCGN-labeled cells near the Purkinje cell layer (pcl). Sagittal sections were immunoprocessed with secretagogin (SCGN; red) and Calbindin-D28k (CB; green). Purkinje cells (PCs) are CB-immunoreactive. **a** SCGN-labeled cells in a low-power photomicrograph of the pigeon cerebellum. **b**–**i** High magnification images of various SCGN-labeled cells near the PCs. Note the CB-immunopositive varicosities surrounding a both fusiform (arrows) and globular (asterisks) cells. This is clearest for the cells in **f**, on the left in **d**, and in **h**, **i**. mcl = molecular cell layer; gcl = granular cell layer. Scale bars = 25 μm in **f**, 50 μm in **b**–**e**, **g**–**i**, 100 μm in **a**

Morphological similarities included fusiform somata and bipolar dendrites extending parallel to the pcl in sagittal sections. Similarities in circuitry were also apparent as the SCGN-ir revealed parallel-fiber-like axons in the mcl in coronal sections, which is characteristic of mammalian Lugaro cells [3–5]. Presumably, these axonal projections originated from the SCGN-labeled Lugaro cells and not from granule cells, as granule cells themselves were not immunoreactive for SCGN. Additionally, PC axon collaterals are known to synapse on mammalian Lugaro cells [6, 12]. Immunoprocessing for CB in the pigeon cerebellum showed CB-labeled varicosities on the SCGN-labeled somata near the pcl (Fig. 4d, f, h). Presumably, these were PC axon collaterals as CB is mainly expressed in PCs.

Additionally, we found that SCGN labeled other large cells in the granular cell layer of the pigeon's cerebellum. These large cells did not resemble typical mammalian Lugaro cells as they either lacked the classic fusiform

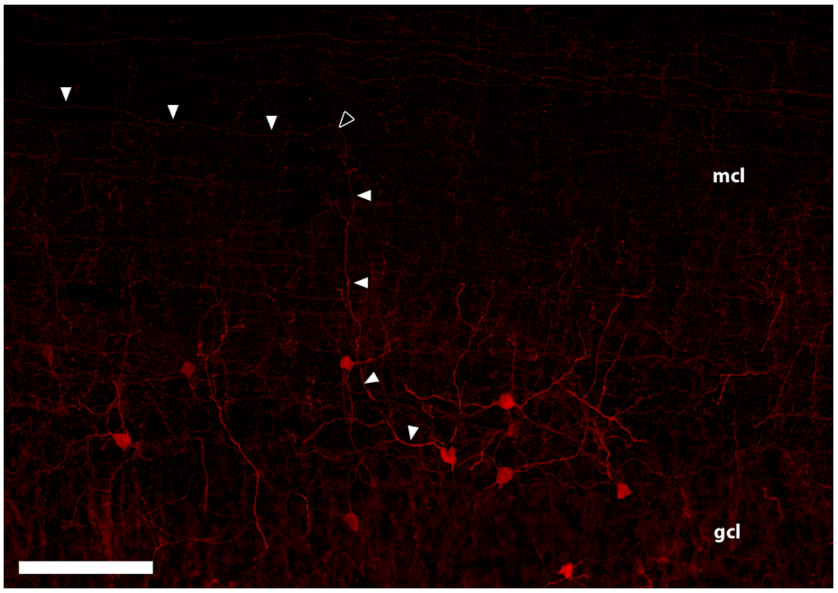

Fig. 5 Coronal section through the pigeon cerebellum. Cell bodies, dendrites, and axons are labeled by SCGN. The labeled cells exhibit axons that project into the molecular cell layer (mcl) and traverse alongside parallel fibers, which is a characteristic of Lugaro cells. White arrowheads follow along a single axon as it ascends into the mcl. The empty arrowhead points out the abrupt turn taken by the axon before projecting transversely along the folium. gcl = granular cell layer. Scale bar = 100 μm

morphology and/or resided deeper in the molecular layer (Figs. 3 and 4). It is possible that these correspond to Golgi cells, but they could also correspond to a recently described subtype of mammalian Lugaro cells described by Lainé and Axelrad [6] in the rat cerebellum. These cells morphologically resemble Golgi cells, as they have

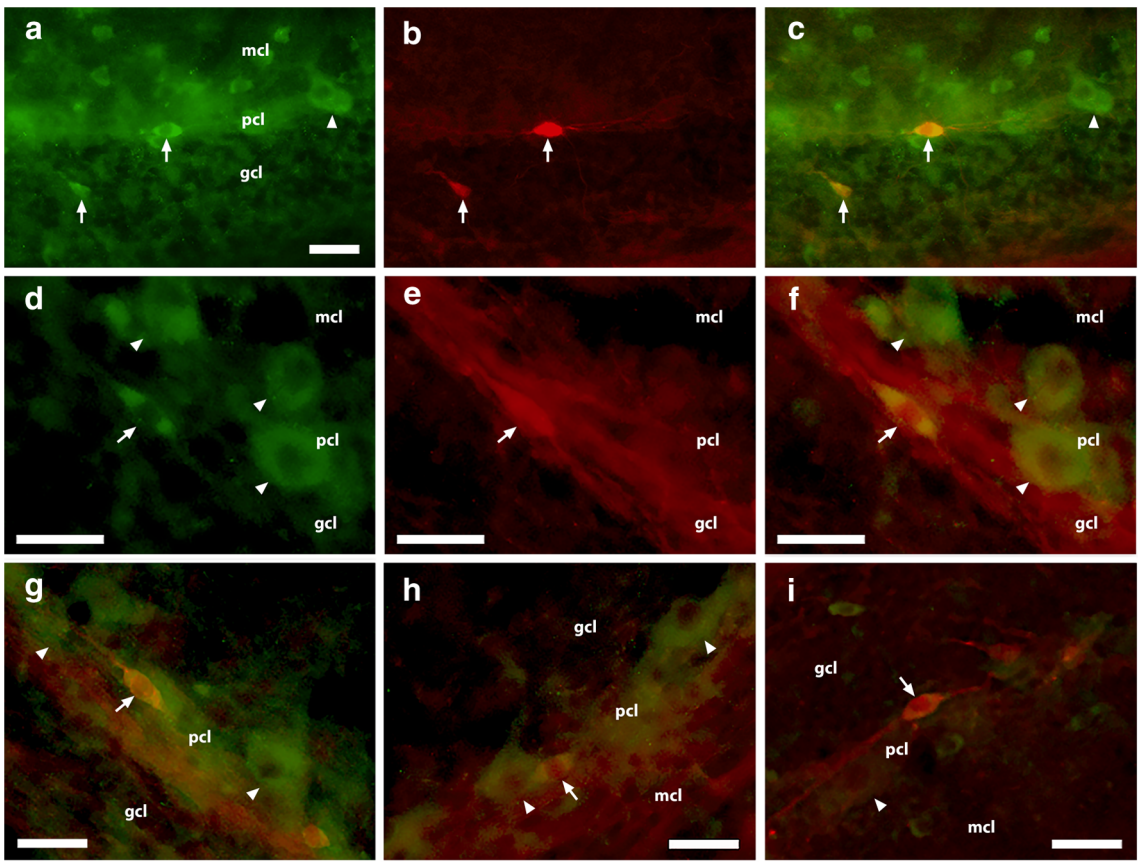

Fig. 6 GAD and SCGN labeling in the cerebellum. **a**–**c**, **d**–**f** are shown as triptychs: GAD (**a**, **d**; green), secretagogin (**b**, **e**; SCGN; red), and the overlays (**c**, **f**). The yellow cells in c and f indicate that SCGN-immunopositive cells co-express GAD. In all others (**g**–**i**), only the overlay is shown. Arrows indicated double-labeled cells whereas arrowheads indicate some Purkinje cells. mcl = molecular cell layer; plc = Purkinje cell layer; gcl = granular cell layer. Scale bars = 25 μm in **a**–**i**

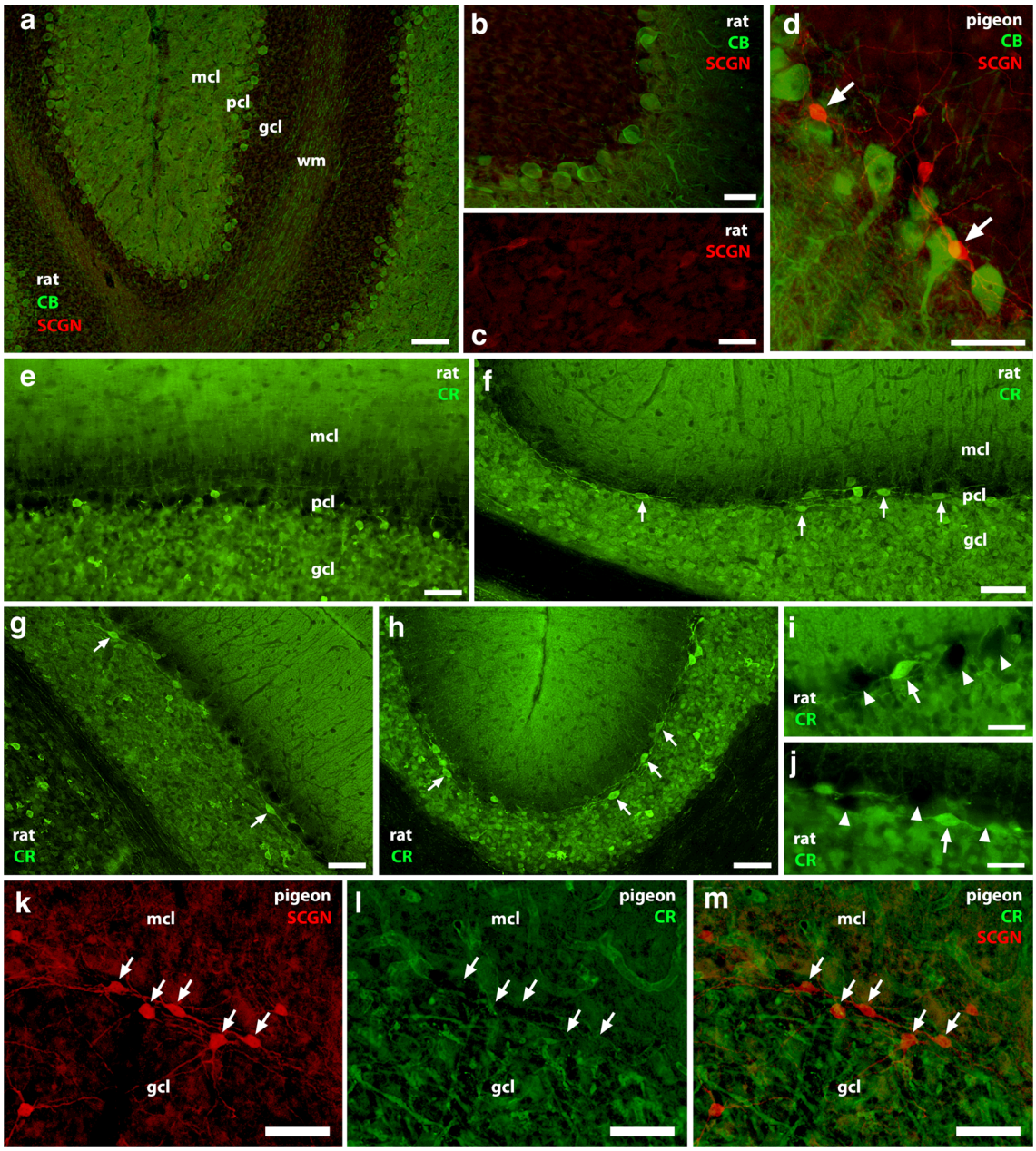

Fig. 7 Calretinin (CR) and secretagogin (SCGN) labeling in rat and pigeon cerebellum. **a**, **b** A coronal section through the rat cerebellum immunoprocessed for secretagogin (SCGN; red) and Calbindin-D28k (CB; green). Purkinje cells (PCs) express CB. There were no SCGN-immunopositive cells in the rat cerebellum. **c** SCGN immunoreactive cells in the central gray of the rat mesencephalon. **d** SCGN and CB immunoreactivity in the pigeon cerebellum. **e**–**j** Calretinin (CR) immunoreactivity in the rat cerebellum. In the sagittal sections (**f**–**j**), note the many fusiform Lugaro cells in the granular cell layer (gcl) below the Purkinje cell layer (pcl) (arrows). **e** A coronal section where parallel-fiber-like axons can be seen in the molecular cell layer (mcl). **k**–**m** A triptych of a sagittal pigeon section immunolabeled with SCGN (red) and CR (green). The SCGN-expressing cells in the pigeon cerebellum do not express CR. Arrows indicate supposed Lugaro cells; arrowheads indicate holes where PCs would be found. Scale bars = 25 μm in **i**, **j**, 50 μm in **b**–**h**, **k**–**m**, 100 μm in a

globular somata, three to four long radiating dendrites, and are located at variable depths in the granular layer. However, they are thought to be Lugaro cells as they have axons that travel into the molecular layer in similar fashion to fusiform Lugaro cells, and also receive axon collaterals from Purkinje cells. At least some of the SCGN-ir cells in the pigeon's cerebellum further from the Purkinje cells closely resemble this description (asterisks in Fig. 4), and in several cases, CB positive varicosities can be seen in close proximity of the cell body (Fig. 4g–i). This suggests that like calretinin in mammals, SCGN labels all types of Lugaro cells in the pigeon cerebellum. There

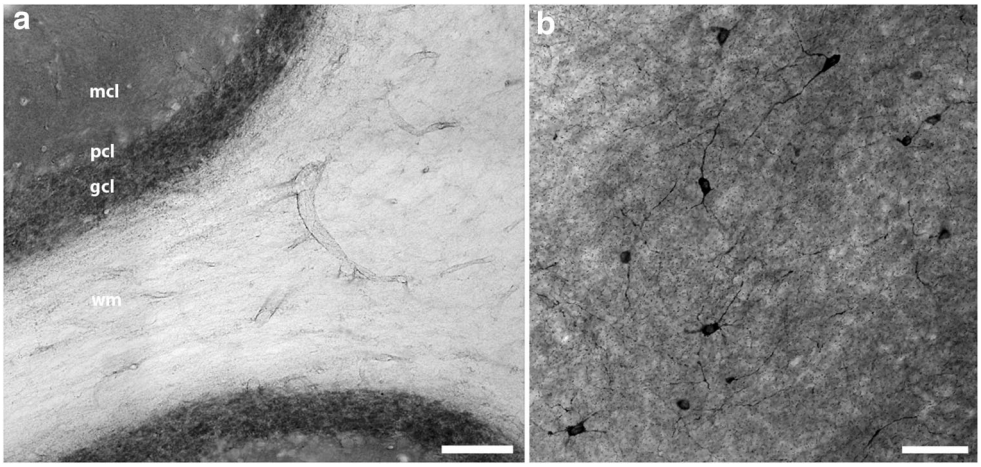

Fig. 8 NADHP-diaphorase (NADPH-d) labeling in the pigeon optic tectum and cerebellum. **a** Staining for nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) in a sagittal section of the pigeon cerebellum. **b** Staining for NADPH-d in the optic tectum of the pigeon. There were no apparent NADPH-d-stained cells in the cerebellum, while the optic tectum did exhibit staining. mcl = molecular cell layer; plc = Purkinje cell layer; gcl = granular cell layer; wm = white matter. Scale bars 50 μm in **b**, 100 μm in **a**

are several SCGN-ir cells in the gcl, including on the border of the white matter, which do not appear to be innervated from PC axon collaterals. As such, we assume these to be Golgi cells [11].

Comparison of Mammalian and Avian Lugaro Cell Characteristics

Though there are several similarities between the proposed avian Lugaro cells and their mammalian counterparts, there were some noted differences (resumed in Table 2). First, mammalian Lugaro cells are known to express CR, whereas pigeon Lugaro cells do not (Fig. 7 k–m). Conversely, pigeon Lugaro cells express SCGN while mammalian Lugaro cells do not. In fact, there was no SCGN immunoreactivity noted in the rat cerebellum at all (Fig. 7a, b). Finally, in some mammals (e.g., rabbits), Lugaro cells contain nitric oxide as shown by staining against NADPH-d, and the Lugaro cells described in the one species of teleost were also detected by NADPH-d staining [23]. In the pigeon, there were no somata stained by NADPH-d (Fig. 8a). This was not surprising as Atoji et al. [24] described a similar lack of NADPH-d-stained cells in the pigeon cerebellum. Thus, despite the striking similarities in circuitry and morphology, the putative Lugaro cells in the pigeon cerebellum have neurochemical differences to those described in other vertebrates (Table 2).

Table 2 Lugaro cell characteristics in different vertebrates

Characteristic	Mammals	Pigeons	Teleost fish
Calretinin-ir	+	–	?
Secretagogin-ir	–	+	?
GAD-ir	+	+	?
NADPH-diaphorase	+/–[a]	–	+
Neurogranin-ir	–	–	?
Fusiform somata	+	+	+
Bilateral horizontally oriented dendrites	+	+	+
Parallel-fiber-like axons	+	+	?
Innervation by Purkinje cell collaterals	+	+	?
Located in the gcl below the pcl	+	+	+

[a] Lugaro cells express NADPH-d in rabbits but not in rats, cats, or humans. *ir* immunoreactivity, *gcl* granular cell layer, *pcl* Purkinje cell layer, "+" present; "–" absent; "?" unknown

The existence of Lugaro cells in aves is not surprising as the basic structure and circuitry of the cerebellar cortex is highly conserved among vertebrates [3]. This includes the connectivity among PCs, granule cells, and Golgi cells, basket and stellate cells, as well as unipolar brush cells (UBCs). Indeed, UBCs were first described in mammals [31], and then were subsequently reported in an avian species [32]. Interestingly, as with Lugaro cells, UBCs in mammalian and avian species are similar in their structure and circuitry, but different in terms of neurochemistry. For example, mammalian UBCs express PLCB4, while their avian counterparts do not.

Calcium Binding Proteins in Lugaro Cells

Although they have several functions, a major task of calcium binding proteins (CBPs) is to regulate the amount of intracellular calcium by either sensing intracellular calcium levels and/or by acting as cytosolic buffers to dissipate local calcium, as an overabundance of calcium results in neuronal death from excessive neuronal excitation [33]. Parvalbumin and Calbindin-D28k (CB) for instance act to buffer cytosolic calcium, while SCGN has been suggested to act as an intracellular calcium sensor as it undergoes structural changes upon binding calcium [33]. Similarly to SCGN, CR may also have calcium-sensing properties as it also undergoes conformational changes

when binding calcium [34], but unlike SCGN, it has also been reported to act as a calcium buffer [35].

CBPs are particularly prevalent in the cerebellum as PCs are at risk for glutamate excitotoxicity [36]. As previously discussed, pigeon Lugaro cells do not express CR, but express SCGN, whereas the contrary is true for their mammalian counterparts. It may be that CR has been replaced by SCGN in avian Lugaro cells, as both of these proteins have the ability to function in sensing intracellular calcium levels [33]. It is possible that calcium buffering in the pigeon Lugaro cells is not as important as in their mammalian counterparts, as SCGN shows evidence for function as a calcium sensor and not a buffer, whereas CR may also be able to buffer [33, 34]. It is also possible that pigeon Lugaro cells use a different mechanism to fulfill the calcium buffering role.

Evolution of Mammalian and Avian Lugaro Cells

As Lugaro cells seem to be present in both aves and mammals, it begs the question as to whether their presence is a case of convergent evolution or homology. If it is a case of homology, it may be that mammalian and avian Lugaro cells have retained the morphological characteristics and connections, but evolved different neurochemical profiles. Alternatively, it is possible that this cell type and circuitry are an example of convergent evolution. In this case, neurochemically different cells evolved to have common morphology and connectivity. Because the presence of Lugaro cells has also seen suggested in one species of teleost [23], we would argue that Lugaro cells are homologous in different species. It is unlikely that they would have evolved similarly in three separate instances and the neurochemical profiles are likely derivative [37].

Conclusion

Lugaro cells, a characteristic feature of the mammalian cerebellar cortex, have not been described in the cerebellum of birds or non-avian reptiles so far. In the present study, using SCGN expression in the avian cerebellum, we observed inhibitory cells in the gcl near the pcl that exhibit the morphological features and connectivity of mammalian Lugaro cells. They are characterized by a fusiform cell body with bipolar dendrites projecting in the sagittal plane, parallel-fiber-like axons projecting transversely through the mcl, and these cells are innervated by PC axon collaterals. We argue that these are indeed Lugaro cells although they have a different neurochemical profile than mammalian Lugaro cells. The presence of Lugaro cells in the avian cerebellum provides an additional piece of evidence towards cerebellar conservation in vertebrate species.

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Compliance with Ethical Standards

The methods used adhere to the guidelines established by the Canadian Council on Animal Care and were approved by the Biosciences Animal Care and Use Committee at the University of Alberta.

Conflict of Interest The authors declare that they have no conflict of interests.

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