Cerebellar distribution of calcitonin gene-related peptide (CGRP) and its receptor components calcitonin receptor-like receptor (CLR) and receptor activity modifying protein 1 (RAMP1) in rat

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Abbreviations

CGRP	Calcitonin gene-related peptide
CLR	Calcitonin receptor-like receptor
RAMP1	Receptor activity-modifying protein 1
RCP	Receptor component protein
PBS	Phosphate buffered-saline
BSA	Bovine serum albumin
PFA	Paraformaldehyde
PBST	Phosphate buffered-saline (PBS) containing 0.25% Triton X-100
PET	Positron emission tomography
GFAP	Glial fibrillary acidic protein

Abstract

Clinical and experimental results have revealed a fundamental role of calcitonin gene-related peptide (CGRP) in primary headaches. CGRP is widely expressed in neurons both in the central nervous system (CNS) and in peripheral sensory nerves. In the CNS there is a wide distribution of CGRP-containing neurons with the highest levels seen in striatum, amygdale and cerebellum. Moreover, in acute attacks of migraine there is evidence of cerebellar activation. To understand the role of CGRP, antibodies towards the CGRP receptor components calcitonin receptor-like receptor (CLR) and receptor activity modifying protein type 1 (RAMP1) have been developed. In the present study we therefore examined immunohistochemically the distribution of CGRP and its receptor components in the cerebellum.

CGRP immunoreactivity was only found intracellularly in the cerebellar Purkinje cell bodies, whereas CLR and RAMP1 were detected on the surface of the Purkinje cell bodies and in their processes. The elaborate dendritic tree of Purkinje cell fibers was distinctly visualized with the RAMP1 antibody. In addition, profoundly stained fibers spanning from the molecular layer into the medulla was observed with the RAMP1 antibody. Judged from the high density of immunoreactive cells expressing CGRP, RAMP1 or CLR, and from the double staining of CGRP and RAMP1 it is likely that most, if not all, Purkinje cells express both the peptide and the receptor components. Double staining with RAMP1 and the glial cell markers glial fibrillary acidic protein (GFAP) and S-100 revealed an almost identical staining pattern of the antibodies in the area of the cell body surfaces. However, as judged by confocal microscopy, no double staining was present. Instead, it was discovered that the glial cells tightly surrounded the Purkinje cells which easily could be interpreted as co-localization in the epifluorescence microscope.

Our observations demonstrate that there is a rich expression of CGRP and CGRP receptor elements in the cerebellum which points towards a functional role of CGRP in cerebellar Purkinje cells. Recent advances in the biology of the cerebellum indicate that there may be a role in nociception; hence a target of the recently discovered CGRP receptor antagonists that have demonstrated improvement in migraine pain and associated symptoms could be cerebellar CGRP receptors.
Key words: CGRP, CLR, RAMP1, rat cerebellum, immunohistochemistry.

Introduction

Positron emission tomography (PET) studies of patients during migraine attacks demonstrated activation of cerebellar regions (May and Goadsby 1999, Weiller et al., 1995, Bahra et al., 2001). However, no explanation for this activation has emerged (Goadsby et al., 2002). Migraine is considered to originate in the central nervous system (CNS), and as a result of an acute attack the trigeminovascular system is activated resulting in both central and peripheral release of calcitonin gene-related peptide (CGRP) (Edvinsson and Uddman, 2005). Increased levels of CGRP occur in the cranial venous outflow following an acute migraine attack (Edvinsson, 2004) and CGRP receptor antagonists have demonstrated improvement in migraine pain and associated symptoms (Ho et al., 2008a, b, Olesen et al., 2004). The site of action of the CGRP receptor antagonists is debated but presently both the peripheral and central ends of the trigeminovascular system are considered likely targets (Edvinsson, 2008a, Edvinsson, 2008b).

Early immunohistochemical studies have demonstrated CGRP in the cerebellum (Kawai, et al., 1985, Lee, et al., 1985). The CGRP immunoreactivity was localized in the Purkinje cell and in its elaborated dendrite tree. Morara and collaborators have since then investigated CGRP distribution in different developmental stages in rats (Morara et al., 1989, Morara et al., 2000, Morara et al., 2001, Morara et al., 1998). It has been shown that CGRP is transiently expressed in cerebellar climbing fibers (Gregg et al., 1999) while its receptor is suggested to be expressed in cerebellar glial cells (Morara et al., 1998). Climbing fiber afferents to the cerebellum from the inferior olivary complex have powerful excitatory effects on Purkinje cells. CGRP may suppress both the spontaneous firing rate of olivary neurons and the enhanced activity induced by excitatory amino acids. The receptor for CGRP is composed of a G-protein-coupled receptor known as the calcitonin receptor-like receptor (CLR), a single trans-membrane domain protein called receptor activity-modifying protein 1 (RAMP1) and an intracellular protein, receptor component protein (RCP) (McLatchie et al., 1998). A previous study on RAMP1 revealed localization in the cerebellum (Ueda et al., 2001) while the detailed localization remains unclear.

The detailed examination of CGRP receptor components have been made possible with the recent development of a series of specific antibodies against CLR and RAMP1 (Eftekhari et al., 2010). This is of particular interest in understanding how the recent CGRP receptor

antagonists exert their antimigraine effects. In the present study we examined the distribution of CGRP and its receptor components in the cerebellum.

Experimental procedures

Adult male Sprague-Dawley rats (weighing 300-400 g; Scanbur, Stockholm, Sweden) were exsanguinated during CO_2 sedation, and the cerebellum removed (n = 8). The experiments were approved by the University Animal Ethics Committee (M161-07), Lund University, Sweden.

The tissue pieces were immersed into 4% paraformaldehyde (PFA) for 2-4 hours and subsequently rinsed in raising concentrations of sucrose in Sörensen´s phosphate buffer, embedded in a gelatin medium (30% egg albumin and 3% gelatin in distilled water) and cryosectioned at 12 µm. The sections were stored at –20°C until use.

Sections were thawed and rinsed in phosphate buffered saline (PBS, pH 7.2) containing 0.25% Triton X-100 (PBST). The sections were then exposed to primary anti-sera against CGRP, CLR or RAMP1 (for detailed description of antibodies, see Table 1) for 16-18 hrs in a moist chamber at 4ºC, following rinsing in PBST. In addition, double immunostainings with CGRP and RAMP1, RAMP1 and CLR, RAMP1 and GFAP, RAMP1 and S-100 were performed. Tissue sections were incubated with secondary antibodies (Table 2) for 1 hrs in the dark at room temperature and thereafter mounted with an antifading mounting medium (Vectashield, Sigma). DAPI (Vectashield medium containing 4', 6-diamidino-2-phenylindole, Sigma) was used to stain nuclei in some sections. Omission of the primary antibody served as negative controls.

In addition, to compare the results achieved with the human RAMP1 and CLR antibodies used, we examined the outcome of rat RAMP1 and CLR immunohistochemistry (for antibody details, see (Eftekhari, et al., 2010).

Specimens were examined using an epifluorescence microscope and co-localization was assessed by super-imposition of separate digital images. In addition, confocal microscopy was performed using Nikon confocal microscope (EZ-cl, Germany) and double staining analysis using imaging software NIS Elements (Nikon).

Results

The negative controls displayed no immunoreactivity.

CGRP immunoreactivity was found in all of the cerebellar Purkinje cell bodies as intracellular granular staining. CGRP immunoreactivity was sometimes identified in combination with thin, twisted and thread-like immunoreactive formations (Figs 1A-C), resembling the appearance of endoplasmic reticulum. No immunoreactivity was found in the dendrites or axons, nor in other neurons or glial cells.

CLR immunoreactivity was found on the surface of the Purkinje cell bodies and in their processes (Figs 1D-F). However, the staining results were somewhat inconsistent compared to the results obtained with the CGRP and RAMP1 antibodies, with weakly stained or even immuno-negative areas.

Similar to the CGRP staining, RAMP1 was found in all of the Purkinje cells. The elaborated tree of Purkinje cell fibers, especially the proximal parts, displayed intense and homogenous RAMP1 immuno-positivity (Figs 1G-I). In addition, profoundly stained fibers spanned from the molecular layer into the medulla.

Staining with rat CLR and RAMP1 displayed similar results as for human CLR and RAMP1, i.e. immunoreactivity localized to the region of the cytomembrane of the Purkinje cells (Fig 2).

Double staining with CGRP and RAMP1 revealed no double-labeling of the peptide and its receptor component, but all cells positive for CGRP seemed to be positive for RAMP1 (Figs 3A-F).

CLR-RAMP1 double staining showed somewhat variable results due to inconsistency with the CLR antibody, as also was the case with the CLR single staining. However, Purkinje cells positive for both CLR and RAMP1 were found in some regions with cell surface and fiber staining for both CLR and RAMP1 (Figs 3G-I).

Double staining with RAMP1 and GFAP disclosed an almost identical staining pattern of the two antibodies in the area of the cell body surfaces, which would suggest RAMP1 in the glial cells (Figs 4A-L). In the molecular layer, RAMP1 positive horizontal fibers, judged as fibers of the Purkinje cells, were a common finding whereas GFAP stained vertical fibers typical for glial cell processes (Figs 4G-I). True double staining could not be established with the epifluorescence microscope (Figs 4J-L), however, confocal microscopy revealed that such double staining did not occur (Fig 4M, video data), which means that RAMP1 is not expressed in the cerebellar glial cells.

Double staining (Fig 5A-I) with RAMP1 and the glial cell marker, S-100, revealed a similar pattern as for RAMP1 and GFAP. In addition, Bergmann glial cell stained positive with the S-100 antibody (Fig 5F and 5J), a staining that was not present with the GFAP antibody. Also in this experiment, the glial cell marker seemed to outline the cell surface of the Purkinje cells (Figs 4G-I), but again confocal microscopy revealed no double staining (Fig 4J, video data).

In summary, CGRP and its receptor components RAMP1 and CLR could be found in an individual Purkinje cell (Fig 6). However, no peptide- or receptor immunoreactivity was found in the glial cells.

Discussion

The aim of the present study was to identify CGRP and its receptor components within the rat cerebellum since there are PET data revealing activation of the cerebellum in migraine attacks. For this purpose, we used a set of newly developed and in depth characterized antibodies against CLR and RAMP1 (Eftekhari et al., 2010).

The study demonstrates for the first time the detailed mapping of CGRP together with its receptor components in the rat cerebellum. The study shows that CGRP is exclusively found in the cytoplasm of cerebellar Purkinje cell bodies, which confirms results from an early study of Kawai and co-workers (Kawai et al., 1985). RAMP1 and CLR, which collectively represent the two components of the mature CGRP receptor, are expressed on the surface of the Purkinje cell bodies and in the fibers, spanning through the entire cerebellum. In the CGRP receptor complex the CLR component is a 7 transmembrane component that needs to be associated with RAMP1 in order to form the functional CGRP receptor. Overall there was a larger RAMP1 distribution compared to CLR. There are several possible explanations for this; (i) it might reflect differences in the quality of the antibodies recognizing the epitopes. (ii) The slightly higher proportion of RAMP1 compared to CLR may also reflect the presence of other RAMP1-containing receptors such as the amylin receptors (Oliver, et al., 2001). Since there are few amylin binding sites in the cerebellum, another receptor could be involved. (iii) Hypothetically, since other receptors use RAMP1 in their formation, RAMP1 may well be functionally rate limiting in receptor formation and an excess would be necessary for high density CGRP binding. This issue may be clarified in future studies.

Double-staining with CGRP and RAMP1 revealed no co-localization between the peptide and its receptor components, although they were expressed in the same cell suggesting that the neuronal messenger might act on sites where it is produced. RAMP1 and CLR double staining revealed that both are expressed in the same cell which indicates that the functional receptor is present. In addition, double staining with RAMP1 and the glial cell markers did not reveal any co-localization. This was studied in detail using confocal microscopy and the NIS Element software; this allows the full use of confocal microscopy where stacks of 100-200 sections can be viewed and analyzed 3-dimensional. Hence, the adjacent glial cells were observed not to be the target of the Purkinje cell CGRP. Glial cells were in the rat cerebellum devoid of both CGRP and CGRP receptors.

CGRP has a key role in migraine, where levels of trigeminal system released CGRP is increased during migraine attacks (Ho et al., 2010). Activation of regions in the cerebellum has been demonstrated by PET in patients during acute migraine attacks (May and Goadsby 1999, Weiller et al., 1995, Bahra et al., 2001). The cerebellum is of interest in migraine research as studies suggest a role of cerebellum in symptoms of migraine such as vertigo (reviewed by Vincent and Hadjikhani 2007) Our observations reveal that there is a rich expression of CGRP and CGRP receptor elements in the cerebellum clearly pointing towards a functional role of CGRP in the cerebellum; this is of course not necessarily related to primary headaches.

Disclosure Statement

All authors participated in design, performing the study, and writing of the manuscript. All authors participated in the writing of the manuscript and approved the final manuscript.

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Figure legends

Figure 1. CGRP (A-C), CLR (D-F) and RAMP1 (G-I) immunohistochemistry. Figures A-C reveal immunoreactivity in the cerebellar Purkinje cell bodies. The staining is exclusively found in the cytoplasm and disclosed as intracellular, granular staining, sometimes in combination with thin, twisted and thread-like immunoreactive formations (arrows). Figures D-F show a punctuate CLR immuno-positivity, seemingly on the Purkinje cell surface (arrow). Figures G-I demonstrate RAMP1 staining. The elaborated tree of Purkinje cells is shown, arrows point at horizontal fibers in the molecular layer. Immuno-stained fibers are spanning from the molecular layer into the medulla.
ml – molecular layer, Pc – Purkinje cells

Figure 2. Montage demonstrating CLR and RAMP1 immunohistochemistry using rat antibodies. The localization of the immunoreactivity resembles the one seen with the human antibodies; i.e. immunoreactivity localized to the region of the cytomembrane of the Purkinje cells.

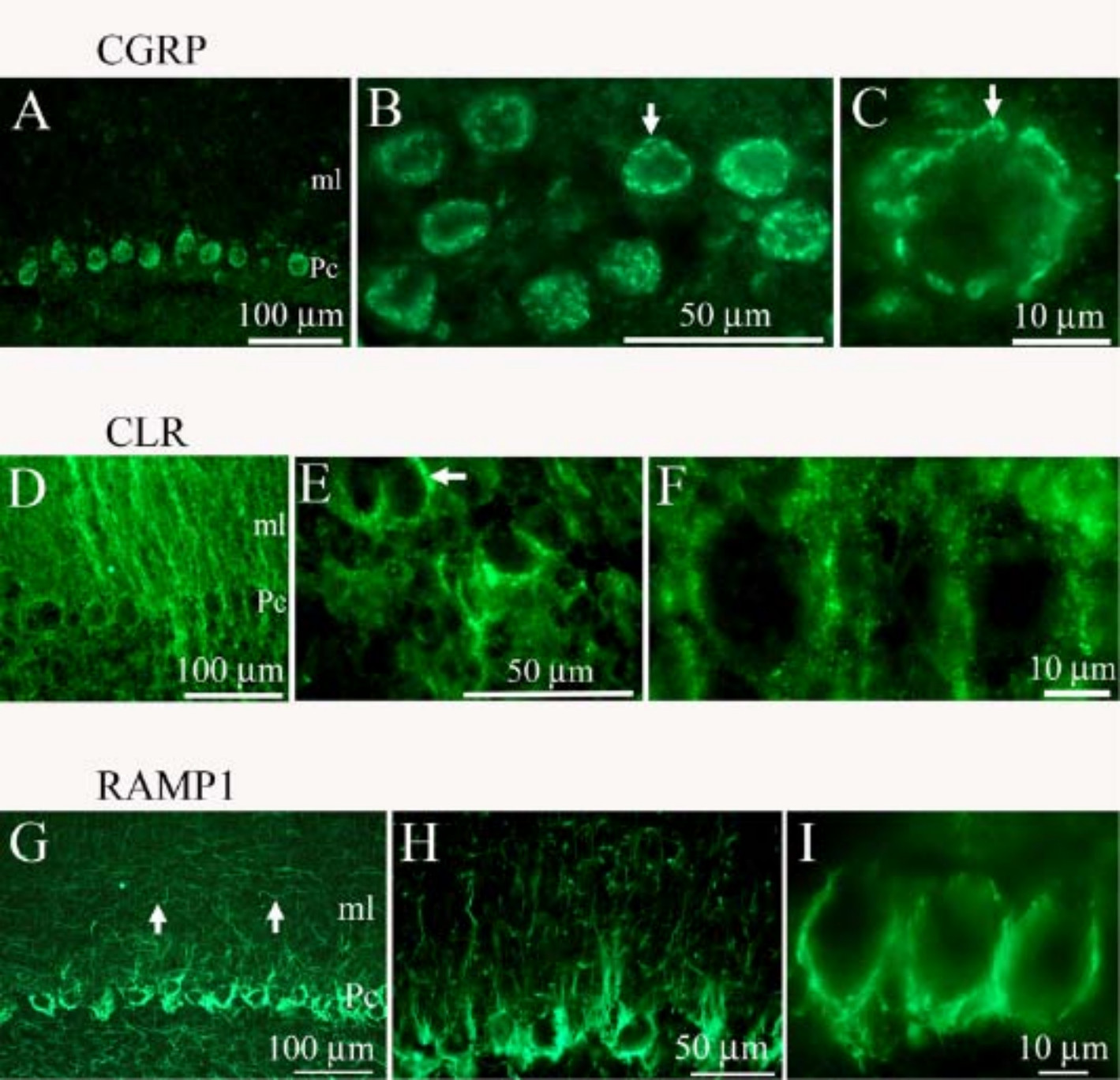
Figure 3. RAMP1 and CGRP, and RAMP1 and CLR double staining. CGRP was exclusively expressed in the cytoplasm of Purkinje cell bodies as opposite to the RAMP1 staining (A-F), which was expressed on the surface of the cell bodies and in the processes throughout all the cerebellar layers. (G-I) CLR and RAMP1 were expressed on the surface of the Purkinje cells
ml – molecular layer, Pc – Purkinje cells, DAPI – blue

Figure 4. RAMP1 and GFAP double staining. Images A-C represent low magnifications of the cerebellar layers. The RAMP1 image demonstrates the distribution of immuno-positive fibers spanning through the cerebellar layers. Also the GFAP staining reveals immunopositivity in all the layers. Images D-E shows the staining of the granular layer and the white matter. Arrow points at RAMP1 a positive fiber and asterisk at a GFAP positive astrocyte. Images G-I show the molecular and Purkinje cell layer. Arrow points at a horizontal RAMP1 positive fiber and asterisk at GFAP positive vertical process. Images J-L demonstrate the Purkinje cell layer. The staining with RAMP1 and GFAP showed similar pattern of cell surface immunoreactivity. However, there was a difference: the GFAP staining distinctly outlined the cell surface whereas the RAMP1 staining seemed more diffuse. Large arrows point at regions where either of the antibodies showed positivity and small arrow at a

Bergmann glial cell outlined with RAMP1 immunoreactivity, which could be mistaken for RAMP1 immunoreactivity in Bergmanns cells. However, confocal microscopy revealed that no co-localization exists (M), which means that RAMP is exclusively expressed in neurons and GFAP in glial cells. Image 3M is also shown as video data, see supplementary data.
ml – molecular layer, Pc – Purkinje cells, gc – granular layer, wm – white matter, DAPI – blue

Figure 5. RAMP1 and S-100 double staining. Images A-C demonstrate low magnifications of the molecular, Purkinje cell and granular layers. Thin arrows point at a Purkinje cell seemingly positive for both antibodies and thick arrow at a fiber traversing through the granular layer. Images D-F show the staining of the Purkinje cells: RAMP1 intense, but diffuse and the S-100 outlining the cells. Thin arrows point at a region where the RAMP1 and S-100 staining went parallel, arrowheads at areas with distinct S-100 staining but diffuse RAMP1. Thick arrows point at Bergmann glial cells. Images G-I demonstrate a high magnification of Purkinje cells. Thin arrows point at the cell surface with distinct RAMP1 and with missing S-100 staining. Arrowheads point at the outlining of a process, the RAMP1 and S-100 running parallel. (J) Confocal microscopy in combination with NIS Element software revealed no co-localization of RAMP1 and S-100. Arrows point at Bergmann glial cells.
ml – molecular layer, Pc – Purkinje cells, gc – granular layer, DAPI – blue. Image 4J is also shown as video data, see supplementary data.

Figure 6. A schematic drawing of CGRP (Purkinje cell cytoplasm), RAMP1 (Purkinje cell surface), CLR (Purkinje cell surface) and glial cell distribution.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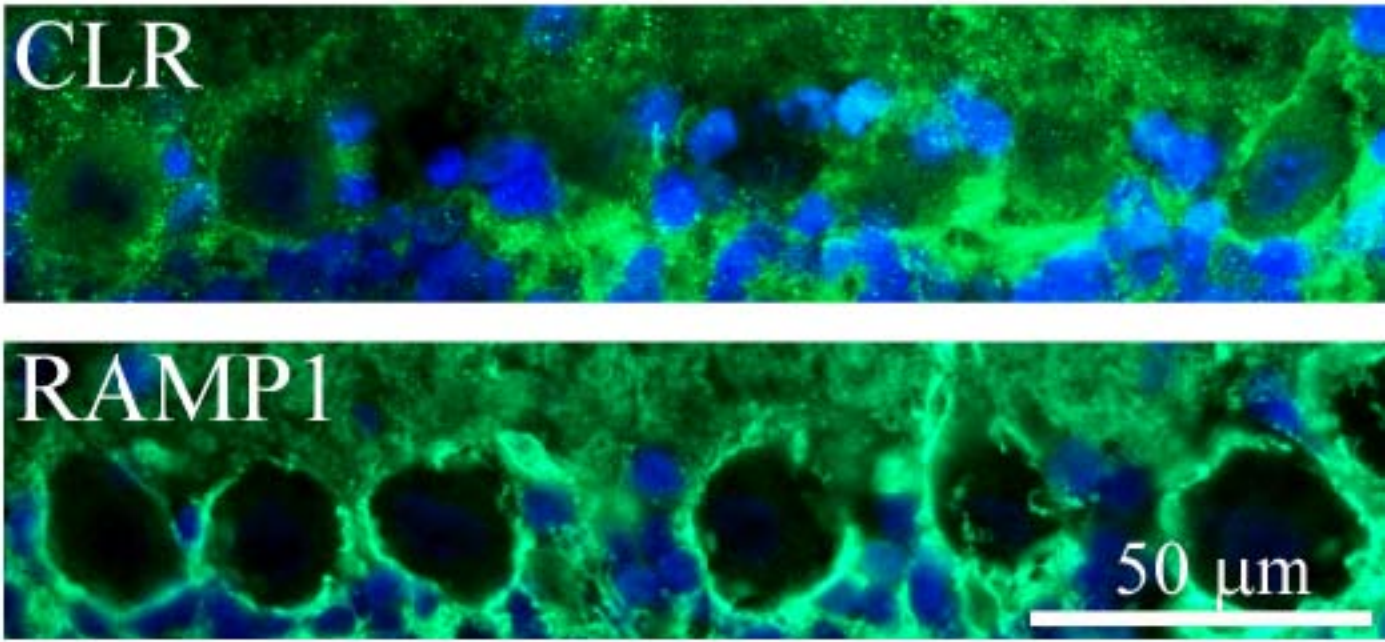
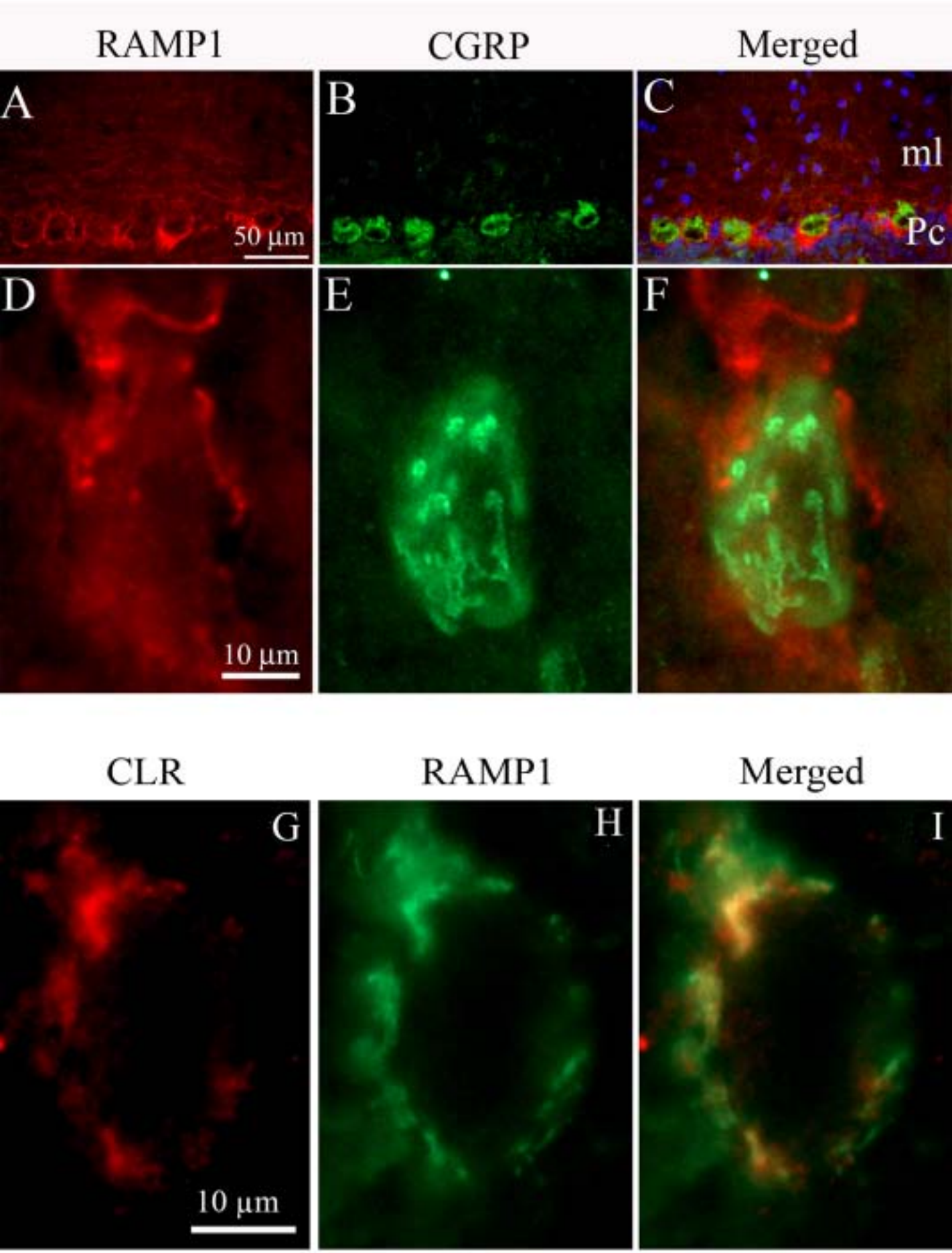
Anti-rat antibodies

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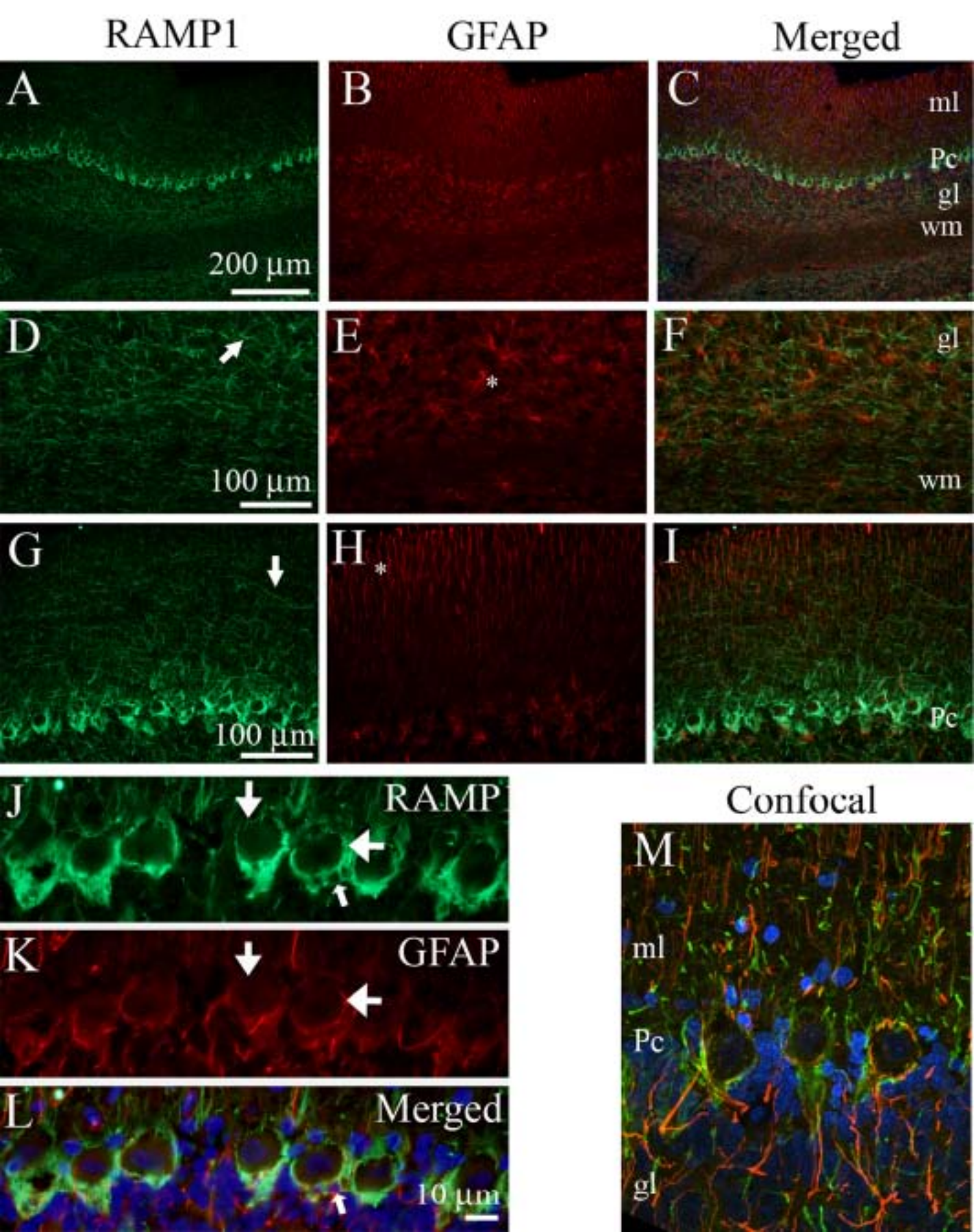
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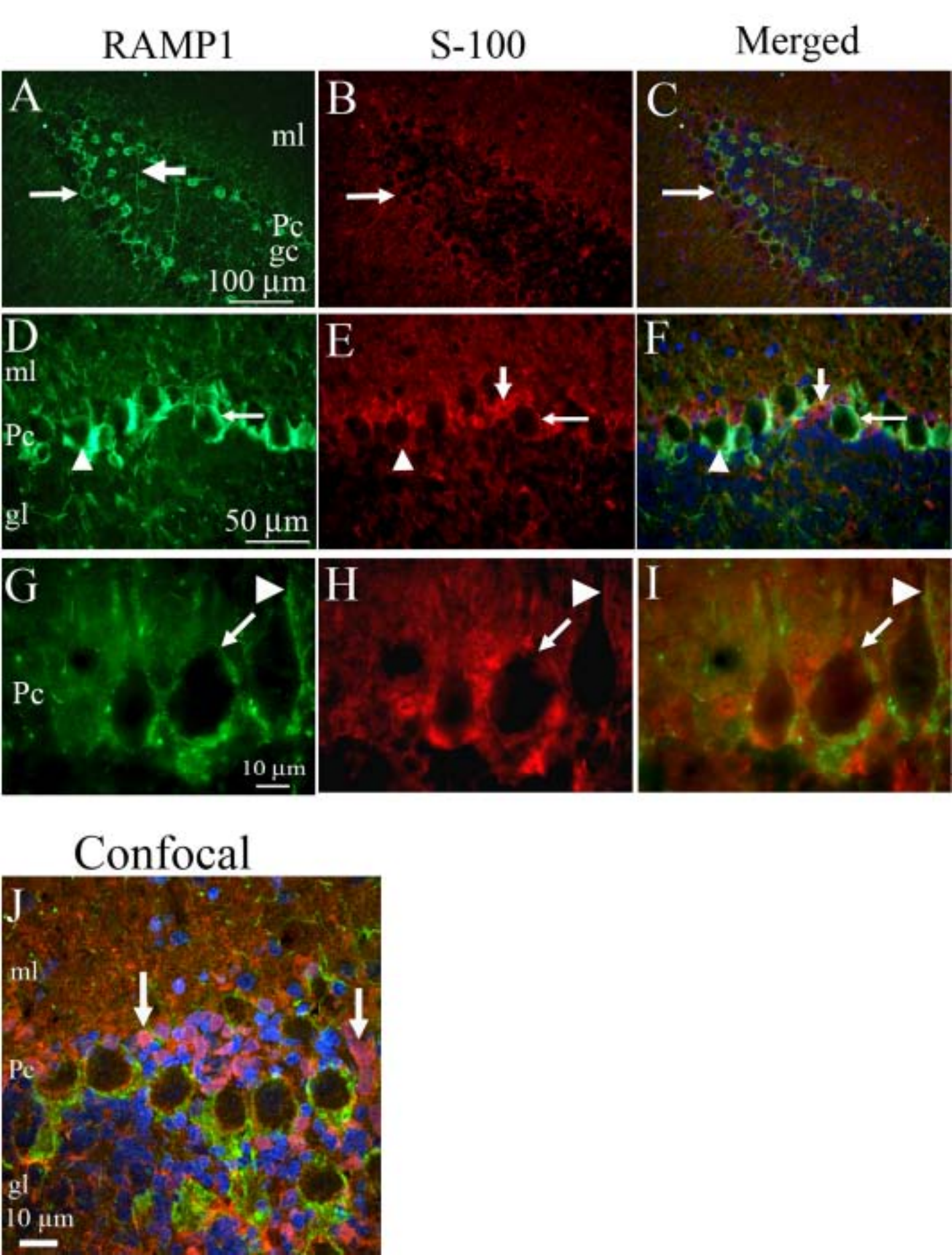
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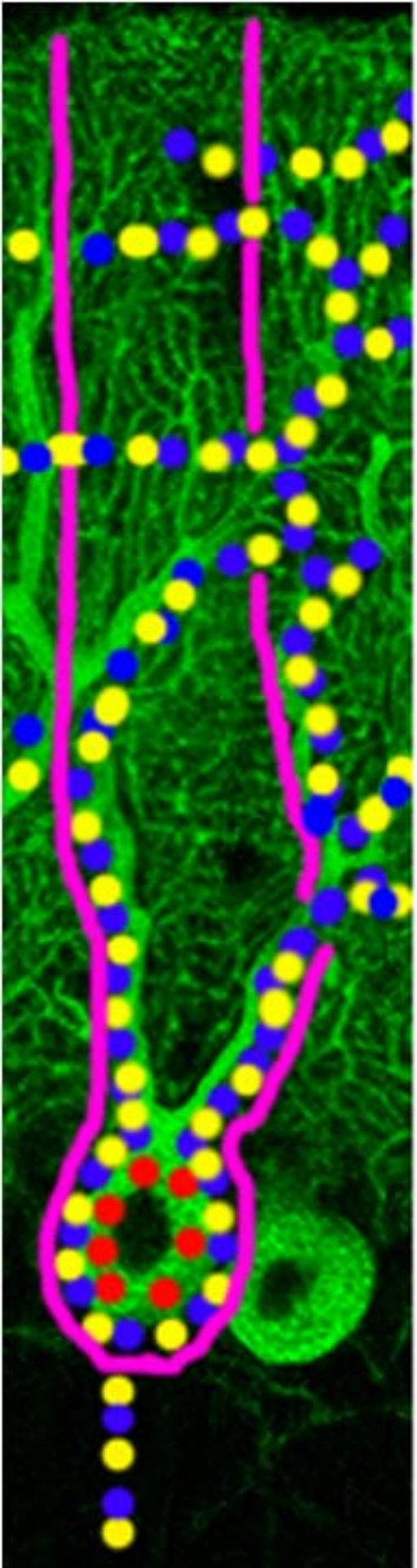

- CGRP
- RAMP1
- CLR
- GFAP

Molecular layer

Purkinje cell layer

Granular layer

Table 1. Details on primary antibodies used for immunohistochemistry

Name and product code	Host	Dilution	Detects	Supplier
Calcitonin gene related peptide (CGRP), B47-1	Rabbit	1:1600	Rat CGRP	Europroxima; Arnhem, The Netherlands.
Calcitonin receptor-like receptor (CLR) 3152	Rabbit	1:500	C-terminal of human CLR	Merck & Co., Inc., (Eftekhari, et al., 2010)
Receptor activity modifying protein (RAMP1) 844	Goat	1:100	C-terminal of human RAMP1	Merck & Co., Inc., (Eftekhari, et al., 2010)
Glial fibrillary acidic protein (GFAP)	Rabbit	1:1500	Glial cells	Dako; Copenhagen, Denmark
S-100, ab66041	Rabbit	1:300	Glial cells	Abcam; Cambridge, UK

Table 2. Secondary antibodies used for immunohistochemistry

Conjugate and host	Against (used with primary ab)	Dilution	Supplier
FITC (goat)	anti-rabbit (CGRP, CLR)	1:100	Cayman Chemical, Ann Arbor,MI
Alexa 488 (donkey)	anti-goat (RAMP1)	1:400	Invitrogen, La Jolla, CA
Texas-Red (donkey)	anti-goat (RAMP1)	1:200	Jackson Immunoresearch, West Grove, PA
Texas-Red (donkey)	anti-rabbit (CLR, S-100, GFAP)	1:200	Jackson Immunoresearch, West Grove, PA