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ORIGINAL PAPER

Localization of CGRP Receptor Components, CGRP, and Receptor Binding Sites in Human and Rhesus Cerebellar Cortex

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Abstract The cerebellum is classically considered to be mainly involved in motor processing, but studies have suggested several other functions, including pain processing. Calcitonin-gene-related peptide (CGRP) is a neuropeptide involved in migraine pathology, where there is elevated release of CGRP during migraine attacks and CGRP receptor antagonists have antimigraine efficacy. In the present study, we examined CGRP and CGRP receptor binding sites and protein expression in primate cerebellar cortex. Additionally, mRNA expression of the CGRP receptor components, calcitonin receptor-like receptor (CLR) and receptor activity modifying protein 1 (RAMP1), was examined. In addition, expression of procalcitonin was studied. We observed high [^{3}H]MK-3207 (CGRP receptor antagonist) binding densities in the molecular layer of rhesus cerebellar cortex; however, due to the limit of resolution of the autoradiographic image the exact cellular localization could not be determined. Similarly, [^{125}I]CGRP binding was observed in the molecular layer and Purkinje cell layer of human cerebellum. CLR and RAMP1 mRNA was expressed within the Purkinje cell layer and some expression was found in the molecular layer. Immunofluorescence revealed expression of CGRP, CLR, and RAMP1 in the Purkinje cells and in cells in the molecular layer. Procalcitonin was found in the same localization. Recent research in the biology of cerebellum indicates that it may have a role in nociception. For the first time we have identified CGRP and CGRP receptor binding sites together with CGRP receptor expression through protein and mRNA localization in primate cerebellar cortex. These results point toward a functional role of CGRP in cerebellum. Further efforts are needed to evaluate this.

Keywords Cerebellum · Nociception · CGRP · CLR · RAMP1 · Primate

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Introduction

The cerebellum is classically considered an area of the brain mainly involved in motor processing, but recent studies have suggested several other functions, including pain processing [1]. The deep cerebellar nuclei receive inhibitory inputs from Purkinje cells in the cerebellar cortex and excitatory inputs from mossy fiber and climbing fiber pathways. The deep cerebellar nuclei project to brainstem nuclei and the thalamus, which relays these projections to different parts of the cerebral cortex [1]. A detailed description of the organization of the connections from the cerebellar nuclei to the brainstem in rat showed that widespread areas in the brainstem receive cerebellar projections [2]. Some of these regions are red nucleus, inferior olive, deep layers of spinal trigeminal complex, periaqueductal grey (PAG), and ventrolateral thalamic nuclei [2]. The pons receives input from cerebral cortical efferents that classically relate to motor function but there are also cortical inputs from association areas involved in cognition, such as the prefrontal cortex [3], and in pain modulation [4],

which distributes the information via mossy fibers to the cerebellum. The inferior olive sends information via climbing fibers from both cortical and subcortical sources to the cerebellum [5].

The cerebellum receives inputs from cutaneous primary afferent connections as based both on neuroanatomical tracing [6] and electrophysiology [7, 8]. Thus, stimulation of cutaneous Aδ- and C-fiber nociceptors activates climbing fibers that terminate on Purkinje cells [8]. Early neurophysiology and anatomy literature report that sensory modalities arise in the trigeminal nucleus in mid pons and end in the anterior lobule and the tonsils [9]. It is possible that both crossed and uncrossed fibers arise in the spinal nucleus of the trigeminal and ascend to the same areas of the cerebellum. It has been suggested that C-fiber nociceptors may act through mossy fibers to reach the Purkinje cells, the final decoders of information in the cerebellar cortex [10]. In particular, the cerebellum may engage pain modulation circuitry in the brainstem that involves PAG and the rostral ventromedial medulla [11].

Pain neuroimaging studies often show cerebellar activation [1]. Stimuli such as thermode, electric, laser, and capsaicin injection evokes cerebellar activation. Neuroimaging studies have demonstrated that, during pathological pain conditions such as neuropathy, cluster headache, back pain, and fibromyalgia, activation of the cerebellum is observed [1]. The first PET study of acute migraine attacks revealed activation both of the cerebellum and brainstem area around the PAG [12]; this finding has been substantiated and extended by others [13]. Additionally, this response was unrelated to pain relief by subcutaneous sumatriptan. In human pain imaging studies, activation of the cerebellum is almost always observed [14] suggesting a role in nociception. However, relatively little is known about the details of signaling and distribution of pain-relevant receptors in cerebellum.

Calcitonin gene-related peptide (CGRP) is a neuropeptide consisting of 37 amino acids and is involved in transmitting nociceptive information to second order neurons within CNS [15]. CGRP is widely expressed in the CNS, in regions such as the striatum, amygdale, hypothalamus, brainstem, trigeminal ganglion, and the cerebellum [16, 17]. CGRP is so far the only neuropeptide consistently shown to be involved in migraine pathophysiology, where there is elevated release of CGRP during migraine attacks [18, 19]. The receptor for CGRP is a G-protein-coupled receptor, consisting of a seven transmembrane-spanning protein, calcitonin receptor-like receptor (CLR), a single transmembrane-spanning protein, receptor activity modifying protein 1 (RAMP1), and an intracellular protein, receptor component protein (RCP) [20, 21]. There are three different RAMP proteins: RAMP1, RAMP2, and RAMP3. Interaction of CLR with RAMP2 or RAMP3 forms adrenomedullin receptors [20, 22]. RAMPs can interact with the calcitonin receptor to form amylin receptors. For a functional CGRP receptor, co-expression of CLR and RAMP1 is required. Recently, CGRP receptor antagonists have been developed with clinical efficacy [23].

In a recent study on rodents, we observed that the Purkinje cells store both CGRP and the CGRP receptor components, CLR and RAMP1 [24]. In support of this observation, Salvatore et al., 2010, reported that tritium-labeled MK-3207, a selective CGRP receptor antagonist with clinical antimigraine efficacy [25], displayed high binding densities in the rhesus cerebellum cortex but the cellular distribution remained unclear. The present study was designed to examine in detail cellular localization of CGRP and CGRP receptors. We addressed this issue in monkey and human, using immunohistochemistry, in vitro autoradiography, and in situ hybridization mapping studies to define the cerebellar localization of CGRP and its receptor binding sites in order to unravel their putative role in cerebellum physiology and in migraine.

Materials and Methods

Post-mortem Human Tissue Samples

Samples of cerebellum were obtained at autopsy from adult subjects in accordance with the University of Szeged guidelines for ethics in human tissue experiments and were approved by the Hungarian Ethics Committee. The tissue was bilaterally removed from six subjects (three female; three male) with an age span of 65 to 86 years. None of the subjects suffered from any central nervous system disease and the causes of death were related to heart failure, septicemia, or cancer. The tissues were collected within 24 to 36 h after death. The samples were immersed overnight in fixative consisting of 4 % paraformaldehyde (PFA) and in 0.1 mol/*l* phosphate buffer, pH 7.2. After fixation, the specimens were rinsed in sucrose-enriched (10 %) Tyrode solution overnight, frozen, and then stored at −80 °C. The samples were 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. In addition, two paraffin-embedded human cerebella were obtained from the Department of Pathology Lund, Lund University (one male 67, years old; one female, 74 years old, cause of death were due to cardiac reasons). This part was approved by the Regional Ethical Review Board in Lund, Sweden (LU-801-01). The tissue was collected at autopsy, prior to which the bodies were kept at refrigerator temperature (+4 °C). The autopsy procedure lasted approximately 2 h, during which the body temperature approached room temperature. Paraffin sections were cut at 5 μm and incubated at +60 °C for 1 h. Human cerebellum (Analytical Biological Services Inc., Wilmington, DE) utilized for autoradiography studies was collected 7 h post-mortem from a brain donor (male, 46 years old) without any known neurological disorders. The procedures for the human

samples were conducted according to the principles outlined in the Declaration of Helsinki.

Rhesus Monkey Tissue Samples

Rhesus cerebellum (*Macaca mulatta*, n=3, age; 13–15 years old, females) was harvested and divided into pieces in accordance with a Merck Research Laboratories Institutional Animal Care and Use Committee approved protocol. The samples were placed in a fixative solution consisting of 4 % PFA in 0.1 mol/L phosphate buffer, pH 7.2, approximately 20 min after sacrifice and fixed overnight. After fixation, specimens were either rinsed in sucrose-enriched (25 %) Tyrode solution overnight, frozen, and stored at −80 °C or paraffin embedded. The tissues were sent to the laboratory in Sweden for immunohistochemistry experiments. The rhesus samples were then gelatin-embedded, sectioned, and stored as the human samples. CITES, import permits, for the Swedish part was approved and given the permit number Dnr34-10088/10 nr 51016-10. CITES permit for export from USA was approved and given the permit number 10US11621A/9. Tissues to be used for in situ hybridization were quickly removed and frozen over dry ice. The samples were cryosectioned at 20 μm (cryostat model CM3050: Leica Microsystems, Inc., Deerfield, IL) and collected on cold Superfrost® Plus slides, and stored at −80 °C until hybridization.

Autoradiography Studies

Autoradiography studies were conducted as previously described [26]. Briefly, rhesus and human cerebellum slices (20 μm) were prepared using a cryostat (model CM3050; Leica Microsystems, Inc., Deerfield, IL) from a fresh frozen rhesus monkey or human brain. Slices were preincubated for 15 min in binding buffer (0.9 % NaCl, 50 mM Tris–HCl, pH 7.5, 2 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$) followed by 90 min incubation with [^{3}H]MK-3207 (0.045 nM; specific activity 73.7 Ci/mmol) or [^{125}I]CGRP (80 pM; specific activity 2,200 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) at room temperature for rhesus and human, respectively. Non-displaceable binding was defined by blocking with 1 μM unlabeled MK-3207 [26] or 30 nM MK-2918 [27] using an adjacent slice for rhesus and human, respectively. Slices were washed three times in ice-cold buffer (0.9 % NaCl and 50 mM Tris–HCl, pH 7.5) followed by an ice-cold rinse. Rhesus brain slices were air-dried and exposed to phosphorimaging plates (TR2025; Fujifilm Medical Systems USA, Inc., Stamford, CT) for 3 weeks and scanned with a BAS 5000 scanner (Fuji, Tokyo, Japan). Image analysis was conducted with MCID software (MCIC, Linton, Cambridge, UK). Human brain slices were air-dried, exposed to X-ray film for 2 days, developed, and scanned images analyzed as above.

Rhesus CLR and RAMP1 cDNA Cloning

A partial rhesus RAMP1 cDNA was previously isolated from rhesus forebrain ([28]; accession number AY587017). Rapid amplification of cDNA ends (RACE) experiments were conducted using the GeneRacer Kit (Invitrogen, Carlsbad, CA) according the manufacturer's recommended protocol. 5′ RACE gene-specific primers (5′-GCAGCCTAGCTTCTC CGCCATGTG and 5′GTGCAGTCGGCCAGCTCCCTGTAG) and for 3′ RACE gene-specific primers (5′-CTGGCCGACTG CACCTGGCACATG and 5′-CTGGCAGTGCACGGGCACT ACTTC) were designed based on the partial RAMP1 sequence.

A partial rhesus CLR cDNA was isolated from rhesus forebrain cDNA using PCR. The PCR primers were based upon human CLR (5′-CCATTCAACAAGCAGAAGGC and 5′-CATCACTGATTGTTGACACTGTG). Amplification reactions consisted of 30 cycles of 30 s at 94 °C, 30 s at 50 °C, and 90 s at 68 °C and were carried out according to the manufacturer's recommended protocol for Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA). Amplification products were subcloned using TOPO TA Cloning Kit (Invitrogen) and multiple clones were sequenced bidirectionally.

In Situ Hybridization

Probes were designed to be specific for CLR and RAMP1. The CLR probe corresponds to bases 1018–1453 of human CLR (accession number L7630) and was designed to equally distribute the human and rhesus non-conserved bps. The RAMP1 probe (447 bp) corresponds to the full length rhesus RAMP1 coding sequence. Both probes were synthesized by GENEWIZ (South Plainfield, NJ) and subcloned into pBluescript SK+ vectors as 5′ EcoRI-3′XbaI fragments.

The CLR and RAMP1 vector constructs were linearized with EcoR1 (antisense) and Xba1(sense) and used to generate ^{35}S-UTP-labeled cRNA probes for in situ hybridization.

The slide-mounted sections were processed for in situ hybridization. Briefly, section-mounted slides were slowly warmed to room temperature, fixed in ice-cold 4 % PFA, pH 9 for 5 min, followed by washes in: 1× PBS (2 min), 100 mM triethanolamine (TEA, 1 min), 0.25 % acetic anhydride in 100 mM TEA (10 min), and 2× saline-sodium citrate (SSC, for 2 min). The sections were then delipidated and dehydrated with chloroform and ethanol and allowed to air-dry. Sections were hybridized overnight at 55 °C with hybridization solution, 200 μl/slide. The hybridization solution was 1/5 a mix of 1 M dithiothreitol (DTT) in TEA combined with ^{35}S-labeled cRNA probe at a concentration of 6×10^6 counts/slide and hybridization buffer. The other 4/5 of the hybridization solution was a mix of 50 % formamide, 5 % dextran, 0.3 M

NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, 1× Denhardt's, and 0.2 M DTT, Q.S. with double-distilled water.

After hybridization sections were washed in 2× SSC containing DTT at room temperature, treated with RNAse (5 ml/L RNAse) for 30 min at 37 °C, and washed in 1× SSC for 15 min at RT, followed by 40 min at 72 °C in 0.1× SSC. Slides were equilibrated to room temperature, dehydrated with increasing concentrations of ethanol, and air-dried. Sections were placed in film cassettes and exposed to Biomax film (Kodak # IB 8715187) for 7 days. The films were developed using a Kodak Min-R mammography processor and digitized using a Vidar film scanner. The tissue sections were then dipped in NTB-2 liquid emulsion (Kodak # IB1654433) and stored at 7 weeks at 4 °C in light-tight desiccated slide boxes. After the 7 weeks, these sections were processed for silver grain deposition with developer and fixer (Kodak #1464593 and 1971746), stained with hematoxylin and cover slipped. Scanned images of the tissue sections were taken using an Aperio brightfield slide scanner.

Hematoxylin–Eosin Staining

Paraffin sections were deparaffinized in xylene followed by serials of alcohol. Human and rhesus monkey sections were stained with hematoxylin–eosin (Htx–eosin) using a standard protocol (Htx 3 min, water rinse, eosin 1 min) for orientation and examination of the tissue condition. Adjacent slides used for in situ hybridization were as well stained with Htx.

Immunohistochemistry

Before the immunohistochemistry experiments, paraffin sections were deparaffinized in xylene followed by serials of alcohol. To unmask the antibody epitopes, heat-induced epitope retrieval were performed, where the sections were heated in a microwave for 10 min in citric acid solution pH 6.0.

All sections were washed for 10 min in PBS pH 7.2 containing 0.25 % Triton X-100 (PBST). The sections were blocked for 1 h in blocking solution of PBS and 5 % normal donkey or goat serum (depending on species origin of the secondary antibody). After blocking, the sections were incubated overnight at +4 °C for single or double immunolabeling with primary antibodies against CGRP, CLR (3152), and RAMP1 (844). The development and specificity of CLR and RAMP1 antibodies have been demonstrated in our previous study, where the specificity of the antibodies was confirmed in HEK293 cells stably expressing the human CGRP receptor and was confirmed by Western blotting [29]. In addition, double immunostaining for CGRP and procalcitonin, GFAP, S-100, GAD67 with CGRP and the receptor components was performed. For detailed description of the primary antibodies, see Table 1. For all double immunostainings, the antibodies were applied separately and not mixed as a cocktail. Omission of the primary antibody served as negative controls for all antibodies. The primary antibodies were diluted in PBST containing 1 % BSA and 3 % normal serum. After incubation with primary antibodies, sections were equilibrated to room temperature, rinsed in PBST for 3×15 min and exposed to secondary antibodies (for details, see Table 2) in PBST and 1 % BSA for 1 h at room temperature. The sections were subsequently washed with PBST for 3×15 min. Vectashield, an anti-fading medium, containing DAPI (Vectashield, Vector Laboratories, Burlingame, CA).

Microscopic Analysis

Immunostained sections were examined and images were obtained using a light- and epifluorescence microscope (Nikon 80i, Tokyo, Japan) coupled to a Nikon DS-2MV camera. Adobe Photoshop CS3 (v.8.0, Adobe Systems, Mountain View, CA) was used to visualize co-labeling by superimposing the digital images and processed for brightness and contrast.

Results

Autoradiographic Studies

High [^{3}H]MK-3207 binding densities were observed in the molecular layer of rhesus cerebellum while the granular cell layer

Table 1 Details on primary antibodies used for immunohistochemistry

Name and product code	Host	Dilution	Detects	Supplier
Calcitonin receptor-like receptor (CLR) 3152	Rabbit	1:500	C-terminal of human CLR	Merck & Co., Inc, USA
Receptor activity modifying protein (RAMP1) 844	Goat	1:100	C-terminal of human RAMP1	Merck & Co., Inc, USA
Calcitonin gene-related peptide (CGRP, PA1-36017)	Guinea pig	1:500	Human and rat CGRP	Thermo Scientific, IL, USA
Calbindin (ab11426)	Rabbit	1:200	Human, mouse, and rat calbindin D28k	Abcam, Cambridge, UK
Procalcitonin (ab53897)	Rabbit	1:500	Amino acids 69–82 of human procalcitonin	Abcam, Cambridge, UK
GFAP (z0334)	Rabbit	1:1,500	Glial cells	Dako, Copenhagen, Denmark
S-100 (ab66041)	Rabbit	1:200	Glial cells	Abcam, Cambridge, UK
GAD67 (MAB5406)	Mouse	1:500	67 kDa isoform of glutamate decarboxylase	Millipore, Billerica, USA

Table 2 Secondary antibodies used for immunohistochemistry

Conjugate and host	Against	Dilution	Supplier
DyLight 549 (goat)	Anti-rabbit	1:200	Jackson Immuoresearch, West Grove, PA
DyLight 549 (donkey)	Anti-goat	1:200	Jackson Immuoresearch, West Grove, PA
DyLight 488 (donkey)	Anti-goat	1:200	Jackson Immuoresearch, West Grove, PA
DyLight 488 (donkey)	Anti-rabbit	1:200	Jackson Immuoresearch, West Grove, PA
DyLight 549 (rabbit)	Anti-mouse	1:200	Jackson Immuoresearch, West Grove, PA
Alexa 488 (goat)	Anti-guinea pig	1:200	Invitrogen, La Jolla, CA

showed no radiotracer binding (Fig. 1). Due to the limit of resolution of the autoradiographic image it was not possible to accurately determine if the radiotracer binds to the Purkinje cell layer. [^{3}H]MK-3207 displayed minimal nondisplaceable binding defined on an adjacent brain slice by 1 μM self-block (Fig. 1). Similarly, high-density [^{125}I]CGRP binding was observed in the molecular layer, in addition to the Purkinje cell layers of human cerebellum (Fig. 2a). Moderate nondisplaceable binding was observed in the granular layer and white matter of the human cerebellum. To identify the granular and molecular layers the slides were counter-stained with Htx-eosin (Figs. 1 and 2b).

Isolation of Rhesus CLR and RAMP1 cDNAs

The full-length rhesus RAMP1 cDNA encodes a protein of 148 amino acids in length, which shares 96.4 % identity with human RAMP1 nucleotide sequence. The partial rhesus CLR cDNA is 1,076 bp in length which shares 98.9 % identity with human CLR on the nucleotide sequence. The rhesus RAMP1 and CLR sequences have been deposited in GenBank with accession numbers KC855758 and KC855759, respectively.

In Situ Hybridization

Expression of CLR and RAMP1 mRNA was localized to the Purkinje cell layer (Fig. 3a, b). It appears that there was less CLR mRNA expression as compared to that of RAMP1 mRNA, but this would need to be confirmed with RT-PCR. CLR and RAMP1 mRNA expression was mostly expressed around the Purkinje cells (Fig. 3a, b). Some Purkinje cells displayed mRNA expression within the cell body. It could not be determined if the expression of CLR and RAMP1 was located on the dendrites/processes of the Purkinje cell and/or other cell types in the Purkinje cell layer. Low mRNA detection within the soma of Purkinje cells could be due to artifactual changes such as cell shrinkage and loss of Purkinje cells in theses slides (as visualized by Htx staining). This may also be due to that the mRNA levels in the Purkinje cells were low at the time of the experiment.

Histology

Some cell shrinkage was observed in the rhesus and human materials used for immunohistochemistry (Fig. 4a, b). Areas where Purkinje cells were absent or lost were seen. Paraffin-embedded cerebellum displayed better morphology.

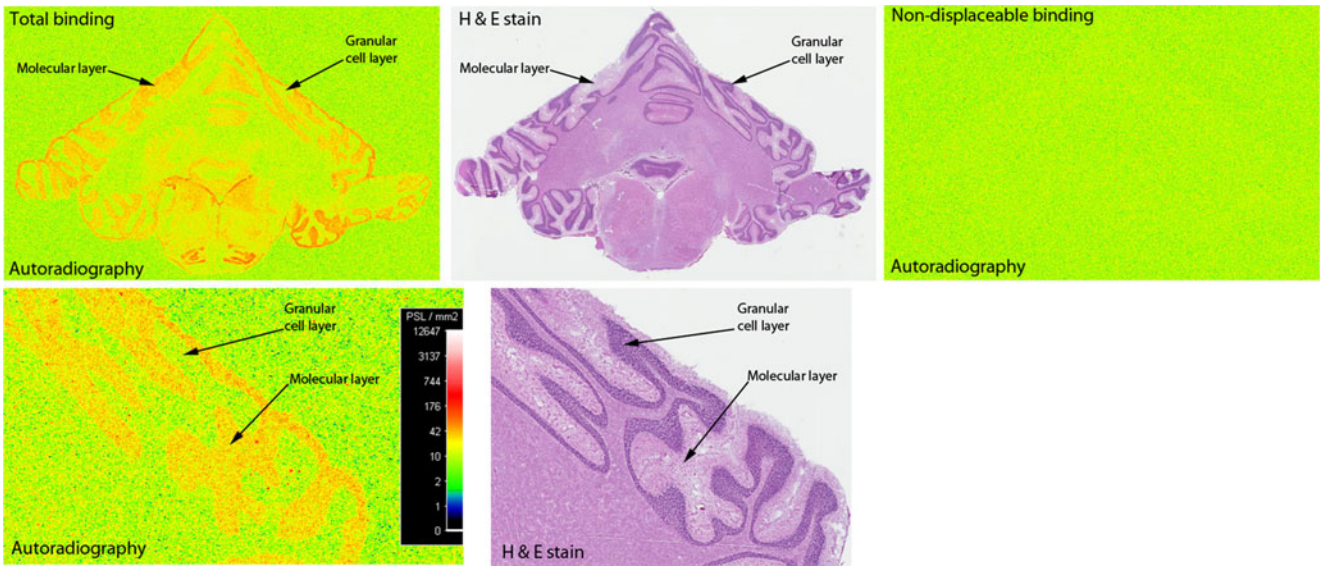

Fig. 1 Distribution of [^{3}H]MK-3207 binding to rhesus monkey coronal brain slices. [^{3}H]MK-3207 displayed high binding density in the molecular layer of the cerebellar cortex with minimal-to-no binding in the granular cell layer and white matter (total binding). Htx–eosin staining of the same slice. Self-block on the adjacent slide with no binding (non-displaceable binding)

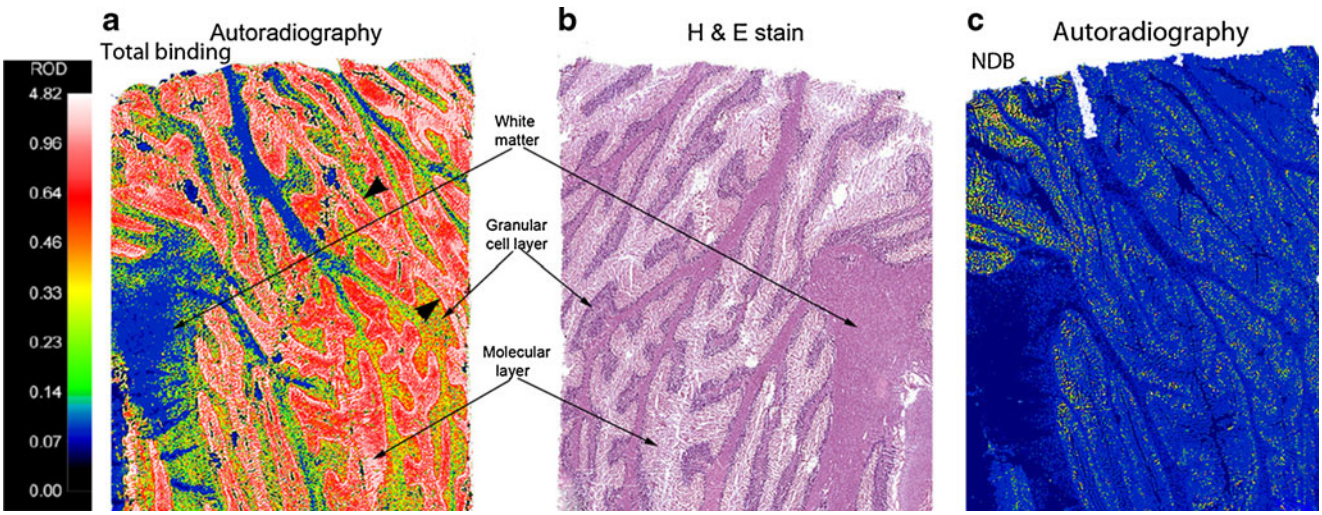

Fig. 2 Distribution of [^{125}I]hCGRP binding to human cerebellum brain slice. **a** [^{125}I]hCGRP displayed high binding density in the molecular layer and Purkinje cell layer (*arrowheads*) of the cerebellar cortex with low-to-moderate binding in the granular cell layer and white matter. **b** Htx–Eosin staining of the same slice. **c** Non-displaceable binding (*NDB*) by 1 μM self-block

Therefore these samples were primarily used for the immunohistochemistry experiments. To examine the condition of the Purkinje cells, a specific marker for these cells calbindin, was used (Fig. 4c, d). The calbindin marker showed strong immunoreactivity in all Purkinje cells and their dendrite elaborated tree of rhesus and human cerebellum which allowed the integrity of these cells to be verified.

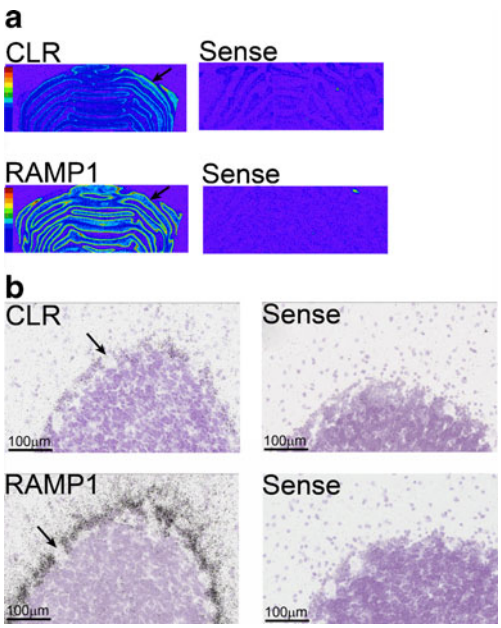

Fig. 3 In situ hybridization, CLR, and RAMP1 expression in rhesus monkey cerebellum. **a** CLR and RAMP1 mRNA expression in the Purkinje cell layer (*arrows*). The respective sense probes displayed no binding. **b** High-resolution localization of CLR and RAMP1 mRNA. The mRNAs were mostly expressed around the Purkinje cells (*arrows*), however, some of these cells displayed mRNA expression within the cell body. The respective sense probes displayed no binding

Immunohistochemistry

The negative controls displayed no immunoreactivity. Some of the secondary antibodies revealed unspecific staining in the white matter. Autofluorescence was observed in basket cells, both in rhesus and human cerebellum. For each primary antibody, at least two secondary antibodies were tested to make sure that the same results were obtained. The human material showed higher background staining with all secondary antibodies as compared to the rhesus material.

CGRP immunoreactivity was found in the Purkinje cell bodies (cytoplasm) and in their branches (Purkinje cell dendrites) of human and rhesus cerebellum (Fig. 5). Not all Purkinje cells expressed CGRP. In some Purkinje cells the CGRP staining was especially strong in the cell nuclei. CGRP positive cells were found in the molecular layer.

Procalcitonin expression was found in the Purkinje cell bodies and in cells in the molecular layer (Fig. 5a, b). Like CGRP, not all Purkinje cells expressed procalcitonin. Often, several positive Purkinje cells were found in row in the same folia. The human cerebellum showed similar staining pattern as observed in rhesus monkey (Fig. 5b). Co-expression was found between CGRP and procalcitonin (Fig. 5a, b).

Expression of CLR and RAMP1 was found in the Purkinje cells of rhesus (Fig. 6a) and human (Fig. 6b) cerebellum. The RAMP1 staining was more prominent compared to that of CLR. CLR and RAMP1 positive cells were also observed in the molecular layer (Fig. 6). Expression of the receptor components were also observed in the processes of the Purkinje cells (Figs. 6 and 7). Co-expression of the receptor components was found in the Purkinje cells and cells in the molecular

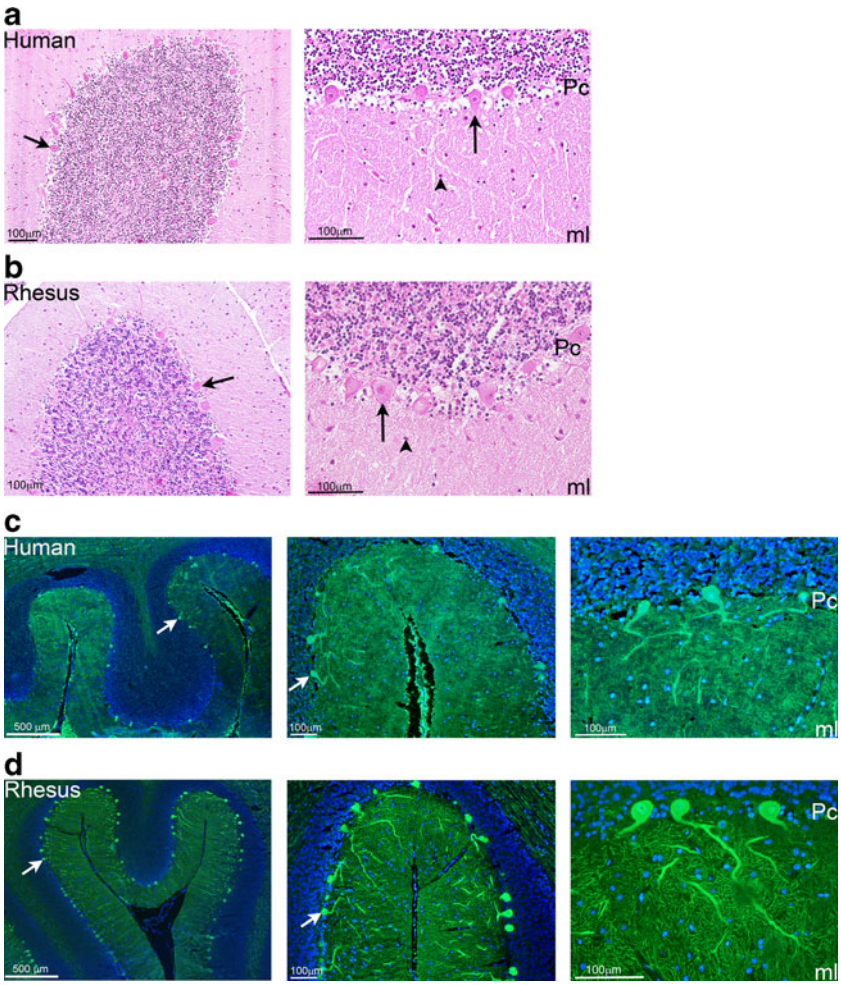

Fig. 4 The histology of human and rhesus monkey cerebellum. Htx–eosin staining of human (**a**) and rhesus monkey (**b**) cerebellum, showing Purkinje cells (*arrows*) and cells in the molecular layer (*arrowheads*). Some cell shrinkage and splits are seen in the material. **c** Calbindin expression in Purkinje cells (*arrows*) and their elaborated tree of human and rhesus monkey cerebellum (**d**). *Pc* Purkinje cells, *ml* molecular layer

layer of rhesus (Fig. 7a) and human cerebellum (Fig. 7b). Some Purkinje cells only expressed RAMP1 (Fig. 7a).

Double-staining with CGRP and CLR (Figs. 8a and 9a) or RAMP1 (Figs. 8b and 9b) showed co-localization in some Purkinje cells and cells in the molecular layer of rhesus (Fig. 8) and human cerebellum (Fig. 9). Almost all CGRP positive cells were also positive for RAMP1 or CLR. However, not all CLR or RAMP1 positive cells showed CGRP immunoreactivity (Figs. 8 and 9).

In order to identify the other cells expressing CGRP and its receptor, double-staining with GFAP, S-100, or GAD 67 was performed. Double-staining with GFAP and S-100 with CGRP or the receptor components revealed no co-expression (data not shown). GAD67 expression was found mostly on the surface of Purkinje cells and neurons in the molecular layer. GAD67 immunoreactivity was found on the surface of some of the cells expressing CGRP or its receptor components in the molecular layer (Fig. 10a, b). Purkinje cells expressing CGRP or its receptor in the cytoplasm showed GAD67 expression on the cell surface (Fig. 10). The GAD67 did not work on the human sections.

Discussion

Clinical and experimental studies suggest that the cerebellum is involved in pain conditions, including migraine [1, 30, 31]. CGRP has an important role in migraine pathogenesis, and the recently discovered CGRP receptor antagonists have demonstrated efficacy in the treatment of migraine [23, 32]. Therefore, we have delineated the expression pattern of CGRP and the CGRP receptor in primate cerebellar cortex. Additionally, CGRP receptor binding sites were defined using both a radiolabeled CGRP receptor antagonist and CGRP itself. This is the first report to show in detail the localization of CGRP and CGRP receptor components with three different methods, in vitro autoradiography, in situ hybridization, and immunohistochemistry in rhesus and human cerebellar cortex.

High-density binding sites of CGRP and the CGRP receptor antagonist (MK-3207) were displayed in the molecular layer of cerebellum. The expression of RAMP1 and CLR mRNA were detected within the Purkinje cell layer and some cells in the molecular layer. With immunohistochemistry it was shown that CGRP and its receptor components are

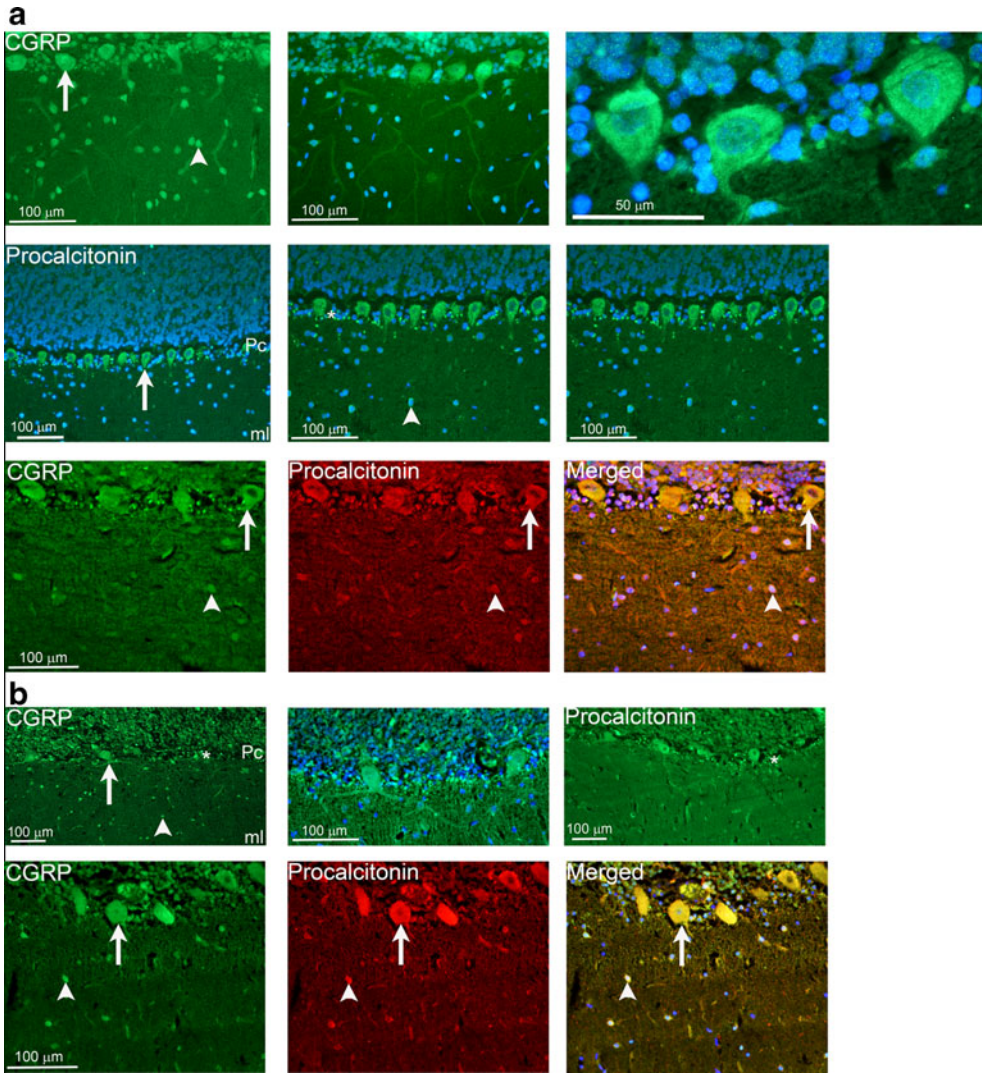

Fig. 5 CGRP and procalcitonin expression in rhesus and human monkey cerebellum. CGRP and procalcitonin immunoreactivity and their co-expression in cerebellar Purkinje cells (*arrows*) and cells in the molecular layer in rhesus monkey (**a**) and human cerebellum (**b**). The CGRP staining is found in the cytoplasm and the elaborated tree of Purkinje cells. The human material contains lipofuscin, which is autofluorescent (*asterisks*). For nuclei staining DAPI (*blue*) is used. *Pc* Purkinje cells, *ml* molecular layer

expressed in Purkinje cells and cells in the molecular layer. These results demonstrate the presence of CGRP and its receptor in primate cerebellar neurons, and suggest a role of CGRP in cerebellum, which could be involvement in pain and migraine pathophysiology.

Pain neuroimaging often show activation in the cerebellum, where patterns of cerebellar responses to innocuous and noxious thermal stimuli have been identified [33, 34]. Also, cerebellar activation is observed in patients who have neuropathic pain that affects the maxillary division of the trigeminal nerve (V2) [14]. Activation of cerebellar regions has been demonstrated by PET in patients during migraine attacks [12, 13]. However, there is at present no explanation for this activation. Interestingly, a recent study showed activation of the cerebellum during the premonitory phases of migraine [35]. The cerebellum is of interest in migraine research since studies suggest a role of cerebellum in migraine pathophysiology. Spreading depression, cerebellar dysfunction, and familial hemiplegic migraine have suggested a connection between the cerebellum and migraines [30]. Symptoms such as vertigo and balance changes may occur in migraine patients, and these suggest that migraine affects cerebellar function [36]. CGRP is an important player in migraine pathogenesis and CGRP receptor antagonists have been shown to be effective in the acute treatment of migraine [23, 37, 38]. In this report binding sites of MK-3207 were examined by in vitro autoradiography. MK-3207 is a selective CGRP receptor antagonist, which in vitro is a potent antagonist of the human and rhesus monkey CGRP receptors [26]. We observed high binding density of [^{3}H]MK-3207 to be mainly located in the molecular layer of rhesus cerebellum. No binding was found in the granular layer. However, due the limit of resolution of the autoradiographic image, it could not be excluded that the antagonist binds to the Purkinje cells as well. In human cerebellum, high-density [^{125}I]CGRP binding was also found in the molecular layer and most likely the Purkinje cell layer. By examining the binding sites of both the small molecule antagonist MK-3207 and the endogenous ligand CGRP, the receptor expression

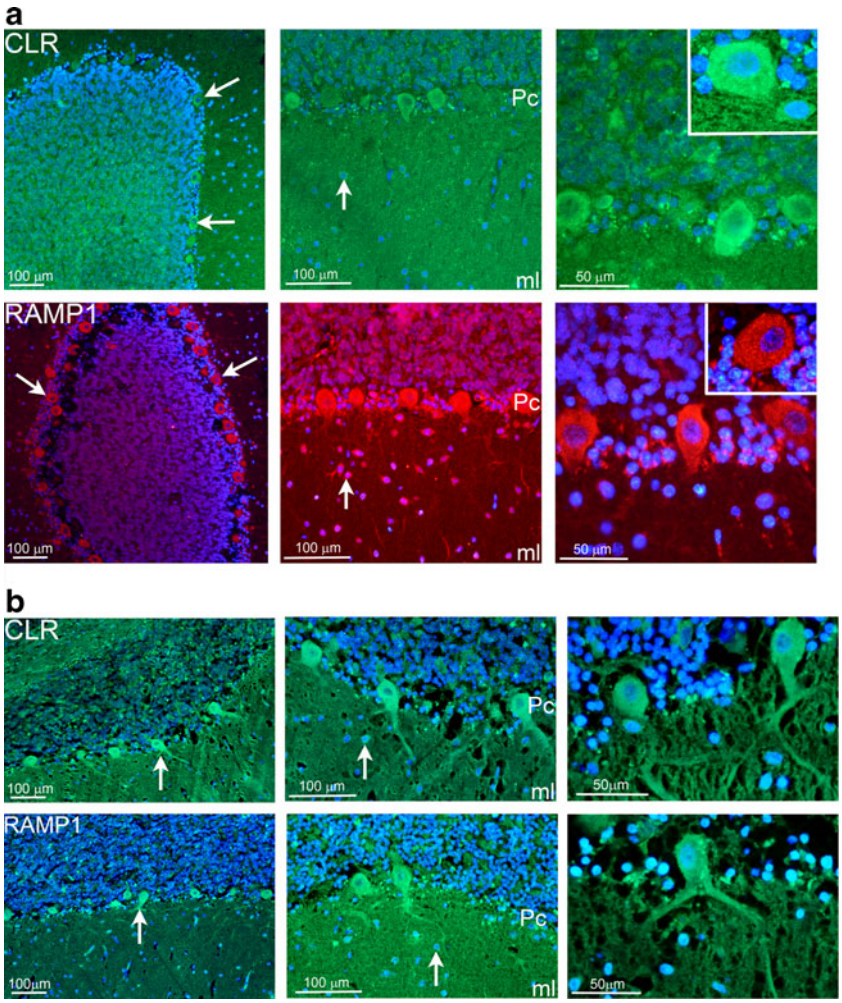

Fig. 6 Expression of CLR and RAMP1 in rhesus monkey (**a**) and human (**b**) cerebellum. CLR and RAMP1 are expressed intracellular in Purkinje cells and cells in the molecular layer (arrows) in rhesus monkey (**a**) and human (**b**) cerebellum. Parts of the elaborated tree of Purkinje cells display CLR and RAMP1 immunoreactivity. Not all Purkinje cells express CLR or RAMP1. For nuclei staining DAPI (blue) is used. Pc-Purkinje cells, ml-molecular layer

pattern could be confirmed. These results suggest the possibility of a functional role of CGRP in primate cerebellar cortex. Due to the low limit of resolution of the autoradiographic image, the exact cellular localization could not be determined. Immunohistochemistry was used to define the cellular/subcellular localization of CGRP and its receptor components.

Earlier studies have shown CGRP immunoreactivity in the Purkinje cells of rat [39] and recently by our group [24]. CGRP has been shown to be expressed in the cerebellar climbing fibers [40]. In primates, we demonstrate that CGRP is expressed in the cytoplasm of Purkinje cells and their branches. The climbing fibers did not show expression of CGRP. Some cells in the molecular layer displayed CGRP immunoreactivity. It cannot be determined if these minor variations between rat and primate are related to species differences or technical reasons such as material collection.

CGRP is produced by alternative mRNA splicing of the calcitonin gene and belongs to the calcitonin family peptides. Procalcitonin, the precursor of calcitonin was co-expressed with CGRP in the Purkinje cell body. This suggests that CGRP is produced in these cells within the cerebellum.

Early studies have revealed expression of the CGRP receptors in several regions of the brain [41] including cerebellar glial cells [42]. However, these studies were based on immunohistochemistry and bindings sites, and were performed before the CGRP receptor was fully characterized. Recently, we found CLR and RAMP1 expression on the surface of rat Purkinje cell bodies and in fibers spanning through the entire cerebellum [24]. Detailed confocal analysis showed no co-expression of the CGRP receptor and glial cell markers in rat cerebellum [24]. In the present study, we observed expression of the receptor components in the cytoplasm of Purkinje cells and in cells within the molecular layer. Compared to our previous study on rat cerebellum, we did not find any fibers expressing CLR or RAMP1 in the primate cerebellum. The expression of the receptors components in primate Purkinje cells appeared to be intracellular rather than only on the surface of Purkinje cells. This might reflect differences in the quality of tissue or that the receptor expression is so high that the immunoreactivity appears as intracellular. No obvious difference in expression pattern of CGRP and its receptor was observed between rhesus and human cerebellum. Similar to the CGRP

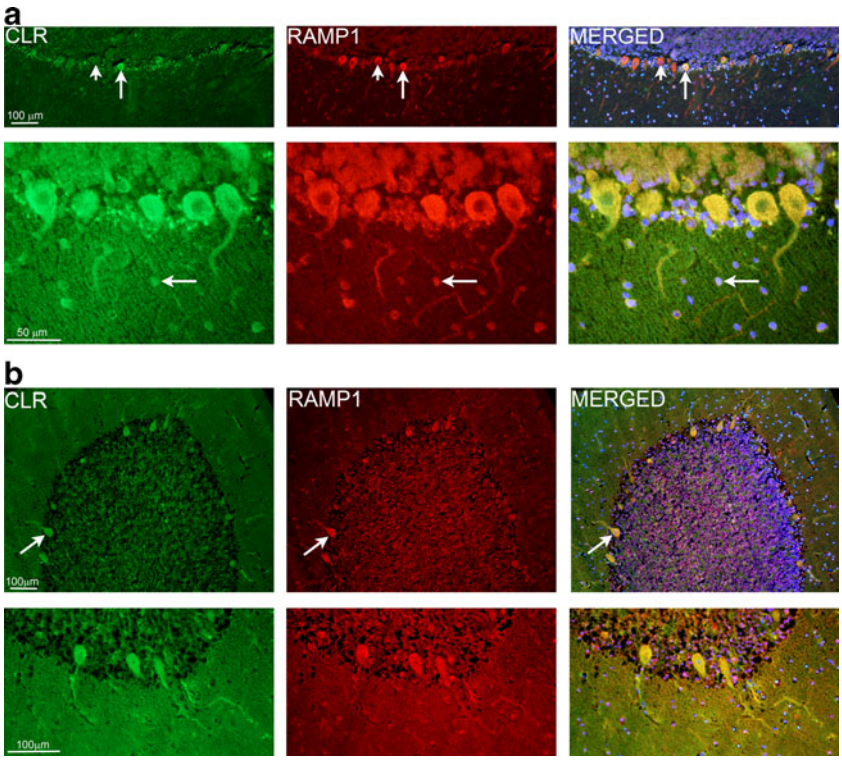

Fig. 7 Double-staining of CLR and RAMP1 in rhesus monkey and human cerebellum. Co-expression of CLR and RAMP1 in rhesus monkey (**a**) and human cerebellum (**b**) in the Purkinje cells (*large arrows*) and cells in the molecular layer (*small arrows*). Not all RAMP1 positive cells expressed CLR (*arrowheads*). DAPI (*blue*), nuclei staining, is used in the merged pictures

staining, the receptor components displayed immunoreactivity in some cells in the molecular layer. We found that some of these cells in the molecular layer expressing CGRP or its receptor expressed GAD67 on their cell surface. GAD67 is one of major isoforms of the enzyme that converts glutamate into GABA, where the interneurons of cerebellum use GABA as neurotransmitter. The interneurons provide feed-forward and lateral inhibition to Purkinje cells, thus controlling their firing rate, the precise timing of action potential firing, and the spread of activity [43]. Functional experiments are needed to evaluate if and how there is a connection between the CGRP and the GABA system in cerebellum. CLR and RAMP1 were co-expressed in the Purkinje cells and cells in the molecular layer, suggesting expression of functional CGRP receptors in

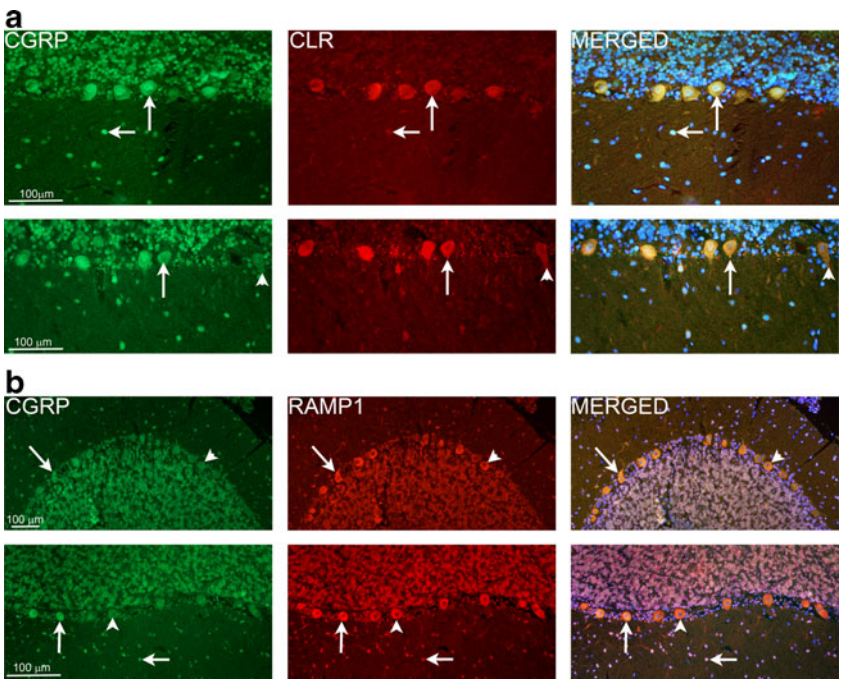

Fig. 8 CGRP and CLR/RAMP1 double-staining in rhesus monkey cerebellum. Double-staining of CGRP and CLR (**a**) or RAMP1 (**b**) in the Purkinje cells (*large arrows*) and cells in the molecular layer (*small arrows*). Not all CLR or RAMP1 positive cells express CGRP (*arrowheads*). DAPI (*blue*), nuclei staining, is used in the merged pictures

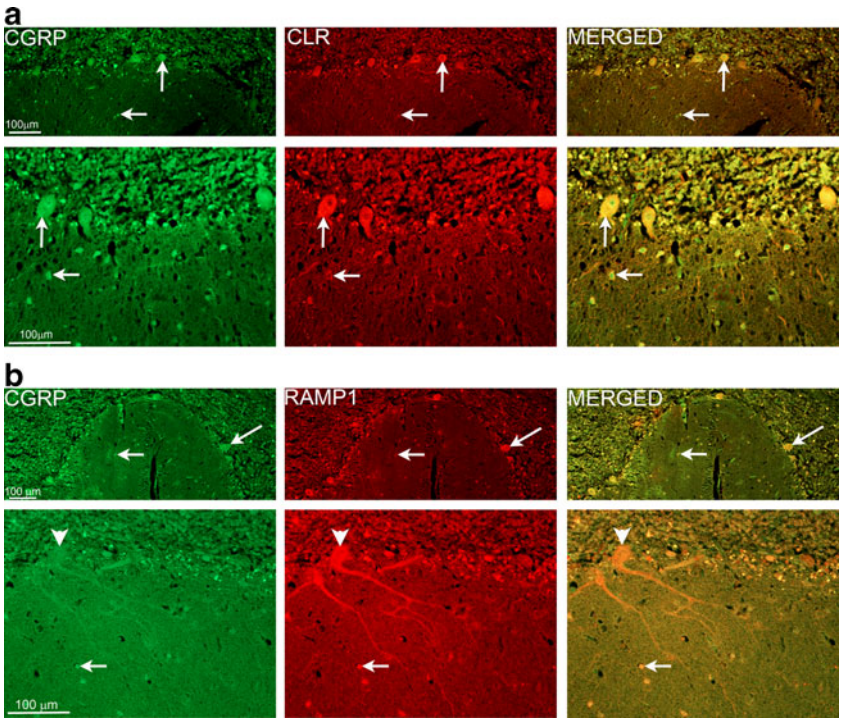

Fig. 9 CGRP and CLR/RAMP1 double-staining in human cerebellum. Double-staining of CGRP and CLR (**a**) or RAMP1 (**b**) in the Purkinje cells (*large arrows*) and cells in the molecular layer (*small arrows*). Not all CLR or RAMP1 positive cells express CGRP (*arrowheads*)

these cerebellar cells. However, this needs to be evaluated with proper functional methods. RAMP1 positive cells, negative for CLR, were also observed. This could be due differences in the quality of antibodies recognizing the epitopes, slightly higher proportion of RAMP1 compared to CLR or the presence of other RAMP1 containing receptors, such as amylin receptors. The latter suggestion is less likely since it has been shown that the cerebellum is devoid of amylin binding sites [44, 45]. The in situ hybridization results also showed slightly higher RAMP1 mRNA expression. One can also speculate that since other receptors rely on RAMP1 in their formation, RAMP1 may be functionally rate-limiting and an excess might be necessary for high-density CGRP binding. This may also be related to how well the probes

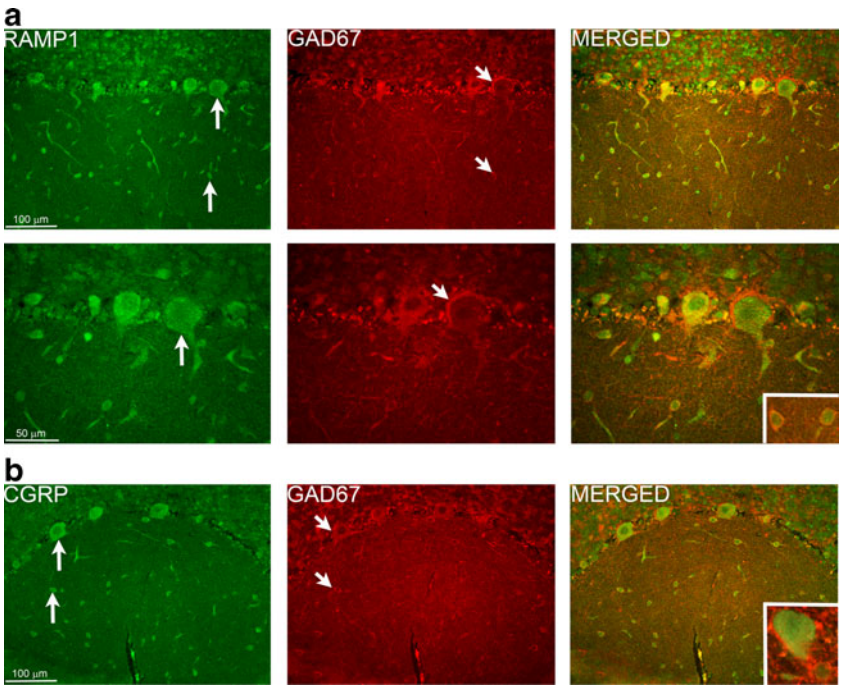

Fig. 10 Double-staining of GAD67 with RAMP1 or CGRP in rhesus monkey cerebellum. RAMP1 with GAD67 (**a**) and CGRP with GAD67 (**b**). RAMP1 and CGRP is found intracellulary in Purkinje cells and cells in the molecular layer (*large arrows*) while GAD67 is expressed on the surface of the same cells (*small arrows*). *Inserts* show higher magnification

recognize the sequences. Further efforts are needed to clarify this issue.

Double-staining of CGRP and the receptor components showed co-localization in Purkinje cells and in cells in the molecular layer. However, not all CLR or RAMP1 immunoreactive cells displayed CGRP expression. This suggests that the neuronal messenger might act on the same cell or on cells only expressing the receptor.

The functional role of CGRP in the cerebellum is unknown. It has been shown that the expression of CGRP and its receptor undergoes marked variations during development in rat. The number of CGRP receptors in the Purkinje cells/molecular layer is low in early stages and greatly increases with maturation and decreases from high to low levels during the same time in the white matter [46]. The same group demonstrated that CGRP stimulates Purkinje cell dendrite growth in vitro, suggesting that CGRP influences Purkinje cell dendrite growth [47]. Further efforts are needed to evaluate the role of CGRP and its receptor in the cerebellum.

Conclusion

Recent studies clearly indicate that the cerebellum might have a role in nociception and primary headache disorders. The present study demonstrates that there is a rich expression of the neuropeptide CGRP and CGRP receptor components in primate cerebellar cortex, which together suggest a functional role of CGRP in cerebellum. The roles of cerebellum and CGRP in response to nociceptive stimuli warrant additional research which may in turn lead to a better understanding of the cerebellum as a pain control center and as a target for new drug therapies.

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Conflict of Interest The authors do not have any conflicts of interest related to this work.

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