Distribution of signaling neurotransmitters and interaction in the sphenopalatine ganglion

Ph.D. Thesis

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"Discovery consists of seeing what everybody has seen and thinking what nobody has thought."

Albert Szent-Györgyi *in Irving Good, The Scientist Speculates (1962)*

CONTENTS

LIST OF PAPERS

This doctoral thesis is based on the following publications:

I. **Csati A**, Tajti J, Kuris A, Tuka B, Edvinsson L, Warfvinge K. Distribution of vasoactive intestinal peptide, pituitary adenylate cyclase-activating peptide, nitric oxide synthase, and their receptors in human and rat sphenopalatine ganglion. Neuroscience. 2012. 202:158-68. IF: 3.122

II. **Csati A**, Tajti J, Tuka B, Edvinsson L, Warfvinge K. Calcitonin gene-related peptide and its receptor components in the human sphenopalatine ganglion -- interaction with the sensory system. Brain Res. 2012. 1435:29-39. IF: 2.879

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Publications not directly related to the thesis:

I. **Csáti A**, Vécsei L, Warfvinge K, Edvinsson L, Toldi J, Fülöp F, Tajti J. Kynurenic acid and kynurenic acid amide 2 modify pERK1/2 associated experimentally induced acute and chronic inflammation in the trigeminal ganglion. Submitted paper

II. Tajti, J., Szok, D., Párdutz, Á., Tuka, B., **Csáti, A.**, Kuris, A., Toldi, J., Vécsei, L. Where does a migraine attack originate? In the brainstem. J Neural Transm. 2012. 119:557-68. IF: 3.052

III. Tajti, J., Szok, D., Tuka, B., **Csáti, A.**, Kuris, A., Majláth, Zs., Lukács, M., Vécsei, L. Botulinum neurotoxin-A terápia migrénben. Ideggyógyászati Szemle. 2012. 65:77-82. IF: 0.348

IV. Vámos E., **Csáti A.**, Vécsei L., Klivényi P. Effects of valproate on the dopaminergic system in mice. Neurol Res. 2009. 31:217-9. IF: 1.28

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LIST OF ABBREVIATIONS

Ach	acetylcholine
cAMP	cyclic adenosine monophosphate
CGRP	calcitonin gene-related peptide
CLR	calcitonin receptor-like receptor
DAPI	4',6-diamino-2-phenylindole
GFAP	glial fibrillary acidic protein
GPCRs	G protein-coupled receptors
GS	glutamine synthetase
NOS	nitric oxide synthase
PACAP	pituitary adenylate cyclase-activating peptide
RAMP1	receptor activity modifying protein 1
RCP	CGRP-receptor component protein
SGCs	satellite glial cells
SPG	sphenopalatine ganglion
SSN	superior salivatory nucleus
VIP	vasoactive intestinal peptide

SUMMARY

Clinical studies have suggested a link between the sensory trigeminal system and the parasympathetic ganglia. Calcitonin gene-related peptide (CGRP) is a sensory neuropeptide which plays an important role in vasodilatation and pain transmission in the trigeminovascular system.

Our work was performed to examine the expression of the parasympathetic signaling transmitters and their receptors in human and rat sphenopalatine ganglion (SPG), and if CGRP and CGRP receptor components are present in the human SPG in order to reveal an interaction between the sensory and parasympathetic systems.

Indirect immunofluorescence technique was used for immunohistochemical demonstration of vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP), nitric oxide synthase (NOS), glutamine synthetase (GS), glial fibrillary acidic protein (GFAP), VIP and PACAP common receptors (VPAC1, VPAC2), PACAP receptor (PAC1), CGRP, the CGRP receptor components as the calcitonin receptor-like receptor (CLR) and the receptor activity modifying protein 1 (RAMP1) in human and rat SPG. In addition, double labeling was carried out to reveal the co-localization of neurotransmitters. Cryostat sections were examined and images were obtained using a light- and epifluorescence microscope coupled to a camera to visualize co-labeling by superimposing the digital images. In addition, Western blot technique was used to demonstrate the existence of VIP/PACAP receptors and CGRP receptor components in rat SPG.

In human SPG VIP immunoreactive neurons as well as fibers were frequently found. Many, homogenously stained NOS immunoreactive cells were found, but no positive fibers. In addition, PACAP immunoreactivity was observed in some of the neurons and in fibers. Co-localization was found between VIP/NOS and PACAP/NOS in human. In rat VIP, NOS and PACAP immunoreactivity were found in many neurons and fibers. Co-localization of PACAP and NOS was observed in rat neurons. PACAP and GS double staining revealed that the PACAP immunoreactivity was localized in/close to the cell membrane, but not in the satellite glial cells (SGCs). PAC1 and VPAC1 immunoreactivity was found in the SGCs, in addition, VPAC1 and VPAC2 in fibers of both human and rat. Western blot revealed protein expression of PAC1, VPAC1 and VPAC2 in rat SPG. CGRP immunoreactive fibers were frequently found intraganglionic in both human and rat SPG in the vicinity of neurons, and in neurons in rat SPG. CLR immunoreactivity was observed in

SGCs as well as in nerve fibers, but not in neurons in both human and rat. RAMP1 immunoreactivity was localized in many neurons (in both human and rat), SGCs (in human) and nerve fibers (in rat). Thus, the two CGRP receptor components together were found in the SGCs in human and in the nerve fibers in rat. Western blot confirmed the presence of RAMP1 and CLR in rat SPG.

We hypothesized that VIP, PACAP, NOS, PAC1, VPAC1, and VPAC2 play a role in the activation of parasympathetic cranial outflow during trigeminal-autonomic reflex. We have revealed that the trigeminal CGRP-containing fibers project to the SPG and act on CGRP receptors on SGCs in human. Therefore, our results suggest a functional coupling between the trigeminal (sensory) and the sphenopalatinal (parasympathetic) system.

1. INTRODUCTION

The head and neck regions and the intracranial circulation are innervated by parasympathetic nerve fibers from the SPG, otic and internal carotid ganglia (Suzuki et al., 1988). The central control emanates in the superior salivary nucleus (CN VII, the facial nerve) with cholinergic fibers that synapse in the SPG. Tracing and denervation studies have revealed that the majority of the intracranial vascular parasympathetic innervation originates in the otic ganglion and SPG (Hara and Weir, 1988, Suzuki et al., 1988, Edvinsson et al., 1989, Hara et al., 1993, Edvinsson et al., 2001). The neuronal cell bodies in the human SPG contain VIP, PACAP and NOS as the main parasympathetic signaling transmitters (Uddman et al., 1999). Traditionally the cell bodies have been considered to be cholinergic, but only a minor subpopulation of cells in the SPG have been reported to contain acetylcholine transferase (Lee et al., 1984). In addition, a large number of VIP and PACAP immunoreactive cell bodies have been shown to co-localize with NOS in rat (Uddman et al., 1999, Edvinsson et al., 2001).

VIP and PACAP belong to the secretin/glucagon/VIP superfamily of neuropeptides. VIP was first isolated from the ovine intestine and found to be a very potent neuropeptide (Said and Mutt, 1970) that consists of 28 amino acids. VIP is widely distributed in central and peripheral nervous system (Loren et al., 1979, Said, 1984). The peptide has various biological effects in mammals such as embryonic brain development, pain perception and inflammation (Harmar et al., 1998). PACAP, the newest member of this family of peptides was originally isolated from the ovine hypothalamus (Miyata et al., 1989), and occurs in two forms: C-terminally truncated PACAP-27 and PACAP-38 (27 or 38 amino acids). PACAP shares two-third sequence homology with the N-terminal domain of VIP. Both forms of PACAP exert a large variety of biological effects including vasodilatation, relaxation of lower airways, immune modulation, stimulation of cell proliferation and differentiation, control of neurotransmitter release and pain transmission (Harmar et al., 1998, Vaudry and Laburthe, 2006). PACAP-38 predominates over PACAP-27 in most studied tissues (Sundler et al., 1996). Both forms are derived from the same precursor and are amidated in the C-terminal.

The actions of VIP and PACAP are mediated through the family of 7 transmembrane G protein-coupled receptors (GPCRs) (Vaudry and Laburthe, 2006, Dickson and Finlayson, 2009). Classically, the GPCR superfamily is divided into three main classes - A, B, and C - with no shared sequence homology between the classes. The largest class by far is class A, which accounts for nearly 85% of the GPCR genes. Uniquely, the VIP and PACAP

receptors belong to the class B family of GPCR. VIP and PACAP act via VPAC1 and VPAC2 receptors with equally high affinity, while PACAP is more potent than VIP at the PAC1 receptor (Harmar et al., 1998).

During the investigation on primary headache pathophysiology a close correlation between the calcitonin gene-related peptide (CGRP) and the head pain was observed in acute attacks of migraine (Juhasz et al., 2005, Ho et al., 2010).

In some cases of migraine and in all cluster headache cases, additional facial symptoms (reddening of sclera, rhinorrhea, nasal congestion, etc.) were associated with co-release of the sensory neuropeptide CGRP from the trigeminal system and VIP from the cranial parasympathetic system into the cranial venous outflow (Goadsby and Edvinsson, 1994a). CGRP consists of 37 amino acids and plays an important role in vasodilatation and pain transmission in craniocervical structures (Ho et al., 2010). Sumatriptan, acting on 5-hydroxytryptamine type 1B/1D subtypes of receptors, aborts not only the CGRP release and the head pain, but also the VIP release and the parasympathetic symptoms (Goadsby and Edvinsson, 1994a). The mechanisms involved are not clear, however, experimental studies have provided some support of a link between the two systems (Goadsby and Edvinsson, 1994b).

Based on experiments in cats, it has been hypothesized that this is an effect at the brain stem level (Goadsby and Edvinsson, 1994b). On the other hand, according to a recent study 5-hydroxytryptamine 1D receptor immunoreactive fibers were found in rat SPG. Double-immunolabelling with CGRP and vesicular acetylcholine transporter, revealed CGRP, but not vesicular acetylcholine transporter immunoreactivity, suggesting a sensory origin (Ivanusic et al., 2011).

The receptor for CGRP was elucidated over a decade ago and belongs to the family of G-protein-coupled receptor of the B-subtype (Hay et al., 2008). The functional CGRP receptor consists of three proteins: (i) the calcitonin receptor-like receptor (CLR) which forms the ligand binding site with (ii) receptor activity modifying protein 1 (RAMP1), and together they determine the specificity of the receptor (McLatchie et al., 1998, Heroux et al., 2007). (iii) The CGRP-receptor component protein (RCP) is essential in coupling the receptor to intracellular signal-transduction pathways and, in particular, to adenylyl cyclase (Evans et al., 2000).

2. AIMS

(i) Reveal the presence and distribution of VIP/PACAP receptors in human and rat SPG using indirect immunofluorescence and Western blot techniques.

(ii) Examine the SGCs and their relation to neurons both in human and rat SPG.

(iii) Study CGRP and CGRP receptor elements (CLR and RAMP1) in human and rat SPG neurons, nerve fibers and SGCs as an indication of putative local function.

(iv) Compare the distribution of examined neurotransmitters between human and rat SPG, using immunofluorescence technique.

3. EXPERIMENTAL PROCEDURES

3.1. Tissue material

3.1.1. Human

Human SPG were obtained at autopsy from 5 adult subjects with an average age of 72.6 years (range 60-81 years). The collection of tissue samples was done in accordance with the University of Szeged, Faculty of Medicine guidelines for ethics in human tissue experiments (6/1996 – 13/12/2010).

For preparation of human SPG, a standardized method was used. After removal of the brain, the overlying dura was removed from the trigeminal impression area in the medial cranial fossa. The trigeminal ganglion was separated and gently excised. Then a 20x25 mm bone window was made, including the trigeminal fossa together with foramina rotundum and ovale. Following the mandibular and maxillary nerve into the infratemporal and pterygopalatinal fossa, the SPG was gently excised. The ganglia were collected within 48 hours of dead. Cause of death was related to cardiac disease and none of the subjects were suffering from central nervous diseases.

The human subjects took antibiotics, cardiovascular drugs (cardiac glycosides, antianginal agents, antiarrhythmic agents, antihypertensive agents, platelet aggregation inhibitors and statins), gastrointestinal drugs (antiulcer drugs and antispasmodics), insulin, and central nervous system agents (analgetics and anxiolytics).

SPG were immersed overnight in a mixture of 2% paraformaldehyde and 0.2% picric acid in phosphate buffer (pH 7.2). After fixation, specimens were rinsed repeatedly in sucrose-enriched (10%) Tyrode solution, snap frozen, embedded in Tissue Tek (Sakura Finetek, Europe) and stored at -80ºC.

3.1.2. Rat

SPG were collected from eight adult male Sprague-Dawley rats (weighing 300-400 g). The animals were raised and maintained under standard laboratory conditions. The study followed the guidelines of the European Communities Council (86/609/ECC) and was approved by the Ethics Committee of The Faculty of Medicine, University of Szeged.

The rats were deeply anesthetized with chloral hydrate (0.4 g/kg bodyweight, Fluka Analytical, Buchs, Switzerland, 23100) and perfused transcardial with 100 mL phosphate-buffered saline (PBS, 0.1M, pH 7.4) followed by 500 mL a mixture of 2% paraformaldehyde and 0.2% picric acid in phosphate buffer (pH 7.2).

After extraction of the rat eye and cut across the zygomatic arch, the maxillary branch of the trigeminal nerve was seen in the posterior part of the cavity. The SPG lies in the pterygopalatine fossa beneath the maxillary branch of the trigeminal nerve. This fossa is localized between the nasal orbital wall and the medial surface of the maxillary branch of the trigeminal nerve. The schematic drawing and detailed anatomical description of rat SPG published by Suzuki N et al. in 1988 was used.

The ganglia were removed and post-fixed overnight in 4% paraformaldehyde. After fixation rat specimens were rinsed repeatedly in sucrose-enriched (10%) Tyrode solution. The ganglia were frozen on dry ice and stored at -80ºC.

Both human and rat specimens were embedded in gelatin medium (30% egg albumin and 3% gelatin in distilled water), cryosectioned at 10 µm, mounted on Superfrost Plus coated slides (Menzel GmbH Co KG, Braunschweig, Germany) and stored at -20ºC until use.

3.2. Regular tissue staining

3.2.1. Hematoxylin-Eosin

For orientation and examination of the tissue condition, human and rat sections were stained with Hematoxylin-Eosin (Htx-Eosin) using a standard protocol (Htx 4 min, water rinse, Eosin 30 sec).

3.3. Immunohistochemistry

Briefly, the sections were rehydrated for 2 x 15 minutes in PBS containing 0.25% Triton X-100 (PBS-T, Chemicon, Sweden), and thereafter exposed to primary antisera (details of the antibodies are given in Table 1) in PBS-T containing 1% bovine serum

albumin (BSA) overnight in a moist chamber at +4ºC. Sections were then rinsed in PBS-T for 2 x 15 min followed by incubation with secondary antibodies (Table 2) for 1 h in dark at room temperature. In addition, double immunohistochemistry was performed using VIP/NOS, PACAP/NOS and PACAP/Vimentin, PACAP/GS, CGRP/CLR, CGRP/RAMP1 and CLR/RAMP1 primary antibodies following the same protocol consecutively repeated twice.

Thereafter, sections were rinsed in PBS-T for 3 x 10 min in room temperature and mounted with mounting medium (Glycerol, Sigma-Aldrich, Munich, Germany) or Vectashield (Vector Laboratories, Burlingame, CA, USA). Vectashield medium containing 4',6-diamino-2-phenylindole (DAPI, nucleus staining) was used in some sections. Omission of the primary antibody served as negative controls. All antibodies were applied at least three independent staining sessions in order to validate reproducibility. The VIP and PACAP immunoreactivity were blocked by addition of 100 µm of VIP or PACAP38, both obtained from Sigma-Aldrich.

Table 1. Details of primary antibodies used for immunohistochemistry

Name	Product code	Host	Dilution	Detect	Source
VIP (M19)	sc-7841	goat	1:100	C-terminus of VIP of mouse	Santa Cruz Biotech, Santa Cruz, CA, USA
VPAC1 (H-130)	sc-30019	rabbit	1:150	N-terminus of VPAC1 of human	Santa Cruz Biotech, Santa Cruz, CA, USA
VPAC2 (H-50)	Sc-30020	rabbit	1:150	Internal region of VPAC2 of human	Santa Cruz Biotech, Santa Cruz, CA, USA
PACAP-38	T-4473	rabbit	1:500	Human and rat PACAP-38	Peninsula Laboratories, LLC, San Carlos, CA, USA
PAC1	ab28670	rabbit	1:800	Human amino-acids 506-525 PAC1	Abcam, Cambridge Science Park, Cambridge, UK
bNOS	N2280	mouse	1:2500	Human and rat bNOS	Sigma-Aldrich, St Louis, MO, USA
GS	MAB302	mouse	1:150	Sheep and rat glial cells	Chemicon Internatonal, Temecula, CA, USA
GFAP	Z0334	rabbit	1:1500	Cytoskeleton in glial cells	Dako, Coppenhagen, Denmark
CGRP	B47-1	rabbit	1:800	Rat CGRP	Europroxima, Arnhem, The Netherlands
CGRP	ab81887	mouse	1:100	Rat α-CGRP	Abcam; Cambridge, UK
RAMP1	844	goat	1:100	C-terminal of human RAMP1	Merck & Co., Inc
CLR	3152	rabbit	1:800[a]	C-terminal of human CLR	Merck & Co., Inc

[a] Cross-reacts with rat receptor at 1:100 dilution.

Table 2. Secondary antibodies used for immunohistochemistry

Conjugate and host	Against	Dilution	Source
FITC (goat)	Anti-rabbit	1:100	Cayman Chemical, Ann Arbor, MI, USA
FITC (donkey)	Anti-rabbit	1:100	Jackson Immunoresearch, West Grove, PA, USA
Alexa 488 (donkey)	Anti-goat	1:400	Invitrogen, La Jolla, CA, USA
Texas-Red (donkey)	Anti-mouse	1:200	Jackson Immunoresearch, West Grove, PA, USA
Texas-Red (donkey)	Anti-rabbit	1:200	Jackson Immunoresearch, West Grove, PA, USA
DyLight 549 (donkey)	Anti-mouse	1:200	Jackson Immunoresearch, West Grove, PA, USA
DyLight 488 (donkey)	Anti-rabbit	1:200	Jackson Immunoresearch, West Grove, PA, USA
Cy2 (donkey)	Anti-goat	1:100	Jackson Immunoresearch, West Grove, PA, USA

3.4. Image analysis

Sections were examined and images were obtained using a light- and epifluorescence microscope (Nikon 80i, Tokyo, Japan) coupled to a Nikon DS-2 MV camera. FITC (480/30X), TRITC (540/24X) and DAPI (360/40X) filters were used (filter specifications are given in nanometers and X denotes excitation center wavelength/bandwidth). Adobe Photoshop CS3 (v.8.0, Adobe Systems, Mountain View, CA, USA) was used to visualize co-labeling by superimposing the digital images.

3.5. Western blot

Adult male Sprague-Dawley rats (500-600g; n=8) were deeply anaesthetized with 4% chloral hydrate and perfused transcardially with 150 ml PBS. Following perfusion, SPG from both sides were dissected and stored at -80°C until use. All collected ganglia were homogenized in cold lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% igepal, 0.1% cholic acid, 2 μg/ml leupeptin, 2 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg/ml pepstatin, 2 mM EDTA and 0.1% sodium dodecyl sulphate (SDS) with ultra-thurrax appliance (all chemicals were from Sigma-Aldrich, Germany). Lysates were separated by centrifugation and supernatants were stored in aliquots at -20°C until total protein concentration was determined according to BCA protein assay method (Novagen®, Germany), with bovine serum albumin (BSA) as a standard.

Protein (10 μg/lanes) were diluted in 2X Laemmli sample buffer and heated at 75°C for 5 min. Thereafter, the samples were separated by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) along with a standard 10-170 kDa molecular weight marker (Fermentas, Germany). The proteins were transferred to a nitrocellulose membrane (Amersham™, England,) followed by incubation in 5% non-fat dry milk in TBS-T for 1 h to avoid unspecific binding. The membrane was then washed for 3 x 5 min in TBS-T and incubated for 2 h at room temperature with PACAP receptors (PAC1, VPAC1 and VPAC2) and CGRP receptor components (CLR and RAMP1) primary antibodies (sc-30018, sc-52794, sc-30020, sc-30028, sc-11379), diluted 1:300 in 1% not-fat dry milk in TBS-T followed by washing in TBS-T 5 x 5 min. Then goat anti-rabbit IgG-HRP conjugated (sc-2030, for PAC1, VPAC2, CLR and RAMP1) and goat anti-mouse IgG-HRP (sc-2031, for VPAC1) at 1:3000 dilution in 1% non-fat dry milk in TBS-T were used as secondary antibody for 1 h at room temperature (all antibodies were from Santa Cruz Biotechnology, Inc., Germany). 5 x 5 min TBS-T washing was followed. The membranes were incubated in enhanced chemiluminescent substrate (Pierce Protein Research Product, Thermo Scientific, Germany) and developed using KODAK light film and reagents (Sigma-Aldrich, Germany).

Omission of primary antibodies were used as negative controls.

4. RESULTS

4.1. Regular tissue staining

4.1.1. Hematoxylin-Eosin

Most of the human material displayed qualitatively adequate morphology as visualized with Htx-Eosin staining (Figures 1a-b). The SPG were found as well-defined ganglia with neurons intermingled within the sphenopalatine branches of the maxillary nerve. Ganglia consisted of neurons of various size enveloped by SGCs (Figure 1b). Minor cell shrinkage was observed.

Rat SPG showed similar morphology with neurons of various size surrounded by SGCs (Figure 1c).
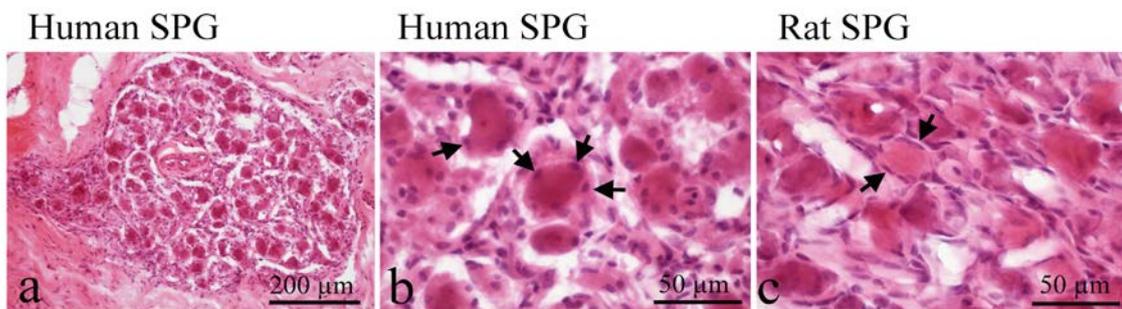
Figure 1 Htx-Eosin staining of human and rat SPG. (a) Overview of a well-defined human SPG. (b) Higher magnification of human SPG demonstrates neurons and satellite glial cells (arrows) surrounding the neurons. (c) Rat SPG showed similar morphology, with satellite glial cells (arrows) enveloping around single neurons forming distinct units.

4.2. Immunohistochemistry

4.2.1. Human SPG

Due to the subject's old age, many neurons in the human material contained lipofuscin granulae in their cytoplasm.

VIP immunoreactive neurons as well as fibers were frequently found in human SPG (Figures 2a-d). The immunoreactivity was granular-like in both neurons (Figure 2d) and fibers. Many NOS (homogenously stained) immunoreactive neurons were found, but no positive fibers (Figures 2e-h). In addition, PACAP immunoreactivity was found in some of the neurons and in fibers (Figures 2i-o). As for VIP, the PACAP staining displayed granular-like immunoreactivity. The neuronal staining was often localized to, or close to, the cell surface (Figures 2m, 2o). In order to reveal if the peptide only was localized to the neurons, and not the SGCs, double-staining with different glial cell specific antibodies

were performed. Double staining with PACAP/Vimentin revealed that PACAP staining was not localized in the SGCs (Figures 3m, 3o).

Double stainings of human SPG – VIP/NOS (Figures 3a-f), PACAP/ NOS (Figures 3g-l) and VIP/PACAP – were carried out. In our hands, co-localization was found between VIP/NOS, and PACAP/NOS. We were not able to establish, with the methods used, if the peptides were present in different neuronal subpopulations.
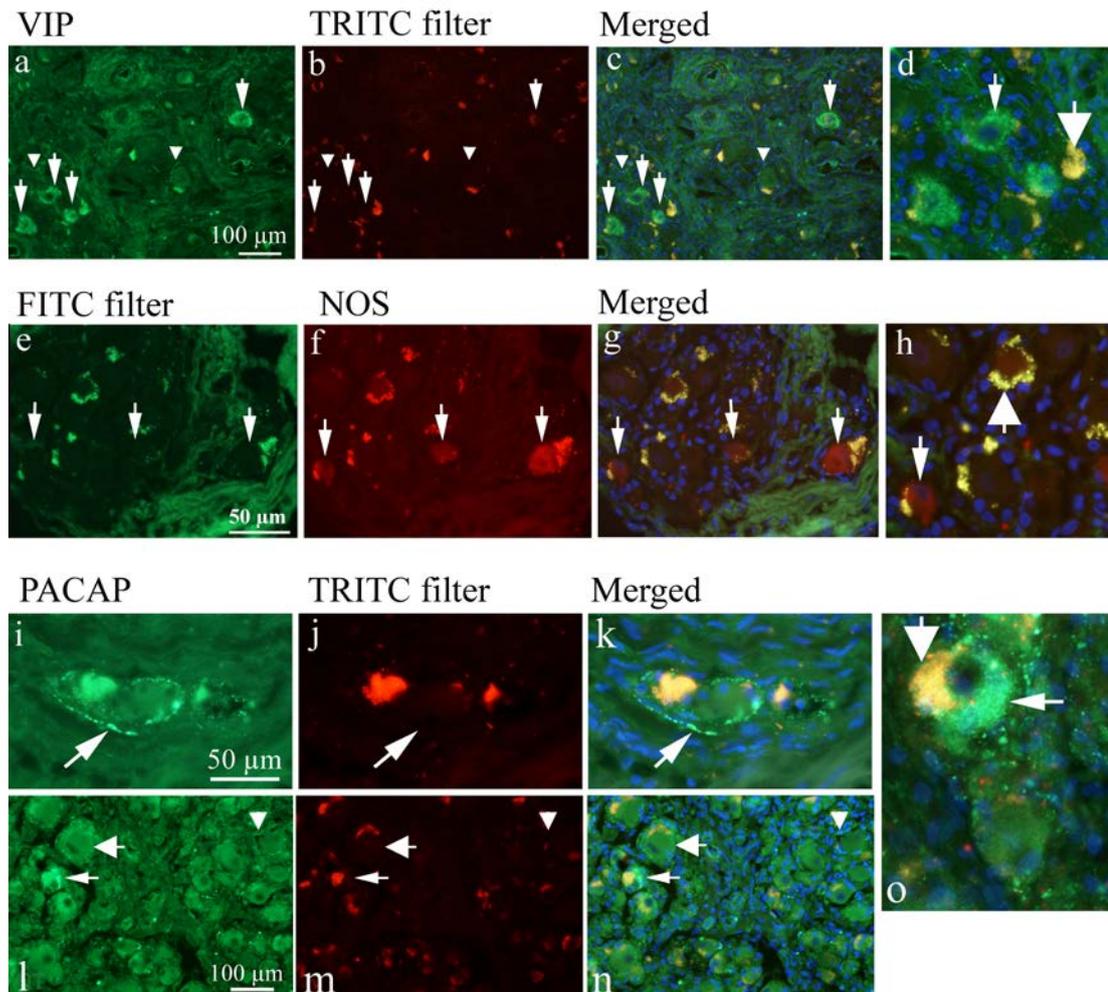

Figure 2 In the montage, TRITC or FITC (depending on the secondary antibody) filters were used to visualize lipofuscin and thereby differentiate the pigment from antibody immunoreactivity. **a-d** VIP (green) immunoreactive neurons (arrows) as well as fibers (arrow heads) were frequently found in human SPG. The immunoreactivity was granular-like in both neurons and fibers. Superimposing green and red images revealed yellowish lipofuscin (thick arrow in 2d). **e-h** Many NOS (red), homogenously stained, immunoreactive cells (arrows) were found in human SPG, but no positive fibers. Thick arrow (h) shows lipofuscin. **i-k** PACAP (green) granular-like immunoreactivity was found in fibers (arrow). **l-n** Some of the neurons (thin arrows) also displayed PACAP immunoreactivity. The neuronal staining was often localized to, or close to, the cell surface (small, thick arrows). Arrow head points at a cell resembling a satellite glial cell seemingly positive for PACAP. However, double staining with glial cell markers could not confirm PACAP expression in satellite glial cell. **o** Higher magnification of a PACAP positive cell (thin arrow). Thick arrow points at lipofuscin.
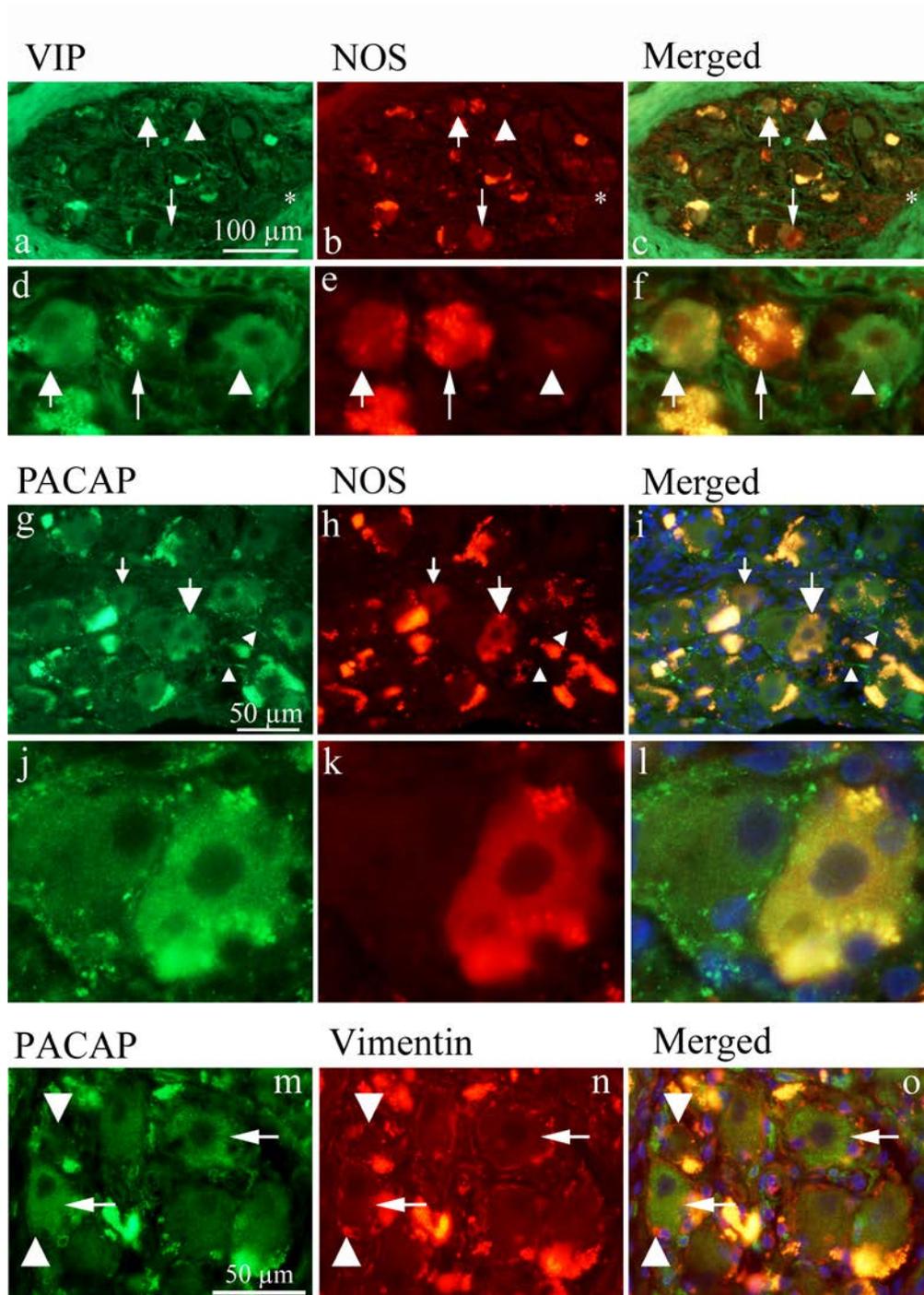
Figure 3 Double stainings VIP/NOS, PACAP/NOS and PACAP/Vimentin. **a-f** VIP (green) and NOS (red) double immunohistochemistry revealed neurons that were only VIP positive (arrow heads), only NOS positive (thin arrows) or double stained (thick arrows). Asterisk - VIP positive fiber. **g-i** The montage demonstrates PACAP (green) and NOS (red) double immunohistochemistry. Thin arrows point at NOS, but not PACAP, positive cells. Thick arrows point at double stained cells. Arrow heads point at PACAP positive fibers. **j –l** The montage shows a double stained neuron. **m-o** Double staining with PACAP/Vimentin reveals that PCAP staining is not localized in the satellite glial cells. Arrows point at PACAP immunoreactive cells and arrow heads at vimentin immunoreactive satellite glial cells.

Pearl-like CGRP immunoreactive fibers were frequently found in human SPG (Figures 4a-b). In order to scrutinize the CGRP immunoreactivity, we used different primary (anti-rabbit and anti-mouse) and secondary (FITC, DyLight 549 and Texas Red) antibodies. More CGRP positive fibers were visible with the use of CGRP anti-mouse primary and DyLight 549 secondary antibodies (Figure 4b) relative to CGRP anti-rabbit and FITC anti-rabbit (Figure 4a). Texas Red secondary antibody revealed the same staining patterns as DyLight 549 (data not shown). CGRP immunoreactivity was not observed in human SPG neurons or SGCs using either of the combination of antibodies.
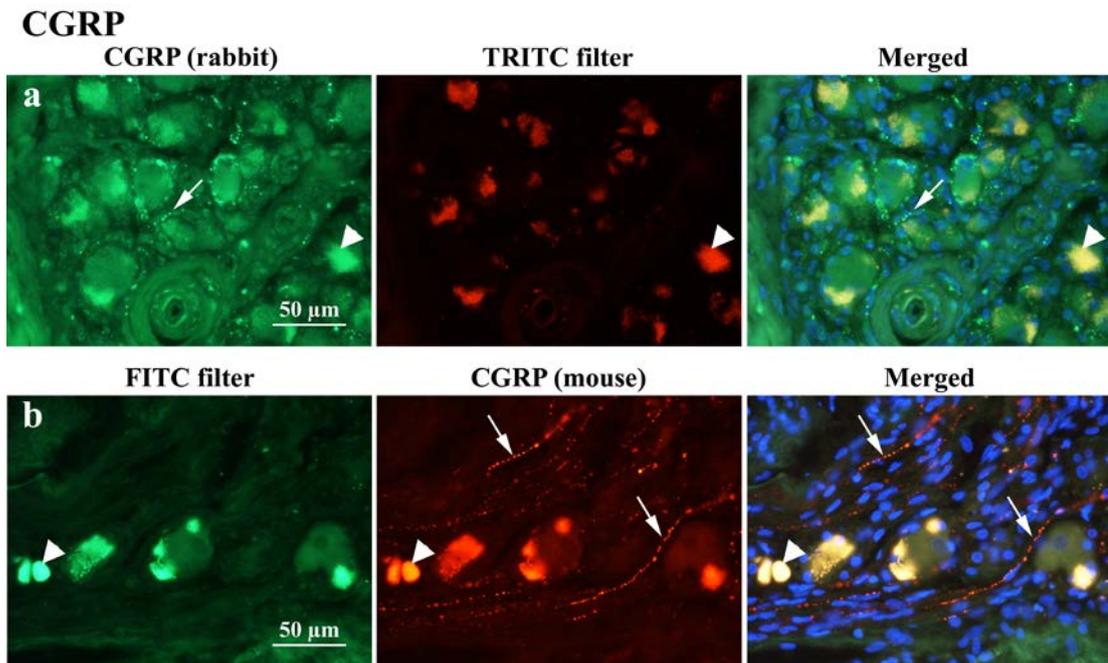
Figure 4 Expression of CGRP in human SPG. **(a)** Some CGRP positive fibers (arrows) were found using CGRP anti-rabbit (green) primary antibody. **(b)** CGRP anti-mouse (red) antibody revealed more CGRP immunoreactive fibers (arrows). The immunoreactivity was granular-like with both primary antibodies. There were no CGRP immunopositive neurons. In the montage, both TRITC and FITC filters were used to differentiate the lipofuscin (arrowheads) from antibody immunoreactivity.

Many CLR immunoreactive SGCs and fibers were found (Figure 5). For the demonstration of CLR, FITC (Figures 5a-b) or DyLight 488 (Figures 5c-e) was used as secondary antibody. Both antibodies visualized immunoreactive SGCs, but only FITC revealed immunoreactive fibers in the SPG. No CLR immunoreactive neurons were found.

For the visualization of RAMP1, the use of Cy2 secondary antibodies (Figure 6a) revealed RAMP1 immunoreactive neurons (homogenously stained). Some large and medium-sized neurons were positive for RAMP1. Furthermore, RAMP1 positive SGCs

were detected with Alexa 488 secondary antibodies (Figure 6b-c). There was no RAMP1 immunoreactivity in the fibers.

In order to examine co-localization between CGRP, CLR and RAMP1, double stainings were performed (Figure 7a-f). No co-localization was found.
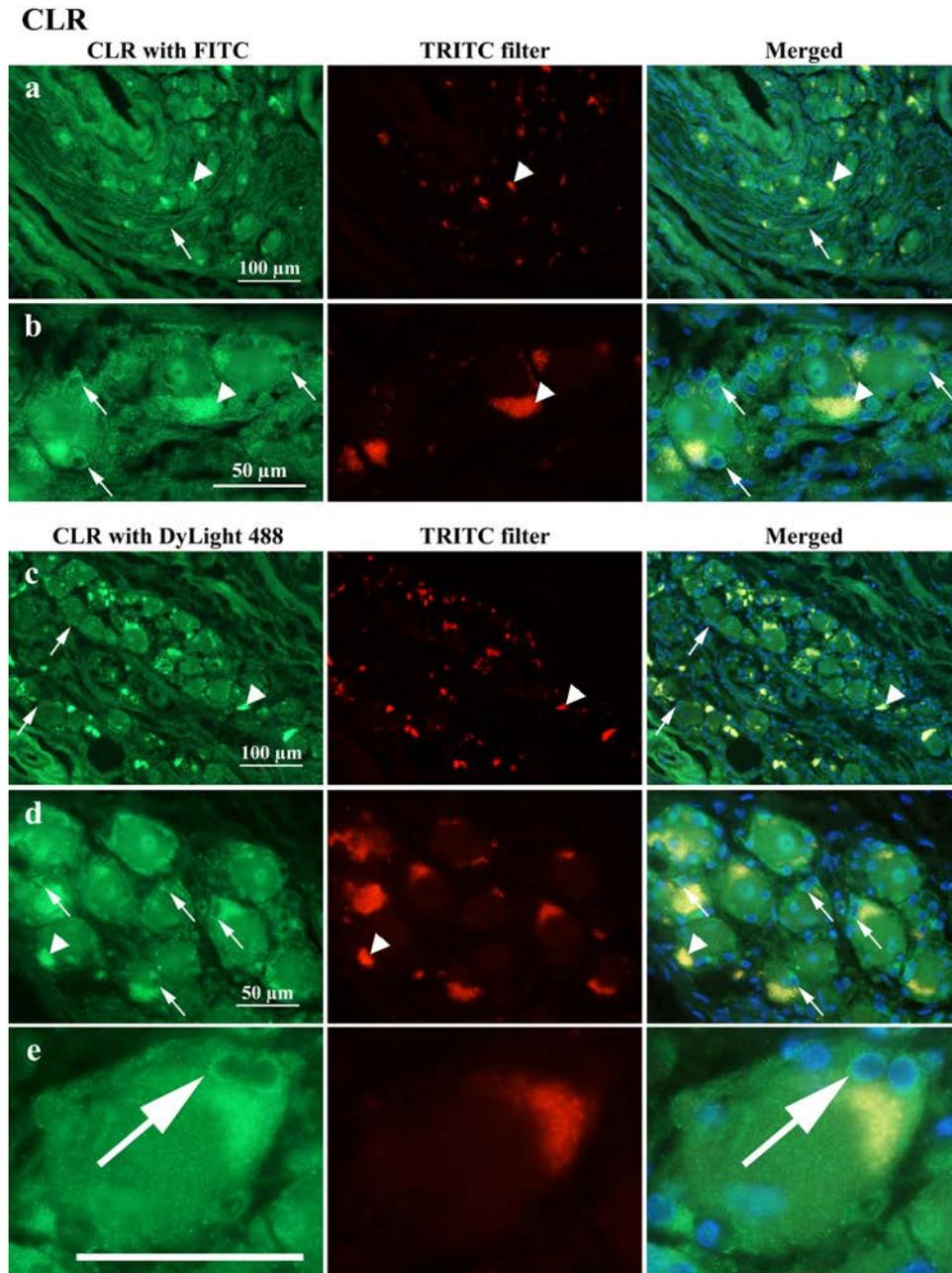

Figure 5 CLR staining in human SPG. **(a)** CLR (green), homogenously stained, immunoreactive fibers (arrows) were visualized with FITC secondary antibody. **(b)** Some CLR positive satellite glial cells (arrows) were found using FITC secondary antibody. **(c-e)** CLR immunoreactivity is shown in lower and higher magnification using DyLight 488 secondary antibody. DyLight 488 disclosed the presence of CLR positive SGCs (arrows), but no immunoreactive fibers. No CLR positive neurons were found. The anti-CLR antibody revealed some nonspecific nuclear staining. Autofluorescent lipofuscin (arrowheads) was demonstrated by superimposing images from both FITC and TRITC filters.
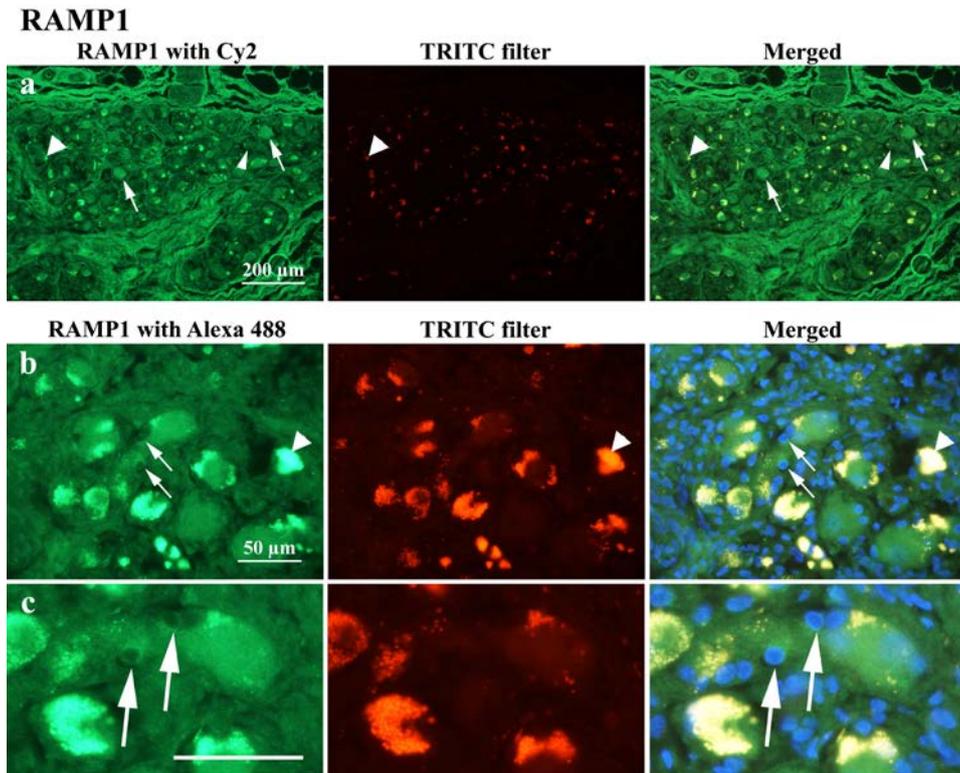
RAMP1

Figure 6 RAMP1 immunoreactivity in human SPG. **(a)** Cy2 secondary antibody revealed RAMP1 (green) immunoreactivity in large and medium-sized neurons (arrows). Thin arrowhead points to a negative neuron. Vectashield with DAPI was not used together with Cy2 secondary antibody. **(b-c)** RAMP1 (green) positive satellite glial cells (arrows) were found using Alexa 488. Thick arrowheads show lipofuscin, visible in both FITC and TRITC filters.
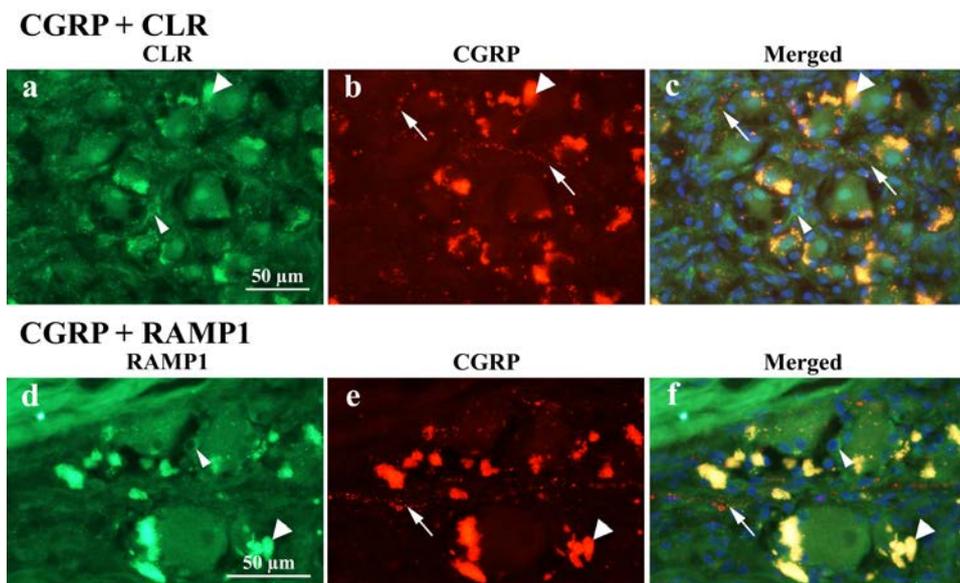

CGRP + CLR

CGRP + RAMP1

Figure 7 Double staining with CGRP/CLR **(a-c)** and CGRP/RAMP1 **(d-f)**. There was no co-localization of CGRP and CLR or RAMP1. **(a)** CLR (green) immunoreactive satellite glial cells (thin arrowheads) were found. **(d)** RAMP1 (green) satellite glial cells (thin arrowheads) were seen. **(b, e)** CGRP (red) positive, granular-like fibers (arrows) are shown. Lipofuscin (thick arrowheads) is present using FITC and TRITC filters.

4.2.2. Rat SPG

In the rat material, VIP, NOS and PACAP immunoreactivity were found in many neurons and fibers (Figures 8a-i). PACAP immunoreactivity was often localized close to the cell membrane (Figure 9), whereas VIP and NOS stainings were more evenly visualized within the cell soma, although somewhat granular-like for VIP (Figure 8). We were also able to observe co-localization of PACAP and NOS (Figures 9a-c), but not between VIP/NOS or PACAP/VIP. PACAP and GS double staining revealed that the PACAP immunoreactivity was localized in or close to the cell membrane, but not in the SGCs (Figures 9d-f).
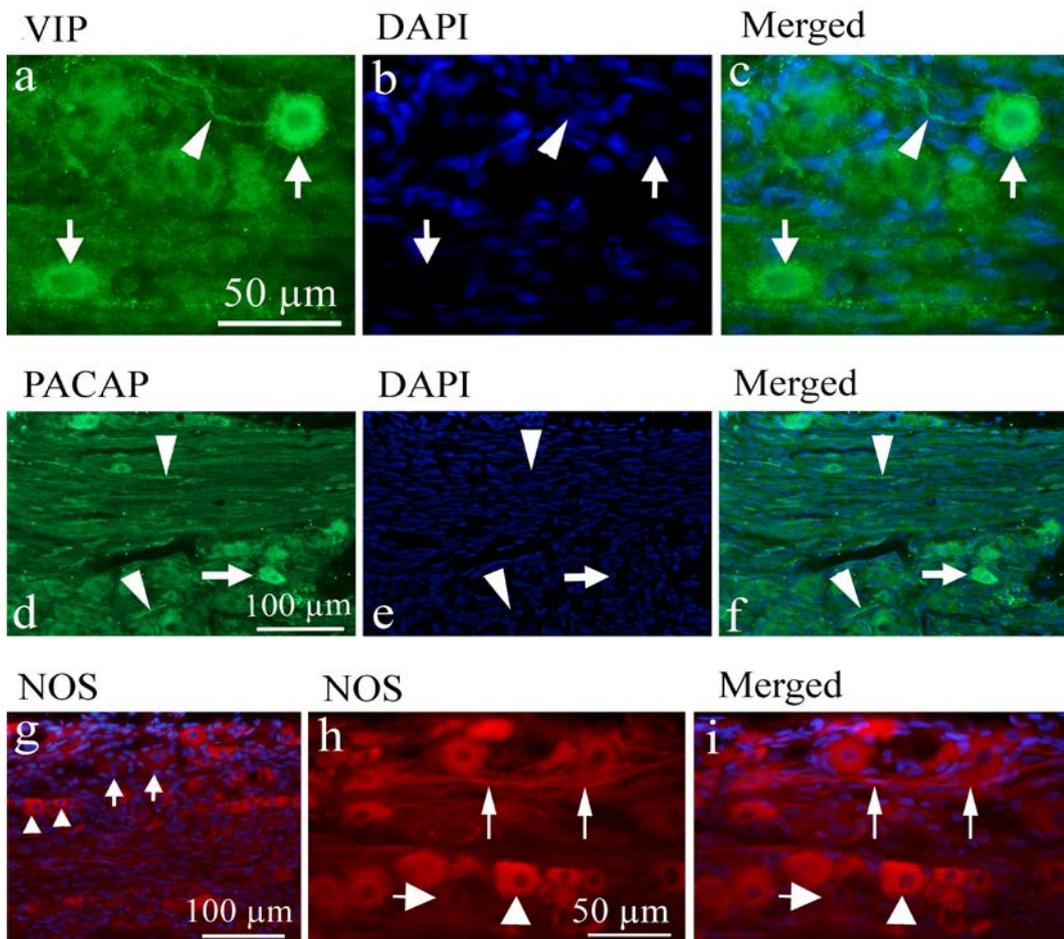
Figure 8 Montage of VIP **(a-c)**, PACAP **(d-f)** and NOS **(g-i)** immunoreactivity in rat SPG. **a-f** Many cells (arrows) and fibers (arrowheads) showed VIP and PACAP immunoractivity. **g-i** Most cells (arrow heads) were NOS positive. Few cells were negative (arrows). Long arrows point at NOS positive fibers.
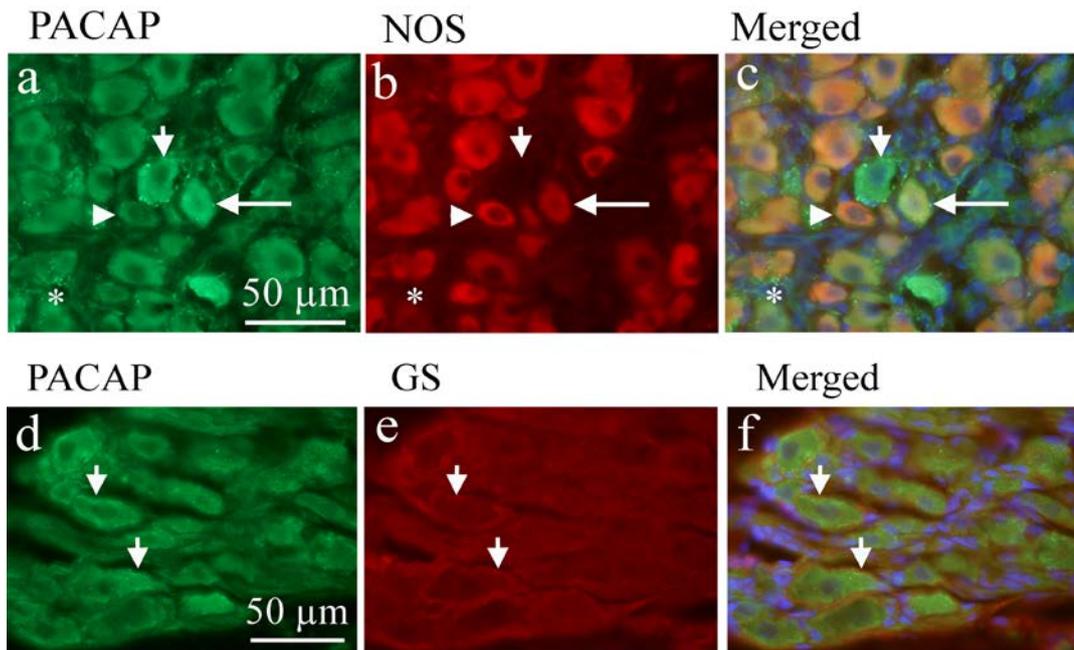
Figure 9 Double stainings PACAP/NOS and PACAP/GS of rat SPG. **a-c** The montage demonstrates PACAP (green)/NOS (red) double immunohistochemistry. Short arrows point at PACAP positive cell, arrow heads at NOS positive and long arrows at a double stained cell. Asterisk – PACAP positive fibers. **d-f** PACAP (green) and GS (red) double staining revealed that the PACAP immunoreactivity was localized in/close to the cell membrane, but not in the satellite glial cells. Arrows point at areas where green PACAP positivity is clearly separated from the GS positivity.

In rat SPG, anti-rabbit and anti-mouse CGRP primary antibodies were used to demonstrate CGRP immunoreactivity. In both cases, CGRP immunoreactive fibers were frequently found (Figures 10a-b). CGRP anti-rabbit antibody disclosed many homogenously stained neurons (Figure 10a), while CGRP anti-mouse antibody revealed only few CGRP positive neurons (Figure 10b). CLR immunoreactive fibers and SGCs were found using FITC anti-rabbit, but not noted in neurons (Figure 10c). Both Cy2 (data not shown) and Alexa 488 secondary antibodies revealed RAMP1 positive fibers and some homogenously stained neurons (Figure 10d). Double staining with RAMP1 and CLR revealed co-localization of the immunoreactive fibers (Figures 11a-c).
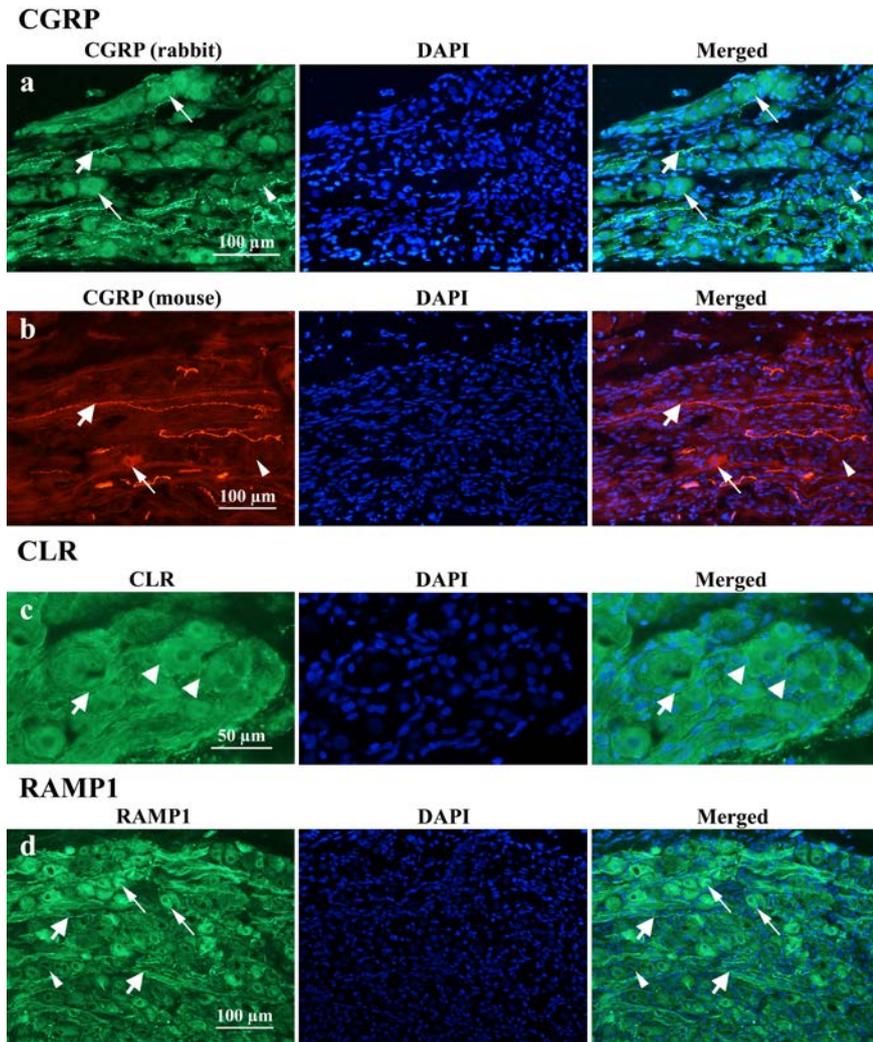
Figure 10 Montage of CGRP, CLR and RAMP1 immunoreactivity in rat SPG. **(a)** Using CGRP anti-rabbit primary antibody, CGRP (green) positive neurons (thin arrows) and fibers (thick arrows) were frequently found. Some neurons were negative (arrowheads). **(b)** Using CGRP anti-mouse primary antibody, a few CGRP (red) immunoreactive neurons (thin arrows) and many fibers (thick arrows) were visualized. Majority of the neurons were negative (thin arrowheads). In both cases, CGRP positive fibers were granular-like and CGRP immunoreactive neurons were homogenously stained. **(c)** CLR (green) positive fibers (thick arrows) and satellite glial cells (thick arrowheads) were revealed. **(d)** RAMP1 (green) positivity was found in fibers (thick arrows) and in many neurons (thin arrows). Both neurons and fibers showed homogenous staining patterns. Some of the neurons were negative (thin arrowheads).
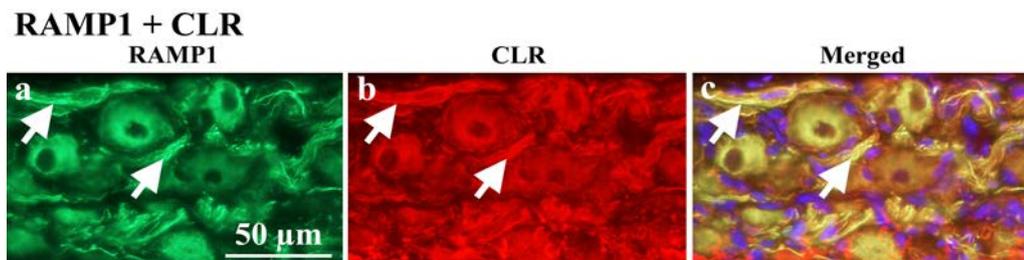

Figure 11 Montage of CLR / RAMP1 double stainings in rat SPG **(a-c)**. Co-localization **(c)** of RAMP1 (green) **(a)** and CLR (red) **(b)** positive fibers (thick arrows).

4.2.3. Human and rat SPG

Distribution of the receptors PAC1, VPAC1 and VPAC2 was investigated in both human and rat. PAC1 and VPAC1 immunoreactivity was found in the SGCs (Figures 12a-f). VPAC1 immunoreactivity was also observed in few fibers in both the human (Figures 12d-f) and rat SPG (Figures 12k). In addition, we observed VPAC2 immunoreactive fibers in both human (Figures 12g-i) and rat specimens (Figure 12l). However, the staining was not as distinct as for PAC1 and VPAC1. No co-localization between the peptides and the receptors were found.
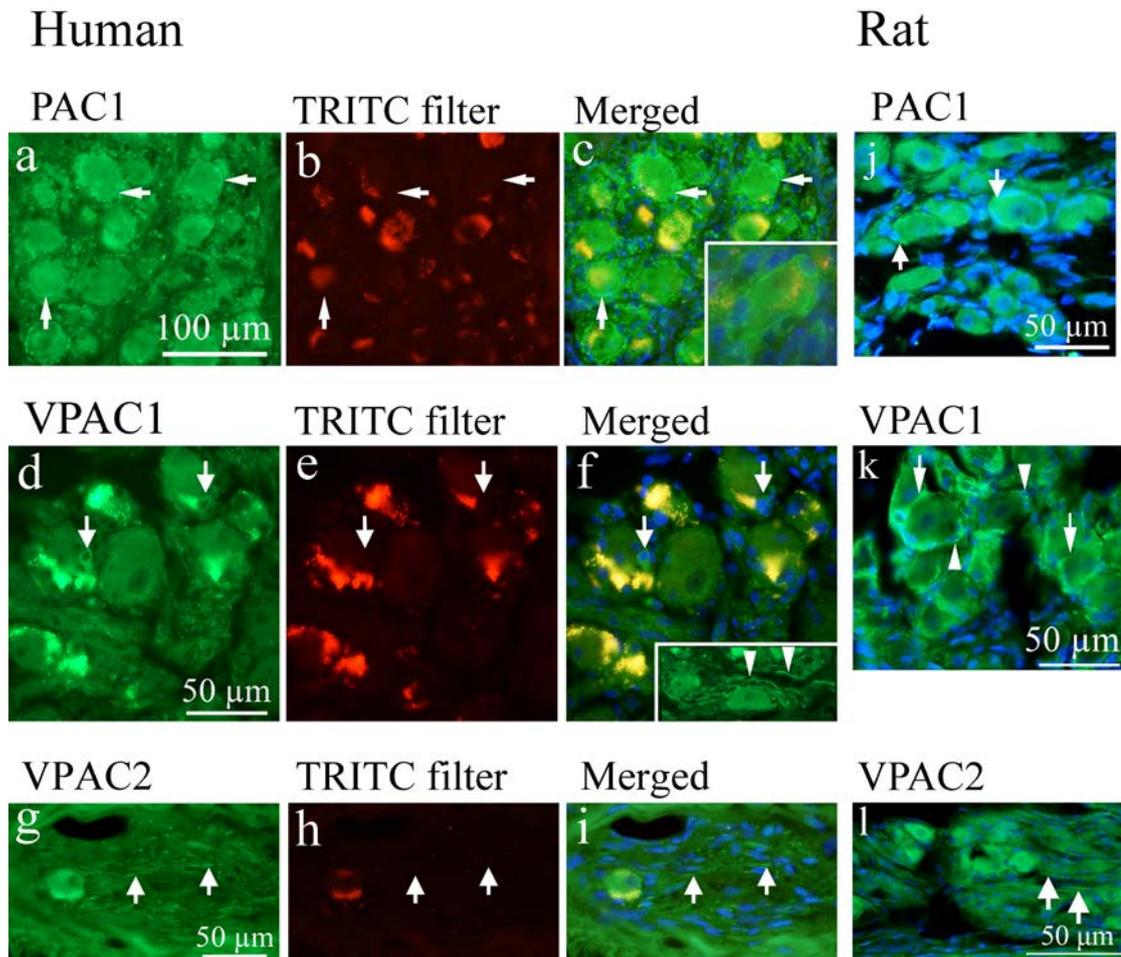

Figure 12 Distribution of receptors PAC1, VPAC1 and VPAC2 in human and rat SPG. Human material **a-c** PAC1 immunoreactivity was found in the satellite glial cells (arrows). Insert: higher magnification of a neuron enveloped by PAC1 positive satellite glial cells. **d-f** VPAC1 immunoreactive satellite glial cells (arrows) were found. Insert: VPAC1 positive fibers (arrowheads). **g-i** Arrows point at VPAC2 positive fibers. Rat material **j** PAC1 immunoreactivity was found in the satellite glial cells (arrows). **k** In addition to VPAC1 positive satellite glial cells (arrowheads), fibers were found positive (arrows). **l** Arrows point at VPAC2 positive fibers.

Overview of CGRP, CLR and RAMP1 immunoreactivity in human and rat SPG is shown in Table 3.

Table 3. Summary of PACAP, VIP, NOS, PAC1, VPAC1, VPAC2, CGRP, CLR and RAMP1 results in human and rat SPG

	Neurons		Satellite glial cells		Nerve fibers	
	Human	Rat	Human	Rat	Human	Rat
PACAP	+	+	-	-	+	+
VIP	+	+	-	-	+	+
NOS	+	+	-	-	-	+
PAC1	-	-	+	+	-	-
VPAC1	-	-	+	+	+	+
VPAC2	-	-	-	-	+	+
CGRP	-	+	-	-	+	+
CLR	-	-	+	+	+	+
RAMP1	+	+	+	-	-	+

4.2.4. Negative controls

Negative controls (omission of primary antibodies) displayed no immunoreactivity, except for autofluorescent lipofuscin.

4.3. Western blot

Western blot revealed protein expression of PAC1, VPAC1, VPAC2, RAMP1 and CLR in rat SPG. As shown in Figure 13, PAC1 receptor gave a 60 kDa band with anti-PAC1 rabbit polyclonal antibody, VPAC1 receptor a 58 kDa band with anti-VPAC1 mouse monoclonal antibody and VPAC2 receptor was visualized as a 65 kDa band with anti-VPAC2 rabbit polyclonal antibody. The homodimeric form of RAMP1 was visualized as a 30 kDa band with anti-RAMP1 polyclonal antibody (Figure 14a). A 60 kDa specific band was observed with anti-CLR polyclonal antibody (Figure 14b). All receptors were analysed in duplicate. Bands were identified by protein molecular weight marker. No bands were visualized after omission of primary antibodies (data not shown).
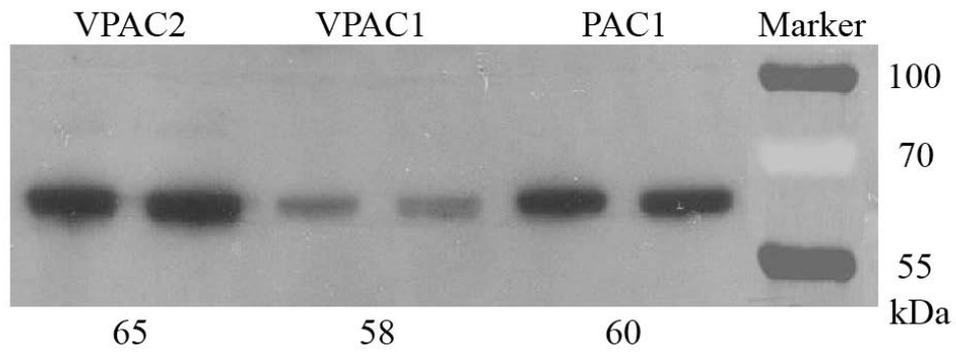
**Figure 13** Expression of PACAP receptors in rat SPG.
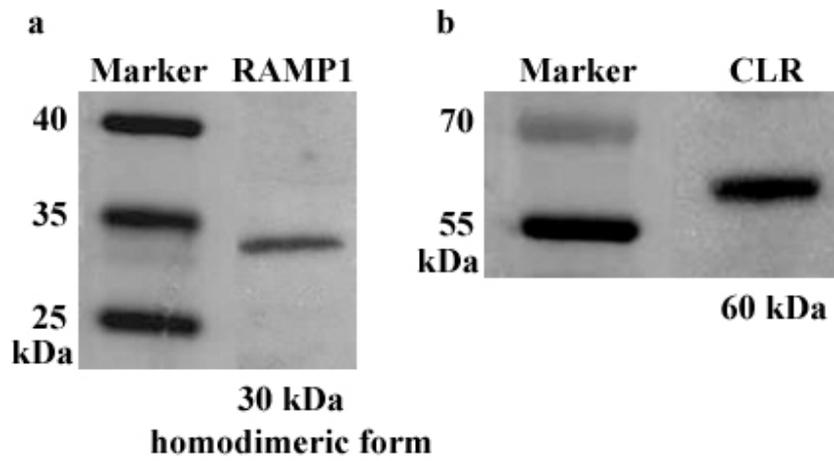
Figure 14 Western blot analysis of RAMP1 **(a)** and CLR **(b)** expressed in rat SPG.

5. DISCUSSION

In our knowledge we were the first who have revealed that VIP/PACAP/NOS co-localize in a large proportion of the human SPG neurons and, in addition, can be seen in some nerve fibers within the ganglion. Furthermore, Western blot revealed that the SPG contains the VPAC1, VPAC2 and PAC1 subtypes of receptor proteins. Immunohistochemistry provided a clear cut demonstration that the PAC1 is localized to the SGCs and VPAC1 receptor to SGCs and fibers. The VPAC2 staining was much weaker than that of the other receptors, although the results were in agreement with Western blot receptor protein results. Moreover, our work has for the first time demonstrated the presence of thin CGRP containing fibers, probably originating from the trigeminal ganglion as C-fibers (Eftekhari et al., 2010), in human and rat SPG. The fibers may modulate the activity in the SPG via CGRP receptors since we in addition found CLR and RAMP1 immunoreactive nerve fibers in rat and immunoreactive SGCs in man.

In recent years there has been considerable interest in the pathomechanism of migraine (Tajti et al., 2011a, Tajti et al., 2011b, Tajti et al., 2012, Vecsei et al., 2013). Clinical observations and experimental studies have suggested a possible role for the SPG in the pathophysiology of migraine (Barbanti et al., 2002, Tepper et al., 2009). Interestingly, systemic administration of PACAP but not VIP has been found to induce "migraine-like" headache in migraine patients, although both peptides elicited similar changes in the vessel tone (Rahmann et al., 2008, Schytz et al., 2009). The present results do not, however, suggest a morphological reason for this differential response. In addition, vascular studies have revealed that VIP is by far a stronger and more potent vasodilator than PACAP of human and rat cerebral and meningeal arteries (Jansen-Olesen et al., 2004, Boni et al., 2009, Chan et al., 2011). *In vivo* studies in man also showed relaxation of cranial vessels by VIP (Hansen et al., 2006, Rahmann et al., 2008) and PACAP (Birk et al., 2007, Schytz et al., 2009). It was argued that dilatation alone could not be the direct cause of the migraine-like attacks after the PACAP-38 infusion but perhaps this response could involve neurons or other cells that contain VIP/PACAP receptors such as cranial ganglia (Schytz et al., 2009). It has been revealed that PACAP-38 is expressed in the trigeminal ganglion (Tajti et al., 1999) and in the caudal trigeminal nucleus (Tajti et al., 2001). It has been demonstrated that blood plasma PACAP-38-like immunoreactivity is increased following the electrical stimulation of the trigeminal ganglion in rat (Tuka et al., 2012). Furthermore, it has been suggested that PACAP is one of the mediators of light aversion, because it elicited photophobia in wild-type mice, while it did not in PACAP-gene deficient mice

(Markovics et al., 2012). Recent data showed that the concentration of PACAP-38 together with CGRP was elevated during the migraine attack period versus to the attack-free periods (Tuka et al., 2013). It has been postulated that elevation of cellular cyclic adenosine monophosphate (cAMP) plays a role in the development of delayed headache via sensitization of trigeminal neurons after CGRP or cGMP since cilostazol administration elicited migraine-like attacks without vascular effects on intracranial vessels (Ingram and Williams, 1996, Lassen et al., 2002, Birk et al., 2004, Birk et al., 2006). PACAP activates VPAC1-2 and PAC1 receptors, which induces cAMP level elevation (Dickson et al., 2006), which more resembles the mode of action of CGRP (Walker et al., 2010).

The cranial parasympathetic outflow is mediated in part through the SPG and in part via the otic ganglion. Cerebral and dural blood vessels are innervated by parasympathetic fibers (from SPG and otic ganglion) and by unmyelinated sensory nerve fibers (from trigeminal ganglion) (Edvinsson and Uddman, 1981, Nozaki et al., 1993, Minami et al., 1994, Edvinsson and Uddman, 2005). The activation of parasympathetic fibers can alter the status of the perivascular sensory pain fibers (Delepine and Aubineau, 1997). During this process acetylcholine (Ach), VIP and nitric oxide are released from the parasympathetic nerve fibers. Ach can activate C fibers which subsequently can modify the sensory nerve release of CGRP and substance P (Tanelian, 1991, Yarnitsky et al., 2003) via muscarinic and nicotinic cholinergic receptors of the peripheral nociceptors (Steen and Reeh, 1993, Hua et al., 1994) and possibly activating or sensitizing (or both) the nociceptors (Yarnitsky et al., 2003).

It has been demonstrated that postganglionic parasympathetic fibers from the SPG mediate meningeal blood flow elevations and meningeal vasodilatation (Bolay et al., 2002) and, in addition, neurogenic inflammation which in turn may sensitize meningeal nociceptors (Burstein and Jakubowski, 2005). The preganglionic fibers to the SPG originate from the superior salivatory nucleus (SSN) and synapse in the SPG. The SSN can be activated/modified by trigeminal sensory nerve fibers. This is a trigeminal-autonomic reflex which may be active in migraine attacks (Zagami et al., 1990).

It seems that the fundament for a trigeminal action is at place by the presence of CGRP receptor components. The role of the SGCs in the SPG is largely unknown. However, increasing glial cell research suggests a ganglion function at many levels (Hanani, 2010), especially the characterized SGCs and neurons forming a morphological unite in the SPG.

There are some functional data which suggest an interaction between the trigeminal and sphenopalatine ganglia. Cluster headache is associated with activation of both ganglia since there is co-release of CGRP and VIP (Goadsby and Edvinsson, 1994a). Treatment with sumatriptan aborts both symptoms of parasympathetic activation and neuropeptide release, presumably by the triptan acting as inhibitor on the sensory nervous system via a presynaptic mechanism or the formation of CGRP (Durham and Russo, 1999). Further, experimental activation of the superior sagittal sinus results in co-release of VIP and CGRP (Zagami et al., 1990). Cutting of the trigeminal nerve abolished not only the CGRP release but also that of VIP which supports an interaction between the two systems. It is tempting to speculate that the present finding reveals a direct link between the trigeminal ganglion CGRP-containing fibers and the SPG. The nature of this is unclear but available data would imply that intense activation of the trigeminal ganglion can result in parasympathetic symptoms (cluster headache, red eye, rhinorrhea, conjunctival injection, and tearing) associated with VIP release (Goadsby and Edvinsson, 1994a). Our data suggest that the CGRP containing fibers end on neurons and SGCs, and, in addition, SGCs express both parts of the functional CGRP receptor, CLR and RAMP1.

There are some immunohistochemical differences between human and rat SPG. CGRP immunoreactive neurons were found in rat and not in human. These results suggest that CGRP can be produced or stored in rat SPG neurons, while in human it is more plausible that CGRP is produced by the trigeminal neurons and transmitted to the SPG through CGRP nerve fibers. In addition, RAMP1 is expressed in human SGCs, while it is expressed in nerve fibers in rat. The data from human material suggest that CGRP acts on the SGCs, since both of the CGRP receptor components are present. In the rat material, RAMP1 was expressed in the nerve fibers together with CLR.

We also show that fibers express CLR and neurons RAMP1 in man; this could suggest that other receptors are involved as RAMP1, with calcitonin receptor (CTR), forms a high affinity amylin receptor (Muff et al., 1999). Dimerization of CLR with RAMP2 or RAMP3 may form adrenomedullin receptors (Hay et al., 2008).

Moreover, we observed distinct PAC1 and VPAC1 receptor immunoreactive SGCs in both human and rat SPG.

According to a recent review (Hanani, 2010), few data have been published on the subject of the properties of SGCs in parasympathetic ganglia. The reason might be that parasympathetic ganglia are located near or within their target and as a result, the access to most of them is difficult. Parasympathetic ganglia in general contain SGCs (Pomeroy and

Purves, 1988). The available data suggest that SGCs are involved in synaptic maintenance and remodeling. In guinea-pig intrinsic ganglia of the urinary bladder, Hanani et al. (1999) reported that the SGCs were positive for S100 and glutamine synthetase but not for GFAP. In the intrinsic ganglia of the cat urinary bladder, SGCs were seen to possess P2X and P2Y receptors but no function was revealed (Ruan et al., 2006). Electrophysiology has demonstrated a higher resting membrane potential in SGCs than in the accompanying neurons (Sha et al., 1996). When nerve trunks were electrically stimulated, a frequency-dependent membrane depolarization was observed in the SGCs (King et al., 1989). It is well-known that in sympathetic ganglia neurons can release ATP, to which SGCs respond via P2Y (metabotropic) and P2X (ionotropic) receptors and releasing ATP and TNF-α (Vizi et al., 1997). These mediators act on neurons, causing profound functional changes in them, e.g. uptake mechanisms controlling the microenvironment (Zhang et al., 2007). The data emphasize that the SGCs are more than cells surrounding the neurons. Our study is the first that examines SGCs in cranial parasympathetic ganglion in man. Future may provide more insight on how SGCs may influence synaptic transmission, and information processing in autonomic and sensory ganglia.

5.1. Methodological considerations

We used anti-human CLR and RAMP1 antibodies in human and rat SPG. These antibodies have been characterized in human and rat trigeminal ganglion (Eftekhari et al., 2010). The study revealed no immunoreactivity with preabsorbed CLR or RAMP1 antibodies, using their respective blocking peptides. In addition, the CGRP immunoreactivity was abolished if pretreated with an excess of unlabeled CGRP (Eftekhari et al., 2010). Thus, these antibodies were considered as specific for the epitope they were raised against. Whether our results reflect difficulties for the antibodies to recognize their epitope in human SPG (which is reflected by the discrepancies in our results using combination of different antibodies), the complexity of protein expression or accurate depiction of CGRP and its receptor components are not known. Nevertheless, we demonstrate CGRP and receptor components in human SPG which may indicate an interaction between parasympathetic and sensory ganglia.

5.2. Conclusion

The present work has revealed the presence of VIP, PACAP and NOS in nerve fibers within the ganglion, in addition, VIP/NOS and PACAP/NOS show co-localization in the human SPG neurons. Western blot verified the presence of VPAC1, VPAC2 and PAC1 receptors in rat SPG. Immunohistochemistry showed that PAC1 and VPAC1 are localized in the SGCs, while VPAC1 and VPAC2 in the nerve fibers in both human and rat SPG. These results suggest that the peptides may be involved in intraganglionic activity.

Moreover, the present finding demonstrates that CGRP-positive fibers, probably originating from the trigeminal ganglion as C-fibers, are present in both human and rat SPG. In rat CGRP positive neurons are found. Since both components of the CGRP receptor, CLR and RAMP1, are present in the ganglion (Western blot) and these are localized to SGCs (human) and fibers (rat), an interaction between parasympathetic and sensory ganglia is plausible.

The immunohistochemical differences between human and rat SPG suggest that in human CGRP is produced by the trigeminal neurons, transmitted to the SPG through CGRP positive nerve fibers and acts on the SGCs in the SPG, since both of the CGRP receptor components are present. In rat CGRP can be produced or stored in SPG neurons and act through the nerve fibers, where both of the receptor components are present.

6. SUMMARY OF NEW FINDINGS

(i) Our work has revealed VIP and PACAP immunoreactivity in nerve fibers besides the neurons but not in satellite glial cells both in human and rat SPG.

(ii) We disclose that the SPG contains the VPAC1, VPAC2 and PAC1 subtypes of receptor proteins. We found PAC1 and VPAC1 immunoreactivity in the satellite glial cells and VPAC1 and VPAC2 immunoreactive nerve fibers of both human and rat.

(iii) We demonstrate that CGRP-positive fibers are present in human and rat SPG and CGRP immunoreactive neurons in rat.

(iv) CGRP receptor components (CLR and RAMP1) are localized to SGCs in human and to fibers in rat SPG.

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8. HUNGARIAN SUMMARY

Idegrendszeri hírvivőanyagok megoszlása és kapcsolatuk a ganglion sphenopalatinum rendszerében

1. BEVEZETÉS

A fej-nyak tájéki és a koponyaűri (agyi, agyburki) vasculatura paraszimpatikus beidegzést kap a ganglion (ggl.) sphenopalatinumból és a ggl. oticumból (Suzuki et al., 1988). A humán ggl. sphenopalatinumban vazoaktív intestinalis peptid (VIP), hypophysis adenilát-cikláz aktiváló polipeptid (PACAP) és nitrogén-monoxid szintetáz (NOS) tartalmú neuronok jelenlétét mutatták ki (Uddman et al., 1999).

A VIP és a PACAP a szekretin/glukagon/VIP peptidcsalád tagjai. A VIP széleskörű biológiai hatásokkal rendelkezik, úgymint az embrionális agyi fejlődés, a fájdalom percepció és a gyulladás (Harmar et al., 1998). A PACAP ezen peptidcsalád legújabb tagja, amelyet legelőször birka hypothalamusból izoláltak (Miyata et al., 1989). Két formája ismert, a 38 aminosavból álló PACAP-38 és a 27 aminosavból álló PACAP-27. Mindkét peptid nagy változatosságot mutató biológiai hatásokkal rendelkezik, mint például a vazodilatáció, a sejtproliferáció és differenciáció stimulálása, a neurotranszmitter felszabadulás szabályozása és a fájdalom transzmisszió (Harmar et al., 1998, Vaudry és Laburthe, 2006). Mind a PACAP, mind a VIP G-proteinhez kapcsolt receptorokon keresztül fejtik ki hatásukat (Vaudry és Laburthe, 2006, Dickson és Finlayson, 2009). A VPAC1 és a VPAC2 receptorok egyenlő erősséggel kötik a VIP-et és a PACAP-ot, míg a PAC1 receptor csak a PACAP-ra specifikus (Harmar et al., 1998).

Az elsődleges fejfájások (migrén és cluster fejfájás) patofiziológiájának vizsgálata során migrénesekben szoros kapcsolatot figyeltek meg a calcitonin gén-rokon peptid (CGRP) és a fejfájás intenzitása között rohamok esetén (Ho et al., 2010, Juhász et al., 2005). A CGRP receptor a G-protein receptor család B-típusába tartozik (Hay et al., 2008). A funkcionális CGRP receptor három fehérjét tartalmaz. A (i) calcitonin receptor-szerű receptor (CLR) és a (ii) receptor aktivitást módosító fehérje 1 (RAMP1) együttesen a ligand kötő oldalt képzi (McLatchie et al., 1998, Heroux et al., 2007). A (iii) CGRP receptor komponens fehérje (RCP) szerepe a receptor intracellularis jelátvivő pályákhoz kapcsolása (Evans et al., 2000).

Cluster típusú fejfájásban szenvedő betegek mindegyikében, míg migrénes páciensek közül csak néhányban a CGRP és a paraszimpatikus rendszerre jellegzetes VIP együttes felszabadulását figyelték meg (Goadsby és Edvinsson, 1994a). Kiemelendő, hogy ezen migrénes betegekben a clusterre jellegzetes kísérő tüneteket (conjunctivalis belövelltség, orrdugulás, orrfolyás) figyeltek meg. A CGRP az érző rendszer jellegzetes, 37 aminosavból álló neuropeptidje, amely jelentős szerepet játszik a craniocervicalis vazodilatációban és a fájdalom transzmissziójában (Ho et al., 2010). A sumatriptán az 5-hidroxitriptamin 1B/1D receptorokon fejti ki agonista hatását. Megszünteti nem csak a CGRP felszabadulást és a fejfájást, hanem a VIP felszabadulást és a paraszimpatikus tüneteket is (Goadsby és Edvinsson, 1994a, Juhász et al., 2005).

Az érintett mechanizmusok nem tisztázottak, ezért terveztük kísérleteinket a ggl. sphenopalatinum rendszerében.

2. CÉLKITŰZÉS

(i) VIP és PACAP receptorok jelenlétének és eloszlásának feltárása humán és patkány ggl. sphenopalatinumban indirekt immunfluoreszcens és Western blot technika használatával.

(ii) A satellita glia sejtek és azok neuronális kapcsolatának vizsgálata mind humán és patkány ggl. sphenopalatinumban.

(iii) A CGRP és CGRP receptor komponensek (CLR és RAMP1) tanulmányozása humán és patkány ggl. sphenopalatinum neuronjaiban, idegrostjaiban és satellita glia sejtjeiben, a feltételezett helyi funkció feltárása céljából.

(iv) A vizsgált neurotranszmitterek eloszlásának összehasonlítása humán és patkány ggl. sphenopalatinumban immunfluoreszcens technika segítségével.

3. ANYAG ÉS MÓDSZER

Kísérleteinkhez boncolás során nyert humán (n=5), illetve hím Sprague-Dawley patkány (n=8, 300-400g) ggl. sphenopalatinumokat használtunk követve az érvényben lévő etikai engedélyek irányelveket.

A minták előkészítését követően 10 µm vastagságú kriometszeteket készítettünk. A tájékozódás és a szövet állapotának vizsgálata céljából Hematoxylin-Eosin festést alkalmaztunk mind a humán, mind a patkány ggl. sphenopalatinum metszeteken. Humán és patkány ggl. sphenopalatinumban a VIP, PACAP, NOS, VIP és PACAP közös receptorok (VPAC1, VPAC2), PACAP receptor (PAC1), CGRP és CGRP receptor komponensek

(CLR, RAMP1) immunhisztokémiai vizsgálata céljából indirekt immunfluoreszcens technikát használtunk, továbbá kettősfestéseket is alkalmaztunk az egyes neurotranszmitterek kolokalizációjának feltárása céljából. A metszetek vizsgálatához és a felvételek elkészítéséhez fény- és epifluoreszcens mikroszkópot használtunk.

Patkány ggl. sphenopalatinumban a VIP/PACAP receptorok és a CGRP receptor komponensek jelenlétének kimutatására Western blot technikát alkalmaztunk.

4. EREDMÉNYEK

4.1. Hisztológia

4.1.1. Hematoxylin-Eosin

Mind a humán, mind a patkány ggl. sphenopalatinum jól körülírt ganglion, melyek satellita glia sejtekkel körülvett különböző méretű neuronokat tartalmaznak.

4.2. Immunhisztokémia

4.2.1. Humán

Az alanyok idős életkora miatt számos neuron tartalmazott citoplazmális lipofuszcin granulumokat.

A humán ggl. sphenopalatinumban granuláris festődést mutató VIP és PACAP immunoreaktív neuronokat és idegrostokat, valamint homogénen festődő NOS immunoreaktív idegsejteket figyeltünk meg. A glia sejt specifikus Vimentin és PACAP antitestekkel végzett kettősfestéssel kimutattuk, hogy a PACAP immunoreaktivitás nem jelentkezik a satellita glia sejtekben. VIP/NOS, PACAP/NOS és VIP/PACAP kettősfestést végeztünk. Vizsgálatainkban kolokalizációt találtunk a VIP és a NOS, valamint a PACAP és a NOS között.

A humán ggl. sphenopalatinum számos gyöngyfüzérszerű megjelenést mutató CGRP immunoreaktív idegrostot tartalmaz. CGRP immunoreaktivitást nem találtunk a humán sphenopalatinális neuronokban és satellita glia sejtekben. Számos CLR immunoreaktív satellita glia sejtet és idegrostot, továbbá RAMP1 pozitív satellita glia sejtet, valamint néhány nagy és közepes méretű neuront figyeltünk meg.

4.2.2. Patkány

A patkány ggl. sphenopalatinum VIP, NOS és PACAP immunoreaktív neuronokat és idegrostokat tartalmazott. PACAP és glutamin szintetáz kettősfestéssel igazoltuk, hogy a PACAP immunoreaktivitás bár közel helyezkedik el a sejtmembránhoz, de nem jelentkezik a satellita glia sejtekben. Továbbá kettősfestéssel mutattuk ki a PACAP és a NOS kolokalizációját.

CGRP immunoreaktív idegrostok, továbbá homogénen festődő neuronok találhatóak a patkány mintákban. A receptor komponensek közül CLR immunoreaktivitást a idegrostokban és a satellita glia sejtekben, míg RAMP1 pozitivitást a idegrostokban és a neuronokban írtunk le. Kettősfestés felfedte, hogy a CLR és a RAMP1 kolokalizációt mutat az idegrostokban.

4.2.3. Humán és patkány

A PAC1, VPAC1 és VPAC2 receptorok eloszlását mind humán, mind patkány ggl. sphenopalatinumban azonosnak észleltünk. PAC1 immunoreaktivitást a satellita glia sejtekben, VPAC1 immunoreaktivitást a satellita glia sejtekben és néhány idegrostban, míg VPAC2 immunoreaktivitást a idegrostokban találtunk.

4.2.4. Negatív kontrollok

A primer antitestek elhagyásával negatív kontroll festéseket alkalmaztunk, amelyek az autofluoreszcens lipofuszcintól eltekintve nem mutattak immunoreaktivitást.

4.3. Western blot

Western blot vizsgálattal igazoltuk a PAC1, a VPAC1 és a VPAC2 receptorok, valamint a RAMP1 és a CLR receptor komponensek fehérje expresszióját patkány ggl. sphenopalatinumban. A PAC1 60 kDa, a VPAC1 58 kDa, a VPAC2 65 kDa, a RAMP1 30 kDa, míg a CLR 60 kDa molekulatömegű sávok formájában jelentek meg.

5. KÖVETKEZTETÉS

Eredményeink igazolják a VIP, a PACAP és a NOS jelenlétét a ggl. sphenopalatinumban az idegrostokban. A VIP/NOS és a PACAP/NOS neuronális kolokalizációját írtuk le a humán ganglionokban. Immunhisztokémiai vizsgálattal PAC1 és VPAC1 aktivitást mutattunk ki a satellita glia sejtekben, míg VPAC1 és VPAC2 immunoreaktivitást a idegrostokban. Western blot vizsgálat megerősítette a VPAC1, a VPAC2 és a PAC1 receptorok expresszióját patkány ggl. sphenopalatinumban. Mindezen eredményeinek alapján feltételezzük, hogy ezen peptidek részt vesznek az intraganglionáris aktivitásban.

Továbbá a vizsgálataink során humán és patkány ggl. sphenopalatinumban CGRP pozitív idegrostokat találtunk, amelyek valószínűleg C-idegrostok formájában a trigeminális ganglionból erednek. A patkány minták CGRP pozitív neuronokat is tartalmaztak. Mivel a CGRP receptor mindkét komponensét kimutattuk a ganglionban Western blot technikával, valamint immunhisztokémiai módszerrel igazoltuk azok

jelenlétét a satellita glia sejtekben (humán) és idegrostokban (patkány), a paraszimpatikus és szenzoros ganglionok közötti kölcsönhatást valószínűsítünk.

A humán és a patkány ggl. sphenopalatinum között észlelt immunhisztokémiai különbségek azt sugallják, hogy humánban a CGRP a trigeminális neuronokban termelődik, és a CGRP pozitív idegrostokon keresztül jut a ggl. sphenopalatinumba, ahol a satellita glia sejteken fejti ki hatását. Ezzel szemben patkányban a CGRP a sphenopalatinális neuronokban termelődhet vagy tárolódhat és az idegrostokon keresztül fejti ki hatását.

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