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First study on the peptidergic innervation of the brain superior sagittal sinus in humans

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ABSTRACT

The superior sagittal sinus (SSS) of the mammalian brain is a pain-sensitive intracranial vessel thought to play a role in the pathogenesis of migraine headaches. Here, we aimed to investigate the presence and the potential co-localization of some neurotransmitters in the human SSS. Immunohistochemical and double-labeling immunofluorescence analyses were applied to paraformaldehyde-fixed, paraffin-embedded, coronal sections of the SSS. Protein extraction and Western blotting technique were performed on the same material to confirm the morphological data. Our results showed nerve fibers clustered mainly in large bundles tracking parallel to the longitudinal axis of the sinus, close in proximity to the vascular endothelium. Smaller fascicles of fibers encircled the vascular lumen in a spiral fashion, extending through the subendothelial connective tissue. Isolated nerve fibers were observed around the openings of bridging veins in the sinus or around small vessels extending into the perisinusal dura. The neurotransmitters calcitonin gene related peptide (CGRP), substance P (SP), neuronal nitric oxide synthase (nNOS), vasoactive intestinal polypeptide (VIP), tyrosine hydroxylase (TH), and neuropeptide Y (NPY) were found in parietal nerve structures, distributed all along the length of the SSS. Overall, CGRP- and TH-containing nerve fibers were the most abundant. Neurotransmitters co-localized in the same fibers in the following pairs: CGRP/SP, CGRP/NOS, CGRP/VIP, and TH/NPY. Western blotting analysis confirmed the presence of such neurosubstances in the SSS wall. Overall our data provide the first evidence of the presence and co-localization of critical neurotransmitters in the SSS of the human brain, thus contributing to a better understanding of the sinus functional role.

1. Introduction

The superficial veins of the brain are mainly fibrous in structure and devoid of parietal valves (Kiliç and Akakin, 2008). Most of these veins drain in a complex of endothelium lined, vessel-like excavations of the dura mater known as dural or cerebral sinuses, which open into the jugular veins at the base of the encephalus. Thus, this complex of cerebral veins and sinuses collects the blood outflowing from the brain and modulates its discharge. However, the regulatory mechanisms that operate to maintain this delicate homeostasis are still poorly understood (Edvinsson and Jansen, 1992; Vignes et al., 2007).

A major component of the brain sinuses is the superior sagittal sinus (SSS) which is a canalicular excavation of the dura mater tracking between the two cerebral hemispheres from the prerolandic to the postrolandic cortex, just upon the attachment of the dura to the falx cerebri. The SSS is roughly triangular-shaped, increases in size craniocaudally, and dichotomizes in the two transverse sinuses at the posterior pole of the brain. The SSS is the initial segment of the brain sinusal complex and collects blood from the veins of the anterior, lateral, and dorsal portions of the cerebral surface.

The role of the SSS in the onset of pain resulting in primary headaches, such as migraines, is currently debated. Studies have shown that stimulation of the human SSS causes pain ascribed to the first division of the trigeminal nerve (Ray and Wolff, 1940). More recently,

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electrical and mechanical stimulation of the sinus were shown to enhance metabolic activity, blood flow, and *c*-fos expression of the trigeminal nucleus and of the C1/C2 segment of the spinal dorsal horn in cat and monkey models (Goadsby et al., 1997; Goadsby and Hoskin, 1997; Kaube et al., 1993; Lambert et al., 1988). When cholera toxin subunit b or wheat germ agglutinin horseradish peroxidase was applied to the rat SSS, nerve structures in the trigeminal and C2 dorsal root ganglia, the medullar trigeminal nucleus, and the C1/C3 segment of the spinal dorsal horn were labeled through retrograde intra-axonal transport of both molecules (Liu et al., 2004). Taken together, these findings suggest that the SSS is a sensitive vessel capable of sending algic sensations to the spinal trigeminal nucleus. Particularly, the SSS is considered the more nociceptive-specific structure of the brain (Goadsby et al., 2009).

Morphological studies of SSS innervation are a necessary complement to the functional ones, but remain largely incomplete. Many decades ago, the fibers innervating the SSS walls were shown to originate from the ophthalmic division of the trigeminal nerve. Particularly, the initial portion of the sinus received fibers through the anterior ethmoidal nerves, while the posterior third was innervated by fibers from the falx (McNaughton, 1966). More recently the rat SSS was found to be encircled by dopamine β hydroxylase-containing nerve fibers which probably originated from neurons of the left and right superior cervical ganglia, which resulted intensely stained when the complex wheat germ agglutinin/horseradish peroxidase was applied to the supratentorial dura (Keller et al., 1989). Further, dense network of nerve fibers containing the peptides calcitonin gene related peptide (CGRP), substance P (SP), neuropeptide Y (NPY), and vasoactive intestinal polypeptide (VIP), and the amine serotonin were described around the SSS of the same animal species (Keller and Marfurt, 1991). These studies were performed by fluorescent and immunohistochemical analyses of whole mount preparations of dural strips and provided an elegant description of the perisinusal innervation, but were unable to disclose the distribution of such fibers within the SSS walls.

Finally, close networks of NPY- and SP-containing nerve fibers were described around the superior sagittal and transverse sinuses in the cat (von Düring et al., 1990), and around the SSS in the guinea pig (Furness et al., 1982), respectively. In particular, the first study showed that all branches of the NPY-containing network comprised small or large bundles of unmyelinated fibers, while the second described perivascular SP-containing innervation of the total animal body.

In this study we investigated the presence of a neurotransmitter pool in the SSS sinusal wall of the human brain using immunohistochemical and double-labeling immunofluorescent techniques. We also investigated the co-localization of the tested molecules in the same nerve fibers of the SSS. Western blotting analysis was used as well to validate the presence of the neuropeptides. Our results may provide further insight to better understand the role of these neurotransmitters in the pathogenesis of migraine headaches.

2. Material and methods

2.1. Samples

The tissues used in this study were collected from 20 human cadavers of both gender humans who had died from causes unrelated to cerebral pathologies and whose brains were found to be free of lesions during necroscopic analysis. All samples were collected at the Hospital Universitario Marqués de Valdecilla, Santander (Spain), carefully following the procedures described by the Declaration of Helsinki for experimental use of biological material of human provenience.

2.2. Tissue collection, fixation, and processing

After craniotomy and dural laminectomy, the SSS of each subject was carefully collected and transversely cut with a razor blade in 0.5–0.7 cm-thick sections, which were immersed in a 4% paraformaldehyde solution in 0.1 M phosphate buffered saline (PBS, pH 7.4) for 18–24 h. Subsequently, the material was washed overnight in cold buffer, dehydrated in a series solutions with increasing ethanol content, kept for 48 h in methylbenzoate, and finally, embedded in paraffin. For the embedding process, samples were oriented to obtain transverse sections of the sinus (7 μm in thickness), which were cut by a Minot type microtome. Sequences of seriated sections were cut and mounted on numbered slides to follow the entire length of long parietal nerve structures. Sections were mounted on poly-L-lysine-coated slides and stained using either the avidin-biotin immunohistochemical technique or the double-labeling immunofluorescence method described by Wessendorf and Elde (1985). Before staining, all sections were subjected to an antigen unmasking procedure, performed by immersion in a citrate buffer (pH 6.0) and double permanence (5 min each) in a microwave oven at 750 W as described elsewhere (Liguori et al., 2017). Other sections were submitted to a protein extraction method utilizing a specific kit in order to perform a Western blotting (WB) analysis of the polypeptide residue.

2.3. Immunohistochemistry

The immunohistochemical procedure was performed as previously described (De Luca et al., 2014; Liguori et al., 2014). After the dewaxing and unmasking steps, the sections were washed and quickly submerged in 3% hydrogen peroxide solution for 30 min at room temperature to inhibit endogenous peroxydases. Afterward, they were washed three times in PBS and blocked with normal serum for 30 min. Sections were incubated with primary antibodies overnight at 6 °C. The following day, they were incubated with the secondary antibody for 30 min at room temperature. The site of the immunological reaction was visualized with an avidin-biotin complex applied for 30 min at room temperature, followed by staining with 3.3-diaminobenzidine. Finally, sections were dehydrated, clarified, and mounted for visualization.

2.4. Double-labeling immunofluorescence

The unmasking procedure was performed as described above. Normal sera from the two donor species of the secondary antibodies were applied in a unique mixture. The two primary antibodies were raised in different donor species and applied simultaneously on each section. Additionally, the two secondary antibodies were applied simultaneously and were conjugated to the brief wavelength fluorochrome fluorescein isothiocyanate (FITC) or to the long wavelength fluorochrome tetramethylrhodamine isothiocyanate (TRITC), respectively. Primary and secondary antibodies were incubated on sections overnight at 6 °C and for 1 h at room temperature, respectively. All sections were kept in a dark and humid environment to prevent photobleaching and evaporation. Stained sections were mounted with glycerol/PBS (1:2) and visualized immediately. FITC was visualized with a 450–490 nm bandpass excitation- and a 525/20 nm bandpass barrier-filter, and TRITC was visualized with a 546/19 nm bandpass excitation- and a 580 nm longpass barrier-filter. These filter sets eliminate any red emission during blue excitation and green-yellow emission during green excitation.

For the immunohistochemistry (IHC), all washes were done with PBS, while those for the immunofluorescence method were done with PBS containing 0.3% Triton X-100. Normal sera were used prior to the primary antibody step as a blocking agent. These sera were obtained by the donor species of the secondary antibody. Both normal and fluorescent light preparations were observed using a Nikon Eclipse E 600 microscope equipped with a reflection system for fluorescence and photographed by a Coolpix 8400 Digital Camera. A 10× ocular lens was used in combination with the following objective lenses: 20× [dry; numerical aperture (NA) 0.50], 40× (dry; NA 0.75), 60× (dry; NA

0.85), and 100 × (oil immersion; NA 1.30). Some sections were stained by EE to obtain general view of the vessel walls and of the structures contained around and within them.

Negative controls for primary antibody specificity were obtained by pre-absorbing the primary antibody with an excess (100 μg/ml) of the relative antigen for use at the primary antibody step. The control for secondary antibody specificity was normal serum. All controls produced negative results. To avoid cross-reaction between tissue antigens and/or immunofluorescence reagents, the following tests were employed as controls: 1) affinity of the primary antibodies to heterologous antigens; 2) affinity of the secondary antibodies to the heterologous primary antibodies; 3) affinity of the primary antibodies to each other; 4) affinity of the secondary antibodies to each other; 5) affinity of the primary antibodies for the heterologous secondary antibodies; 6) emission of green and red light by one of the two fluorochromes. The protocols for these controls are described in detail by Wessendorf and Elde (1985).

2.5. Image processing

The light and fluorescent images were photographed and analyzed on a personal computer. Some of them were adjusted for contrast, brightness, and uniformity of illumination. Using Adobe Photoshop CS5, images for each filter were superimposed, and then the scale bars were applied before preparing the final figure layout.

2.6. Protein extraction and Western blotting analysis

The proteins contained in sections from paraffin-embedded SSS samples were extracted using a Qproteome Formalin Fixed Paraffin-embedded (FFPE) Tissue Kit (Qiagen, Hilden, Germany) which provides optimized conditions for non-degraded total protein extraction from FFPE tissues (Becker et al., 2007). Briefly, 12 tissue sections (15 μm thick) were deparaffinized in xylene, rehydrated in graded alcohol series and mixed with 100 μl of extraction buffer EXB supplemented with β-mercaptoethanol and 1 × Protease and Phosphatase Inhibitor Cocktail (Sigma). The samples were cooled on ice for 5 min, and were successively incubated at 100 °C for 20 min and at 80 °C for 2 h on a heating block, under continuous shaking. Total proteins were recovered after centrifugation at 1400 × *g* at 4 °C for 15 min and their concentration was determined with the Bradford protein assay using Bovine Serum Albumin (BSA) as standard.

For WB analysis 1 × Laemmli buffer and β-mercaptoethanol were added to 38 μg and 22 μg of proteins obtained from two subjects defined as subject 1 (S1) and 2 (S2), respectively. The proteins were denatured by heating at 95 °C for 5 min and separated on 12% SDS-PAGE Polyacrylamide gel in Running Buffer (1 × Tris-Glycine, 1% SDS), for 1–3 h at 100 V. Then the proteins were transferred onto 0.45-m-pore size Immobilon-P membranes (Millipore, Bedford, MA) by using the "transfer sandwich" procedure performed in transfer buffer (1 × Tris-Glycine, 20% Methanol), at 4 °C for 1 h at 100 V. Membranes were incubated with the blocking buffer (5% nonfat dried milk powder in 1 × TBS, 0.05% Tween 20) for 1 h under continuous shaking at room temperature. Finally, the proteins were incubated in blocking buffer diluted primary antibodies over night at 4 °C and then in blocking buffer diluted secondary antibodies for 1 h at room temperature. The proteins were visualized using Advansta ECL and X-ray analysis. This latter was performed using a CAWOMAT 2000 IR machine. All neurosubstances investigated by IHC and IF have been searched by WB analysis.

2.7. Antibodies

The primary antibodies used in this study were monoclonal or polyclonal peptides whose characteristics were carefully checked for specificity before acquiring them commercially. They are listed below.

Protein gene product (PGP) 9.5 was used as a marker for nerve structures. PGP 9.5 was detected with the same antibody used here at the expected size of 27 kDa in rat brain cell lysates examined by WB analysis. The antibody also detected PGP 9.5 in neuronal cell bodies and axons in the CNS and periphery, nerve fibers in peripheral tissues, neuroendocrine cells in the pituitary gland, thyroid gland, and pancreas, and tumors of the diffuse neuroendocrine system. The nerve structures stained here were morphologically similar to those described by others using a different anti-PGP 9.5 antibody in other organs (Xue et al., 2000).

The guinea pig anti-α- CGRP antibody demonstrated high specificity for the α and β variants of human CGRP [100% binding specificity for human α-CGRP, α-CGRP(8–37), and β-CGRP] as shown using the radioimmunoassay (RIA) application. This antibody also exhibited binding specificity for similar antigens (displayed as binding specificity percentages): chicken CGRP (30%), rat α-CGRP (25%), human amylin (8%), rat amylin (0%), human calcitonin (0%), and salmon calcitonin (0%). A second anti-α-CGRP antibody (6006 T 4032), which was produced by the same manufacturer, displayed similar staining patterns and specificities as those described above (Lennerz et al., 2008).

The goat polyclonal anti-CGRP antibody detected CGRP 1 (α-CGRP) and CGRP 2 (β-CGRP) of mouse, rat and human origin by WB, IF, and solid phase ELISA. This antibody is also recommended for the detection of the two molecular variants in equine, canine, bovine, porcine and avian species.

The anti-SP antibody detected SP of rat, mouse, and human origin by WB, immunofluorescence (IF), IHC (formaldehyde fixation/paraffin embedding), and solid-phase ELISA techniques. SP from equine, canine, bovine, porcine, and avian species was detected as well. The antibody specifically stained human pancreatic Langherans islet cells by means of indirect IHC.

The anti-neuronal nitric oxide synthase (nNOS) antibody detected human nNOS by WB, immunoprecipitation, IHC (including paraffin-embedded material), IF, and solid-phase ELISA. The antibody revealed band at 155 kDa in lysates from in vitro cultured rhabdomyosarcoma cells (A673) examined by WB analysis and stained human cerebellar Purkinje cells using the immunoperoxidase technique applied to sections from formalin-fixed/paraffin-embedded material.

The anti-VIP antibody showed high reactivity for VIP and, to a lesser extent, for pituitary adenylate cyclase-activating polypeptide (PACAP) of mouse, rat, and human origin as shown in WB, IF, and solid-phase ELISA techniques. The antibody was used for VIP detection in horse, dog, cattle, pig, and avian tissues as well. This antibody detected a 20 kDa polypeptide in lysates from neuroblastoma cells (SK-N-SH) and mouse brain extracts and specifically stained the cytoplasm of the same cells when tested by indirect IF.

The enzyme tyrosine hydroxylase (TH) is considered a molecular marker for sympathetic nerve structures. The monoclonal antibody that we used here detected TH of mouse, rat, and human origin by WB, immunoprecipitation, IF, and solid-phase ELISA. The antibody detected a 60 kDa protein by WB analysis in PC-12 whole cell lysates, consistent with the theoretical molecular weight of TH: 56 kDa.

Finally, the anti-NPY antibody specifically detected NPY of mouse, rat, and human origin by WB, IF, and solid-phase ELISA. This antibody also bound NPY from equine, bovine, porcine, and avian species.

All the antibodies described above have been used to perform IHC, IF and WB analysis with the exception of those directed against CGRP of which, the first (second in the list) was utilized for IHC and IF, and the second (third in the list) was used for WB. The peculiarities of all primary antibodies are described in Table 1.

The secondary antibodies used in this work were also commercially acquired and are immunoglobulins derived from different animal species conjugated to biotin, fluorochromes FITC or TRITC and horseradish peroxidase. The antibodies used for IHC and IF are listed in Tables 2 and 3, respectively, while those used for WB are listed in Table 4.

Table 1
List of the primary antibodies used.

Name	Host	Antigen characteristics	Source	Dilution
Protein gene product 9.5	Rabbit (P)	Protein gene product 9.5 from human brain	AbD Serotec Zeppelinstr,Puchheim, Germany; 7863–0504	1:200
α-Calcitonin gene related peptide	Guinea Pig (P)	α-Calcitonin gene related peptide synthetic peptide	Peninsula Laboratories, San Carlos, CA, USA; T-5027	1:250
CGRP (N-20)	Goat (P)	Peptide mapping near the N-terminus of CGRP of human origin	Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-8856	1:200
Substance P (N-18)	Goat (P)	Synthetic peptide corresponding to the structure of substance P of human origin	Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-9758	1:300
Tyrosine hydroxylase (A-1)	Mouse (M)	Epitope mapping between amino acids 500–526 at the C-terminus of tyrosine hydroxylase of human origin	Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-374,047	1:200
Neuropeptide Y (E-17)	Goat (P)	Peptide mapping within an internal region of the molecule of human origin	Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-14,727	1:200
Vasoactive intestinal peptide (M-19)	Goat (P)	Peptide mapping at the C-terminus of the molecule of mouse origin	Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-7841	1:200
Nitric oxide synthase 1 (H-7)	Mouse (M)	Epitope mapping between amino acids 2–300 of the molecule of human origin	Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-55,521	1:200

P: polyclonal.
M: monoclonal.

Table 2
List of secondary antibodies used for Immunohistochemistry.

Secondary antibodies	Conjugated To	Source	Dilution
Goat anti-rabbit	Biotin	Vector Laboratories, Burlingame, CA; BA-1000	1:200
Goat anti-guinea pig	Biotin	Vector Laboratories, Burlingame, CA; BA-7000	1:200
Rabbit anti-goat	Biotin	Vector Laboratories, Burlingame, CA; BA-5000	1:200
Goat anti-mouse	Biotin	Vector Laboratories, Burlingame, CA; BA-9200	1:200

3. Results

3.1. Immunohistochemical and immunofluorescent findings

Using the antibody directed against the neuronal marker PGP 9.5, we visualized the nerve structures contained in the walls of the SSS through a series of coronal sections. The sinusal wall can be challenging to define in a morphological study of the cerebral sinuses because of the lack of the wall musculature. However, data produced through the experimental application of retrograde neuronal tracers to the dura encircling the SSS lumen of the rat allowed Liu et al. (2004) to mark the medullary areas of sensitive fiber reception that were functionally related to the sinus. When the neuronal tracer application was enlarged to a dura more distant from the vessel, the field of reception was extended. Therefore, we defined the nerve structures contained within the range of 1000 μm from the vessel endothelium as “parietal.”

The parietal nerve fibers extended primarily parallel to the longitudinal axis of SSS and were frequently grouped in large bundles whose components appeared as small and round structures in coronal sections of the vessel. These bundles lacked a connective envelope and their fibers ran discretely, but in close proximity, to each other (Fig. 1a, d, f). Small clusters of fibers wrapped by a thin connective envelope were observed rarely in parietal structures (Fig. 1b), and became more frequent as more they were distant from the SSS lumen in the supratentorial dura. Single fibers were seen around small blood vessels tracking along the sinusal wall (Fig. 1c). Sometimes such vessels were supposed to be the final portion of venules confluent into the SSS because showed large dimension and close proximity to sinusal lumen (see beyond the Fig. 2f). Occasionally, fascicles of fibers, likely originating from a longitudinal bundle, seemed to change direction and encircle the sinusal lumen. This pattern gave rise to a small group of subendothelial nerve fibers obliquely or longitudinally cut in coronal sections of the vessel (Fig. 1e, g). The fibers of a subendothelial fascicle traversed at a short distance from each other, and the most superficial of them seemed to be close in contact with the endothelium.

Nerve fibers containing the neurotransmitters CGRP, SP, nNOS, VIP, TH, and NPY were observed all along the length of SSS, although their abundance varied widely over the field of view. For each neurotransmitter, areas of high density, low density, and absence of signal were observable. The localization of the different fiber types was fairly uniform, with some exceptions. CGRP- (Fig. 2a–c) and TH- (Fig. 3a–c) containing nerve fibers appeared to be more numerous than the other types. The two neurotransmitters were present both in parietal (Fig. 2a, c and Fig. 3a, c, respectively) and subendothelial (Fig. 2b and Fig. 3b, respectively) nerve fibers. The parietal fibers were mainly organized in large bundles traversing parallel to the SSS lumen. This pattern was followed by long series of consecutive sections. Areas of the SSS walls that completely lacked of fibers containing these two neurotransmitters were rare. SP- (Fig. 2e–g), nNOS- (Fig. 2i–k) and NPY- (Fig.3e–g) containing nerve fibers showed similar patterns, but were less abundant. SP-containing fibers were the least abundant of all the tested types, and were often localized around the opening of bridging veins

Table 3
List of secondary antibodies used for immunofluorescence.

Secondary antibodies	Conjugated to	Source	Dilution
Goat anti-rabbit	FITC	Jackson ImmunoResearch Laboratories, Baltimore Pike, West Grove, PA; 111-095-144	1:100
Goat anti-guinea pig	TRITC	Jackson ImmunoResearch Laboratories, Baltimore Pike, West Grove, PA; 106-025-003	1:100
Donkey anti-goat	TRITC	Jackson ImmunoResearch Laboratories, Baltimore Pike, West Grove, PA; 705-025-003	1:100
Donkey anti-mouse	TRITC	Jackson ImmunoResearch Laboratories, Baltimore Pike, West Grove, PA; 715-025-150	1:100
Donkey anti-guinea pig	FITC	Jackson ImmunoResearch Laboratories, Baltimore Pike, West Grove, PA; 706-095-148	1:100
Donkey anti-goat	FITC	Jackson ImmunoResearch Laboratories, Baltimore Pike, West Grove, PA; 705-095-003	1:100

Table 4
List of secondary antibodies used for Western blotting.

Secondary antibodies	Conjugated to	Source	Dilution
Goat anti-mouse IgG_1	HRP	Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-2060	1:5000
Rabbit anti-goat IgG (H + L)	HRP	Bio-Rad, Laboratories, Milano, Italy; 172-1034	1:1000
Goat anti-rabbit IgG (H + L)	HRP	Biorad Laboratories, Milano, Italy 170-6515	1:1000

into the SSS lumen (Fig. 2f). As with the other types, VIP-containing nerve fibers (Fig. 2m–o) were frequently seen extending longitudinally in the sinusal wall (Fig. 2m), but were not present in the subendothelial connective tissue. Moreover, these fibers often encircled blood vessels contained within the supratentorial dura, distant from the SSS lumen (Fig. 2n, o). The images of the negative controls contained in Fig. 1 (h), Fig. 2 (d), (h), (l), (p), and Fig. 3 (d), (h) were obtained as described above.

Furthermore, we tested the co-localization of the neurotransmitters within the nerve structures of the sinusal wall. The following pairs of

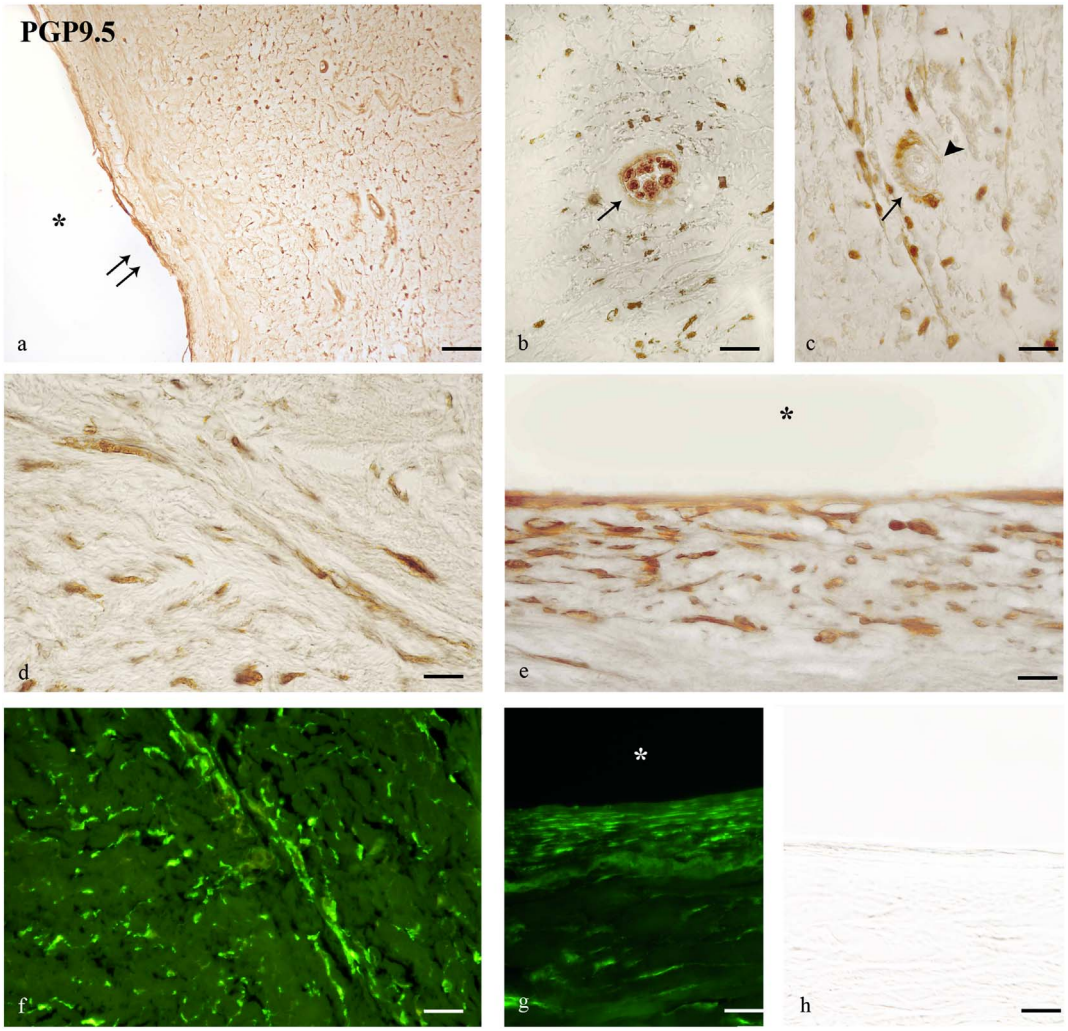

Fig. 1. PGP 9.5-immunoreactive nerve structures contained within the SSS wall. (a) Transverse section of a large nerve fiber bundle extending parallel to the longitudinal axis of the vessel in the SSS wall. The nerve fibers appear as punctiform structure at a low magnification. On the left, the sinus endothelium (double arrow) and lumen (asterisk) are visible. (b) Small bundles of parietal fibers (arrow) encircled by a connective envelope can be seen in proximity of the sinusal lumen. (c) Perivascular nerve fibers (arrow) adjacent to a small blood vessel (arrowhead) extending parallel to the SSS major axis and in proximity to its lumen. (d) and (f) Parietal bundles of nerve fibers transversely or obliquely cut. Each bundle comprises many fibers not enveloped by a connective sheet. The fibers are distinct from each other in close proximity. (e) and (g) Subendothelial fascicles of nerve fibers encircle the SSS lumen (asterisks) in a spiral fashion, appearing obliquely cut in a transverse section of the sinus. (h) negative control for anti-PGP 9.5 immunoreaction. Images were produced using avidin-biotin immunohistochemical and double-labeling immunofluorescence techniques. Scale bars: a = 100 μm; b–d = 20 μm; e–f = 40 μm; g, h = 30 μm.

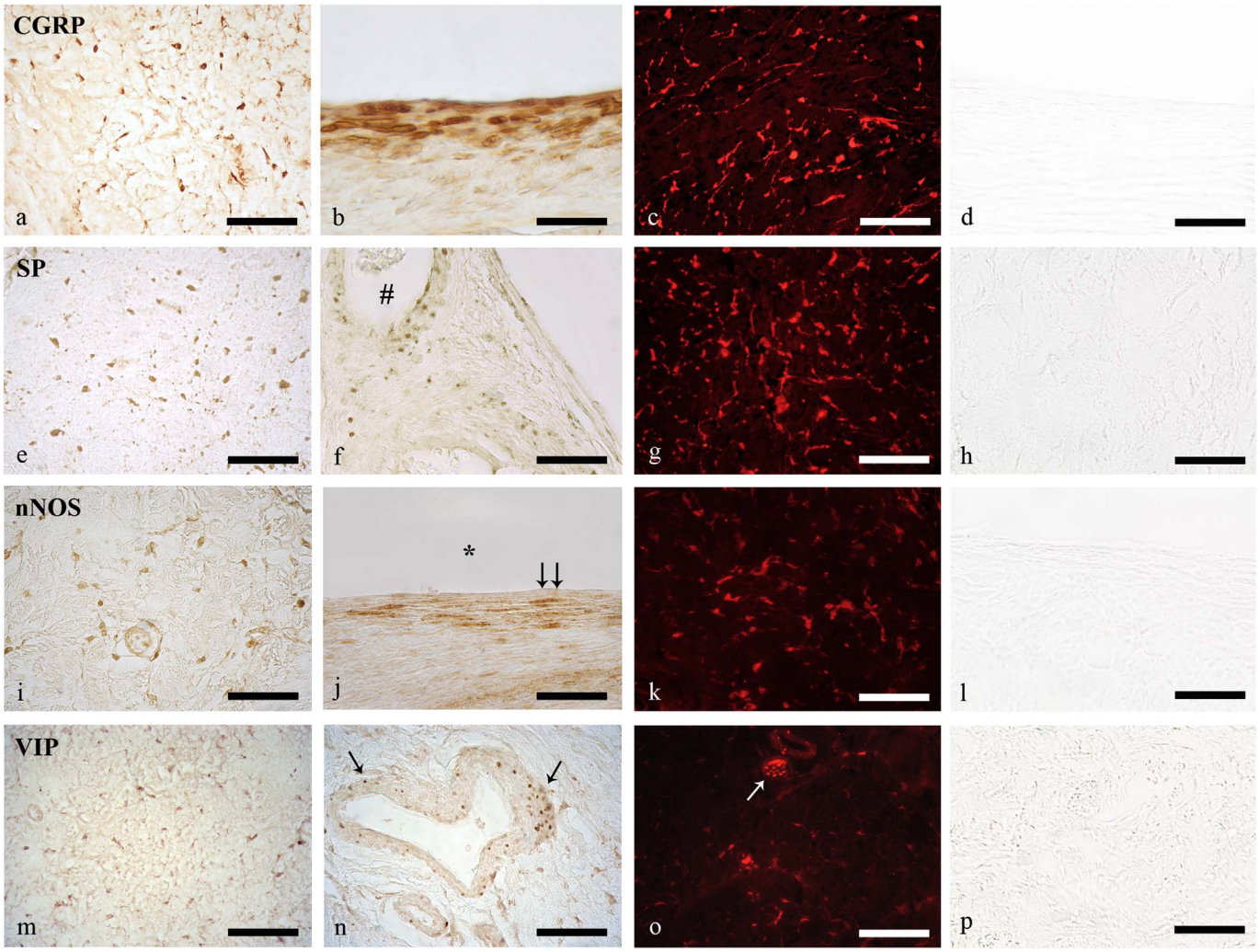

Fig. 2. Presence of neurotransmitters in parietal nerve fibers of the SSS. (a), (b), and (c) CGRP- and (e), (f), and (g) SP-containing nerve fibers were observed frequently clustered in bundles of parietal (a, c, e, and g) or subendothelial (b) structures. SP-containing nerve fibers (f) were observed in proximity of the openings of bridging veins (hash) into the SSS lumen (asterisk). (i) and (k) nNOS- and (m), (n), and (o) VIP-containing nerve fibers were observed tracking longitudinally along the SSS walls. Small fascicles of nNOS-expressing fibers (j) also encircled the sinusal lumen (asterisk) below the endothelium (double arrow), while isolated (n) or clustered (o) VIP-expression fibers (arrows) were observed in close contact with the blood vessels in the perisinusal dura. (d), (h), (l) and (p) negative controls for anti-CGRP, -SP, -nNOs, -VIP immunoreactions, respectively. Images were produced using avidin-biotin immunohistochemical and double-labeling immunofluorescence techniques. Scale bars: a, c, e, f, h = 80 μm; b, d = 50 μm; g = 100 μm; i, k = 40 μm; j, l = 25 μm; m–p = 60 μm.

neurotransmitters were found to co-localize in parietal fibers: CGRP/SP (Fig. 4a-c), CGRP/nNOS (Fig. 4d–f), CGRP/VIP (Fig. 4g–i), and NPY/TH (Fig. 4j–l). The co-localization between the two neurotransmitters of each pair was always high, as shown in the merged images of Fig. 4 (right column).

3.2. Neuropeptide detection by Western blotting analysis

In order to confirm the presence of the selected neuropeptides in the SSS, WB analysis was performed on proteins extracted by paraffin embedded tissues. Protein expression was confirmed for all the analyzed substances which were present in samples from both the subjects examined. The molecular weights assessed for the single neuropeptides are consistent with those currently attributed to the respective molecules contained in nerve tissues of mammals. As expected the protein band obtained for PGP 9.5, already reported as a marker of nerve structures, is the most prominent and can be considered as a warranty of the presence of such structures in the samples examined. The double band obtained for CGRP are consistent with the theoretical molecular weights of the two variants of the substance. The results obtained for the WB analysis performed in this study are shown in Fig. 5.

4. Discussion

4.1. CGRP/SP-containing nerve fibers

Although the morphological studies have shown the presence of CGRP- and SP-containing nerve fibers in the SSS wall (Furness et al., 1982; Keller and Marfurt, 1991), our results provide the first evidence of co-localization of these two neuropeptides in the same perisinusal fibers of human SSS. The origin of these fibers is probably represented by the CGRP- and SP-producing nerve cell bodies contained in the trigeminal ganglion, which are known to project their peripheral axons toward cerebral arteries (Hanko et al., 1986) and various orofacial targets (Gibbins et al., 1985; Lee et al., 1985). CGRP and SP encourage vasodilation, the first being the most potent known vasodilating agent (Brain et al., 1985). The CGRP/SP-containing fibers innervating the SSS walls should act as nociceptor neurons. Electrical or mechanical stimulation of the SSS or medial meningeal artery causes pain similar to that perceived during headache in humans and this sensation must be referred to the first division of the trigeminal nerve (Ray and Wolff, 1940). The previously mentioned studies in cat (Goadsby et al., 1997) and monkey (Goadsby and Hoskin, 1997) SSS models, as well as tracing the medullary target of the sinus-sensitive nerve fibers in the rat (Liu

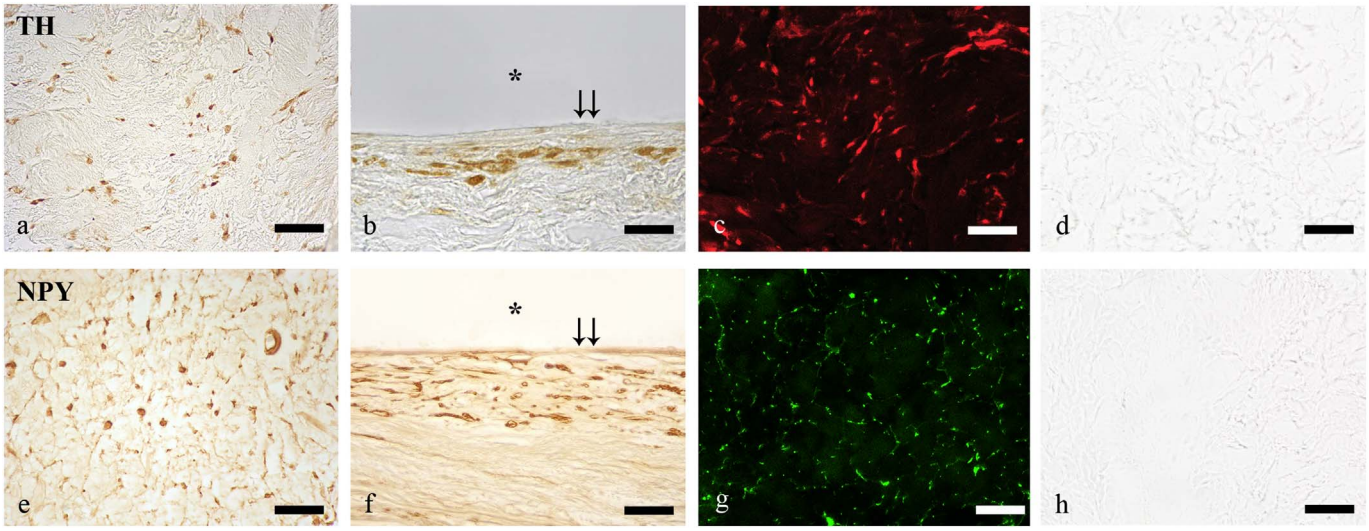

Fig. 3. Presence of neurotransmitters in parietal nerve fibers of the SSS. (a), (b), and (c) TH- and (e), (f), and (g) NPY-containing nerve fibers were similar to those described for the other four types both in the parietal (a, c, e, and g) and subendothelial (b and f) connective tissue of the SSS. TH-containing fibers were spread diffusely along the sinusal walls. (d) and (h) negative controls for anti-TH and -NPY immunoreactions, respectively. In (b) and (f) the asterisks indicate the SSS lumen and the double arrows the SSS endothelium. Images were produced using avidin-biotin immunohistochemical and double-labeling immunofluorescence techniques. Scale bars: a, c, d, f = 100 μm; b = 50 μm; e, h = 80 μm; g = 40 μm.

et al., 2004), clearly demonstrate the nociceptive function of a portion of sinus wall innervation. Nociceptive nerve fibers of the cat SSS, classified morpho-pharmacologically as Aδ, Aβ, or C types, respond to electrical, mechanical, and chemical stimuli and project to the ipsilateral trigeminal spinal nucleus (Davis and Dostrovsky, 1988).

The CGRP/SP-containing neurons of the SSS walls could also function as mechano-receptors. The muscular component of the walls of the cerebral sinuses is minimal, and blood influx coming from the bridging veins could easily cause sinus dilatation and rise of blood pressure. Muscle sphincters encircling the vein openings in the sinus have been thought to regulate blood influx and pressure (Vignes et al., 2007). Interestingly, Ruffini-like nerve terminals have been detected by electron microscopy around the vein openings (Andres et al., 1987) and were thought to function as stretch receptors to monitor blood pressure. Notably, several types of stimuli excite the meningeal sensory nerve fibers including, mechanical (e.g., punctuate probing, stroking, traction), thermal (i.e. cold or warm stimuli), chemical (e.g. hypertonic saline), algesic (caused by potassium chloride), low/high osmolarity buffer solutions, and a mixture of inflammatory substances (bradykinin, prostaglandin E2, serotonin, and histamine) (Messlinger et al., 2006). If these nerves function as mechano-receptors, they may help to modulate responses to these stresses and regulate blood influx and pressure.

Some capsaicin-sensitive sensory neurons that project toward the wall of blood vessels can drive an efferent impulse (Maggi and Meli, 1988). This phenomenon is analogous to the “axon reflex mechanism” of cutaneous tissue and consists of antidromic activation of collateral branches of sensory neurons following the peripheral stimulation of their axon. Morpho-functional studies on the trigeminal vascular complex have highlighted that the CGRP/SP-containing neurons of the complex are examples of capsaicin-sensitive sensory neurons (Escott et al., 1995). Electrical stimulation of the trigeminal ganglion caused CGRP and/or SP release from the relative sensory terminals (Goadsby et al., 1988) and enhancement of extracerebral blood flow (Goadsby and Duckworth, 1987). Moreover, electrical (Zagami et al., 1990) or capsaicin (Geppetti et al., 1990) stimulation of the SSS dural wall caused CGRP release from the surrounding nerve fibers. Based on these findings, the CGRP/SP-secreting neurons of the trigeminal vascular complex are thought to play a pivotal role in the algic phase of migraine pathogenesis. CGRP/SP-secreting neurons may cause a diffuse vasodilatation through neurotransmitter release and drive the stimuli that cause the pain (Edvinsson and Linde, 2010; Keller and Marfurt, 1991; Tajti et al., 2015). The experimental dilatation of a superficial vessel of the brain (artery or vein) is a well-known source of pain, in this case attributable to the ophthalmic division of the trigeminal nerve (Martins et al., 1993). The intensity of pain may be a direct function of the amount and diffusion of vasodilatation (i.e. vasodilator liberation) because CGRP levels are elevated during high levels of pain, including during the headache phase of migraines (Gallai et al., 1995), cluster headaches (Fanciullacci et al., 1995), chronic paroxysmal hemicranias (Goadsby and Edvinsson, 1996), and throbbing exacerbations of chronic tension-type headaches (Ashina et al., 2000). In contrast, CGRP levels are low during less-developed migraine (Tvedskov et al., 2005). The hypothesis is also supported by the clinical finding that, during nitroglycerin-induced migraine attacks, the intensity of pain decreases in parallel with blood CGRP levels after sumatriptan administration (Juhasz et al., 2005). In cat models, CGRP release upon SSS stimulation is not inhibited by administration of CP122288 (Knight et al., 1999a) or 4991w93 (Knight et al., 1999b), which are structurally modified analogues of sumatriptan and zolmitriptan respectively, ineffective in migraine sufferers. The physiological level of CGRP grew during a migraine attack and caused a typical algic phase in migraneurs alone (Kaiser and Russo, 2013). These finding further support the importance of CGRP as mediator of vasodilatation in the headache phase of migraines. On the contrary, the administration of the specific CGRP antagonist, BIBN 4096 BS, is a valid treatment for acute migraine headaches (Petersen et al., 2005).

Recently, CGRP release within the trigeminal ganglion was shown to stimulate the secretion of cytokines from gangliar satellite glial cells, which synthesize both the subunits of the CGRP receptor, the calcitonin receptor-like receptor (CLR), and receptor activity-modifying protein (RAMP) 1 (Eftekhari et al., 2010). Hypothetically, these cytokines could modulate the activity of the CGRP-containing neurons, thus suggesting an intraganglionic crosstalk between the two cellular populations (Vecsey et al., 2015). CGRP-containing nerve fibers and CLR/RAMP 1 immunoreactivity have been found around nerve cell bodies and within satellite glial cells of the sphenopalatine ganglion, respectively. This finding led to the hypothesis that CGRP release in the trigeminal vascular complex can influence not only the sensitive component of the complex, but also its parasympathetic counterpart (Csati et al., 2012).

In trigeminal nerve fibers, CGRP and SP exhibit close relationships at subcellular level by co-localizing in the same secretory vesicles. In spite of this, SSS stimulation did not cause SP plasma level elevation (Zagami et al., 1990), and blockade of the neurotransmitter receptor was ineffective to treat acute migraine headaches (May and Goadsby,

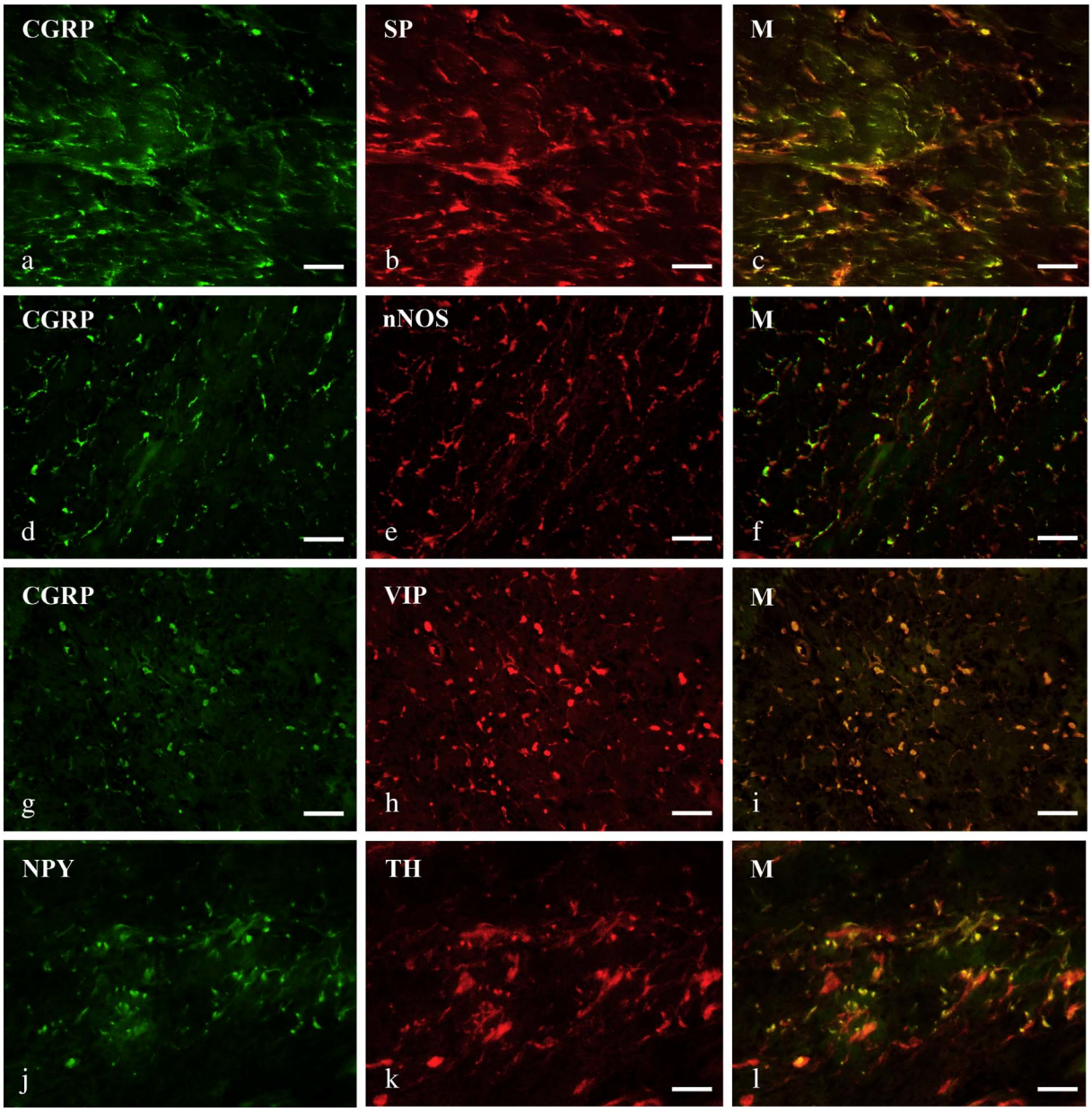

Fig. 4. Co-localization of neurotransmitters in parietal nerve fibers of the SSS wall. The first two images in each row were taken of the same microscopic field at a constant focal length using FITC (yellow/green, left) and TRITC (red, center) filter columns. The third image of each line (right) is the merged image (M), showing areas of yellow-orange fluorescence to represent sites of co-localization. The observed neurotransmitters that co-localized include: (a) CGRP and (b) SP, (c) displays the merged image; (d) CGRP and (e) nNOS, (f) displays the merged image; (g) CGRP and (h) VIP, (i) displays the merged image (j) NPY and (k) TH, (l) displays the merged image. All images are representative examples of nerve fiber bundles that simultaneously contain two neurotransmitters. Areas of co-localization for each pair of neuropeptides were widely diffuse. Double-labeling immunofluorescence method. Bars: a, b, c, g, h, i, j, k, l = 5 μm; d, e, f = 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2001). On the other hand, SP causes plasma protein extravasation and vasodilatation during trigemino-vascular complex activation (Moskowitz, 1993), and SP receptor antagonists inhibited the response of the second order sensory neurons of the trigeminal nucleus caudalis after trigeminal ganglion electrical stimulation (May and Goadsby, 2001). Such conflicting results remain unexplained, and the role of the neuropeptide in human vascular nociception is still unclear (Holthusen et al., 1997; Tajti et al., 2015).

In this study, the neuropeptide CGRP was found to co-localize with the enzyme NOS in nerve fibers of the SSS walls. The origin of these fibers may be the trigeminal ganglion cell bodies, which contain both CGRP and NOS (Edvinsson et al., 1998). In vitro studies showed that NO increased the production of CGRP from trigeminal ganglion neurons (Eberhardt et al., 2009), while a vasodilatory effect of the substance during the algic phase of migraine was doubtful (Edvinsson et al., 1993). Nothing is known about a potential synergistic action of NO and CGRP peripherally released by the relative nerve fibers.

4.2. *Parasympathetic nerve fibers*

The sphenopalatine and otic ganglia were shown to be the source of the parasympathetic nerve fibers that project toward the superficial vascular bed of the brain (Edvinsson et al., 2001). These ganglia contain cell bodies that produce acetylcholine (Ach), the well-known mediator of the parasympathetic nervous impulse, and the neurotransmitters NO, VIP, and PACAP (Uddman et al., 1999). ACh causes vasoconstriction of endothelium-deprived cerebral vessels in several species (Lee, 1980), but a wide array of experimental data, also obtained at ultrastructural level, led to the hypothesis that, in the cerebrovascular complex, Ach is probably co-released with NO, VIP, or PACAP which act as the last link

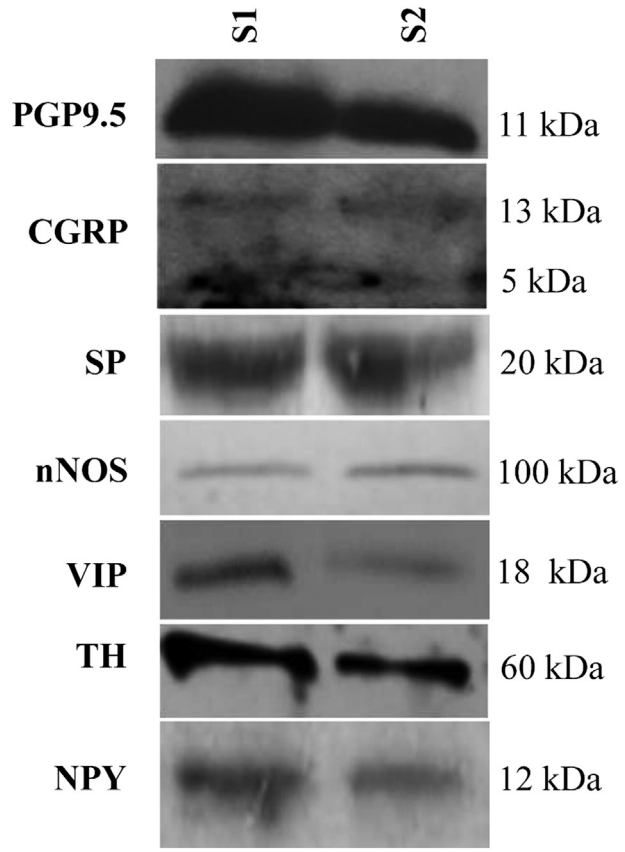

Fig. 5. Western blotting analysis. Protein expression in samples from the SSS of two human subjects (S1 and S2). The blots of the two lanes (S1 and S2) report the presence of all neuropeptides studied in the SSS walls. The peptides are reported on the left of the figure and the sizes on the right.

in nerve impulse transmission. Thus, stimulation of the parasympathetic nerve fibers of the brain surface vessels led to different vasodilatation kinetics, based on the characteristics of these three vasodilators (Jansen-Olesen et al., 1994). Alternatively, these neuropeptides may act as post-junctional messengers, released through the cholinergic stimulation of a pre-junctional receptor (Lee, 2000).

The labile molecule NO, produced from NOS, gained importance in the headache pathogenesis when research showed that administration of glyceryl nitrate, an NO donor molecule, caused a migraine attack in human healthy volunteers and migraine sufferers (Olesen et al., 1995). However, the explanation of this finding remains obscure. NO can be liberated, in the trigemino-vascular complex as elsewhere in the body, not only by nitrergic nerve fibers but also from the vascular endothelium and from tissue mast cells. NO causes decrease of intracellular Ca^{+} $^{+}$ levels and vasodilatation (Olesen et al., 1995). Nerve fibers containing nNOS are widespread and diffuse in the cerebral vessel network (Bredt et al., 1990), and nNOS was found to co-localize with VIP and acetylcholinesterase (AChE) in perivascular cerebral nerve fibers of rat and cattle brains. Thus, nNOS is putatively considered parasympathetic in origin (Edvinsson et al., 2001). Although the vasodilatory effect of NO on cerebral blood vessels has been experimentally proven (Olesen et al., 1995), its role in migraine pathogenesis remains debated.

VIP is a neuropeptide known to cause a long lasting, diffuse vasodilatation when systemically administered to the body (Said and Mutt, 1972). Particularly, VIP induces relaxation of the cerebral vessel musculature through an endothelium-independent mechanism (Edvinsson et al., 1981). VIP-containing nerve fibers are present around blood vessels of the brain surface (Edvinsson and Kruse, 2002). This neuropeptide sometimes co-localizes with AChE in perivascular nerve fibers of parasympathetic origin (Gulbenkian et al., 1991). Plasma VIP levels are elevated during migraine headaches in patients who have previously showed parasympathetic type symptoms (Goadsby et al., 1990), and cat SSS stimulation strongly enhanced the quantity of VIP circulating in cranial blood vessels (Zagami et al., 1990).

To our knowledge, CGRP and VIP co-localization in nerve structures of the superficial brain vessels has never been described. The presence of CGRP has been detected immunohistochemically in nerve cell bodies of the sphenopalatine ganglion (Silverman and Kruger, 1989) which contains, as described above, also a good amount of VIP- positive pirenophora (Uddman et al., 1999). On the other hand, the presence of VIP-containing nerve cell bodies has never been described in the trigeminal ganglion (Jansen-Olesen et al., 2014). On this basis, a hypothetical source of CGRP/VIP-containing nerve fibers could be a nerve cell body localized in the sphenopalatine ganglion, which is capable of producing both substances. However, further studies are needed to test this hypothesis and establish the precise role of the CGRP/VIP-containing fibers here described.

4.3. TH/NPY-containing nerve fibers

Keller et al. (1989) described close, but separate, networks of dopamine β hydroxylase (DBH)- and NPY-immunoreactive nerve fibers around the walls of the rat SSS using whole mount preparations of the supratentorial dura mater. In both cases, the immunoreactive fibers most likely originated by sympathetic cell bodies contained in the superior cervical ganglion of each side because their ablation almost completely removed the immunopositivities around the sinus. The common sympathetic origin of the DBH- and NPY-containing nerve fibers and their similarity of distribution around the sinus led to hypothesize that the two nerve networks were effectively the same (Keller et al., 1989). It was, in fact, already known that NPY-containing nerve fibers are diffusely distributed around the walls of cerebral arteries (Edvinsson et al., 1983) and that DBH and NPY co-localize in perivascular nerve fibers at the surface of the brain (Edvinsson et al., 1987). Later, also the inferior cervical ganglia of both sides and the stellate ganglion were recognized as sources of the sympathetic nerve supply to superficial blood vessels of the brain (Edvinsson et al., 2012). Our results concretely demonstrate that dense nerve networks containing the neuropeptides TH and NPY encircle the SSS walls and that the two molecules often co-localize in the same fibers.

Superior cervical ganglion-derived sympathetic nerve fibers innervating the cerebral blood vessels are vasomotor in function (Edvinsson and Kruse, 2002) and the two mediators of this effect, noradrenaline (NA) and NPY, act synergistically on the vascular musculature (Ekblad et al., 1984). However, the actions of these two compounds appear to be different, because the former is prominent under normal body conditions, while the latter is more active during elevated sympathetic activity (Lundberg et al., 1985). The vasomotor action of NPY on the extracerebral vasculature seems to be exerted at level of pial arterioles, which contain the Y1 receptor of the neuropeptide in large amounts just before entering the brain. On the contrary, wider superficial vessels, although enveloped by a network of sympathetic fibers, are devoid of the Y1 receptor or show its presence at a lesser extent. Thus, these major vessels can only internalize the neuropeptide whose target is located elsewhere. More recently, an alternative source of NPY-containing nerve terminals surrounding the pial arterioles was found inside the brain and is putatively represented by local interneurons producing the peptide. Such control of the brain on regulation of its own vasculature is suggestive (Hökfelt et al., 1998).

Currently, the purpose and function of TH/NPY-containing nerve fibers in the SSS wall is not known also because the presence of a vasomotor innervation in the walls of a vessel-like structure almost completely devoid of a muscle layer seems to be ineffective. However, as reported above, actin has been detected around the openings of bridging veins in the sinus, forming a muscle sphincter in which Ruffini-like structures, supposed to be stretch receptors, have been described

(Andres et al., 1987; Vignes et al., 2007). In this study the presence of SP-containing nerve fibers has been often observed around the terminal portion of bridging veins in the sinus and a possible CGRP/SP and TH/NPY innervation of this portion could result in an antagonistic regulation of blood influx in the SSS. If extended to the openings of the major bridging veins in the cerebral sinusal complex, such a study could disclose the regulatory mechanism of blood efflux from the brain and, consequently, of the cerebral blood homeostasis.

In conclusion, this work describes the existence of ramified networks of nerve fibers within the walls of the SSS in the human brain. Several neurotransmitters have been identified in such structures and some of them were found to be co-localized in the same fibers. Although the neurotransmitter combinations recognized can be functionally ascribed to different components of the nervous system, further studies are needed to clarify their function in SSS. Such acquisition could translate in a better comprehension of the role played by the sinus in regulation of the brain-blood homeostasis and in determinism of the migraine and other primary headache pathogenesis.

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Conflicts of interest: none.

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