Neuropeptides xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Neuropeptides

journal homepage: www.elsevier.com/locate/npep

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

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ARTICLE INFO

Keywords:
Human brain
Superior sagittal sinus innervation
Immunohistochemistry
Double-labeling immunofluorescence
Western blotting analysis
Neurotransmitter co-localization

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, electrical and mechanical stimulation of the sinus were shown to enhance metabolic activity, blood flow, and *c*-fos expression of the

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http://dx.doi.org/10.1016/j.npep.2017.04.008
Received 13 October 2016; Received in revised form 15 April 2017; Accepted 22 April 2017

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