Neuroanatomical mapping of spexin and nesfatin-1-expressing neurons in the human brainstem

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ABSTRACT

Neuropeptides are involved in numerous brain activities being able to control a wide spectrum of physiological functions. In recent years, a number of novel pleiotropic regulatory peptides have been discovered in animal brain structures. The purpose of this descriptive neurochemical investigation was to detect the possible expression of the novel multifunctional neuropeptides spexin (SPX) and nesfatin-1 within the human brainstem. Using immunohistochemical and fluorescence techniques, neuroanatomical analysis of the SPX and nesfatin-1 expression and distribution was performed in selected sections of the human midbrain and medulla oblongata. The presence of SPX-positive neurons in the human brainstem was revealed for the first time and previous reports on the expression of nesfatin-1 were additionally confirmed. The research results suggest that SPX and nesfatin-1 are new regulatory neuropeptides of the human brainstem potentially involved in the regulation of key autonomic activities of this brain region.

Key words: spexin; nesfatin-1; brainstem; neuropeptides; neuromodulators

1. Introduction

The human brainstem plays a crucial role in integrating sensory and motor pathways and is necessary for autonomic and regulatory processes that are important for life (Smith et al., 2013). It serves as a conduit between the brain and spinal cord, coordinating a variety of physiological processes such as eye movement, pain, heart rate, sleep, vocalisation, analgesia, visual, auditory, motor, respiratory, and cardiovascular systems (Kandel et al., 2000). Neuropeptides are a diverse group of signaling molecules found in both the central nervous system (CNS) and peripheral nervous system (PNS) (Jakob et al., 2021). They have significant effects on several brainstem activities, including synaptic transmission, gene expression, excitability of cells, neural growth and development, and the structure and function of both neurons and glial cells (Teleanu et al., 2022). Although neuropeptides are the fundamentally important, our understanding of their neurochemical composition and specialised functions within the brainstem is still inadequate. Several multifunctional regulatory neuro-peptides have been identified and characterized, primarily using animal models. Recently, two neuropeptides, Spexin (SPX) (Pałasz et al., 2021) and nesfatin-1, (Zhou et al., 2024) have gained attention for their important roles in several biological activities. As a result, they are considered promising candidates for future investigations in human brainstem.

Spexin (SPX) is a naturally occurring neuro-peptide that has effects on both CNS and PNS (Mirabeau et al., 2007). The *Ch12orf39* gene, situated on chromosome number 12 within human genome, is responsible for coding pre-prospexin (Wan et al., 2010). After undergoing a number of protein synthesis steps, pre-prospexin is transformed into SPX, which is a highly efficient form for cellular physiological activities. The amino acid sequence of the SPX peptide, consisting of fourteen amino acids, has been evolutionarily conserved in both invertebrates and vertebrates (Ma et al., 2018; Lv et al., 2019). SPX-expressing neurons in the rat brain have been identified with the hypothalamic magnocellular nuclei showing the highest expression (Porzionato et al., 2010; Pałasz et al., 2021). The chemical composition of SPX in rat is different from the human form by a single C-terminal amino acid (Porzionato et al., 2010). Although there have been several animal research on SPX biology, its distribution and physiology are so far lacking within the human brain. SPX has been identified as a natural ligand for GalR2/3 galanin receptors and further investigations have validated the gene expression of SPX in the

brain and peripheral tissues of humans, rats, mice and fish (Kim et al., 2014; Ma et al., 2017). Expression in the CNS involves specific areas such as hippocampus, cortex, and the brainstem (Porzionato et al., 2010). The presence of multiple secretion sites indicates that SPX has many physiological activities. For instance, its impact on fish reproduction and appetite has been confirmed. SPX leads to a reduction in secretion of luteinizing hormone and inhibits the appetite (Liu et al., 2013; Wong et al., 2013). Furthermore, it has been observed that elevated levels of insulin, which are known to influence the feeling of fullness, leads to an upregulation of the SPX gene in the brain (Ma et al., 2017). The disadvantage of these reports, which may restrict their applicability to human physiology, is the predominant focus on research conducted on fish. The confirmed roles of SPX in mammals include controlling pain responses, suppression of the growth of adrenal cortex cells, regulating stomach contractions, influencing cardiovascular reactions and, notably, promoting weight loss (Rucinski et al., 2010; Toll et al., 2012; Walewski et al., 2014).

Another neuro-peptide nesfatin-1 molecule is composed of eighty-two amino acid residues and immunohistochemical studies have revealed that its precursor, nucleobindin-2 (NUCB-2), is found in various locations including the brainstem, forebrain, pituitary gland, ventrolateral medulla, hypothalamus, midbrain nuclei, central amygdaloid nucleus, and cerebellum (Ayada et al., 2015, Stengel 2015, Pałasz et al. 2012). The amino acid sequence of nesfatin-1 is consistent across different vertebrate species, and the presence of the *NUCB-2* gene has been verified in both the adipose tissue and brain. Nesfatin-1 primarily acts by supressing food consumption. It has been proven that the NUCB-2 hydrolysis takes place naturally in the brain. This is supported by the detection of nesfatin-1 and the N-terminal fragment of NUCB-2 in the cerebrospinal fluid (Shimizu et al., 2009). The ability of nesfatin-1 across the blood-brain barrier (BBB) has been confirmed (Pan et al., 2007). The varied activities of nesfatin-1 underscores its importance in maintaining overall physiological balance. The recent findings suggest the evidence for nesfatin-1 involvement in other important brain functions such as reproduction, cognition and anxiety- or stress-related responses. (Xiao et al., 2018, Xu et al. 2015, Weiber and Hofmann 2019, Gołyszny et al. 2022). It should be emphasized that, a putative G-coupled nesfatin-1 receptor is so far uncloned (Pałasz et al. 2012).

The specific distribution of SPX and nesfatin-1 inside the human brainstem, as well as their potential interactions, have not been well characterised, despite their known peripheral functions. Therefore, through a current research and experimentation, this investigation demonstrates the presence of SPX and nesfatin-1 in the human brainstem. Using

immunohistochemical and fluorescence techniques, a neuroanatomical, descriptive analysis of the expression of SPX and nesfatin-1 was performed in selected sections of the human midbrain and medulla oblongata. It took into account the distribution of immunopositive cells in the brainstem structures, their morphology and intra-cellular reaction parameters. The presence of SPX receptor in the human brainstem was revealed for the first time and previous reports on the expression of nesfatin-1 were additionally confirmed. The research results suggest that SPX and nesfatin-1 are new regulatory neuro-peptides of the human brainstem potentially involved in the regulation of key autonomic activities of this brain region.

The primary goal of the presented research work in the field of neuroanatomy and brain biochemistry was to demonstrate the possible presence of SPX and nesfatin-1 expressing neurons in selected sections of the human midbrain and medulla oblongata and a descriptive cytoarchitectonic analysis of their distribution in key structures of the human brainstem. The main reason for choosing these two neuropeptides was their analogous distribution profile in the rat brainstem and similar physiological effects (Porzionato et al. 2010, Pałasz et al. 2012, Liu et al. 2013, Stengel 2015). Both SPX and nesfatin-1 are strongly anorexigenic factors, and their involvement in the regulation of key autonomic functions of the body has been recently demonstrated. It is worth emphasizing that the implemented project is the first attempt to detect SPX in the human brainstem and is one of the few studies concerning the mapping of nesfatin-1 expression in this brain region.

2. Materials and methods

Human brainstem specimens with no neuropathological findings (2 males, aged 65-67 years, died due to severe cardiovascular collapse) were obtained from the Conscious Body Donation Program conducted by the Department of Anatomy at the Medical University of Silesia in Katowice. Brains were *post mortem* perfusion-fixed with 4% paraformaldehyde buffered solution (pH 7.2-7.4) and then immersion-fixed over a period of two weeks. The mesencephalon and medulla oblongata were precisely excised from 2 diencephalic slices (n=2) according to the referenced human brain atlases (Mai et al. 2015, Cho 2014). Samples were dehydrated, embedded in paraffin wax and finally sectioned by microtome (Leica Microsystems, Germany) at 10 μm thick serial slices. The brainstem was cut transversely at the height of the nucleus of the oculomotor nerve (CN III) and the middle part of the inferior olivary

nucleus, respectively. Topographic coordinates of the sections were based on the reference neuroanatomical atlas: Paxinos, Furlong & Watson; Human Brainstem, 2020, Academic Press. They were expressed numerically as the distance from the obex anteriorly: mesencephalon (+40.5 mm), medulla oblongata (+7.5 mm). In the case of medulla oblongata, the analysis involved serial sections taken at a distance of approximately 1 mm between the boundary planes determined according to Coulombe et al. (2021). The border points: 30.94 and -31.9 were measured from the plane of the pons-midbrain junction (PMJ) defined by a virtual line connecting the lower edge of the lamina tecti with the foramen cecum of the interpeduncular fossa.. After rehydration and subsequent antigen retrieval with citrate buffer (pH 4.0) solution (Vector Laboratories) at 60 ^{0}C sections were rinsed three times for 5 min in 0.05 M TBS-saline (pH 7.6) and placed in PBS with 0.1% Triton X-100 (Sigma). They were blocked with 10%, goat serum and incubated overnight at 4^{0}C with with rabbit anti-rat spexin polyclonal antibody (1:2000, Phoenix Pharmaceuticals, Burlingame, CA, USA, H-023–81, RRID: AB2923380) or with rabbit polyclonal antibody against rat nesfatin-1 (1:1000, Bioss Antibodies Inc., Woburn, MA, USA, bs-3552R, RRID: AB_10855692). Both antisera were characterized by reactivity with human tissue antigens. Primary antibodies were followed by biotinylated goat anti-rabbit secondary antibody and then an avidin-biotin-horseradish peroxidase complex (Vectastain ABC kit, Vector Labs) and visualised with3,3'-diaminobenzidine (DAB). Alternatively, following incubation with primary antibodies brain sections were kept in darkness with secondary antibodies labeled with Alexa Fluor 488 or 594 (1:200, Abcam) for SPX, nesfatin-1 respectively, and mounted on slides with DAPI-containing medium. All sections were treated with TrueBlack® (Biotium, Hayward, CA, USA) to remove unwanted lipofuscin autofluorescence. Because rodent and human SPX molecules differ in only one amino acid we checked the SPX antibody specificity with an absorption test with human SPX (Phoenix Pharmaceuticals) as previously reported (Gu et al. 2015). The crucial point of this test was a preincubation of the SPX (pure antigen) with the antibody before the essential IHC reaction. Moreover, this antiserum has also been verified using series of human positive controls (stomach and intestine tissues). All sections were mounted on glass slides, dehydrated and coverslipped.

In order to generally assess the cytoarchitectonics of the brainstem and check the examined tissue for possible structural signs of necrosis and tigrolysis, staining with cresyl violet according to Nissl method and with gallocyanin-phloxin according to Lapham method was performed. The assessment of myeloarchitectonics and the normal structure of nerve fibers in order to exclude potential demyelinating changes was performed using Luxol Fast Blue

staining with Li_2CO_3 differentiation according to Klüver-Barrera method and with eriochromocyanin R (solochrome cyanine) with ammonia water differentiation.

All slides were scanned with tissue scanner Hamamatsu NanoZoomer S360 (Hamamatsu Photonics, Japan) or captured with microscope Olympus BX43 fluorescent optic systems and processed using CellSens standard software (Olympus Inc., Japan).

3. Results

3.1. Mesencephalon

3.1.1. SPX and nesfatin-1

SPX and nesfatin-1 were expressed primarily in the neurons of the oculomotor nucleus (CN III) and a few spindle-shaped or multipolar cells of the red nucleus and an unspecified population of neurons in the substantia nigra (Fig. 1, 3 , 4 and 5.). Neuromelanin granules filling the cytoplasm of substantia nigra neurons significantly overlapped the histochemical reaction, masking the DAB reaction, which made the assessment of SPX expression significantly difficult (Fig. 2.). Small number of loosely scattered SPX and nesfatin-1 immunopositive polygonal or elongated perikarya were found in the periaqueductal gray (Fig. 2.). In the structure of the central part of the nucleus of the oculomotor nerve, clusters of immunopositive cells were revealed, mainly round, less often oval or polygonal, with medium reaction intensity. They were mainly oval in the ventral part, while in the central nucleus of Perlia they were long and spindle-shaped (Fig. 4 and 5.). DAB cells of the reactive Edinger-Westphal nucleus were round or tear-shaped. The distribution of the granular reaction with varying intensity was usually very uneven and its concentrations were usually located at one pole of the cell, the opposite part of the neuroplasm and neuron processes were immunonegative. In general, the expression profiles of SPX and nesfatin-1 in the oculomotor nuclei were almost identical (Fig. 4 and 5.). It seems that many cells may co-express SPX and nesfatin-1, but only the use of double fluorescent staining can verify this hypothesis.

3.2. Medullla oblongata

3.2.1. SPX

SPX expression was characteristic of neurons building the nuclei of the following cranial nerves: hypoglossal (CN XII), vagus (CN X), trigeminal (CN V, nucleus of the solitary tract, nucleus ambiguus, nucleus cuneatus, olivary nuclei, raphe nuclei and nuclei of pre-Bötzinger i complex (Fig. 1.).

Nucleus of hypoglossal nerve (CN XII)

A population of loosely scattered immunopositive neurons was observed, mainly oval in shape, sometimes elongated or spindle-shaped. A small number of perikarya took the form of a pallet (the proximal part of the projection is visible) or a round shape. The granular, medium intense reaction is dispersed evenly in the neuroplasm, showing no clear areas of density or local enhancement (Fig. 6.)

Dorsal nucleus of vagus nerve (CN X)

The presence of a large group of loosely scattered immunopositive perikarya with various morphological forms was found. Cell bodies were mostly spherical or polygonal in shape, and spindle-shaped or oval cells were also visible. The immunohistochemical reaction of medium intensity was granular and evenly distributed in the neuroplasm (Fig. 6.)

Cuneate nucleus

Scattered immunopositive cells were observed, mainly oval in shape, less often polygonal with medium intensity of reaction. It was extremely uneven in nature and its densities were usually located at one pole of the cell, the opposite part of the neuroplasm was usually immunonegative, as were all visible processes (Fig. 10.).

Spinal nucleus of trigeminal nerve (CN V)

Clusters of immunopositive cells were visible, mainly round, less often oval or spindle-shaped, with medium staining intensity. Its distribution was often very uneven and its concentrations were usually located at one pole of the cell, the opposite part of the neuroplasm and neuron processes were immunonegative (Fig. 10.).

Inferior olivary nucleus. Dorsal and medial olivary nuclei.

Only large paddle-shaped, round or oval perikarya were characterized by a very intense immunohistochemical reaction. The distribution of DAB reactions in the neuroplasm was uniform, evenly distributed over the entire area of the perikaryon and the dendritic processes (Fig. 7 and 9.).

Nucleus ambiguus

The presence of few, compactly located, round or polygonal neurons manifesting strong SPX expression was revealed. Axonal hillocks of these cells were also often visible - completely immunonegative - and few dendritic trunks showing a moderately intense DAB reaction (Fig. 8.).

Nucleus of the solitary tract

In all areas constituting the nucleus, there were small, scattered immunopositive cells of a round or slightly oval shape, less often polygonal. The distribution of the immunohistochemical reaction in the neuroplasma was heterogeneous - there were densities and places with very weak intensity of the DAB reaction (Fig 8.).

Nucleus of the pre-Bötzinger complex

Few, scattered spherical immunopositive perikarya with medium staining intensity were visible. Its distribution was most often insular. There were concentrations of coarse-grained DAB reaction in the peripheral part of the neuroplasma or it appeared in the form of linear clusters located parallel to the neuron's cell membrane (Fig. 9.).

Nucleus raphe obscurus

Large, spherical or oval SPX-positive perikarya with weak immunohistochemical staining intensity and scattered cytoplasmic distribution were observed. However, areas of neuroplasm with a slightly greater intensity of DAB reaction were sometimes visible (Fig 7.).

3.2.2. Nesfatin-1

Nesfatin-1 was expressed in neurons forming the nuclei of the cranial nerves: oculomotor (CN III), hypoglossal (CN. XII) and vagus (CN X)) and subhypoglossal nucleus of Roller, nucleus of the solitary tract, nucleus ambiguus, cuneate nucleus and complex of olivary nuclei.

Nucleus of hypoglossal nerve (CN XII)

Numerous, loosely scattered immunopositive neurons are visible, mainly oval in shape, sometimes elongated or spindle-shaped. A small number of perikarya took the form of a pallet (the proximal part of the projection is visible) or a round shape. The granular reaction was intense and dispersed evenly in the neuroplasm, showing no clear areas of density or local enhancement (Fig.11.).

Subhypoglossal nucleus of Roller

Few, scattered, polygonal, oval, sometimes pyramidal or elongated immunopositive pericaryons with high staining intensity were visible. Its distribution was most often polar. There were concentrations of granular DAB reaction in a specific area of the neuroplasma, the opposite pole was characterized by the absence of reaction (Fig. 11.)

Dorsal nucleus of vagus nerve (CN X)

The presence of a large group of loosely distributed round or slightly oval immunopositive perikarya with a weak intensity of fine-granular cytoplasmic reaction and even distribution in the neuroplasm was detected (Fig. 12.).

Nucleus of the solitary tract

n all areas constituting the nucleus, there were small, scattered immunopositive cells of a round or slightly oval shape, less often polygonal. The distribution of the immunohistochemical reaction in the neuroplasm was usually even, sometimes there were concentrations and places with very weak intensity of the DAB reaction (Fig. 12.)

Cuneate nucleus

Scattered immunopositive cells were observed, mainly oval in shape, less often polygonal with high intensity of granular reaction. It was very uneven in nature and its concentrations were usually located at one pole of the cell, the opposite part of the neuroplasm was usually immunonegative (Fig. 11.).

Inferior olivary nucleus. Dorsal and medial olivary nuclei.

Only large perikarya with a paddle-like, round, polygonal or oval shape were characterized by a very intense immunohistochemical reaction. The distribution of DAB reactions in the neuroplasm was uniform, evenly distributed over the entire area of the perikaryon and the dendrites (Fig. 13 and 14).

Nucleus ambiguus

The presence of few, compactly located polygonal or spindle-shaped, sometimes pyramidal or tear-shaped neurons manifesting strong SPX expression was revealed. Axonal hillocks of these cells were also often visible - completely immunonegative - and few dendritic trunks showing a moderately intense DAB reaction (Fig. 14.).

4. Discussion

Research on new regulatory neuropeptides, which include nesfatin-1 and spexin (SPX), may provide a range of potentially valuable information about brainstem function allowing for a more complete understanding of its key role in the regulation of fundamental physiological and behavioral processes.

Nesfatin-1 is a peptide that plays an important role in the central control of appetite and energy homeostasis. In the brainstem, nesfatin-1 affects nuclei associated with the control of food intake, such as the nucleus of the solitary tract (NTS) and the dorsal nucleus of the vagus nerve (DMV). The mechanism of action of nesfatin-1 in the CNS is based primarily on its modulatory role on the activity of orexigenic and anorexigenic neurons of the hypothalamus, which leads to a reduction in food intake. Moreover, nesfatin-1 has an anxiolytic effect, suggesting its potential role in the treatment of anxiety disorders (Xu et al. 2015) . Spexin (SPX) is a multifunctional neuropeptide that has very diverse physiological activities, including the regulation of appetite, metabolism and reproductive functions. Spexin receptors, identical to galanin receptors Gal2 and Gal3, are expressed in numerous structures in the brainstem and hypothalamic nuclei, including: in the arcuate and paraventricular nuclei. The physiological effects of SPX include: inhibition of food intake by modulating the synthesis and release of hormones regulating energy homeostasis, such as insulin and leptin. Moreover, SPX has the potential to regulate blood pressure by influencing the central autonomic system, which may be of key importance in the future treatment of hypertension. Previous research indicates significant interactions between nesfatin-1, SPX and other neurotransmitter systems, such as the dopaminergic, serotonergic, noradrenergic and glutamatergic systems. Understanding these functional interdependencies at the molecular, synaptic and cellular levels may contribute to the development of new therapeutic strategies for the treatment of a number of neurological and psychiatric disorders.

In the presented research work, thanks to the use of modern immunohistochemical techniques supported by canonical Nissl topographic staining, the expression of new regulatory neuropeptides spexin (SPX) and nesfatin-1 was analyzed in selected sections of the human brainstem. It is worth emphasizing that the description of the neuroanatomical distribution of SPX-synthesizing neurons in the human brainstem has been made for the first time, which may be an interesting and important addition to the current state of knowledge about neuropeptide

signaling in the CNS from a pharmacological point of view. The research was carried out on sections of the human diencephalon, representing a cross-section of this structure at the level of the exit of the oculomotor nerves (CN III) and on sections of the medulla oblongata, cut transversely at the level of the olivary nuclei. The result of immunohistochemical staining, both classical using diaminobenzidine (DAB) and fluorescent (using secondary antibodies conjugated with Alexa Fluor 488 and 594), was the precise identification, localization and morphological description of neurons expressing the tested neuropeptides in a number of brainstem structures. In this respect, the populations of neurons building the initial and terminal nuclei of the cranial nerves, the nuclei of the reticular formation and selected subcortical nuclei representing the extrapyramidal pathways of the brain were analyzed.

Particularly noteworthy is the discovery of a large, morphologically diverse population of neurons expressing SPX in all subunits of the human oculomotor nerve nucleus (CN III), including the accessory nucleus of Edinger-Westphal (EW) and the medial nucleus of Perlia, as well as confirmation of the presence in this area of nesfatinergic neurons, recently described by Psilopanagioti et al. (2020). The expression of SPX and nesfatin-1 in the human nucleus III is also analogous to the distribution of these neuropeptides in the same region of the rat midbrain (Porzionato et al 2010), suggesting that the neuronal signaling processes carried out by these new regulatory factors are phylogenetically conserved. This assumption is confirmed by the identification of neurons expressing SPX in the midbrain of evolutionarily lower vertebrates, in particular fish species: *Dicentrarchus labrax* (Paullada-Salmeron et al. 2023), *Danio rerio* (Kim et al. 2019) and *Caurasius auratus* (Wong et al. 2013). However, the functions performed by SPX in the n. III nucleus are currently not clarified; it is possible that this peptide is a local co-transmitter involved in the control of the discharges of motor neurons supplying the muscles that determine eye movement. Numerous neuronal populations of the brain releasing classic excitatory and inhibitory neurotransmitters synthesize slow-acting neuropeptides in parallel. The potential involvement of SPX in autonomic activities controlled by the accessory nucleus of Edinger-Westphal is also interesting, as there are reports suggesting that neuropeptide signaling may play an important role in the activities of this structure. It has been observed that most noncholinergic neurons of the mouse ECT nucleus, especially those sending axonal projections to the telencephalon, express the CART peptide (cocaine and amphetamine regulated transcript), urocortin and cholecystokinin (Che Ngwa et al. 2014). Connectomic and chemogenetic studies have proven that these cells are selectively activated in response to the loss of motor function and generate anxiety behaviors. SPX may be another brainstem neuropeptide that is functionally part of the above regulatory pathway, but there is currently no

data confirming this possibility. A similar model of interpretation of the obtained results is in the case of nesfatin-1, as there are a number of studies showing the presence of neurons expressing this peptide in the Westphal-Edinger nucleus of rats (Goebel-Stengel and Wang 2013, Foo et al. 2008), mice (Goebel-Stengel et al. 2011, Okere et al. 2010) and humans (Bloem et al. 2012).

A particularly intriguing report that sheds new and unobvious light on the function of nesfatin-1 in the human brainstem is the original work of Bloem et al. (2012). It showed that nesfatin neurons of the human Edinger-Westphal nucleus are characterized by coexpression of the CART peptide (cocaine and amphetamine-regulated transcript), and the expression of CART mRNA was higher in male (almost 4-fold) and female (almost 6-fold) suicide victims relative to controls. The same pattern occurred in the case of nesfatin-1; its level was 2 and 3 times higher in men and women, respectively. It can therefore be assumed that the Edinger-Westphal nucleus is involved in the process of adaptation to stress and the pathogenesis of mood disorders, perhaps also depression, in a significantly gender-dependent manner. This may have an important practical implications because women have a higher incidence of mood dysfunctions. Studies in animal models seem to indirectly confirm this hypothesis. It was observed that both acute and chronic stress activates nesfatin-1/CART neurons in the Edinger-Westphal nucleus of rats of both sexes, while only long-term stress leads to an increase in the immunoexpression of nesfatin-1 and CART in these cells with a stabilized mRNA level of both neuropeptides (Xu et al. al. 2010).

Spexin may be a new neuropeptide involved in the control of the activity of the glossopharyngeal (CN. IX), vagus (CN X), accessory (CN XI) and hypoglossal (CN XII) nerves. This is supported by the presence of neurons expressing SPX in the initial, terminal and autonomic nuclei of the mentioned nerves in the human brainstem. Numerous multipolar motor neurons of the nucleus ambiguus manifest strong SPX expression, which allows us to assume that this neuropeptide may be classified as a previously unknown factor regulating the functioning of the muscles of the larynx, pharynx, palate and upper part of the esophagus. The potential involvement of SPX in the regulation of myocardial function cannot be ruled out. The stimulation of baroreceptors caused by an increase in the frequency of contractions stimulates the neurons of the nucleus of the solitary tract (NTS) via afferent fibers n. IX and X), which causes the heart rate to slow down.

It is also worth noting that NTS neurons expressing neuropeptide Y (NPY) mRNA are depolarized under the influence of adiponectin, which suggests that this neuropeptide acts at the level of subtle cell populations of the brainstem as an element of central mechanisms

controlling blood pressure (Hoyda et al. 2009). On the other hand, the expression of the NPY gene in the rat NTS cells is activated under conditions of food restriction but not during a two-day period of starvation (Ishizaki et al. 2003). Therefore, the hypothesis that neuropeptide signaling may play an important role in the physiology of the NTS seems to be justified. This is supported by the fact that neurons in this area of the cat brainstem express a number of multifunctional peptides and neurohormones: oxytocin, vasopressin, substance P, Met- and Leu-enkephalin, β-endorphin, cholecystokinin, neurotensin, somatostatin, FMRMamide, angiotensin II, VIP, TRH and even ANF and LH (Maley 1996). Recently, there have also been suggestions that glucagon-like peptide (GLP-1) and prolactin-releasing peptide (PrRP) play a key role in organizing the response of NTS neurons to a wide range of stress factors in animal models (Holt and Rinaman 2022). Nesfatin-1 also seems to play a key role in autonomic activities carried out at the NTS level. This neuropeptide may be an important element of the hormonal regulatory axis: digestive system-brain, responsible for, among other things, generating the feeling of hunger and satiety and controlling food intake. It was observed that gastric muscularis membrane tension activated nesfatin-1 expressing cells in the rat NTS (Bonnet et al. 2013). These were mostly GABAergic inhibitory interneurons, also present in the dorsal nucleus of the vagus nerve. It was also revealed that cholecystokinin 8 (CCK-8) can significantly stimulate the activity of nesfatin cells in the rat NTS (Noetzel et al. 2009).

The presence of SPX-expressing neurons in the nucleus of the pre-Bötzinger complex (pre-BötC) may suggest the involvement of this neuropeptide in the central mechanisms of respiratory movement control. Therefore, a functional correlation of SPX with somatostatin (SST) and neurokinin 1 receptor (NK1R) - molecules that are considered histochemical markers of pre-BötC (Stornetta et al. 2003) cannot be excluded. However, the potential coexpression of SPX with SST is debatable, because the presence of SST is characterized by small spindle-shaped glutamatergic neurons generating the respiratory rhythm (Wei et al. 2012), while the expression of SPX was characterized primarily by large multipolar neurons. Only double immunofluorescence staining can solve this problem.

Neurons building the olivary nuclei complex, both the main nucleus and the dorsal and medial accessory nuclei, manifested strong expression of SPX and nesfatin-1 in the human brainstem. This may suggest the potential involvement of these new neuropeptides in the processes of coordination of voluntary movements, related to the activity of climbing fibers running to the cerebellum, which originate in the cells of the inferior olive nucleus. However, information on the structure and function of peptidergic pathways in this region of the human brain is so far very limited. However, it seems that neuropeptide signaling is not without

significance for the proper function of the olivary testes in rats, as the cells of this structure were characterized by the expression of neuromedin U (Honzawa et al. 1987), Met-enkephalin (Sanchez et al. 2013) and the CGRP peptide (Sanchez et al. 2014).

Also noteworthy is the demonstration of the presence of neurons expressing SPX in the human spinal nucleus of trigeminal nerve (CN V). It corresponds to previous reports revealing the expression of numerous neuropeptides in cell populations of the trigeminal nuclei, including CGRP, PACAP and VIP in the human spinal nucleus (Uddman et al. 2002), NPY, parvalbumin and calbindin in the midbrain nucleus V of the cat and rat (Wakisaka et al. 1996, Lazarov 1995). It is also worth noting in this case that numerous peptidergic neurons were also identified in the trigeminal nucleus of mammals, they were characterized by the expression of CGRP, substance P (SP), NPY, galanin and enkephalin (Gaspersic et al. 2006, Elcock et al. 2001). SPX-expressing cells were also numerous in the cuneate nucleus, which may suggest the involvement of this neuropeptide in motor activities carried out by the posterior cord pathways of the spinal cord. Little is known about peptidergic signaling in this nucleus, but the presence of NPY-positive neurons has been demonstrated in the rat and raccoon cuneate nucleus (Lin et al. 2010, Dick et al. 1998).

It is worth emphasizing that the results obtained are the first data on the expression and distribution of SPX in the human brainstem and one of the few data on the presence of nesfatin-1 in this brain region. However, it is necessary to clearly highlight certain inevitable limitations of the conducted experiment, which do not allow for the formulation of final and conclusive judgments about the role of these new neuropeptides in the functions of the human brainstem. Firstly, the research was conducted on a very small number of samples, de facto covering 2 brains. Secondly, no analysis of SPX and NUCB2 gene expression was performed in brain tissue samples. Obtaining this type of preparations is an extremely difficult task because they cannot be subjected to preservation processes and must be frozen as soon as possible after the cessation of brain function. We encounter similar difficulties when examining tissue protein expression using Western blotting. The immunohistochemical tests performed may, of course, carry a certain risk of incomplete antibody specificity and selectivity. However, in this case, an anti-SPX antibody was used, verified in previous research and additionally verified by preincubation with pure SPX. In the case of SPX and nesfatin-1 appropriate negative controls were performed each time, omitting the primary antibody. The results presented in this paper may therefore constitute an interesting starting point for further, more extensive studies of the expression of new regulatory neuropeptides in the human brain. A valuable complement to the obtained results would be the examination of the potential co-expression of SPX and nesfatin-

1 in brainstem cell populations and the quantitative assessment of the number of immunopositive cells.

Although the confirmation of SPX and nesfatin-1 functions in the human brainstem requires numerous further studies including analysis gene expression, even at the present stage of knowledge these novel neuropeptides can be considered as an intriguing and potentially important regulatory factors in this brain region. Given the scarcity of human brain tissue, we believe that this first report will offer a much-needed initial neurochemical map of SPX and nesfatin-1 expression in the human brainstem. Taking into account the small number of brain samples, it is definitely worth considering expanding the study to complement our initial report. To date, a role SPX and nesfatin-1 in the human brainstem has not yet been precisely determined, but undoubtedly further structural and functional studies on these novel neuromodulators e.g. on their coexpression with other neuropeptides, classical neurotransmitters and receptors definitely merits attention. It should also be emphasized that the nesfatin-1 receptor remains so far unidentified. Therefore, the further progress of neurophysiological pharmacological studies on nesfatin-1 signaling is still substantially difficult. Another limitation of the study is that there was only two male brains examined and further studies on female brainstem are therefore urgently required. The conducted experiment is so far a modest introduction to more advanced research on the neuropeptide signaling in the human brainstem. However, it is possible that this work will provide a range of information shedding new light on the understanding of the mechanisms of brainstem function and will open previously unknown perspectives for new pharmacotherapeutic strategies in psychiatry and neurology.

5. Concluding remarks

Spexin (SPX) and nesfatin-1 are novel, phylogenetically conserved regulatory neuropeptides of the human brainstem potentially involved in the execution of key autonomic activities of this brain region.

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Ethical statement This is a statement, that all data published in the following manu script: were reviewed in-house and no ways of manipulating research materials were used.

Declaration of Competing Interest

This material has not been submitted elsewhere while under consideration. All authors declare no conflict of interest.

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Figure captions

Fig. 1. Schematic representation of SPX and nesfatin-1 neurons distribution in the human brainstem. Figure shows core neuroanatomical localization and relative density of peptidergic neurons identified in several nuclei of medulla oblongata (A,C) and mesencephalon (B,D). Dots outline general spatial distribution of SPX (black) and nesfatin-1 (red) immunopositive perikarya but do not reflect actual cell sizes. Scale bars: 2 mm. Abbreviations: 3N, nucleus of oculomotor nerve; 10N, nucleus of vagus nerve; 12N, nucleus of hypoglossal nerve; Amb, nucleus ambiguus, Ecu, external cuneate nucleus; IOD, dorsal olivary nucleus; IOM, medial olivary nucleus; IOPr principal nucleus of inferior olive; PAG, periaqueductal gray; PrBo, nucleus of pre-Bötzinger complex; RN, red nucleus; Ro, subhypoglossal nucleus of Roller; ROb, raphe obscurus nucleus; SNpc, substantia nigra-compact part.

Fig. 2. Neurons with SPX and nesfatin-1 expression in red nucleus, substantia nigra and periaqueductal grey (PAG). Nissl staining (A,B,G,H). Red nucleus: SPX (C-D), nesfatin-1 (E-F), substantia nigra – compact part: SPX (I-J), nesfatin-1 (K-L), PAG; SPX (M-O), nesfatin-1 (P-S). Scale bars: 100 μm (P), 50 μm (A,C, E, G, I, K, M, N, R), 20 μm (D, F, H, J, L, O, S).

Fig. 3. Neuroanatomy of the human nucleus of oculomotor nerve (CN III) and its topographic relations in the cross sectioned mesencephalon stained with Nissl (A,D-I) Klüver-Barrera (B.) and Lapham (C.) method. Histological division of the nucleus according to Coulombe et al. (2021). Cytoarchitectonic subgroups: dorsomedial (DM), dorsolateral (DL), central (Cen), ventral (Ven), Edinger-Westphal accessory nucleus (EW), nucleus of Perlia (NP). Neurons of the central part (C-F), ventral part (G-H), E-W nucleus (K-L) and nucleus of Perlia (I-J.). Scale bars: 200 μm (A, B), 100 μm (C-L, K), 50 μm (J, L).

Fig. 4. Neurons with SPX expression in the nucleus of oculomotor nerve. Cytoarchitectonic subgroups: dorsomedial (DM), dorsolateral (DL), central (Cen), ventral (Ven), Edinger-Westphal accessory nucleus (EW), nucleus of Perlia (NP). Immunopositive cells in the central part (C-F), ventral part (G-H), E-W nucleus (K-L) and nucleus of Perlia (I-J.) Scale bars: 200 μm (A.), 100 μm (C-G), 50 μm (H-M), 20 μm (N-O).

Fig. 5. Neurons with nesfatin-1 expression in the nucleus of oculomotor nerve. Cytoarchitectonic subgroups: dorsomedial (DM), dorsolateral (DL), central (Cen), ventral (Ven), Edinger-Westphal accessory nucleus (EW), nucleus of Perlia (NP). Immunopositive cells in the central part (B), ventral part (C), E-W nucleus (K-L) and nucleus of Perlia (I-J.) Scale bars: 200 μm (A.), 100 μm (C-F, H-I), 50 μm (J, K., M,N), 20 μm (G, L, O).

Fig. 6. Neurons with SPX expression in the nucleus of hypoglossal nerve (A, C-F) and vagus nerve (B, G-J). Fluorescence: immunopositive cells labeled with Alexa Fluor 488 (green) or Alexa Fluor 594 (red), nuclei counterstained with DAPI. Scale bars: 3 mm (A,B), 100 μm (G), 50 μm (C,D,H), 20 μm (E, F, I, J).

Fig. 7. Neurons with SPX expression in the nucleus raphes obscurus (A, C-F) and inferior olivary nucleus (B, G-J). Nissl staining: (B). Fluorescence: immunopositive cells labeled with Alexa Fluor 488, nuclei counterstained with DAPI. Scale bars: 3 mm (A), 200 μm (B) 100 μm (G,I), 50 μm (C,H,J), 20 μm (D-F).

Fig. 8. Neurons with SPX expression in the nucleus ambiguus (A, C-F) and nucleus of solitary tract (B, G-J). Fluorescence: immunopositive cells labeled with Alexa Fluor 488, nuclei counterstained with DAPI. Scale bars: 3 mm (A,B), 100 μm (G-I), 50 μm (C, E, J), 20 μm (D,F).

Fig. 9. Neurons with SPX expression in the nucleus of pre-Bötzinger complex (A, C-F) and dorsal accessory olivary nucleus (B, G-J). Additional Nissl staining (E-F, I-J). Scale bars: 3 mm (A, B) 100 μm (I), 50 μm (C-E, I, G), 20 μm (F, H, J).

Fig. 10. Neurons with SPX expression in the cuneate nucleus (A, C-F) and spinal nucleus of trigeminal nerve (B, G-J). Additional Nissl staining (E-F, I-J). Scale bars: 3 mm (A, B) 100 μm (E, G), 50 μm (C, F, I), 20 μm (D, H, J).

Fig. 11. Neurons with nesfatin-1 expression in the nucleus of hypoglossal nerve (A-F) and subhypoglossal nucleus of Roller (G-L). Scale bars: 3 mm (A,G), 500 μm (B,C,I), 100 μm (D, E, J,K), 50 μm (D,H).

Fig. 12. Neurons with nesfatin-1 expression in the dorsal nucleus of vagus nerve (A-F) and nucleus of solitary tract (G-L). Scale bars: 3 mm (A,G), 500 μm (B,C), 100 μm (D, E, H-K), 50 μm (F,L).

Fig. 13. Neurons with nesfatin-1 expression in the cuneate nucleus (A-F) and inferior olivary nucleus (G-L). Scale bars: 3 mm (A,G), 200 μm (B,C,H,I), 100 μm (D, E, J , K), 50 μm (L), 20 μm (F).

Fig. 14. Neurons with nesfatin-1 expression in the medial accessory olivary nucleus (A-F) and nucleus ambiguus (G-L). Additional Nissl staining (G-H). Scale bars: 3 mm (A,G), 500 μm (C, H), 200 μm (D), 100 μm (B, E, I), 50 μm (F), 20 μm (J,K,L).