Spexin and nesfatin-1-expressing neurons in the male human claustrum

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Abstract

Neuropeptides are involved in numerous brain activities being responsible for a wide spectrum of higher mental functions. The purpose of this concise, structural and qualitative investigation was to map the possible immunoreactivity of the novel regulatory peptides: spexin (SPX) and nesfatin-1 within the human claustrum. SPX is a newly identified peptide, a natural ligand for the galanin receptors (GALR) 2/3, with no molecular structure similarities to currently known regulatory factors. SPX seems to have multiple physiological functions, with an involvement in reproduction and food-intake regulation recently revealed in animal studies. Nesfatin-1, a second pleiotropic neuropeptide, which is a derivative of the nucleobindin-2 (NUCB-2) protein, is characterized by a wide distribution in the brain. Nesfatin-1 is a substance with a strong anorexigenic effect, playing an important role in the neuronal circuits of the hypothalamus that regulate food intake and energy homeostasis. On the other hand, nesfatin-1 may be involved in several important brain functions such as sleep, reproductive behaviour, cognitive processes, stress responses and anxiety. For the first time we detected and described a population of nesfatin-1 and SPX expressing neurons in the human claustrum using immunohistochemical and fluorescent methods. The study presents the novel identification of SPX and nesfatin-1 immunopositive neurons in the human claustrum and their assemblies show similar patterns of distribution in the whole structure.

Key words;

claustrum; nesfatin-1; spexin; brain; neuropeptides

1. Introduction

The claustrum, a thin leaflet of gray matter located in the human basolateral forebrain (Fig 1.) was first identified in 1786 by Felix Vicq d'Azyr, a physician to queen Marie Antoinette, but a detailed description of this structure was made by the German neuroanatomist Karl Friedrich Burdach in 1819. In the second half of the 19th century, the claustrum was repeatedly presented in an increasingly more perfect way in numerous anatomical atlases, however no attempt was made to explain the functions of this mysterious structure. Moreover, many leading anatomists of that period, including even the famous pioneers of brain cytoarchitectonics research such as Korbinian Brodmann and Carl W. Wernicke, incorrectly considered the claustrum to be the inner, compact layer of the insular cortex. Against this background, the figure of the Viennese anatomist and neurologist Theodor Meynert stands out, as he was the first to suggest that the claustrum may play an important role in multimodal signal processing (Smythies and Edelstein 2013). The claustrum is an intriguing subcortical brain structure bidirectionally connected with almost all neocortical areas (Jackson et al. 2020, Torgerson et al. 2015) therefore it seems to play an important role in the multimodal and crossmodal integration of diverse sensory and cognitive functions (Smythies and Edelstein 2013, Ham and Augustine 2022). Recent hypotheses suggest that the claustrum neurons are involved in generation and maintaining of consciousness (Liaw and Augustine 2023, Crick and Koch 2005). It is also speculated that the claustrum plays a key role in the spatial information processing and decision making (Smith et al. 2017). Its neuronal populations receives afferent signals from many sensory-related areas of the brain, including the auditory cortex, which may suggest that the claustrum plays a role in integrating information across sensory modalities (Remedios et al. 2010).

Spexin (SPX) is a newly discovered multifunctional neuropeptide acting at both central and peripheral levels. SPX was identified in 2007 by Mirabeau and colleagues (2007) as a transcript of the *Ch12orf39* gene. In the rat and human brain, SPX-expressing neurons have been detected with the highest expression being in the hypothalamic magnocellular nuclei (Porzionato et al. 2010, Pałasz et al. 2021). The chemical structure of SPX is distinctly conserved among species, the rat molecule differs from the human form by only one C-terminal amino acid (Porzionato et al. 2010).

SPX is an alternative endogenous ligand for the GALR2/3 receptors, with even higher affinity toward GALR3 than galanin itself (Kim et al. 2014). SPX has recently been linked to multiple physiological functions such as reproduction, food-intake regulation (Ma et al. 2018), cardiovascular/renal function and nociception (Toll et al. 2012). Due to potent anorexigenic properties of SPX, it has been suggested that a potential excess in hypothalamic SPX signaling may be involved in the pathogenesis of anorexia nervosa (Pałasz et al. 2018). A recent study therefore suggests a possible application of SPX for obesity therapy (Walewski et al. 2014). A role of SPX as a potential biomarker of glucose metabolism in humans has been also suggested (Hodges et al. 2017). Nesfatin-1 is an anorexigenic peptide derived from nucleobindin 2 and involved in the regulation of food intake and glucose homeostasis (Stengel 2015, Algul et al., 2016, Li et al., 2013). Nesfatin-1 is posttranslationally cleaved from the 396-amino acid protein, nucleobindin 2 (NUCB2) by the pro-hormone-convertase into the active N-terminal nesfatin-1, nesfatin-2 and the C-terminal nesfatin-3 (Oh-I et al., 2003, Stengel et al., 2013). The production of NUCB2 occurs initially in the hypothalamic nuclei (Folgueira et al., 2014). Nesfatin-1 is released from endocrine cells such as gastric and intestinal mucosal cells and pancreatic beta cells and is present in peripheral tissues such as muscle and adipose tissues (Chen et al., 2015). Nesfatin-1 may be a regulator of appetite, energy and glucose homeostasis, and insulin secretion. Thus, it can participate in the etiology of various metabolic diseases including obesity and diabetes (Stengel et al., 2011). However, 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. For example, the plasma nesfatin-1 level is positively correlated with the severity of depression (Xiao et al., 2018; Palasz et al., 2012). The neurophysiological effects of nesfatin-1 being distinctly "sex-related". For example, elevated nesfatin-1 expression was noted in the brainstem of male suicide victims (Bloem et al. 2012).

Despite the accumulating animal studies on nesfatin-1 and SPX neurochemistry, their distribution and physiology are so far lacking within the human claustrum. In the present study we aim to provide a structural investigation of the human claustrum to reveal the first outline for the neurochemical map of SPX and nesfatin-1 expression within this brain structure, allowing a potential deeper

mechanistic understanding of SPX and nesfatin-1 neurobiology to aid future pharmacological study.

2. Materials and Methods

Human brain tissue specimens (2 males, aged 65-67 years, died due to circulatory failure) with no neuropathological findings were obtained from the Conscious Body Donation Program conducted by the Department of Anatomy at the Medical University of Silesia in Katowice. Brains were perfusion-fixed up to 12 hours *post mortem* with 4% paraformaldehyde buffered solution (pH 7.2-7.4) and then immersion-fixed over a period of two weeks. The brain regions comprising claustrum were then excised from two separated slices that were sectioned in the horizontal orientation perpendicularly to the insular branch of middle cerebral artery (M2) according to the referenced human brain atlases (Mai et al. 2015, Cho 2014) as visualized in Figure 2. Rostral edge of the short insular gyrus (b1) and caudal surface of the long insular gyrus (l1) were established as anterior and posterior boundaries respectively. The medial boundary of the section examined in the study was identified in the horizontal plane by the lateral medullary lamina. The free surface of the insular cortex was taken as the lateral edge. Samples were dehydrated, embedded in paraffin blocks and finally sectioned by microtome (Leica Microsystems, Germany) at 10 μm thick serial slices. The sections were deparaffinized with xylene and rehydrated to 50% ethanol via graduated alcohol solutions. 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 rabbit anti-rat spexin polyclonal antiserum (1:2000, Phoenix Pharmaceuticals, Burlingame, CA, USA, H-023-81, RRID: AB2923380) or with rabbit polyclonal antibody against rat nesfatin-1 (1:1000, Phoenix Pharmaceuticals, Burlingame, CA, USA, H-003-22, RRID: AB2313672). Both antibodies were characterized by reactivity with human tissue antigens. Primary antibodies were followed by biotinylated secondary antibodies (ImmPress-HRP horse anti-rabbit IgG polymer reagent, Vector Laboratories) and then an avidin-biotin-

horseradish peroxidase complex (Vectastain ABC kit, Vector Laboratories) and visualised with 3,3'-diaminobenzidine (DAB). Alternatively, following incubation with primary antibody against rat nesfatin-1 (the same as for IHC) brain sections were kept in darkness with goat anti-rabbit secondary antibody labeled with Alexa Fluor 488 (1:200, Invitrogen, A11008, RRID: AB143165) and mounted on slides with DAPI-containing medium. All sections were previously treated with TrueBlack® (Biotium, Hayward, CA, USA, No; 23007) to remove unwanted neural lipofuscin autofluorescence. Sections incubated with IgG instead of primary antibodies were used as negative controls. 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, Burlingame, CA, USA, No; 023-81, RRID: AB2923380) 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. For basic neurostructural evaluation, representative sections were stained via Nissl method in 1% Cresyl violet for 60 mins. After rinsing and differentiation by acetic acid and mounting with DPX, sections were coverslipped. All images were captured with Nikon Eclipse E600 fluorescent optic systems and processed using CellSens Entry software (Olympus, Japan). The cyto- and chemoarchitecture was analyzed and immunopositive cells were counted using ImageJ 1.43u software. The number of SPX and nesfatin-1-positive cells was counted in both anterior (A.) and posterior (B.) claustrum region to obtain the density of these cells per standardized area (0.16 mm^2, frame 400 x 400μm). Data are presented as a mean ± standard error of the mean (SEM).

3. Results and Discussion

We have demonstrated for the first time SPX and nesfatin-1 immunoreactive neurons in the human claustrum, suggesting that these novel neuropeptides may be involved in autonomic functions mediated by this brain region. The disclosure of the expression of SPX and nesfatin-1 in the human claustrum and the preliminary analysis of distribution and cytoarchitectonics performed for the first time seem to be interesting and important for a more complete understanding of the neurochemical basis of the functioning of this particular structure. So far, the expression of these two new regulatory neuropeptides has been demonstrated in numerous structures of the rat brain, especially in the hypothalamus, structures of the limbic system and the brainstem, while the claustrum has not yet been studied in this respect. In case of the human brain, the number of available data on the expression of nesfatin-1 and SPX and other newly discovered peptides is even more limited. The presence of nesfatin-1 neurons has so far been revealed only in the human hypothalamus (Psilopanagioti et al. 2019), the nucleus of the lamina terminalis (BNST) (Pałasz et al. 2019) and the brainstem (Bloem et al. 2012). A significant population of neurons expressing SPX has been described in the magnocellular nuclei of the hypothalamus (Pałasz et al. 2021).

Immunohistochemical studies revealed the presence of a diffuse population of neurons expressing SPX and nesfatin-1 (positive DAB reaction) in both the anterior and posterior segments of the human claustrum. Most of their perikarya were relatively small, polygonal, oval or round (Fig. 4 to 6), and the neuroplasm was sometimes shrunken, which is often the case with human nervous tissue, which usually reaches the fixing medium with a significant delay and stays there for a relatively long time. Cytoplasmic processes were usually invisible. It is worth emphasizing that the specificity of the antibodies used was confirmed in previous studies on neuropeptides, and the negative controls performed each time were characterized by the absence of immunopositive cells in the claustrum. It can therefore be assumed that labeling of a significant number of false-positive cells was avoided, and glial cells and blood vessels did not show any color reaction. During the quantitative analysis of cell distribution, it

was found that their number slightly changes with the highest values in the most rostral and caudal areas of the claustrum. The spatial distribution of neuropeptide expressing cells is relatively uniform along the entire length of the anterior and posterior claustrum, no compact clusters of cells or their ordered orientation are observed. The total number of SPX-expressing neurons was significantly higher in the rostral part of the posterior claustrum (Fig 3.). In the case of nesfatin-1, a slightly higher density of immunopositive cells was found in the caudal part of the posterior claustrum (Fig 3.). In the anterior part of the structure, there were no significant changes in the number of cells expressing both neuropeptides. Three-dimensional analysis of cell distribution revealed a slightly higher number of nesfatin-1 expressing neurons in the superior region of both anterior and posterior claustrum (Fig 7.). Initially, 5 different types of neurons were distinguished in the human claustrum (Braak and Braak, 1982), but over time their classification was corrected (Moryś et al. 1996) and narrowed to 2 basic categories: projection and intercalate neurons. Projection neurons are cells with long axons that form connections between distant areas of the brain. Intercalated neurons are key neurons for the regulation of local claustrum neural networks. These are small cells that mediate the transmission of information between projection neurons. There is ample evidence that interneurons in the claustrum act as a synchronous inhibitors on the excitation of large neural populations, which likely serves as a regulator supporting the process of segregation of cortico-claustral information (Kim et al., 2016). At present, it is not possible to determine which category the identified SPX and nesfatin-1 expressing neurons belong to. It can be suggested that this fact has some impact on the density of peptidergic innervation (SPX and nesfatin-1) in these areas, but verification of this assumption requires a number of further neurochemical studies. The functional interpretation of the distribution profile of cells expressing SPX and nesfatin-1 in the anterior and posterior claustrum described in this study is extremely difficult and is, at the moment, entirely speculative. First of all, we do not yet know the connectomics of these cells, it remains unclear what afferents reach them and where their axons go. It also remains unknown what neurotransmitters and synaptic modulators are coexpressed with SPX and nesfatin-1. Answering the above questions is certainly an important research challenge that should be undertaken in subsequent work on the neurochemistry of the claustrum. However, given the fact that the observed population of peptidergic neurons is quite large and appears to constitute a significant percentage of all claustrum neurons, some highly cautious, preliminary considerations

are possible regarding the functional, spatially determined implications of the existence of these cells in the cortico-claustral loop. Tractographic studies based on diffusion tensor imaging (DTI) prove (Milardi et al. 2015) that the anterior section of the human claustrum is connected to the Broca speech motor center (Brodmann's area: 44, 45) as well as the regions of the premotor (fields 9, 10) and orbitofrontal cortex (areas 11, 12). The posterior part of the claustrum is, in turn, connected to the Wernicke speech sensory center (field 39), the primary and association auditory cortex (fields 41, 42), the cingulate cortex (field 31) and the visual cortex (areas 17, 18). It can therefore be assumed that the neurons expressing nesfatin-1 and spexin present in these areas of the claustrum play an unexplained at present, highly selective role in the phenomenon of creating and uttering words and their understanding, in initiating certain motor activities, in cognitive processes, perception of auditory and visual signals as well as feeling and processing emotional stimuli. It is also possible that the variable number of peptidergic cells in individual sections of the rostral and caudal parts of the claustrum is reflected in the myeloarchitecturally different density of nerve fibers heading to specific cortical and subcortical structures. These fibers may originate in zone-limited, relatively small populations of nesfatin-1 and spexin neurons and participate in subtle regulatory processes in the cortico-claustral pathways.

However, in order to identify the route of these fibers more advanced connectomic studies including retrograde tracing, single axon neurochemical analysis and precise evaluation of the neuropeptide receptors expression are required. Obviously, in case of human brain all aforementioned procedures are substantially difficult or even impossible to carry out and only animal models are therefore accepted reference points. Numerous interspecies differences in the morphology and cytoarchitectonics of the claustrum should definitely be taken into account. All the aforementioned limitations significantly hamper a broad and multi-dimensional interpretation of the results obtained in this study, which does not change the fact that the description of neurons expressing nesfatin-1 and SPX in the human claustrum for the first time is an important contribution to the study of the structure and function of the brain. It is worth noting here that recent research on the role of nesfatin-1 in the CNS has provided a number of evidence that this initially unknown, unique, anorexigenic hypothalamic neuropeptide is an important element of numerous neuronal loops that perform key functions at the level of a number of brain structures. The presence of nesfatin-1 in

midbrain neurons and other sections of the brainstem does not seem surprising, considering the important role this neuropeptide plays in the hypothalamic control of energy homeostasis and metabolic activities. The executive stage of central, nesfatin-1-related regulation of food intake and other autonomic functions is carried out through brainstem centers including solitary tract nuclei (Psilopanagioti et al 2020). Against this background, the expression of nesfatin-1 in neurons of the vagus nerve (n. X) nuclei deserves special emphasis (Rupp and Stengel 2022). The vagus nerve is increasingly appearing in discussions on the effectiveness of stimulation of brain structures in modulating the state of consciousness and contemporary therapies for its disorders (Wang et al. 2023). A recently published clinical research paper presents the physiological effects of long-term n. X stimulation in a patient with profound dementia (Osińska et al., 2022). Its results indicate a significant improvement in the clinical condition of the subject after the use of the transcutaneous auricular vagus nerve stimulation (taVNS) therapeutic model on the CRS (Coma Recovery Scale), which is used to assess the state of consciousness. Therefore, the postulated role of vagus nerve in regulating the state of consciousness is a common point between this structure and the claustrum. The identification of nesfatin-1 in claustrum neurons results in a new, this time neurochemical coincidence between both structures. However, the potential role of nesfatin-1 in neuronal pathways related to the process of generating and stabilizing the state of consciousness is only hypothetical and has not yet been confirmed by experimental results. It is worth noting, however, that a number of research studies suggest the involvement of nesfatin-1 in the implementation of higher nervous activities, including affective and cognitive processes (Weibert et al. 2019, Pałasz et al. 2018). Although the primary arena of action of both neuropeptides remains the hypothalamus, they are also expressed in numerous neurons in other areas of the brain, especially those involved in the implementation of the body's autonomic activities. Confirmation of the participation of nesfatin-1, SPX and phoenixin (PNX) in the functioning of the claustrum requires numerous, advanced molecular and neurostructural studies, but even at the current, initial stage of knowledge, these new peptides should be included among the group of interesting new regulatory factors of the claustrum.

Although the confirmation of nesfatin-1 and SPX-related signaling in the human claustrum requires numerous further studies including analysis of NUCB2 and SPX gene expression, even at the present stage of knowledge these novel neuropeptides can be considered as an interesting and potentially important regulatory factors in this brain region. Nevertheless, given the scarcity of human brain tissue, we believe that this first report will offer a much-needed initial neurochemical map of nesfatin-1 and SPX expression in the human claustrum. Taking into account the relatively small number of brain samples, it is definitely worth considering expanding the study to complement our initial report. At present, a role of nesfatin-1 and SPX in the human claustrum still remains an area of speculation, but undoubtedly further structural studies on these factors, e.g. on their possible coexpression with other brain neuropeptides (NPY, somatostatin, parvalbumin) and neurotransmitters definitely merits attention. The main limitation of the study is that there was only two male brains examined and further studies on female claustrum are therefore definitely required. The conducted experiment is so far a modest introduction to more advanced research on the neuropeptide signaling in the human claustrum and adjacent brain structures.

Conclusions

The new regulatory neuropeptides nesfatin-1 and SPX may play a role in the activities carried out by human claustrum neurons. The spatial distribution of neurons expressing nesfatin-1 and SPX in the human claustrum is similar, suggesting the existence of potential functional correlations between these peptides.

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CRediT authorship contribution statement

A. Pałasz, A. Lipiec-Borowicz, P. Horka: Conceptualization, Investigation, Data curation, Writing - original draft**. A. Lipiec-Borowicz, A. Suszka-Świtek, A. Kaśkosz, J. Kistowska:** Methodology, Immunohistochemistry, Tissue acquisition **A.**

Piwowarczyk-Nowak, K. Mordecka-Chamera: Resources. **J.J. Worthington**: Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing interest

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Figure captions

Fig.1. Neuroanatomy of the human claustrum and its topographic relations in the coronally sectioned brain hemisphere stained with Weigert method (A.) Morphology of the claustrum and adjacent brain structures (B.). Histological division of the claustrum according to Mai et al. (2022). (C.) Cla, claustrum; dCl, dorsal claustrum, DiCl; diffuse insular claustrum; e, external capsule; ex, extreme capsule; GPL, lateral globus pallidus; GPM; medial globus pallidus, IC, internal capsule; InC, insular cortex; LiCl, limiting claustrum;; lml, lateral medullary leaflet; lmm, medial medullary leaflet; M2, insular branch of middle cerebral artery; Pu, putamen; vCl, ventral claustrum.

Fig.2. Fragment of a horizontal section of the human brain used for immunohistochemical studies. Topographic coordinates are present (arrows). The yellow ribbon marks the claustrum, the dashed line indicates the border between the white matter capsules (external and extreme), striatum and insular cortex. Boxes indicate areas: anterior (A.) and posterior (B.) subjected to quantitative analysis of the

number of cells expressing nesfatin-1 and spexin (SPX) immunoreactivity. Paraffin blocks were sliced from top to bottom, in the plane shown in the right three-dimensional drawing. Cla, claustrum; Ctx, insular cortex; e, external capsule; ex, extreme capsule; Gb, short insular gyrus; Gl, long insular gyrus; Pu, putamen.

Fig.3. Number of SPX and nesfatin-1 immunopositive cells counted from rostral to caudal region of anterior and posterior claustrum.

Fig. 4. Neurons with SPX and nesfatin-1 expression in the anterior part of human claustrum. Sequence of images from rostral to caudal side: left column → right column. Negative controls with omission of primary antibodies against spexin and nesfatin-1. Scale bars: 100 μm (M), 50 μm (A-I, N), 20 μm (J-L,O).

Fig 5. Neurons with SPX and nesfatin-1 expression in the posterior part of human claustrum. Sequence of images from rostral to caudal side: left column → right column. Scale bars: 100 μm (M), 50 μm (A-I, N), 20 μm (J-L,O).

Fig 6. Nesfatin-1 expressing neurons in the anterior claustrum. Fluorescence: immunopositive cells labeled with Alexa Fluor 488 (arrows), nuclei counterstained with DAPI. Negative control with omission of primary antibody against nesfatin-1. Scale bars: 20 μm.

Fig. 7. Spatial representation of the intensity of SPX and nesfatin-1 expression in the human claustrum. The heat maps show mean number of neuropeptide expressing cells in the anterior and posterior parts of the claustrum. The subsequent series correspond to the movement from the rostral to caudal region of a specific section of the claustrum (anterior and posterior) presented in Fig. 2.