Neuropeptides in the rat claustrum – an immunohistochemical detection

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Abstract

Neuropeptides are involved in numerous brain activities and are responsible for a wide spectrum of higher mental functions. The main purpose of this outline structural qualitative study was to identify the possible immunoreactivity of classical neuropeptides, as well as novel ones such as nesfatin-1, phoenixin (PNX), spexin (SPX), neuromedin U (NMU) and respective receptors within the rat claustrum for the first time. The study shows the novel identification of peptidergic neurotransmission in the rat claustrum which potentially implicates a contribution of this neuropeptide to numerous central neurosecretory mechanisms.

Keywords;

claustrum, neuropeptides, nesfatin-1, phoenixin, spexin, brain

1. Introduction

Claustrum is a thin layer of gray matter, which is located in a part of the insula, between the external capsule and the extreme capsule of the brain. An important feature of this structure is a bilateral connection with the neocortex – and similarly to the cerebral cortex, the claustrum has sensory, auditory and visual areas (Wang et al. 2023, Torgerson et al. 2015). Claustrum has been demonstrated in the brains of all mammalian species studied so far (Kowiański, et al.1999). However, its anatomy, cytoarchitecture and connectomics are characterized by very significant variability across mammalian species. In lissencephalic animals (e.g. rodents) this structure appears to be small and is difficult to distinguish from the adjacent insular cortex due to the weakly developed capsula extrema. The claustrum of phylogenetically lower mammals is divided into two parts: the insular claustrum and prepiriform. However, an alternative division does assume that only the dorsal part is a true claustrum. The ventral part forms a separate endopiriform nucleus, which is also divided into a ventral and dorsal part (Krettek and Price 1978). The five structural models of the claustrum were distinguished based on its shape and volume (Kowiański, et al., 1999). The claustrum of rodents and insectivores (type I) is often difficult to identify due to its dense structure, almost indistinguishable from the insular cortex. In addition, its ventral part is divided into the minor portions: ventral and dorsal endopiriform nuclei. Type 2 occurs in lagomorphs and guinea pigs, in this case, the claustrum is cut off from the insular cortex and the transition area between the ventral and dorsal parts (Kowiański et al. 1999) where becomes more noticeable and elongated than in type 1. A third type of claustrum with the triangular shape of its insular part is characteristic for carnivore species (Pirone at al. 2015). In the claustrum's development hierarchy, primates that are assigned to the 4th type are the highest ones. The primate claustrum is thin, elongated on the dorsal side, and widens in the medial anterior-posterior part. The last type of claustrum that is the most diverse is the human one (Moryś et al. 1996), on existing mainly of an insular part about 2 mm-thick while the nucleus prepiriformis is rudimentary (Dillingham et al. 2017).

The types of neurons present in the claustral area are varied depending on the subarea and species. Neurons occurring in the area of this structure are characterized by bidirectional transmission. This indicates that connections from specific cortical areas reach the appropriate areas of the claustrum, with this organization of connections, signals are transmitted both from the cortex to the claustrum and in the opposite direction. This feature is necessary to produce fully integrated information. There are two types of spiny neurons in the claustrum, differing in size and morphology of the perikaryon (Mamos et al. 1984, Wójcik et al. 2006). Neurons with smaller cell bodies are characterized by a compact arrangement of processes, as opposed to those with larger ones, in which axons and dendrites are more branched. Since the axons of these cells do not leave the claustrum they are classified as interneurons. It is suggested that these neurons may be particularly sensitive to the synchronization of input data, which exposes the claustral predisposition to integrate various types of information received at single time points and also to generate a pre-processed response. Three types of nerve cells have been identified in the rat claustrum (Mamos et al. 1984). The first type (MS) is the most numerous, medium-sized (15-23 μm) spiny neurons. Based on the morphology of the perikaryon and dendrite, the following three subtypes were distinguished within it: MSI - neurons with an oval or pyramidal perikaryon and a single long dendrite, MSII - round or oval neurons with a short axon, MSIII - oval or round neurons without a dendrite. Type two (MA) are medium-sized, (19-15 μm) spindle-shaped spineless neurons with two main dendrites. The third type (SA) consists of small (12-15 μm) oval or round spineless neurons. A distinct population of somatostatin (SOM), cholecystokinin (CCK), and vasoactive intestinal polypeptide (VIP) expressing interneurons has been found in the rat claustrum. (Eiden et al. 1990). The nitric oxide synthase (NOS) immunoreactive neurons were also detected, many of them exhibit colocalization with SOM and NPY (Kowiański et al. 2008), while the majority of claustral cells are glutamatergic projecting neurons.

Multifunctional, highly anorexigenic neuropeptide nesfatin-1 is considered to play an important role in several brain signaling processes (Pałasz et al. 2012) including mechanisms underlying the generation of anxiety symptoms in animals (Friedrich and

Stengel 2021). Newly discovered hypothalamic neuropeptide phoenixin (PNX), a novel regulator of the gonadoliberin (GnRH) releasing neurons (Treen et al. 2016), has also manifested potential anxiolytic properties in the animal model (Jiang et al. 2015), and its multifaceted modulatory role in the brain has recently been suggested (Schalla nad Stengel 2019). Spexin (SPX) is a newly discovered multifunctional neuropeptide acting at both central and peripheral levels. The highest expression of SPX-expressing neurons in the rat brain has been detected in the hypothalamic magnocellular nuclei (Porzionato et al. 2010). SPX has recently been linked to multiple physiological functions such as reproduction, food-intake regulation (Ma et al. 2018, Wong et al. 2013), cardiovascular/renal function, and nociception (Toll et al. 2012, Porzionato et al. 2010). To date, there is no information available about nesfatin-1, PNX, SPX, and neuromedin U (NMU) expression in the rodent claustrum. The presence of orexin, melanocortin, and NPSR receptors in this brain region remains also understudied. In the present qualitative and descriptive study we aim to provide a structural investigation of the rat brain to reveal the first outline for the neurochemical map of novel neuropeptides expression within the claustrum allowing a more detailed mechanistic understanding of peptidergic regulatory circuits.

2. Materials and Methods

Animals

Male adult Sprague-Dawley rats (n=5) from the Medical University of Silesia Experimental Centre were housed at 22ºC with regular 12/12 light-darkness cycle, access to standard Murigran chow and water *ad libitum*. The research was approved by the Local Ethical Commission for Animal Experimentation at the Medical University of Silesia (agreement No; 36/2012) and all experimental procedures were conducted according to the NIH Guide for Care and Use of Animals.

Immunohistochemistry

Animals were quickly anesthetized with isoflurane inhalation and killed. Brains were excised, fixed by immersion for 48 hrs in 4% paraformaldehyde PBS (pH 7.2-7.4) at 40ºC, dehydrated via graded alcohols at room temperature, cleared in xylene, embedded in paraffin and finally sectioned on a microtome (Leica Microsystems, Germany) in the coronal plane (0.12 to 1.80 mm from bregma) at 7 μm thick slices, according to Paxinos & Watson The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson 2007).

For DAB-single staining, after blocking with 0.1% Triton X-100 (Sigma, T-7878) and 10% serum (horse normal serum for orexin Vector Labs), sections were incubated overnight at 4^0C with the rabbit antibodies against the following rat neuropeptides or their membrane receptors; NPY (monoclonal, ABS 028-08-02, Invitrogen, RRID:AB_1077304, 1:500), nesfatin-1 (polyclonal, H-003-22, Phoenix Pharmaceuticals, RRID: AB_2313672, 1:10000), PNX (polyclonal, H-023-81, Phoenix Pharmaceuticals, RRID: AB_2923380, 1:500), SPX (polyclonal, H-023-81, Phoenix Pharmaceuticals, RRID: AB_2923380, 1: 2000), NMU (polyclonal, A01919-2, Boster,1:200), Y1 receptor (polyclonal, AB 216966, Abcam, 1:500), orexin-1 receptor, OX_1R (polyclonal, PA5-33837, Thermo Fisher Scientific, RRID: AB_2551206, 1:2000), melanocortin receptor 4, MC_4R (polyclonal, AB 24233, Abcam, RRID: AB_2250589 1:800), neuropeptide S receptor, NPSR (polyclonal, BS-11430R, Bioss Antibodies, 1:500) and neuronal marker protein NeuN (polyclonal, AB 128886, Abcam, RRID: AB_2744676, 1:1000). Primary antibodies were followed by biotynylated anti-rabbit/anti-goat secondary antibodies for 30 min, and then an avidin-biotin-horseradish peroxidase complex (Vectastain ABC kit, Vector Labs) for another 30 min, before DAB was used (for 1-2 min) to complete the reaction. Brain sections were dehydrated, mounted on glass slides with medium and coverslipped. Sections incubated with rabbit/mouse IgG instead of primary antibodies were used as negative controls in order to check the specificity of primary antibodies. Additionally, following incubation with primary antibodies against rat nesfatin-1, PNX, SPX and NPSR (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. For basic neuromorphological 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 same planes of the brain were chosen from each slide and morphology of DAB stained perikarya from total areas was analyzed. The number of immunopositive cells was counted in the claustrum to obtain the density of these per standarized area (0.16 mm^2 , frame 400 × 400μm). Data are presented as a mean ± standard deviation (SD).

Results and Discussion

Neuropeptide Y (NPY) expression is characterized by a large population of oval or round neurons (MS neurons) and scattered single polymorphic perikaryal (Fig 2., Tb 1.). The granular neuroplasmatic reaction shows a medium density and its distribution is even, in some cells there is a tendency to concentrate the granulations around the nuclear membrane. Since, NPY is the most common pleiotropic brain regulatory factor (Cerdá-Reverter and Larhammar 2000) a confirmation of its expression in the rat claustrum is highly expected. The results correspond to previous studies reporting NPY and NPY1 receptor expression in the rodent and cat claustrum (Larsen et al. 1993, Kowiański et al. 2008, Reuss et al. 1990, Hinova-Palova et al. 2014). Nevertheless, it is still difficult to identify the function of NPY at the level of claustrum

A similar pattern of expression is characteristic of the Y1 receptor, round and oval perikarya predominate, although there are also a few polygonal and pyramidal cells (MS neurons), granular varicosities are also noticeable in axons (Fig 2., Table 1). However, unlike NPY expression, the DAB reaction density is very high and the neuroplasm is completely filled with granules, taking a uniform brown color. Numerous claustrum neurons of various morphology are characterized by the presence of nesfatin-1. The most intense, dense cytoplasmic reaction is revealed by medium-sized, elongated, spindle-shaped perikarya (MA neurons), sometimes polygonal or pyramidal (MS neurons). A dispersed, although approximately equal in number, population of

larger round or oval cells is characterized by a less pronounced histochemical reaction, the granules are clustered in a ring around the vesicular cell nuclei (Fig 1 and 3, Table 1.). In case of phoenixin (PNX) and spexin (SPX) a completely different pattern of expression occurred. Only a few, scattered oval or polygonal cells of the middle part of the claustrum (MS neurons) with weakly marked expression of PNX (Fig 1 and 3, Table 1). Cells expressing SPX are more numerous, they are oval or spindle-shaped with a medium-density reaction. Perinuclear and reticular DAB staining indicate labeling of the endoplasmic reticulum and Golgi apparatus, especially within the oval interneurons (Table 1). Within some of these cells, a slightly thickened nuclear membrane was distinctly DAB stained.

The presence of nesfatin-1, PNX, and SPX immunoreactive neurons in the rat claustrum does suggest that these novel neuropeptides may act as local neurotransmitters involved in some integrative functions mediated by this brain region. However, it is not yet known, whether nesfatin-1, PNX, and SPX can play a significant role in the claustro-cortical projection but are probably involved in the information transfer and modulation of signal processing in the claustrum. The neurochemical functions of SPX are not fully understood, the neuropeptide may affect serotonin signaling and regulate anxiety behavior in zebrafish (Jeong et al. 2019). Furthermore, chronic treatment with selective serotonin reuptake inhibitors (SSRI) such as escitalopram decreased the SPX expression in the rat hypothalamus but increased its levels in the hippocampus and striatum (Pałasz et al. 2016). Although confirmation of SPX neurochemistry requires numerous further neurostructural and molecular studies, even at the present stage of knowledge this regulatory peptide can be considered as an interesting and potentially important regulatory factor in the rat claustrum. A distribution profile similar to SPX in the claustrum is also presented by neuromedin U. Still, in this case, the reaction density is higher and the immunopositive cells are almost exclusively spindle-shaped (MA neurons), with a slight representation of small polygonal cells (Fig 1., Table 1). NMU-expressing neurons were identified in several rat brain structures such as hypothalamus, nucleus accumbens, hippocampus, amygdala and brainstem (Teranishi and Hanada 2021) but its potential role in the claustrum in not known. The claustrum is a structure rich in neurons expressing OX_1R, that are visible throughout the area, characterized by significant morphological diversity. Polygonal and pyramidal cells (MSI neurons) with high staining density predominate, and round perikarya of smaller diameter are OX_1R-positive (Fig. 2, Table

1). The presence of these OX_1R-expressing neurons is in line with a previous report showing hypothalamo-claustral orexinergic projections in the rat brain (Yoshida et al. 2006), however, function of this pathway remains unclear. Interestingly, the structural and functional MRI imaging of patients suffering from narcolepsy (a disease strictly related to loss of orexinergic neurons in the lateral hypothalamus) has shown an impairment of the fibers connecting lateral hypothalamus to the claustrum (Balotta et al. 2021). Structural abnormalities of pathways connecting hypothalamus to some brain structures involved in sleep-related memory consolidation such as hippocampus and possibly claustrum can probably be related to the loss of orexinergic neurons. On the other hand, the presence of orexinergic innervation in the rat claustrum is called into question when the role of neurons with melanin concentrating hormone (MCH)-expression is pinpointed (Barbier et al. 2017). The cells with MC4R-expression were characterized by numerous, large perikaryon of oval, polygonal, or round shape, but the staining density is relatively low (Fig. 2., Table 1). A very strong histochemical reaction is manifested by smaller polygonal, pyramidal and spindle-shaped perikarya scattered between them. The NPSR distribution model in the claustrum is largely analogous to SPX, but in this case, few diffusely distributed, almost exclusively round cells are characterized by a weak cytoplasmic reaction (Fig. 2 and 3, Table 1).

At present, the role of nesfatin-1, PNX, SPX as well orexinergic, melanocortin, and neuromedin signaling in the claustrum neurophysiology remains an area of speculation, but undoubtedly further studies on this regulatory factor, e.g. coexpression with other neuropeptides and neurotransmitters are definitely overdue and necessary. Moreover, the 3-D imaging of the aforementioned molecules distribution in the whole rat claustrum, as well as the study of potential interactions between these factors is of paramount importance.

CRediT authorship contribution statement

AB and AP came up with a research idea. AB, ASS. ŁF and AP-N conducted animal experiments. ASS conducted statistical analysis. AP, ADV, JJW contributed to the interpretation of the results. AB and AP made significant contributions to the manuscript. AP oversaw the conduct of this study. All authors reviewed drafts of the manuscript and critically revised it for intellectual content. All authors approved the final version of the manuscript for publication.

Declaration of Competing Interest

The authors declare that they have no known competing interests.

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Figure captions

Fig.1. Neuroanatomy of the rat claustrum. A simplified structural grid is partly based on Paxinos et al. (2007); coronal sections at the following levels; 1.80 (A.) and 0.48 (B.) mm from bregma. Mean number of cells with the neuropeptide and receptor expression in the rat claustrum, n=5 (C.) Standard Nissl staining (A-D). Expression of nesfatin-1, phoenixin (PNX), spexin (SPX) and neuromedin U (NMU) in the rat claustrum. Scale bars: 200 µm (a), 100 µm (b,e,n), 50 µm (c, f, g, h, i, k, m, n, o, p), 20 µm (d, l, j). Claustrum area is marked with a dashed line. Abbreviations: AID, agranular dorsal insular cortex; AIV, agranular ventral insular cortex; DCl, dorsal claustrum; DEn, dorsal endopiriform nucleus; DI; dysgranular insular cortex; IEn, intermediate endopiriform nucleus; GI, granular insular cortex; lo, lateral olfactory tract; pir, piriform cortex; S2, secondary somatosensory cortex; VCl, ventral claustrum.

Fig. 2. Expression of NeuN, neuropeptide Y and selected neuropeptide receptors: Y1R, MC4R, OX1R and NPSR in the rat claustrum. Negative controls with omission of primary antibodies anti-nesfatin-1 and SPX. Scale bars: 200 μm (A, N), 100 μm (B, E, H, K, L, M, O), 50 μm (C, F, I, K, M), 20 μm (D, G, J, L, N, P). Claustrum area is marked with a dashed line.

Fig. 3. Nesfatin-1, SPX, PNX and NPSR expressing neurons in the rat claustrum. Fluorescence: immunopositive cells labeled with Alexa Fluor 488(green) and 555 (red), nuclei counterstained with DAPI. Scale bars: 20 μm.

Table 1. Morphological characteristics of neuropeptides-expressing perikarya in the rat claustrum.