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Immunolocalization of different neuropeptides in human interthalamic adhaesion indicates its functionality

Imunolokalizacija različitih neuropeptida u intertalamičkoj adheziji čoveka ukazuje na njenu funkcionalnost

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Abstract

Background/Aim. The interthalamic adhaesion (IA), gray matter connecting both thalami, is absent in about a quarter of human brains. Controversies are present about the nature and functional significance of the human IA. **Methods.** In six adult human brains we investigated the expression of different neuropeptides: somatosatin (SOM), neuropeptide Y (NPY), ghrelin, neurotensin (NT), adrenocorticotropic hormone (ACTH), substance P (SP) and L-enkephalin (L-Enk) in neurons and/or neuropil of the IA, using immunohistochemistry (streptavidin-biotin technique). **Results.** In neurons, as well as in fibers, we found immunoreactivity for ghrelin, SOM, L-Enk and NT. However, reactivity for NPY, SP and ACTH was present only in fibers within the IA. Fusiform neurons were immunoreactive for SOM, Ghrelin, L-Enk, and NT, neurons with oval perikaryon for SOM, and L-Enk, triangular neurons showed immunoreactivity mainly for NT and multipolar neurons for NT and L-Enk. **Conclusion.** These findings can contribute to the understanding of the function of interthalamic adhaesion, and to resolving the question whether it is a vestigial structure. No mather if the interthalamic adhaesion is vestigial structure or not, its presence or absence could be a marker for other, genetic or functional differences between human brains. Our findings indicate the presence of certain neuronal organization in the human interthalamic adhaesion which could have functional significance, and do not support its vestigial nature.

Key words:
brain; humans; thalamus; neuropeptides; immunohistochemistry.

Apstrakt

Uvod/Cilj. *Adhaesio interthalamica* (AI), siva masa koja povezuje oba talamusa, odsutna je kod oko jedne četvrtine ljudi. Postoje različita mišljenja o prirodi i funkcionalnom značaju AI čoveka. **Metode.** Na šest mozgova odraslih osoba imunohistohemijskim metodama (tehnika streptavidin-biotin) istraživali smo prisustvo različitih neuropeptida: somatosatin (SOM), neuropeptid Y (NPY), grelin, neurotenzin (NT), adrenokortikotropni hormon (ACTH), supstanca P (SP) i L-Enkefalin (L-Enk) u neuronima i/ili neuropilu AI. **Rezultati.** Našli smo pozitivnu imunoreaktivnost na grelin, SOM, L-Enk i NT u neuronima i vlaknima. Reaktivnost na NPY, SP i ACTH bila je prisutna samo u vlaknima unutar AI. Fuziformni neuroni su bili imunoreaktivni na SOM, grelin, L-Enk i NT, neuroni sa ovalnim perikarionom na SOM i L-Enk, triangularni neuroni su, najvećim delom, bili imunoreaktivni na NT, dok su multipolarni neuroni bili imunoreaktivni na NT i L-Enk. **Zaključak.** Dobijeni nalazi mogu da doprinesu razumevanju funkcije *adhaesio interthalamica* čoveka, kao i razmatranju pitanja da li je *adhaesio interthalamica* vestigijalna struktura ili ne. Nezavisno od toga da li je *adhaesio interthalamica* vestigijalna struktura ili ne, njeno prisustvo ili odsustvo može, takođe, biti i marker za druge, genetske ili funkcionalne razlike između ljudskih mozgova. Rezultati ukazuju na postojanje određene neuronske organizacije u *adhaesio interthalamica* čoveka koja bi mogla imati funkcionalni značaj, i ne govore u prilog njene vestigijalne prirode.

Ključne reči:
mozak; ljudi; talamus; neuropeptidi; imunohistohemija.

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Introduction

Interthalamic adhesion (massa intermedia; middle commissure; gray commissure), as a specific part of human diencephalon connects the medial surfaces of the left and right thalamus within the third ventricle. Specific is the fact that the human interthalamic adhesion (IA) is variable in the presence (absent in 22–30% of subjects), localization, size, and density, as well as in the number of its neurons [1]. Males without the IA die earlier than males with it, but such finding has not been reported for females [2]. Male neurological patients with the IA have relatively lower nonverbal factor scores [3]. Identification of nuclei within the human IA based on their homology to mammalian thalamic midline nuclei is not clearly established, so that the controversial and non-uniform terminology complicates the studies of IA. Various midline nuclei in the IA (paratenial, paraventricular, reuniens, rhomboidal, median central and intermediodorsal nucleus) [4, 5], are actually very small and difficult to demarcate in humans [2, 6–9]. Human IA contains only nucleus reuniens and should not be unconditionally compared with the IA of animals and can thus be considered as a specific finding [2, 8]. Within the IA there are four types of neurons on Golgi sections: fusiform (most characteristic for human IA), oval, triangular, and multipolar [10]. In addition, the presence or absence of the IA in humans can be an indicator for some other genetic and/or functional differences between the persons with and without the IA. For example, the absence of the IA in schizophrenia could be a marker of developmental abnormalities in the neural network including the thalamus and connected amygdala regions [11].

The aim of this study was to investigate the pattern of the expression of different neuropeptides in the human IA, in order to contribute to better understanding of this structure in the human brain.

Methods

In this study 6 human brains with the IA (4 males and 2 females; age 45 to 65 years), and without any visible pathological changes or neuropsychiatric history were examined. The brains were obtained during the routine autopsies at the Institute of Pathology of Faculty of Medicine in Belgrade with postmortem intervals from 4 to 7 hours. The tissue blocks of thalamus containing the IA with adjacent paraventricular regions were dissected and fixed by immersion (10% formalin solution in isotonic phosphate buffer) during 3 weeks, dehydrated, and embedded in paraffin. Frontal serial sections (4 mm thick) for immunohistochemistry were deparaffinized in xylene and rehydrated through decreasing concentrations of ethanol. Afterwards, the slides were immersed in citrate buffer (pH 6.0) (Target Retrieval Solution, ready-to-use; DAKO) and heated for 21 min in a microwave oven at 680 w (except for neurotensin and L-Enk). After cooling, slides were rinsed in distilled water and treated with 3% H_2O_2 in distiled water for 10 min to reduce endogenous peroxidase activity. Immunostaining was performed by incubating tissue sections with appropriate sera for 60 min at room temperature in a humid chamber or overnight at +4°C, using the streptavidin-biotin technique (LSAB+ Kit, Peroxidase Labeling, K0690, DAKO Cytomation, Denmark). The list of the primary antibodies is shown in Table 1.

Table 1

Antibodies used in the present study

Antibody	Monoclonal (Mo)/Polyclonal (Po) Antigen unmasking technique	Manufacturer Code No. or received from	Dilution	Detection system
Mo mouse anti-hu Adrenocorticotropin (ACTH), Clone 02A3	Mo Not recommended	DAKO Cytomation Denmark M3501	1:50–1:75	LSAB
Po goat anti-Ghrelin	Po Microwave-20min, 0.01M citrate retrieval solution pH6.0 or DAKO cytomation target retrieval solution No. S1700	Santa Cruz Biotechnology, INC Sc-10368	1:100	LSAB+
Po rabbit anti-hu Substance P, 4-11	Po Not recommended	MP Biomedicals, USA 11845	1:400–1:800	LSAB+
Po rabbit anti-hu Somatostatin	Po Microwave-20min, 0.01M citrate retrieval solution pH6.0 or DAKO cytomation target retrieval solution No. S1700	DAKO A/S Denmark A 0566	1:200–1:300	LSAB+
Po rabbit anti-hu neuropeptid-y (NPY)	Po Microwave-20min, 0.01M citrate retrieval solution pH6.0 or DAKO cytomation target retrieval solution No. S1700	Euro-Diagnostica B 48-1	1:400–1:800	LSAB+
Neurotenzin (NT)		R. L. Eskay, Bethesda, MD, USA	1: 15 000	
L-enkephalin		R. L. Eskay, Bethesda, MD, USA	1:10 000	

After washing in 0.01M phosphate buffered saline (PBS, pH 7.2) specimens were incubated with biotinylated anti-mouse, anti-rabbit and anti-goat immunoglobulins for 30 min at room temperature in a humid chamber, and subsequently incubated with peroxidase-conjugated streptavidin–biotin for another 30 min. After incubation, the sections were rinsed in 0.01M PBS. Antigen-antibody complexes were visualized with 3-amino-9-ethylcarbasole (AEC, No. K3469, DAKO Cytomation, Denmark) or diaminobenzidine hydrochloride (DAB, No. K3468, DAKO Cytomation, Denmark) substrate solution and afterwards washed in distillated water. The cell nuclei were contrastained with Mayer's haematoxylin. Control stainings included omission of the primary antisera and replacement of the primary antibody by non-immune serum diluted 1:10 and by the diluent alone.

Immunoreactive neurons and fibers were studied and photographed on the light microscope (Olympus) under different magnifications.

Results

Both, immunoreactive fibers and neurons were found for NT, ghrelin, L-Enk and SOM. However, immunoreactivity to SP, NPY and ACTH was found only in fibers, and not in neurons of human IA.

Neurons in the human IA immunoreactive for SOM, NT, ghrelin, and L-Enk

Ghrelin immunoreactivity (IR) was found in medium sized oval and fusiform neurons, but IR granules, accumulated in one part of soma opposite to the nucleus were sparse (Figure 1 A). Ghrelin IR fibers were found in all cases.

Somatostatin IR of ependyma was intense, while there was no reaction in the subependimal region. SOM IR neurons were numerous and large, and their bodies were fusiform or oval (Figure 1 B and C). Somatostatin IR fibers were closely related to non-reactive neurons.

L-Enk IR neurons were very rare and of fusiform, oval and multipolar shape, with some reactivity present also in their dendrites (Figure 1 D). In human IA also L-Enk imunoreactive fibers were present.

NT IR neurons were grouped (Figure 1 E and F), but not all neurons in such groups were immunoreactive. The majority of NT IR neurons were triangular, but NT IR was found also in fusiform neurons. Varicose NT fibers were related to other non-reactive neurons (Figure 1G).

Fiber networks in the IA immunoreactive for NPY, SP and ACTH

ACTH IR fibers were rare, branched and often cut in their course directed supraependymaly. In IA they were located around cell bodies and ramified around the neurons (Figure 2A).

SP – long varicose fibers were commonly located around non-reactive neurons (mainly around fusiform neurons) (Figure 2B).

NPY – varicose fibers were relatively rare and when present they were not distant from each other (Figure 2C).

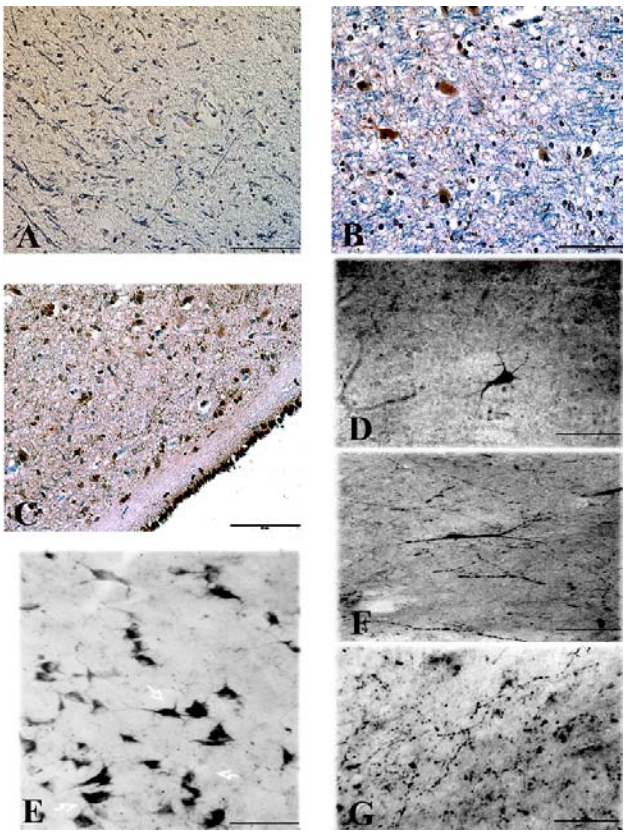

Fig. 1 – Human interthalamic adhesion, frontal section: immunoreactivity both in neurons and fibers.
A – ghrelin; B and C – somatostatin; D and F – L-enkephalin; E – Neurotensin (NT) positive neurons in groups, and G – NT positive fibers. (Scale bars in A and C = 50 µm; B = 40 µm; in D = 100 µm; E and = 100 µm; G = 20 µm).

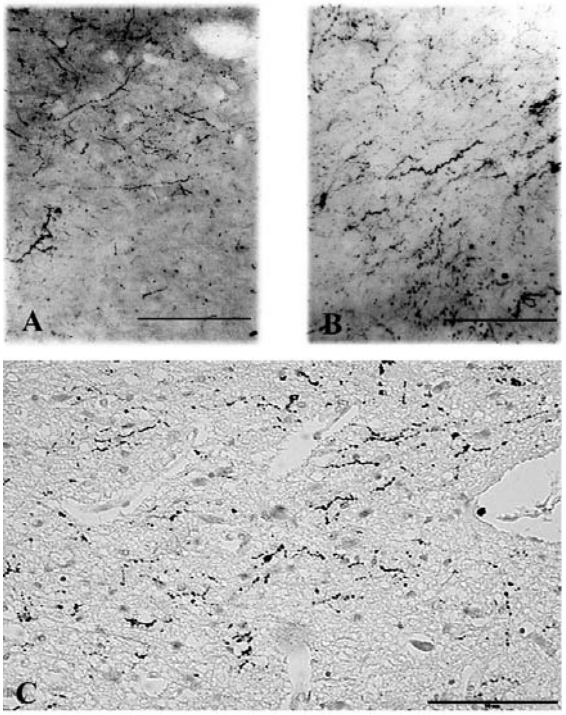

Fig. 2 – Human interthalamic adhesion, frontal section.
Immunoreactivity for adrenocorticotropic hormone (ACTH), substance P (SP) and neuropeptide Y (NPY) is present exclusively in fibers. A – ACTH; B – SP; C – NPY. (Scale bars in A and B= 50 µm; C= 100 µm).

Specific IR and different neuronal types in the human IA

Considering potential neuronal forms representing specific neuropeptide IR, we can conclude that fusiform neurons showed IR for SOM, ghrelin, L-Enk, and NT, neurons with oval perikaryon for SOM and L-Enk. Grouped triangular neurons showed IR mainly for NT, and multipolar neurons for NT and L- Enk (Figure 1).

Discussion

Comparing to other mammals, the human IA is specific in its considerably smaller size, and in its variability, including its absence in a considerable percent of cases [12]. Contrary to the human brain, IA in diversity of mammalian species is generally of considerable size and leaves only smaller and narrow space of the third ventricle [4, 13, 14]. The question is whether IA in humans, with its neurons and fibers, is simply vestigial structure or not. The midline nuclei, variously defined and designated by different investigators, include the diverse *nn. reunientes*, and seem particularly related to the periventricular fiber system, as well as to other intrinsic diencephalic connections. While some authors interpret these grisea as phylogenetically "very ancient", the mammalian *nn. reunientes* may also be interpreted as manifestations of "progressive differentiation" [12]. Even if IA is a vestigial structure, its significance for the human brain cannot be necessarily ascribed exclusively to its specific function, but its presence or absence can somehow indicate different genetics, development or different modes of human brain functioning. However, it should not be neglected that the comparative small size of the IA structure, is not the exclusive indicator of its function, and that even small structures in the brain can have important functions [15].

For a long time the midline thalamic nuclei were considered nonspecific, but their designation as a part of "diffuse", "nonspecific", "generalized" or "commissural" systems, institutes misleading simplifications [16]. Dense nerve cell and/or neuropil immunoreactivity of human midline nuclei characterized by calbindin-D–28K, and calretinin indicate their limbic connections [17]. Executive deficits in humans may arise from combined lesion of several structures, including midline nuclei and in monkeys IA is involved in motor functions [18, 19]. Anatomical relationships, combined with functional studies in animals and in humans, lead to propose that the midline, and intralaminar nuclei of the thalamus, as a whole, play a role in awareness, with each of the groups having a role in a different aspect of awareness [5].

In previous studies [20] we found the differences in modalities of functioning of human intelligence in persons with and without IA. In subjects with the IA, the complex simultaneous processing (the ability of spatial visualization in particular, which means the capacity for 3D mental manipulation of objects) is more developed. On the other hand, the simpler perceptive processing (which includes perceptive search, identification, visual attention and 2D object manipulation) is more developed in subjects without IA [20]. The presence of neuropeptides reported here in specific circular formations of IA neurons, together with the uncertain vestigial nature of IA in human, suggest the necessity for further comparative studies.

During this study we were not able to clearly delineate any of nuclei on frontal sections of the human IA what corresponds to the statement of Gottschick [21]. Within the human IA, in addition to four types of neurons, five fiber systems were found: from paramedian, dorsomedial, and ventral posterolateral nucleus of thalamus, from nucleus centromedianus, intralaminar, and neighboring nuclei [10, 22]. Very high densities of histamine immunoreactive fibbers mostly oriented sagittally were found in the human thalamus midline nuclei [23]. It cannot be excluded that the circular formations that we previously described in the human IA represent a kind of "bed nucleus" for some of fiber tracts within the human IA [24]. The modulatory neuropeptides as modulatory transmitters are released from both synaptic terminals and axonal varicosities, providing not only 'point to point' contact (pre- to postsynaptic), but also extending integrative potential, 'volume control', which could be considerably different in human brains with and without IA [25].

Conclusion

Contributing to the elucidation of the function of the human interthalamic adhaesion, we can conclude that even if interthalamic adhaesion is a vestigial structure in humans, our previous and current results do not exclude a certain degree of neuroanatomical organization within the human interthalamic adhaesion.

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