Functional anatomic examination of the dorsolateral hypothalamus in rats

PhD thesis

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1. INTRODUCTION

The dorsolateral hypothalamic area (DLH) covers a relatively large, but not strictly outlined area in the posterior part of the hypothalamus. This area extends rostro-caudally from the level of the caudal aspect of the paraventricular nucleus (about 2.2 mm caudal to the bregma level, in adult rats) until the level of the caudal end of the third ventricle (3.4 mm caudal to the bregma) in rats.

Based on our previous quantitative histological studies by measuring cell density on coronal serial sections of the hypothalamus in rat and on quantitative topographical analysis of orexin and MCH-expressing neurons in the DLH, we divided the DLH into three areas with a total of 8 subdivisions. The *dorsomedial hypothalamic area* incorporates the upper portion of the posterior periventricular area (PeVe), the lateral portion of the dorsal part of the dorsomedial nucleus (DMN), and the area between the dorsomedial and perifornical area, called dorsomedial hypothalamic subdivision (DMH). The *perifornical area* (PeF) includes the rostral (PeFr), the caudomedial (PeFcm) and the caudolateral (PeFcl) subdivisions of the perifornical area. In adult rats, the PeFr occupies a territory between the fornix and the mamillothalamic tract from the dorsomedial nucleus until the medial border of the lateral hypothalamic area. Rostro-caudally, it extends between 2.2 and 2.8 mm caudal to the level of the bregma. A large area over the fornix with a rostro-caudal extension between 2.8 and 3.4 mm caudal to the bregma represents the caudal portion of the perifornical area. An imaginary vertical line through the fornix divides this field into medial and lateral areas. The *lateral hypothalamic area* (LH) consists of two subdivisions in the posterior hypothalamus, one ventral (LHv) and one dorsal (LHd) lateral hypothalamic subdivision separated by a virtual line between ventral edge of the fornix and the medial edge of the pedunculus cerebri/capsula interna.

Orexin A and B, also called hypocretin 1 and 2, are a pair of neuropeptides that were discovered simultaneously in 1998 in two independent laboratories. Orexin-expressing neurons exist exclusively in the DLH within the central nervous system. From these neurons four orexinergic pathways originated and the fibers of these pathways project across the entire neuraxis from the cortex to the spinal cord. Within the rat brain, the orexin neural field extends from just caudal to the paraventricular hypothalamic nucleus to just rostral to the tuberomammillary nucleus; and from the third ventricle to the optic tract. The distribution pattern of orexin-containing neurons is unique, the neurons do not form a distinct nucleus; do not respect the anatomical borders of the nuclei and subdivisions visible on coronal sections of the hypothalamus. Based on the topographical distribution and density of orexin-containing

neurons, we outlined three areas with five subdivisions within the orexinergic neural field as follows: the dorsomedial area has been divided only two (PeVe and DMNd), the posterior lateral hypothalamus also two (LHV and LHd) subdivisions, while the perifornical area remained undivided.

The location of the orexin-containing neurons and fibers indicates their regulatory role in several physiological functions.

Orexin neuropeptides have orexinergic effect and this was the reason why these peptides were named „orexin" after the Greek word orexis, meaning appetite. As premotor neurons, orexin-containing cells can affect the energy balance and food intake throughout their relations with sympathetic and parasympathetic preganglionic cell groups.

Orexin neurons are thought to sustain wakefulness and suppress REM sleep by activating the histaminergic neurons in the tuberomamillary nucleus, noradrenergic neurons in the locus coeruleus, serotonergic neurons in the raphe nuclei and the cholinergic cells in the basal forebrain.

In the course of stress-response, orexin-containing neurons are capable of influencing the hypothalamic-pituitary-adrenal axis, as well as the sympathetic-adrenomedullary system.

2. AIM OF EXPERIMENTS

To the better understanding the functional anatomy of the dorsolateral hypothalamus, its connections and regulatory roles were examined by applying two different approaches.

1. First, Fos-activation of the orexin-containing neurons was examined in three different experimental models: following fasting, REM sleep-deprivation and acute pain-evoked stress. The goals were the following:
    a. comparing the Fos-activations to determine the extent of participation of the orexin-containing neurons in control of the three examined functions;
    b. comparing the percentages of the Fos-activated orexin-containing neurons located in the different DLH subdivisions to determine whether there are function-specific orexin-containing neuronal cell groups or subdivisions.
2. Second, the brainstem projections of neurons located in various subdivisions of the DLH were examined, especially the biogenic amine cell groups of the brainstem to find out through which lower brainstem structures take part in the orexin neurons in the control of food intake, sleep-wake cycle and stress-response. The goals were the following:

a. to determine which brainstem nuclei, subdivisions and areas innervated by fibers originated from the DLH; and the participation of DLH subdivisions in the innervations of the lower brainstem;
b. to value the participation of DLH subdivisions in the innervation of the brainstem cell groups by determining the density of anterogradely labeled, DLH originated fibers (projections of eight subdivisions in 7 brainstem cell groups, e.g. monoamine cell groups);
c. to determine which adrenaline and noradrenaline cell groups of the brainstem innervated by the orexin-containing neurons of the DLH.

3. MATERIAL AND METHODS

3.1. Experimental animals

Adult male Wistar rats (250–300 g) and C57/Bl6 mice (30 g) were used. Animals were housed three per cage, at a temperature of 22±1◦C, with 12h light and 12h dark periods. All efforts were made to minimize the number of animals used and also their suffering. Experiments were performed according to the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the National Institutes of Health "Principles of Laboratory Animal Care" (NIH Publications No. 85-23, revised 1985), as well as specific national laws (the Hungarian Governmental Regulations on animal studies, December 31, 1998). All experiments were approved by the National Scientific Ethical Committee on Animal Experimentation, and permitted by the government (Food Chain Safety and Animal Health Directorate of the Government Office for Pest County, Permit Number: XIV-I-001/2263-4/2012).

3.2. Experimental models

Three different models have been applied in our experiments: 24h fasting, 72h REM sleep-deprivation and pain-induced acute stress. They proved appropriate to qualitative and quantitative evaluations of the activity of orexin-expressing neurons in response to the experimentally altered homeostasis. On the basis of information obtained in previous studies, optimal parameters (doses, timing, experimental conditions) in each experimental model were chosen.

3.2.1. Fasting-refeeding experimental protocol

Rats were randomly divided into three major groups. Controls (n=4) were fed *ad libitum*. Fasted rats (n=7) were food-deprived for 48h. Refed rats (n=6) were initially fasted for 48h, then refed for 2h before having been sacrificed. All of the rats had continuous *ad libitum* access to drinking water.

3.2.2. REM sleep-deprivation protocol

Animals were randomly divided into two groups: home cage (HC) and REM sleep-deprived (SD) group. HC animals (n=8) kept under undisturbed conditions during the whole experiment. Animals of the SD group (n=6) spent 72h on small platforms, situated in the middle of a round water tank (41 cm diameter) at lights on (each rat in individual water tank and platform). The diameter of the platforms was 6.5 cm and their height was 18.5 cm. The surface of the platform was 0.5 cm above the water level. Food and water was available *ad libitum* for all animals throughout the experiment.

3.2.3. Formalin injection

Rats were randomly divided into formalin-injected (n=5) and intact control (n=4) groups. Sixty minutes after injections rats were sacrificed. As a short-term nociceptive stressor, formalin (0.2 ml/100 g body weight) was injected subcutaneously into the hind limb of the animals. By formalin injections, we did not want to investigate the painful effect of formalin, rather the effect of the complete stress situation, including handling, the painful injection procedure itself, *plus* the pain-elicited acute effect of formalin. Therefore, we compared the effect of stress to intact control animals.

3.3. Stereotaxic application of biotinylated dextran-amine

Under anesthesia, rats received a single stereotaxically placed iontophoretic injection of biotinylated dextran-amine (BDA – 25 mg/250 μl PB, 10000 MW) into one of the eight subdivisions of the DLH unilaterally. Glass capillaries were used with 18-22 μm tip diameter for the electrophoresis by applying 7 mA positive current pulses (7 sec on, 7 sec off) for 20 min; the capillaries were then left in place for 10 min before retraction. The animals were killed 14 days after surgical intervention, brains were removed. The BDA-containing cells and fibers were visualized by ABC method.

3.4. Histological protocols

Perfusion, fixation, sectioning: Rats were deeply anesthetized, fixed and perfused transcardially with 50 ml 0.9% saline followed by 300 ml 4% paraformaldehyde (pH 7.4). For electron microscopic experiments, the fixative solution contained additional 0.08% of glutaraldehyde. Brains were removed and postfixed in 4% paraformaldehyde solution and transferred to PB containing 20% sucrose for 24 hours. Serial coronal sections were cut on a frigomobile. The section thickness was 40 μm for the experimental models and in the case of mice brains, and 50 μm thick in the other cases. For electron microscopy, 50 μm thick serial coronal sections were cut on a vibratome.

Immunohistochemistry: Fos protein, orexin A, tyrosine hydroxylase (TH), phenylethanolamine N-methyltransferase (PNMT) and synaptophysin were visualized in the following immunostainings:

Orexin A and TH double labeling for light microscopic (LM) investigations

Orexin A and PNMT double labeling for LM investigations

Orexin A, TH and synaptophysin triple labeling

Orexin A, PNMT and synaptophysin triple labeling

Orexin A and TH double labeling for electron microscopic (EM) investigations

Orexin A and PNMT double labeling for EM investigations

Orexin A and Fos double labeling

3.5. Documentation

Double labeled sections were examined by using an Olympus BX60 microscope, and photomicrographs were taken by a SPOT Xplorer digital CCD camera, and SPOT Advanced software. Confocal images were acquired with a Nikon Eclipse E800 confocal microscope equipped with a Bio-Rad Radiance Laser Scanning System. Ultrathin sections were examined by using a JEOL 1200 EX electron microscope and and photographs were taken by an Olympus Morada digital camera.

The contrast, brightness and grayscale of the images were adjusted by Adobe Photoshop CS 8.0. The photomontages were prepared by the same software.

3.6. Evaluation and statistics

3.6.1. Analysis of orexin and Fos double immunostainings

Orexin neurons with and without Fos-immunostaining were counted in each DLH areas in all of the sections bilaterally on every 5th sections from 2.00 mm till 3.50 mm caudal to the level of the bregma. The percentage of double-labeled cells was calculated in each examined dorsolateral hypothalamic area. The effect of the applied treatments on Fos-activation in orexin-expressing neurons in the DLH and in the investigated areas was analyzed, statistically. Values obtained in each area were evaluated using factorial analysis of variance (ANOVA) with the factors „treatment" (fasting, SD and formalin-stress). *Post hoc* analysis was performed using Tukey's *post hoc* test in case of significant main effect in ANOVA. The percentual distribution of the double and single labeled orexin neurons in the DLH subregions was analyzed statistically in seven rostro-caudal planes using Student's t-test. All data were presented as the mean ± SEM. The statistical significance level was set at $p < 0.05$.

3.6.2. Verification of the BDA applications and histological analysis of BDA labeling

A total number of 42 rats were BDA-injected. Serial coronal sections were cut and stained for the topographical determination of the sites of the injections. The correct sites and topography within the proper DLH subdivisions consisted the basic criteria for acceptance. Finally, 22 brains of 42 were considered acceptable for further cut and mapping BDA projections in the lower brainstem. The relative abundance of BDA-labeled fibers was evaluated by the following grading: absence of labeled fibers (-), very low (±), low (+), moderate (++) and high (+++).

The data from individually evaluated brains were grouped based on the given subdivisions. The data were estimated in the following aspects: 1) which and how many of the brainstem areas/nuclei/subdivisions showed labeled fibers after injections into the 8 investigated DLH subdivisions; 2) the participation of the 8 DLH subdivisions in the innervations of the 116 brainstem areas/nuclei/subdivisions (how many subdivisions project to a particular brainstem subdivisions); 3) which brainstem areas/nuclei/subdivisions showed up high densities of labeled fibers; 4) evaluation of BDA fiber density in the special cell groups of the brainstem.

3.6.3. Quantitative analysis of the confocal microscopic photographs

The relationship of orexin A-containing axons and varicosities with PNMT-containing cells and dendrites in the C1, C2 and C3 adrenaline cell groups, as well as their synaptophysin content was examined on 216 confocal micrographs. The same examination was made in the A1 and A2 noradrenaline cell groups with TH cells and dendrites on 131 confocal

micrographs. Every labeled axon, dendrite and soma was determined and counted. Close contacts were considered when there were close appositions with no discernable gap between the two profiles (orexin-containing axon and PNMT/TH-positive neuron), in the same focal plane. The percentage of synaptophysin-containing close contacts was determined in the C1, C2 and C3 adrenaline, as well as in the A1 and A2 noradrenaline cell groups.

4. RESULTS

4.1. Activation of orexin A-containing neurons in different functional models

4.1.1. Number, neural density and distribution of orexin-containing neurons in the DLH

4.1.1.1. Quantitative data of the DLH

The orexin-neuron containing area of DLH was outlined into five regions based on the density and topography of the orexin-immunoreactive (ir) neurons: PeVe, PeF, DMNd, LHv and LHd. The total number of quantified orexin-ir neurons was 1332.3±22.6 (n=40). The *total volume* of the DLH in the investigated coronal sections represented about 20% of the total DLH (every fifth rostro-caudal sections have been quantified). Thus, the total number of orexin-ir cells per animal could be between 6500 and 7000. In the control rats, the experimental conditions did not lead to any significant differences in the total number of orexin-ir neurons, or in the number of orexin-containing neurons counted the five DLH areas, individually. High but not significant differences were found in the number of orexin-ir cells between the three experimental models. Few orexin-expressing neurons were seen out of the DLH area in the anterior hypothalamic area, or in the subthalamus. Their total percentage in the counted orexin cells was less than 1%. This small number can not take account in the statistical analysis of the data. The orexin-containing neural field was divided into seven rostro-caudal planes. The most rostral plane (between 2.04-2.28 mm caudal to the level of the bregma) contained only 1.26% of the total orexin neurons. The most orexin-immunopositive cells (29.99%) were located in the middle portion (between 2.76-3.00 mm caudal to the level of the bregma) of the DLH.

4.1.1.2. Quantitative data of the DLH subdivisions

Orexin-ir neurons of the *PeVe* located along and over the dorsal part of the caudal portion of the third ventricle. The volume of this area was quite small, with a relatively high density of

orexin-ir neurons (68.48±3.11 cell/mm^3). In spite of this, only the 4.25±0.40% of the hypothalamic orexin-ir neurons located here.

Most of the orexin-ir neurons (44.26±3.63%) were situated in the *PeF*, where the neuronal density was the highest in the DLH: 222.65±12.94 cell/mm^3. This density of orexin neurons showed significant alterations through the rostro-caudal sections.

The DMNd is the second densest area regarding orexin-ir neurons (88.99±2.46 cell/mm^3), it contains 12.84±0.75% of the orexin-ir cells in the DLH.

In the *LHv* the density of orexin-ir neurons was relatively low (39.89±1.73 cell/mm^3); 15.24±0.90% of the orexin-ir neurons located here. In contrast, the *LHd* contains 23.00±1.85% of the total orexin-stained neurons with a relatively high cell density (84.70±4.16 sejt/mm^3).

4.1.2. Fos activation of the orexin-containing neurons in different experimental models

In general, each of the three interventions activated a fairly high number of orexin-ir neurons in the DLH. The topographical patterns of activated cells showed some diversities on the basis either of subdivisions, or their rostro-caudal locations, or the impact of the experimental models.

4.1.2.1. Fos activation of orexin neurons in the DLH subdivisions after fasting

In control animals, 6.19±3.53% of the orexin-containing neurons was Fos-positive. The distribution of the activated neurons was somewhat inhomogeneous. The highest (PeVe: 8.18±4.14% of the orexin-neurons of this area) and the lowest (LHd: 4.65±2.40%) values were comparable.

In response to fasting, 32.15±3.92% of the orexin-containing neurons expressed Fos in the DLH. The highest number (14.53±2.60% of the total orexinergic neurons) was located in the PeF. The DMNd and the PeVe showed also strong response to fasting (in both of them the percentage of the Fos-activated orexin neurons was more than 60%). The activation of orexin-ir cells in the lateral hypothalamic area was relatively low; the number of Fos-positive neurons did not reach the 20% of the total number of orexin-ir cells in the LHv and LHd.

The 2h-long refeeding did not alter the fasting-induced activation in the DLH significantly.

According to our statistical analysis, Fos-positivity in orexin-expressing neurons increased markedly as a result of fasting ($p < 0.0001$, $F_{1,45} = 93.7151$). The result of Tukey's *post hoc* comparison showed significant difference between treated and control groups in the PeF ($p < 0.05$), DMNd and PeVe (both of them $p = 0.0002$). Considering refeeding, Fos expression

in orexin-containing neurons increased markedly ($p < 0.0001$, $F_{1,40} = 141.09$). Tukey's *post hoc* comparison showed significant difference in PeF, DMNd and PeVe (all of them $p < 0.001$), compared to controls.

4.1.2.2. Fos activation of orexin neurons in the DLH subdivisions after sleep-deprivation (SD)

In this experimental group, home cage (HC) animals served as controls. Due to the special experimental conditions, the animals showed relatively high Fos activation: 20.79±5.51% of the orexin-ir neurons was Fos-labeled.

The 72h-long SD elicited a robust Fos expression (54.65±2.37%; $p < 0.0001$, $F_{1,50} = 105.8929$) in the orexinergic neurons. This value represented an additional 33.86% activation to the high "control" values in HC rats. The most activated orexin neurons (26.11±2.19%) were in the PeF. The percentages of double-labeled neurons in the most responding areas exceeded the 60% (DMNd and PeVe). The percentage of double-labeled neurons in the lateral hypothalamic area reached over 40%. Tukey's *post hoc* comparison showed significant increase in Fos-positivity of orexin-neurons in all investigated areas, compared to HC controls (in all cases $p < 0.001$).

4.1.2.3. Fos activation of orexin neurons in the DLH subdivisions after formalin injection

In intact control animals, 16.92±6.68% of the orexin-ir neurons showed Fos positivity. Formalin injections increased Fos-positivity of orexinergic neurons markedly, 45.68±1.31% of the orexin-ir cells were double-labeled ($p < 0.0001$, $F_{1,35} = 59.3332$). The PeF contained the most activated orexin neurons (23.80±1.54%), while neurons in the DMNd and the PeVe presented the highest activity rate to acute pain (more than 70% was Fos-positive). Tukey's *post hoc* comparison revealed significant difference between treated and control groups in the PeF, PeVe (both of them $p < 0.01$) and DMNd ($p < 0.005$).

4.1.2.4. Rostro-caudal distribution of the activated orexin neurons

Regarding the rostro-caudal distribution of double-labeled neurons, fasting resulted significant ($p < 0.05$) increase in the rostral portion of the PeF. In contrast, activated orexin-ir neurons were found in higher number more caudal in the LHv, the PeVe and the DMNd.

The response of the orexin-ir neurons on SD was remarkable in the DLH but rostro-caudal activation pattern of orexin-ir cells showed fairly high individuality according to the subdivisions. Significant Fos induction was seen at 2.52 mm caudal from bregma in DMNd. In the PeVe and the LHv high plateau was seen between 2.52-3.00 mm, as well as high percentage between 2.76-3.00 in the PeF and LHd.

Rostro-caudally, the DMNd, PeF and LHd showed high activation at 2.76 mm caudal to the bregma. In addition, relatively high number of double-labeled cells was seen in the LHv at the caudal portion of the DLH 3.00-3.48 mm caudal to the bregma.

4.2. Lower brainstem connections of the subdivisions of the dorsolateral hypothalamus

4.2.1. Localization of the injection sites

4.2.1.1. Hypothalamic dorsomedial area

Totally, six rats were found with topographically correct injection sites: two in each of the 3 subdivisions of the dorsomedial hypothalamic area.

In the case of *periventricular area*, the injected areas were relatively small, their average mediolateral diameters did not exceed 250 μm in the coronal plane, but they extended more in rostro-caudal and posteroventral directions. The injected area was located immediately adjacent to the third ventricle. They extended over the ventricle, dorsally. This division showed a small overlap with the dorsal part of the dorsomedial nucleus.

Regarding to the *dorsal part of the dorsomedial nucleus*, the sizes of injections varied between 400 and 500 μm. In one case, the injection spread in a relatively large area, covering almost the entire dorsomedial part of the dorsomedial nucleus. In the second rat, the injection's site was smaller. In both cases, the tracer spread slightly into the posterior periventricular area.

The sizes of the injections in the *dorsomedial hypothalamic subdivision* were small, between 350 and 400 μm average diameters. The injections centered in the dorsomedial hypothalamic area between the dorsomedial nucleus and the medial border of the perifornical area. BDA spread slightly into these two neighboring areas.

4.2.1.2. Perifornical area

Eleven injections were classified into the perifornical area: two injections in the rostral (PeFr), six in the caudomedial (PeFcm) and three in the caudolateral (PeFcl) perifornical areas.

The sizes of injections in the *PeFr* were 350 and 450 μm. In both cases, the injected BDA spread entirely within the boundaries of the area.

Regarding to the *PeFcm*, the average diameter of the injections varied between 250 and 700 μm. In three cases, BDA-positive neurons were located mainly within the boundaries of the area, whereas in the other 3 rats BDA spread also to neighboring portion of the lateral area.

In the case of the *PeFcl*, the sizes of injections varied between 200 and 450 μm. Two smaller injections centered within the area, whereas the third, a large one, spread partly into the neighboring LHd.

4.2.1.3. Lateral hypothalamus

Five injections were centered in the lateral hypothalamic area: two injections in the ventral (LHv) and three in the dorsal part (LHd). (In this study, the term of "LH" is restricted to the posterior portion of the "hypothalamus-long" lateral hypothalamic area that extended between 2.2 and 3.4 mm caudal to the bregma level.).

Both injections into the *LHv* were located entirely inside the boundaries of the area. Their sizes were moderate with average diameters around 400 μm.

The sizes of injections into the *LHd* varied between 250 and 350 μm. In two rats, all of the BDA-labeled cells were within the boundaries of the area, whereas, in another case, a small spread of label was seen in the LHv.

4.2.2. Distribution of BDA-positive nerve fibers in the lower brainstem

4.2.2.1. Midbrain

In general, the locations of BDA-positive fibers in the midbrain showed ipsilateral dominance to the unilateral application of the tracer in the DLH.

The most densely network of labeled fibers in the midbrain appeared in the periaqueductal central gray (PAG). They arise mainly in the three subdivisions of the perifornical area and the periventricular subdivision of the dorsomedial hypothalamic area. Dense network of BDA-positive fibers was seen in the ventrolateral and the ventral parts, moderate to weak in the other parts of the PAG. Although, a substantial portion of the fibers appeared to be 'axons of passage', there were also interspersed numerous axons with varicosities, indicating possible synaptic contacts.

Numerous axons fanned out in the midbrain lateral and ventral to the PAG, predominantly in the deep mesencephalic (also called cuneiform) nucleus, the precuneiform area and in the isthmic reticular formation.

The dorsal raphe nucleus established moderate to heavy distribution patterns of BDA-positive fibers originated from the PeF and LHv subdivisions of the DLH. In the median raphe nucleus only light or very low projections were observed. The rhabdoid and paramedian raphe nuclei showed moderate density of BDA fibers.

The other investigated midbrain nuclei and areas, including the oculomotor and trochlear

nuclei showed low fiber density or were devoid of BDA-positive fibers.

4.2.2.2. Pons

Like in the midbrain, the distribution of the BDA fibers was bilateral but with strong ipsilateral dominance.

A moderate density of partly *en passant* BDA-positive fibers was apparent within the oral part of the pontine reticular formation after injections into the PeFcm and PeFcl. Light to moderate density of labeling was observed in both the oral and caudal parts of the pontine reticular formation especially after injections into the PeVe, PeFr and LHv.

Labeled fibers were present in the pedunculopontine tegmental nucleus after all injections into the 8 subdivisions, as well as in the laterodorsal tegmental nucleus after injections into 6 subdivisions. Their density was very high after LHv injections.

Dense network of BDA-positive fibers was seen in the Barrington's nucleus but only in PeVe injected rats. The density of the BDA-labeled fibers in the locus coeruleus was almost equal to those in the Barrington's nucleus. The PeVe and the subdivisions of the PeF generated moderate, while the other subdivisions of the DLH only light (or sparse in the case of DMH) projections to the locus coeruleus. In the caudal portion of the locus coeruleus, from where neurons project to the spinal cord, the labeling of fibers was rare. Ventral to the locus coeruleus, in the subcoeruleus area, especially in its dorsal part, moderately dense fiber network was present. The fibers were seen mainly in rats with injections in the PeFr, PeFcm and LHv. BDA-positive fibers were present in very light to moderate densities in the lateral and medial parabrachial nuclei. The neurons of the PeFr and PeFcl innervated moderately both parabrachial nuclei.

In the ventrolateral area of the pons, few fibers were spread throughout the area of the A5 noradrenaline cell group. Few labeled fibers were seen in the pontine raphe and pontine nuclei, some in the sensory and motor nuclei of the pons, while in many others (like sensory trigeminal, cochlear and lateral vestibular nuclei) were devoid of BDA-labeled fibers or terminals.

4.2.2.3. Medulla oblongata

Although generally, sparse labeled fibers could be traced all along the medulla, their topographical distributions and density were heterogeneous. They showed generally bilateral appearance with ipsilateral dominance.

The subdivisions of the DLH generated light labeling in the gigantocellular reticular nucleus. In the vicinity, somewhat lesser density of fibers was observed in the lateral

paragigantocellular and the intermediate reticular nuclei. Except neurons in the DMN, labeled fibers arise in the other seven subdivisions of the DLH, but in different densities.

From all of the DLH divisions (with exception of DMN) projections were generated to the nucleus of the solitary tract: light ones from neurons of the PeVe, PeFr and LHv, and sparse projections from the other areas. It was not the case for the dorsal motor nucleus of the vagus, where the BDA-labeling was low or sparse.

In the territory of the medullary catecholamine cell groups, despite of the gap of heavy innervations, the BDA fibers originated from more individual subdivisions of the DLH. The source of the fibers was especially wide in the case of A2 noradrenaline cell group, which is innervated by *all of the investigated* DLH subdivisions. BDA-labeled fibers in the caudal part of the A1 noradrenaline and C1 adrenaline cell groups arised in 7 subdivisions, whereas the rostral part of the A1 noradrenaline and the C2 adrenaline cell groups received fibers from 5 different subregions of the DLH.

Light to moderate density of varicose fibers was present in the raphe magnus nucleus originating from all the subdivisions of the DLH. In the raphe pallidus and obscurus nuclei delicate varicose fibers were apparent after the tracer injections within 7 different subregions of the DLH. Some of the BDA-positive fibers traveled in the raphe obscurus and the paramedian reticular nucleus nearly perpendicular to the plane of the sections.

In most cases, after perifornical or lateral hypothalamic injections, BDA-positive fibers were identified among fibers of large neuronal tracts or fasciculi. Their number was low, except in the medial lemniscus, the medial longitudinal fascicle and the superior cerebellar peduncle, where the density of BDA fibers could be rated as moderate.

Several, more than one third of recognized lower brainstem areas/nuclei/subdivisions were devoid of BDA-labeled fibers or terminals.

4.3. Connection of orexin A-containing neurons with the cathecolamine cell groups of the lower brainstem

4.3.1. Orexin A network of fibers in brainstem adrenaline cell groups

Double immunohistochemistry for phenylethanolamine-N-methyltransferase (PNMT) and orexin A showed that all of the adrenaline cell groups in the brainstem receive orexin innervation. In the ventrolateral medulla, among the neurons of the C1 adrenaline cell group, fine, varicose orexin-containing fibers were observed in juxtaposition to PNMT-containing

perikarya and dendrites. Such close interactions were detectable along the entire rostro-caudal extension of this cell group.

The C2 adrenaline cell group, located in the rostral part of the nucleus of the solitary tract also contains delicate, varicose orexin-ir fibers, many of them just on the surface of PNMT-ir perikarya and dendrites.

The C3 adrenaline cell group contains few PNMT-positive neurons located mainly in the dorsomedial and paramedian portions of the rostral medulla. Long, thin, varicose orexin-ir fibers run among the PNMT-containing neurons establishing close contacts with their perikarya and dendrites.

4.3.2. Orexin A network of fibers in brainstem noradrenaline cell groups

Fine orexin-positive neuronal networks were seen in all of the noradrenaline cell groups in the brainstem of the rat and the mouse. The innervation pattern of orexin-ir network in brainstem noradrenaline cell groups showed high level of similarity in these two species.

A very dense network of orexin-ir fibers was seen in the caudal ventrolateral medulla, in and around the A1 noradrenaline cell group. Two fascicles of fibers seemed to be directed to the A1 cell group, one from lateral and the other from dorsomedial direction. In this area, TH-ir neurons established close contacts with varicose orexin-ir fibers, and some orexin-positive varicosities were observed in juxtaposition to TH-immunostained dendrites.

The A2 noradrenaline cell group, mainly in the territory of the nucleus of the solitary tract, received numerous varicose orexin-positive fibers. An increasing fiber density was observed rostro-caudally inside the nucleus. In the commissural subdivision of the nucleus of the solitary tract, a network of orexin fibers occupied mainly in the dorsal and lateral parts of the subdivision, just in the vicinity of the gracile nucleus. Horizontally orientated fibers were crossing over inside the nucleus, just dorsal to the central canal. Orexin-ir fibers that spread through the entire nucleus of the solitary tract contained a number of varicosities and established numerous close contacts with TH-positive cells. NE neurons received delicate orexinergic innervation throughout the entire rostro-caudal length of the A5 cell group. Long, thin, varicose fibers surrounded TH-positive neurons and dendrites. Two orexin-ir fascicles reached the A5 cell group, one through the subcoeruleus area and one from a ventrolateral direction.

The highest density of orexin-containing fibers in the lower brainstem was observed in the locus coeruleus, i.e. A6 cell group. Thick, varicose orexin-positive fibers formed delicate networks inside the locus coeruleus, along the medial edge of the nucleus, an area that

contains dendrites of locus coeruleus neurons, and also in the subcoeruleus area, between the locus coeruleus and the A5 noradrenaline cell group. Long, parallel running orexin-ir fibers were observed under the fourth ventricle, very close to the surface. Parallel varicose orexin-ir fibers can be seen also in the wall of the fourth ventricle between subependymal noradrenaline neurons (A4) and ependymal cells.

Close contacts could be seen between TH-immunoreactive perikarya or dendrites and varicose orexin-ir fibers in the entire locus coeruleus. Ventral to the locus coeruleus, in the subcoeruleus area, where the density of TH-immunoreactive cells is somewhat lower than inside the locus coeruleus, the orexin-positive network of fibers was more delicate, therefore the arrangement of the varicose orexin-containing fibers were well observable around the TH-ir neurons.

The A7 cell group is constituted by relatively few TH-positive noradrenaline neurons. Like in other noradrenaline cell groups, a fine orexin-containing network was present created by fibers that reached this cell group from a ventral direction. Rare, but close contacts were observed between TH-positive perikarya or dendrites and orexin-ir fibers.

4.3.3. Demonstration of orexin-TH and orexin-PNMT close contacts in C1, C2, C3 adrenaline and A1, A2 noradrenaline neurons

Double immunostainings clearly indicate close relationship between orexin-ir fibers and adrenaline or noradrenaline neurons. The question has risen whether these connections are only topographical or there are synaptic specializations? We analyzed double immunostainings with a confocal microscope and extended this confocal analysis on triple immunostained sections when synaptophysin was used in addition to adrenaline and orexin, or noradrenaline and orexin.

Using double confocal microscopic immunostaining, axo-dendritic and axo-somatic close appositions were observed between varicose orexin-ir axons and PNMT-, or TH-containing neurons and dendrites. A high number of confocal micrographs was taken from the C1, C2, C3 cell groups and analyzed for determining the percentage of synaptophysin-containing close contacts. Similarly, confocal micrographs were analyzed in the A1 and A2 cell groups.

In these cell groups 69.2% of the orexin+PNMT and 70.6% of the orexin+TH double-labeled close contacts showed also synaptophysin immunoreactivity.

4.3.4. Demonstration of orexin-PNMT and orexin-TH synaptic contacts

4.3.4.1. Orexin-PNMT synaptic contacts in the C1 and C2 noradrenaline cell groups

Electron microscopic analysis of double-stained material in the C1 cell group demonstrated typical synaptic contacts between orexin A-containing axon terminals and PNMT-positive perikarya and dendrites. Among all orexin-ir axons, a few established close contact with PNMT-labeled dendrite without synaptic specializations. Some orexin-ir terminals made synaptic contact with non-labeled dendrites. Most synapses that were terminated on PNMT-containing neurons were non-labeled for orexin. Similar observations had been made on the C2 cell group.

4.3.4.2. Orexin-TH synaptic contacts in A1, A2 and A6 noradrenaline cell groups

As it is shown in representative electron micrographs from the A2 and A6 noradrenaline cell groups, axo-dendritic asymmetrical synapses were detected between orexin-ir axon terminals and TH-containing dendrites of noradrenaline neurons in the investigated cell groups, as well as between orexin-ir axon terminals and non-labeled dendrites. Orexin-ir axons frequently were in close apposition to TH-positive dendrites. Majority of synapses that was terminated on TH-labeled neuronal elements was non-labeled.

The detected synapses were asymmetrical, that is generally associated with excitatory synapses. The presynaptic boutons contained many small clear vesicles in addition to larger dense core vesicles. Some of orexin-ir fibers projecting to adrenaline and noradrenaline cell groups in the medulla appear to pass through these nuclei without synapsing. Orexin-ir varicosities that pass these nuclei are surrounded by bundles of unlabeled axons and by glial processes. The varicosities were never observed to form synaptic contacts.

5. CONCLUSIONS

5.1. Functional heterogeneity of orexin-containing neurons in the DLH

- Orexin-containing neurons in the DLH respond with Fos-activation to fasting, sleep-deprivation and acute pain stress. Sleep-deprivation elicited a relatively high c-fos expression, somewhat less was seen after acute formalin stress, while a lower number was found in the food intake experiment suggesting that orexin-containing neurons take part in different extent in the regulation of these functions.
- The topographical distribution of activated orexin cells was similar in the three functional models suggesting that there are no function-specific orexin-containing cell groups.
- The summarized percentage of orexin/Fos cells in the three experimental models exceeded 100% suggesting that a single neuron should participate in more than one functional mechanism.

5.2. Brainstem connections of the DLH

- The 60% of the brainstem structures receive inputs from the dorsolateral hypothalamus. The investigated eight subdivisions of the DLH take part in this innervation in different extent. There are differences between the subdivisions concerning the brainstem projections. The most dense projections from the investigated 8 subdivisions of the DLH arisen partly from the three perifornical ones. From the 3 subdivisions of the perifornical area twice as many brainstem areas/nuclei received fibers than from the subdivisions of the dorsomedial hypothalamic area. From the lateral hypothalamic area, neurons from the ventral part projected to more brainstem areas than neurons from the dorsal one.
- The density of the fibers of DLH origin in *all* of the noradrenaline, adrenaline and serotonin cell groups of the lower brainstem, which are important regulatory parts of the food intake, sleep-wake cycle and stress response, is higher than the average in the lower brainstem, suggesting that neurons of the DLH take their functional effect through these cell groups.
- About 70% of the orexin-PNMT or orexin-TH immunopositive close contacts contained synaptophysin, a presynapse-specific protein in cathecholamine cell groups of the lower brainstem indicating the existence of synaptic contacts.
- Synaptic contacts were demonstrated between adrenaline- and noradrenaline-containing neurons and orexin terminals in the territory of some catecholamine cell groups of the lower brainstem. These data suggest the existence of a hypothalamic feedback signal, and also possible regulatory pathway on which the hypothalamic orexin neurons take their functional effect.

6. LIST OF PUBLICATIONS

6.1. Publications related to the theses

1. Puskás N, **Papp RS**, Palkovits M. (2010) Interactions between orexin-immunoreactive fibers and adrenaline or noradrenaline-expressing neurons of the lower brainstem in rats and mice. Peptides, 31: 1589-97.
   Impact factor: 2.654
2. **Papp RS,** Palkovits M. (2014) Brainstem projections of neurons located in various subdivisions of the dorsolateral hypothalamic area – an anterograde tract-tracing study. Front Neuroanat, 8: 34.
   Impact factor: 4.058

6.2. Publications not related to the theses

1. Könczöl K, Bodnár I, Zelena D, Pintér O, **Papp RS,** Palkovits M, Nagy GM, Tóth ZE. (2010) Nesfatin-1/NUCB2 may participate in the activation of the hypothalamic-pituitary-adrenal axis in rats. Neurochem Int, 57: 189-97.
   Impact factor: 3.601
2. Kitka T, Adori C, Katai Z, Vas S, Molnar E, **Papp RS,** Toth ZE, Bagdy G. (2011) Association between the activation of MCH and orexin immunoreactive neurons and REM sleep architecture during REM rebound. Neurochem Int, 59: 686-94.
   Impact factor: 2.857
3. Ruisanchez É, Cselenyák A, **Papp RS,** Németh T, Káldi K, Sándor P, Benyó Z. (2012) Perivascular expression and potent vasoconstrictor effect of dynorphin A in cerebral arteries. PLoS One, 7: e37798.
   Impact factor: 3.730
4. Vas S, Ádori C, Könczöl K, Kátai Z, Pap D, **Papp RS,** Bagdy G, Palkovits M, Tóth ZE. (2013) Nesfatin-1/NUCB2 as a potential new element of sleep regulation in rats. PLoS One, 8:e 59809.
   Impact factor: 3.730

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