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Hypothalamus in Health and Diseases

Edited by Stavros J. Baloyannis and Jan Oxholm Gordeladze

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Meet the editors

Stavros J. Baloyannis, Professor Emeritus of Neurology at Aristotelian University, Thessaloniki, Greece, was born in Thessaloniki. He graduated from the School of Medicine, Aristotelian University, and trained in neurology at Aristotelian University and the Institute of Neurology, London, neuropathology at the Institute of Neurology, London, Catholic University of Louvain, and University of Pennsylvania, neuropathology of the auditory pathway at Harvard University, electron microscopy at the University of Pennsylvania, and neuroimmunology at Yale University. His research interests are the blood–brain barrier, mitochondria in Alzheimer's disease, synaptogenesis, neuronal apoptosis, dendritic and synaptic pathology, and Golgi apparatus in dementias. His special interests include neuroethics, neurolinguistics, neurophilosophy, history of neurosciences, neurology, and art. He is a member of 57 scientific societies, honorary member of the Academy of Hellenic Air Forces, President of the Society for the Amelioration of the Quality of Life in Chronic Neurological Diseases, President of the Orthodox Association for Medical Education and Health in Africa, and visiting professor at Tufts University, Democritus University, Aristotelian University, School of Theology, and School of Philosophy. He is the author of 28 textbooks and 722 papers on neurology and neurosciences. He has organized 26 international congresses and participated in 612. He was head of the Department of Neurology (1992–2011) and Director of the Research Institute for Alzheimer's Disease.

Dr. Jan O. Gordeladze, Ph.D. (born 25th of April, 1950), holds a triple professor competence (Medical Biochemistry, Physiology, and Pharmacology), and is presently working as a Professor Emeritus at the Department of Biochemistry, Institute of Basic Medical Science, University of Oslo, Norway. He has previously been employed as the Medical Director of MSD, Norway, serving two years as a Fulbright scholar at the NIH, Bethesda, Maryland, USA. From 2006-2009 he was employed as Associate Professor at the University of Montpellier, France. He is a member of the Norwegian Stem Cell Center, and his research has over the past 7-10 years been devoted to differentiation of osteochondral cells from stem cells focusing on the impact of transcription factors and microRNA species constituting regulatory loop interactions with functional target genes. He has published more than 120 scientific articles, reviews/book chapters and presented more than 250 abstracts/posters/talks at conferences worldwide. Dr. Gordeladze has served as a Fulbright Scholar at The National Institute of Health, Bethesda, Washington DC during the years 1990-91.

Contents

Preface

The human hypothalamus, a small structure at the base of the brain, has strategic importance for the harmonic function of the human body. It controls the autonomic nervous system, neuroendocrine function, circadian and circannual rhythms, somatic activities, and behavior, and is situated at the borders between the brain and the body and the brain and the soul, meeting points for mind (νους) and body (σώμα).

The Greek pre-Socratic philosopher Anaxagoras of Clazomenae claimed in the sixth century BC that the mind controls the physical existence of the human being: "...καί όσα γε ψυχήν έχει...πάντων νους κρατεί" (DK B12.12). Galen, the great Greek neuroscientist from Pergamum (AD 130–200), claimed, on the other hand, that from the base of the brain (the hypothalamus) the animal spirit is transmitted to the body, infiltrating, vitalizing, and activating all the organs, inducing also the phenomenon of "sympathy," which means the harmonious coordination and cooperation of all the organs of the body.

Today, we know definitely that the hypothalamus, "the very main spring of primitive existence" according to Cushing, is the principal regulatory center for autonomic and endocrine homeostasis. In addition, we know that the hypothalamus is involved in a wide range of higher mental functions, including attention, learning and memory, reinforcement of mnemonic processes, emotional control, mood stability, and cognitive–emotional interactions, since it is a core structure of the limbic system with connections to the orbitofrontal cortex, amygdala, and thalamus.

It is reasonable that the hypothalamus, as homeostatic regulator, is closely involved in autonomic and neuroendocrine disorders, disorders of development and growth, disorders of drinking and eating, disorders of thermoregulation, sleep disorders, and autoimmune dysfunction.

However, it is important that clinical observations, neuroimaging data, and neuropathological studies plead in favor of hypothalamic involvement in behavioral disorders, such as psychomotor agitation, aggressive and disruptive behavior, obsessive–compulsive disorder, panic reactions, pain and addiction, fatigue syndromes, as well as in neurological conditions, such as cluster headaches, episodes of migraine, hypnic headache, gelastic epilepsy, mental deficiency, periodic disorders, and in a substantial number of neurodegenerative diseases.

The possible involvement of the hypothalamus in depression, schizophrenia, and autism, a fact that enlarges greatly the essential contribution of the hypothalamus in controlling psychosomatic equilibrium and retaining the internal unity of the human existence, poses an additional reason for attracting the attention of neuroscientists, who by applying modern neurobiological techniques may proceed to a further clarification of the complex, substantial, and multidimensional strategic role that the hypothalamus plays in the human brain.

I extend my gratitude to the authors and the editorial and secretarial staff who worked with much devotion and enthusiasm to publish this volume. I wish that it might be one more step for further fruitful research activity in the immense field of the human hypothalamus.

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Section 1

Neuroanatomy

Chapter 1

Anatomy and Function of the Hypothalamus

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Additional information is available at the end of the chapter

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Abstract

The hypothalamus is a small but important area of the brain formed by various nucleus and nervous fibers. Through its neuronal connections, it is involved in many complex functions of the organism such as vegetative system control, homeostasis of the organism, thermoregulation, and also in adjusting the emotional behavior. The hypothalamus is involved in different daily activities like eating or drinking, in the control of the body's temperature and energy maintenance, and in the process of memorizing. It also modulates the endocrine system through its connections with the pituitary gland. Precise anatomical description along with a correct characterization of the component structures is essential for understanding its functions.

Keywords: anatomy, structure, function

1. Embryological development of the hypothalamus

At the end of the fourth week of embryological development, the neural tube is organized in primary vesicles: the forebrain vesicle or prosencephalon, the midbrain vesicle or mesencephalon, and the hindbrain vesicle, also called rhombencephalon. Prosencephalon further divides into two secondary vesicles, the telencephalon that will form the cerebral hemispheres and the diencephalon which gives rise to the diencephalon. Mesencephalon forms the midbrain, structure involved in the processes of vision and hearing. The hindbrain vesicle or rhombencephalon divides in metencephalon, which further forms the pons and the cerebellum and the myelencephalon that forms the medulla.

Embryological concepts regarding the development of the hypothalamic region are over 100 years old. Since Herrick [1] first proposed the columnar model of the forebrain organization,

the anatomical description was accepted *per se* and very few research papers have questioned its validity.

The columnar morphologic model is based on the division of the forebrain in functional longitudinal units, placing the telencephalon in the most rostral region and the diencephalon caudally, in between the telencephalon and the midbrain, while the hypothalamus if formed from the ventral most part of the diencephalic vesicle [2].

In the last decades, mapping of the genes involved in hypothalamic development allowed the identification of a disparity between the morphological, classic boundaries of this region and the molecular ones. According to Puelles' Prosomeric model [3], the initially proposed longitudinal axis of the brain is bent due to the first mesencephalic flexure of the embryo. This condition puts the diencephalon rostrally between the telencephalon cranially and the midbrain caudally and sets the hypothalamus independent from the diencephalon as a distinct posterior part of the forebrain [2, 3].

An important role in hypothalamic development is assigned also to the presence of specific signaling centers (Wingless-Int protein family–Wnt, Hedgehogs family–Hh, and Bone morphogenetic family–FgF) that modulates cell proliferation and neurulation [4].

2. Definition and localization

The hypothalamus is a small, central region of the human brain formed by nervous fibers and a conglomerate of nuclear bodies with various functions. The hypothalamus is considered to be a link structure between the nervous and the endocrine system, its main function being to maintain the homeostasis of the body.

The hypothalamus is located under the thalamus from which it is separated by the hypothalamic sulcus of Monro. The sulcus is located at the lateral wall of the third ventricle and extends anteroposteriorly from the interventricular foramen of Monro (that assures the communication between the third, diencephalic ventricle and the frontal horn of each lateral ventricle) up to the level of Sylvius cerebral aqueduct. The hypothalamus is limited anteriorly by the *lamina terminalis*, a gray matter layer of triangular aspect extended above the chiasma optique, in between the two anterior horns of the fornix. *Lamina terminalis* also forms the anterior wall of the third ventricle and contains the organum vasculosum, a circumventricular structure characterized by the absence of blood–brain barrier and thus highly sensitive to osmotic variations of the blood [5]. The superior wall of the hypothalamic region participates in the formation of the inferolateral wall of the third ventricle of the brain and has close relations with the white matter structure that surrounds it, called the fornix. The fornix is a C-shaped white cerebral structure that connects various parts from the brain (hypothalamic nuclei with hippocampal region, thalamic nuclei with hypothalamus's mammillary bodies). Even if its function is not clearly understood, its relation with memory is known, and recent studies are testing its deep brain stimulation as a treatment in advanced Alzheimer's disease [6]. Posteriorly, the hypothalamus extends up to the periaqueductal gray substance and the tegmentum of the superior part of the brainstem.

Only on the inferior surface of the brain, the hypothalamus can be visualized from the optic chiasm and the anterior perforated substance anteriorly to the posterior cerebral peduncles of the midbrain and the mammillary bodies, dorsally (**Figure 1**). The mammillary bodies are small, round white-matter structures that belong to the limbic system. They are involved in memory due to their connections with the hippocampal region and also in maintaining the sense of direction [7]. The hypothalamus is limited laterally by the optic tracts in their direction toward the lateral geniculate bodies, an important relay of the optical pathway. Inside the delimited area on the exterior surface of the brain, a small prominence, called tuber cinereum or infundibulum connects the hypothalamus with the posterior lobe of the underneath pituitary gland. The pituitary or the hypophyseal gland is located at the base of the brain, in a depression of the sphenoid bone called the sella turcica.

2.1. The hypothalamus—hypophyseal complex

The pituitary gland is a three-lobe structure: anterior, posterior and intermediate lobe, with different embryological origin.

The anterior lobe, pars anterior, or adenohypophysis is derived from the anterior wall of Rathke's pouch, an ectodermal structure that also forms the primitive oral cavity and the pharynx [8]. The anterior gland contains a heterogeneous cellularity that synthesized and secreted hormones in the blood stream: the majority of the cells are somatotrope cells that produced the human growth hormone (hGH) or somatotropin hormone (STH), a peptide that promotes growth in childhood. The production of the somatotropic hormone is under the control of the hypothalamic growth-releasing hormone (GRH) produced by the arcuate nucleus. The next hormones produced in high quantity by the anterior gland of the hypophysis are the corticotrope ones (adrenocorticotropic hormone—ACTH, melanocyte-stimulating hormone—MSH, and beta-endorphins). This group of hormones is under the control of the hypothalamic corticotropin-relasing hormones (CRHs) derived from the paraventricular nuclei. In smaller percentages, the adenohypophysis has population of cells that produced

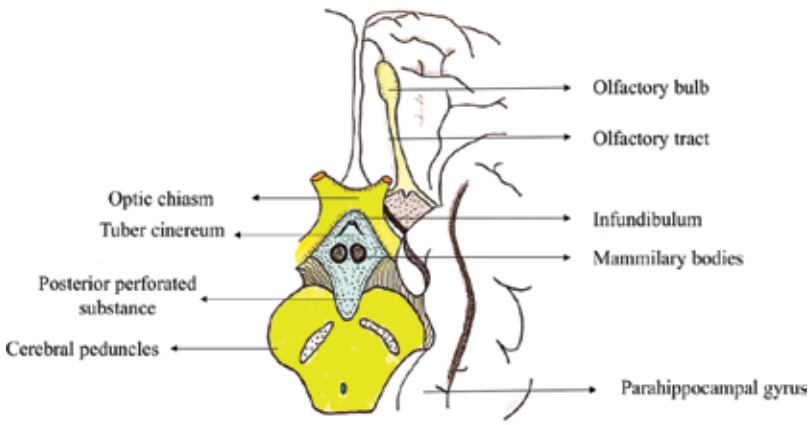

Figure 1. Inferior surface of the brain with hypothalamic visualization at this level.

thyrotropes, gonadotropes, and lactotropes. Thyrotropes respond to signals from the hypothalamic thyrotropin-releasing hormone (TRH) produced in the paraventricular nuclei and further synthesize the hormone responsible for thyroid hormones production—thyroid stimulating hormone (TSH). Luteinizing hormones (LHs) and follicle stimulating hormones (FSHs) are secreted by gonadotrope cells of the gland under the influence of pulsatile secretion of gonadotropin-releasing hormone (GRH) produced in hypothalamus preoptic area. The secretion of prolactine (PRL) from the lactotropes is stimulated by hypothalamic thyrotropin-releasing hormone (TRH) and inhibited by the dopamine [9].

Hypothalamic hormones reach the adenohypophysis through a vascular system. Hypothalamus exerts its effects over the anterior part of the gland through the hypothalamo-hypophyseal portal system, a special vascular system formed by fenestrated capillaries. The proximal vascular structure of the portal system is the anterior hypophyseal artery, branch from the ophthalmic segment of the internal carotid artery [9]. Through it, hypothalamic hormones are transported to the primary plexus, located near the infundibulum of the hypothalamus. From this region, hormones are drained into the second vascular venous plexus of the hypothalamo-hypophyseal portal system that surrounds the adenohypophysis [9]. This vascular system allows hormones to diffuse through the wall, inside of the gland. The hypophyseal vein further drains the blood into the venous sinuses of the dura mater and from here in the venous system of the body.

The posterior wall of Rathke's pouch forms the intermediate lobe of the gland [8]. It is absent or of small size in adults. In children, it is the part of the gland responsible for skin pigmentation through the secretion of the melanocyte stimulating hormone (MSH) or "intermedins" [9]. Pars intermedia also produces corticotrophin-like intermediate lobe peptide (CLIP) and adrenocorticotrophic hormone (ACTH) [9].

The posterior lobe of the gland, pars distalis or neurohypophysis derives from the neuroectoderm [9]. It is an inferior extension of the hypothalamus and is mainly from its neural fibers. The connection between the hypothalamus and the posterior lobe of the gland forms the infundibular stalk. Through this complex, hormones synthetized in the hypothalamus nuclei are transported and deposited in the posterior gland where they are stored in presynaptic vesicles and then released into the blood stream. The supraoptic nuclei of the hypothalamus are responsible for the secretion of antiduretic hormone (ADH) or vasopressin, the hormone involved in maintaining the water balance in organism and thus in preventing dehydration. The paraventricular nuclei produce oxytocin, a hormone released during labor, in the presence of uterine contractions.

The hypothalamus intervenes along with the pituitary gland the majority of the endocrine and metabolic functions of the body through a double-sense transport of hormones between the two structures.

3. Structure of the hypothalamus

The hypothalamus is divided by the anterior horns of the fornix in a lateral, medial, and periventricular (median) region and by a coronal plane passing through the infundibulum in

an anterior and posterior region. The anterior region is also referred to as the prechiasmatic region, due to its location above the chiasma optic, while the posterior region is called the mammillary region. The infundibular region is situated between the previous two regions.

From a structural point of view, the hypothalamus is formed by gray matter conglomeration of neurons that organize in nuclei and also by white-matter substance formed by myelinated nervous fibers.

The anterior region of the hypothalamus is located above the optic chiasm and is referred to as the supraoptic area. It contains the following nucleus: supraoptic, preoptic and medial preoptic, the suprachiasmatic and the anterior hypothalamic nucleus, alongside with the paraventricular one (**Figure 2**). The supraoptic nucleus produces vasopressin or the antidiuretic hormone (ADH) that is stored in the posterior lobe of the pituitary gland and is responsible for blood pressure control and water balance of the organism. The preoptic region alongside with the anterior hypothalamic nucleus is involved in cooling (thermoregulation) of the body through the sweating process. The preoptic nucleus is also involved in the habit of eating and in reproduction while the medial preoptic region is involved in cardiovascular control as a response to stress [10]. The suprachiasmatic nucleus is situated above the optic chiasm and is involved in the circadian rhythm. The paraventricular nucleus (named after its location near the third diencephalic ventricle) represents an important autonomic center of the brain involved in stress and metabolism control [11].

The central part as the hypothalamus is located above tuber cinereum and is named the tuberal area. It is composed of two parts, anterior and lateral, and contains the following nucleus: dorsomedial, ventromedial, paraventricular, supraoptic, and arcuate (**Figure 2**). The ventromedial area is involved in controlling the habits of eating and the feeling of satiety [12]. The arcuate or infundibular nucleus is responsible for orexigenic peptides secretion: ghrelin, orexin, or neuropeptide Y [11].

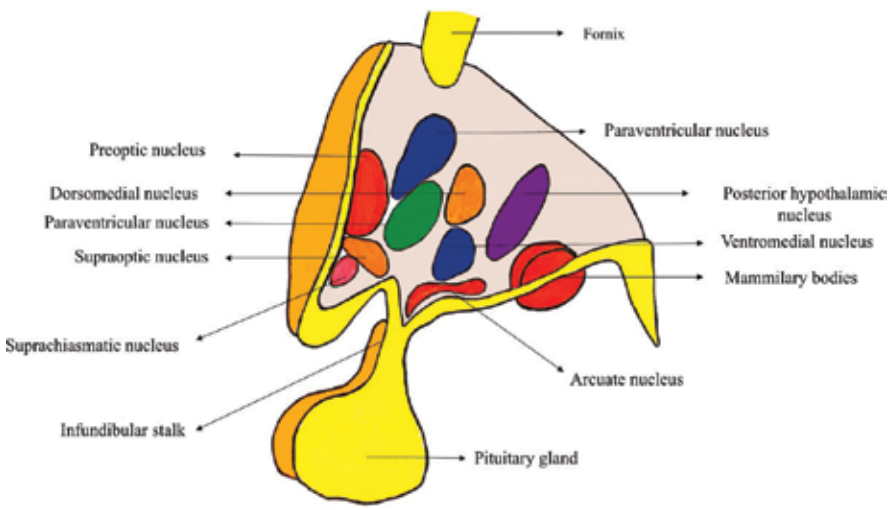

Figure 2. Schematic representation of hypothalamic nuclei (sagittal section).

The posterior region is formed by a medial and, respectively, lateral area. The medial region contains the mammillary nucleus alongside with the posterior hypothalamic nucleus, the supramammillary and the tuberomammillary ones. The nucleus of the lateral region contains the hypocretins (orexin) peptides that control feeding behavior, thermoregulation, gastrointestinal motility [13], and cardiovascular regulation and are also involved in sleep regulation [14]. Lesions of the lateral region lead to the refusal to feed or aphagia. The posterior part of the hypothalamus is involved overall in energy balance, blood pressure, memory, and learning. The posterior hypothalamic nucleus has a major role in controlling the body temperature [12]. The tuberomammillar nucleus is involved in memory due to their connection with the hippocampus and Papez memory circuit [9].

4. Connections of the hypothalamus

The hypothalamus is a small region of the brain connected with numerous, various cerebral structures that allows it to intervene in many regulatory processes of the organism. It has an important role in the optimal, normal functioning of the body, and it controls the endocrine system, the metabolism, and it is involved in stress control and in other different actions that modulates a person's behavior. More, the hypothalamus is involved in the homeostasis of the organism in terms of body temperature, blood pressure, fluid balance, and body weight.

The connections of the hypothalamus are made with the following structures.

4.1. The midbrain

The ascending reticular activating system represents a structure composed by neural fibers passing from the reticular formation of the midbrain, through the thalamus, reaching the cerebral cortex [15]. The system is responsible for concentration, attention, and for maintaining the awakening state. Through it, the reticular formation is connected with the hypothalamic nuclei: the lateral mammillary bodies [12], the tuberomammillar nuclei, and the periventricular ones. The periventricular nuclei receive information about the general visceral sensibility [16] while the two others mediate behavior and are involved in consciousness [17]. Information from the solitary tract nucleus passing from the reticular substance of the midbrain can also reach the hypothalamus. The nucleus of the solitary tract is connected with the hypothalamus through either the solitarohypothalamic tract or through colaterales from the solitariothalamic tract.

4.2. The thalamus

The anterior hypothalamus has connections with the intralaminar nucleus and the nucleus of the median line. Recent studies described that lesions of the intraluminal group of nucleus can lead to Parkinson's disease [18] or even schizophrenia [19]. The mammillothalamic fascicle of Vicq d'Azyr connects both the medial and lateral mammillary nuclei with the anterior part of the thalamus [20]; its destruction in case of a cerebral hemorrhage is associated with memory loss [17, 20].

4.3. The amygdala

The amygdala represents a conglomerate of perykarions located in the temporal lobe. Efferent fibers from this region project directly to hypothalamus or neural fibers can detach from the amygdala-thalamic fascicle and reach the anterior hypothalamus [12]. It is involved in body's response to fear and rewards but also in memory [21]. Direct connections of amygdala with the hypothalamus are either through the ventral amygdalofugal pathway or through the stria terminalis.

4.4. The hippocampal region

The hippocampus is a curved-shaped cerebral structure located in the temporal lobe. It is formed by the dentate gyrus and different regions called Cornus Ammonis (CA): CA1, CA2, CA3, and CA4 [22]. CA1 and CA3 are connected with the infundibular and the ventromedial nuclei of the hypothalamus [22].

According to a recent study [23] CA2 area lighted that also CA2 area, a small region in the hippocampus composed from pyramidal neurons, is involved in memory and learning through its connections with the supramammillary nuclei of the hypothalamus.

4.5. The olfactory bulb

Fibers from the olfactory bulb reach the periamigdalian region (the entorhinal and periamygdaloid cortex) and then the lateral hypothalamus through either the amigdalian or the accumbens nucleus [12].

4.6. The retina

Visual information from the retinal neuroepithelium through the lateral geniculate body of the mesencephalon and then the superior colliculus reach the suprachiasmatic and supraoptic nuclei of the hypothalamus and are involved in circadian rhythm [12]. The hypothalamus can receive direct fibers from the retina through a retinohypothalamic tract that reach the suprachiasmatic nuclei. The connections are involved in the circadian rhythm.

4.7. Cerebral cortex

There is a double sense connection between the cerebral cortex and the hypothalamus. The hypothalamus projects on the surface of the cortex diffuse, in a poorly defined area over the cortex and transmits information that maintain the cortical tonus while from the gray matter of the cerebral cortex, neural fibers projects over the hypothalamus and triggers visceral response according to the affective state (sweating in case of fear, intestinal manifestations in case of stress). Neural fibers from the lateral hypothalamus project in the prefrontal cortex while the frontal lobe also has efferent for all the hypothalamic regions [24]. Through these connections, the autonomic control is assured in the organism. More, from the paraorbital gyrus, fibers project into the paraventricular and ventromedial nuclei.

Axons from the spinal cord can project in the hypothalamic region using the path of the spinohypothalamic tract. They carry out pain and temperature information. The hypothalamus exerts its effects within two projections: the spinothalamic tract reaching the lateral horn of the spinal cord of T1-L2 segments regulates the sympathetic autonomic response; the mammillotegmental tract and the dorsal longitudinal fasciculus carry out information from the posterior region of the hypothalamus while the anterior one connects with the thalamus (mammillothalamic tract) and the above fornix.

5. Functions of the hypothalamus

The hypothalamus is involved in different daily activities like eating or drinking, in the control of the body's temperature and energy maintenance, and in the process of memorizing and in stress control. It also modulates the endocrine system through its connections with the pituitary gland.

5.1. Thermoregulation

Thermoregulation is the process that allows maintenance of the body's temperature within normal ranges. In case of high body temperature, the hypothalamus responds through thermoregulatory heat loss behavior (either sweating or vasodilatation). If the body needs to be warm up, hypothalamus can determine heat production behavior (vasoconstriction, thermogenesis—heat production from muscles, brain or other organs, including the thyroid gland) [25].

They are of the hypothalamus responsible for controlling this process is the anterior one, more specific the preoptic nucleus.

5.2. Regulation of food intake

The hypothalamus controls appetite and food intake through the ventromedial, dorsomedial, paraventricular, and lateral hypothalamus nucleus. The ventromedial nucleus is referred to as the appetite-suppressing or anorexigenic center. Destruction of this nucleus leads to hyperpolyphagia, obesity, and to an aggressive behavior.

Contrary, the appetite-increasing or orexigenic center is considered to be the lateral hypothalamic nucleus that can lead to aphagia and cashexy in case of its destruction and to hyperphagia or polyphagia in case of its stimulation.

Appetite control is modulated by the leptin hormone released by the fatty cells that binds to specific hypothalamic receptors.

5.3. Regulation of body water content

Water control in the living organism is assured by the hypothalamus through the antidiuretic hormone (ADH) secretion. In cases of blood volume loss and dehydration, the ADH hormone

is secreted from the supraoptic nucleus–that have osmoreceptor cells–and released in the circulation. The peptide is directed toward the specific receptor from kidneys and decreases the urine production with subsequent water retention in the organism.

5.4. Center for autonomic nervous system

The hypothalamus regulates both sympathetic and parasympathetic systems. The anterior region of the thalamus has an excitatory effect over the sympathetic system while the posterior and lateral ones have an excitatory effect over the parasympathetic system.

5.5. Endocrine control

The endocrine control is realized through the pituitary gland or the hypophysis situated below the tuberal region of the hypothalamus. The hypothalamus is connected with the posterior lobe of the gland through the hypothalamo-hypophyseal tract. Along these fibers, the AHD and oxytocin hormones are transported into the neurohypophysis where they are stocked in vesicles.

Hormones secretion in the body is regulated by the hypothalamus through the releasing and inhibitor factors: thyrotropin-releasing, gonadotropin-releasing, corticotrophin-releasing, somatostatin, and dopamine. These hormones are involved in the process of growth, in the reproduction, in the metabolism of the body, and also can assure the homeostasis of the body.

5.6. Reproduction

The reproduction function of an organism is assured by the hypothalamic-pituitary-gonadal axis. The gonadotropin-realizing hormone (GnRH) secreted by the hypothalamus stimulates the production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the anterior subdivision of the pituitary gland. Action of these two hormones on the gonads determines the estrogen and testosterone production.

Behavior in males and females is influenced as well by the sex steroids. The neurons in the preoptic are involved in the male sexual behavior while the ones from the tuberal regional exert their properties in females [26].

5.7. The circadian rhythm

The photosensible suprachiasmatic nucleus is involved, along with is connections with the pituitary gland, in the circadian rhythm. The suprachiasmatic nucleus receives electro-chemical information from the stimulated retina. The circadian rhythm represents the endogenous clock of an organism that is involved in the well-being of the body due to keeping within normal limits the major functions.

Despite its reduced size, the hypothalamus represents an important, integrative region of the brain with complex functions and multiple connections with essential cerebral structures.

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Section 2

Neurphysiology

Chapter 2

Studies on the Character of Hypothalamic GnRH Neurons and Kisspeptin Neurons Using Hypothalamic Cell Models

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Additional information is available at the end of the chapter

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Abstract

The hypothalamic-pituitary-gonadal (HPG) axis controls the hormonal network responsible for reproductive functions. In the past, hypothalamic gonadotropin-releasing hormone (GnRH) neurons have been positioned at the highest level in the HPG axis. After the discovery of the indispensable roles of hypothalamic kisspeptin in GnRH neurons, our understanding of the neuroendocrine regulation of the HPG axis was reconfirmed, and it is now recognized that hypothalamic kisspeptin neurons are positioned at the summit of the HPG axis. Accumulating evidence shows that kisspeptin neurons are responsible for the onset of puberty and sex steroid feedback mechanisms by modulating the activity of GnRH neurons. Furthermore, the identification of kisspeptin in the hypophyseal portal circulation suggests that this peptide has some direct roles in the pituitary gland. The detailed mechanisms underlying the regulation of GnRH by kisspeptin and the regulatory control of kisspeptin neurons are still largely unknown because of the limitations of the experimental models. The establishment of GnRH-expressing and kisspeptin-expressing cell models has enabled us to examine the character of these neuronal cells. In this chapter, we describe our in vivo studies examining the character of GnRH neurons and kisspeptin neurons in the hypothalamus using hypothalamic GnRH- and/or kisspeptin-expressing cell models.

Keywords: kisspeptin, GnRH, gonadotropin, HPG axis

1. Introduction

The hypothalamus maintains the homeostasis within the body and controls endocrine functions. The hypothalamic-pituitary-gonadal (HPG) axis is a hormonal network responsible

for female reproductive function. After the discovery of inactivating mutations in the gene encoding the kisspeptin receptor (Kiss1R) in patients with idiopathic hypogonadotropic hypogonadism [1, 2], a new concept of the HPG axis was established. Now, it is generally agreed that kisspeptin produced from kisspeptin neurons, which are located in different regions of hypothalamus, stimulates gonadotropin-releasing hormone (GnRH) synthesis and release through Kiss1R within the GnRH neurons.

2. Kisspeptin as a regulator of the HPG axis

Kisspeptin is positioned upstream of GnRH in the HPG axis. Kisspeptin, which is encoded by the Kiss1 gene, was first discovered as a peptide that has potency to suppress metastasis of malignant melanoma and was initially named metastin [3]. The Kiss1 gene product is translated into a 145-amino acid precursor protein and further cleaved into a 54-residue peptide (kisspeptin 54), which can be further cleaved into 14-, 13-, and 10-amino acid peptides [4]. Kiss1R was discovered 4 years after kisspeptin, and it was found that Kiss1R is a member of the G protein-coupled receptor superfamily and is structurally similar to the galanin receptor [4, 5]. Discovery of loss-of-function mutations in Kiss1R in the family of a hypogonadotropic hypogonadism patient [1, 2] clearly linked kisspeptin and reproduction, and these observations indicated that kisspeptin acts as a major stimulator of the HPG axis.

Two different populations of kisspeptin-expressing neurons have been identified in rodents. The most predominant population is located in the arcuate nucleus (ARC) of the hypothalamus, where kisspeptin-expressing neurons co-express dynorphin and neurokinin B (NKB). Another kisspeptin-expressing cell population is located in the anteroventral periventricular nucleus (AVPV) of the hypothalamus. In humans and primates, kisspeptin is expressed predominantly within the infundibular nucleus, which is equivalent to the ARC in rodent [6]. Kisspeptin neurons in both populations make direct synaptic contacts with GnRH neurons and their terminals in the median eminence [7].

3. GnRH release is influenced by kisspeptin

In rodents, GnRH is released into the portal circulation by GnRH neurons located in the preoptic area and eventually reaches the anterior pituitary. GnRH is released in a pulsatile manner, and the pulse frequency and amplitude of GnRH release vary physiologically during reproductive cycles in females. Secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) is maintained by pulsatile, not continuous, release of GnRH [8]. Moreover, the frequency of the GnRH pulse differentially regulates the production and release of FSH and LH from the anterior pituitary [9]. Administration of high-frequency GnRH pulses increases the secretion of LH, whereas a lower frequency of GnRH pulses shifts the gonadotropin secretion to more FSH dominant [10]. Taken together, these

observations show that the secretory mode of pituitary gonadotropins is controlled by the so-called GnRH pulse generator.

The neuronal mechanisms underlying the pulsatile release of GnRH are still not fully understood, but at present it is agreed that kisspeptin neurons located in the ARC of the hypothalamus may be involved. Previous studies recorded the electrical activity of neurons, measured as multiunit activity (MUA), within the ARC region of the hypothalamus and reported that MUA correlates with pulsatile secretion of LH in several animal models [11, 12]. The neuronal population of kisspeptin-expressing cells is called KNDy neurons because kisspeptin-expressing cells located in the ARC region of the hypothalamus co-express kisspeptin, neurokinin B, and dynorphin. KNDy neurons generate synchronized oscillatory patterns of neuronal activity by sharing excitatory and inhibitory input from NKB and dynorphin produced within themselves [13, 14]. Selective and synchronized activation of KNDy neurons induces pulsatile release of LH in a mouse model [15]. Furthermore, exogenous kisspeptin administration can increase LH pulse frequency and amplitude in healthy women [16]. Because kisspeptin antagonism suppresses both mean GnRH and GnRH pulses [17], it is natural to think that kisspeptin neurons in the ARC of the hypothalamus (KNDy neurons) comprise the GnRH pulse generator.

The pattern of GnRH release and subsequent LH release across the reproductive cycle is modulated by gonadal steroid feedback. In rodents, the estrogen-induced GnRH/LH surge is mediated by kisspeptin neurons in the AVPV region (positive feedback). However, KNDy neurons in the ARC region of the hypothalamus are sensitive to estradiol (E2) and reduce the GnRH/LH secretion (negative feedback). This concept is based on the observations that kisspeptin expression was increased in the AVPV region at the time of the estrogen- and progesterone-induced LH surge in ovariectomized rats, whereas kisspeptin expression levels in the ARC were lowest during this time [18]. Another experiment showing that Kiss1 gene expression in the kisspeptin neurons in the AVPV is upregulated by E2, whereas those in the ARC are inhibited by E2 [19], also supports the current understanding that kisspeptin neurons in the AVPV play a role in the GnRH/LH surge, and kisspeptin neurons in the ARC region maintain the pulsatile release of GnRH and also play a pivotal role in the negative feedback control by E2.

4. Hypothalamic cell models, GT1-7 and rHypoE8, for investigating neuroendocrine mechanisms of the HPG axis

The hypothalamus is the control center for the HPG axis; however, it has been difficult to study in detail the GnRH neurons as well as the kisspeptin neurons because of the inherent heterogeneity of this brain region. The hypothalamus is composed of a complex network of neurons, and there are different neuronal phenotypes that express a specific complement of neuropeptides, neurotransmitters, and receptors [20]. Immortalized, clonal cell lines represent a relatively homogeneous population of specific neuronal cells and can be used as

experimental models. For the study of the character or the functions of hypothalamic GnRH neurons, we are using GT1-7 cell lines, which have proven to be a valuable GnRH-expressing cell model for GnRH neurons. These cells were created from the hypothalamic tumor cells in a transgenic female mouse that expressed the SV40 T-antigen under the control of the GnRH promoter [21]. GT1-7 cells display neuronal morphology and secrete GnRH; therefore, these cells have become one of the most highly utilized neuronal cell models for studies related to GnRH neurons.

The embryonic rat hypothalamic cell line R8 (rHypoE8) consists of hypothalamic neurons from rat embryonic day 18 hypothalamic primary cultures immortalized by retroviral transfer of SV40 T-antigen. These cells express neuroendocrine markers such as kisspeptin, GnRH, and RF-amide-related peptide-3 (RFRP-3, the mammalian ortholog of the avian gonadotropin-inhibiting hormone, GnIH). Because the expression of kisspeptin or RFRP-3 is functionally altered by physiological neuropeptides, these cells serve as tools for the analysis of the cellular and molecular mechanisms involved in the hypothalamic control of a number of physiological processes [22].

5. Effect of kisspeptin on hypothalamic GT1-7 cells

It is generally agreed that hypothalamic kisspeptin regulates GnRH release from GnRH neurons by kisspeptin binding the Kiss1R that is expressed by GnRH neurons [23]. A previous study by Novaira et al. demonstrated a functional role for kisspeptin in GT1-7 cells, in which they showed that kisspeptin stimulates the expression and secretion of GnRH [24]. Similarly, Terasaka et al. demonstrated the stimulatory effect of kisspeptin on GnRH gene expression, and they also found that this stimulatory effect was antagonized in the presence of bone morphogenetic protein in these cells [25]. In our study using GT1-7 cells, we did not observe any effect of kisspeptin on GnRH expression [26]. Because GT1-7 cells express Kiss1R, we suspected that Kiss1R function was lost or diminished in our GT1-7 cells, probably because of a change in cell character due to cell immortalization or multiple passages. On the other hand, when GT1-7 cells overexpressed Kiss1R after transfection with a Kiss1R expression vector, exogenous Kiss1R was absolutely functional. Furthermore, both extracellular signal-regulated kinase (ERK) and cAMP/protein kinase A (PKA) pathways were activated by kisspeptin in Kiss1R-overexpressing GT1-7 cells. These observations suggested that overexpression of exogenous Kiss1R could lead to activation of the intracellular signaling pathways mediated by kisspeptin stimulation in these cells. It is also noteworthy that, even when GT1-7 cells overexpressed Kiss1R, GnRH expression was not stimulated by kisspeptin [26]. It is still unclear why kisspeptin did not increase GnRH expression in our GT1-7 cells even when Kiss1R was overexpressed; instead, we clearly observed that kisspeptin could stimulate the expression of the GnRH receptor (GnRHR) in GT1-7 cells overexpressing Kiss1R [26] (**Figure 1**). GnRH-producing cells have been reported to respond to GnRH and modify their GnRH expression levels [27]. Furthermore, GnRHRs within GnRH neurons were reported to be involved in the pulsatile secretion of GnRH by an autocrine or paracrine interaction between GnRH and GnRHR [28, 29]. These

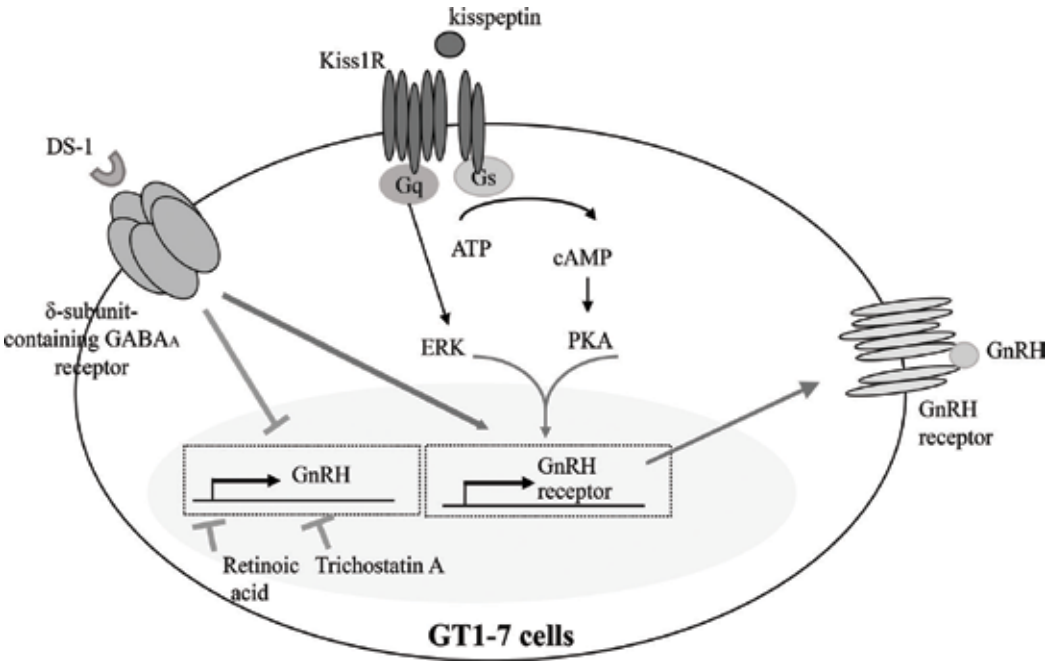

Figure 1. Schematic summary of the regulation of GnRH in GT1-7 cells. GT1-7 cells express Kiss1R, but endogenous Kiss1R does not respond to kisspeptin. Therefore, we used GT1-7 cells overexpressing Kiss1R. GT1-7 cells overexpressing Kiss1R did not show an increase in GnRH mRNA expression upon kisspeptin treatment. However, kisspeptin increased GnRH receptor expression in these cells. We also found that a γ-subunit-containing $GABA_A$ receptor agonist, DS1, as well as histone deacetylase inhibitor trichostatin A, reduces GnRH mRNA expression. Retinoic acid also has an inhibitory effect on GnRH expression.

observations implied that kisspeptin could affect the function of GnRH neurons by changing their expression levels of GnRHR.

6. Effect of kisspeptin on primary cultures of fetal rat brain

Although GT1-7 cells endogenously express Kiss1R, kisspeptin does not affect these cells. When GT1-7 cells overexpress Kiss1R, kisspeptin stimulates intracellular signaling pathways and increases GnRHR, but not GnRH expression. To determine the character of kisspeptin neurons in their original, non-transformed state, we used primary cultures of fetal rat brain that contain both GnRH and kisspeptin neurons. GnRH neurons in these cells did not respond to E2, which failed to stimulate GnRH mRNA expression. This observation was consistent with a previous study that revealed a lack of estrogen receptor immunoreactivity in GnRH neurons, raising doubts about the role of E2 in GnRH neuronal function [30]. In contrast, kisspeptin neurons in these primary cultures responded to E2, and Kiss1 mRNA expression was upregulated by E2 [31], suggesting that kisspeptin neurons, but not GnRH neurons, could be a target of E2 in neuronal cells in the fetal brain. GnRH mRNA expression within these primary cultures of fetal rat brain containing GnRH-producing neurons was

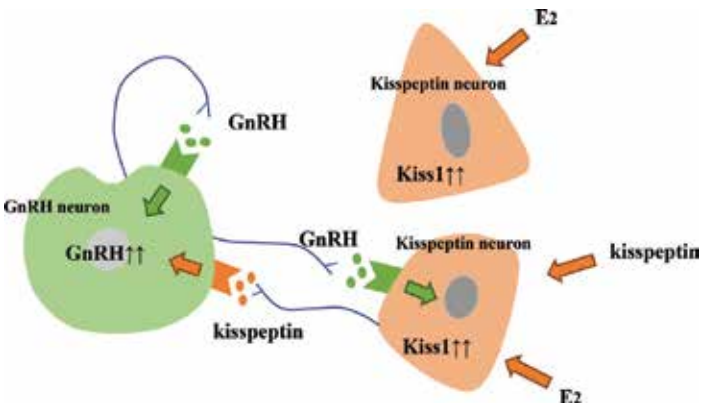

Figure 2. Schematic summary of the regulation of Kiss1 mRNA and GnRH mRNA expression in primary cultures of fetal rat brain and the proposed interaction between kisspeptin neurons and GnRH neurons. In experiments using primary cultures of fetal rat neuronal cells, Kiss1 mRNA, but not GnRH mRNA expression, was upregulated by estradiol (E2). GnRH mRNA expression was clearly increased by treatment with kisspeptin. GnRH stimulation increased the expression of both Kiss1 and GnRH mRNAs, and kisspeptin itself was found to increase the expression of the Kiss1 gene. We postulate that GnRH neurons reversibly interact with kisspeptin neurons and also form an autocrine interaction with kisspeptin neurons.

clearly increased by treatment with kisspeptin. Therefore, we could conclude that kisspeptin can stimulate GnRH synthesis in GnRH-expressing neurons in vivo. However, kisspeptin increased GnRH mRNA expression only up to about 1.5-fold [31]. In addition, we have found that GnRH stimulation increased the expression of the Kiss1 gene as well as that of the GnRH gene and also found that kisspeptin itself increased the expression of the Kiss1 gene. We postulate that GnRH neurons reversibly interact with kisspeptin neurons and also form an autocrine interaction with kisspeptin neurons (**Figure 2**).

7. Trichostatin A, a selective inhibitor of mammalian histone deacetylase, reduces GnRH expression in GT1-7 cells

Observations from the studies using GT1-7 cells and primary cultures of fetal rat brain imply that kisspeptin could affect GnRH neurons and increase GnRH expression. In addition, kisspeptin may change the GT1-7 cells' expression levels of Kiss1R. GnRH synthesis is not only regulated by kisspeptin, but several experimental reagents can modify the GnRH synthesis in GnRH-producing cells. Trichostatin A (TSA), a selective inhibitor of histone deacetylase, is an experimental reagent that modifies gene expression by opening chromatin structure through hyperacetylation of histones [32]. The structural change in chromatin allows transcription factors to bind DNA to modify gene expression. In GnRH-producing GT1-7 cells, TSA significantly reduced GnRH expression, with a concomitant increase in the gene encoding retinaldehyde dehydrogenase, which catalyzes the oxidation of retinol to retinoic acid [33]. Because retinoic acid also reduces GnRH expression in these cells, epigenetic mechanisms modified through retinaldehyde dehydrogenase, and retinoic acid might have an inhibitory effect on GnRH production (**Figure 1**).

8. DS1, a δ-subunit-containing $GABA_A$ receptor agonist, reduces GnRH mRNA expression and increases that of GnRHR in GT1-7 cells

It is well documented that the neurotransmitter γ-aminobutyric acid (GABA) can modulate the activity of GnRH neurons. GnRH neurons possess functional $GABA_A$ receptors [34], and GABAergic neurons establish synapses with GnRH neurons [35]. GABA neurons predominantly exert their inhibitory effect on GnRH neurons in rodents and sheep. GT1-7 cells also express functional $GABA_A$ receptors [36]. $GABA_A$ receptors are multimeric proteins that are composed of five subunits drawn from a repertoire of several homologous protein groups (α1-6, β1-3, γ1-3, δ, ε, θ, and π); the majority of $GABA_A$ receptors in the central nervous system are composed of α, β, and γ subunits, and less abundant populations of $GABA_A$ receptor contain the δ subunit [37]. DS1, an α4β3δ $GABA_A$ receptor agonist, reduces GnRH mRNA expression in GT1-7 cells, although DS1 can exert a stimulatory effect on signal transduction systems, such as ERK and cAMP/PKA [38]. The δ-subunit-containing α4β3δ $GABA_A$ receptor was found in extra-synaptic sites and is known to control neuronal excitability [39]. Interestingly, although GnRH mRNA expression was decreased, GnRHR expression within GT1-7 cells was significantly increased by DS1 stimulation [38] (**Figure 1**). At present, it is still unknown why δ-subunit-containing $GABA_A$ receptor agonism decreases the production of GnRH in spite of increasing GnRHR expression. We currently speculate that GABA could modulate GnRH-producing neurons through δ-containing $GABA_A$ receptors and deplete their GnRH content by modulating gene expression and secretory function in association with the expression of their GnRHR within the cell (**Figure 1**).

9. Kisspeptin expression is induced by glucagon-like peptide-1 in rHypoE8 cells and GT1-7 cells

As described above, we used GT1-7 cells as a model for GnRH-producing neurons; however, GT1-7 cells also express the Kiss1 gene, which encodes kisspeptin [40]. rHypoE8 cells, another hypothalamic model that was developed from rat embryonic hypothalamic primary cultures, express the Kiss1 gene, and they also express the GnRH gene [22]. Because both rHypoE8 and GT1-7 are immortalized cell lines derived from heterogeneous hypothalamic cell populations, they express several types of neuropeptides. Using these hypothalamic cell models, we found that Kiss1 mRNA was regulated by several metabolic factors. Glucagon-like peptide-1 (GLP-1) is a gastrointestinal hormone produced by the small intestine and colon in response to food intake [41]. GLP-1 is also expressed in the central nervous system, and its expression in the brain is altered during fasting or feeding [42], suggesting that GLP-1 plays a role as a satiety factor. We found that GLP-1 increased the expression of Kiss1 mRNA in rHypoE8 cells as well as GT1-7 cells [43]. Moreover, leptin, which is an anorexigenic factor that is released from adipocytes, can also stimulate Kiss1 mRNA expression in these cells (**Figure 3**). These observations suggest that the levels of metabolic factors such as GLP-1 or leptin, which change during a state of starvation or negative energy balance, can critically influence the HPG axis by changing kisspeptin expression.

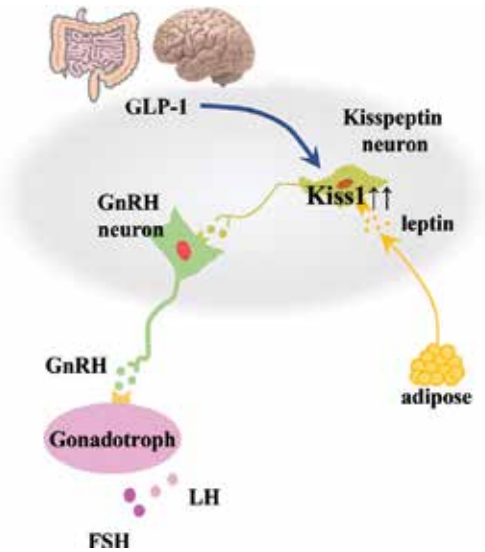

Figure 3. The metabolic factors glucagon-like peptide-1 (GLP-1) and leptin can affect the HPG axis via kisspeptin neurons. From experiments using the hypothalamic cell models rHypoE8 and GT1-7 cells, we found that metabolic factors such as GLP-1 and leptin had the ability to stimulate Kiss1 gene expression. These metabolic factors may affect the HPG axis by modulating the synthesis and release of kisspeptin.

10. Conclusion

Within the past decade, our understanding of the hypothalamic control of female reproductive function has matured considerably. The identification of hypothalamic kisspeptin, a regulator of GnRH, has provided us decisive insight into previously unanswerable questions. Kisspeptin neurons within the hypothalamus play a pivotal role in the control of the HPG axis, but it is still not entirely clear how kisspeptin release and expression are regulated in the brain during the reproductive cycle. Furthermore, the precise biology of kisspeptin and GnRH neurons remains unknown because of the difficulty of isolation of these neurons from heterogeneous neuronal populations of the hypothalamus.

In this review, we described our observations concerning the regulation of kisspeptin and GnRH neurons using hypothalamic cell models. Because we believe these cell models may reflect the original character of genuine kisspeptin and GnRH neurons, future studies using these cells are likely to contribute to our understanding of the mechanisms of regulation of the HPG axis.

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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Chapter 3

Hypothalamic Control of Sleep-Wake Circadian Cycle

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Additional information is available at the end of the chapter

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Abstract

Sleep-wake cycle is probably the most truthful signature of life. These unavoidable interchangeable states are together the matrix for all that occurs in physiology, and its rhythms are regulated by homeostatic and circadian processes involving different neuronal structures and distinct neural substrates. Hypothalamic regulation of sleep-wake cycle becomes of relevance as several neuropeptide-producing neurons involved in sleep and wakefulness regulation are located there. In this chapter, we provide a review of the hypothalamic regulation of sleep-wake cycle, focusing on the hypocretin system and melanin-concentrating hormone (MCH)-producing neurons located in the lateral hypothalamic area (LHA).

Keywords: hypothalamus, sleep-wake, circadian rhythm, hypocretin, orexin

1. Hypothalamus as a sleep-wake cycle regulator aside the RAS

The invention of the EEG by Hans Berger was a landmark in the history of sleep science. Until then, sleep was primarily considered to be a passive state, resulting from an exhaustion-modulated partial disconnection of sensory-motor circuitry from the higher-level neural regulators [1]. When early and after the first recordings of brain electrical activity, Berger established the alpha and beta waves as the EEG-dominant oscillations in healthy subjects [2]; he was proposing the electrophysiological definition of being awake. Later developments of Berger research allowed Frédéric Bremer, who was studying the physiology of the cerebellum and the neural control of muscular tone, to further investigate on the side effects of sleepiness after a lesion was produced on the hypothalamus. Although not precisely involved in sleep research, Bremer curiosity on exploring the functional effects of lower brain damages

further led him to perform cats' decerebration by which the forebrain was left in situ after a mesencephalic transection at intercollicular level. The results of this approach—the "cerveau isolé" model—leading to a persistent and indefinite condition with the brain deprived from the ascending sensory information, except for olfaction and optical ones, led Bremer to consider the hypothesis of sleep being a consequence of a complete deprivation of a sensory input arriving from the spinal cord. In this model, the cortical EEG pattern was dominated by a high-amplitude, low-frequency activity, like that observed in the slow-wave sleep (SWS). The following experiments, where the brain transection was performed at the level of the meeting point between the brain stem and the spinal cord, revealed very different results. In this "encephale isolé" model, an interchangeable oscillation between the sleep and the wake states, with an EEG pattern varying from the spontaneous low-frequency, high-amplitude activity usually observed in SWS, and high-frequency, low-amplitude activity, typical of wakefulness and rapid eye movement, was observed, not different from what can be noticed in a healthy condition. Although, at this time, Bremer was unaware of the reticular activating system (RAS), the assumption taken from his work that sleep was derived from a reduction in cortical tone while wakefulness resulted from the maintained sensorial flow to the brain served as the basis for later developments on sleep-wake cycle neurophysiology [3].

RAS was identified about 14 years later by Moruzzi and Magoun who significantly contributed to sleep-wake physiology by showing that brainstem reticular formation stimulation abolished EEG low-frequency activity and induced high-frequency activity in the cortical recordings [4]. Further experiments using the transection technique concluded that RAS underlies wakefulness, while its absence or its "silence" precipitates sleep [5]. These results were, however, obtained in acute experiments when EEG was assessed almost immediately after the brain damage. However, Villablanca [6] observed that, in the animals transected and maintained alive days or weeks after the surgical procedure, a waking-like EEG activity characterized by low-amplitude high-frequency waves was observed, suggesting that the forebrain could be involved in this partial recovery of the normal rhythm, in particular, its magnocellular region which contains cholinergic, GABAergic, and glutamatergic neurons. This allowed conceptualizing that the wake-state modulation may also be dependent regions located rostral to RAS, in particular, of the forebrain. Some studies showed that the electrical stimulation of the posterior hypothalamus and the basal forebrain in the isolated cat forebrain induced fast cortical EEG rhythms [7]. On the other hand, the cholinergic stimulation of these areas was shown to induce arousal, suggesting a role in the modulation of a wakening mechanism.

In a "diencephalic model," resulting from the removal of the cortex and striatum, leaving the thalamus, hypothalamus, and basal forebrain connected to the brain stem, animals became hyperactive, hyperreactive to sensory stimuli, and with a low-amplitude, high-frequency activity in the thalamus. In "athalamic animal" in which the thalamus was removed, they were also hyperactive and reactive to sensory stimuli, but they could not localize the stimuli and do not show very much awareness with only brief periods of low-amplitude, high-frequency activity.

To evaluate how close is the relationship between the structure and the elicited command to develop wake, we can infer using the latency of a stimuli to induce awake EEG. The stimulation of RAS-thalamic pathway is several times faster on inducing a wake-like pattern than stimulating basal forebrain or lateral hypothalamic/orexin pathways, thus meaning that for both regions, there is a need to project elsewhere to induce such a wake EEG pattern.

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In the 1920s, during the influenza epidemic, a new type of encephalitis, attacking brain regions and regulating sleep and wakefulness, was described by Constantin von Economo. This disorder, which was eventually called encephalitis lethargica or von Economo's sleeping sickness, swept through Europe and North America, with some patients exhibiting severe insomnia, while others slept for 20 or more hours per day, arising only briefly to eat and drink. The postmortem autopsies of these patients indicated that those with an insomnia-like phenomenon had a damage in the anterior hypothalamus, whereas those with abnormally increased sleep periods showed an abnormal posterior hypothalamus. In view of that, an ascending arousal system originating in the brainstem that kept the forebrain awake was proposed and later described by Moruzzi and Magoun as the ascending reticular activating system. Later studies, during the 1980s, clarified the nature of this pathway.

Although Von Economo's work represented a crucial achievement for sleep research, the seminal studies of the hypothalamic-hypocretin system were performed by Lecea and Kilduff who characterized the mRNA-encoding hypocretin and identified that the neurons were responsible for its production [8]. Soon after their findings, the relationship between hypocretin/orexin neurons and narcolepsy was established with a mutation in the orexin-2/hypocretin-2 receptor observed in a narcoleptic dog [9]. Symptoms of narcolepsy, a disorder characterized by hypersomnolence and muscle weakness (cataplexy) triggered by emotion, were also associated to the absence of orexin/hypocretin [10] to the lack of orexinergic/hypocretinergic neurons [11] or orexin/hypocretin 2 receptor [12]. Cell bodies of those neurons are in the perifornical area and lateral hypothalamus (LH), responsible for RAS and tuberomammillar nucleus (TMN) neurons activation and are active during wake state and rapid eye movement (REM) sleep [13].

2. Orexinergic neurons, their receptors, and physio-pharmacological aspects of orexinergic system related to the sleep-wake cycle

Prepro-orexin protein is the precursor protein, generating the excitatory neuropeptides orexins A and B (hypocretins 1 and 2). Orexin A (hypocretin 1), with a structure of 33 amino acids and 3.5 kDa, is completely conserved among different mammals which reflects its physiological relevance. Orexin B (hypocretin 2) is a 28-amino acid peptide with 2.9 kDa with 46% similarity to orexin A [14]. Their neurons, located on the LH, project widely throughout the brain and spinal cord [15]. Orexin excites target neurons through two types of expressed G-protein-coupled receptors. Orexin 1 receptor (OX1R) is dominantly expressed in the locus coeruleus (LC) and orexin 2 receptor (OX2R) is dominantly expressed in the arcuate nucleus (Arc), ventrolateral hypothalamus (VMH), LH, and TMN. Both OX1R and OX2R are expressed in the raphe nucleus and ventral tegmental area (VTA).

Similar to other wake-promoting neurons, orexin neurons fire mainly during active wakefulness when orexin levels are highest and are silenced during NREM and REM sleep, concurring with the lowest levels of orexin [16].

Different neuronal pathways involving orexin and neurotransmitters affecting its activity were identified. Neuropeptide Y (NPY) and agouti-related peptide (AgRP) in the Arc project to orexin neurons [17]. Also, serotoninergic neurons in the median/paramedian

raphe nucleus and GABAergic neurons in the ventrolateral preoptic (VLPO) nucleus send axons to orexin neurons [18]. VLPO is of major importance on initiating and maintaining NREM sleep as their neurons are activated by the somnogens adenosine [19] and prostaglandin D2 [20], and VLPO damage reduces NREM and REM sleep [21]. Orexinergic neurons are also targeted by neuronal projections from the bed nucleus of the stria terminalis (BST), supraventricular zone, and dorsomedial hypothalamus (DMH) [18] and receive neuronal projections from the suprachiasmatic nucleus—the human master circadian clock [22]. A direct neuronal pathway between SCN and orexinergic neurons was not identified until now.

Since orexinergic neurons in LH are scarce and difficult to distinguish from other neurons just by morphology, a slice-path clamp technique, an electrophysiological method based on the expression of enhanced green fluorescent protein (EGFP) under the control of orexin promoter in transgenic mice, has been used in order to identify substances affecting orexinergic neuron activity [23, 24]. For instance, this allowed to assume the effects of distinct neurotransmitters on orexin neurons: glutamate receptor agonists AMPA and NMDA depolarize orexin neurons, while GABAA and GABAB receptor agonists muscimol and baclofen hyperpolarize those cells. Serotonin and noradrenaline hyperpolarize all orexin neurons through two receptors coupled to inhibitory Gi proteins (5HT1A and alpha 2A receptors, respectively) and subsequently activate protein-coupled inwardly rectifying potassium channels. Recent optogenetic methods allowed to confirm that the activation of serotoninergic neuron terminal inhibits orexin neurons either directly (via 5-HT1A receptor) or indirectly (via facilitation of GABAergic-inhibitory inputs) [25]. Dopamine also hyperpolarizes orexin neurons possibly by an indirect action through alpha 2A receptor [26], and glycine inhibits the activity of orexin neurons either directly and indirectly [27].

One complementary method to study the function of orexinergic neurons is to look for the physiological consequences of its ablation. Hara and coworkers generated transgenic mice, in which orexin neurons are ablated, and showed a phenotype similar to human narcolepsy [11], which also occurred in OX1r and OX2r knockout mice [28]. In transgenic mice, experimentally induced gradual ablation of orexin neurons using a specific "time-controlled death" technique was associated to a fragmentation of the usual sleep-wake cycle [29]. The anatomical proximity and the genetic co-localization of the orexin neurons regulating sleep-wake state have recently benefitted from optogenetics. Using this kind of approach, Adamantidis and collaborators showed that by increasing the activity of orexin neurons, there was also an increased probability of transition to wakefulness from either NREM or REM sleep [30]. On the other hand, results from Zhang group using the same kind of approach indicate that the acute inhibition of orexinergic neurons leads to a time-of-day-dependent induction of NREM sleep [31]. To overcome some difficulties related to the study of neuronal networks located deeper in the brain, several new-generation optogenetic tools are being developed with an expected great impact on the near future in the areas of chronobiology and sleep physiology.

3. Melanin-concentrating hormone (MCH) and MCH neurons

The melanin-concentrating hormone is a 19-amino acid peptide predominant in specific neurons with the cell body located in the lateral hypothalamus and incerto-hypothalamic area of mammals. Apart from the sleep-active neurons in the preoptic area, these groups of neurons are also active during sleep, especially in REM sleep [32]. MCH neurons project throughout the brain with a dense innervation of the cholinergic and monoaminergic arousal centers [33]. MCH decreases cAMP levels in the cell through the MCH receptor 1(MCHR1), a G-protein-coupled receptor linked to Gq, Gi, and Go subunits which are expressed widely in the brain [34], and cellular electrophysiological studies showed that MCH has both presynaptic and postsynaptic strong inhibitory effects [35, 36]. The evidence that MCHR1 is expressed in several areas of the brain including those which are part of physiological pathways within sleep-wake control mechanisms (hippocampus, subiculum, basolateral amygdala, shell of the nucleus accumbens, ventromedial nucleus, arcuate nucleus, tuberomammillary nucleus, dorsolateral pons including dorsal raphe, and locus coeruleus) [37] supports that MCH neurons must play an essential role on sleep-wake physiology.

Furthermore, while intracerebroventricular infusion of MCH peptide facilitates REM and NREM sleep [38], knockout of MCH is associated to a more active wakefulness state [39] and to a reduction on either REM or NREM sleep. Optogenetically selectively activated MCH neurons generally increase REM sleep duration [40–42]. Consistent results have shown that MCH neurons are strongly activated on REM sleep and de-activated during NREM, suggesting that MCH neurons promote REM sleep [32]. However, studies with timing-controlled ablation of MCH neurons revealed an increase in wakefulness and a reduction in NREM sleep, showing that MCH is also involved in the regulation of NREM sleep.

MCH neurons seem to inhibit some awake center neurons through GABAergic-inhibitory synapses onto histaminergic neurons of tuberomammillary nucleus. Recent work showed that the acute activation of MCH neurons, at the onset of REM sleep, extended the duration of this sleep stage but not that of the NREM sleep [42]. The inhibition of MCH neurons on the other hand reduces the frequency of theta rhythms from the hippocampus without interfering on REM sleep duration [41].

MCH neurons are excited by orexin, AMPA agonists, NMDA, and cannabinoid type-1 receptor agonists [43–45] and inhibit orexinergic and adjacent GABAergic neurons [46]. It is clear, however, that orexin may also inhibit MCH neurons via GABAa receptors [47]. Dopamine is also an MCH neuronal inhibitor either via alpha-2 receptor [48] or via D1- and D2-like receptors [49]. Furthermore, MCH neurons are inhibited by MCH itself and by GABA, noradrenaline, serotonin, acetylcholine, neuropeptide Y, and histamine [50]. This mutual inhibitory interaction between orexin neurons and MCH neurons in the LH is crucial for the regulation of sleep-wake physiological cycle [51–53].

4. Circadian regulation of sleep-wake cycles and some of its disturbances

Sleep disorders are complex phenomena. A detailed correlation of sleep-wake regulation and clinical states is beyond the scope of this chapter, but a few examples can help to bridge the basic science concepts to everyday clinical scenarios. Since the first description of the hypocretin/orexin system 20 years ago, a body of literature investigating the physiologic and pathophysiology role of this system, as well as the potential for drug development, has emerged. Disruption of this system has been linked to pathological sleep-wake states such as insomnia and narcolepsy. A role for the hypocretin/orexin system in other sleep disorders and in sleepiness associated with other neurological disorders has also deserved some investigation. Recent results indicate that subjects with head trauma or encephalitis may have moderately but significantly decreased hypocretin levels. A few selected subjects with Guillain-Barré syndrome, Parkinson's disease (PD), multiple system atrophy, and other neurodegenerative disorders have also been found to have shallow hypocretin levels. Importantly, central actions of orexin regulate motivated behaviors, stress response, and energy/glucose metabolism by coordinating regions of the central autonomic network and the endocrine system, these multiple actions of orexin being critical to maintaining life.

Considering these putative clinical targets, there has been an ongoing research in the development of selective hypocretin/orexin receptor agonists and antagonists. Recently, suvorexant became the first US Food and Drug Administration (FDA)-approved hypocretin/orexin receptor antagonist for the treatment of insomnia [54], and Nagahara and coworkers published a work on the first hypocretin/orexin agonist with good potency and pharmacological selectivity [55].

4.1. Primary hypersomnias

4.1.1. Narcolepsy

As previously mentioned, narcolepsy has been associated with changes in the orexinergic/hypocretinergic neurons. It is a disabling neurologic condition affecting around 1 in 2000 individuals, characterized by excessive daytime sleepiness, frequently running with sudden muscle paralysis (cataplectic attacks), and transitions from wakefulness into REM sleep [56]. Human narcolepsy is a genetically complex disorder and environmentally influenced. The association of HLA with human narcolepsy suggests that it may have an autoimmune origin. Available treatment strategies are mainly symptomatic and include amphetamine-like stimulants and antidepressants, being met with unsatisfactory results.

Canines with narcolepsy were found to have a mutation in the orexin-2 (hypocretin-2) receptor [57] while mice lacking the orexin peptide or the neurons containing orexin (hypocretin) displayed behavioral and EEG signs of narcolepsy [11, 58]. Human subjects with narcolepsy have been found to have a lack or very low levels of hypocretin neurons (with an 85–95% reduction in the number of neurons) and orexin-A in the CSF [59]. These findings have been corroborated by postmortem examination of brain tissue of subjects with narcolepsy,

depicting massive losses of orexin neurons [60]. It is not yet entirely clear what leads to this massive loss of the orexin neurons. By contrast, the number of melanin-concentrating hormone (MCH) neurons is not reduced in number, indicating that the cell loss is relatively specific for hypocretin neurons.

4.1.2. Idiopathic hypersomnia

Idiopathic hypersomnia is characterized by excessive daytime sleepiness, without sudden muscle paralysis (cataplectic attacks) nor abrupt transitions from wakefulness into REM sleep but with a dopaminergic and overall aminergic impairment associated with this condition. Some authors have described low but detectable levels of hypocretin in these patients [61], while others reported normal levels [62, 63]. Postmortem studies are not available yet.

4.2. Hypocretin studies in neurodegenerative disorders

4.2.1. Parkinson's disease

Sleep disturbances often occur in patients with Parkinson's disease (PD) and can even precede the motor symptoms, showing, in this way, the close relation at a central level between autonomic (non-motor symptoms) and sleep centers. Excessive daytime sleepiness has been reported in almost half of the PD patients [64, 65]. In postmortem brain studies, hypocretin-1 tissue concentrations in the prefrontal cortex were almost 40% lower in these patients, with the total number of hypocretin neurons being almost half compared with controls [66, 67]. A progressive loss of MCH neurons has also been described, increasing with the disease progression [67].

4.2.2. Multiple system atrophy

Sleep disturbances occur in 70% of patients with multiple system atrophy (MSA), a progressive neurodegenerative disease of undetermined etiology, characterized by parkinsonian features, cerebellar, autonomic, and urogenital dysfunction and corticospinal disorders [68]. The clinical features include reduced and fragmented sleep, excessive daytime sleepiness, rapid eye movement (REM), sleep behavior disorder (RBD), stridor, and sleep-disordered breathing [69, 70]. In these patients, Benarroch and coworkers found up to 70% reduction in the total number of hypocretin neurons in these populations of patients and described abundant glial cytoplasmic inclusions in the hypocretin distribution area [71].

4.3. Immune-mediated neurological disorders

4.3.1. Guillain-Barré syndrome

Guillain-Barré syndrome is a post-infectious polyradiculopathy affecting mainly the peripheral nervous system, frequently presenting also with autonomic nervous system failure symptoms. Not infrequently, these patients also show other signs of hypothalamic disturbance. Guillain-Barré syndrome has been the only disorder besides narcolepsy in which undetectable levels of

hypocretin have been consistently observed [63, 72]. Patients with the lowest levels tend to have a more severe and rapid disease course, running with tetraplegia and respiratory failure. The mechanism underlying the lack or very decreased levels of hypocretin in Guillain-Barré syndrome remains unknown, but an immune-mediated hypothalamic dysfunction has been hypothesized.

4.4. Orexin and sleep-related physical disorders: cardiovascular disease

Almost all bodily functions are dependent on the autonomic nervous system (ANS), which exerts precise control over visceral functions. Sleep disruption causes an increased activity of the sympathetic nervous system in association with an elevated blood pressure, and the risks of hypertension and cardiovascular disease are increased as a consequence of either strong acute or long-term sleep disruption [73]. The hypocretin/orexin system also contributes to the regulation of cardiovascular functions via the autonomic nervous system. Hypocretin/orexin neurons project to several brain regions involved in the regulation of cardiovascular activity, namely the paraventricular nucleus (PVN), nucleus tractus solitarius, and the rostral ventrolateral medulla (RVLM), all areas of the central autonomic network [74].

Over-activation of the hypocretin/orexin system has been implicated in the pathogenesis of hypertension. It has been shown that the central administration of orexins A and B increases arterial blood pressure and elicits tachycardia in animal models [74]. Conversely, orexin/ataxin-3 transgenic rats, lacking orexin neurons, have a significantly reduced sympathetic nervous system tone and a lower systolic blood pressure when compared with controls [75]. In addition, spontaneously hypertensive rats (SHRs) have increased levels of hypocretin/orexin [74] that, when blocked by the oral administration of almorexant or by intracerebroventricular injections of TCSOX229, led to a significant reduction of systolic blood pressure while not affecting arterial blood pressure in normotensive animals [76, 77]. These data suggest that hypocretin/orexin may play a significant role in the pathogenesis of hypertension. In humans, Dauvilliers and coworkers reported a lower cardiac activation associated with periodic leg movements during sleep in narcoleptic patients which was proposed to be related to changes in baroreflex sensitivity [78]. The same group found a large percentage of diastolic non-dippers, with 64% failing to achieve the 15% fall point on diastolic blood pressure [79], and recent data suggested that narcoleptic patients displayed a nighttime non-dipping blood pressure pattern with increased systolic blood pressure during nighttime REM sleep [80].

The blunted cardiac activation and sleep-related blood pressure fall in narcoleptic patients may be clinically relevant and may indicate an increased risk for cardiovascular events among attributable to a potentially clinically significant hypocretin/orexin deficiency.

5. Conclusion

In summary, despite being present throughout the animal kingdom, the precise sleep function is still relatively elusive. However, it is evident that sleep regulation is fundamental for survival having the hypothalamus a significant role in those modulatory processes through the orexin/hypocretin and the MCH neurons. Nevertheless, further studies on sleep physiology

are needed to determine the inner mechanisms associated with sleep-wake cycle and their regulatory processes.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 4

Role of the Dorso- and Ventrolateral Pons in Cardiorespiratory Hypothalamic Defense Responses

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Additional information is available at the end of the chapter

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Abstract

Stimulation of discrete sites throughout the hypothalamus elicits autonomic and somatic responses. This chapter will stand out the cardiorespiratory changes evoked from stimulation of specific areas within the caudal hypothalamus: the perifornical area and the dorsomedial nucleus. The stimulation of these regions, known as the hypothalamic defense area (HDA), produces a pattern of visceral and somatic changes characteristic of the defense reaction, which includes tachypnea, tachycardia and a pressor response. A close review of the literature demonstrates that the changes observed during this defensive behavioral response are partially mediated by the interactions with pontine regions. These include the parabrachial complex, located in the dorsolateral pons, and the A5 region, located in the ventrolateral pons. Specific glutamatergic stimulation of cell bodies located within the parabrachial complex and A5 region evokes cardiorespiratory responses similar to those observed during stimulation of the HDA. This functional interaction suggests a possible role of glutamate pontine receptors in the modulation of the HDA response. This chapter describes the most important evidences confirming the implication of the dorso- and ventrolateral pons in the control of cardiorespiratory autonomic responses evoked from the perifornical and dorsomedial hypothalamus and the role of glutamate in this interaction.

Keywords: caudal hypothalamus, parabrachial complex, A5 region, cardiorespiratory responses, glutamate receptors, defense response

1. Introduction

Brief alerting stimuli such as an unexpected noise or light will evoke in animals immediate cardiovascular and respiratory responses, including strong cutaneous vasoconstriction and

respiratory activation [1–5]. Consistent with this, alerting stimuli in humans reliably increase cutaneous sympathetic activity [6]. Brief alerting stimuli also evoke variable changes in heart rate due to the fact that there is an activation of cardiac sympathetic and vagal parasympathetic activity [5, 7–10].

The initial response to alerting stimuli is a reflex termed "defense reaction" or "visceral alerting reaction" [11]. It is known that alarming stimuli evoke a characteristic autonomic response that includes tachypnea, accompanied by an increase in heart rate and blood pressure. A vasoconstriction in renal and mesenteric vascular beds with vasodilatation of skeletal muscle vessels is also observed in humans [12–22] and animals [23–27]. These cardiovascular changes are accompanied by a marked increase in total norepinephrine spillover in humans, indicative of an overall increase in sympathetic activity [28]. Research carried out in both, humans and animals, shows that stress elicits a typical pattern of catecholaminergic responses, with significant increases in sympathetic activity to the heart, kidney, skin, adrenal medulla and mesenteric beds and with a variable effect to the skeletal muscle.

Previous studies, using c-Fos expression, have identified several brain regions that are activated during stress. These morphological studies show that most of these regions also play a crucial role in respiratory and cardiovascular sympathetic regulation. These regions include, among others, the dorsomedial hypothalamus (DMH), the perifornical area (PeF), the paraventricular nucleus (PVN), the parabrachial complex (PBc), the periaqueductal gray (PAG), the nucleus tractus solitarius (NTS) and the ventrolateral medulla (VLM) [29–37].

The stimulation of specific areas within the caudal hypothalamus in rat, such as the PeF and DMH, classically known as hypothalamic defense area (HDA) (**Figure 1**), produces a pattern of visceral and somatic changes characteristic of the defense reaction [23]. The cardiorespiratory changes observed during the defense response are partially mediated by a facilitation of the chemoreceptor reflex and an attenuation of baroreceptor [38, 39] and laryngeal reflexes [40, 41] involving a GABAergic mechanism in the NTS [42]. The cardiovascular response is also mediated by direct descending projections from the PVN to sympathetic preganglionic

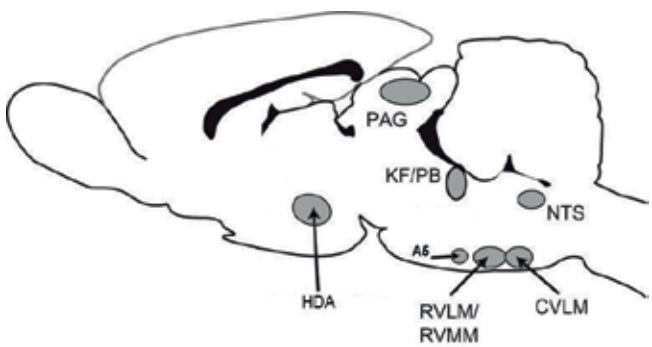

Figure 1. Semischematic line drawing of the parasagittal section through the rat brain showing the location of the hypothalamic defense area (HDA) and periaqueductal gray matter (PAG). The dorso- and ventrolateral pons shows the parabrachial complex (PB), Kölliker-Fuse (KF) and A5 region (A5). In the brainstem, nucleus of the solitary tract (NTS), rostroventrolateral medulla (RVLM), rostroventromedial medulla (RVMM) and caudalventrolateral medulla (CVLM) are shown.

neurons of the intermediate lateral cell column in the thoracic spinal cord (IML) [43], the rostral ventrolateral medulla (RVLM) [44] and the A5 catecholaminergic region of the pons [45].

Several observations clearly demonstrate the critical importance of the DMH in mediating stress-evoked cardiovascular and respiratory responses. The inhibition of neurons within the DMH greatly reduces the pressor response and tachycardia evoked by air jet stress [46, 47]. In addition, activation of somata of the DMH evokes a pattern of autonomic and respiratory effects, including a resetting of the baroreceptor reflex, which are similar to naturally evoked stress responses [48–55].

Interestingly, there are also evidences showing that the cardiovascular effects elicited by the activation of the pontine parabrachial nucleus are partially generated by a similar control of the function of the baroreceptor reflex at the level of the NTS [56–58].

The PBc lies at the junction between the rostral dorsolateral pons and the mesencephalon (**Figure 1**). The PBc contains three main subdivisions: the medial parabrachial nucleus (mPB), the lateral parabrachial nucleus (lPB) and Kölliker-Fuse area (KF) [59]. This region has been considered the site of the "pneumotaxic center" controlling inspiratory duration and is now often referred to as the pontine respiratory group [60]. The PBc modulates respiration in two different ways. Neurons located in the mPB and KF are implicated in the increase of expiratory time observed during bradypnea. On the contrary, somata located within the lPB elicit the classical tachypnea, characterized by a decrease of expiratory duration with an inspiratory facilitation [61–63]. The PBc is also related to a topographical organized regulation of bulbar laryngeal motoneurons regulating subglottic pressure [63]. Moreover, activation of these regions, typically considered as "respiratory areas," also produces cardiovascular changes including an increase of heart rate and arterial blood pressure [63, 64].

Electrical stimulation or microinjections of excitatory amino acids within the PBc [63, 65, 66] show different modulatory respiratory responses depending on the location of PBc-stimulated neurons. At all locations where respiratory responses are elicited by stimulation of PBc somata, a cardiovascular response is also observed. Similar cardiorespiratory effects are observed when glutamate is microinjected within these sites. The response comprises an increase in blood pressure with a small increase in heart rate. The cardiovascular response evoked by the stimulation of cell bodies located within the PBc resembles the response evoked on HDA stimulation [63].

The dorsolateral pontine modulation of the arterial baroreflex primarily originates from ventrolateral regions of the lPB and involves descending projections to both the NTS [56, 67] and the VRLM [67–69]. In the early 1980s, it was established that electrical stimulation of the PBc attenuates baroreflex responses [69]. The functional importance of PBc modulation of baroreflex function has been linked to the simultaneous pressor response and tachycardia evoked during the defense response, which indicates a resetting of the barorreceptor reflex. Chemical lesions of the PBc eliminate the descending modulation of the baroreflex control of heart rate and mean arterial pressure evoked from at least one "brain defense region," the dorsal PAG [70]. Blockade of neurons located in lPB, using bilateral microinjections of muscimol, a $GABA_A$ receptor agonist, or kynurenic acid, an unspecific glutamate receptor antagonist, decreases but not abolishes the attenuation of the cardiac baroreflex response evoked from the

dorsal PAG [71]. These data support the hypothesis that lPB is also a crucial pontine region implicated in the descending modulation of cardiac brainstem baroreflex function during the stress reaction evoked from hypothalamic stimulation.

In addition, the PBc is an important pontine secondary relay from the NTS, because it is involved in the modulation of this arising cardiorespiratory information [72]. The PBc, mainly its lateral part, is reciprocally connected with forebrain structures involved in cardiorespiratory regulation [59]. The activation of neuronal somata of the lPB with glutamate elicits a cardiorespiratory response that includes hypertension, tachycardia and tachypnea, while activation of cell bodies located within the mPB and KF produces a similar cardiovascular response, increase in blood pressure and heart rate, but on the contrary, accompanied with bradypnea [63]. Thus, the integrity of PBc neuronal circuits seems to be essential for the modulation of baroreflex function and appears to represent an important relay between midbrain and medulla for the coordination of autonomic defense responses.

On the other hand, the PBc is connected with another crucial area in cardiovascular control, the A5 region [73]. Electrical stimulation of the mPB or lPB produces an increase of c-Fos-like protein immunoreactivity within the A5 pontine catecholaminergic region [74].

The A5 group of catecholamine-containing neurons is located in the ventrolateral pons, between the root of the facial nerve and the superior caudal olivary nucleus (**Figure 1**). Classically, the A5 has been defined as a catecholaminergic region. It is known to provide the major component of the noradrenergic input to the sympathetic preganglionic neurons of IML [75–77], whereby it is implicated in cardiovascular control [41, 65, 78–82]. It also contains noncatecholaminergic neurons, which are mainly located at the level of the most caudal part of the A5 region [83]. These neurons seem to have properties similar to the respiratory chemoreceptors identified in the rostral medulla oblongata [84]. The A5 region has connections with the NTS, RVLM, caudal ventrolateral medulla (CVLM), caudal pressor area and the retrotrapezoid nucleus in the medulla oblongata; with the mPB, lPB and KF in the pons; and with the PeF, the PVN and the amygdala in the hypothalamus [85–90]. These connections with regions of the central nervous system involved in cardiorespiratory regulation are indicative for a role of the A5 region in the control of both sympathetic activity and cardiorespiratory function [81, 91, 92]. Moreover, A5 neurons are activated during baroreceptor unloading [81] and stimulation of carotid chemoreceptors [93, 94]. Thus, it has been proposed that A5 neurons may play an important role in the carotid sympathetic chemoreflex triggered by hypoxia [95–97]. Furthermore, the A5 region plays an important role in respiratory control, modulating the activity of respiratory neurons [98]. These cells are synaptically connected to phrenic motoneurons [99] and contribute to the respiratory responses evoked by hypoxia and hypercapnia [96, 97, 100–102]. A5 cells also modulate the cardiorespiratory response evoked by activation of the PBc [65], which is a critical component of the brainstem respiratory network required for eupnea [103].

Stimulation of A5 neurons with glutamate produces cardiorespiratory and laryngeal responses similar to those observed with mPB stimulation. That is, an expiratory facilitatory response associated with an increase in blood pressure, heart rate [104] and subglottic pressure [41]. In the same way as with PBc stimulation, the cardiovascular response is similar to that obtained during electrical stimulation of the HDA.

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The similarity of the responses to stimulation of the mPB and the A5 region suggests a possible interaction between these two pontine regions. In fact, studies from the literature demonstrate a role for the A5 region in the cardiorespiratory responses evoked on PBc electrical

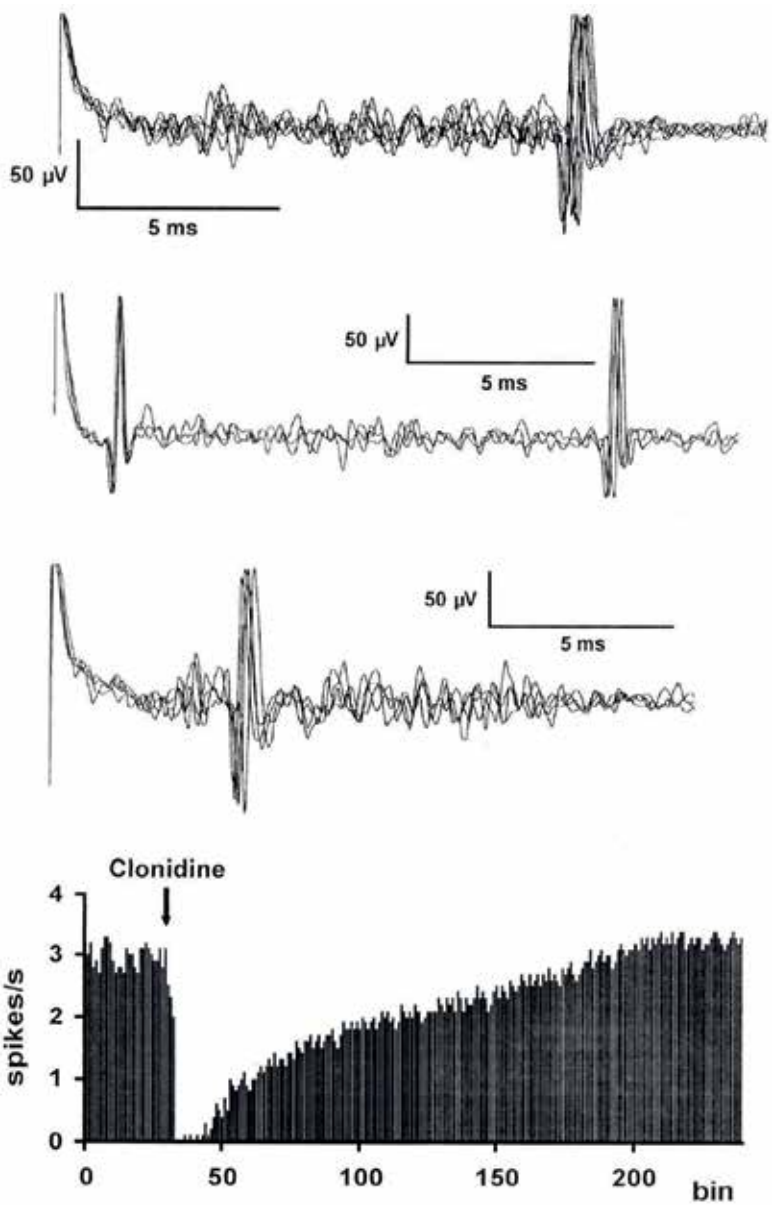

Figure 2. Neurophysiological interactions between PBc and A5. Extracellular recording (superimposed sweeps) of three A5 putative cells activated from the PBc. Effect of clonidine i.v. injection (10 µg/kg) on the discharge rate of a putative A5 neuron. Arrow shows drug injection. Firing rate histogram of a parabrachial-activated A5 putative neuron (bin size 5 s). Authors´ figure modified from Ref. [65].

and chemical stimulation [65]. The microinjection of muscimol or lidocaine within the A5 region modifies the pattern of the cardiorespiratory responses evoked from PBc stimulation [65]. The expiratory facilitatory response elicited from mPB-KF activation is reversed to an inspiratory facilitatory response. Nevertheless, when the lPB is activated, no changes are observed in the inspiratory facilitatory response. The magnitude of the increase of the pressor response and the tachycardia observed during PBc stimulation decreases significantly after A5 blocking microinjections. Moreover, a high number of extracellularly recorded neurons in the A5 region are activated on electrical stimulation within the mPB-KF nuclei [65] (**Figure 2**).

These functional connections suggest a possible interaction between PBc and A5 pontine regions in mediating the defense response evoked from the HDA. This statement will be discussed deeply in the following sections.

2. Dorsolateral pons in cardiorespiratory hypothalamic defense responses: role of the Parabrachial complex

Recent data show that neurons located within the PBc play a role in the cardiorespiratory response evoked from HDA. As previously mentioned, the stimulation of cell bodies located within the PBc resembles the cardiovascular response elicited by HDA stimulation, thus evoking tachycardia and hypertension [63].

Neuropharmacological studies show that the inhibition with muscimol of somata located within the main subdivisions of the PBc, lPB and mPB-KF produces two different patterns of cardiorespiratory responses evoked to HDA stimulation [105].

The inhibition with muscimol of neurons located within the mPB-KF reduces the tachycardia and the pressure response evoked by HDA stimulation [105] (**Figure 3A**). It is known that neuronal activity of the parabrachial nuclei can modify the effectiveness of the baroreflex in rat, rabbit and cat [56, 106] and that the PBc is essential for a full expression of the bradycardia that typically accompanies the initial hypotensive response to blood loss and for the normal rate of blood pressure recovery [107, 108].

The decrease in the cardiovascular response to HDA stimulation seems to be an indication of a resetting of the baroreceptor reflex. The normal cardiovascular response to hypothalamic stimulation, tachycardia and pressor response is due to direct activation of neurons from the RVLM, which send direct projections to sympathetic preganglionic neurons of the IML. The inhibition or the resetting of the baroreceptor reflex is the origin of the tachycardia observed during the activation of the HDA. This inhibition seems to be partially mediated by $GABA_A$ receptors located within the NTS, which produces a hyperpolarization of baroreceptor cells [42, 58].

The reset of the baroreceptor response partially explains the decrease of the tachycardia observed during the stress reaction evoked from the activation of the HDA. It could also explain, through an indirect modulatory pathway, the decrease of the magnitude of the

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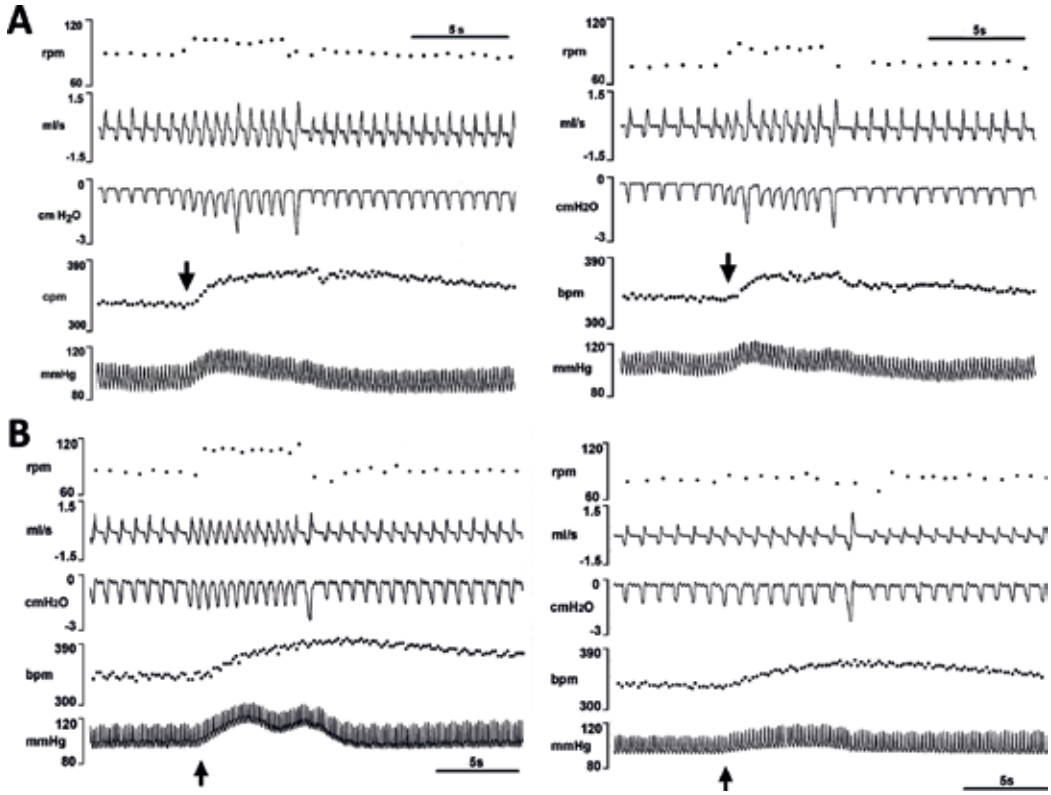

Figure 3. Neuropharmacological interactions between HDA and PBc. From top to bottom, instantaneous respiratory rate (rpm), respiratory flow (ml/s), pleural pressure (cm H_2O), instantaneous heart rate (bpm) and blood pressure (mmHg). Cardiorespiratory response evoked to HDA stimulation before (left) and after (right) muscimol microinjection within the mPB-KF (A) and lPB (B). The arrows show the onset of the HDA electrical stimulation. Authors′ figure modified from Ref. [105].

hypertensive response, although, and probably, the most important factor is the inhibition of the excitatory projections from the PBc to the IML. The most relevant conclusion from this data is the suggestion that the reset of the barorreceptor reflex elicited by HDA activation could be also mediated though a secondary indirect pathway using the PBc of the pons [105].

Therefore, the activity of mPB-KF makes an important contribution to the modulation of the intensity of the cardiovascular response evoked on HDA stimulation through an indirect pathway to both the IML and the NTS.

On the other hand, the inhibition of neurons located within the lPB with muscimol abolishes the respiratory response evoked to HDA stimulation [105]. Similar to mPB-KF inhibition, the increase of blood pressure evoked to HDA stimulation decreases after the microinjection of muscimol within the lPB; however, no significant changes of the heart rate response were observed (**Figure 3B**).

Similar results are observed with PAG stimulation, thus indicating that the PBc is also a critical relay in mediating dorsal PAG-evoked sympathoexcitation and baroreflex modulation [109]. In addition, neurons localized in the lPB are involved in mediating the defense-like behavior response during the stimulation of the dorsal PAG, modulating the arterial baroreflex [71]. This inhibitory effect is more evident from the mPB-KF than from lPB.

Therefore, the pressor response evoked during the stimulation of the HDA and PAG may involve the recruitment of neurons of both the lPB and mPB-KF subdivisions, which, using an indirect pathway, activate the IML.

Morphological studies have confirmed the presence of reciprocal connections between the PBc and different hypothalamic regions [110]. It has been also described that the PBc projects widely to areas of the forebrain involved in cardiovascular regulation and defense reactions [111]. It also projects, via descending fibers, to brainstem nuclei including the A5 region, the NTS and the IML of the spinal cord [112].

It is important to stand out the complete abolishment of the respiratory response to HDA stimulation after the inhibition of lPB somata with muscimol. The lPB is part of the neuronal pathways involved in the sympathoexcitatory component of the chemoreflex [113]. Fos protein expression studies show that the tachypnea evoked on HDA stimulation is produced by activation of carotid chemoreceptors within neurons of the lPB [94]. Moreover, neuronal recordings show that during chemoreflex stimulation, neurons of the lPB are activated and that this increase in firing precedes the classical hypertensive response to chemoreceptor stimulation, thus showing the relevance of lPB neuronal circuits on the central modulation of chemoreceptor inputs and reflex [114].

There are also indications that HDA stimulation may facilitate the chemoreceptor reflex by means of a group of intrinsic excitatory neurons localized within the NTS [115]. These cells are activated or facilitated by HDA-NTS direct excitatory connections. These neurons are also the main targets of excitatory inputs from the lPB [56]. The inhibition of these lPB excitatory projections with muscimol leads to the abolishment of the tachypneustic response evoked on HDA stimulation.

Electrophysiological studies using neuronal recordings support the above. A significant number of mPB-KF and lPB neurons are affected from HDA stimulation, confirming the importance of the functional correlation between the HDA and these pontine regions. The presence of anti-/orthodromic activations, short and long latency excitations, and inhibitions and excitatory/inhibitory activities gives electrophysiological evidence of reciprocal connections between these regions. It is also an index of the complexity of the different types of synaptic interactions between both areas (**Figure 4**) [105].

Studies related to glutamate receptors suggest that this neurotransmitter plays a crucial role in mediating the functional relation between the PBc and the HDA [116]. Glutamate activates metabotropic and ionotropic (NMDA and non-NMDA) receptors [117]. By employing immunocytochemical and in situ hybridization techniques, studies have demonstrated the presence of both metabotropic and ionotropic receptors in different nuclei of the PBc and KF [118–120]. Activation of vagal afferent fibers releases glutamate within the PBc [121]. An ascending excitatory pathway involving glutamate from the NTS to the PBc has been described [122]. In

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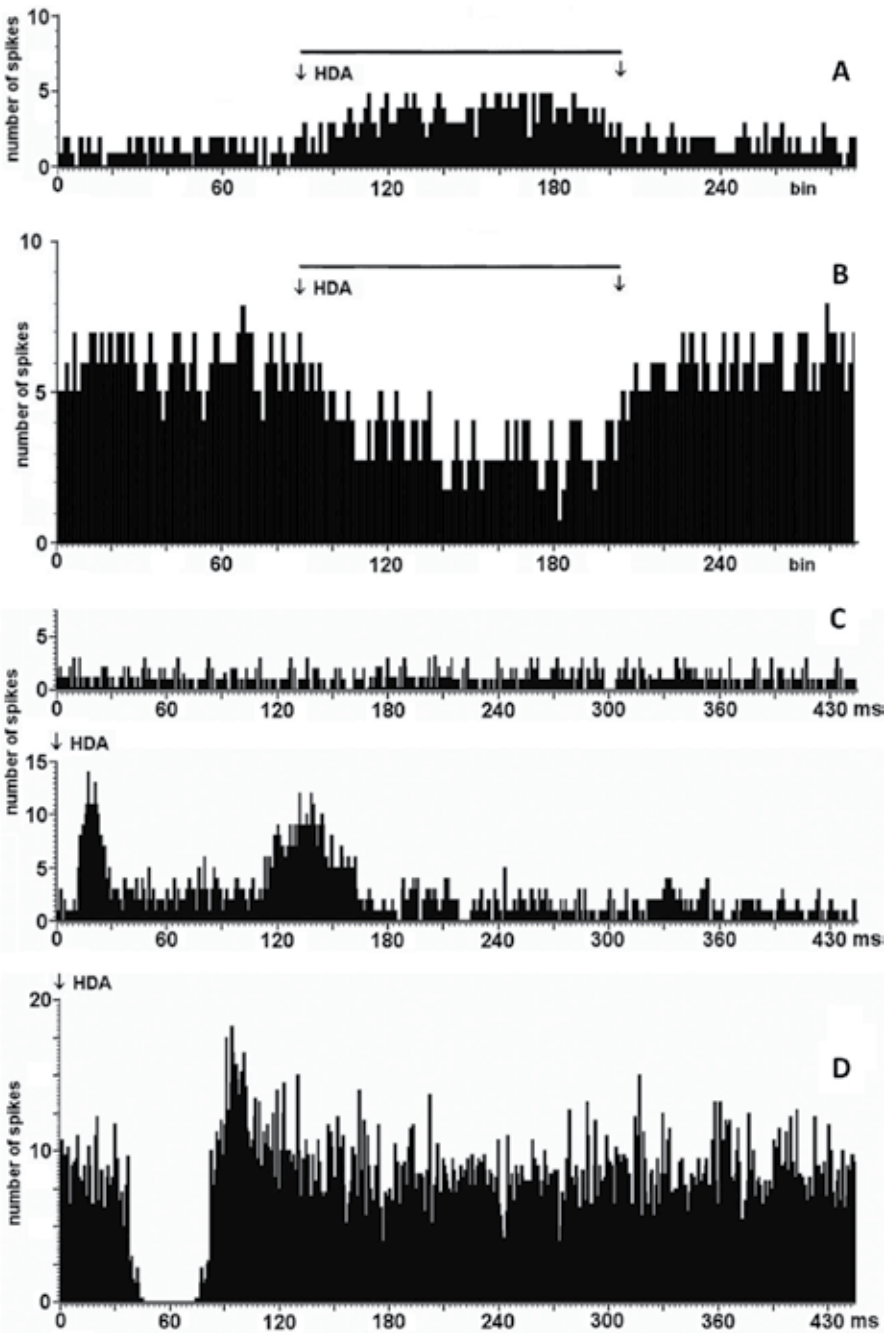

Figure 4. HDA and PBc neurophysiological interactions. (A) Shows a rate histogram (bin size 2 s) representing the firing of an lPB cell not excited nor inhibited during HDA stimulation that increased the activity during HDA stimulation. (B) Shows a rate histogram (bin size 2 s) of an mPB-KF cell not excited nor inhibited during HDA stimulation showing a decrease of activity during HDA stimulation (0.1 ms given at 1 Hz). (C) The poststimulus time histogram shows spontaneous activity of an lPB neuron and double excitation after HDA stimulation. (D) The poststimulus time histogram shows an inhibition of an mPB neuron after HDA stimulation (100 stimuli, 1 Hz). Authors´ figure modified from Ref. [105].

vitro studies also show that glutamate agonists depolarize neurons of the PBc [123], and lPB stimulation causes local glutamate release, which depolarizes lPB neurons through NMDA and non-NMDA receptors [124].

Moreover, the blockade of glutamate receptors and the microinjections of glutamate into the PBc and KF elicit a variety of cardiovascular and respiratory responses indicating that this amino acid is an important neurotransmitter for mediating autonomic functions in these regions [61, 63, 64, 122–127].

The pattern of the cardiorespiratory response evoked from HDA is modified by the microinjection of different glutamate antagonists into the PBc [116]. Kynurenic acid, a nonspecific ionotropic glutamate receptor antagonist, microinjected into the lPB and mPB abolishes the tachycardia and decreased the pressor response to HDA electrical stimulation (**Figure 5A** and **B**). The respiratory response is only abolished when kynurenic acid is microinjected into the lPB (**Figure 5A**) [116]. These results suggest that ionotropic glutamate receptors located within the lPB region are involved in both the respiratory- and the cardiovascular-evoked responses from the HDA, whereas ionotropic glutamate receptors located in mPB seem to be only involved in the modulation of the cardiovascular response.

The effectiveness of the modulation is depending on the distribution of these receptors within the PBc and these findings suggest that lPB appears to exert a more efficient modulation on the cardiovascular response to HDA stimulation compared with mPB. This cardiovascular response seems to be mediated by a direct activation of neurons located within the RVLM, which send direct efferences to sympathetic preganglionic neurons of the IML [128–130]. The activity of the RVLM can be also modulated via indirect projections. The changes in heart rate and blood pressure evoked from "defense" regions of the brain may use separate efferent pathways [51]. The blockade of the PBc attenuates the dorsal PAG-evoked changes in blood pressure [109], thus indicating that the cardiovascular changes observed during the stimulation of the HDA could be partially modulated by "direct" efferences to the RVLM but also by indirect projections, which involve the activation of ionotropic glutamate receptors located in the PBc [116].

It is known that the PBc is crucial mediating the changes of heart rate appearing during baroreceptor reflex activation [105]. The fall in the magnitude of the cardiovascular changes to HDA stimulation observed after the microinjection of kynurenic acid could indicate that neurons of the lPB and mPB exert an inhibition of tonic excitatory inputs, at the level of the NTS, on inhibitory mechanism of the baroreceptor reflex [40]. This hypothesis is also supported by the observation that the blood pressure response also tends to disappear with the decrease and/or the abolishment of tachycardia.

Another fact that could explain the more efficient modulation exerted from lPB on the cardiovascular response elicited by HDA stimulation is the specific expression of glutamate subtype receptors located within this region. A very different profile is observed when compared with the mPB or with other subnuclei of the PBc. GluR4 non-NMDA receptor subunits predominate in the internal lPB [118]. These subunits are characterized by a high sensitivity for glutamate. There is also evidence that the external and internal lPB express specific subunits of NMDA receptors, which are different to that of the mPB [119]. NMDA receptors can be quite different with respect to their physiological and pharmacological channel properties, such as differences in glutamate affinity and glycine sensitivity, crucial coagonist for glutamate

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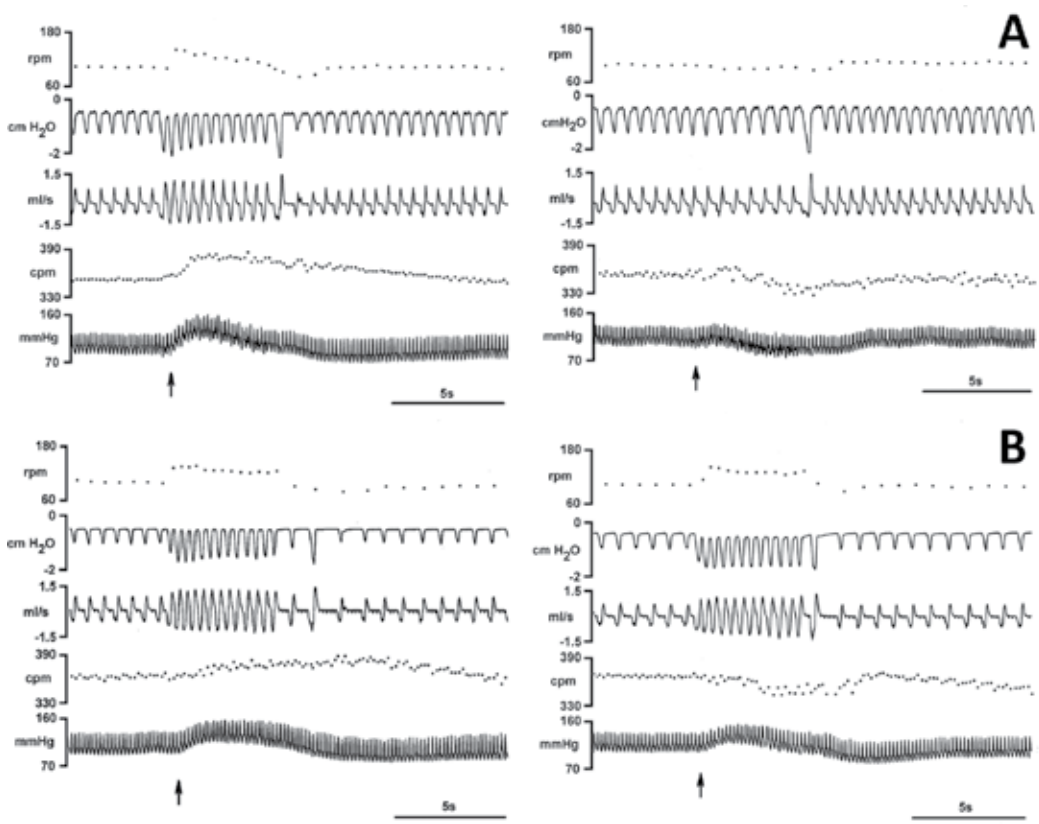

Figure 5. Neuropharmacological interactions between HDA and PBc, role of glutamate. From top to bottom, instantaneous respiratory rate (rpm), respiratory flow (ml/s), pleural pressure (cm H_2O), instantaneous heart rate (bpm) and blood pressure (mmHg). The cardiorespiratory responses evoked on HDA stimulation before (left) and after (right) kynurenic acid microinjection within the lPB (A) and mPB-KF (B) are shown. The arrows show the onset of the HDA electrical stimulation. Authors´ figure modified from Ref. [116].

efficacy [131], in calcium currents and deactivation kinetics as well as other single channel characteristics [132]. NMDA receptors of lPB are composed of NR2A and NR2B subunits, which are characterized by high affinity for glutamate and long mean open time. NMDA receptors located within the mPB are composed of NR2D subunits, which exhibit low affinity for glutamate [119, 132].

In summary, the arterial blood pressor response observed during HDA stimulation could be mediated by the activation of neuronal glutamate ionotropic receptors located in both lPB and mPB somata, which exert an indirect excitation to sympathetic preganglionic neurons at the level of the IML. The inhibitory mechanism of the baroreceptor reflex seems to depend more on the activation of lPB glutamate ionotropic receptors than mPB receptors, because tachycardia associated to the pressor response is only suppressed after lPB microinjections [116].

With respect to the changes of respiratory rate observed during the stimulation of the HDA, we have to highlight that are only abolished when the microinjection of kynurenic acid is delivered within the lPB (**Figure 5A**). Nevertheless, the respiratory response remains unchanged when

kynurenic acid is microinjected into the mPB (**Figure 5B**) [116]. The result suggests that only glutamate receptors of the lPB modulate the respiratory response to HDA stimulation.

It has been shown that the lPB is an important part of the neuronal pathways for the modulation of the respiratory response evoked on HDA stimulation. Muscimol microinjections within the lPB have similar effects to kynurenic microinjections [105]; tachypnea observed during HDA stimulation is abolished. This observation gives a role for the described lPB afferent connections from several hypothalamic nuclei involved in the defense reaction [110].

Hayward et al. obtained similar results with the blockade of glutamate receptors with the microinjection of kynurenic acid into the lPB during the dorsal PAG stimulation, one of the so-called secondary brain defense regions, confirming the importance of lPB in the integration of tachypneic responses from supraencephalic regions [133].

There are indications that HDA stimulation may facilitate the chemoreceptor reflex at specific cells located within the NTS [115]. These neurons are activated by HDA-NTS direct excitatory connections and are also the main targets of excitatory inputs from the lPB [56]. Glutamate seems to activate these excitatory inputs. The inhibition of the activation of these lPB projections with kynurenic acid leads to the abolishment of tachypnea evoked on HDA stimulation [116].

According to these observations, the cardiovascular component of the response to HDA stimulation seems to be modulated by glutamatergic neurons located in both the lPB and the mPB, whereas the respiratory component seems to be only mediated by glutamate receptors of the mPB. Moreover, different subnuclei within the lPB are involved in this cardiorespiratory modulation, which includes the crescent, ventral, central and external subnuclei. It is interesting to note that microinjections into the internal subnucleus of the lPB have no effects on this cardiorespiratory response. This result is an indication of the specificity and complexity of this region. Nearby areas, separated only by microns, such as the external and internal subnuclei of the lPB, show very different effects in the cardiorespiratory response to HDA stimulation. In contrast, all mPB microinjections, including external mPB, have an effect. These results give us clear evidence that glutamatergic neurons of the PBc are essential intermediaries for the modulation of the descending pathways for cardiovascular sympathetic and respiratory control mechanisms [116]. The impact of these projections on overall cardiorespiratory function is highly dependent on convergent inputs from specific subnuclei of the lPB region and from alternate pathways outside the PBc. Direct projections to the RVLM are also involved in HDA-evoked changes in arterial pressure [128–130], thus supporting those changes in heart rate and blood pressure evoked from "defense" regions of the brain that may travel via separate pathways [51].

3. Ventrolateral pons in cardiorespiratory hypothalamic defense responses: role of the A5 region

As previously mentioned, there are data suggesting the functional connections between the HDA and the A5 region. Fos protein expression studies, neuronal recording and neuropharmacological experiments confirm this hypothesis [23, 65, 104].

Some studies in rats have used HDA electrical stimulation to map methodically populations of neurons within the brainstem and other areas, which are excitated by changes in arterial blood pressure [134, 135]. In the A5 region, blood pressure changes cause a specific and consistent pattern of c-Fos expression.

A c-Fos-ir expression is induced during HDA stimulation in both A5 noncatecholaminergic (TH-negative) and A5 catecholaminergic (TH-positive) cells of the pons [136]. This increase in c-Fos expression is higher in noncatecholaminergic than in catecholaminergic neurons [136]. In addition, in both populations of neurons of the A5 region, this activation seems probably to be due to a direct activation from the HDA and not due to a secondary activation to the pressure response elicited during stimulation of the HDA.

This result is further confirmed with neuronal recordings. It is described as the possible role of A5 neurons in respiratory modulation [65, 93]. Moreover, there are electrophysiological evidences of interactions between HDA and A5 catecholaminergic neurons. The importance of the connections between both regions is confirmed with the observation that a significant number of these A5 neurons are activated from HDA stimulation [136]. In the same way as with PBc, antidromic and orthodromic activation are observed in A5 neurons. Cells that are antidromically activated are spontaneously active, while cells orthodromically activated are silent, indicating the origin of the somata (**Figure 6**). After clonidine, A5 cells are active and decrease their frequency of discharge while, in all cases, hypothalamic fibers are silent [136]. The presence of activations or facilitations indicates the existence of polysynaptic pathways acting on the A5 region. The complexity of the different types of synaptic connections is illustrated by the association of these activations with inhibitions or disfacilitations.

On the other hand, as previously mentioned, the stimulation of cell bodies located within the A5 region resembles the cardiovascular response elicited by HDA electrical stimulation, thus eliciting an increase in heart rate and blood pressure [104] and suggesting the possible interaction between both cardiorespiratory regions. In order to evaluate this possible modulation, microinjection of muscimol also has been made into the A5 region [136].

Muscimol microinjection within the A5 region does not produce changes in the respiratory response to HDA electrical stimulation; however, a clear decrease is observed in the cardiovascular response (**Figure 7**). The increase in heart rate and the hypertension evoked to HDA activation involve a direct excitation of neurons located in the RVLM, which send direct projections to the preganglionic neurons of the IML that are responsible for the acute pressor response [137]. Also, the release of adrenaline by a direct activation of the adrenal medulla provides a secondary increase of blood pressure contributing to the hypertensive response.

Indirect forebrain projections can also modulate the activity of the RVLM. Furthermore, HDA stimulation activates the chemoreceptor reflex by means of the excitation or facilitation of chemoreceptor neurons located in the NTS, in a parallel circuit to the activation of the RVLM and the preganglionic neurons in the IML [38]. An inhibition of the baroreceptor

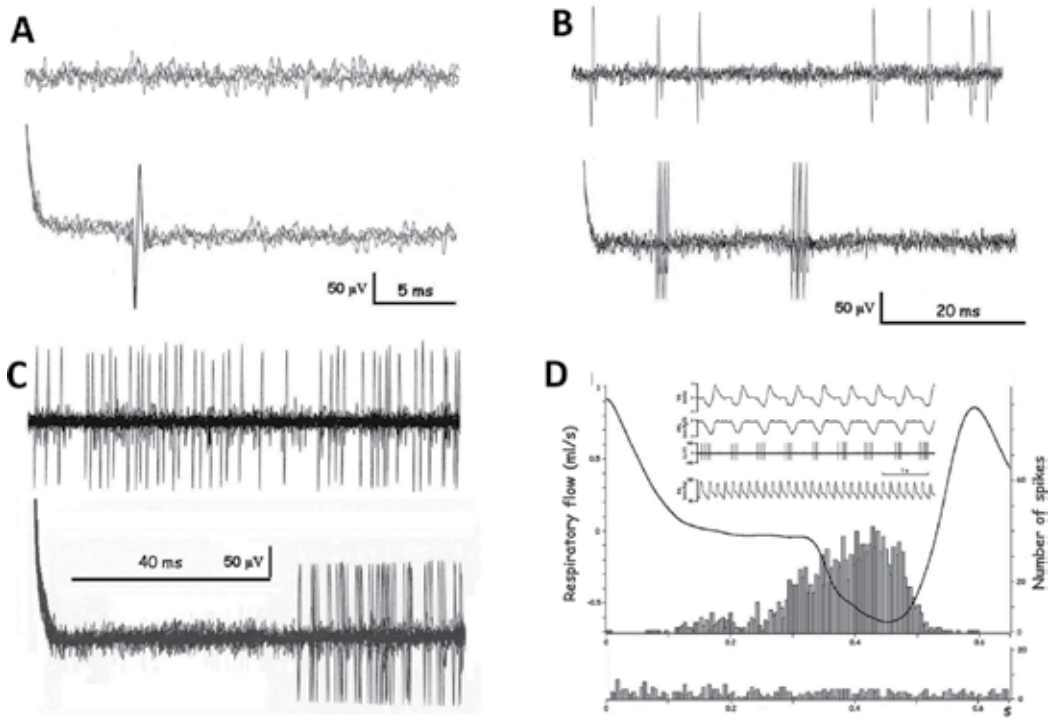

Figure 6. HDA and A5 neurophysiological interactions. Extracellular recordings (superimposed sweeps) of four putative cells recorded form the A5 region. (A) Silent neuron (upper trace) with constant latency responses to the HDA (lower trace). The cell was demonstrated to be orthodromically activated from the HDA. (B) Spontaneously active cell (upper trace) excitated with short and long latency responses from HDA stimulation (lower trace). (C) Spontaneously active cell (upper trace) inhibited from HDA stimulation (lower trace). (D) Recording of respiratory flow, pleural pressure, neuronal activity and blood pressure of a putative respiratory-modulated A5 cell with respiratory flow (ml/s, inspiration downwards) and HDA-triggered histograms (lower trace). This respiratory putative A5 neuron shows no modulation from the HDA. Authors´ figure from Ref. [136].

response is also produced, in another parallel pathway, by the inhibition or disfacilitation of baroreceptor neurons located within the NTS [42, 58], inhibition that seems to be mediated through GABAergic interneurons in the NTS [42].

In conscious rats, stress produces tachycardia and hypertension together with a resetting, rather than an inhibition, of the baroreceptor reflex. Thus, heart rate control is reset to higher levels of blood pressure without decrease in the gain of the reflex [54, 138].

The activation of A5 somata with glutamate also produces tachycardia and hypertension [104]. The increase in heart rate, blood pressure and sympathetic vasomotor activity at the same time indicates a baroreceptor reflex reset but without reduction in sensitivity of the reflex.

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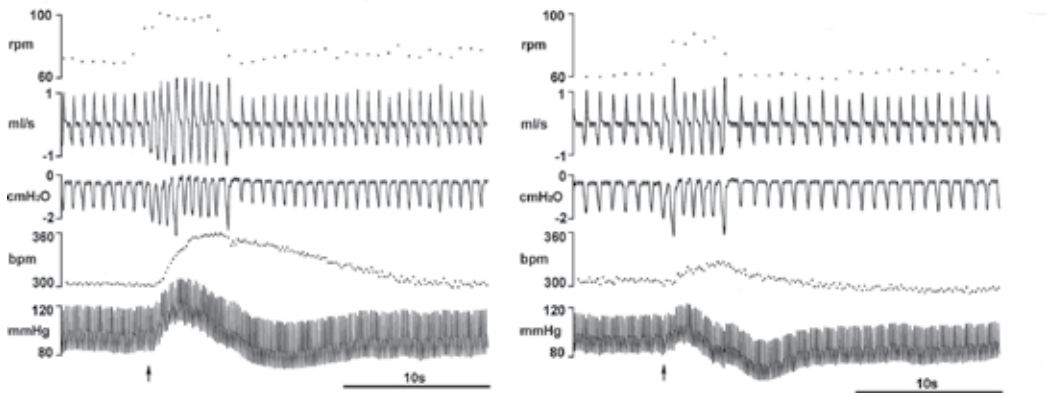

Figure 7. Neuropharmacological interactions between HDA and A5 region. Instantaneous respiratory rate (upper trace, rpm), respiratory flow (ml/s), pleural pressure (cm H_2O), instantaneous heart rate (bpm) and blood pressure (mmHg) showing the cardiorespiratory response evoked on HDA stimulation before (left) and after (right) the microinjection of muscimol in the A5 region. Authors´ figure from Ref. [136].

The inhibition of A5 neurons with muscimol microinjections attenuates the cardiovascular response elicited by the stimulation of the HDA (**Figure 7**) [136]. This attenuation can be an indication of an incomplete resetting of the baroreceptor reflex. This effect can explain the decrease in the magnitude of the tachycardia and the hypertension, through an indirect pathway. But the most relevant aspect of this response is probably the inhibition of the excitatory projections from the A5 region to the IML. These findings suggest that an indirect pathway through the A5 region could also mediate the resetting of the baroreceptor reflex evoked by HDA stimulation. The activity of neurons of the A5 region modulates the intensity of the cardiovascular response evoked on HDA stimulation through an indirect pathway to both the IML and the NTS.

In summary, the A5 region seems to be an important component of those brainstem pathways known to be involved in mediating autonomic changes associated with the defense response elicited from the PeF and the DMH. This response involves also the integrity of the circuits located within the PBc. It is not possible to separate the activity of the PBc and the A5 region; thus, dorso- and ventrolateral pons act together to mediate the cardiorespiratory response evoked on HDA stimulation.

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Section 3

Neuropathology

Chapter 5

The Hypothalamus in Alzheimer's Disease

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Abstract

Alzheimer's disease is a progressive, irreversible neurodegenerative disorder, characterized by gradual decline of mental faculties, including learning capacity, emotional and behavioral alterations, serious decline of motor skills, and dysfunction of the autonomic nervous system with disruption of circadian rhythms. Among the potential modifiable risk factors, diabetes and obesity may play a considerable role in the pathogenetic background of the disease. We describe some of the morphological alterations of the hypothalamic nuclei in early cases of Alzheimer's disease, using silver impregnation techniques and electron microscopy. The morphological and morphometric study revealed substantial decrease of the neuronal population, which was particularly marked in the suprachiasmatic, the supraoptic, and the paraventricular nuclei of the hypothalamus. The silver staining demonstrated an obvious shortage of the dendritic arborization of neurons, associated with marked spinal pathology and axonal dystrophy. It must be underlined that Alzheimer's pathology, such as neuritic plaques and neurofibrillary degeneration, was minimal in the hypothalamus in comparison with other cortical and subcortical areas of the brain. Mitochondrial alterations and fragmentation of Golgi complex were observed by electron microscopy in a substantial number of neurons and astrocytes in the hypothalamic nuclei. The hypothalamic pathology may be related to instability of autonomic regulation which occurs gradually in Alzheimer's disease.

Keywords: Alzheimer's disease, hypothalamus, Golgi staining, electron microscopy, autonomic dysfunction

1. Introduction

Alzheimer's disease (AD) is a progressive, devastating, irreversible neurodegenerative disorder of the central nervous system, which has been recognized as the most common cause of serious cognitive decline in elderly people, resulting in profound dementia [1, 2] with no effective therapeutic intervention [3]. It is reasonable that AD induces a huge social burden and has a serious economic impact, since it starts frequently as mild cognitive impairment, resulting eventually in dementia, as the time advances [4, 5], affecting over 26 million people worldwide [6, 7].

The pathogenesis of AD involves a considerable number of cellular and molecular underlying mechanisms, as well as many genetic or acquired overlapping risk factors [8], such as diabetes, obesity, and psychosocial stress, which although are among the modifiable factors, may contribute substantially in the rapid mental deterioration, aggravating the clinical phenomenology of the disease [9].

A substantial number of clinical observations and laboratory investigations plead in favor of brain injury [8], stress [10–12], or stress-related psychiatric disorders [13, 14], type 2 diabetes [15, 16], insulin resistance [17, 18], inflammation [19] and depression [20] which may be considered, as probable predisposing factors for AD [21].

The neuropathological findings in AD are numerous. Among them, the amyloid containing neuritic plaques, the neurofibrillary tangles, which consist of intraneuronal aggregation of highly phosphorylated tau proteins, the morphological alterations of dendrites and spines, the synaptic pathology, and the increased neuronal loss in limbic structures and the cortex of the brain hemispheres are considered as hallmarks of the disease [22–24]. The gradual accumulation of Aβ peptide in the brain may induce inflammatory reactions, in which activated microglial cells are mostly involved [24]. It is important that the aggregation of Aβ amyloid peptide may promote selective degeneration of neurons, which are particularly vulnerable to age-related procedures, to oxidative stress, and any other type of energy deficiency [25]. The disruption of the blood brain barrier and the pathology of capillaries play a substantial role in shaping the neuropathological pattern of AD [26, 27], since they can facilitate the infiltration of immune cells promoting the exacerbation of inflammatory reactions in the brain.

The initial clinical manifestations of AD are subtle. However, as the time advances, progressive memory and learning impairment [28]; language disturbances; visuospatial disorientation; ideomotor apraxia; behavioral disturbances; depressive symptoms [29–32]; personality changes [33–35]; and a multitude of non-cognitive symptoms, such as sleep disruption, circadian dysrhythmia, changes in body weight, and autonomic dysfunction, are progressively established as dominant deficits in AD [36]. Sleep disturbances, on the other hand, might have a negative impact on the amyloid burden and the cognitive capacity of the patients, though the entire pathogenetic mechanism in sporadic cases remains unclear and is only approached by various hypotheses. The study of familial cases of AD, on the other hand, advocates in favor of the heterogeneity of the disease, and suggests that the morphological alterations in AD follow an eventual common pathway with many other degenerative conditions of the CNS [37, 38].

Oxidative stress seems to contribute substantially in the pathogenesis of AD [39, 40]. In addition, electron microscopy revealed serious morphological alterations of mitochondria in nerve

cells, astrocytes, and endothelial cells in various brain structures, including the cerebellum [40, 41], which are associated with tremendous spinal loss and loss of dendritic branches. It is important that morphological changes of the Golgi complex [42] have been observed in early cases of AD, in areas of the brain with minimal Alzheimer's pathology, suggesting that the protein trafficking might be impaired from the initial stages of AD, since Golgi apparatus plays a crucial role in trafficking and targeting of the plasma membrane proteins [43, 44].

Autonomic disorders have frequently been observed in patients who suffer from AD. Particularly, autonomic failure frequently occurs under strong emotional or cognitive stimuli during the disease, since the hypothalamus may be seriously involved even in the early stages of the neurodegenerative diseases, including AD [45–49], whereas the suprachiasmatic nucleus (SCN), the main circadian pacemaker, undergoes several continuous alterations during the course of the disease [50].

Stress, which is presumably a potential risk factor, mediated via the hypothalamic-pituitary-adrenal (HPA) pathway, may induce a substantial increase of glucocorticoids [49, 50], affecting seriously the homoeostatic equilibrium of the patients.

An evidence of the involvement of the hypothalamus in cases of AD is the increased volume of the third ventricle, seen in neuroimaging. In addition, there are substantial molecular and cellular differences in the morphological elements in the hypothalamus in cases of AD [51, 52], in correlation with the hippocampus and the involved cortical structures [53]. In addition, they do not contain tau-, neurofilament-, or microtubule-associated protein-reactive epitopes, and do not disrupt the neuropil or induce gliosis [53]. Numerous diffuse neuritic plaques in the hypothalamus in cases of AD are labeled with an antiserum to the Aβ peptide, of the beta-amyloid precursor proteins (beta APPs), whereas Aβ peptide-immunoreactive plaques were uncommon in the hypothalamus of non-AD patients [54]. It was also noticed that the neurofibrillary degeneration in the hypothalamus involves primarily those neurons, which are associated with cortical areas seriously affected by Alzheimer's pathology [55].

We proceeded in studying the morphological changes of the neurons and the neuronal networks of the hypothalamus in early cases of Alzheimer's disease, focusing our observations mainly on the suprachiasmatic (SCN), the supraoptic (SON), and the paraventricular nuclei (PVN) of the hypothalamus.

We described the alterations of dendrites, spines, and dendritic arbors in specimens impregnated by silver nitrate, using light microscope, whereas the mitochondrial alterations as well as the morphological and morphometric changes of Golgi apparatus have been studied and described in electron microscopy.

2. Material and methods

2.1. Material

Our morphological observations are based on the study of 14 brains of patients, aged 54–82 years, who suffered from AD. The brains were excised at autopsy, performed between

4 and 8 hours post mortem at a room temperature of 4°C. All of the patients fulfilled the clinical, neurological, neuropsychological, and neuropsychiatric criteria of AD. All of them died 24–46 months following the clinical diagnosis of the disease (**Table 1**).

Twelve additional macroscopically intact brains of apparently healthy individuals, aged 50–80 years, who died accidentally, were used as normal controls. The definite diagnosis of AD was based on NINCDS-ADRDA criteria [54].

2.2. Methods

Samples from the hypothalamus were excised and processed for electron microscopy and silver impregnation techniques, including rapid Golgi's method, Golgi-Nissl method, and Rio Hortega and Bodian techniques [55, 56].

2.2.1. Electron microscopy

For proceeding to electron microscopy, the specimens were immediately immersed in Sotelo's fixing solution, composed of 1% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer adjusted at pH 7.35. Then, they were post fixed in 1% osmium tetroxide for 30 minutes at room temperature. After fixation, the specimens were dehydrated in graded alcohol solutions

Gender	Age at death (years)	Duration of the disease	Length of brain fixation in months	Braak and braak stage
M	55	3 years	1	II/III
F	62	28 months	1	II/III
M	63	37 months	1	II
F	66	40 months	1	II/III
M	72	3 years	1	III
M	74	38 months	1	II/III
F	75	42 months	1	II/III
F	76	46 months	1	III
M	78	42 months	1	II/III
F	80	2 years	1	II/III
M	78	42 months	1	II/III
F	76	36 months	1	III
M	54	2 years	1	III
M	65	37 months	1	II/III

The hypothalamus was excised and studied from 1974 to 2011.
AD, Alzheimer's disease; F, female; M, male. Fixation for silver impregnation techniques.

Table 1. List of the AD brains.

and twice in propylene oxide. Thin sections were cut in a Reichert ultratome, which were contrasted with uranyl acetate and lead citrate and studied in a Zeiss 9aS electron microscope.

2.2.2. Light microscope

2.2.2.1. Silver impregnation techniques

The hypothalamus was processed for silver impregnation techniques, according to rapid Golgi method and Golgi-Nissl method. After a 4-week fixation in solution of 10% fresh formalin, the specimens were immersed in potassium dichromate (7 g potassium dichromate in 300 mL water) for 10 days at room temperature. Then, they were immersed in a solution of 1% silver nitrate for 10 days in a dark environment at a temperature of 16°C. Following rapid dehydration in graded alcohol solutions, the specimens were embedded in paraffin and cut, some of them at 100 μ and some at 25 μ, alternatively. Many sections of 25 μ were stained also with methylene blue, according to Golgi-Nissl technique [57, 58]. Then, the sections were mounted in Entellan (Merck-Millipore, Darmstadt, Germany), between two cover slips and studied in a Zeiss Axiolab Photomicroscope, equipped with digital camera and computer.

We studied extensively, mostly, the suprachiasmatic (SCN), the supraoptic (SON), and the paraventricular nuclei (PVN) of the hypothalamus [45]. For the calculation of the volume of the nuclei, we applied the Cavalieri principle [59, 60]. We estimated the dendritic arborization as a whole and subsequently we described the morphology and calculated the number of the dendritic branches. We studied, in a detailed way, the morphology of the dendritic spines in light microscope, on sections stained according to rapid Golgi and Golgi-Nissl methods.

2.2.3. Morphometry

Morphometric studies were performed with an image analyzer (Image J program). The surface of the neurons and the dendritic arbors of the hypothalamic nuclei were calculated in specimens stained with silver nitrate, according to rapid Golgi method [61].

The morphology and the morphometry of the neurons, the dendrites, and the dendritic spines were estimated, according to Jacobs et al. [62] principles, which concern: (a) the quality of silver impregnation of neurons and dendrites and (b) the sufficient contrast between stained neurons and neuropile space.

Dendritic arbores were quantitatively estimated in a centrifugal way, according to Uylings et al. [63]. The diameter of the neurons was precisely measured, as well as the total length of the apical and basal dendrites. The number of dendritic bifurcations was enumerated as well as the length and number of dendritic segments per dendritic order, and the density of spines on each one of dendritic segments. The dendrites that arise from the neuronal body up to their first symmetrical bifurcation are considered as first-order branches. Subsequently, the dentritic branches, which are located distantly, are considered as second-order segments, third-order segments, and so on. For the morphometric analysis, we applied Image J program after a calibration for the specific types of microscope (Carl Zeiss Axiolab Photomicroscope) and we counted the number and estimated the order of the dendritic branches according to Sholl's

method of concentric circles [64], which were drawn, at intervals of 15 μm, centered on the soma of the neuron. The dendritic spines were counted on three segments of the dendritic field. Thus, we calculated those, which were located: (a) on primary dendrite, 20–30 μm in length; (b) on the secondary dendrite, 20–30 μm in length; and (c) on the tertiary dendrite, 40–50 μm in length.

In electron microscopy, we performed stereological analysis following the Nyengaard [65] and West [66, 67] principles. The number, the length, the total surface area, the volume, the circulatory ratio, and the spatial distribution of mitochondria [68] were precisely counted and estimated as well as the cisternae and vesicles of the Golgi apparatus [69].

We also estimated the mean nuclear area, the dendritic profiles [70], the total number of the dendritic spines per dendritic segment, the pre- and post-synaptic components [71–73], and the number of synaptic vesicles per presynaptic terminal [73].

The statistical analysis of the data was evaluated by Student t tests. p-Values below 0.05 were considered statistically significant, and those below 0.01 were considered as highly significant.

3. Results

3.1. Silver impregnation technique

Topographically, the human hypothalamus is located between the lamina terminalis anteriorly and the posterior commissure and the posterior edge of the mammillary bodies, posteriorly. By rapid Golgi staining, the Golgi-Nissl method, and the other silver impregnation techniques, we could visualize the hypothalamic nuclei entirely and clearly. However, we focused our detailed description and measurement mostly on the suprachiasmatic (SCN), the supraoptic (SON), and the paraventricular nuclei (PVN).

The morphological and morphometric study of the hypothalamic nuclei revealed a substantial decrease of the number of neurons and an impressive loss of dendritic branches in the brains of the patients who suffered from AD (**Figures 1** and **2**), as compared with normal controls (**Figures 3** and **4**). Abbreviation of the dendritic arborization was prominent mostly in the neurons of suprachiasmatic nucleus (SCN). The dendritic alterations were associated with marked decrease in the number of dendritic spines (**Figures 5** and **6**) in comparison with the normal control brains (**Figure 7**). The same morphological alterations concerning the dendritic branches and the spines were also observed in the supraoptic (SON) and paraventricular nuclei (PVN) of the hypothalamus in AD (**Figure 8**).

The morphometric estimation of the dendritic spines of neurons of the SCN and SON revealed a dramatic decrease of spines in AD brains, in comparison with normal controls (**Table 2**).

3.2. Electron microscopy

Detailed study on electron microscope demonstrated substantial morphological changes of the dendritic arbors, concerning mostly the secondary and tertiary dendritic branches, in a substantial number of neurons of the suprachiasmatic (SCN), supraoptic (SON), and

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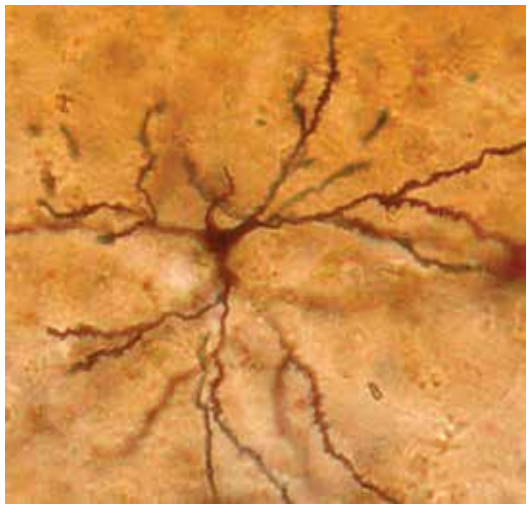

Figure 1. Neuron of the SCN in AD brain. Golgi staining, 1200×.

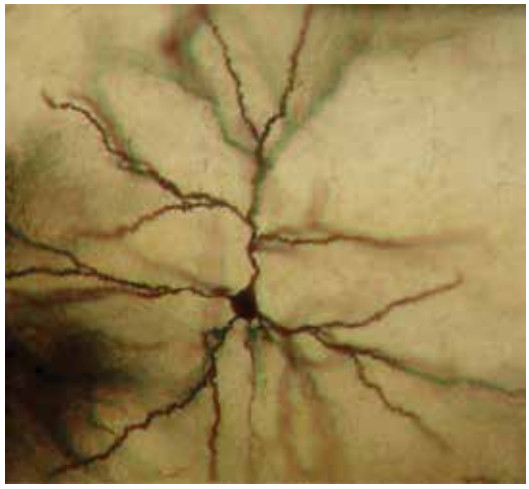

Figure 2. Neuron of SCN of the hypothalamus in a case of AD. The loss of the dendritic branches is obvious. Golgi staining Mag. 1200×.

paraventricular nuclei (PVN) of the hypothalamus in AD brains, in correlation with normal controls. Considerable decrease in spine density was mainly noticed in the secondary and tertiary dendritic branches, which was particularly prominent in the suprachiasmatic nucleus. Small spines and giant spines were also observed in a large number of neurons of the suprachiasmatic nucleus. Many giant spines included large multivesicular bodies.

In a considerable number of dendritic profiles, in the suprachiasmatic and the paraventricular nuclei, the mitochondria demonstrated marked morphological alterations, consisted of wide size diversity, disruption of the cristae, and accumulation of fibrillary material (**Figure 8**).

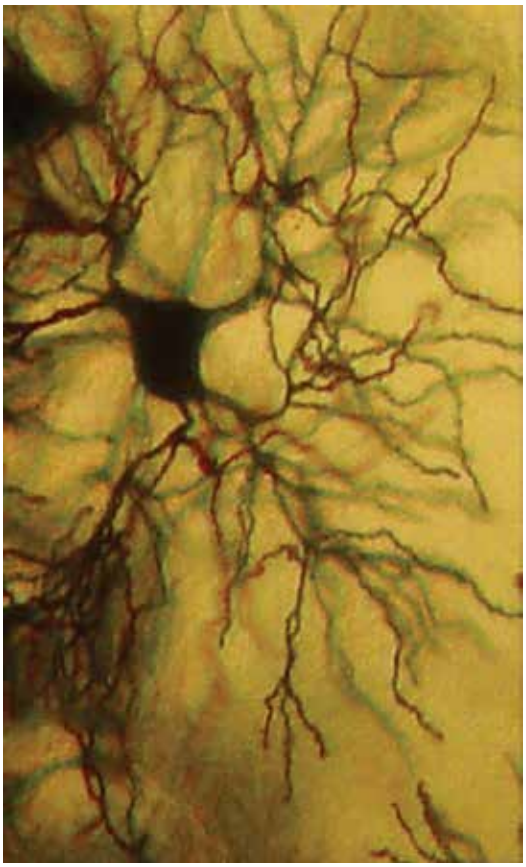

Figure 3. Neuron of the SCN of the hypothalamus of a normal brain aged 75 years.

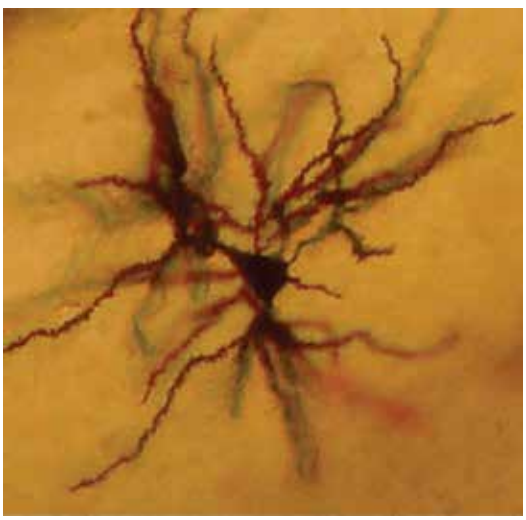

Figure 4. Neuron of the SON of the hypothalamus of a normal brain aged 80 years. The dendritic branches have numerous spines. Golgi staining. Mag. 1200×.

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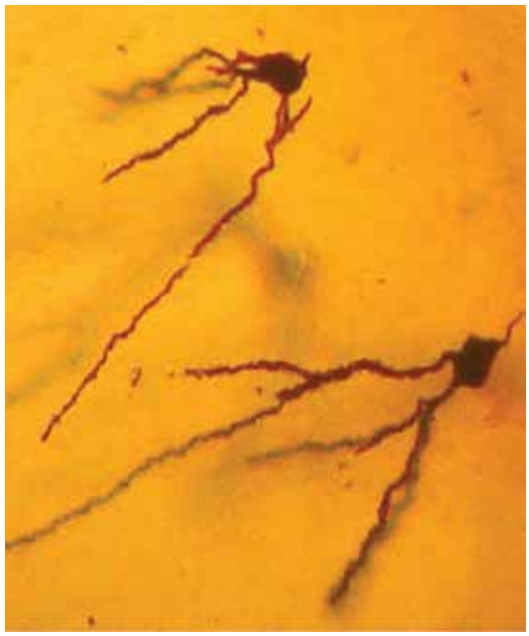

Figure 5. Abbreviations of the dendritic arborization is prominent in the neurons of suprachiasmatic nucleus (SCN) which is associated with marked decrease in the number of dendritic spines. Golgi staining. Mag. 1200×.

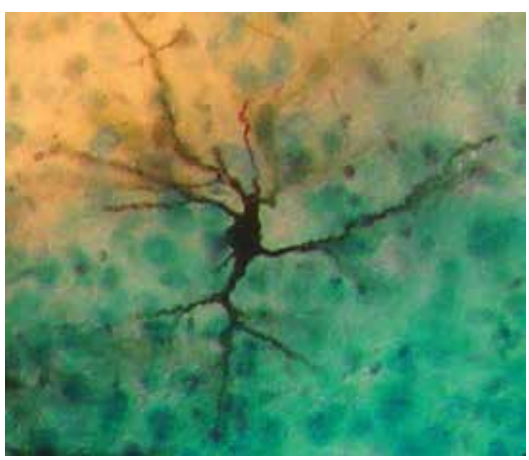

Figure 6. Neuron of the SCN of the hypothalamus of a case of AD. The abbreviation of the dendritic arborization and the poverty of dendritic spines are obvious. Golgi-Nissl staining. Mag. 1200x.

In a morphometric estimation of the mitochondria in dendrites, dendritic spines, and cell body of neurons of the suprachiasmatic nucleus in normal control brains, we concluded that the ellipsoid mitochondria of the spines appear to have an average diameter of 650 ± 250 nm and a mean axial ratio of 1.9 ± 0.2. In addition, the round mitochondria appeared to have a mean diameter of 350 nm. In AD brains, the mitochondria in neurons of

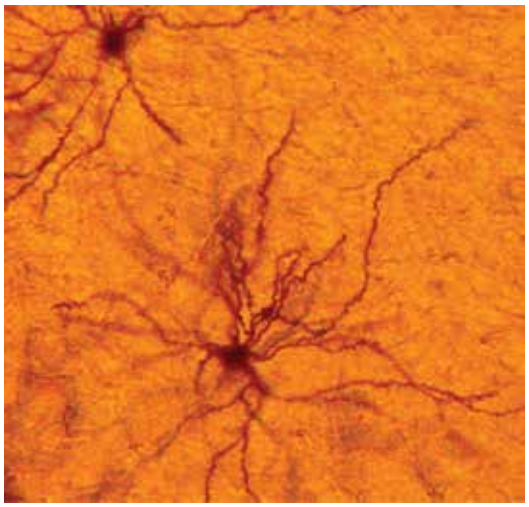

Figure 7. Neuron of the SCN of the hypothalamus of a normal brain aged 80 years. The dendritic branches are covered by spines. Golgi staining. Mag. 1200×.

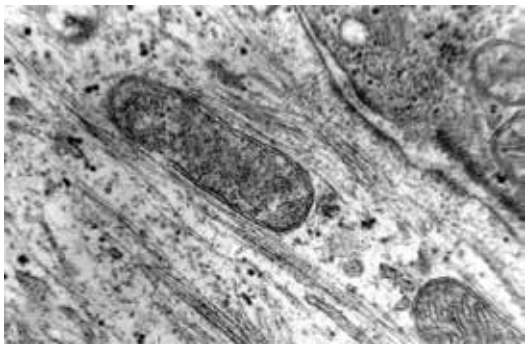

Figure 8. Mitochondrial alterations of a dendritic profile of a neuron of SCN of the hypothalamus of a case of AD. Electron micrograph Mag. 124,000×.

the suprachiasmatic nucleus were estimated as having an average diameter of 440 ± 250 nm and a mean axial ratio of 1.7 ± 0.2 (**Table 3**). The round mitochondria appear to have a mean radius of 235 nm. The changes in the morphology of the cristae were also frequent in the mitochondria of hypothalamic neurons in AD, in comparison with normal controls. Morphological alterations of the mitochondria were also seen in a considerable number of astrocytes and pericytes in AD brains.

In a substantial number of neurons of the suprachiasmatic and paraventricular nuclei of the hypothalamus, the Golgi apparatus appeared to be fragmented and atrophic (**Figure 9**). It

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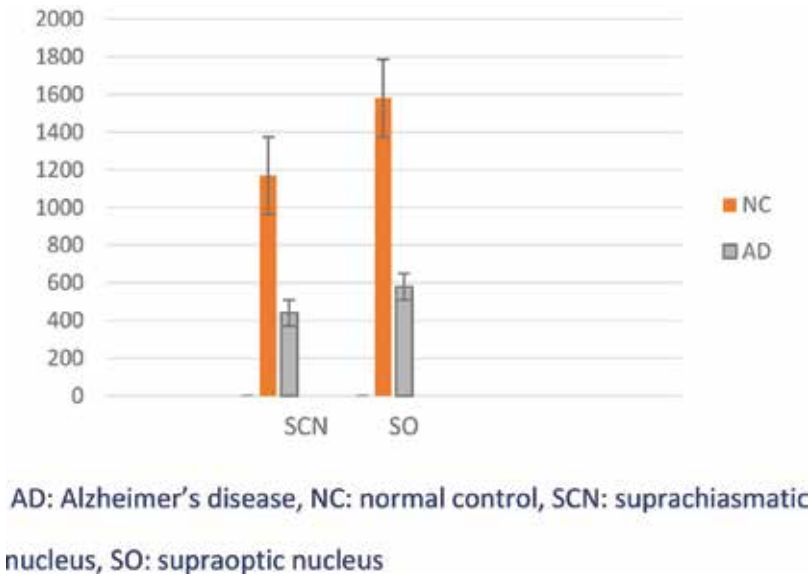


Table 2. Average dendritic spines per dendritic arbor in SCN and SO neurons, based on measurements of 100 neurons ($p < 0.005$).

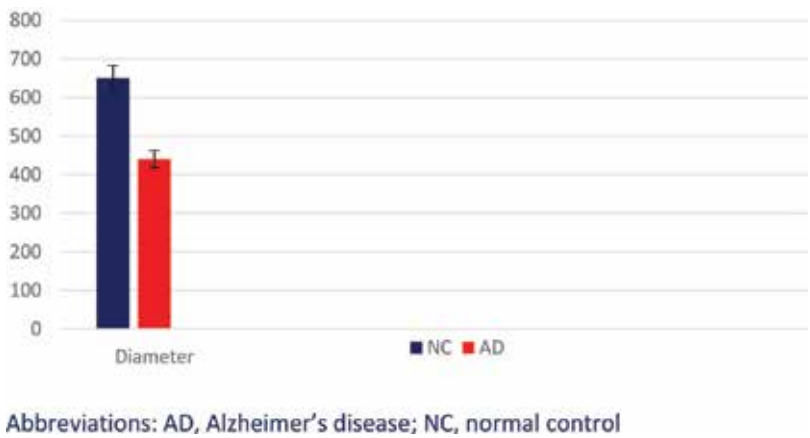


Table 3. Mean diameter (in nm) of mitochondria in neurons of mammillary bodies, based on estimation of 500 mitochondria ($p < 0.05$).

was noticed, that the atrophy and fragmentation of Golgi apparatus (**Table 4**) and the mitochondrial alterations coexisted frequently with dendritic and spinal pathology in the majority of neurons.

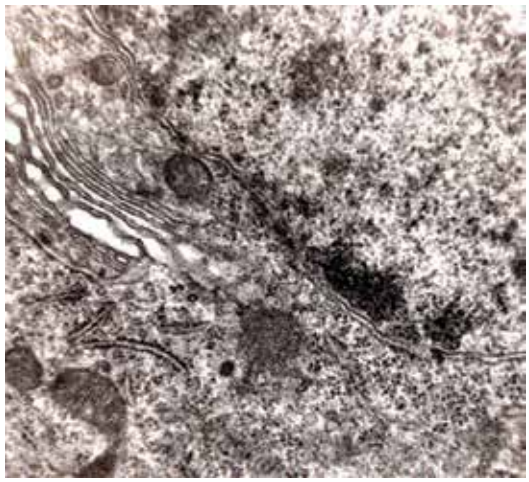
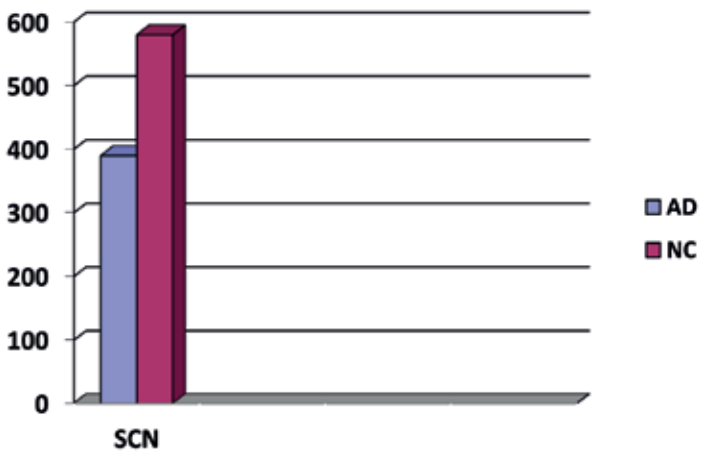


Figure 9. Alteration of Golgi apparatus of a neuron of the SCN of the hypothalamus of a case of AD. Electron micrograph. Mag. 124,000×.



Abbreviations: AD, Alzheimer's disease; NC: normal control
SCN, suprachiasmatic nucleus.

Table 4. The volume of Golgi apparatus in nm^3 based on measurements of 100 neurons of SCN ($p < 0.005$).

4. Discussion

Hypothalamus is a crucial brain structure for the regulation and control of essential homeostatic functions, including the circadian rhythms (CRs) and the sleep-wake cycle. In Alzheimer's disease and other neurodegenerative disorders [74–76], several hypothalamic nuclei are affected. It seems that the hypothalamic nuclei are not involved simultaneously at

the early stages of AD. The suprachiasmatic nucleus seems to be more seriously affected than the others in aging [76] and degenerative disorders. In previous studies, it was clearly revealed that the total cell population of the suprachiasmatic nucleus is decreased both in aging and in AD [76], in which the hypothalamic dysfunction is closely related to sleep disturbances [77].

The hypothalamic nuclei seems to be involved in those neurodegenerative alterations, which would progressively result in AD. In addition, the comparison of the morphological and morphometric alterations of the dendrites in the hypothalamic nuclei with those observed in the cortex of the brain hemispheres and the cerebellum disclosed that the alterations in the hypothalamus were rather modest, in correlation with those of the acoustic and visual cortices, the prefrontal areas of the brain, and the cerebellar cortex [78–81].

The fact that the hypothalamus is the principal subcortical center for the homeostatic and autonomic processes may explain the reason that the supraoptic and the periventricular nuclei, among others, reserve substantial synaptic density, even at the advanced stages of AD.

However, the suprachiasmatic nucleus demonstrated more severe dendritic alterations and synaptic loss than the supraoptic and paraventricular ones, a fact which might explain the phenomenon of desynchronization of circadian rhythms in the majority of the patients, who suffer from AD [82] and cognitive decline [83] in the spectrum of other degenerative brain disorders [84], given that the suprachiasmatic nucleus is indispensable for the generation and synchronization of circadian rhythms in man [85, 86]. It is reported that changes of the circadian rhythm (CR), arterial blood pressure, and body temperature may occur in AD patients [87] especially during the night time [88–90]. Changes also of the melatonin levels are not an unusual phenomenon in advanced senility and AD [91–93]. Sundown syndrome, on the other hand, frequently associated with increased motor activity, is a rather common phenomenon in advanced AD cases [93].

In the majority of neurons of hypothalamic nuclei, mitochondrial alterations were prominent in the cell body as well as in dendrites and synaptic components. As the mitochondria play an essential role in the energy supply of the cell, as ATP-generating organelles, their role is of utmost importance in the alteration of reduction-oxidation potential of the cell, in the free radical formation and scavenging, in the intracellular calcium control and the eventual activation of apoptotic chain [94–96]. Normally, the number of dendritic, axonic, and synaptic mitochondria is very high, especially in pre- and post-synaptic components, since they are the major energy contributor for the synaptic activity.

Mitochondrial dysfunction might induce Aβ peptide neurotoxicity, whereas enhancing mitochondrial proteostasis may reduce amyloid-β proteotoxicity [97]. In addition, impaired mitochondrial biogenesis contributes to mitochondrial dysfunction [98], which is directly associated with oxidative stress, activating furthermore the pathogenic cascade of AD [99–101]. Mitochondrial motility and accumulation are related to the functional state of the neuron, since mitochondria are transported to regions where the necessity for energy is particularly high, as it occurs in the dendritic and axonal profiles and the synapses [102–104]. The shape and size of mitochondria are not stable, since they undergo continual fission and fusion, which are necessary both for the survival of the cell and the harmonious adaptation to changing conditions.

Recent studies reported increased mitochondrial fission and decreased fusion, due to increased Aβ peptide interaction with the mitochondrial fission protein Drp 1, inducing increased mitochondrial fragmentation, impaired axonal transport of mitochondria, and

synaptic degeneration in AD [99]. The consequence of the dynamic fusion and fission processes is the eventual mitophagy of the damaged mitochondria.

A prominent decrease of the size of the mitochondria is observed in aging-related neurodegenerative diseases [95, 96], as well as at the early stages of AD, prior to the onset of a noticeable cognitive dysfunction [105]. Normally, a limited number of dendritic spines contain small and round mitochondria, which are increased in number in the dendritic profiles during the synaptogenesis and hormonal instability [102, 104]. It is important to underline that mitochondrial alterations are mostly associated with synaptic loss in AD patients, due to impairment of mitochondrial energy production [106], seen even before the typical generation of the neuritic plaques and tau pathology [105, 107].

The morphological alteration of the mitochondria, seen in the hypothalamic nuclei in early cases of Alzheimer's disease, pleads in favor of a generalized mitochondrial dysfunction in AD, which may be associated with the dendritic pathology, the tremendous loss of spines, and the marked synaptic alterations [108–110].

The density of the spines on the dendritic branches of a considerable number of neurons of the suprachiasmatic nucleus was decreased. The loss of the dendritic spines causes substantial impairment in neuronal communication and also induces reasonable dysfunction of the neuronal circuits in AD. Previous observations revealed that the loss of dendritic spines coincides with the morphological alterations of the mitochondria and the fragmentation of the cisternae of Golgi apparatus [25, 102, 109, 110]. In an experimental mouse model of Aβ peptide deposition, it was revealed that nonfibrillar Aβ peptide may exert toxicity on the spines, resulting in dramatic decrease of spine density [108, 111].

The role of the hypothalamus in the harmonization of circadian rhythms is crucial for the maintenance of energy homeostasis [25]. The feeding behavior [111–113] and the thermoregulation of the body become gradually unstable during the clinical course of AD [114–116], a fact which was also noticed in experimental models of AD [117] as well as in the behavioral variant of fronto-temporal dementia [118].

In conclusion, the hypothalamic nuclei are involved in AD, inducing autonomic dysfunction and homeostatic disequilibrium, phenomena which are clearly noticeable at the advanced stages of AD.

5. Conclusions

In Alzheimer's disease, silver impregnation technique and electron microscopy revealed a substantial decrease of the neuronal population, which is particularly obvious in the suprachiasmatic nucleus of the hypothalamus.

The silver staining technique demonstrated a marked shortage of the dendritic arborization of neurons, associated with spinal pathology and axonal dystrophy.

It must be underlined that Alzheimer's pathology, such as neuritic plaques and neurofibrillary degeneration, is minimal in hypothalamus in comparison with other areas of the brain.

Mitochondrial alterations and fragmentation of Golgi complex are observed by electron microscopy in a substantial number of neurons and astrocytes in the hypothalamic nuclei.

The hypothalamic pathology may be related to instability of autonomic regulation and homeostatic disequilibrium, which are gradually established in Alzheimer's disease.

Conflict of interest

No conflict of interest.

Nomenclature and abbreviations

AD	Alzheimer's disease
SCN	superchiasmatic nucleus of the hypothalamus
SON	supraoptic nucleus of the hypothalamus
PVN	paraventricular nucleus
HPA	hypothalamic-pituitary-adrenal pathway

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Chapter 6

The Apoptosis Regulation Mechanisms in Hypothalamic Neurons in Physiological and Pathological (Overexpression of Oncogene HER-2/Neu) Aging

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Additional information is available at the end of the chapter

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Abstract

This study reveals the molecular regulation mechanisms of neurosecretory cell apoptosis in physiological and pathological (oncogene human epidermal growth factor receptor (HER)-2/Neu overexpression) aging. As we have shown previously, apoptosis level in hypothalamic neurosecretory centers increases in aging, and a low level of apoptosis in aged HER-2/Neu transgenic mice is associated with p53-dependent cascade suppression. In this chapter, we consider the participation of p53-regulating genes and p53 target genes in activation of this cascade during physiological aging, as well as suppression under HER-2/Neu overexpression. However, cell resistance to apoptosis may also be due to the activity of cytokine-dependent STAT-signaling pathway, including the high expression of survivin belonging to the family of inhibitors of apoptosis proteins (IAP). Also, another cytokine-dependent signaling, an extrinsic apoptosis pathway associated with the family of tumor necrosis factor (TNF) receptors, has been investigated. Thus, in the present work, three signaling cascades are considered: p53-dependent (the expression and interaction of apoptosis-associated proteins p53, WRN, pin1, p21, and caspase-3), STAT-mediated (STAT1, 3, 5, 6, and survivin), and TNF-dependent (CD95 (FAS), Fas-associated death domain (FADD), TNF receptor–associated death domain (TRADD), and caspase-8). These cascades are involved in both the activation of apoptosis and its suppression. This will reveal the general trends of regulation of neurosecretory cell apoptosis during aging.

Keywords: hypothalamus, neuron, aging, apoptosis, signal cascades

1. Introduction

This study reveals the molecular regulation mechanisms of neurosecretory cell apoptosis in physiological and pathological (oncogene human epidermal growth factor receptor (HER)-2/Neu overexpression) aging. In this chapter, we consider the participation of p53-regulating genes and p53 target genes in activation of this cascade during physiological aging, as well as suppression under HER-2/Neu overexpression. In addition, we consider cytokine-dependent pathways of apoptosis regulation (STAT-signaling pathway and tumor necrosis factor (TNF)-dependent pathway).

As shown at present, the process of apoptosis is an integral part of involutional tissue changes [1]. However, the mechanisms of senile apoptosis differ from the mechanisms of cell death in the early stages of ontogenesis. P53-mediated pathway of apoptosis plays a special role in aging, and its changes (excessive activation or suppression) cause severe pathologies, including neurodegenerative diseases and carcinogenesis. This is especially important for neurons and neurosecretory cells, which, as is known, are not capable of proliferation in the mass, so apoptosis is the only way to regulate their amount. Since the hypothalamic neurosecretory system is one of the main regulatory systems, the decrease in the amount of neurosecretory cells significantly changes the function of the hypothalamus and, consequently, of the target organs. Thus, the study of regulation of the p53-signaling path upon aging has particular importance.

DNA damage leads to the phosphorylation of p53 at the Ser/Thr-Pro sites, so the interaction of p53 with Ser/Thr-Pro sites of peptidyl-prolyl isomerase pin1 becomes possible [2–4]. Several studies have shown the suppressive role of prolyl isomerase pin1 in oxidative stress-induced apoptosis [5]. Pin1 involves in the stabilization of Mcl-1 and, further, in the prevention of apoptosis [6]. However, most authors report a high proapoptotic activity of pin1. Pin1 is necessary for the timely activation of p53, leading to apoptosis or cell arrest [4, 7–9]. It is known that pin1 participates in the realization of the cell cycle and mitosis in mammalian cells, transcription, and differentiation [10, 11]. In addition, overexpression of pin1 in various types of cancer tissue has been demonstrated [12, 13]. Pin1 plays an important role in cellular response to DNA damage [14], by conformational transformations participating in the transfer of the apoptotic stimulus and further initiation of the apoptotic cascade [15]. Pin1 isomerizes the bonds between molecules that are important for a variety of oncogenic and other signaling pathways in the cell, including Bcl-2, p53, c-Jun, beta-catenin, nuclear factor-kappa B (NF-kappaB), cyclin D1, c-Myc, and Raf-1. This can cause conformation changes leading to damage to catalytic activity, protein-protein interactions, subcellular localization, and protein stability. Similar changes have been shown to be associated with cell transformation and cancer development [3, 12, 13]. In addition, the absence of pin1 affects embryonic fibroblasts, leading to rapid genomic instability and the so-called immortality due to inactivation of p53 and further to aggressive transformation and carcinogenesis [16].

After attachment to pin1, p53 changes its transcriptional activity and increases the p21 transcription, which facilitates the cell arrest [3, 17]. Moreover, pin1 regulates the stability and transcriptional activity of p53 in relation to the p21 gene promoter [17]. P21 is known to be an inhibitor of cyclin-dependent kinases. Regulation of the cell cycle is an important part of development,

differentiation, DNA repair, and apoptosis. P53-dependent expression of p21 due to DNA damage causes the arrest of the cell cycle in the late G1 phase [3, 18, 19]. In addition, p21 participates in cell aging [20, 21]. Besides, one of the genes, which play an important role in pathological involution process and take part in p53 regulation, is WRN, gene of Werner's syndrome.

However, neuron resistance to apoptosis may also be due to the activity of cytokine-dependent STAT-signaling pathway. Various molecules, including hormones, cytokines, and so on, participate in the regulation of apoptosis. As it is known, a significant change in the level of cell death is an important biological problem. Thus, a decrease in the level of apoptosis leads to oncogenesis, and an increase in the proportion of dying cells is the cause of diseases associated with tissue degeneration. Currently, it is considered a proven close connection between apoptosis and aging. To study the mechanisms of apoptosis associated with the aging, we used a line of transgenic mice with overexpression of the oncogene HER-2/Neu.

HER2, or ERBB2, belongs to the family of transmembrane tyrosine kinase receptors. After connecting to the ligand, members of this family form homo- or heterodimers and transmit the signal forward for activating a significant amount of cascades. Normally, ERBB receptors are involved in the processes of growth, differentiation, migration, and apoptosis. The extracellular domain of ERBB2 (HER2), unlike the HER1, 3, and 4 domains, has an open conformation and is normally capable of forming functionally active heterodimers with other HER receptors by carrying out and amplifying the signal, without preliminary binding to the ligand.

The HER-2/Neu overexpression in pathology promotes the formation of functionally active homo- and heterodimers, and, so, uncontrolled signal transduction [22] and it is characteristic of a number of tumors. The signal network, initiated by the interaction of ERBB family receptors with ligands, and its key elements regulating the direction and speed of signal transmission play an important role in the pathogenesis of tumor diseases [22].

We have previously shown that a low level of apoptosis of neurons upon aging in HER-2/Neu transgenic mice is associated with the suppression of the p53-dependent cascade. However, cell resistance to apoptosis may also be due to the activity of cytokine-dependent apoptosis-limiting systems (STAT-signaling pathway); these members are synthesized in various cell types, including neurons. At present, there is a report on the presence of a pro-inflammatory component in involutional changes of various tissues, including the brain tissues. It is shown that cellular stress and an inflammatory environment can trigger an immune response and provoke cell aging through epigenetic regulation involving STAT signaling [23]. However, there are almost no data characterizing age-related changes in the expression and activity of cytokines, the main mediators of inflammation. Some authors reported an increased pro-inflammatory reaction accompanying the involution processes, and other ones reported suppression of the inflammatory response at the cellular level, and about a decrease in neuroimmune interactions.

STAT proteins (signal transducers and activators of transcription) are transcription factors that conduct a signal from the cytoplasm to the nucleus. For the first time, proteins of this family were described in the 1990s of the twentieth century. Their usual ligands are cytokines, including interferons. In addition to cytokines, STAT are also activated by growth factors and growth factor receptors (a family of tyrosine kinase receptors, including HER-2) that stimulate STAT factors directly or indirectly through JAK kinases.

After activation, phosphorylation of the C-terminal domain of the STAT factors occurs, the homo- or heterodimers form, translocate into the nucleus, and activate DNA regions [24]. It is shown that STAT signaling is negatively regulated by two groups of proteins, one of them is suppressors of cytokine signaling (SOCs) and STAT-induced STAT inhibitors [25], or "proteins that inhibit activated STAT" [26]. The STAT family includes at least seven members—STAT1, 2, 3, 4, 5a, 5b, and 6 [27].

Although all members of the STAT family are structurally similar, they perform various biological roles, participating in such processes as embryonic development, inflammation, organogenesis, cell differentiation and control of cell growth [28, 29], regulation of immune processes, control of proliferation, and apoptosis (STAT3, 5) [30–32]. In many studies, it has been established that STAT proteins play a critical role in the activation of pro-inflammatory and antiproliferative processes (primarily, by the factor STAT1). Members of this family participate in the interferon gamma-induced response [33–35]. Information on the important role of STAT factors in the regulation of cell proliferation, differentiation, and survival suggests the active role of these proteins in malignancy.

However, most studies report about the participation of STAT factors in oncogenesis, but there are almost no data concerning the expression of STAT proteins in neurons with aging and their participation in the regulation of apoptosis. So, the purpose of the next part of the work was to reveal the role and molecular mechanisms of cytokine-dependent signaling in the mechanism of p53-dependent apoptosis suppression in the physiological and pathological (overexpression of the HER-2/Neu oncogene, epidermal growth factor receptor (EGFR)) aging and to investigate the causes of neuronal resistance to apoptosis with aging, possibly due to overexpression of HER-2/Neu.

Also, another cytokine-dependent signaling, an extrinsic apoptosis pathway associated with the family of tumor necrosis factor receptors, has been investigated.

Changes in cytokine expression are observed in various pathological conditions, so, TNF-alpha, a protein that plays a role in apoptosis, increases with oxidative stress [36].

As is known, tumor necrosis factor has a high antitumor and pro-inflammatory activity. Expression of the *tnf* gene activates the synthesis of the cytokine TNF-alpha (TNF-α), which regulates the processes of proliferation, apoptosis, immune cell activity, inflammation, embryo-, and carcinogenesis. Binding of TNF-α to cell death receptors leads to the activation of caspase-8, which initiates an apoptosis program. One of the receptors is the Fas-receptor (CD95, APO-1), whose main function is signaling to the development of apoptosis. In the case of Fas-dependent apoptosis, binding of the Fas ligand to the Fas receptor leads to conformational changes in the cytoplasmic domain of Fas receptor. This makes it possible to bind it to FADD-adapter molecule (Fas-associated death domain), and then to the same domain of the RIP protein (receptor-interacting protein). This complex activates caspase-8 (FLICE protease (FADD-like IL-1b-converting enzyme)), which means the development of apoptosis.

We supposed that the activity of a cytokine-dependent pathway associated with a family of tumor necrosis factor—the TNF-signaling pathway—can also be altered by aging. Only a few authors associate the TNF-dependent apoptotic pathway with aging or with the pathological

processes accompanying aging [37, 38]. So, it is reported that Fas signaling is activated in aged oocytes [39]; the extrinsic apoptotic pathway plays an important role in the development of macular degeneration in accelerated aged OXYS rats while the synthesis of proteins such as Fas, caspase-8, TRAIL increased [40], and in neurodegeneration in ischemia-reperfusion [41]. Apoptosis of the heart cells upon aging is induced via the Fas-FADD pathway, with a significantly suppressed survival signaling pathway which is associated with the insulin-like growth factor receptor (IGF1R), PI3K, and AKT kinases [42]. The senile neurodegeneration is an important clinical pathology, but there are almost no works devoted to the role of Fas-FADD and TNF-receptor-associated death domain (TRADD) pathway in neurons during physiological aging.

Thus, the aim of the present work is investigation of age-related changes of three signaling cascades, which are involved in both the activation of apoptosis and its suppression—p53-dependent (p53, WRN, pin1, p21, caspase-3), STAT-mediated (STAT1, 3, 5, 6, survivin), and TNF-dependent (FAS, FADD, TRADD, caspase-8). This will reveal the general trends of regulation of neurosecretory cell apoptosis during physiological and pathological aging.

2. Mechanisms of apoptosis regulation in ontogenesis: apoptosis signaling cascades in hypothalamic neurons in physiological and pathological (overexpression of oncogene HER-2/Neu) aging

2.1. Research methods

2.1.1. Animals

We studied a mouse model of accelerated aging, namely transgenic HER-2/Neu female mice, at the age of 2 and 10 months [43], obtained from the Italian National Research Center of Aging; the breeding is maintained at the Petrov Research Institute of Oncology (St. Petersburg, Russia). Outbred FVB/N female mice, descending from mice of the Swiss line (Rappolovo Nursery, Russian Academy of Medical Sciences, St. Petersburg, Russia), were 2 and 18 months of age (four to five mice in each group). The animal room was equipped with a 24-h light-dark cycle with 12:12 period.

The model of our study is transgenic mice with the overexpression of the transmembrane tyrosine kinase receptor HER-2/Neu, the wild type is the FVB/N line. The FVB/N line mice are often used to produce models of transgenic mice, since this line is characterized by high fertility and good survival of the embryo after injection. Overexpression of activated HER-2/Neu oncogene in transgenic female FVB/N mice leads to malignant transformation of mammary epithelial cells, followed by development of several breast adenocarcinomas, as well as hyperinsulinemia, hyperglycemia, and a decrease in the activity of the antioxidant system, which are biomarkers of the premature aging of transgenic mice (**Figure 1**). The lifespan of these mice is about 11–12 months. Thus, overexpression of HER-2/Neu causes hormone-metabolic changes, which are characteristic of accelerated aging, simultaneously with carcinogenesis, in this line of mice.

Figure 1. HER-2/Neu transgenic old mice.

2.1.2. Sample preparation

The brains from animals were fixed in 4% paraformaldehyde for morphological and immunohistochemical assays. Fresh-frozen brain sections containing supraoptic and paraventricular nuclei (SON and PVN) of the hypothalamus were prepared.

The fresh-frozen brain region containing SON and PVN were homogenized separately for further biochemical analysis.

2.1.3. TUNEL

The terminal deoxynucleotidyl transferase-biotin dUTP-nick end labeling (TUNEL) assay was used to detect 3′ hydroxyl ends in fragmented DNA in the hypothalamic neurosecretory cells. In brief, after rehydration, cryo-sections (5–7 mkm) were processed according to the manufacturer's instructions for the TUNEL assay using the detection kit (Sileks, Russia) and then stained with diaminobenzidine (DAB).

2.1.4. In situ hybridization

We identified the transcripts of genes involved in the signaling cascade of p53 protein (*p53*, *WRN*, *pin1*, *p21*) in fresh-frozen brain sections containing neurosecretory nuclei (SON and PVN) (in situ hybridization using a riboprobe labeled with digoxigenin). Riboprobes for the detection of mouse p53, WRN, pin1, and p21 mRNA were prepared by in vitro transcription method using a synthetic template, as recommended in the DIG-11-UTP guide. To mark the riboprobes, we

used modified NTP, DIG-11-UTP (Roche Applied Science, USA), which can be combined with growing T7 RNA polymerase transcripts and serve as a highly specific antigen with subsequent immunohistochemistry coloration [44]. We used a standard protocol published earlier [45] followed by densitometry to determine the level of expression of apoptosis-associated molecules (VideoTest Morphology).

2.1.5. Immunohistochemistry

The expression of WRN and survivin was examined immunohistochemically. WRN expression was detected for the determination of protein proportion in the nucleus or in the cytoplasm of cell (standard streptavidin-biotin-peroxidase method) [46]. The fresh-frozen brain sections containing SON and PVN of the hypothalamus cut sections were incubated with primary rabbit polyclonal antibody to WRN (NBP-23002, 1:100; Novus Biologicals, Inc., USA) and rabbit monoclonal antibody to survivin ([EPR17358], 1:500; Abcam, USA), and then with ABC elite complex (Vector Laboratories Inc., Peterborough, Cambridgeshire, UK). The peroxidase reaction of the avidin-biotin complex was revealed in the buffer containing 3,30 diaminobenzidine (0.05%) and hydrogen peroxide (0.01%). Additionally, reactions lacking primary antibodies were performed to ensure the specificity of the observed staining.

2.1.6. Western blot analysis

Tissues were homogenized in lysis buffer containing protease inhibitors and phosphatase inhibitor cocktail (both from Sigma-Aldrich, St. Louis, MO, USA). The total protein concentrations were determined by Bio-Rad protein assay (Bio-Rad Laboratories Inc., Hercules, CA, USA). Equal amounts of protein (15 lg per line) in sample buffer (Bio-Rad Laboratories Inc.) were denatured at 95°C for 5 min and separated on 10% acrylamide gel. The proteins from the gel were transferred to a nitrocellulose membrane. The membranes were incubated in 3% non-fat milk in Tris buffer (0.1% Tween 20, 0.2 mM Tris, 137 mM NaCl) for 30 min and then incubated overnight with primary antibodies against STAT1 (9172, 1:1000; Cell Signaling, USA); STAT3 (4904, 1:2000; Cell Signaling, USA); STAT5 (9363, 1:1000; Cell Signaling, USA); STAT6 (5397, 1:1000; Cell Signaling, USA); caspase-3 (4904, 1:2000; Cell Signaling, USA); FAS (ab82419, 1:1000; Abcam, USA), FADD (24,533, 4 mkg/ml; Abcam, USA), TRADD ([EPR3604] ab110644, 1:500; Abcam, USA), caspase-8 (25,901, 0.5 mkg/ml; Abcam, USA), (Abcam); p53 ([PAb 240] (ab26), 5 mkg/ml; Abcam, USA), and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) (1:2000; Abcam, USA) or actin (1:1000; Abcam, USA). Subsequently, the membranes were incubated with secondary anti-rabbit (1:8000; Sigma-Aldrich, USA) or anti-mouse (1:80,000; Sigma-Aldrich, USA), followed by chemiluminescent detection by enhanced chemiluminescenceplus (Amersham, GE Healthcare, Little Chalfont, Buckinghamshire, UK).

2.1.7. Evaluation of sections and statistical analysis

The positive TUNEL staining cells in hypothalamic SON and PVN were counted under a high-power magnification (20×) field of light microscope (Zeiss Axiolab, Carl Zeiss Inc., Germany) [47–49]. At least five fields were sampled in a section and data were expressed as the number of TUNEL-positive counts.

The semi-quantitative analysis of protein amount in the histological slices was processed by measurement of optical density [50]. Five sections at the same level of the studied zones were analyzed from each animal. The relative optical density of immuno-positive substances in the SON and PVN of the hypothalamus was estimated, and the average and standard deviation were calculated. Optical density reflecting the content of immuno-positive substance was calculated as the 'gray level' (GL) of immunoreactive field of tissue minus background GL. Optical density of the background was estimated at the same slice in non-immunoreactive brain tissue field. Results are presented in relative units of optical density per lm2.

Immunohistochemical study showed that in the neurosecretory centers there are three types of cells—with WRN-immuno-positive cytoplasm, with WRN-immuno-positive nucleus, and cells where the immune reaction took place in the nucleus and cytoplasm simultaneously, and each type of cells was counted under a high-power magnification (20×) field of light microscope. At least five fields were sampled in a section.

The optical density of the bands (Western blotting) was measured and quantified by ImageJ.

Statistical analysis was carried out by Student's *t*-test ($p \leq 0.05$) (Microsoft Excel 5.0a), and values are expressed as mean SE for immunohistochemistry, TUNEL, in situ hybridization, and for Western blot analysis.

2.2. Expression of Werner syndrome genes in hypothalamic neurons in physiological and pathological (HER-2/Neu overexpression) aging

It is known that changes in the p53-mediated pathway of apoptosis with aging cause severe pathologies, including neurodegenerative diseases and cancer. DNA damage leads to the phosphorylation of the p53 protein and allows the interaction of p53 with the peptidylpropyl isomerase pin1. After being connected to pin1, the tumor suppressor p53 increases the expression of p21 (a cyclin-dependent kinase inhibitor), which helps stop the cell cycle [18, 19].

It was shown that p21 limits proliferation in cell culture and also this protein is an internal suppressor of neuronal regeneration in brain damage [51]. The process of differentiation of neuroendocrine cells is associated with up-regulation of the p21, p53, and activation of MAPK and STAT pathways [52]. Stable expression of p53 leads to the onset of p21 synthesis [53], and the role of p21 in p53-dependent cancer protection is shown. The absence of p21 significantly impairs the p53-mediated arrest of the cell cycle, without affecting apoptosis [19]. Other studies report an increase in p21 expression preceding TNF-induced necrosis-like cell death [54]. Most authors consider p21 (Waf1/Cip1) as one of the main mediators of p53 tumor suppressor [55].

According to other data, p21 acts as an oncogene, reducing the level of apoptosis in various tissues, including tumors [56]. Thus, a decrease in the level or the absence of p21 results in a significant increase in the lifespan of p53-deficient mice due to a decrease in the number of spontaneously occurring or induced tumors. It was shown that the reason for this is a higher apoptotic tissue index during the absence of p21 [55, 57]. The involvement of p21 in apoptosis depends on its interaction with the PCNA protein (proliferating cell nuclear antigen, the nuclear antigen of proliferating cells), which is an auxiliary factor in reparative DNA polymerase delta [58, 59].

According to the data of other authors, the p53-p21-dependent pathway determines the choice between apoptosis and cell aging [20]. As is known, the suppression of apoptosis significantly increases the number of aging cells.

In addition, other genes participate in the aging process. Thus, the Werner syndrome gene (WRN) encodes DNA-helicases and endonucleases. WRN mutation causes Werner's syndrome, a progeria, when the characteristic signs of aging appear at an early age. It is known that aging triggers by two mechanisms—telomere shortening and DNA damage. These mechanisms are interrelated, shortening telomeres and their dysfunction can lead to DNA damage; accumulation of DNA damages leads to genomic instability and accelerated cellular aging. Both of these mechanisms are mainly dependent on p53 status [6]. The function of WRN is closely related to the p53 protein and its participation in apoptosis [60]. The destruction of the WRN gene or its mutation leads to spontaneous carcinogenesis, which is also characteristic of Werner's syndrome [61, 62]. Deficiency of the WRN protein reduces the phosphorylation of p53, as shown on the cell lines of normal fibroblasts and osteosarcoma cells [63]. The effects of p53 and WRN on each other are mutual. The absence of p53-WRN interaction can disrupt the signal for apoptosis and lead to genomic instability and carcinogenesis [64]. Many authors show depletion of p53-dependent apoptosis in the cell lines from patients with Werner's syndrome. The physical interaction between p53 and WRN was identified, that suggests functional interaction [65–68]. In addition, overexpression of WRN induces p53 expression, and further, p21, which indicates their overall involvement in premature aging and cancer processes [69]. However, the role of the WRN gene in the regulation of physiological aging is still unknown.

So, one of the aims of this study was determining the role of the *WRN* gene in the apoptosis in the physiological and pathological (HER-2/Neu overexpression) aging. One of the important regulatory systems of organism, the neuroendocrine system of the hypothalamus, was used as a model of the investigation.

The results of our studies showed that the level of apoptosis of the hypothalamus neurons in transgenic animals is low and does not increase with aging, in contrast to mice FVB/N (**Figure 2a** and **b**). Expression of p53 in young FVB/N mice is low and increases with aging (**Figure 2a**). In HER-2/Neu mice, the amount of p53 mRNA is significantly lower in both neurosecretory centers and does not change with aging (**Figure 2a**). Obviously, a decrease in p53 expression is the main reason for the low level of apoptosis in older transgenic mice.

According to our data, WRN is synthesized in the neurosecretory cells of the hypothalamus at a sufficiently low level, and there are no differences in the young animals FVB/N and HER-2/Neu (**Figure 3a**). With aging, WRN expression increases in SON and PVN in wild-type mice (**Figure 3b** and **c**), while overexpression of HER-2/Neu suppresses WRN expression (**Figure 3a**). A low level of WRN does not lead to the activation of p53 and thus suppresses the subsequent cascade of apoptosis. In addition, the WRN protein, which is synthesized in the neurosecretory cells of transgenic mice, is not functionally complete, as revealed in our experiments by immunohistochemistry (a cytoplasmic localization is characteristic of the defective protein [70]).

Immunohistochemical study showed that in the neurosecretory centers, there are three types of cells—with WRN-immuno-positive cytoplasm, with WRN-immuno-positive nucleus, and cells

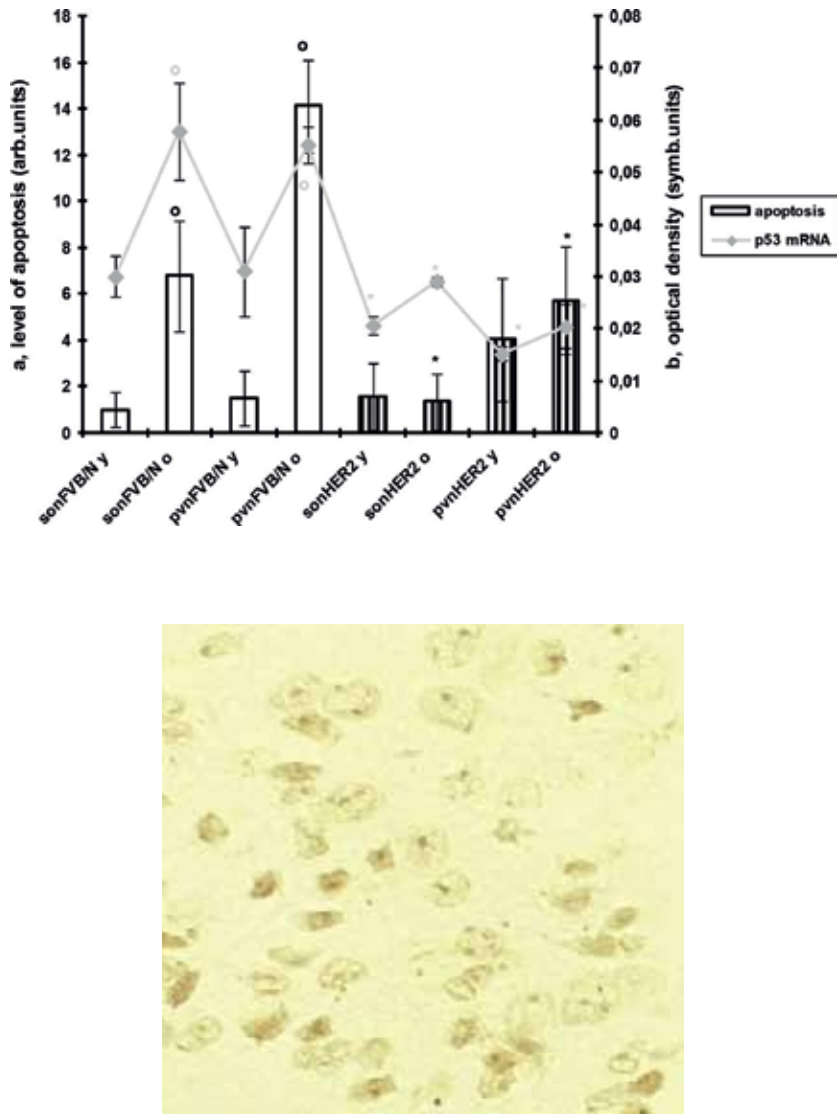

Figure 2. (a) The level of apoptosis and expression of p53 in neurosecretory centers (supraoptic (SON) and paraventricular (PVN) nuclei) in FVB/N and HER-2/Neu mice. The left ordinate axis shows the number of TUNEL-positive (apoptotic) cells ($M \pm m$, $n = 6$) in neurosecretory cells of SON and PVN of FVB/N and HER-2/Neu mice. Designations: (y)—young mice, (o)—old mouse. The right ordinate axis shows the content of p53 mRNA ($M \pm m$, $n = 6$) in the neurosecretory cells of SON and PVN of FVB/N and HER-2/Neu mice. Here and in other figures: optical density presented in arbitrary units. (o) is the significance of differences between the indices for young and old mice of the same group ($p \leq 0.05$), and (*) is the significance of differences between the indices of FVB/N and HER2/Neu mice of the same age ($p \leq 0.05$). (b) PVN of old FVB/N mice, cells with dark-stained nuclei are apoptotic (TUNEL).

where the immune reaction took place in the nucleus and cytoplasm simultaneously. With aging, there are significant changes in the composition of immuno-positive cells. Thus, in the SON of FVB/N old mice the amount of cells with colored nuclei decreases with a simultaneous increase in the amount of cells with cytoplasmic coloring; in PVN, the amount of all three cell types increases. In old transgenic mice, the amount of neurosecretory cells with a nuclear-cytoplasmic

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color increases, with an increase in the amount of cells with nuclear localization WRN. There were significant differences between wild-type mice and transgenic mice—a smaller amount of all types of cells in the HER-2/Neu mice, both young and old.

Thus, the results showed the activation of the p53-dependent pathway of apoptosis upon aging in the hypothalamic neurons of wild-type mice. Werner's syndrome gene was found to be involved in physiological aging. Previously, fibroblast culture showed that cell aging is associated with a decrease in the WRN protein [71]. The increase in WRN expression in the late stages of ontogenesis plays a decisive role in the induction of p53-mediated apoptosis of neurosecretory cells (**Figures 2a** and **3a**). The functional relationship of p53 and WRN found in our experiments can confirm the data obtained by other authors on cell cultures and on a model of mice with Werner's syndrome [66, 68, 72]. Increased expression of WRN in wild-type animals leads

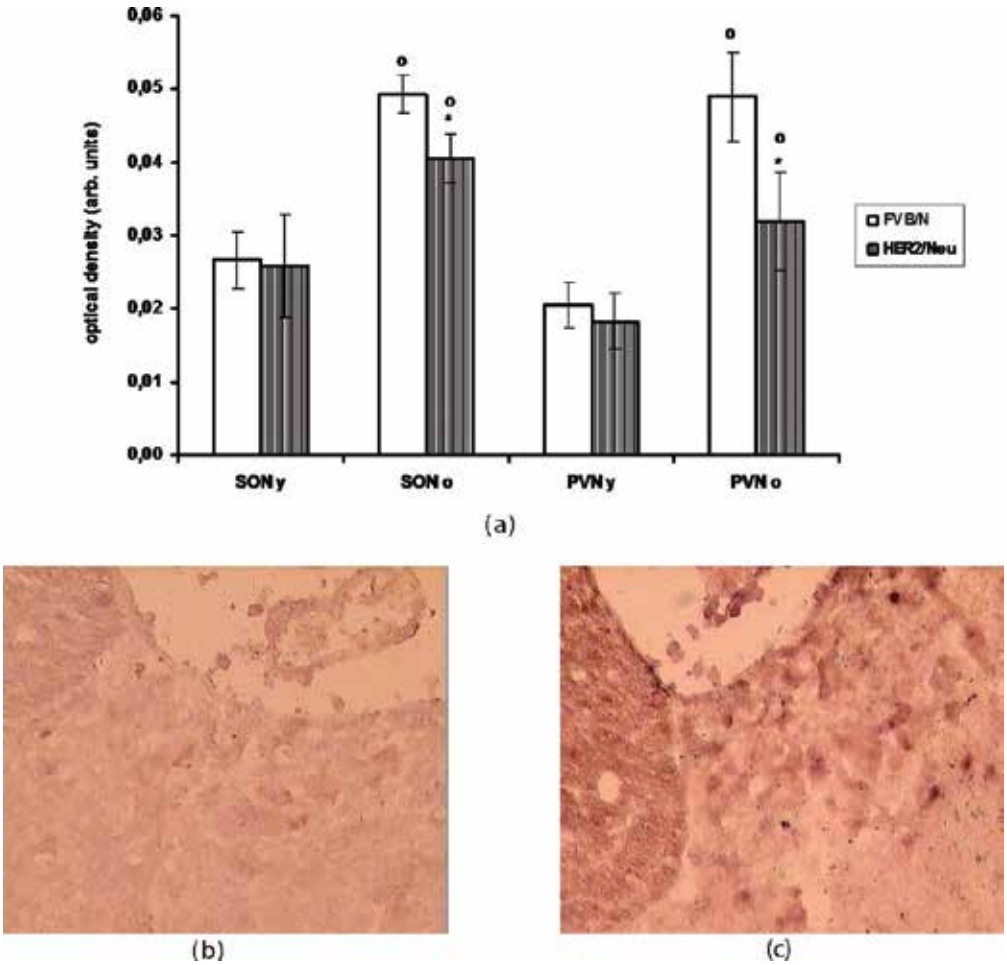

Figure 3. (a) Content of WRN mRNA in neurosecretory cells of the SON and PVN of FVB/N and HER-2/Neu mice of different ages. (b) SON of young FVB/N mice, immunostaining with antibody to WRN. (c) SON of old FVB/N mice, immunostaining with antibody to WRN.

to an increase not only in the synthesis of p53 but also in an increase in p53-dependent transcriptional activity and induction of pin1 expression [69], which is shown in our experiments.

It is known that p21 is the transcriptional target of the tumor suppressor p53, but these proteins mutually affect each other. There is evidence that p21 can induce cell arrest irrespective of the involvement of p53 [21]. In addition, p53 can act on the p21-independent mechanism [73]. P21 can both activate apoptosis and inhibit this process, depending on the involvement of other proteins, in particular PCNA [59]. In the works of many authors, the enhancement of the synthesis of p21 is shown in rest cells, that is, in highly differentiated cells, due to the ability of p21 to reduce proliferation and to participate in cellular aging [20, 51].

In our experiments, the level of the studied genes was higher in both age groups in FVB/N mice (p53, pin1, and p21) or only in older animals (WRN) compared to transgenic mice (**Figures 2a–4**). The high level of expression of p21 in young wild-type mice and its decrease in aging correlates with the level of apoptosis of neurosecretory cells.

The decrease in p21 synthesis in HER-2/Neu mice (as a result of low p53 level in these mice) indicates the possibility of maintaining a balance of cell death survival with the p53-p21 pathway; however, overexpression of the HER-2/Neu oncogene results in the suppression of the apoptotic cascade. Dynamics of the synthesis of pin1 corresponds to a change in the synthesis of p53, a significant level of pin1 expression in young FVB/N mice, increasing with aging, and a decreased content of pin1 mRNA without response to aging in HER-2/Neu mice. Increasing WRN expression leads to not only increased synthesis of p53 but also the induction of pin1 expression in FVB/N animals [69], as shown in our experiments.

Thus, the relatively high level of p21 mRNA that we detected in young wild-type mice is consistent with data of some investigators [55]. The simultaneous enhancement of p53 and p21 expression in aging in various tissues has been reported. The activation of the p53-mediated

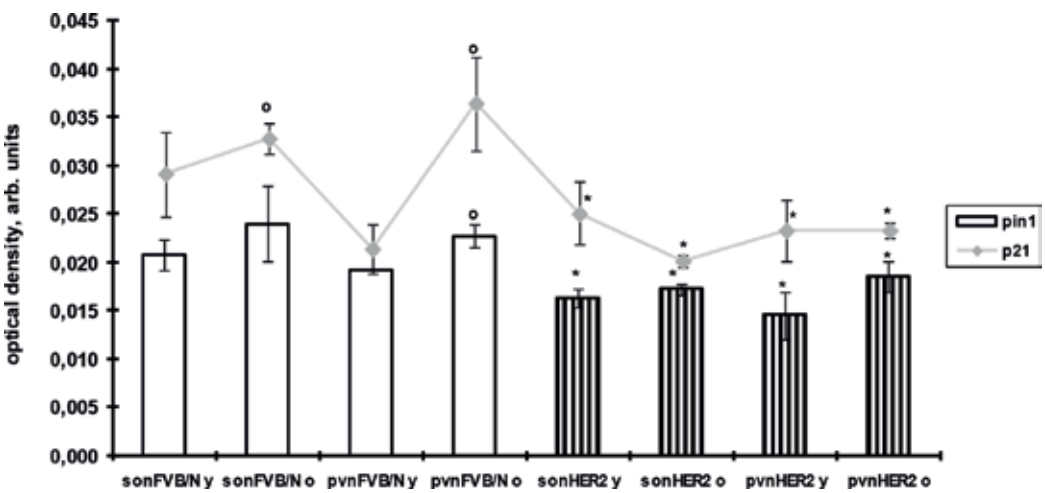

Figure 4. Content of pin1 mRNA and p21 mRNA in neurosecretory cells of the supraoptic and paraventricular nuclei of FVB/N and HER-2/Neu mice of different ages.

transcriptional program may be a common symptom of aging of different tissue types, but the expression pattern of p53-dependent genes is tissue-specific [74]. In our experiments, the level of the p21 protein, an inhibitor of cyclin-dependent kinases, necessary to lower the level of apoptosis [56], is high in neurons of the hypothalamus of young FVB/N mice and decreases with aging, inversely correlating with the expression of p53 and the level of apoptosis.

The data obtained in our experiments showed significant damage to the regulation of the p53-dependent cascade of apoptosis during overexpression of the HER-2/Neu oncogene. The expression of the studied p53-signaling genes (p53, p21, WRN, and pin1) in the hypothalamic neurons is significantly lower in HER-2/Neu transgenic mice, regardless of the ontogenesis stage, compared to wild-type mice (**Figures 2a–4**). An insufficient increase in WRN expression in transgenic mice results in a low synthesis of p53 and, correspondingly, a low level of programmed cellular cell death in aging. In addition, it was found that in HER-2/Neu mice irrespective of the ontogeny stage, the functionally defective WRN protein is synthesized, which is confirmed by immunohistochemistry. Changes in pin1 expression in transgenic mice correspond to the dynamics of p53 synthesis; it is a low level of p53 expression and no response to aging. It confirms the functional relationship of these genes, shown in the works of other authors [9], and supports the suppressive effect of the HER-2/Neu oncogene on various links of p53-mediated signaling.

P21 plays an important role in tumor suppression [19]. The decrease in p21 synthesis in HER-2/Neu mice (as a result of low p53 level in these mice) indicates attempts to maintain a balance of cell death survival with p53-p21 pathway; however, the expression of the HER-2/Neu oncogene results in the suppression of the apoptotic cascade.

2.2.1. Conclusion

This study for the first time revealed the involvement of the Werner syndrome gene in the physiological aging of neurons. The age-related increase in WRN expression promotes the activation of p53-dependent apoptosis of hypothalamic neurosecretory cells in wild-type mice (an increase in the expression of members in this cascade—pin1, p53, and, indirectly, p21). In transgenic animals with overexpression of the oncogene HER-2/Neu, the synthesis of pin1, p53, and p21 is low and there is no reaction to aging. The result of suppression of apoptosis in HER-2/Neu mice is increased carcinogenesis and reduced life expectancy.

Thus, it is shown that the WRN gene not only determines the pathological premature aging (Werner's syndrome) but also plays an important role in the mechanisms of physiological aging.

2.3. The role of STAT transcription factors in apoptosis regulation of hypothalamic neurons in aging in wild-type FVB/N mice and HER-2/neu transgenic mice

The interferon gamma is the main potential activator of STAT1, but STAT1 is also activated by growth factors and their receptors [75, 76]. According to some data, induction of STAT1 leads to a decrease in proliferation and an increase in p53-dependent apoptosis of cells of hepatocellular carcinoma [77]. The protein-protein interaction between p53 and STAT1 is shown. This interaction occurs via the C-terminal domain of STAT1, which is critical for the stress-induced apoptotic response. It was shown that the induction of expression of p53 and its target genes

in response to DNA damage is significantly reduced in STAT1-deficient cells [75]. As is known, Mdm2 is a key regulator of p53 expression. The stabilization of the p53 protein level is regulated by the Mdm2 protein, which interacts with the p53 protein and promotes its degradation by ubiquitination [78]. It is shown that the level of Mdm2 expression is increased in STAT1-deficient cells, and STAT1 represses the promoter of the gene encoding the Mdm2 factor, and STAT1 inhibits the p53-mediated activity of the Mdm2 promoter. Therefore, an elevated level of Mdm2 in STAT1-deficient cells may be responsible for a low level of p53 after DNA damage. These data indicate that STAT1-p53 interaction can have both positive and negative effects on various gene promoters. Evidence of this relationship is also the fact that tumors develop significantly faster in p53-STAT1-knockout mice than in mice knocked out by one of these genes [79]. In many tumors, the p53 function is reduced by 50%, in most cases due to overexpression of Mdm2 [80]. In experiments, it has been shown that the administration of interferon gamma restores p53-induced apoptosis by inhibiting the expression of Mdm2 via the STAT1-mediated mechanism, which is of great importance for the therapy of cancer. Thus, STAT1 is a checkpoint protein and also acts as an oncosuppressor.

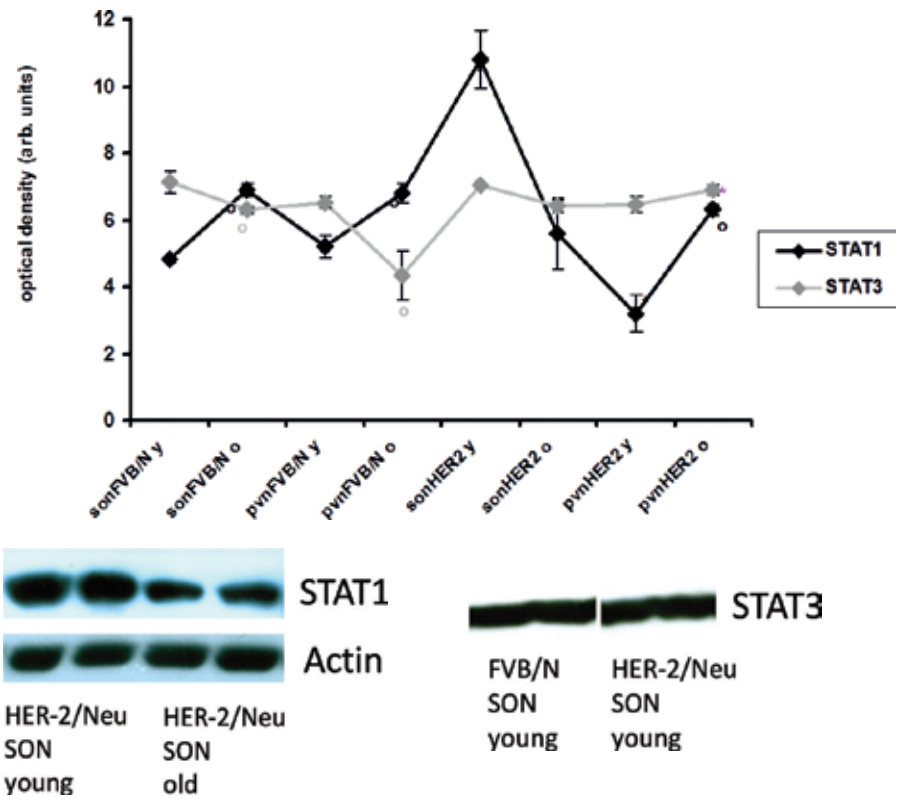

Figure 5. (a) The expression level of STAT1 and STAT3 in the neurosecretory cells of SON and PVN of FVB/N and HER-2/Neu mice of different ages. (b) STAT1 expression in SON of young and old HER-2/Neu mice (Western blotting). (c) STAT3 expression in SON of young FVB/N and HER-2/Neu mice (Western blotting).

In our experiment, the STAT1 level in young FVB/N mice is low, approximately the same in the SON and PVN (**Figure 5**). With aging, an increase in the expression of STAT1 in wild-type mice was found, which correlates with an increased level of neuronal apoptosis and overexpression of p53, caspase-3, and -8 (**Figures 2a**, **5a**, and **6**). The levels of expression of caspase-8 and caspase-3 are similar. The expression of caspases is low and the same in hypothalamic neurons in young mice of both lines (**Figure 8b**). In aging, we obtained the increase of caspases synthesis only in hypothalamus in wild-type mice (**Figure 6**). Some authors too reveal the proapoptotic function of this transcription factor. In young HER-2/Neu mice, STAT1 expression is high in SON and decreases with aging (**Figure 5b**), although some activation of synthesis is observed in the PVN (**Figure 5a**).

In young mice of both lines, STAT3 expression is approximately at the same level. With aging, the synthesis of this factor decreases only in mice FVB/N. In transgenic animals, there is no change in the synthesis of STAT3 in the late stages of ontogenesis (**Figure 5a**).

It was previously shown that STAT1 and STAT3 have the opposite effect on the apoptotic death of various cells [81]. STAT1-deficient cells are resistant to TNF-alpha-induced apoptotic death [82]. By contrast, STAT3 has oncogenic characteristics; its overexpression is observed in many types of tumors [83]. Significant activation of STAT3 is often observed in different types of cancer, including breast cancer. STAT3 plays a role in the progression of tumors and their resistance to anti-cancer treatment by regulating the survival of cancer cells [84]. Activation of STAT3 in glioblastoma multiforme correlates with malignancy and poor prognosis. The phosphorylating signal transducer JAK2 activates STAT3 in response to cytokines and growth factors [85].

It is known that overexpression of STAT1 induces the induction of apoptosis, for example, of cardiomyocytes in ischemia-reperfusion, while overexpression of STAT3 decreases STAT1-induced cell death [86].

One of the targets of STAT3 is survivin, an antiapoptotic protein belonging to the family of IAP (inhibitors of apoptotic proteins) [87]. It is known that HER-2 initiates oncogenic cascades. It has now been shown that HER-2 promotes the activation of STAT3 and, further, survivin [88]. Thus, it is obvious that members of the STAT family are involved in modulating the expression of apoptotic genes.

The problems of neuronal survival or death during aging are very important, since the regulation of the amount of neurons is carried out unilaterally (by cell death). The role of survivin and STAT factors in the regulation of apoptosis in aging is currently unclear. Survivin is almost not expressed in normal differentiated tissues. It is known that overexpression of survivin leads to an increase in proliferation in the hippocampus [89]. Recently, it has been shown that the synthesis of survivin decreases with aging and neurogenesis decreases. In addition, increased expression of survivin induces a significant reduction in β-galactosidase activity; thus, survivin allows cells to avoid aging [90].

It should be noted that in our experiment, STAT3 expression is the same in neurosecretory cells in FVB/N and HER-2/Neu mice (**Figure 5a** and **b**). The age-related changes in the expression of STAT1 and STAT3 are opposite—overexpression of STAT1 with a simultaneous decrease in the synthesis of proapoptotic factor STAT3 in aging in wild-type mice (**Figure 5a**). According to other investigations, STAT1-activated apoptosis proceeds via

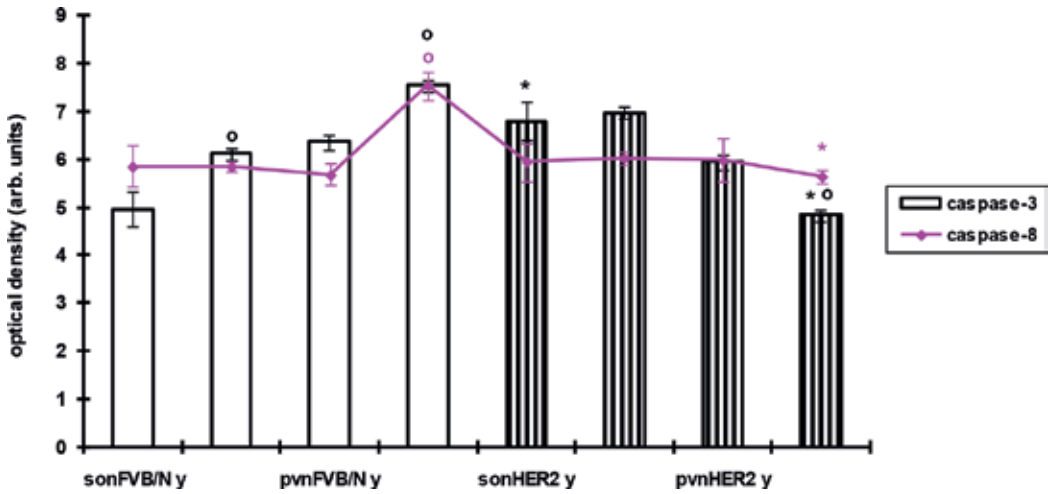

Figure 6. The expression level of caspase-3 and caspase-8 in the neurosecretory cells of SON and PVN of FVB/N and HER-2/Neu mice of different ages.

TNF- and p53-signaling pathways, with activation of caspase-3. Our results also showed an increase in the synthesis of p53 and caspase-3 (**Figures 2a** and **6**). Some authors report a suppression of STAT3 expression in an increased synthesis of STAT1; probably, this mechanism is present in our experience.

The level of survivin in hypothalamic neurons in young FVB/N mice was quite high in comparison with transgenic mice. With aging, survivin expression decreased in both nuclei in wild-type mice. In old HER-2/Neu mice, survivin synthesis did not change in SON and increased in PVN (**Figure 7**). These changes correspond to the dynamics of STAT3 expression (**Figure 5a**).

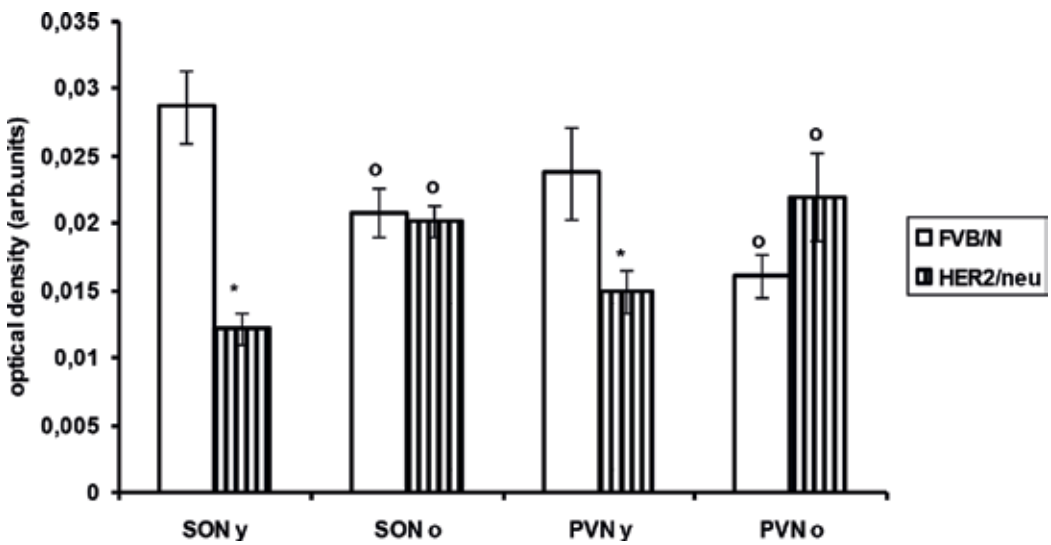

Figure 7. The expression level of survivin in the neurosecretory cells of SON and PVN of FVB/N and HER-2/Neu mice of different ages.

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Other members of this family, STAT5 and 6, are involved in differentiation and cell survival process [91, 92]. STAT5 has pleiotropic functions for the regulation of cell proliferation, differentiation, and apoptosis. There is evidence of a pro-apoptotic activity of the JAK/STAT5 pathway in neurons [93], but most of the work reports an antiapoptotic orientation of STAT5 [94]. The STAT5 transcription factors are essential for both lymphocyte development and acute immune responses [95]. STAT5 is a regulator of cyclin D, Myc, and Bcl-2 in non-neuronal cells and thus is involved in the prevention of apoptosis [92]. It has been shown that STAT5 and STAT6 antiapoptotic cascades [91, 94], and antiapoptotic activity of the JAK/STAT5 pathway are carried out through Bcl-2 [96]. The problem of survival and death of neurons is especially important in aging. According to some studies, the Jak/STAT pathway is involved in the regulation of cytokine-dependent apoptosis and the activity of growth factors and their receptors.

Expression of STAT5 is approximately the same in young HER-2/Neu and FVB/N mice. With aging in FVB/N mice, this factor decreases in both neurosecretory centers (**Figure 8a**). In transgenic mice, in SON, there is no change in aging, and in PVN, age-dependent overexpression of STAT5 is observed (**Figure 8a** and **b**).

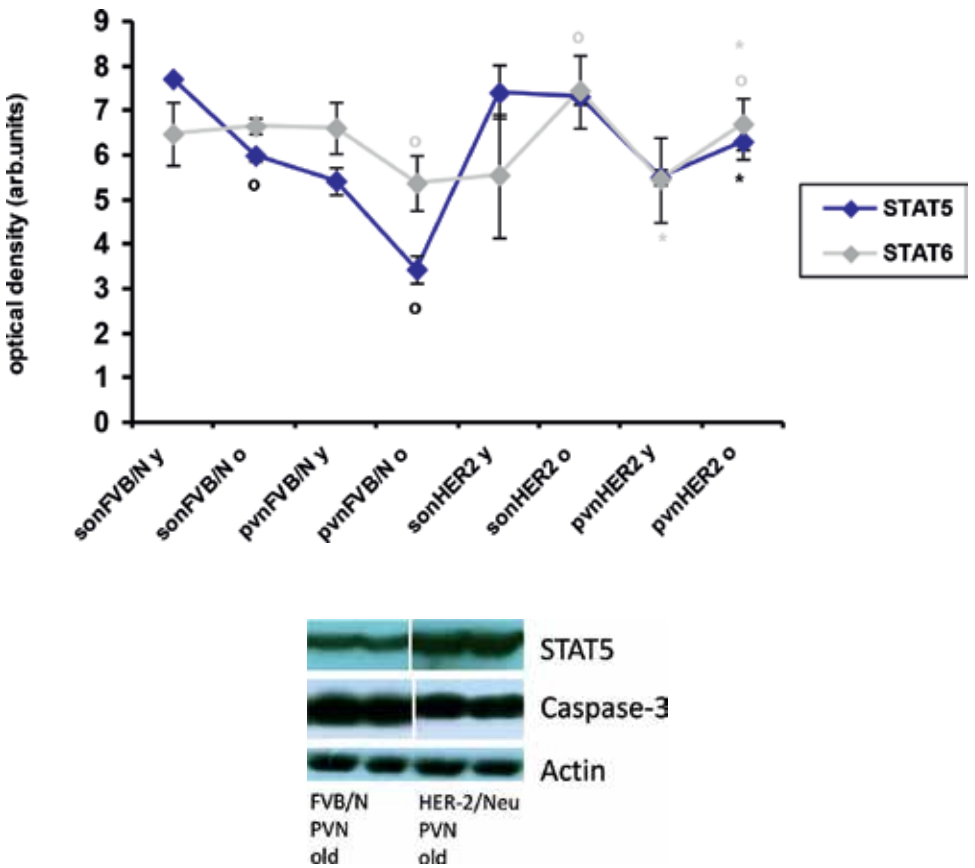

Figure 8. (a) The expression level of STAT5 and STAT6 in the neurosecretory cells of SON and PVN of FVB/N and HER-2/Neu mice of different ages. (b) STAT5 and caspase-3 expression in PVN of old FVB/N and HER-2/Neu mice (Western blotting).

Expression of STAT6 is almost the same in SON and PVN of young FVB/N mice. With aging in FVB/N mice, there are no changes in the SON, and a decrease in STAT6 synthesis is observed in the PVN (**Figure 8a**). In SON in transgenic mice there are no age-related changes, and the age-dependent overexpression of STAT6 is observed in the PVN (**Figure 8a**).

2.3.1. Conclusion

Thus, the synthesis of the studied transcription factors, which show antiapoptotic activity—STAT3, 5, 6, survivin—decreased in the late stages of ontogeny in the hypothalamic neurosecretory centers of wild-type mice. It can be concluded that the suppression of antiapoptotic factors STAT3, 5, and 6 and overexpression of the proapoptotic factor STAT1 is one of the reasons for the increase of the amount of dying neurons during physiological aging.

In young HER-2/Neu mice, the antiapoptotic factors STAT3, 5, and 6 are synthesized at a sufficiently high level. With aging, there is no change in the synthesis of STAT3 and an increase in STAT5, and 6 and survivin expression is observed. These factors are activated, in addition to cytokines, by growth factors and their receptors. Accordingly, overexpression of the HER-2/Neu receptor tyrosine kinase receptor results in cell survival by activating the STAT-signaling pathway, while suppressing the proapoptotic factor STAT1.

Thus, in this study, the participation of the STAT pathway in the regulation of neuronal apoptosis in physiological aging and in old mice with overexpression of the HER-2/Neu oncogene was studied for the first time. Active participation of this signaling pathway in the regulation of neuronal apoptosis during aging was observed.

2.4. The role of TNF-dependent way in apoptosis regulation of hypothalamic neurons in physiological and pathological (HER-2/Neu overexpression) aging

Most of the works is associated HER-2- and TNF-signaling in malignancy tissues [97, 98]. But some investigations demonstrate that HER-2 and TNF can interrelate in normal tissues. TNF-α, a pro-inflammatory and apoptosis-inducing cytokine, stimulates several intracellular signaling pathways. TNF-α can promote cell survival using activation of TAK1 kinase, which is especially important for cancer cells. As is shown, on the other hand, TNF-α induces apoptosis via formation of the death-inducing signaling complex (DISC), which consists of trimerized receptors, the death domain-containing adaptor protein FADD and caspase-8 [99]. HER is a member of the receptor tyrosine kinase family and plays a critical role in a wide variety of cellular functions, including proliferation, differentiation, and apoptosis. At present, the interaction of these factors (HER-2 and TNF) at various pathological conditions is described [100]. However, there is almost no data on the involvement of the TNF-signaling pathway in the regulation of age-related neuronal apoptosis during overexpression HER-2 in vivo.

So, we studied the role of a cytokine-dependent cascade—the TNF-mediated pathway in the regulation of apoptosis of neurosecretory cells of the hypothalamus in physiological aging and in old HER-2/Neu transgenic mice. Expression of the members of the TNF-dependent cascade was assessed at different levels: receptor perception of the apoptotic signal—expression of the Fas receptor (CD95), signaling—adapter expression: FADD and TRADD, and implementation:

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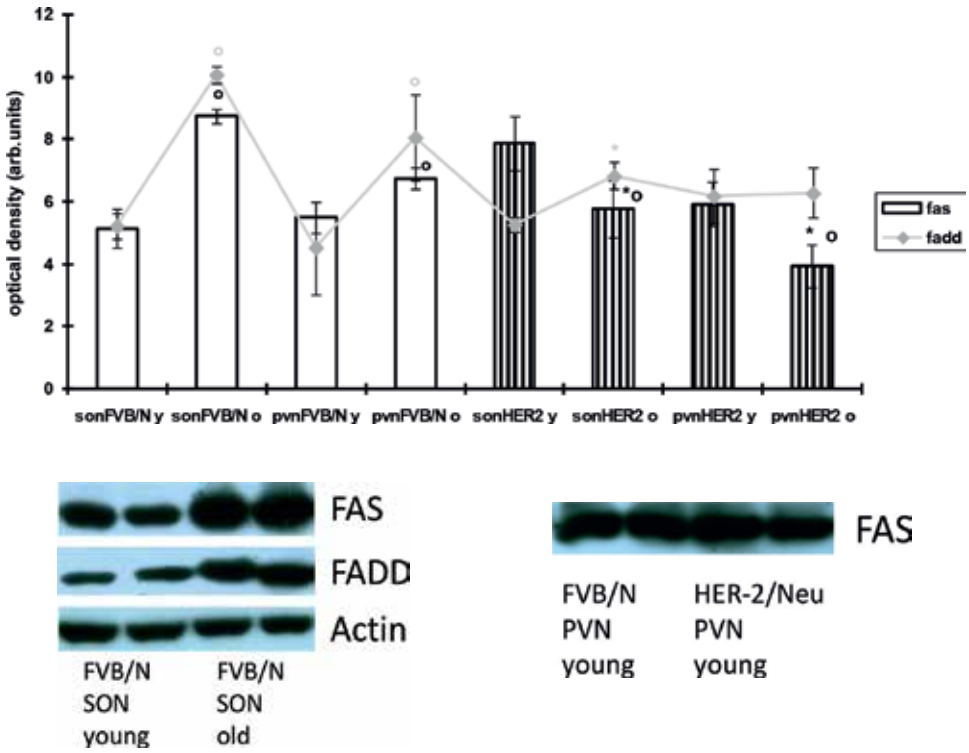

Figure 9. (a) The expression level of Fas and FADD in the neurosecretory cells of SON and PVN of FVB/N and HER-2/Neu mice of different ages. (b) Fas and FADD expression of SON in young and old FVB/N mice (Western blotting). (c) Fas expression in PVN of young FVB/N and HER-2/Neu mice (Western blotting).

the level of caspase-8 expression and, further, the level of neuronal apoptosis in the nuclei of young and old mice.

Fas (CD95) expression was increased significantly in both hypothalamic nuclei in old wild-type mice compared to young ones (**Figure 9a** and **b**). A high level of Fas is correlated with an increase of the synthesis of adapter proteins FADD and TRADD (**Figures 9a**, **b** and **10**). The expression of caspase-8 and -3 and the level of apoptosis were also increased in old wild-type mice (**Figures 2a** and **6**). Thus, in this case (in wild-type mice, i.e., in physiological aging), TNF signaling is an apoptosis-activating pathway and plays an important role in the mechanism of neuronal death.

Expression of the tyrosine kinase receptor HER-2/Neu causes significant changes in intracellular regulatory mechanisms, which is developed in the increased survival of cells, and it is the basis for possible oncogenesis. Our results show that HER-2/Neu expression at late stages of ontogenesis suppresses the main apoptotic cascades—p53- and TNF-dependent (**Figures 2a**, **9a**, and **10**). It was found that in young transgenic mice, the synthesis of the Fas receptor, the adapter proteins FADD and TRADD, is at the same level as in the young wild-type mice (**Figure 9c**) or exceeds it (in case of Fas and TRADD expression in SON). With aging, the expression of these proteins either decreases (Fas) or does not change (FADD, TRADD) in the SON and PVN in hypothalamic nuclei in transgenic mice. Accordingly, significant differences are noted between the studied

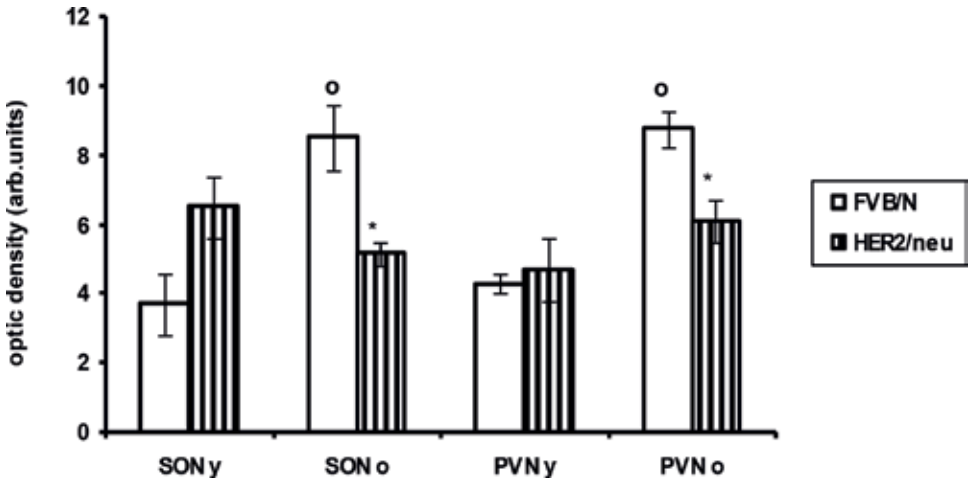

Figure 10. The expression level of TRADD in the neurosecretory cells of SON and PVN of FVB/N and HER-2/Neu mice of different ages.

protein levels in old wild-type and in old transgenic mice (**Figures 2a**, **9a**, and **10**). We show any age-related dynamics in the expression of initiator caspase-8 and effector caspase-3 in neurosecretory cells in young and old HER-2/Neu mice (**Figure 6**).

It can be concluded that one of the ways to ensure increased cell survival in aging in transgenic HER2 animals is the suppression of the TNF-dependent apoptotic cascade.

TNF was previously considered a proapoptotic factor. Recently, it has now been shown that the role of the TNF pathway in apoptosis is ambiguous—first, the canonical pathway of apoptosis activation involving caspase-8 and -3 [36, 99, 101–104] and the second, the anti-apoptotic effect of TNF are associated with the activation of independent survival ways—IkB (inhibitor of kappa-beta kinase-nuclear factor kappa B) pathway (signaling cascade associated with NF-kB) [103, 104] and MAPK-EGFR pathway [99, 100, 105], or the PI3K-AKT pathway [105].

These signaling pathways (PI3K-AKT and MAPK/ERK) are well known as survival. It is known that the main function of transcription factor NF-kB is the coordination of immune and pro-inflammatory cellular responses. However, it has now been shown that, in addition, members of the NF-kB family are factors in cell survival, and some data indicate the importance of NF-kB as a survival factor in the central nervous system. One of the key kinases that activate both NF-kB and MAPK pathways is the TAK1 kinase, which is capable of regulating the phosphorylation and endocytosis of EGFR, regardless of its tyrosine kinase activity. Some authors consider that the TRAIL factor (TNF-related apoptosis-inducing ligand), Fas ligand, Fas, and FADD are proapoptotic factors, whereas TNF-receptor 1 and TRADD may have an antiapoptotic effect, acting through the survival pathway is the NF-kB and the p38-MAPK-EGFR pathway [106].

A high level of Fas in hypothalamic neurons in young HER-2/Neu mice does not lead to an increase in the level of apoptosis, since FADD expression is low (**Figure 9a**). At the same time, increased expression of TRADD may further activate any of the above-mentioned survival

ways—NF-kB, MAPK/ERK, or PI3K-AKT cascade (**Figure 10**). Perhaps, HER-2/Neu expression and TNF receptor 1 and TRADD factors stimulate the phosphorylation of ERK and AKT cascades, which leads to an increase of cell survival. Possibly, these signaling pathways prevent proapoptotic cleavage of caspases mediated by the DISC [99]. A similar mechanism exists in epithelial non-transformed breast cells, when inhibition of EGFR signaling causes up-regulation of the inhibitor of caspase-8 FLICE-inhibiting protein (FLIP(L)) and makes cells more sensitive to TRAIL-induced apoptosis, and the ERK cascade played an important role [107]. In addition, it is known that TNF-α induces the formation of membrane-bound complexes, which include, among other components, IAP, which are triggers of the NF-kB cascade [104]. We have shown an increase of the expression of the antiapoptotic protein survivin, a member of the IAP family, in hypothalamic neurons in aged HER-2/Neu mice (**Figure 7**). We can suppose that the increase of survivin expression is caused by a high content of TNF-alpha in transgenic mice, and this probability will be investigated.

2.4.1. Conclusions

Thus, we showed the participation of the pro-inflammatory component in the aging process (**Figure 11**). Fas expression, adapter proteins associated with the death domain (FADD and TRADD), and caspase-8 expression are activated in the hypothalamic neurons in FVB/N line mice (wild type) during aging. It correlates with an increase of caspase-3 expression and an increase of the apoptosis level of the hypothalamic neurons (**Figure 11**). It can be assumed that

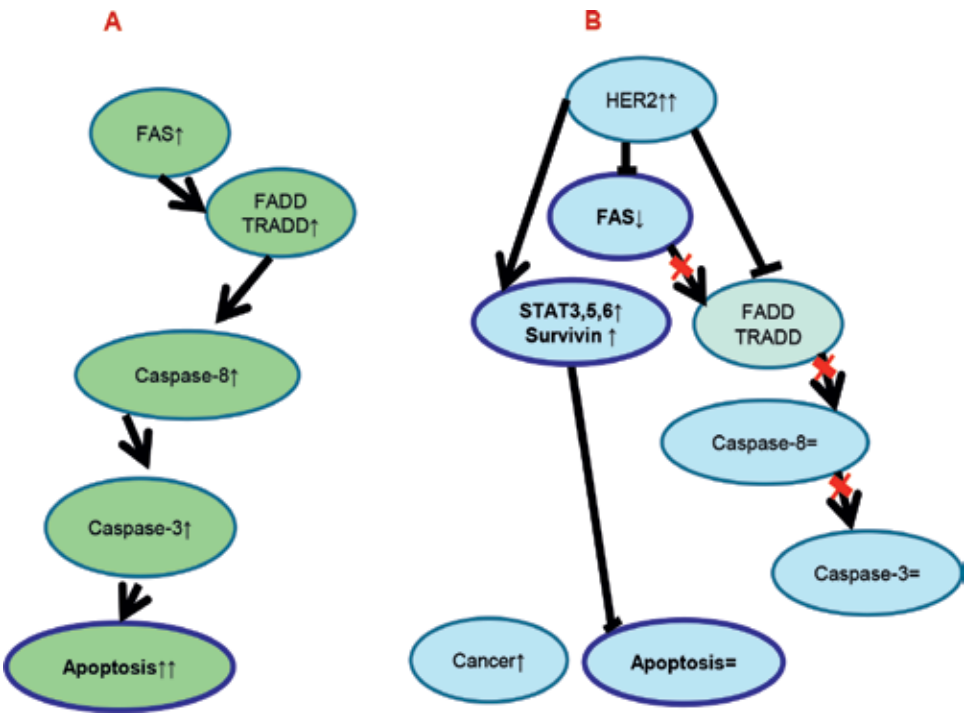

Figure 11. TNF-signal cascade of apoptosis in physiological aging (A), in aged HER-2/Neu transgenic mice (B).

one of the reasons for this is a possible suppression of the survival ways (AKT and ERK cascades) or an increased content of proapoptotic factors, for example, FasL in physiological aging.

HER-2/Neu expression causes suppression of the extrinsic pathway of apoptosis (TNF-dependent). In this case, both the reception of an apoptotic signal (Fas receptor expression) and its further carrying out (FADD and TRADD expression) are suppressed. However, in young transgenic mice, the increased TRADD expression may activate one of the survival ways—NF-kB, MAPK/ERK, or PI3K-AKT cascade (**Figure 11**). Thus, HER-2/Neu tyrosine kinase receptor plays an important role in the mechanism of cell resistance to age-dependent apoptosis, and TNF-signaling pathway is one of the targets of HER-2/Neu.

3. Conclusions

The aging process remains one of the most intriguing problems of biology and medicine. Recent advances in molecular biology make it possible to achieve an understanding of the fundamental foundations of this complex process. An intensive study of time- and tissue-specific gene expression is a tool that should lead us to a tangible control over age-dependent lesions.

Hypothalamic neurosecretory centers have been an object of deep interest since its role in the regulation of many body functions: adaptation, stress response, food and sexual behavior, emotions, thermoregulation, cognitive processes, and circadian rhythms have been discovered.

Aging disrupts vital activity, as noted in many cases. The first sign of aging, discovered by those who studied aging in vivo, is a disruption in the regulation of functions in almost all body systems. We know that the regulation of all processes is at least duplicated, so, the cell has not only an internal genome-dependent development program but also a subject to the influence of the nervous and endocrine systems. So, it can be concluded that the age-dependent changes are found in the central part of endocrine system, that is, in the hypothalamic neurosecretory centers. Indeed, our preliminary study showed that in old mice there is a significant loss of hypothalamic neurosecretory cells by apoptosis [1]. Obviously, a reduced amount of neurosecretory cells cannot maintain the previous level of functional regulation for a long time. This functional stress in the hypothalamic neurosecretory cells can be the cause of avalanche-like morphofunctional changes in the body caused by aging.

So, the results obtained in our studies allow to propose a possible scheme of apoptosis regulation of the hypothalamus neurons in physiological aging and in aged transgenic mice with HER-2/Neu overexpression (**Figure 12**). Thus, in the late stages of ontogeny, we observe an increase in the synthesis of proteins involved in the induction of apoptosis, only in a group of wild-type mice. At physiological aging, we observed increased level of hypothalamic neuron apoptosis mediated by the p53- and Fas-dependent pathways, with caspase-8 and -3 activation. As we have shown, the WRN gene also participates in the regulation of physiological aging. The synthesis of

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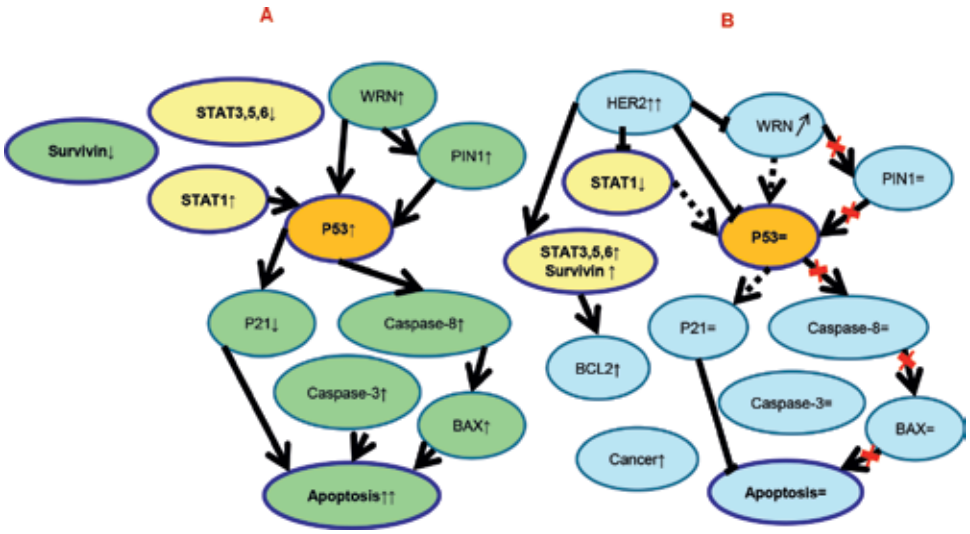

Figure 12. The apoptosis regulation in neurons in aging. (A) Signal cascade of apoptosis in physiological aging; (B) signal cascade of apoptosis in aged HER-2/Neu transgenic mice.

WRN and pin1 proteins increases and stimulates the expression of p53. The p21 protein requires for stable low level of apoptosis. The level of p21 protein is high in young FVB/N mice and decreases with aging. In transgenic animals with overexpression of the oncogene HER-2/Neu, the synthesis of these proteins is low, and there is no reaction to aging (**Figure 12**).

Thus, overexpression of HER2 blocks the signal pathway of p53, affecting both the p53 regulating proteins and the targets of p53.

In addition, we investigated age-related changes of STAT-signaling pathway. We revealed that in hypothalamus of wild-type mice, the synthesis of STAT1 increases and activates p53-mediated way. The expression of antiapoptotic factors STAT3, 5, 6, and survivin decreases in the studied neurosecretory centers. By contrast, in aged HER-2/Neu mice the expression of these factors increases, and STAT1 synthesis was low (**Figure 12**).

We showed the involvement of cytokine-dependent pathways in the mechanisms of apoptosis during aging. The realization of TNF-dependent apoptosis in hypothalamic neurons during physiological aging shows an increase of Fas receptor expression and expression of adapter proteins associated with the death domain (FADD and TRADD). In older transgenic animals, the expression of HER-2/Neu causes suppression of the extrinsic pathway of apoptosis—the TNF-dependent pathway (**Figure 12**). In other side, in the hypothalamus of young transgenic mice, the high TRADD expression may activate one of the survival ways (NF-kB, MAPK/ERK, or PI3K-AKT). So, HER-2/Neu tyrosine kinase receptor plays an important role in the mechanism of cell resistance to apoptosis in aging, and one of the targets of HER-2/Neu is TNF-signaling cascade.

The result of such suppression of the apoptotic cascade in transgenic mice is increased carcinogenesis and a half-reduced life expectancy, compared to the control.

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The human hypothalamus, a small structure at the base of the brain, has strategic importance for the harmonic function of the human body. It controls the autonomic nervous system, neuroendocrine function, circadian and circannual rhythms, somatic activities, and behavior, and is situated at the borders between the brain and the body and the brain and the soul, meeting points for mind and body. The hypothalamus is involved in a wide range of higher mental functions, including attention, learning and reinforcement of mnemonic processes, emotional control, mood stability, and cognitive–emotional interactions. It also has a role to play in behavioral disorders, panic reactions, cluster headache, gelastic epilepsy, mental deficiency, periodic disorders, depression, autism, and schizophrenia, and in a substantial number of neurodegenerative diseases. It enlarges greatly the dimensions of the hypothalamic contribution in controlling psychosomatic equilibrium and retaining internal unity of the human existence.

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