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ROLE OF NEUROPEPTIDE QRFP IN THE HYPOTHALAMUS

PhD thesis

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Pécs, 2021

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Everything we do, every thought we've ever had, is produced by the human brain. But exactly how it operates remains one of the biggest unsolved mysteries, and it seems the more we probe its secrets, the more surprises we find.

(Neil deGrasse Tyson, American astrophysicist)

1. Introduction

Rephrasing L. N. Tolstoy, all healthy people are alike, every unhealthy person is unhealthy in his/her own way. Being a part of a basic research team, we aim to provide an innovative data and strategical solutions for the healthcare issues.

In the course of this paper, the problem of food consumption and weight regulation will be addressed. According to recent WHO statistics, 7.8% of population worldwide (including 10% of Europeans) suffer from eating disorders, such as anorexia nervosa, bulimia nervosa or binge eating [1]. In 70% such patients reveal associated psychiatric conditions: mood and anxiety disorders, substance abuse [2, 3], not mentioning frustratingly high suicide incidence [4, 5]. At the same time, up to 40% of world's adult population are overweight, of these 13% were diagnosed with obesity [6]. Metabolic comorbidities, such as diabetes mellitus type 2, dysfunctions of gastro-intestinal tract and cardiovascular diseases, often accompany weight gain or loss and require special treatment [7-11]. Despite thousands of research initiatives on this topic, the perfect solutions for the prevention and treatment of eating disorders are still to be discovered, the exact causes and mechanisms are to be identified.

The topic of memory and learning processes will be another focus of this research. "There are over 9.9 million new cases of dementia each year worldwide, implying one new case every 3.2 seconds" – states Alzheimer's disease international. The economic impact of dementia is greater than cancer and heart disease combined [12]. For the person with dementia, the diagnosis attracts other health impacts (depression, anxiety, stress, physical problems, sleep disruption), as well as social isolation. Dementia has a profound impact, not only on the life of the affected person but on the lives of those around them.

So, what could be in common between a young lady, who refuses to eat her lunch day by day, and a pensioner, who forgot his address and experiences difficulties finding the way home? It appears, more than it seems at first.

According to rough estimations, a well-coordinated, precise functioning of over the 100 trillion synapses in a typical brain ensure human wellbeing. Minimal breakdowns in this finely tuned system may lead to severe problems. A great role in neuronal communication within the brain, as well as communication between central neurons and cells of different origin at the

periphery, play numerous neuropeptides. By today, over 100 of them are already known, and probably many more are still to be identified from over the 1000 predicted peptides encoded by the genome [13]. The neuropeptides regulate multiple vitally important, sometimes even seemingly unrelated physiological functions.

In this paper, we explore the properties of the novel pyroglutamylated RFamide neuropeptide QRFP. Despite the substance was identified in the early 2000s, its role and mechanisms of action are still under research. Probably, due to its tissue distribution primarily in the hypothalamus, neuropeptide QRFP and corresponding receptors first have been implicated in the regulation of feeding [14-18]. This proposition led to a series of diverse experiments (discussed later in detail), which did not give a clear image though. Besides investigations of QRFP's role in feeding, anxiety, and motivation regulation, here we present unique evidence regarding the previously unknown aspects of neuropeptide's activity, i.e., in the consolidation of spatial memory.

1.1. The hypothalamus

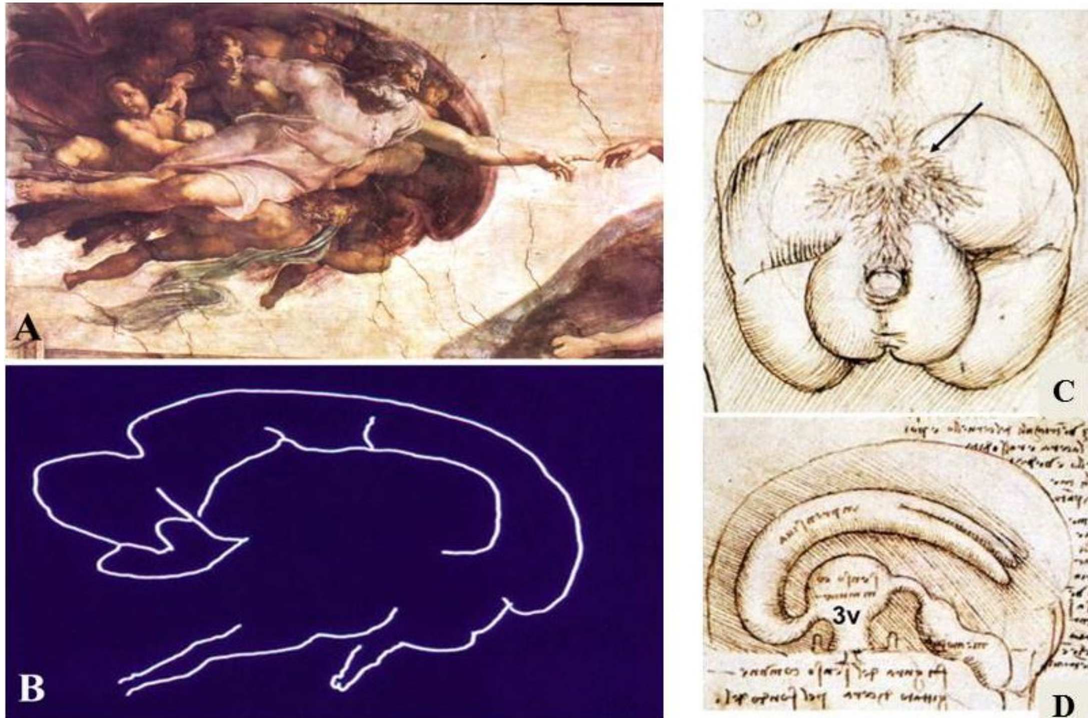
1.1.1. Gross anatomy of the hypothalamus

From an evolutionary perspective, the hypothalamus is one of the most ancient parts of the brain. The hypothalamus develops from the anterior end of the neural tube. Despite it accounts for less than 1% of the brain weight, the hypothalamus is involved in a great number of essential metabolic and behavioral functions.

The hypothalamus is a heterogeneous part of the diencephalon situated right below the thalamus, as it is derived from its name (hypo = below, thalamus = bed) [19]. The hypothalamus itself and its close relation to the pituitary have been under focus of the scientists from ancient times. First described by Galen in the 2nd century AD, the hypothalamus was recognized as an important coordinator, that collects signals from the third ventricle and forwards them to the pineal gland, described at the same time [20]. Much later, in the 14th century Mondino de'Liuzziand and Andreas Vesalius further developed the concept of the third cerebral ventricle as an "integrator" of body functions, including psychic, emotional, and behavioral responses. The most famous researchers and artists of the Renaissance period - Leonardo da Vinci and Michelangelo Buonarroti – have been attracted by the potential of the hypothalamo-pituitary region (Fig. 1). At the end of the 19th century, Wilhelm His introduced the term "hypothalamus" and provided the first anatomical subdivision based on the ontogenesis of the human brain. Soon after, E. Scharrer introduced a concept of neurosecretion determining a new era in brain research.

Starting from the 1950th, connections of the hypothalamus with the other brain regions have been gradually discovered, and the concept of hypothalamic integration as part of the limbic system has been accepted. Twenty years later, R. Guillemin and A. Schally isolated the first hypothalamic releasing factor (for review see [21-24]).

Fig. 1. Images of the hypothalamus by renaissance artists



A: Detail from the fresco, "Creation of Adam," by Michelangelo Buonarroti, visible on the ceiling of the Sistine Chapel in the Vatican at Rome, Italy, painted between 1508-1512.

B: The contour of the same image is reminiscent of a midline saggital section of the brain and includes the hypothalamus, pituitary and brainstem.

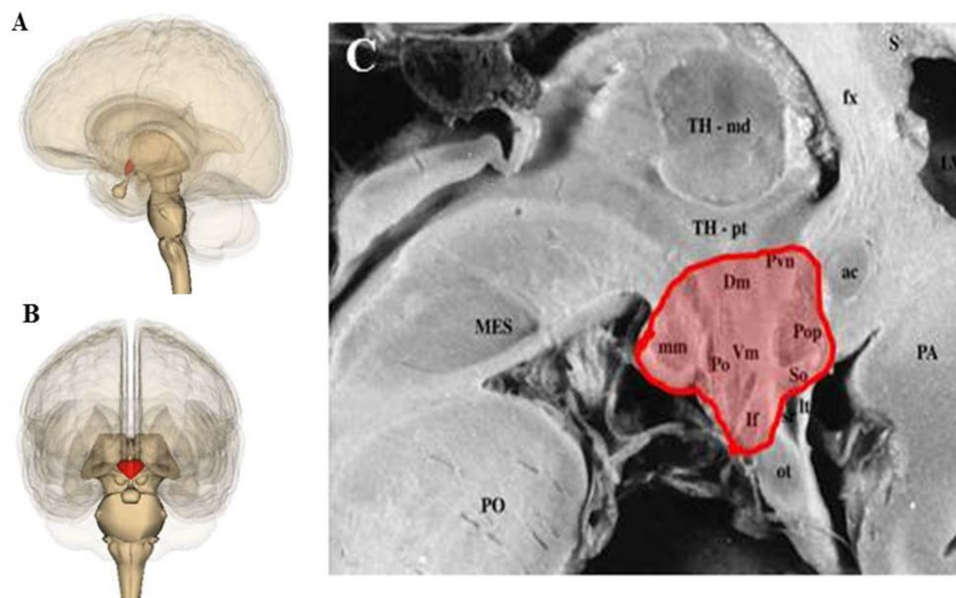
C, D: Drawings by Leonardo da Vinci (1508-1509) taken from the Codici di Anatomia of the Windsor's Collection (Courtesy of the Library of the Department of Human Anatomy of the University of Parma, Italy). C: Inferior surface of the brain, showing the rete mirabilis (arrow) that surrounds the pituitary gland; D: three-dimensional representation of the cerebral ventricles. Reprinted from [25].

The hypothalamus is situated in the ventral diencephalon symmetrically around the third ventricle. In the sagittal section (Fig. 2) the hypothalamus extends from the optic chiasm, lamina terminalis, and anterior commissure rostrally to the cerebral peduncle, interpeduncular fossa, and mammillary bodies caudally. In the coronal section, the boundaries of the hypothalamus are well distinct. Superiorly the hypothalamus is separated by the hypothalamic sulcus from the central mass of the thalamus.

Laterally the hypothalamus relates to the thalamus and subthalamus and is bordered by the internal capsule (IC) and optic tracts (tr.). The medial border is connected to the ependyma of the third ventricle. The inferior surface forms a continuation of the floor of the third ventricle. Hypothalamus is surrounded by the blood vessels of the circle of Willis.

The external surface of the hypothalamic floor continues into the tuber cinereum, which extends anteriorly and dorsally into the infundibulum or median eminence, terminating inferiorly on the pituitary gland. Two additional symmetric eminences: the lateral eminences, corresponding to the most lateral portion of the hypothalamic wall, and the postinfundibular eminence, as well as the symmetric mammillary bodies, complete the macroscopic morphology of the hypothalamic floor (for general review see [26]).

Fig. 2. Image of the human hypothalamus



A, B: Three-dimensional image of human brain with hypothalamus emphasized with red color. Reprinted from [27].

C: Magnified view of a fixed human brain in midsagittal orientation (hypothalamus emphasized with red color). The third ventricle makes up the core of the hypothalamus and extends into the pituitary, creating the infundibular recess. Observed hypothalamic nucleal groups from rostral to caudal: the preoptic nucleus (Pop), paraventricular nucleus (Pvn), dorsomedial nucleus (Dm), ventromedial nucleus (Vm), arcuate (or infundibular) nucleus (If), posterior hypothalamic nucleus (Po), and medial mammillary nucleus (mm). Ac = anterior commissure, fx = fornix, lt= lamina terminalis, ot = optic tract and chiasm, Lv = lateral ventricle, MB = midbrain, PN = pons, Sr = supraoptic recess, T = thalamus. Reprinted from [28]

1.1.2. Microscopic anatomy of the hypothalamus

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

Based on the morphological and functional features, the hypothalamus is divided into three general areas: the periventricular, medial, and lateral hypothalamus. The periventricular region, as the most medial part of the hypothalamus, consists of a large number of neurons organized in separate nuclei: periventricular nucl. (PeVN), suprachiasmatic nucl. (SCh), paraventricular nucl. (PaVN) and arcuate nucl. (Arc). Similarly, the medial hypothalamus (MH) represents a collection of closely situated and functionally interconnected cell groups: anterior hypothalamic nucl. (AHN), medial preoptic nucl. (mPON), dorsomedial nucl. (DMN), ventromedial nucl. (VMN), premammillary nucl. (PMN), mammillary nucl. (MN), posterior hypothalamic nucl. (PHN). The lateral hypothalamic area (LHA), extending till the IC, is not that well-structured, as it is largely composed of a massive bidirectional fiber pathway, the medial forebrain bundle (mfb), and contains only a few nuclear groups: lateral preoptic nucl. (lPON), lateral hypothalamic nucl. (LHN), supraoptic nucl. (SON). These data are summarized in Table 1.

Further, each of the hypothalamic areas can be divided into three zones along the sagittal axis: anterior, median, and posterior. The anterior, or chiasmatic zone (extending from the anterior boundary of the hypothalamus till the infundibular recess) is responsible for thermoregulation, electrolyte balance, wake-sleep, circadian rhythms, and sexual behavior. The median, or tuberal zone (extending between the infundibulum and the anterior column of the fornix) contains integrative circuitry for feeding, as well as output circuitry for sexual behavior, aggressiveness, and many autonomic and endocrine responses. The posterior part of the hypothalamus, or mammillary region (extending between the fornix and the posterior boundary of that hypothalamus), provides intense outputs to the arousal system and hippocampus, regulating wakefulness, memory, and stress responses.

Table 1. Major hypothalamic nuclei (rostral to caudal)

PERIVENTRICULAR REGION	MEDIAL REGION	LATERAL REGION
Periventricular nucl.	Medial preoptic nucl.	Lateral preoptic nucl.
Suprachiasmatic nucl.	Anterior hypothalamic nucl.	Lateral hypothalamic nucl.
Paraventricular nucl.	Dorsomedial nucl.	Supraoptic nucl.
Arcuate nucl.	Ventromedial nucl.	
	Premammillary nucl	
	Mammillary nucl.	
	Posterior hypothalamic nucl.	

Based on the anatomical classification of Nauta W.J.H. and Haymaker W.[29].

1.1.3. Pathways and connections of the hypothalamus

The hypothalamus has dense connections with various cerebral structures that allow realizing its regulatory functions as an integrating center. Inputs to the mammalian hypothalamus arise primarily from the limbic system, brainstem reticular formation (BRF), thalamus, subthalamus, basal ganglia, retina, cerebellum, and the neocortex. Outputs from the mammalian hypothalamus include fiber pathways toward the anterior and posterior pituitary gland, limbic system, BRF, thalamus, subthalamus, basal ganglia, superior colliculi, substantia nigra (SN), cerebellum, and neocortex (for reviews see [30, 31]. Besides orchestrating multiple metabolic functions, the hypothalamus represents a subcortical portion of the "feeling and reacting brain", i.e. the limbic system. Moreover, the hypothalamus as a limbic center is thoroughly connected to the other limbic structures.

The hippocampal complex interacts with the hypothalamus via fornix tr., which is arching into the substance of the hypothalamus to terminate along with its entire extent. Hippocampal cornu ammonis fields CA1 and CA3 are directly connected with the infundibular [32]. The direct pathways between CA1 and VMN [32, 33], as well as between CA1, CA2, and DMN [34], have been described. According to the recent study [35] CA2 area of the hippocampus, composed of pyramidal neurons, is involved in memory and learning through its connections with the supramammillary nuclei. The circuit between the hippocampus and the LH was described in connection with the orexin pathways [36].

Another key limbic structure, the amygdaloid complex (Amy), developed multiple communication pathways with the hypothalamus. The main route is stria terminalis, which is accompanied by subcapsular fiber components of the ventral amygdalofugal tr., amygdaloseptal fibers of the diagonal band, and medial amygdalohypothalamic tr.. Some fibers of stria terminalis follow the fornix and reach the tuberal hypothalamic nuclei, while other components are incorporated into the medial forebrain bundle (mfb) [37, 38]. There were described principal projections connecting the PVN and the central nucleus of the amygdala (CeA) [39, 40], as well as the rich input system from the medial, amygdalohippocampal (AHi), CeA, and basolateral (BLA) nuclei of the Amy towards both the MH and the LHA [41, 42].

The reticular activating system (RAS) is a network of neurons located in the brainstem that projects anteriorly to the hypothalamus to mediate behavior, as well as posteriorly to the thalamus and directly to the cortex. Through it, the reticular formation (RF) is connected with the hypothalamic nuclei: the lateral mammillary bodies, the tuberomammillary nuclei, and the PeVN [38]. Connecting pathways with the BRF include the dorsal longitudinal fasc. (dlf), the periventricular fiber system and the mfb. The dlf receives primarily autonomic inputs from centers in the mesencephalic tegmentum (the limbic midbrain area), reticular raphe nuclei in the pons, and the viscero-sensitive nuclei (e.g., the nucleus tr. solitarii (NTS)) in the medulla oblongata. The periventricular system carries fibers ascending from both the central grey (including the raphe nuclei) and medial nuclei of the RF in the mesencephalon or dorsal nucleus of the mesencephalic tegmentum. Collectively, these fibers enter the hypothalamus next to the wall of the third ventricle. The mfb also receives a well-defined fiber tr., the mammillary peduncle, originating from the medial nuclei of the mesencephalic RF.

Information from the NTS can reach the hypothalamus through either the solitariohypothalamic tr. or through collaterals from the solitariothalamic tr.. Besides the well-known NTS/PVN and Arc projections, there have been described the pathways from/to the LHA [43, 44].

Fibers from the olfactory bulb reach the LH across the periamygdaloid region and then the Amy or the nucl. accumbens (NAcc) [38].

Afferents from the retinal neuroepithelium reach the SCh of the hypothalamus through the lateral geniculate body of the mesencephalon and the superior colliculus by means of a retinohypothalamic tr. [45]. However, it was shown that many fibers reach MH nucll. and even LHA [46].

There is a bidirectional connection between the cerebral cortex and the hypothalamus. The hypothalamus diffusely projects over the cortex and transmits information that maintains the cortical tonus. The cortical grey sends towards the hypothalamus the information regarding the

current state of the organism, and thus triggers the visceral responses. Some neural fibers from the LH project to the prefrontal cortex. At the same time, direct projections arise from the frontal cortex, course in the mfb to the most lateral part of the ventricular wall [47]. The paraorbital gyrus sends fibers projections towards the PVN and VMN [48].

The PVN and LHA send descending projections towards the spinal cord [49]. The axons from the dlf and hypothalamospinal tr. are coursing towards the thoracolumbar and sacral lateral horn, thus providing control of both sympathetic and parasympathetic functions [50].

Afferents from the subthalamus are believed to originate in the nucleus subthalamicus and zona incerta (ZI), and directly enter the hypothalamus (PVN, LHA) along the lateral aspect of the hypothalamic wall [51-53].

Connections with the basal ganglia (globus pallidus, corpus striatum, putamen) arise from the NAcc, and via the substantia innominata, course in the lateral aspect of the ventricular wall in the mammillothalamic tr. (mtt), and reach the posterior and lateral portions of the hypothalamus [54-56].

The mtt connects the posterior hypothalamus (i.e., the LMN, MMN) with the anterior part of the thalamus [57]. The anterior hypothalamus has bidirectional connections with the medial nucleus and the nucleus of the median line through the periventricular fiber system, as well as epithalamic habenula through the stria medullaris. Finally, a part of the mfb courses in the inferior thalamic peduncle of the ansa peduncularis to reach the medial thalamic nuclei.

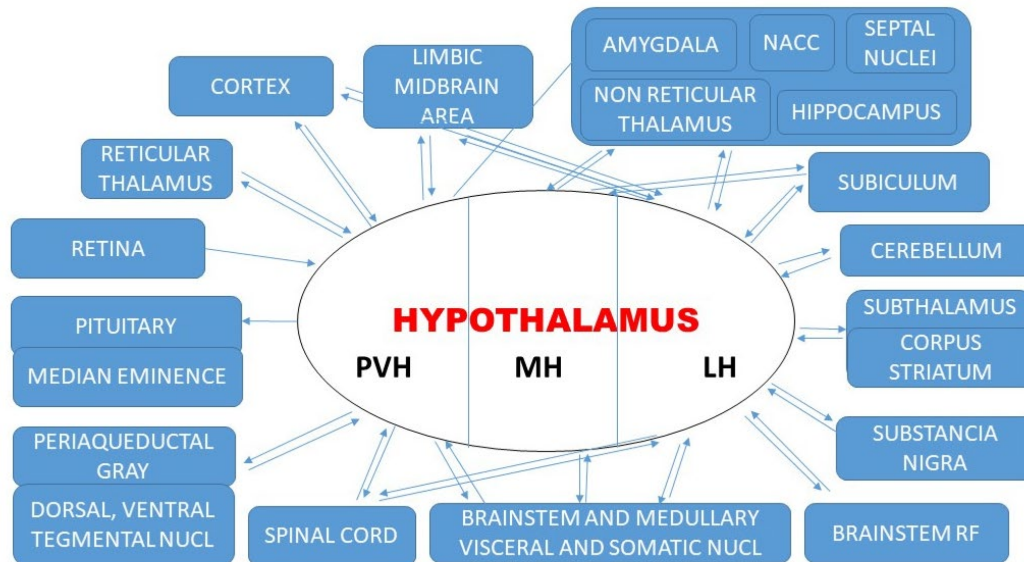
The temporal part of the subiculum was shown to project towards the medial preoptic region of the hypothalamus, whereas the anterior, tuberal, and mammillary regions received those from the full longitudinal extent of the subiculum. The medial corticohypothalamic tr. is the main route taken by fibers from the ventral subiculum to the hypothalamus, where they innervate the medial preoptic area, VMN, DMN, vPMN, as well as the zone of the SCh, AHN, and PVN. [58, 59].

The hypothalamocerebellar connections arise from lateral, posterior, and dorsal hypothalamic areas, the DMN, VMN, SM, tuberomammillary and LMN, and the periventricular zone. The direct or indirect (via BR nuclei) pathways terminate in the neurons of the cerebellar cortex and nuclei [60].

One of the crucial connections is represented by the hypothalamic neurohypophysial tr.. It arises primarily from the magnocellular neurons of the PaVN and SON [61]. Multiple neuropeptides are involved in communication between the hypothalamus and the pituitary gland. Vasopressin-containing axon terminals mostly arise from a population of PaVN neurons that contain also corticotropin-releasing hormone (CRH) [62] and have also been demonstrated in the ME [63]. Next to vasopressin and oxytocin, there was shown a co-localization in magnocellular neurons and a co-transportation toward the posterior pituitary for numerous other peptides, such as

dynorphin, enkephalin, galanin, cholecystokinin (CCK), vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), substance P (SP), thyrotropin-releasing hormone (TRH), CRH, and endothelin-1 [64-67]. Parvocellular neurosecretory cells originating from several hypothalamic nuclei were shown to transport the following neuropeptides: gonadotropin-releasing hormone (GnRH), TRH, CRH, somatostatin (SST), enkephalin, neurotensin (NT), GHRH, and dopamine (DA) [68].

Fig. 3. Schematic representation of the hypothalamic connections



The structure and the rich connection network of the hypothalamus stipulate its pivotal role in multiple physiological functions. The background of those, which are in the focus of the present study, will be discussed in more detail.

1.1.4. Role of the hypothalamus in feeding

Decades ago, back in 1940th, the critical role of the hypothalamus in appetite and food intake was first recognized. Experiments on rodents demonstrated that following the large lesions in the VMN, the massive hyperphagia and obesity occurred, while the lesions of the adjacent LH region led to hypophagia and inanition [69-75]. Later electrical stimulations of the hypothalamic nuclei contributed to the concept of distinct „feeding centers", controlling hunger and satiety. Stimulation of the VMN led to a decrease in food intake, while stimulation of the LHA drastically increased the consumed amount of meals [76-78] even in satiated animals [79]. Numerous peripheral stimuli, including retinal light/dark signals, gastric wall distension and motility, blood glucose concentration, etc., were shown to stimulate the satiety center in the VMH and inhibit the activity of the hunger center in the LH, or vice versa to maintain energetical balance [80-82]. This theory provided a clear and logical explanation for the regulation of appetite mechanisms.

But the more diverse data were collected, the more evident was that the regulation of feeding behavioral reactions is rather complex and multilevel [83, 84].

Next to this, a crucial role of monoamines in the control of hunger and satiety has been described. Normally, noradrenaline (NA) is the dominant monoamine in rats' and in the human hypothalamus [85, 86]. The major NA projections toward the hypothalamus arise from the lateral tegmental brainstem via the ventral NA bundle [87]. At the same time, the dorsal NA bundle originating from the locus ceruleus (LC) courses towards the hippocampus, cerebellar and cerebral cortex across the LHA [88, 89]. The PaVN, DMN, PeVN, and SON receive some direct LC projections as well [87, 90, 91]. Here NA regulates the feeding patterns in correlation to consumed meal size, food content, and energy value, dark/light phase, and production of other food-regulating neuropeptides [92-94].

The nigrostriatal DA pathway, which arises from SN and courses towards neostriatum (caudate, putamen) across the LHA and internal capsule, [90, 95-97] was shown to be involved in the regulation of feeding. Namely, its electrical or chemical (6-OHDA) lesions lead to the lateral hypothalamic syndrome, characterized by aphagia, adipsia, and motor dysfunction [98-100]. Another major brain DA circuitry arises from the VTA and explores its action towards the NAcc, or Amy and hippocampus via the mesolimbic pathway; or projects directly to the prefrontal cortex via the mesocortical pathway [101, 102]. The mesocorticolimbic DA system, implied in mechanisms of drug addiction, exerts direct connections with the LH. Thus, it may play a pivotal role in addiction to palatable foods, binge-eating, and obesity [103]. DA-containing cell bodies are found within the hypothalamus in the PeVN, Arc, and somewhat around the DMN and PoN [104-106]. Hypothalamic own native dopaminergic neuronal network consists of tuberoinfundibular (TI) and incertohypothalamic (IH) dopaminergic systems, periventricular–hypophysial (PHDA), and periventricular dopaminergic neurons [107]. It is hard to overestimate the importance of motivation and reward mechanisms in application to feeding and satiety regulation. Either the lesions of the feeding centers, or the damage of DA and NA pathways, both lead to severe changes in feeding behavior [108-110]. A body of evidence exists showing that DA release is regulated in part by serotonin activity [111-113].

It was shown that serotonin (5-HT) and the serotoninergic system exert an inhibitory effect on food intake [114, 115]. Serotonergic projections from the raphe nucll. and medial lemniscus of mesencephalon reach the hypothalamic Arc, PVN, and LHA [116]. Moreover, there are indications of the intrinsic 5-HT cells [117]. By interaction with AgRP/NPY and α-MSH, the 5-HT accelerates satiation and prolongs satiety. Numerous studies suggest that the interaction of DA and 5-HT within the LHA influences meal size [118, 119], while VMN is rather responsible for meal number [120-122]. At the same time, brainstem 5-HT structures, such as NTS and PBN,

tightly connected to the hypothalamus, represent an anatomical substrate for satiety regulation and integration of the peripheral signals (e.g. carbohydrates, leptin, CCK) (for review see [123, 124]).

In this matter, it is impossible not to mention such a phenomenon as a glucose-monitoring neuronal network. It is known that the LHA integrates via the NTS and PBN relevant metabolic data [125-130]. A specific population of neurons within the LH, as well as VMN and other involved brain areas (the orbitofrontal and cingulate cortex, NAcc, globus pallidus), i.e. so-called glucose-sensitive (GS) neurons, are responsible for analysis of glucose concentration, and contribute to the regulation of feeding behavior [131-136]. It is also suggested that DA, NA, opioids, encephalins, as well as taste and odor sensations, have a regulatory role on GS [137, 138].

Nevertheless, since the late 1980th/early 90th a new period was opened by the discovery of the neuropeptide Y and its involvement in the regulation of feeding [139, 140]. According to the present view, it is the Arc nucleus, situated right by the ME and leaky blood-brain barrier, which integrates peripheral and central signals to generate a coordinated feedback response. Leptin, produced by white adipose tissue, as well as gastric mucosa peptide, ghrelin, are the key substances providing the metabolic inputs to the Arc from the periphery. There are two distinct, functionally antagonistic types of neurons in the Arc: the orexigenic NPY and agouti-related peptide (AgRP)-expressing neurons on one side, and the anorexigenic pro-opiomelanocortin (POMC)-expressing neurons on the other. In response to food consumption, the POMC cells produce α-melanocyte-stimulating hormone (α-MSH) which activates melanocortin 3 and 4 receptors (MC3/4R) on second-order neurons in the PVN, DMH, VMH, LH [141, 142]. These second-order neurons further process the received information and project to multiple extrahypothalamic neurocircuits, leading to an integrated response on energy intake and expenditure. There are numerous downstream mediators likely to be involved in transducing the effects of MC4R activation on food intake, such as brain-derived neurotrophic factor (BDNF), CRH, TRH, some of the RFamide peptides. On the other hand, fasting induces activation of the AgRP/NPY neurons that have similar projections but exert opposite effects: NPY directly stimulates food intake via NPY Y1 and Y5 receptors, and reduces energy expenditure, while AgRP acts as an inverse agonist of MC3/4R. Furthermore, AgRP/NPY neurons directly inhibit POMC neurons by the means of γ-aminobutyric acid (GABA) signaling within the Arc, as well as in the parabrachial nucl. (PBN). The LH also has inhibitory projections towards the PVN, while the PVN and VMH send glutamatergic feedback to the Arc POMC and AgRP/NPY neurons. It is suggested that these state-dependent changes in NPY and POMC neuron activities are responsible for homeostatically appropriate changes in hunger and energy expenditure (for review see [143]).

1.1.5. The hypothalamus from the prospective of memory and learning

Since 1950th, beginning with K. Lashley and his monumental work "In search of the engram" [144], scientists keep searching for the physical trace of memory. Lesioning experiments on multiple brain areas resulted in a view that the "engram" is widely spread across the brain. Several structures have been recognized to play specific roles in complex processes of memory and learning.

The Amy has compiled in the emotional component of the memorization, fear memories [145, 146]. A neural circuit between the Amy and the VMH, along with its downstream effector, the dorsal periaqueductal grey (PAG), are essential for the acquisition and recall of predator fear memory [147]. Besides that, the hypothalamus as an integrator of stress signals is responsible for increased or decreased learning performances depending on the intensity and timing of stress factors [148]. The hippocampus is associated with declarative and recognition memory [149]. The cerebellum plays a role in neurocognitive development, language function, working memory, the processing procedural tasks [150], while the prefrontal cortex appears to be involved in remembering semantic tasks, decision making, recognition memory [151, 152]. These data point towards the limbic system and the hypothalamus as a coordinating center.

Nuclei of the PVH and MH synthesize numerous neuropeptides, which were recognized to regulate memory and learning next to other essential physiological functions. Alterations in CRH release along with the whole hypothalamic-pituitary-adrenal axis (HPA) have been noticed in such mental disorders as major depressive disorder, posttraumatic stress disorder, and borderline personality disorder, all characterized by memory disruptions (for review see [153]). Also, stress can disrupt memory and contribute to cognitive impairments in schizophrenia and attention deficit hyperactivity disorder [154]. CRH overexpression in transgenic mice may disrupt spatial memory [155], and central infusion of CRH in rats leads to attention disruption [156]. The circulating glucocorticoids counterbalance higher centers by negative feedback. It was shown that administration of glucocorticoids impairs working memory and long-term memory retrieval [157-159], while on the other hand, memory consolidation seems to be promoted [158, 160], simple emotional learning (fear conditioning), as well as habit learning, are enhanced [161, 162]. The TRH, whose receptors are highly expressed through the limbic system [163], is another hypothalamic candidate for the regulation of cognitive processes. It was shown to facilitate cholinergic and noradrenergic neurotransmission in animal and human experiments [164-168]. Recent studies revealed that TRH depresses glutamate responses in the hippocampal synapses by multiple mechanisms [169]. The TRH and TRH-R1 mRNA levels within the limbic structures,

including the hypothalamus, correlate with performance in MWM [170]. Acute treatment with GnRH in male rats affects extinction memory consolidation, perhaps through modification of neuronal activity within the infralimbic cortex and Amy [171]. At the same time, via regulation of the luteinizing hormone synthesis in the anterior pituitary, GnRH may interact with the hippocampus thus affecting spatial memory performance and playing a role in the pathophysiology of AD and other diseases with memory impairment [172, 173]. Recently, it has been shown that estrogens may rapidly promote social recognition through interaction with the oxytocin (Oxt) system in the hypothalamus and in the medial Amy (MeA) [174-176]. Indeed, the majority of the immunoreactive Oxt and vasopressin (VP) fibers were detected within the PaVN and SON of the hypothalamus. In addition, Oxt and VP synthesizing neurons were scattered in the preoptic area, AHA, DMN, stria terminalis, bed nucleus of the stria terminalis, and MeA [177, 178]. The role of Oxt in social and emotional behavior is well established [179-181]. In turn, data regarding the memory effects of the neuropeptides are contradictory. Based on the experiments with *Oxt−/−* mice, it appears that Oxt does not play a key role in spatial memory, as the results in Morris water maze, Y-maze, and T-maze tasks remained similar to wild type [182, 183]. Accordingly, chronic central administration of Oxt had no effect on performance in a radial maze task [184]. However, there is a body of evidence suggesting that under certain conditions, Oxt and VP may improve memory consolidation in regard to spatial and episodic memory [184-187], as well as in passive avoidance and taste aversion paradigms [188-191].

In addition to multiple neuropeptides of the MH, the laterohypothalamic MCH's role in memory processes was described. By the means of chemogenetics, it was suggested that activation or inhibition of hypothalamic MCH neurons during the rapid eye movements (REM) phase of sleep affected hippocampus-dependent memory, thus being involved in active forgetting [192]. Kosse and Burdakov [193] identified an upstream inhibitory microcircuit from hypothalamic GAD65 neurons to MCH neurons, which constrained the memory-promoting MCH cell bursts, and demonstrated that object recognition through MCH receptor-dependent pathways can be improved by silencing GAD65 cells. Tyner et al. [194] proposed that disturbances in the hypothalamus-pituitary-adrenal (HPA) axis caused hypertension, and secondarily also may modulate cognitive functions.

These complex interconnections are hard to imagine without the regulatory role of monoamines [195-198]. Generally, the NE is associated with cognitive processes, such as memory, learning, and selective attention [199, 200]. In particular, it was suggested that NE enhances the firing of neurons that registered salient information and decreasing spontaneous firing in response to irrelevant events, thus contributing to selective attention and working memory capacity [201-203]. NE also activates both pre- and post-synaptic adrenergic receptors at central synapses with

different functional outcomes. Via the Amy, NE accelerates memorization of emotionally significant experiences, including conditioned fear memory (for reviews see [204-206]). It was shown that β-adrenergic receptors play role in membrane activation of hippocampal neurons and participate in contextual fear memory retrieval [207]. The NA next to DA seems to affect episodic memory as well. A putative arousal-induced memory-boosting effect was noticed to be sensitive to NA β -adrenergic receptors, while memory selectivity was affected by D2/D3 receptor antagonists [208]. Both NA and DA play important roles in reward and salience recognition. The DA neurons specifically are known for their indispensable role in reward-supported learning as error predictors (for review see [209]). Nevertheless, animal and human researches discovered the role of DA in behavioral vigor and effort [210-213], response for novelty, and surprising events [214-216]. Thus, it seems that DA neurons provide multiple mechanisms for signaling the motivational significance and forwarding them to target regions to learn and obtain goals. Serotonin, as well as its transporter SERT and multiple receptors, have been identified in the brain regions involved in memory [217-222]. It was shown that several 5-HT receptor subtypes in the hippocampus express modifications following maze swimming and passive avoidance tasks [223]. 5-HT 1a receptors are responsible for performance in a novel object pattern separation task (relative to episodic memory) [224]. The memory formation during the water maze and autoshaping tasks affects the 5-HT1a receptor expression in more than 20 brain areas [217]. Central administration of 5-HT2a receptor agonist accelerated working memory [225]. Tomie et al. [226] demonstrated an association between 5-HT2a receptor expression and memory formation in the Pavlovian autoshaping task. While stimulation of serotonergic axons in CA1 was shown to improve spatial memory by means of 5-HT4 receptors [227]. These and multiple other examples (for review see [198]) suggest a pivotal role of serotonin in memorization processes. Moreover, next to 5-HT/DA interaction, there is growing evidence regarding the role of 5-HT and acetylcholinergic systems common dysfunction in age-related cognitive deficits [228-232], GABA, and glutamate [233-235].

1.1.6. The hypothalamus and anxiety

Currently, anxiety disorders contribute by 7,3% prevalence to all mental disorders worldwide [236]. The hypothalamus, as anatomical coordinator of fear and stress regulation networks, must be strongly involved. Traditionally, fear and anxiety are linked to the activity of the Amy region [237]. For example, it was shown that fear and reward are encoded by phasic activation of distinct neuron populations in BLA, while anxiety results in persistent BLA activity [238]. The Amy presents massive projections towards the hypothalamus mainly through the fornix and stria terminalis. Besides the Amy, anxiety cells were reported within a hippocampal-

hypothalamic circuit. CA1 subregion turned to be enriched not only in place cells, but also in anxiety cells, that were activated by anxiogenic behaviors, and projecting directly to the LHA [239]. Also, earlier studies suggest that while lesions of the dorsal hippocampus affect spatial learning, ventral hippocampal lesions rather lead to anxiolytic behavior [240, 241]. The anterior cingulate cortex, lateral prefrontal cortex, and insula are also important contributors to the fear regulating network [242, 243]. Even though not a part of the "classical" limbic system, these cortical areas proceed highly important input to hypothalamic integration of autonomic, behavioral, and emotional information [244, 245].

Multiple neurotransmitters were implicated in the complex regulation of anxiety. First of all, the hormones of HPA are the important markers of anxiety disturbances [246]. Next to this, the hypothalamic-pituitary-thyroid cascade affects behavioral reactions, and its disbalance may lead to panic attacks [247]. The Oxt, known for its pivotal role in sexual and maternal behavior, is also implicated in trust, anxiety, and sociability [248, 249]. The NPY, which primarily regulates feeding and metabolism, has stress-relieving, anxiolytic and neuroprotective properties [250]. Naturally, global regulatory systems including 5-HT, DA, NE, GABA were studied in multiple paradigms and confirmed their effectiveness as pharmacological treatment of anxiety disorders (for review see [251]).

1.2. Neuropeptide QRFP

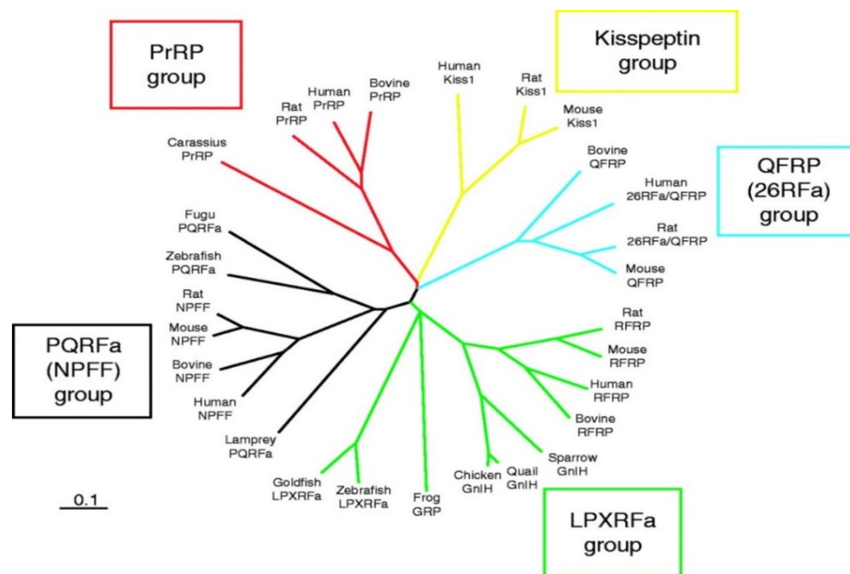
1.2.1. Family of the RFamide peptides

The RFamide peptides represent one of the largest and most widespread families of biologically active peptides characterized by carboxy-terminal arginine (R) and amidated phenylalanine (F) residues (hence RFamide). Since the discovery of the tetrapeptide FMRFamide from the venus clam [252], a number of peptides sharing RF motif at their C-terminal end have been described in all major phyla [253-258]. To date, five genes (farp-1 to 5) encoding five groups of RFamide peptides have been described in vertebrates [257, 259]. These include the prolactin-releasing peptide (PrRP) group, the group of neuropeptide FF (NPFF, PQRFa, NPAF), RFamide-related peptides (RFRPs (also termed NPSF and NPVF), LPXRFamides, GnIH), kisspeptin (metastatin) group, and pyroglutamylated RFamide peptide (QRFP) group.

Neuropeptide QRFP was identified and described simultaneously by three independent teams [17, 260, 261]. The cDNAs encoding the 26RFa/QRFP precursors have been characterized in various species belonging to diverse vertebrate phyla [262]. In mammals, the QRFP sequence is generally flanked at its N-terminus by a single Arg residue [17, 260, 261].

The mature RFamide peptide consisting of 43 amino acids (43RFa, QRFP-43) was identified from the rat hypothalamus and from the culture medium of CHO cells which express the human peptide precursor [18, 255]. Concurrently, a bioinformatic search has led to the identification of the 26-aminoacid residue (QRFP-26, also referred to as 26RFa or P518) gene [17, 260, 261]. Both forms exert similar physiological effects, even though some studies suggest an elongated form of the peptide to be more potent [18, 263].

Fig. 4. Family of the RFamide peptides



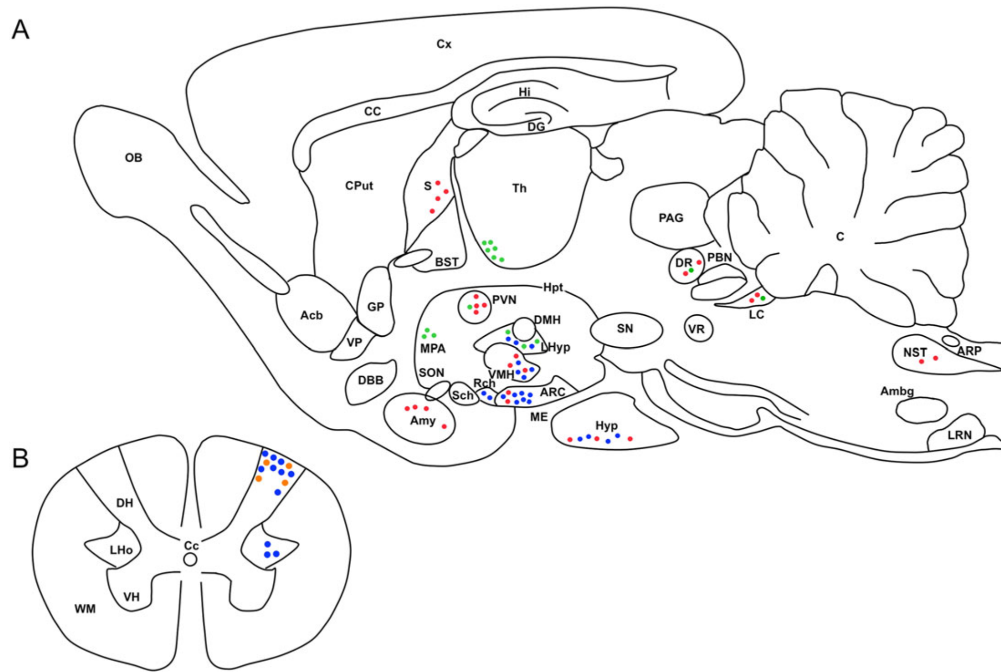
Reprinted from [15]

1.2.2. QRFP tissue distribution

In rodents, the QRFP gene is highly expressed over the body: in the CNS, eye, trachea, mammary gland, and testis. Moderate expression was found in the thymus, salivary gland, duodenum, pancreas, uterus, and adrenal gland [18, 260, 261, 264]. In humans, the QRFP gene was detected rather in endocrine glands. Especial interest is drawn to the endocrine islets of the pancreas [265, 266] and the adrenal cortex [261, 264] due to their clinical importance.

The brain, notably the diencephalon, showed the highest concentration of QRFP transcript in rodents and humans [18, 260, 261, 267]. QRFP-expressing neurons are almost exclusively localized in the hypothalamus, specifically in the VMN, DMN, Arc, PeVN, PVN along the third ventricle, the LHA, and the RCh area [17, 259, 267]. Besides that, QRFP mRNA was detected in the human spinal cord, in the dorsal and somewhat lateral horns [255].

Fig.5. Distribution of the neuropeptide QRFP and receptors in the central nervous system



A: Parasagittal section of the rat brain and pituitary depicting the localization QRFP (●), QRFP receptor 1 (●) and QRFP receptor 2 (●) mRNAs.

B: Coronal section of the human spinal cord depicting the localization of QRFP-like immunoreactivity and/or QRFP mRNA (●), QRFP receptor mRNA and/or binding sites (●). Reprinted from [268]

1.2.3. QRFP receptors

QRFPs have been suggested as the endogenous ligands of the previously orphan G protein-coupled receptor GPR103 (also referred to as AQ27 or SP9155) [260, 261, 269]. While humans possess only one QRFP (GPR103) receptor isoform, two distinct homologs were identified in mouse and rat genomes (termed GPR103 a and b, or QRFPR1 and 2, respectively) [18, 267]. Rat QRFPR1 shares 96 and 84% amino acid identity with the mouse and human homologs, respectively. Rat QRFPR2 shares 82 and 78% amino acid identity with human QRFP receptor and rat QRFPR1, respectively, and also 78% amino acid identity with rat QRFPR1. Moreover, QRFP receptors have been revealed to share sequence identities with receptors of the various closely related neuropeptides: NPFFR1 (49% amino acid identity), NPFFR2 (48%), orexin OX R1 (48%), OX R2 (47%), somewhat NPY2, galanin GalR1, cholecystokinin CCKa, and CCKb [260, 269, 270].

In rats, the QRFPR1 gene is intensely expressed in the adrenal gland and, somewhat, in the eye, kidney, and testis [261]. In humans, the highest level of QRFP receptor mRNA is found in fetal bone [271]. The QRFP receptor gene is also expressed in the heart, thyroid, and parathyroid glands, kidney, prostate, and testis [260, 271], as well as in the pituitary [269]. In human osteoblasts

culture, dexamethasone induces a concentration-dependent decrease of the expression levels of QRFP receptors [271]. QRFP receptor mRNAs were not initially detected in rat and mouse pancreas [260]. However, RT-PCR analyses and immunohistochemical studies revealed that QRFP receptor mRNA is expressed in cultured rat INS-1E beta cells, as well as in cultured human pancreatic islets [265]. In the human adrenal gland, the QRFP receptor is exclusively expressed during embryogenesis in the fetal zone but not in the zona glomerulosa of adrenal medulla, whereas in the adults QRFP receptor mRNA is present in all three zones of the cortex. As for the rat adrenal gland, QRFP receptor mRNA in the medulla is absent [264].

In the human brain, the QRFP receptor is primarily expressed in the cerebral cortex, the hypothalamus, the thalamus, the vestibular nucleus, and the trigeminal ganglion [260, 269]. The moderate expression also occurs in the Amy, the caudate nucleus, the hippocampus, and the VTA [260]. Studies regarding QRFP receptors mRNA expression in rodents suggest a broad receptor distribution within the CNS. The highest concentration of QRFPR1 mRNA in rats was observed in the olfactory bulb, piriform cortex, retrosplenial, entorhinal cortex, Amy complex, hippocampal area with an especially high concentration in presubiculum, some thalamic nuclei, ventral pallidum, ZI, hypothalamic nuclei, namely the MPON, RCh, VMN, LHA, anterior hypothalamic area (AHA), DMN, PaVN, Arc and posterior hypothalamic area (PHA); LC, raphe nuclei, NTS, the superior and inferior colliculus and the vestibular nucleus and the dorsal horn of the spinal cord [259, 267, 272]. Interestingly, the distribution of QRFPR2 mRNA does not match that of QRFPR1 mRNA. Thus, the highest density of QRFPR2 in the rat brain is observed in the medial part of the MPON, the AHA, the reuniens and parafascicular thalamic nuclei, the lateral paragigantocellular nucleus, the facial and the hypoglossal nuclei ([267]; Fig. 8A).

Consistent with these data, Bruzzone et al. [272] reported that QRFP binding sites in the rat's CNS have a much wider distribution than areas of QRFP receptor mRNA expression. Such findings suggest that the neuropeptide QRFP might be involved in activation of other than QRFP receptors thus inducing multiple pathways of action.

The QRFP receptors exert their signaling via heterotrimeric guanine nucleotide regulatory proteins (G proteins). It was shown in the experiments on cultured rat anterior pituitary cells preincubated with forskolin that QRFP provokes a dose-dependent increase in cAMP production, suggesting that the QRFPR is primarily coupled to adenylyl cyclase (AC) through a stimulatory Gα subunit (Gαs) [17]. This proposal has been confirmed in INS-1E beta cells, in human islet cells [265], and H295R adrenocortical cells [264]. The QRFPR is also coupled to Gαq/11, leading to activation of the mitogen-activated protein kinases (MAPK) pathways. In particular, in H295R cells, QRFP causes calcium influx via mibeframil-sensitive T-type voltage-operated Ca2+ channels, leading to PKC activation and, subsequently, to phosphorylation of ERKs ½ [264]. In INS-1E beta cells, QRFP also stimulates phosphorylation of ERK 1/2 [265]. In the

QRFP-orexin (OX) receptor functional heterodimer, QRFP, like orexin-A or orexin-B, induces ERK 1/2 phosphorylation [273]. It thus appears that QRFP receptors, like most GPCRs, displays multiple signaling pathways resulting from multiple G protein couplings (for review, see [274]) that might account for the versatile activities of QRFP (general review by [268]).

1.2.4. QRFP receptor antagonists

Since the discovery of QRFP neuropeptide and the corresponding GPCR receptor in 2001, the synthesis of the potent antagonist was in high priority. Nevertheless, it took almost a decade until the first successful attempts in this field have been published.

Numerous indole derivatives [275, 276] have been suggested to inhibit [125I–Tyr32]QRFP binding to QRFP receptors. Unfortunately, most of them were not suitable for application in experimental work and drug development due to disadvantageous physiological effects and difficulties in solubility. Later on, the compounds with indole replaced by pyrrolo[2,3-c]pyridines as low MW antagonists of QRFP receptors have been developed [277, 278]. These antagonists seem to mimic the C terminal Arg25–Phe26 residues of QRFP, and revealed good results in preclinical tests: in a 3 day automated food intake measurement study, compound provoked a significant and dose-dependent reduction in food intake compared to vehicle-treated animals [277]. Another potential human QRFP receptor antagonists are presented by 2-aryl-imidazoline derivatives [279]. For the sake of an expanded field of view, a thorough screening of the in-house library has been performed by Nordqvist et al. [280], which led to the discovery of carboximidamide derivatives as another promising direction toward antagonist search.

Nevertheless, by the time of our experimental work, none of the specific rat QRFP receptor antagonists have been freely available on the market. For this reason, we have applied a non-peptide receptor antagonist BIBP3226 ((R)-N2-(diphenylacetyl)-N-[(4-hydroxyphenyl)-methyl]-argininamide), which previously had already been confirmed to prevent the orexigenic activity of the QRFP [18].

1.2.5. Role of QRFP and RFamide peptides in feeding and metabolic homeostasis

Members of the RFamide peptide family reveal a remarkable diversity in the N-terminal sequence, which probably determines a wide range of biological activities. These peptides are involved in the regulation of multiple functions such as control of locomotor activity, pain transmission, cardiovascular function, stress responses, regulation of sexual function, maintenance of water balance [259, 281-283], and not least of all in the regulation of feeding (for reviews see [15, 284]).

Experiments on rodents have shown that some of the RFamide peptides, such as NPFF [285, 286], PrRP [287, 288], RFRP-1 [289], and kisspeptin [290, 291] demonstrate central anorexigenic effects, while RFRP-3 is rather considered to enhance feeding [292-295].

QRFPs are thought to be involved in the regulation of feeding behavior as well. Acute i.c.v. administration of QRF peptides dose-dependently increased food consumption in mice [17, 18, 263, 296], in rats [297], and in birds [298]. But some of the previous studies indicated unsuccessful attempts to detect QRFP-induced effects on feeding behavior [267, 299]. Another approach involving macronutrient selection criterion revealed an attenuating effect of a fat-rich diet. Chronic injections of QRFP-43 in mice induced hyperphagic behavior associated with a significant increase in body weight and fat mass with much more pronounced effects when offered a moderately fat diet [296]. In agreement with the previous results acute i.c.v. injections of both QRFP-43 and QRFP-26 in rats led to significant augmentation of high-fat food consumption, while lack of appetite-modifying effects was observed when food with low-fat content was introduced [14, 300]. Consistent with these observations, prepro-QRFP-26 mRNA levels were found to be up-regulated in genetically obese ob/ob and db/db mice [18]. Also, QRFP-43 treated mice exhibited high plasma glucose, insulin, cholesterol, and liver triglyceride suggesting an obese phenotype [296].

QRFP mRNA and corresponding receptors have been identified in multiple peripheral tissues involved in the regulation of metabolism. In adipocytes, QRFP was shown to induce triglyceride and free fatty acid accumulation, to activate lipid-uptake responsible genes, and to inhibit isoproterenol (ISO)-induced lipolysis in a dose-dependent manner [301]. The same study showed on the mouse model that diet-induced obesity leads to increased expression of QRFPRb and decreased levels of QRFP in fat depots. Consistently, a clinical study proposed an adaptive role of QRFP in malnutritive states based on higher plasma levels detected in anorectic patients [302]. Prepro-QRFP mRNA and QRFPRa mRNA were detected in another major contributor to metabolism, in skeletal muscle. QRFP-26 (but not QRFP-43) enhanced the effects of insulin on glucose uptake and glycogenesis in L6 cells [303]. Also dietary fat may modulate prepro-QRFP mRNA and QRFPRa mRNA expression in several rodent models (unpublished observations mentioned by [303]).

In addition to fat and skeletal tissues, QRFP performs regulatory metabolic action within the pancreas. Expression of QRFP gene and receptors was detected within mouse MIN6 beta cells, rat INS-1E beta cells, and human pancreatic islets [265, 304]. Infusion of the QRFP in the perfused rat pancreas inhibited the insulin responses to glucose, arginine, and exendin-4 without affecting basal insulin output. At the same time, the neuropeptide did not modify the basal or arginine-induced glucagon output [305]. Interestingly, the subsequent studies revealed the diverse role of

QRFPs on insulin secretion, suggesting insulinostatic action of QRFP-26 and insulinotropic effect of QRFP-43. Moreover, only the elongated form of the neuropeptide exerted its action via specific QRFPR [265]. Further reports suggest the ability of QRFP to promote insulin secretion in response to low glucose levels via QRFP receptor-mediated mechanisms. In mice, QRFP attenuated hyperglycemia induced by a glucose load, potentiated insulin sensitivity, and increases plasma insulin concentrations [304]. The same research group also reported the abundance of QRFP and receptors all along the GIT with high expression in gastric glands, duodenal, ileal, and colonal enterocytes and goblet cells, indicating role of QRFP as an incretin hormone [306]. Consistently, the glucose load induced a massive secretion of QRFP-26 by the small intestine in mice, both in vivo and in vitro.

This way, there is collected a body of evidence suggesting the strong involvement of neuropeptide QRFP in the regulation of feeding behavior and metabolic homeostasis via central and peripheral mechanisms. The majority of findings (weight gain, the increase of appetite, promotion of fat storage, and glycogenesis) indicate rather anabolic effects of QRFP. Nevertheless, plenty of blind spots in this area require further clearance.

1.2.6. Role of QRFP and RFamide peptides in cognitive functions

Scientific data suggest an involvement of the RFamides in the regulation of higher brain functions. Significant amounts of immunoreactive PrRP, kisspeptin, NPFF, NPAF, and NPSF fibers were detected in the hypothalamic nuclei of different phyla. Data originating from behavioral experiments also confirm the assumption that members of the RFamide peptide family might be involved in the regulation of cognitive functions. NPFF was shown to modify short- and long-term memory depending on dose and paradigm [307, 308]. Kisspeptin improved memory formation and revealed neuroprotective activity in the passive avoidance paradigm, as well as novel object recognition and object location tasks [309, 310]. Similarly, NPAF and RFRP-1 peptides enhance learning and memory in aversive situations [311, 312].

For QRFP, the hypothalamus is the major synthetizing area within the CNS. Prepro-QRFP mRNA in the rat CNS is mostly concentrated in the medial hypothalamic nuclei (VMN, DMN, Arc) and the LHA. But not much is known regarding the effects of QRFP on cognitive functions. Some results indicate the possible role of QRFP signaling in the regulation of sleep in fish and hypothalamic sleep control [313]. Also, there is one report suggesting the involvement of QRFP in AD. There was registered a down-regulation of QRFP and orexin receptors in the hippocampal cells of AD patients, and the neuroprotective role of both QRFP and orexins [273].

Interestingly, the effects of glucose- and fat metabolic disorders on cognitive decline have been noticed already more than two decades ago [314, 315], but the underlying mechanisms remain barely understood. It was proposed that anorectic adipokine leptin might be involved in such phenomenon, as leptin resistance occurs not only in obesity but also in AD and in normal ageing [316, 317]. Leptin was proven to induce plays neuroprotective effect [318, 319] and to enhance spatial memory and learning [320, 321]. Also, the correlation between QRFP concentration and plasma leptin was observed [296, 322]. Thus, it is tempting to speculate that QRFP may be one of the molecular links between metabolic state and cognition.

To our best knowledge, by today no specific research regarding the role of neuropeptide QRFP in memory and learning processes was conducted. Taking into consideration the rich connection network of the hypothalamic synthesizing areas, wide QRFP receptors distribution within the CNS, and confirmed role in cognitive processes of cousin neuropeptides, the investigation of QRFP's role in cognition seems to be a propitious research area.

1.2.7. Role of QRFP and RFamide peptides in anxiety

The members of the RFamide peptide family are known to regulate multiple vital systems. Their role in defending mechanisms of fear and anxiety was proposed as well.

Hypothalamic neuropeptide RFRP-3 may promote stress response [323, 324]. One of the possible mechanisms is the activation of the HPA axis [325, 326]. The RFRPs have even been proposed as stress-induced infertility reasons. Another possibility might be an oxytocin pathway, as central administration of RFRPs induced anxiety-related behavior in rats in open-field tests via activation of Oxy neurons [324]. Consistently, restraint and foot-shock stress stimuli were shown to up-regulate RFRP neurons [324, 327, 328]. Actions of cousin neuropeptide kisspeptin remain contradictory. Several behavioral studies revealed that central administration of kisspeptin induces anxiety via GABAergic transmission and activation of the HPA axis [329-332]. While other findings suggest anxiolytic and antidepressant-like effects of kisspeptin. The interactions with adrenergic and serotoninergic pathways are suggested in this matter [333, 334]. The NPFF and corresponding NPFF2 receptor activation lead to anxiogenic effects. Similarly, multiple mechanisms participate in this action, including the functioning of the HPA axis, GABA signaling, and opioid system [335-337].

QRFP knockout mice, next to changes in feeding, revealed anxiety-like behavior [338]. In agreement with these data, another detailed study established anxiolytic effect of GPR103 (QRFP) receptor activation in mice, and involvement of GABAergic and adrenergic neurotransmission was suggested [339].

2. Objectives

Considering multiple physiological functions of QRFP and involvement of cousin RF peptides in homeostatic and behavioral processes, with the knowledge of binding sites within the CNS, the present study was designed to investigate the possible role of QRFP in feeding behavior, motivation and rewarding mechanisms, processes of learning and memory consolidation. Due to the contradictory data regarding the effects of QRFP on locomotor activity and anxiety level, these parameters have been considered in the present study as well. Herein we have employed a unique experimental design with direct peptide microinjection into the brain parenchyma. The rat medial hypothalamic area (including closely situated VMN and DMN), as well as LH, -the areas with QRFP-synthesizing neurons and binding sites presented in high density, have been chosen for treatment.

To illuminate the designated aspects of QRFP activity, the following experiments have been executed:

1. One of the major purposes of the research was to investigate the possible effects of direct intrahypothalamic administration of QRFP on feeding. Measurement of liquid food intake was chosen as the most appropriate and advantageous (comparing to the dry chow) method. It allowed us a frequent a precise monitoring of milk consumption without disturbing the animals.
2. In the case of modulation of feeding behavior, it is reasonable to investigate the possible rewarding/aversive effect of QRFP. The conditioned place preference test (CPP) was employed to answer this question.
3. A further aim was to investigate an unexplored but promising aspect of QRFP activity – the peptide's effects on memory and learning. Well acknowledged paradigm, the Morris water maze (MWM), was applied to shed light on this topic.
4. It was important to clarify whether QRFP affects general locomotor activity since this parameter could shade the results of other experiments. For this purpose, the open field test (OFT) was employed. Next to that, specific parameters from the EPM, CPP, and MWM were analyzed.
5. Another strategically important goal was to examine whether neuropeptide QRFP alters the anxiety level. The elevated plus maze (EPM) test was recalled answering this question. Specific parameters collected during the OFT and MWM were analyzed as well.
6. In the case of modifications in any of the abovementioned aspects, it was vitally important to determine whether the corresponding receptor system is involved in the observed effects. Examination of non-peptide antagonist BIBP3226 pre-treatment served this purpose.

3. Materials and methods

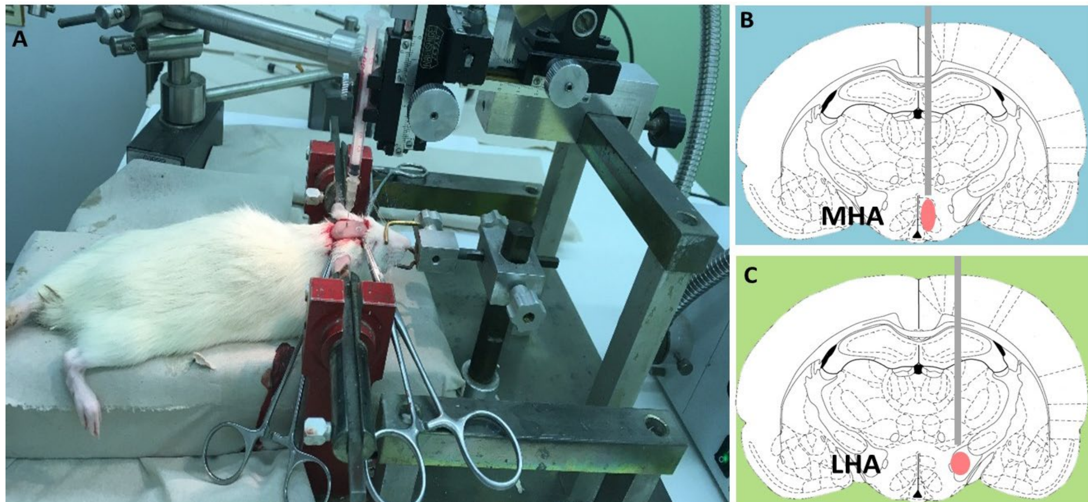
3.1. Subjects

In the present study, 398 adult male Wistar rats (LATI, Gödöllő, Hungary) were used weighing 270–320 g at the beginning of experiments. Animals were housed individually in a temperature- and light-controlled room (22 ± 2◦C, 12-12 h light-dark cycle with lights on at 06:00 a.m.). Rats were cared for in accordance with institutional (Pécs University Medical School, BA02/2000-8/2012), national (Hungarian Government Decree, 40/2013 (II.14.)) and international standards (European Community Council Directive, 86/609/EEC, 1986, 2010). In behavioral experiments tap water and standard laboratory food chow (CRLT/N standard rodent food pellet, Charles River Laboratories, Budapest, Hungary) were available *ad libitum*. In feeding experiments water and food pellets were available *ad libitum* before and after the experimental measurements. The body weight, food, and water consumption were measured daily to the nearest grams and milliliters, respectively. All the tests were performed during the rats' daylight period between 08:00 and 14:00 h.

3.2. Stereotaxic surgeries

Rats were anaesthetized i.p. with ketamine supplemented with diazepam (Calypsol, 80 mg/kg bw and Seduxen, 20 mg/kg bw; Richter Gedeon Ltd., Hungary). Stainless steel guide tubes (22-gauge) were implanted into the MHA of the right hemisphere (coordinates referring to the *bregma*: AP: -2.8 mm, ML: 0.6 mm and DV: 7.0-8.5 mm ventral from the surface of the *dura mater*) or LHA (AP: -2.8 mm, ML: 1.3 mm, and DV: 7.5-8.3 mm) according to the stereotaxic rat brain atlas of Paxinos and Watson [340]. The tips of the cannulae were positioned 0.5 mm above the intended injection site. The cannulae were fixed to the skull with acrylic cement (Duracryl) and stainless-steel screws (so-called „crown"). When not used for injection, the guide tubes were occluded with stainless steel obturators (27-gauge). During the operations, animals received antibiotic prophylaxis (G-penicillin). Following surgery, animals could have a minimum of 6 days for postoperative recovery, during that time they were frequently handled. Before the testing began, each animal underwent a general (preoperative values of body weight, physiological skin and fur condition), as well as neurological examination (intact sensory and motor functions).

Fig. 6. Stereotaxic technique employed for targeting the brain areas in rats



A: Picture of rat fixed in the stereotaxic apparatus, final stage of the operation.

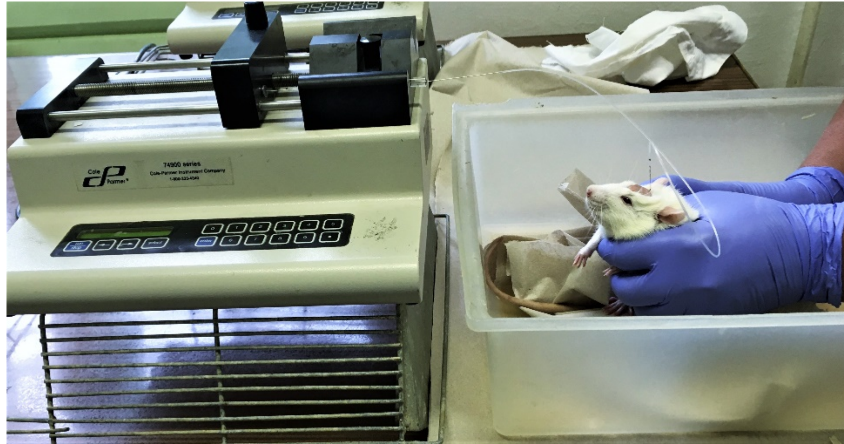
B, C: Schematic illustration of cannulae placement and size of microinjections in the medial (MHA), and in lateral hypothalamic area (LHA), respectively, based on stereotaxic rat brain atlas of Paxinos and Watson.

3.3. Drug injections

During the experiments rat 26-amino acid residue of rat QRFP (048-72, Phoenix Pharmaceuticals Inc., USA) in 100 ng, 200 ng, or 400 ng (35, 70, 140 pM, respectively) doses, and receptor antagonist BIBP3226 (B174, Sigma-Aldrich Kft., Hungary) in 18 ng, 35 ng and 70 ng (38, 74 and 148 pM, respectively) doses were employed (further in the text referred as QRFP and Ant, respectively). The concentrations of peptide microinjections have been determined based on our previous studies with cousin peptides [311, 341, 342] and according to the findings in pilot experiments; while concentrations for Ant treatment have been determined as equimolar to the effective QRFP dose. The drugs were dissolved in 0.15 M sterile saline for intrahypothalamic microinjections in a volume of 0.4 µl. For control measurements, animals received the same volume of vehicle solution (Control 1, 0.15 M sterile saline).

When studying the effects of antagonist, the experimental procedure implicated double injection volume (0.4 µl + 0.4 µl) to each animal with 15 min interval. For control values, rats were treated with the aforementioned vehicle solution (Control 2, vehicle + vehicle). The second group received double volume QRFP treatment: an effective dose of peptide and vehicle injection (vehicle + QRFP). Two other animal groups received Ant treatment. In the third group BIBP3226 was applied prior to QRFP (Ant + QRFP), while BIBP3226 administration followed by vehicle injection (Ant + vehicle) was performed in the last group.

Fig. 7. Microinjections of drugs using a Hamilton microsyringe



During the microinjections, awake, well-handled rats were gently held by hand. Before the experiment, the obturators were removed from the guide tubes. All substances were injected through stainless steel injection tubes (27-gauge) extending 0.5 mm below the tips of the implanted guiding cannulae. The injection cannula was attached via polyethylene tubing (PE-10) to a Hamilton microsyringe (10 µl, Bonaduz, Switzerland). Drugs were injected during 60 sec by automated syringe pumps (Cole Parmer, USA), and the injection cannula was left in place for an additional 60 sec to avoid the backflow and to allow diffusion into surrounding tissues. After that the obturators were replaced.

In the case of feeding experiments, drugs and vehicle injections were separated by at least a 3-day period to prevent cumulative effects. Solutions were applied in a counterbalanced manner, i.e., applications randomly started with vehicle or drugs within groups. Also, important to note, that pursuant to ethical principles in biological research we tended to reduce the involvement of animals. Rats who participated in the EPM test received only one microinjection, this way they were employed in other experiments as well.

The treatments (and the animal number) applied in the different experiments are summarized in the Table 2.

Table 2. Summary of injected drugs and animal numbers in all experiments

Paradigm	MHA	LHA
Feeding experiments	Control 1/100 ng QRFP (n=11) Control 1/200 ng QRFP (n=11)	Control 1/100 ng QRFP (n=11) Control 1/200 ng QRFP (n=9)
	Control 2 / veh + 100 ng QRFP (n=12) Control 2 / Ant + QRFP (n=9) Control 2 / Ant + veh (n=12)	Control 2 / veh + 100 ng QRFP (n=10) Control 2 / Ant + QRFP (n=7) Control 2 / Ant + veh (n=8)
CPP	Control 1 (n=7) 200 ng QRFP (n=6) 400 ng QRFP (n=6)	Control 1 (n=6) 200 ng QRFP (n=6) 400 ng QRFP (n=7)
	Control 2 (n=6) veh + 400 ng QRFP (n=6) Ant + QRFP (n=6) Ant + veh (n=5)	Control 2 (n=7) veh + 400 ng QRFP (n=8) Ant + QRFP (n=8) Ant + veh (n=8)
MWM	Control 1 (n=8) 200 ng QRFP (n=9) 400 ng QRFP (n=7)	Control 1 (n=7) 200 ng QRFP (n=7) 400 ng QRFP (n=8)
	Control 2 (n=9) veh + 400 ng QRFP (n=8) Ant + QRFP (n=9) Ant + veh (n=8)	Control 2 (n=6) veh + 400 ng QRFP (n=7) Ant + QRFP (n=8) Ant + veh (n=8)
EPM	Control 1 (n=8) 200 ng QRFP (n=10) 400 ng QRFP (n=7)	Control 1 (n=7) 200 ng QRFP (n=8) 400 ng QRFP (n=7)
	Control 2 (n=6) veh + 400 ng QRFP (n=6) Ant + QRFP (n=7) Ant + veh (n=8)	Control 2 (n=6) veh + 400 ng QRFP (n=7) Ant + QRFP (n=7) Ant + veh (n=8)
OFT	Control 1 (n=8) 200 ng QRFP (n=8) 400 ng QRFP (n=7) Ant (n=8)	Control 1 (n=6) 200 ng QRFP (n=7) 400 ng QRFP (n=7) Ant (n=6)

3.4. Liquid food intake measurements

For the measurements of food consumption, liquid food (milk) was used. Previously it has been shown in numerous experiments that the liquid food paradigm has several advantages against the standard chow measurements [341, 343-347].

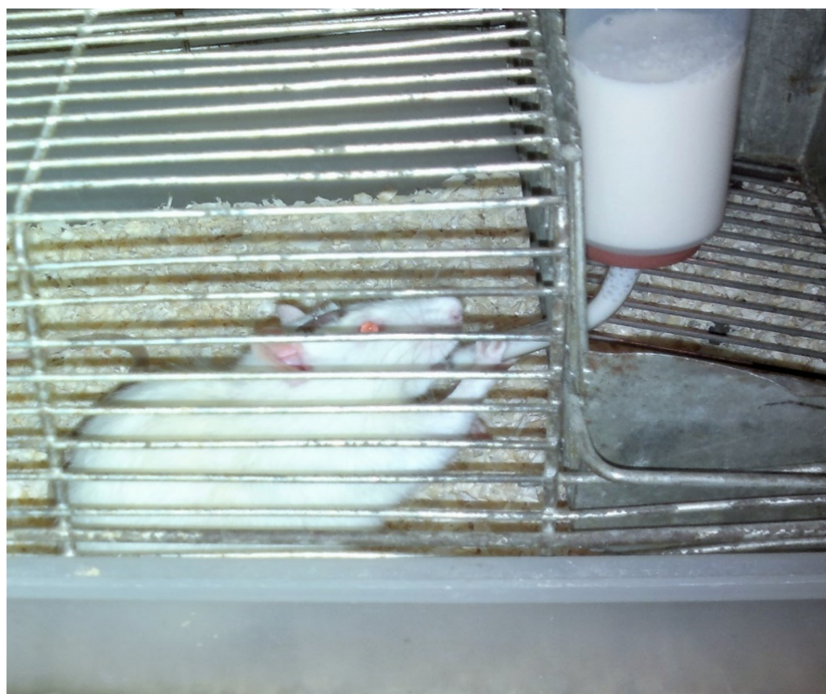
To evaluate changes in liquid food amount, there is no need to remove the food from the cage; all the measurements can be performed in situ. This way it allows frequent and precise monitoring of food consumption without interrupting physiological eating act. Moreover, there is no time wasted for removing and measuring the food on the weights, which could shade the results. Finally, in this paradigm, all the experimental animals can be provided with food of the same taste and energy value.

To overcome neophobia and to accustom rats to the palatable complex food, one week prior to the operation animals were trained to consume the liquid diet. Liquid food with normal fat content (3%) was introduced to animals (Milk, Isosource Standard Natur, Nestle). Graduated drinking cylinders with 1.0 ml divisions fitted with a glass sipper spout attached to a permanent point at the front of each home cage were used for measuring milk ingestion. Milk was available for three hours between 08:00 a.m. and 11:00 a.m., in the remaining time water and standard laboratory food pellets were available *ad libitum*. This feeding schedule was maintained until the end of the experiments. Rats, whose liquid food intake did not show a stable baseline during habituation, were excluded from any experiments.

One-hour prior to the drug administrations, food pellets and water have been removed from the rats. Following the microinjections liquid food intake was measured at milliliters accuracy every 5 min for the first half-an-hour and every 10 min for the following half-an-hour, so the 60-minutes measurement data are presented [342, 348].

The body weight was monitored daily, starting from the day of surgery until the end of the experiment. In these experiments animals served to their own control, i.e. food consumption of the same rat was compared after either vehicle or drug (one dose of QRFP or Ant) administration.

Fig. 8. Measurement of liquid food intake



3.5. Conditioned place preference test (CPP)

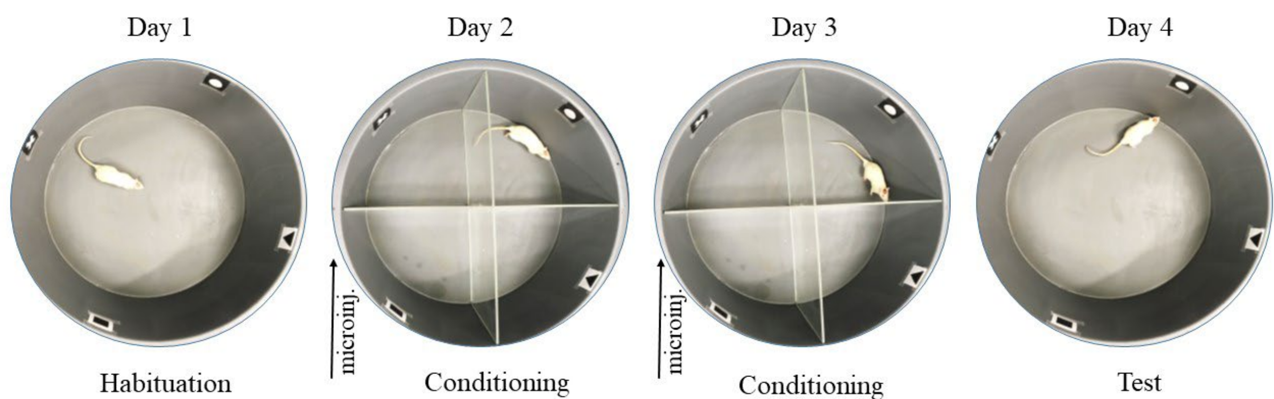
The CPP test was employed to test the rewarding, positive reinforcing, or aversive effects of the drugs [349, 350]. The CPP apparatus consisted of a circular open field (85 cm diameter, 40 cm height). The walls and the floor of the apparatus were made of grey-colored plastic. The floor was divided by thin black lines into four quadrants, which could be separated from each other by removable plexiglass barriers during conditioning. Visual cues in the surroundings assisted to distinguish the quadrants and helped the spatial orientation of animals within the apparatus [349]. The apparatus was provided with homogenous illumination by a 40 W bulb and performance in the field was recorded by a video camera. The arena was cleaned and deodorized with acetic acid after each animal. The animals' performance in the CPP test, as well as other behavioral examinations described further, was recorded by a video camera and registered by special software (EthoVision; Noldus Information Technology, The Netherlands).

The place preference procedure was performed for four days: habituation (1st day), two days of conditioning (2nd, 3rd days), and one test (4th day) trial. Each lasted for 900 sec (15 min). On the first day (Habituation), animals were placed into the apparatus and had free access to all quadrants. The time that the rats had spent in each of the four quadrants was measured. The treatment quadrant (TQ) was determined to be one of the quadrants, in which the animal had spent neither the longest nor the shortest time during the habituation.

On the following two days (Conditioning trials) the quadrants were physically separated from each other by the plexiglass barrier. Animals were introduced into the TQ ten minutes following the microinjections and were restricted there for 15 min. During the conditioning sessions, animals could link the rewarding/aversive effects of the drug with the cue present in the TQ. On the 4th day, when the Test trial was conducted, the separating barriers were removed. Animals were placed into the center of the apparatus (without drug administration) and allowed to move freely around the field.

The time spent in each of the four quadrants was recorded; the place preference was established if the animals spent significantly more time in the TQ. Nevertheless, it is important to consider that increased latency in TQ besides may also occur due to disrupted motor activity or increased anxiety, instead of real preference and rewarding effect. With the help of other behavioral paradigms, these options need to be verified.

Fig. 9. Procedure of the conditioned place preference test



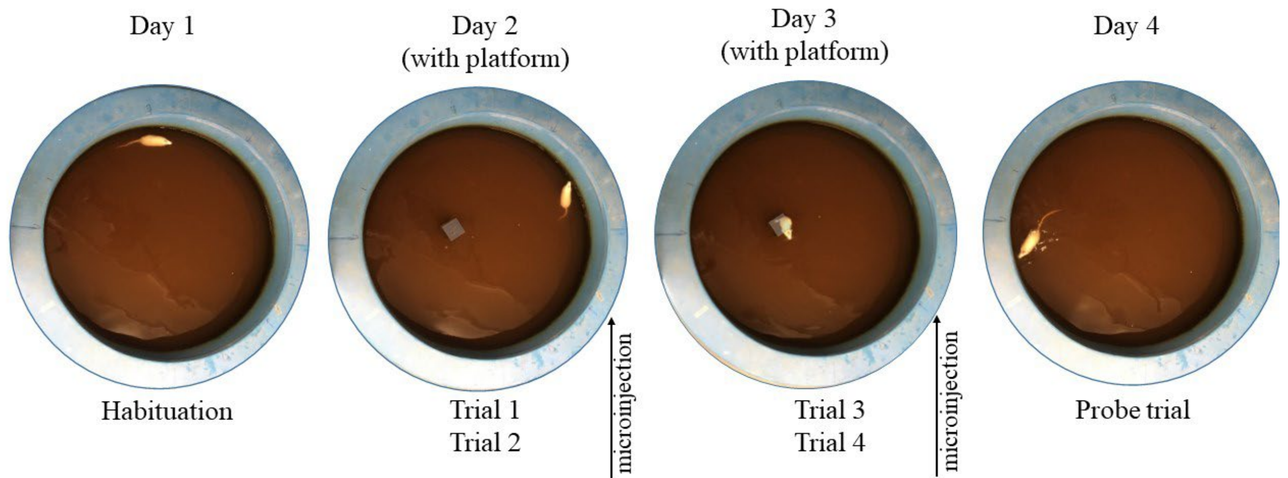
3.6. Morris water maze test (MWM)

MWM experiments were carried out in a circular pool (150 cm diameter, 60 cm height), virtually divided into four quadrants. One of the quadrants was chosen to place a square (10 cm × 10 cm) plexiglass target platform. The location of the platform was fixed during the experiments, except for the habituation and probe trials. The water was kept at a constant temperature (23 ± 1 ◦C) and was colored with Potassium permanganate, so the platform was not visible to the animals. The surface of the water was kept 2 cm above the platform. Spatial reference cues around the pool were maintained in their fixed positions throughout the MWM experiments. The animals' behavior was recorded by a video camera and registered by special software (EthoVision; Noldus Information Technology, The Netherlands).

On the first day of the experiment, rats could get acquainted with the surrounding environment and the pool (without platform) in a habituating session lasting 180 sec. On the second day of conditioning two trials for spatial learning, separated by 60-sec interval, were performed (Trial 1 and Trial 2). This short interval ensured the possibility to observe the short-term memory trace formed during the first trial. On the third day, 24 h later, training was continued on the same schedule (Trial 3 and Trial 4). In these four trials, the latency to finding the safe platform (escape latency) was measured. The four training trials were conducted as follows: rats were placed into the water maze at randomly assigned but predetermined locations to avoid the egocentric orientation. The task required the animals to search for the hidden platform guided by external spatial cues. Each trial lasted until the rat found the platform or for a maximum duration of 180 sec. Animals who failed to find the platform within the allocated time were gently guided to the platform. By finding the platform, the rat could stay there for 60 sec to memorize the surrounding ques. Drug or vehicle treatment was applied by the end of each conditioning day, i.e., emergently after the Trial 2 and Trial 4.

On the fourth day of the experiment, 24 h following the last swimming training, a Probe trial was performed: the platform was removed, and the latency to the first crossing of the platform's place was measured. In addition to the latency to the first occurrence, also distance and the route trajectory were analyzed. The target annulus surrounding the platform and the opposite annulus in the opposite quadrant (in both cases the diameter was 37,5 cm, a quarter of the pool's diameter) were determined [351]. The time spent in those annuli, as well as the number of entries, were analyzed (with the assistance of Noldus software) during the two swimming trials without platform (i.e. habituation and probe trials). The normalized data have been calculated, meaning that in the case of each animal, the data in the given annulus during the Habituation trial have been subtracted from data achieved during the Probe trial. If the animal's preference for the given annulus increased, then the normalized time and the number of entries were positive, and if it decreased then parameters had negative values [352]. An additional parameter, indirectly indicating the signs of anxiety, i.e. time spent by the animals at the walls, was evaluated during the Probe trial.

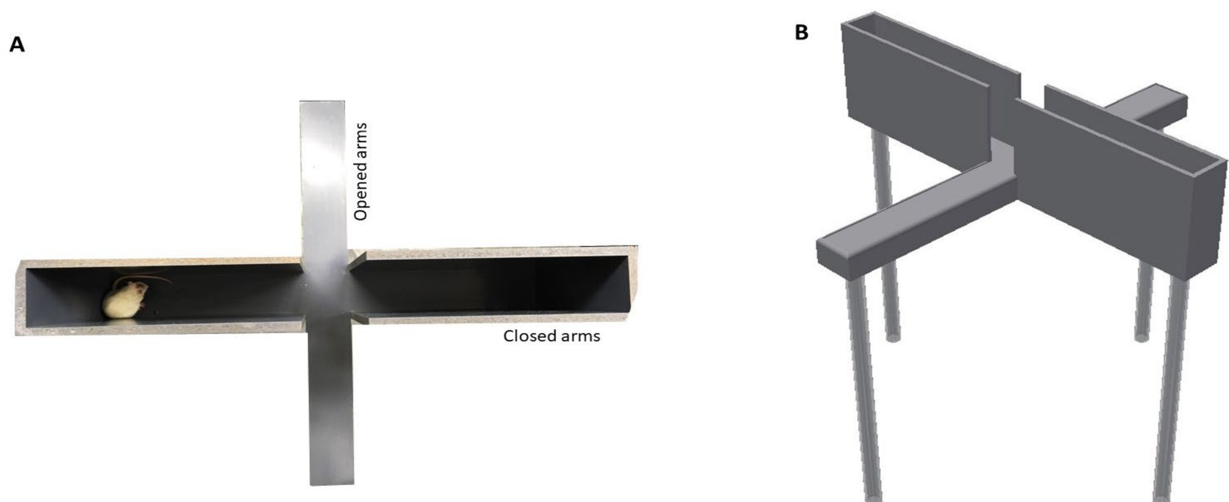
Fig. 10. Procedure of the Morris water maze



3.7. Elevated plus maze (EPM)

The main paradigm for the evaluation of anxiety was the EPM test. The apparatus was constructed of grey colored wooden planks. The equipment consisted of two opposite open arms (50 cm × 10 cm) and two opposite closed arms (50 cm × 10 cm × 40 cm) with walls and open roof. The maze was elevated to a height of 100 cm above the floor. Ten minutes following the drugs administration, animals were placed into the center of the maze (central platform), facing one of the closed arms. Each rat was tested only once. The arena was cleaned and deodorized with acetic acid after each animal. Trials lasted for 5 min, and during this period the time spent on the opened and closed arms and at the ends of the opened arms was recorded.

Fig. 11. Procedure of the elevated plus maze test



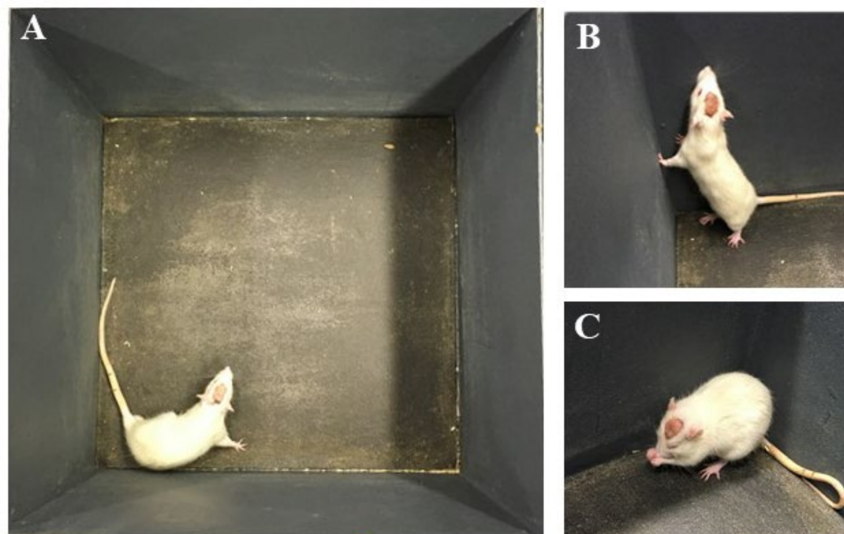
A: General view of the EPM apparatus, with opened and closed arms marked.

B: Schematic drawing of the apparatus (Reprinted from [353])

3.8. Open field test (OFT)

OFT was employed for measuring a spontaneous motor activity and exploration behavior in response to QRFP administration. The experimental arena presented itself as a 50 x 50 x 50 cm gray painted box with a floor virtually divided into 16 identical squares thus marking central and peripheral zones of the field. The apparatus was provided with homogenous illumination. Naive rats were placed in the center of the arena and allowed to explore the environment for 5 minutes (Habituation), afterwards, they were returned to their home cages. In the following two days, the procedure has been repeated for the sake of recording the level of basal activity. On the last day (Test) animals received microinjection of QRFP, Ant, or vehicle and after 10 minutes experimental procedure was repeated. The arena was cleaned and deodorized with acetic acid after each animal. The distance moved in the arena was analyzed by Noldus EthoVison System (Noldus Information Technology, The Netherlands). Behavioral patterns, such as grooming activity and rearing, were analyzed on video recording. Time spent by the animals around the walls of the apparatus was recorded as an indirect indicator for anxiety.

Fig. 12. Open field test



A: General view in the apparatus. B: Rearing. C: Grooming

3.9. Data analysis

3.9.1. Statistical analysis

All results were expressed as a mean ± standard error of the means (S.E.M.). Cumulative food intake per 100 g bw in feeding-related experiments was evaluated by repeated-measures analysis of variance (IBM SPSS Statistics 20 data analysis program). When the analysis of the main effect and/or the interaction showed significance, ANOVA was followed by paired-samples t-test analysis. Choice of statistical methods was determined by the experimental design, implicating that each animal served as its own control (within-subject design).

Due to the between-subjects experimental design, the data from behavioral experiments were evaluated by two-way and one-way ANOVA, followed by Tukey post hoc test in case of significant effect. The statistical rejection criterion for all the experiments was established at $p < 0.05$ level.

3.9.2. Histology

To verify cannulae placements, animals received an overdose of urethane (20%) and were perfused transcardially with isotonic saline followed by 10% formaldehyde solution. Brains were sliced with a freezing microtome in 40 μm sections and stained with Cresyl-violet. Injection sites were reconstructed according to the stereotaxic atlas [340]. The track of cannulae and the tips were determined based on the existence of debris and moderate glial proliferation. Only data from the rats with correctly placed cannulae were analyzed.

4. Results

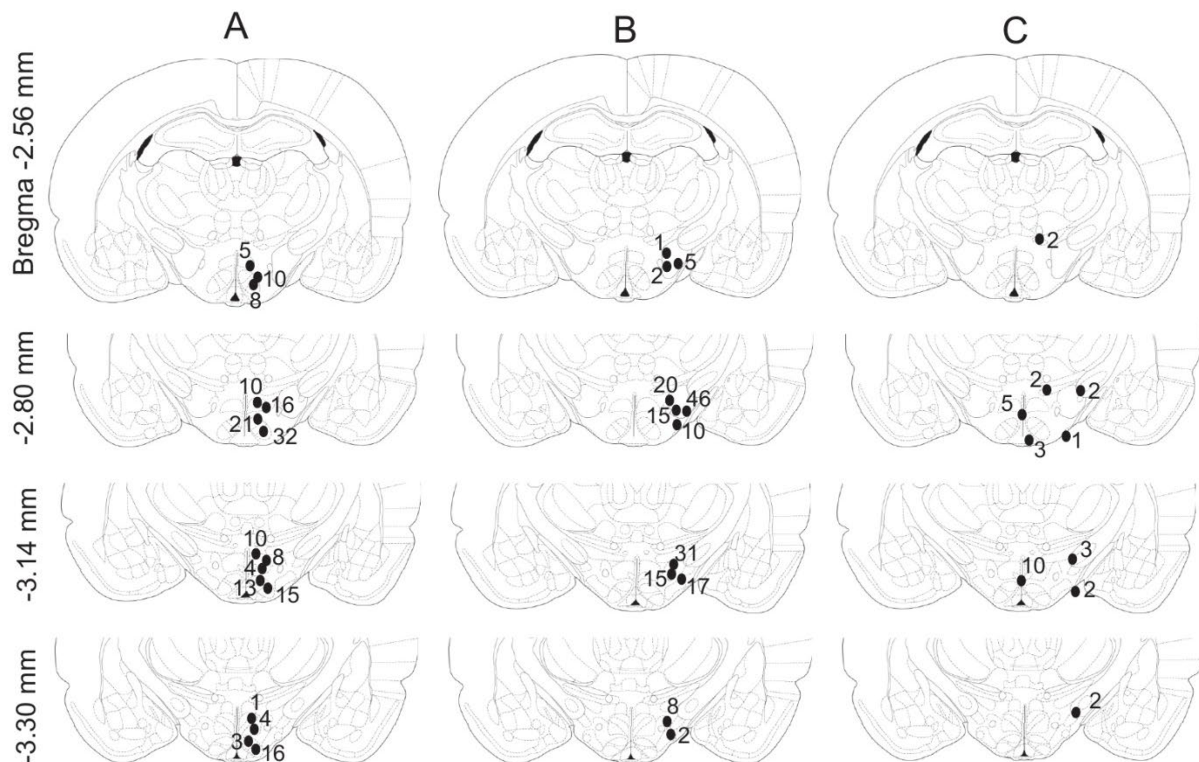
4.1. Histology

The stereotaxic operations were performed based on Paxinos and Watson's atlas of the rat brain. Following the histological examination 32 of 398 operated animals were excluded from data analysis. A schematic illustration of cannulae placements is shown in Fig. 13. In 186 cases the targeting of the cannulae was precisely tipped to the MHA, of which 118 injections reached the DMN and in 68 rats cannulae were placed to the VMN (Fig. 13A). In 172 brains, the LHA was reached (Fig. 13B).

In the other 32 animals, cannulae were not correctly positioned in the target area (Fig. 13C). Among them in 4 cases cannulae were led to the ZI, in 7 animals to the entopeduncular nucl., in 3 cases towards Arc, 3 other cannulae went out of the brain, in 15 rats' cannulae tips entered into the liquor space of the 3rd ventricle.

Another 8 of 398 animals have been excluded from the experimental analysis due to their special characteristics: 6 animals repeatedly jumped out from the experimental arenas (MWM, EPM), and 2 rats' crowns were damaged making the microinjecting impossible.

Fig. 13. Schematic illustration of reconstructed injection sites from all experiments.



Panel A: correct injection placements in the MHA (n = 186).

Panel B: correct injection placements in the LHA (n = 172).

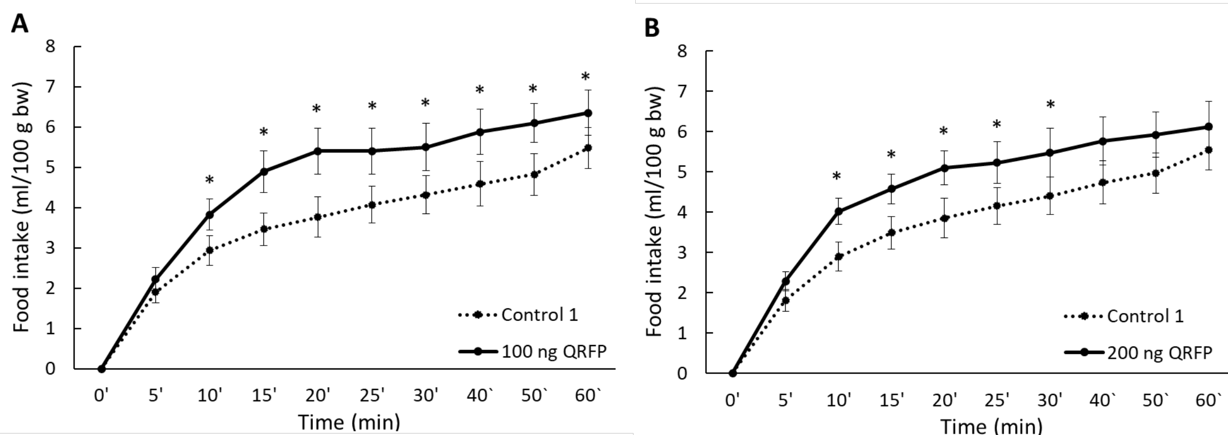
Panel C: incorrect injection placements (n = 32). Brain structure diagrams of coronal sections are adapted from the stereotaxic atlas of Paxinos and Watson [340]. The numbers on the left refer to anterior-posterior distance from bregma in mm. The numbers above circle symbols on panels A, B and C indicate numbers of animals.

4.2. Results of the feeding experiments

4.2.1. Results of the feeding experiments in MHA

Feeding tests began from the fifth postoperative day when all animals reached the preoperative level of body weight and food intake. Figures represent mean cumulative liquid food consumption in ml/100 g body weight (±S.E.M.) during 60 min period. The effect of QRFP microinjections into the MHA on food intake is shown in Fig. 14. **Administration of 100 ng dose of QRFP into the MHA induced significant orexigenic effect** (Fig. 14A, n = 11). ANOVA analysis yielded significant effect of time ($F [8,80] = 38.917$, $p < 0.01$), treatment ($F [1,10] = 12.833$, $p < 0.01$) and significant effect of time × treatment ($F [8,80] = 4.473$, $p < 0.01$). Paired-samples t-test analysis showed a significant increase in liquid food consumption at each time point from 10th to 60th min ($p < 0.03$). In case of **200 ng QRFP treatment, food consumption was markedly increased as well** (Fig. 14B, n = 11), ANOVA indicated a significant effect of time ($F [8,80] = 38.056$, $p < 0.01$) and treatment ($F [1,10] = 8.284$, $p < 0.02$), but not time × treatment interaction. Paired-samples t-test analysis showed significant raise in liquid food intake from 10th to 30th min ($p < 0.05$).

Fig. 14. Feeding-related effects of QRFP microinjections into the MHA



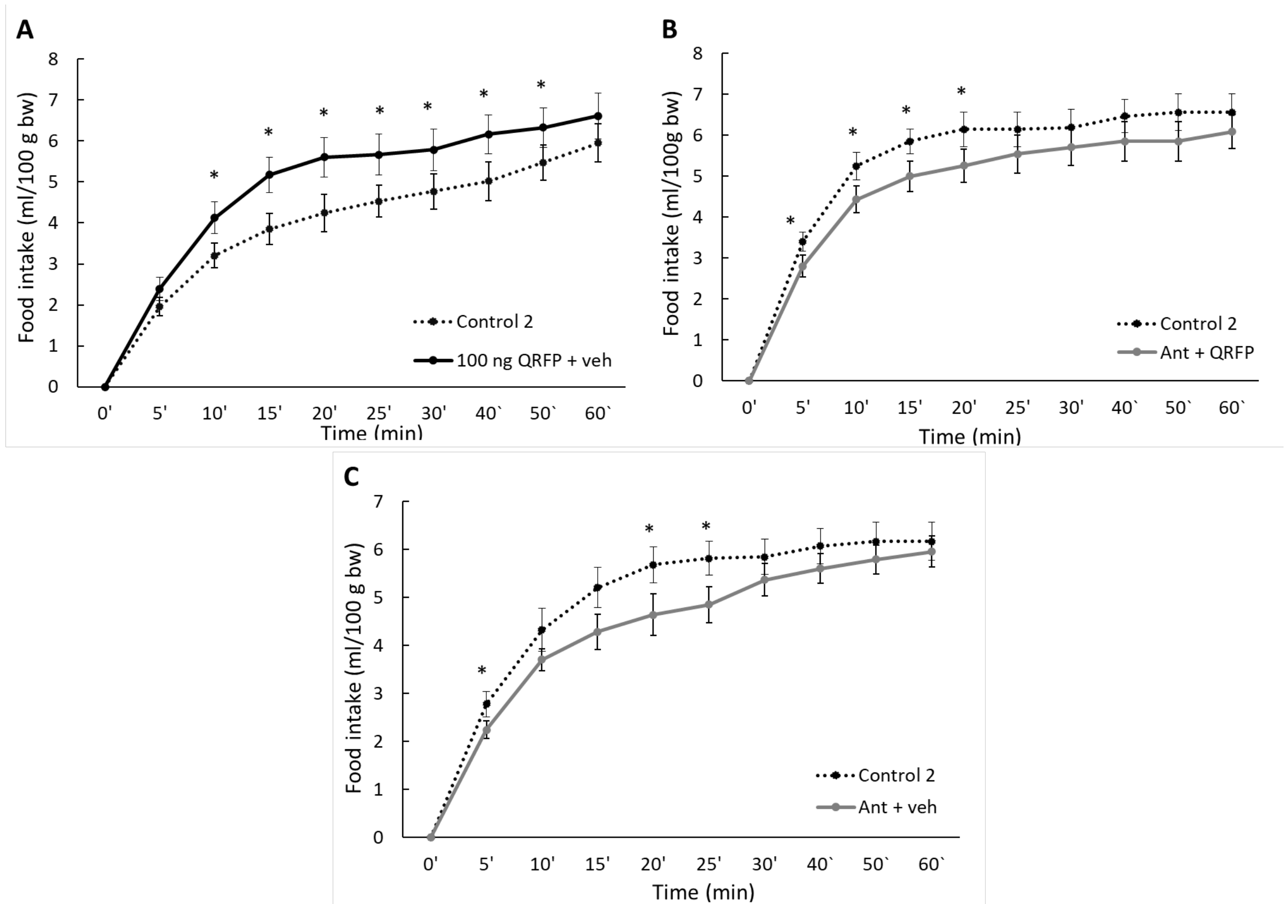
Lines with symbols represent cumulative mean food intake in ml/100 g body weight (±S.E.M.) after application of the peptide in different doses or vehicle microinjections (0.4 µl).

A: Control 1 vs 100 ng QRFP (n = 11).

*B: Control 1 vs 200 ng QRFP (n = 11). * Symbols above the lines indicate significant difference (p < 0.05).*

Data from the antagonistic experiment, where the ability of BIBP3226 was examined, are presented in Fig. 15. The **vehicle + 100 ng QRFP administration into the MHA led to a significant increase in food intake as well** (Fig. 15A, n = 12). ANOVA analysis revealed a significant effect of time ($F [8,88] = 50.466$, $p < 0.01$), treatment ($F [1,11] = 13.450$, $p < 0.01$) and time × treatment interaction ($F [8,88] = 2.225$, $p < 0.05$). According to paired-samples t-test analysis, significant rise in food consumption was detected at each time point from 10th to 50th min ($p < 0.03$). **When animals received combined antagonist and peptide treatment with a 15 min interval (Ant + QRFP), the food consumption not only returned to the control level but also was transiently depressed** (n = 9). The data are presented in the Fig. 15B. According to ANOVA analysis there was significant effect of time ($F [8,64] = 42.995$, $p < 0.01$), significant effect of treatment ($F [1,8] = 8.715$, $p < 0.02$), but not time × treatment interaction. Paired-samples t-test recognized significant depression of milk consumption during the first twenty minutes ($p < 0.01$, $p < 0.05$, $p < 0.01$, $p < 0.01$, respectively). **BIBP3226 microinjections into the MHA inhibited orexigenic features of QRFP, and transiently decreased liquid food consumption compared to control treatment** (Fig. 15C, Ant + veh, n = 12). ANOVA analysis yielded significant effect of time ($F [8,88] = 79.139$, $p < 0.01$), not significant effect of treatment, but significant effect of time × treatment ($F [8,88] = 2.597$, $p < 0.02$). Paired-samples t-test analysis showed a significant fall in food consumption at 5th, 20th and 25th min ($p < 0.03$ at all three time points). During the interval between 5th and 20th minutes tendency for lower milk consumption also can be observed, but not reaching statistically significant values.

Fig. 15. Feeding-related effects of BIBP3226 microinjections into the MHA



Lines with symbols represent cumulative mean food intake in ml/100 g body weight (±S.E.M.) following the microinjections (0.4 µl + 0.4 µl).

A: Control 2 (vehicle +vehicle) vs 100 ng QRFP + vehicle (n = 12).

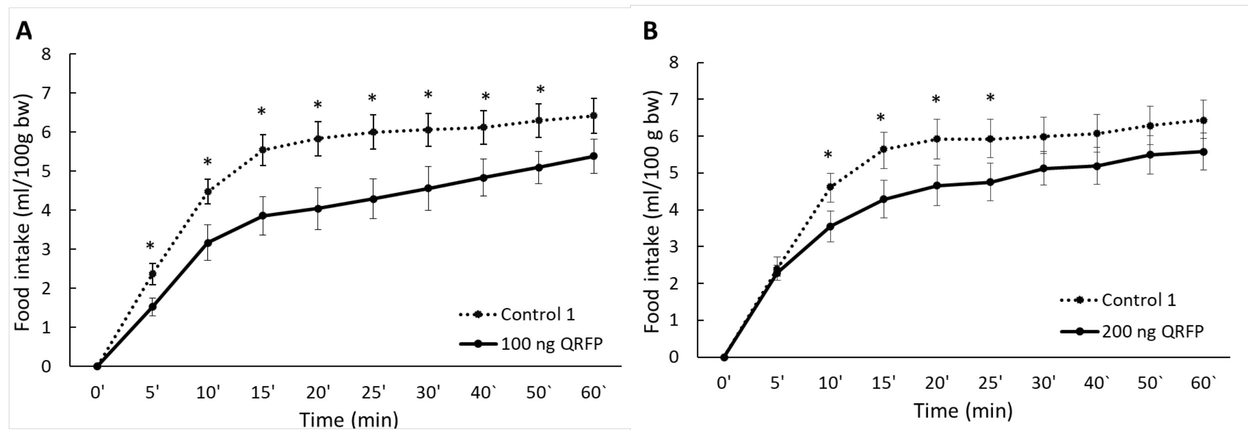
B: Control 2 vs Ant pretreatment followed by QRFP microinjection (Ant + QRFP, n = 9).

*C: Control 2 vs Ant + vehicle (n = 12). *Symbols above the lines indicate significant difference (p < 0.05).*

4.2.2. Results of the feeding experiments in LHA

Effects of QRFP microinjections into LH on food intake are shown in Fig. 16. The **100 ng dose of the peptide injected into the LHA led to a significant anorexigenic effect** (Fig. 16A, n=11). ANOVA analysis revealed significant effect of time ($F_{[8,80]} = 79.326$, $p < 0.01$) and treatment ($F_{[1,10]} = 11.271$, $p < 0.01$), but not time × treatment interaction. According to paired samples t-test, the food consumption was significantly lower at each time point during the first 50 minutes ($p < 0.04$). **Following the application of QRFP in 200 ng dose** (Fig. 16B, n=9)**, anorexigenic effects were registered as well.** ANOVA showed significant effect of time ($F_{[8,64]} = 51.469$, $p < 0.01$) and treatment ($F_{[1,8]} = 8.113$, $p < 0.03$), but not time × treatment interaction. Data from paired samples t-test say that the food consumption was significantly lower from 10th till 25th minutes ($p < 0.04$).

Fig. 16. Feeding-related effects of QRFP microinjections into the LHA



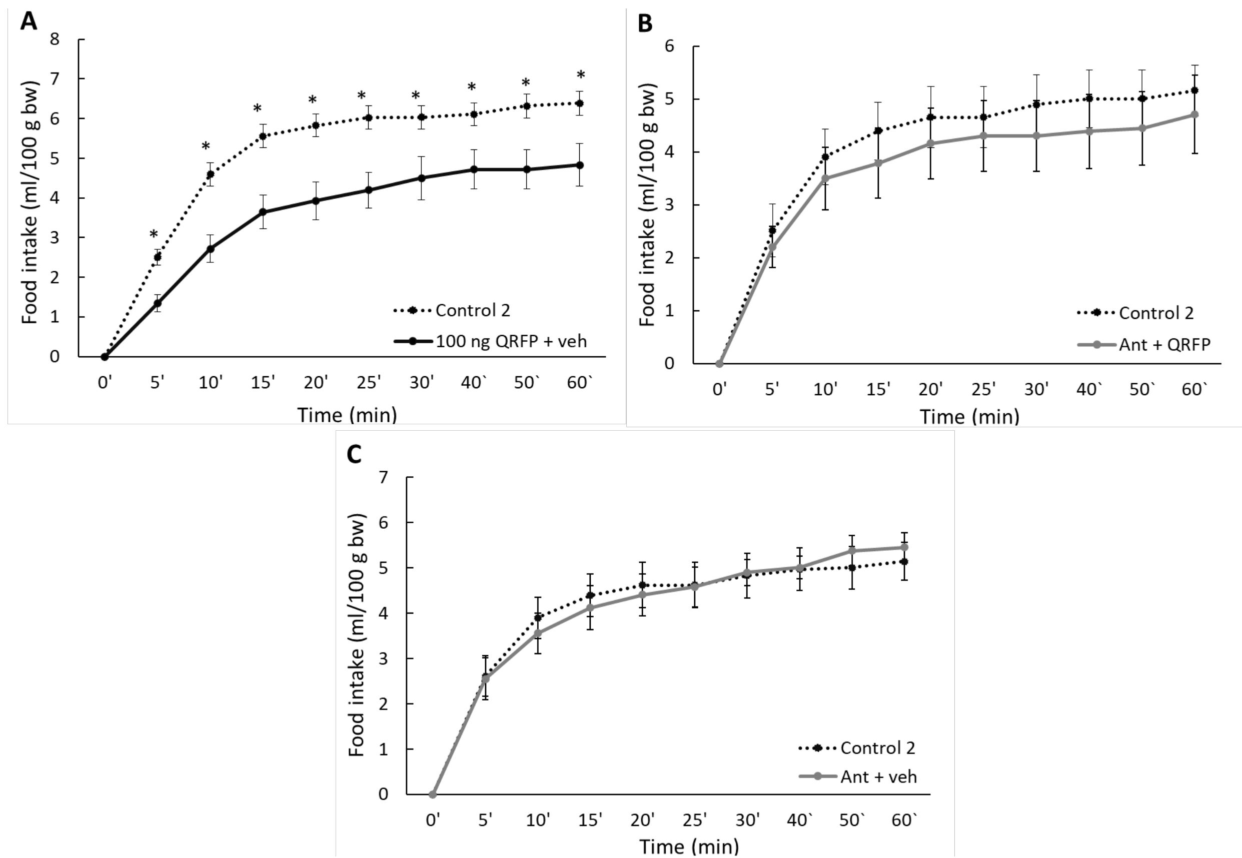
Lines with symbols represent cumulative mean food intake in ml/100g body weight (±S.E.M.) after application of the peptide in different doses or vehicle microinjections (0.4 µl).

A: 100 ng QRFP vs Control 1 (n = 11).

*B: 200 ng QRFP vs Control 1 (n = 9). * Symbols above the lines indicate significant difference ($p < 0.05$).*

Data regarding the antagonistic activity of BIBP3226 microinjected into the LH are presented in Fig. 17. **The effective dose of QRFP applied in double volume** (Fig. 17A, n=12) **confirmed the previous data by decreasing food intake**. ANOVA yielded a significant effect of time ($F [8,72] = 71.800$, $p < 0.01$), treatment ($F [1,9] = 20.882$, $p = 0.01$), but not time × treatment interaction. The t-test showed a significant difference at each time point during the first hour ($p < 0.03$). **Combined antagonist and neuropeptide** (Ant+ QRFP, Fig. 17B, n=7), **as well as antagonist and vehicle treatments** (Ant + veh, Fig. 17C, n=8) **inhibited anorexigenic effects induced by QRFP**. The food consumption in control and antagonist treated groups was identical ($p > 0.05$).

Fig. 17. Feeding-related effects of BIBP3226 microinjections into the LHA



Lines with symbols represent cumulative mean food intake in ml/100 g body weight (±S.E.M.) following the microinjections (0.4 µl + 0.4 µl).

A: Control 2 (vehicle +vehicle) vs 100 ng QRFP + vehicle, n = 10).

B: Control 2 vs Ant pretreatment followed by QRFP microinjection (Ant + QRFP, n = 7).

*C: Control 2 vs Ant + vehicle (n = 8). * Symbols above the lines indicate significant difference ($p < 0.05$).*

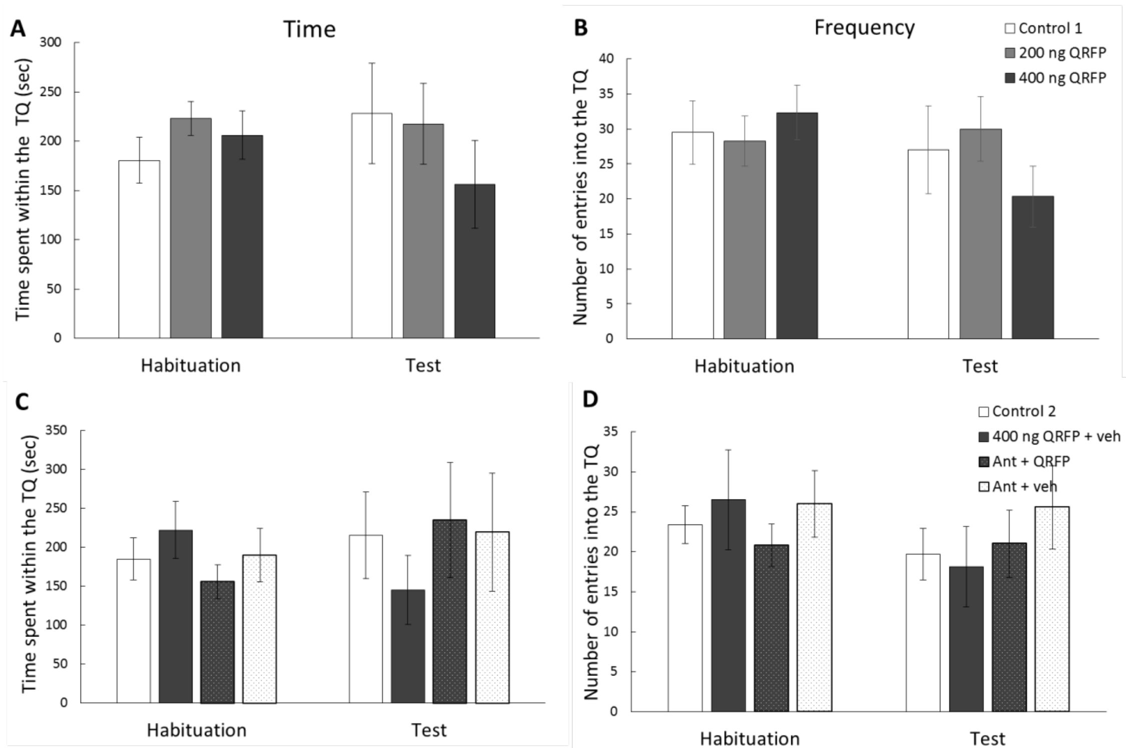
4.3. Results in Conditioned place preference paradigm

4.3.1. Results of the CPP in MHA

Results of the neuropeptide QRFP microinjections on learning in the CPP test are present in Fig. 18A and B. According to the two-way ANOVA, rats treated with **low and high doses of QRFP** into the MHA have shown identical results in both analyzed parameters. **Neither the time spent within the TQ, nor the frequency of entries into the TQ was of significant difference.**

Similar data have been collected during the **Ant experiment**, which can be observed in Fig. 18C and D. **There was no significant difference** regarding the time spent within the TQ. Also, all the groups during the habituation and test trials had a similar average frequency of entries into the TQ.

Fig.18. Effects of QRFP and BIBP3226 microinjections into the MHA on CPP



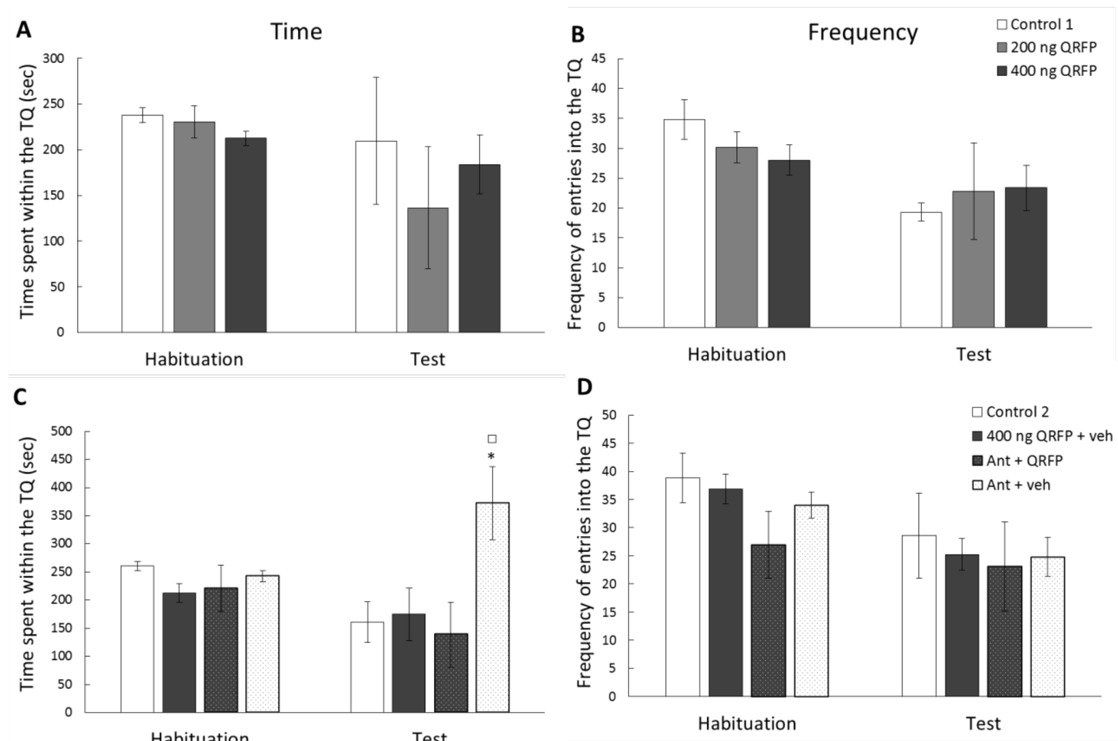
A, B: Effects of QRFP microinjections (0.4 µl): Control 1 (vehicle, n=7), 200 ng QRFP (n=6), 400 ng QRFP (n=6).

C, D: Effects of BIBP3226 (Ant) microinjections (0.4 µl+0.4 µl): Control 2 (vehicle +vehicle, n =6), 400 ng QRFP + vehicle (n =6), Ant pretreatment followed by QRFP microinjection (n =6), or Ant + vehicle (n =5). Time: Columns represent the time spent by the animals within the treatment quadrant (TQ) during habituation and test trials, respectively (±S.E.M.). Frequency: Columns represent number of entries into the TQ during habituation and test trials, respectively (±S.E.M.).

4.3.2. Results of the CPP in LHA

QRFP microinjections had no effect on conditioned learning when injected into the LHA (Fig. 19A, B). Surprisingly, **Ant led to longer latency spent in TQ** (Fig. 19 C, D). The two-way ANOVA suggests a significant effect of treatment ($F [3,54] = 5.248$, $p < 0.01$), and treatment x trial ($F [3,54] = 4.209$, $p = 0.01$), but not trial ($F [1,54] = .764$, $p > 0.05$). One-way ANOVA showed a significant effect of treatment groups during the test trial ($F [3,29] = 5.603$, $p < 0.01$). Post hoc test indicates that group of animals treated with Ant spent significantly more time within the TQ in comparison to Control, QRFP and combined Ant + QRFP treated groups ($p<0.04$, $p < 0.03$ and $p < 0.01$, respectively). At the same time, according to one-way ANOVA, there is a significant difference between the trials ($F [1,14] = 4.479$, $p = 0.05$), indicating that Ant provoked rats to spend more time within the TQ during the Test comparing to the naïve state during the Habituation trial. Further experiments clarify whether the observed phenomenon is a result of real conditioned place preference learning or refers to increased anxiety.

Fig. 19. Effects of QRFP and BIBP3226 microinjections into the LHA on CPP



A, B: Effects of QRFP microinjections (0.4 µl): Control 1 (vehicle, n=6), 200 ng QRFP (n=6), 400 ng QRFP (n=7).

*C, D: Effects of BIBP3226 (Ant) microinjections (0.4 µl+0.4 µl): Control 2 (n =7), 400 ng QRFP + vehicle (n =8), Ant pretreatment followed by QRFP microinjection (n =8), or Ant + vehicle (n =8). Time: Columns represent the time spent by the animals within the treatment quadrant (TQ) during habituation and test trials, respectively (±S.E.M.). Frequency: Columns represent number of entries into the TQ during habituation and test trials, respectively (±S.E.M.). Symbols above the columns indicate significant difference: □ refers to between-trial difference, * refers to between-group difference within one trial (p < 0.05).*

4.4. Results in Morris water maze paradigm

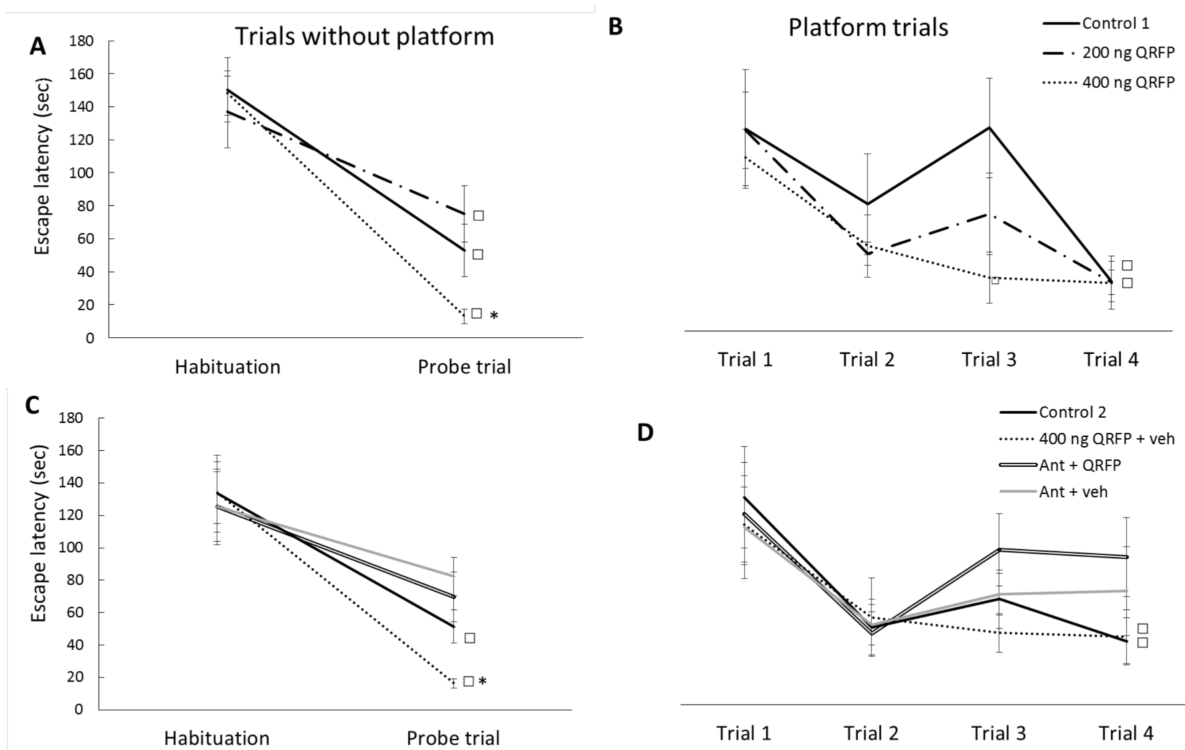
4.4.1. Results of the MWM in MHA

At first, the effect of **MHA QRFP treatment** on the escape latencies of rats was investigated (Fig. 20A, B). Swimming trials without the platform, i.e. Habituation and Probe trial, were evaluated separately from the training trials (1-4). In regard to the trials without the platform, two-way ANOVA analysis revealed that there was a significant effect of trials ($F\ [1,42] = 40.110$, $p < 0.001$), but no significant effect of treatment or interaction between trials and treatment. According to one-way ANOVA, there were significant differences within each treatment group: control, 200 ng and 400 ng ($p = 0.01$, $p = 0.04$, $p < 0.001$, respectively), as well

as between the groups during Probe trial ($p = 0.03$, $p = 0.01$). Concluding from these findings, **by the day of testing (Probe trial) rats treated with 400 ng QRFP found the platform significantly faster.** Nevertheless, all the animals have learned that there was an escape platform in the pool. For a better understanding of the learning dynamics, the training trials 1-4 were analyzed by two-way and one-way ANOVA. Significant difference has been found for the training trials: $F [3,84] = 7.651$, $p < 0.001$, but not treatment or their interaction. During the first two trials (1 and 2) animals did not show significant learning results. Trial 2 was followed by QRFP microinjections in corresponding doses. Twenty-four hours later, in trial 3, mean latencies of control and 200 ng treated animals raised back, but 400 ng treated rats found the platform even faster than the previous day, and their latencies became significantly shorter compared to trial 1 ($p = 0.02$). Similar to the previous experimental day, the next swimming trial (trial 4) followed one minute later. This time for all the animals time to finding platform was approximately the same, but due to minor differences in SEM, the analysis registered that 200 ng and 400 ng groups (but not control) have found the target significantly faster compared to trial 1 ($p = 0.01$, $p < 0.02$, respectively). After trial 4, all the animals received second microinjections.

Data from the subsequent experiment, where the antagonistic ability of BIBP3226 was examined, are presented in Figures 20C and D. Experimental procedure implicated double volume injection to each animal with 15 minutes intervals. The two-way ANOVA analysis of trials without platform indicated a significant effect for trials ($F [1,60] = 35.799$, $p < 0.001$), however the effect for treatment and the interaction between trials and treatment was not significant. According to one-way ANOVA, when comparing Habituation and Probe trials, there were significant differences registered within control and 400 ng treated animals ($p < 0.01$, $p < 0.001$, respectively), but not Ant or combined Ant + QRFP treated rats. Similar to the first experiment, in the Probe trial, the one-way ANOVA indicated significant differences among the groups ($F [3,30] = 6.082$, $p = 0.002$). The Tukey's post hoc test confirmed that **the mean latency of the vehicle + QRFP group was significantly lower compared to that of the control, Ant + Vehicle, and Ant + QRFP treated groups** ($p = 0.05$, $p = 0.01$, $p < 0.003$, respectively). **Meanwhile, pre-treatment with Ant prevented this effect.** Two-way ANOVA analysis was applied to compare means of four training trials to each other within each group and revealed significant differences among trials ($F [3,120] = 7.284$, $p < 0.001$), but not treatment, or trial x treatment. The vehicle + QRFP treated animals (400 ng QRFP), similarly to the previous experiment, have shown shorter latencies, and by trial 4 have learned to find the platform significantly faster than in the first trial (Trial 1), $p = 0.05$. Control animals have shown good results at Trial 4 ($p < 0.03$) as well. By contrast, means of the Ant + QRFP and the Ant + Vehicle - treated groups remained similar to their latencies during Trials 1 and 3, so the progress in learning was not registered by Trial 4.

Fig. 20. Effects of QRFP and BIBP3226 microinjections into the MHA on the platform finding latency (escape latency) in Morris water maze.



The drugs were microinjected immediately following the trials 2 and 4.

A, B: Effects of QRFP microinjections (0.4 µl): Vehicle solution (Control 1, n = 8), 200 ng (n = 9), 400 ng QRFP (n= 7).

C, D: Antagonist experiment with corresponding drugs (0.4 µl + 0.4 µl) delivered with 15 min interval: double volume of saline treatment (Control 2, n = 9), 400 ng QRFP followed by vehicle (n = 8), BIBP3226 (Ant) pretreatment followed by 400 ng QRFP (n = 9), Ant followed by vehicle (n = 8).

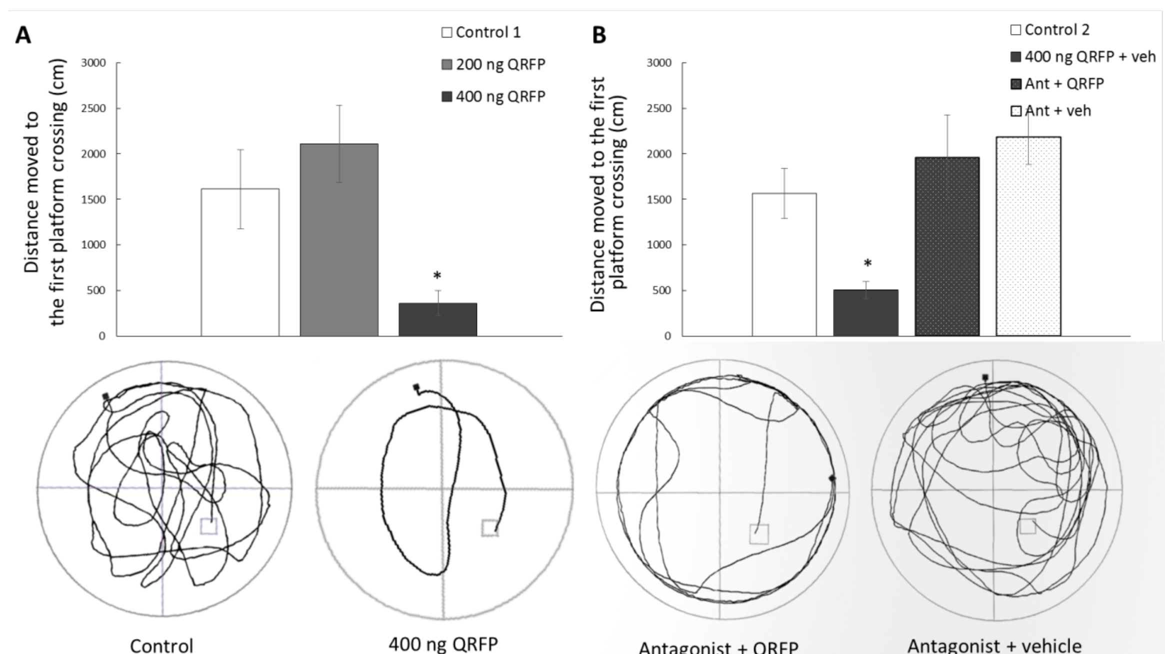
A, C: Trials without the platform, lines represent the mean latencies to finding the place of the removed platform (±S.E.M.),

*B, D: Training trials, lines represent the mean latencies to finding the hidden platform (±S.E.M.). Symbols next to the graphs indicate significant difference: □ refers to between-trial difference, * refers to between-group difference within one trial ($p < 0.05$).*

Considering the mean swimming velocities of the animals, there were no significant differences registered in any trial during both experiments (data not shown).

The distances that rats have covered during the Probe trial until they crossed the place of the removed platform are presented in Fig. 21. Following the tendency, **400 ng QRFP treated rats made shorter routes to the target compared to all other groups**, which was proved by ANOVA analysis ($F [2,19] = 5.673$, $p < 0.02$; $F [3,28] = 5.012$, $p < 0.01$, for the first and the second experiments, respectively). Images of representative trajectories for each group are present in the Fig. 21C.

Fig. 21. Effects of QRFP and BIBP3226 microinjections into the MHA on average distance to finding platform place during the Probe trial in Morris water maze.



A: Columns represent distance moved to the first platform crossing during the experiment with QRFP microinjections (±S.E.M.),

B: Columns represent distance moved to the first platform crossing during the experiment with Ant (±S.E.M.),

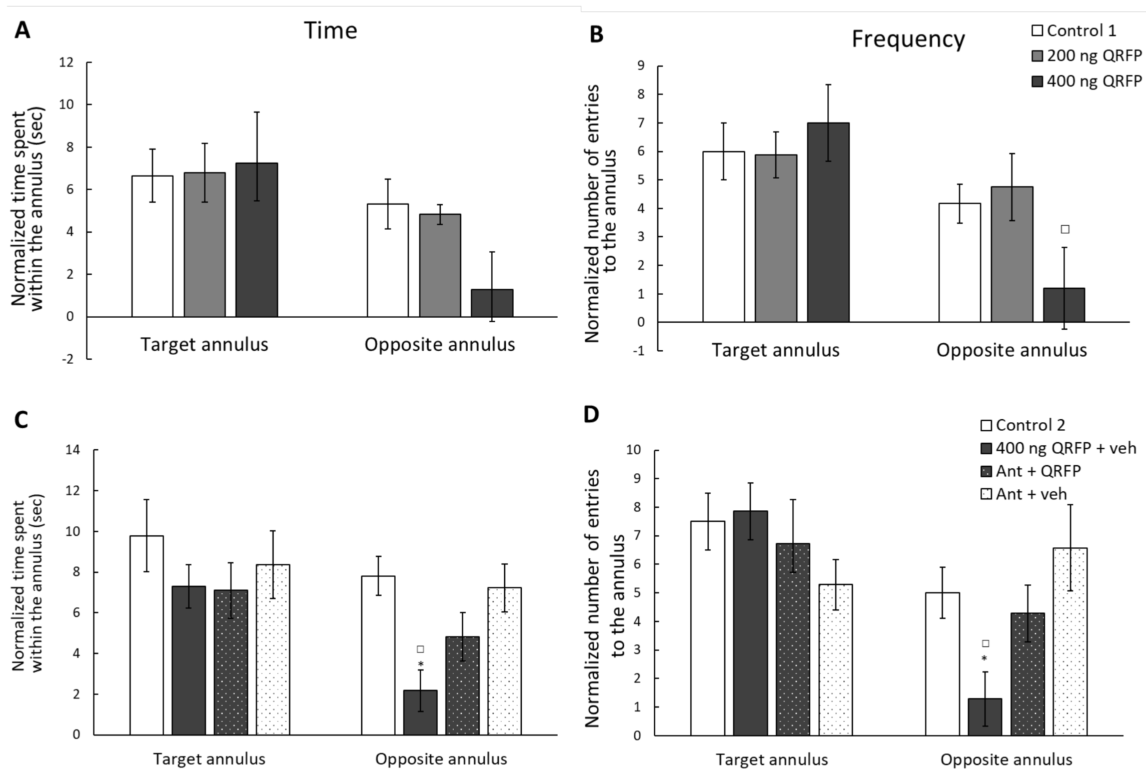
*C: Illustrative images of rats' trajectories during the Probe trial in Morris water maze. The groups and the number of animals are identical to those present in the Fig. 20. Symbol * above columns indicates significant difference ($p < 0.05$).*

Additional analysis of the Probe trial (Fig. 22) revealed a tendency for 400 ng QRFP treated group to spend less time in opposite annulus while searching for escape platform (Fig. 22A). But according to the two-way ANOVA, it did not reach significant level. Corresponding parameter, normalized number of entries to annuli, indicated significant effect for annuli (F [1,34] = 7.891, $p < 0.01$), but no significant effect for treatment or interaction between the treatment and annuli (Fig. 22B). One way ANOVA test revealed that **animals treated with 400 ng QRFP had lower number of entries into the opposite annulus comparing to target annulus** ($p = 0.02$).

In the experiment with Ant **normalized time** spent in the target and in the opposite annuli had significant effect for annuli (F [1,50] = 6.789, $p < 0.02$), a significant effect for treatment (F [3,50] = 3.309, $p < 0.03$), but no significant interaction between the treatment and annuli (Fig. 22C). Tukey's post hoc test demonstrated that the means of the control group significantly differ from those of QRFP treated animals ($p < 0.03$). The one-way ANOVA indicated a significant difference among the groups only **in the opposite annulus** (F [3,25] = 4.796, $p < 0.01$). The Tukey's post hoc test proved that **QRFP treated animals spent there significantly less time compared to the control and Ant groups** ($p = 0.01$, $p < 0.03$, respectively). The time spent in the target and opposite annuli within each treatment group was compared applying one-way ANOVA as well. The analysis showed that the **animals treated with 400 ng QRFP spent much more time searching in the target annulus comparing to the opposite one** ($p < 0.01$).

Another analyzed parameter, the **normalized number of entries** to the target and opposite annuli, indicated similar results (Fig. 22D). The two-way ANOVA revealed significant effect for annuli (F [1,50] = 8.819, $p = 0.005$), but not for treatment, and significant effect for treatment and annuli interaction (F [3,50] = 3.374, $p < 0.03$). According to one-way ANOVA, the difference between the groups reached a **significant level in the opposite annulus** (F [3,25] = 3.366, $p < 0.04$). The Tukey's post hoc test showed that **QRFP treated animals had also a lower number of entries compared to those treated with Ant** ($p < 0.03$). Analysis by one-way ANOVA, of the number of entries into the target and opposite annuli within each group revealed that the **animals treated with 400 ng QRFP appeared in the target annulus significantly more often comparing to the opposite one** ($p = 0.001$).

Fig.22. Effects of QRFP and BIBP3226 microinjections into the MHA on the normalized time and number of entries to the target and opposite annuli during the Probe trial in Morris water maze.



A, B: Effects of QRFP microinjections in two doses,

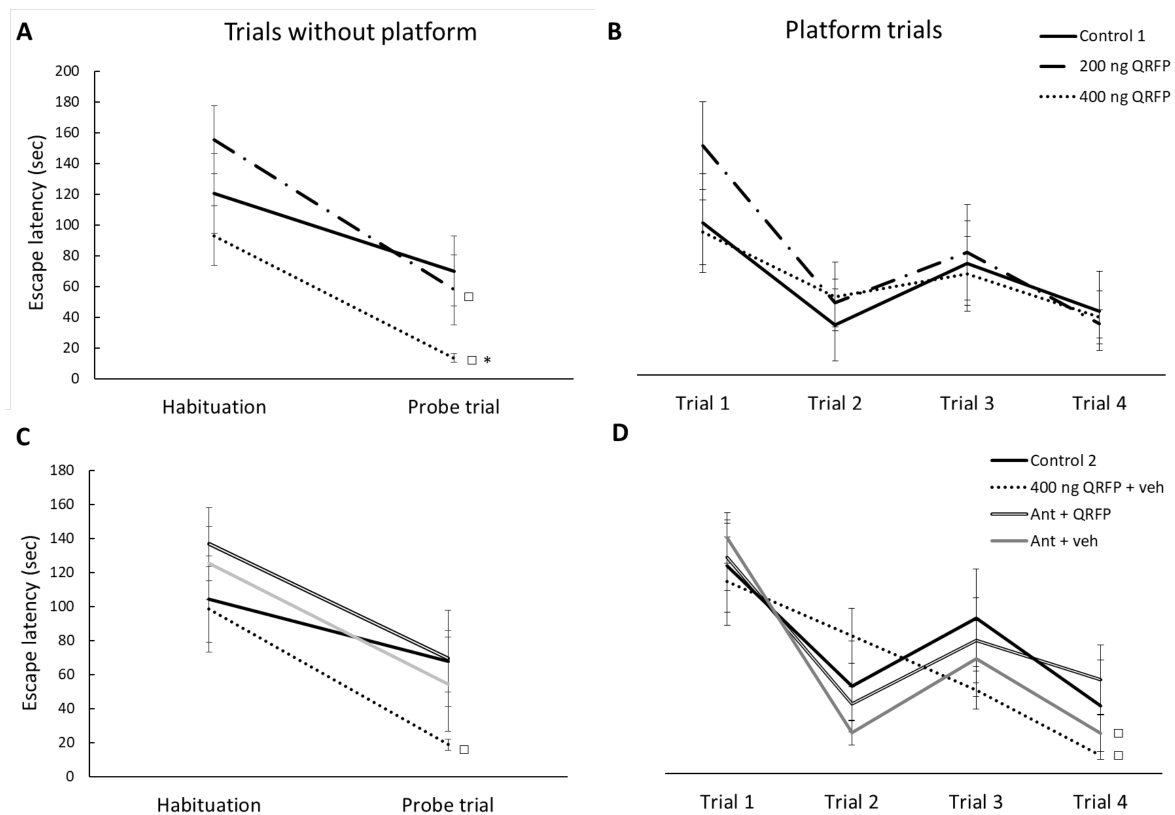
*C, D: Effects of BIBP3226 (Ant) microinjections. Time: Columns represent normalized time spent within the annuli (±S.E.M.), Frequency: Columns represent normalized number of entries to the annuli (±S.E.M.). The groups and the number of animals are identical to those present in the Fig. 20. Symbols above columns indicate significant difference: □ refers to difference for the same treatment group between two annuli, * refers to difference between the groups within one annulus (p < 0.05).*

4.4.2. Results of the MWM in LHA

The QRFP effects on escape latency in MWM following the **LHA administration** are presented in Fig. 23A, B. Analyzing the trials without the platform, two-way ANOVA revealed that there was a significant effect of trials ($F [1,38] = 21.450$, $p < 0.001$), but no significant effect of treatment or interaction between trials and treatment. According to one-way ANOVA, there were significant differences between the trials within **200 ng and 400 ng, i.e., QRFP decreased searching latencies in these groups** ($p = 0.01$, $p = 0.002$, respectively), but not for the control animals. A significant difference between the groups during the Probe trial was not registered. When the training trials 1-4 were analyzed by two-way and one-way ANOVA, significant difference could not be recorded. These findings may suggest that by the day of testing (Probe trial) the learning abilities have improved, but not as drastically as in MHA.

In the course of the following experiment the ability of BIBP3226 antagonist was examined (Fig. 23C and D). Experimental procedure implicated double volume injection to each animal with 15 minutes intervals. The two-way ANOVA analysis of trials without platform indicated a significant effect for trials ($F [1,50] = 12.475$, $p = 0.001$), however the effect for treatment and the interaction between trials and treatment was not significant. According to one-way ANOVA, when comparing Habituation and Probe trials, there were significant differences registered only within 400 ng treated animals ($p < 0.01$). **As for the Probe trial, the tendency for shorter escape latency in case of QRFP treatment in double volume was recorded, a significant difference between the groups was not found though.** Two-way ANOVA analysis was applied to compare means of four training trials to each other within each group and revealed significant differences among trials ($F [3,100] = 18.220$, $p < 0.001$), but not treatment, or trial x treatment. The QRFP + vehicle-treated animals, similarly to the first experiment, have shown shorter latencies, and by trials 3 and 4 have learned to find the platform significantly faster than in trial 1 ($p < 0.05$ and $p = 0.001$, respectively). By contrast, means of the Control, Ant + QRFP, and the Ant + Vehicle-treated groups remained similar, so the progress in learning was not registered.

Fig. 23. Effects of QRFP and BIBP3226 microinjections into the LHA on the platform finding latency (escape latency) in Morris water maze



A, B: Effects of QRFP microinjections. The drugs were microinjected immediately following the trials 2 and 4 (0.4 µl): vehicle solution (Control 1, n=7), 200 ng (n=7), 400 ng QRFP (n=8).

C, D: Effects of BIBP3226 (Ant) microinjections. Corresponding drugs (0.4 µl + 0.4 µl) were microinjected immediately following the swimming trials 2 and 4, with 15 min interval: double volume of saline treatment (Control 2, n = 6), 400 ng QRFP followed by vehicle (n=7), Ant pretreatment followed by 400 ng QRFP (n=8), Ant followed by vehicle (n=8).

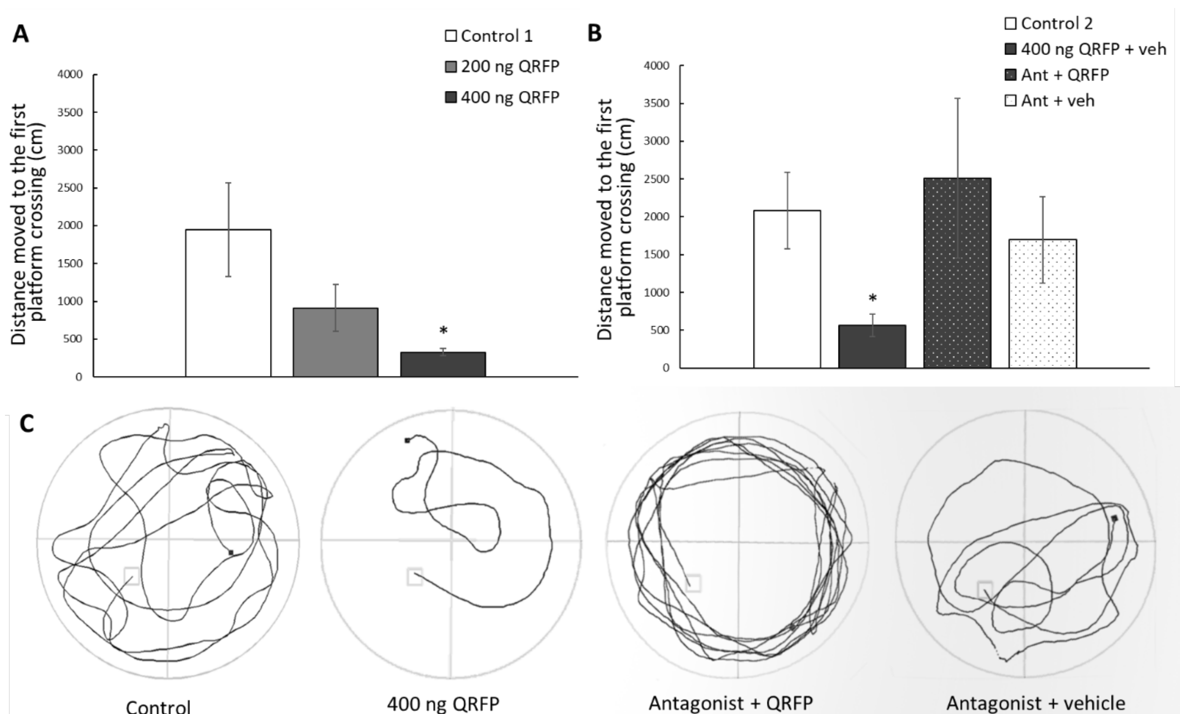
*Platform trials: line graphs represent the mean latencies to finding the hidden platform (±S.E.M.). Trials without the platform: line graphs represent the mean latencies to finding the place of the removed platform (±S.E.M.). Symbols next to the graphs indicate significant difference: □ refers to between-trial difference, * refers to between-group difference within one trial ($p < 0.05$).*

Considering the mean swimming velocity of the animals, there were no significant differences registered in any trial during both experiments (data not shown).

The distances that rats have covered during the Probe trial until they crossed the place of the removed platform are presented in Fig. 24. **Rats treated with 400 ng QRFP made significantly shorter routes compared to the control group while searching the target platform** ($F [2,18] = 4.195$, $p = 0.03$, Fig. 24A). Animals treated by the lower dose of the

neuropeptide also revealed the tendency for faster search, not reaching significant values though. Similar observations have been registered in case of double-volume microinjections during the antagonistic experiment ($F[3,24] = 3.949$, $p < 0.04$, Fig. 24B). This time **QRFP-treated rats have shown better results than the controls and significantly shorter distance comparing to Ant + QRFP group**. Images of representative trajectories for each group are present in Fig. 24C.

Fig. 24. Effects of QRFP and BIBP3226 microinjections into the LHA on average distance to finding platform place during the Probe trial in Morris water maze.



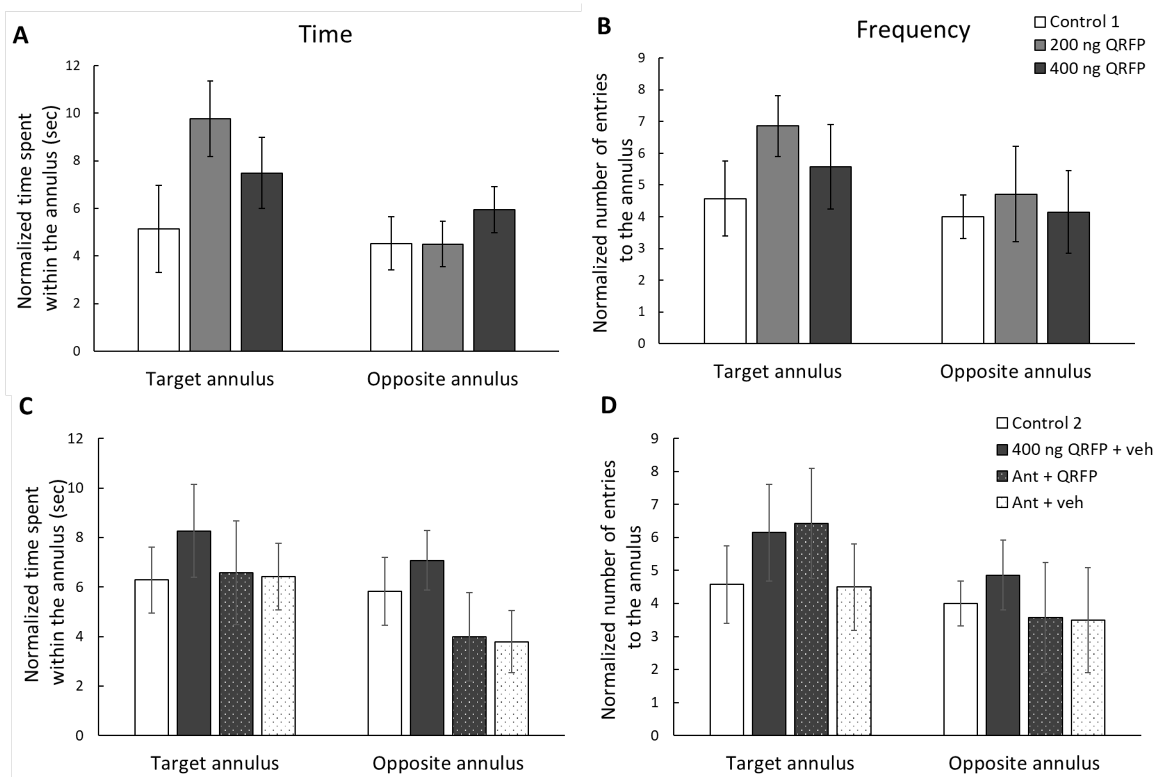
A: Columns represent distance moved to the first platform crossing during the experiment with QRFP microinjections (±S.E.M.),

B: Columns represent distance moved to the first platform crossing during the experiment with Ant (±S.E.M.),

C: Illustrative images of rats' trajectories during the Probe trial in Morris water maze. The groups and the number of animals are identical to those present in the Fig. 23. Symbol above the columns indicates significant difference ($p < 0.05$).*

The analysis of the additional parameters during the Probe trial i.e. normalized time spent within the target and the opposite annuli, as well as a number of entries, did not reveal significant difference between the groups (Fig. 25). In case of 200 ng treatment there was tendency for longer searching latency within the target annulus comparing control group and comparing to opposite annulus searching time (Fig. 25A). Observed changes did not reach significant level.

Fig. 25. Effects of QRFP and BIBP3226 microinjections into the LHA on the normalized time and number of entries to the target and opposite annuli during the Probe trial in Morris water maze



A, B: Effects of QRFP microinjections,

C, D: Effects of BIBP3226 (Ant) microinjections. Time: Columns represent normalized time spent within the annuli (±S.E.M.), Frequency: Columns represent normalized number of entries to the annuli (±S.E.M.). The groups and the number of animals are identical to those present in the Fig.23.

Altogether these results indicate the **promoting effect of hypothalamic QRFP administration on spatial memory. Both regions of the hypothalamus exerted similar changes; nevertheless, the MHA treatment had a more pronounced effect in comparison to the LHA.**

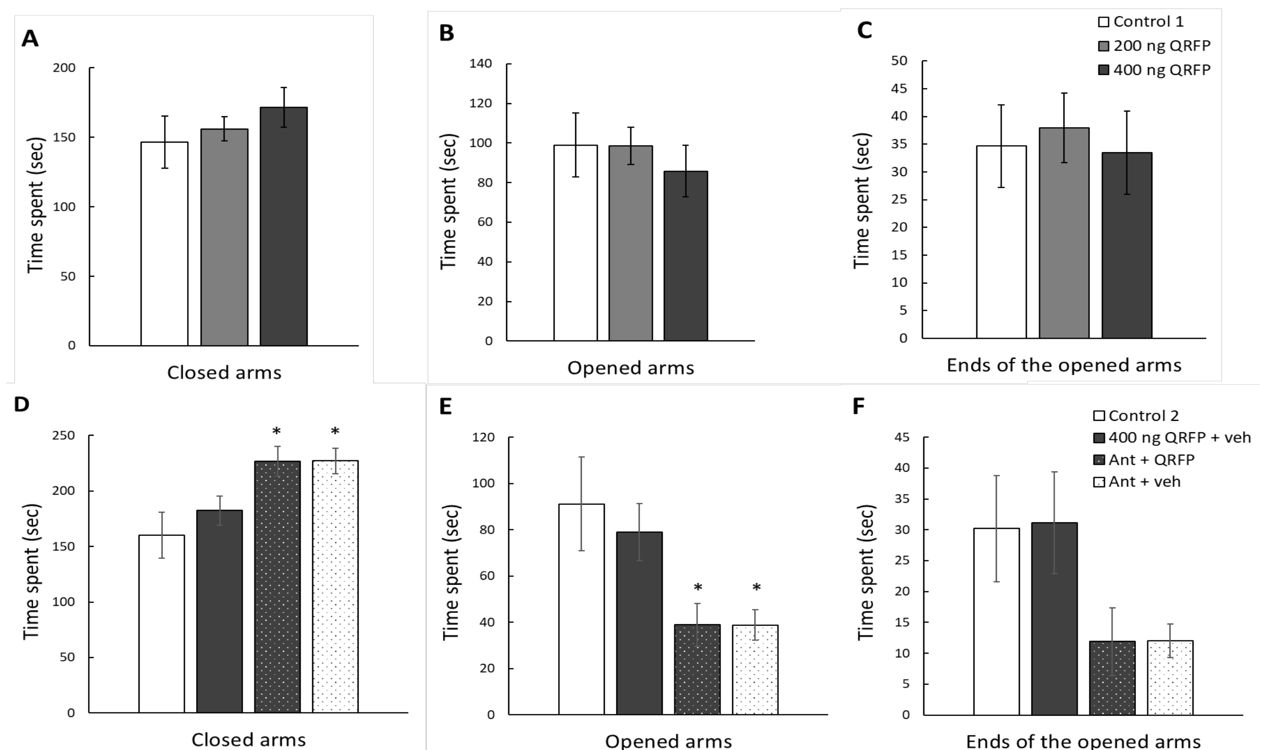
4.5. Results of the experiments on anxiety

4.5.1. Results of the Elevated plus maze in MHA

Effects of any drugs on behavioral parameters might be altered by changes in the anxiety level. The effects of hypothalamic QRFP and BIBP3226 microinjection have been investigated in the EPM paradigm. According to one-way ANOVA **the time spent** by the rats, treated with **200 and 400 ng QRFP into the MHA, in opened and closed arms of the maze did not differ significantly from the data of the control group or each other** (Fig. 26A-C).

In turn, Ant experiments indicated marked changes. One-way ANOVA analysis of closed arms yielded significant difference ($F [3,23] = 5.025$, $p < 0.01$). Post hoc test confirmed that **Ant treatment combined with QRFP, as well as Ant and vehicle treatment led to increase in time spent by rats within the closed arms** ($p < 0.03$ and $p < 0.02$, respectively, Fig. 26D). Corresponding data from the opened arms confirm significant difference between the treatment groups ($F [3,23] = 4.835$ $p < 0.01$) and suggest shorter periods of investigating opened arms following the Ant treatments ($p < 0.04$ and $p < 0.03$, respectively, Fig. 26E). Similar tendency may be observed regarding the ends of the opened arms, nevertheless, data did not reach significant level (Fig. 26F). **These changes may be interpreted as signs of increased anxiety level induced by the Ant treatment**.

Fig. 26. Effects of QRFP and BIBP3226 microinjections into the MHA on the anxiety level in the Elevated plus maze



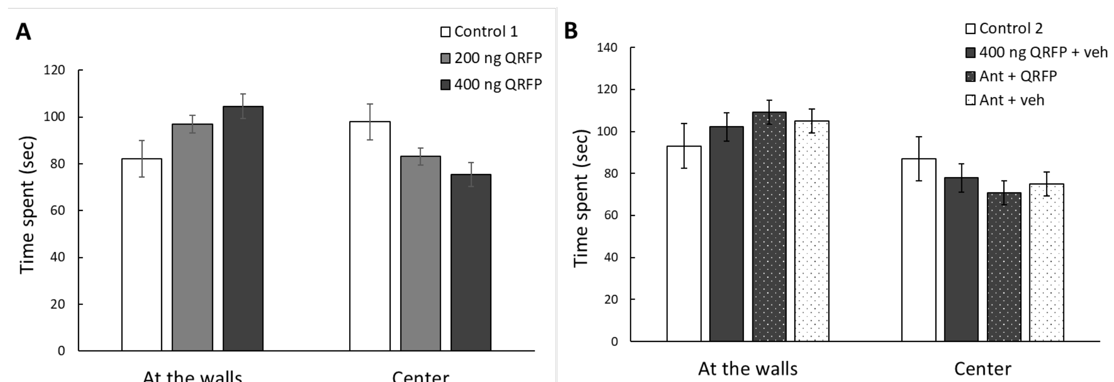
A, B, C: Effects of QRFP microinjections (0.4 µl): Vehicle treated rats (Control 1, n = 8), 200 ng QRFP (n = 10), 400 ng QRFP (n = 7).

*D, E, F: Effects of BIBP3226 (Ant) treatment (0.4 µl + 0.4 µl): Vehicle treated rats (Control 2, n = 6), 400 ng QRFP + veh (n = 6), Ant + QRFP (n = 7), Ant+veh (n = 8). Columns represent mean time spent in the closed arms, in the opened arms, and at the ends of the opened arms, respectively (±S.E.M.). Animals were tested 15 min after the corresponding treatment. * Symbols next to the graphs indicate significant difference (p < 0.05).*

4.5.2. Results of the MWM and OFT in MHA on anxiety

Besides the specific test, the performance in other paradigms was analyzed for the signs of the anxiety deviations. **In the MWM test the time spent by rats during the Probe trial in the outer area and in the central part of the pool did not differ between the treatment groups** (Fig. 27).

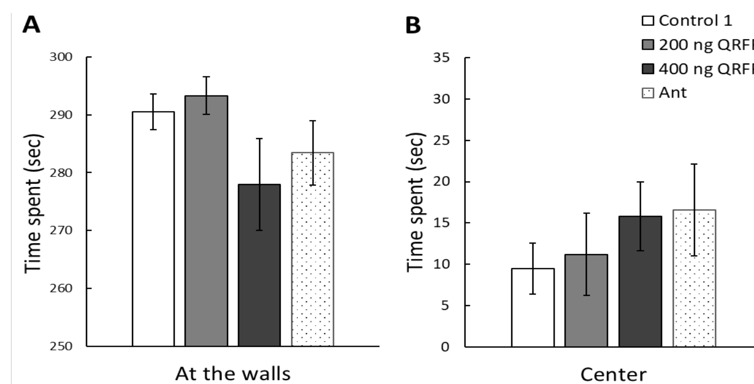
Fig. 27. Effects of QRFP and BIBP3226 microinjections into the MHA on the anxiety level during the Probe trial in Morris water maze



Columns represent the time spent at the walls, or time spent in the central area of the pool, respectively (±S.E.M.). The groups and the number of animals are identical to those present in the Fig.20. There was no significant difference recorded.

These findings correspond with the negative data received in the OFT (Fig. 28).

Fig.28. Effects of QRFP and BIBP 3226 microinjections into the MHA on the anxiety level in the Open field test



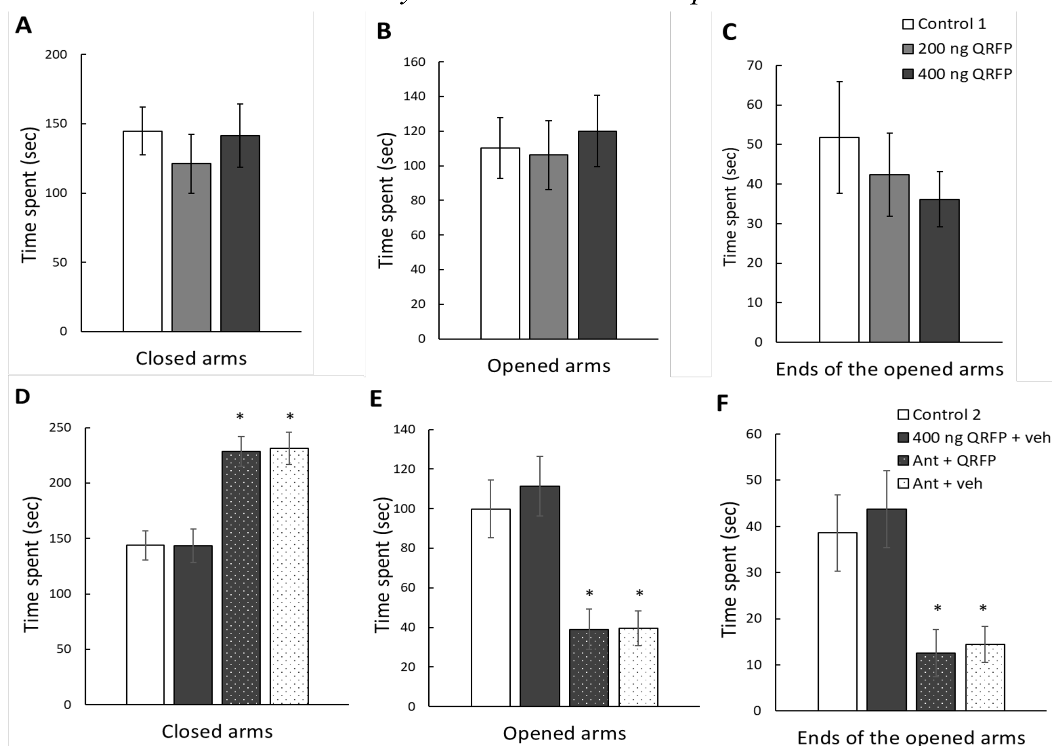
Columns represent the time spent at the walls, or time spent in the central area of the pool, respectively (±S.E.M.). The groups and the number of animals are identical to those present in the Fig. 32. There was no significant difference recorded.

4.5.3. Results of the Elevated plus maze in LHA

EPM-derived data with anxiety records following the QRFP and BIBP3226 microinjections into the LHA are presented in Fig 29. According to one-way ANOVA, **control animals, as well as low and high- dose QRFP treated rats spent similar time in the closed and opened arms of the maze** (Fig. 29A-C).

One-way ANOVA analysis of the experiment with Ant suggested significant difference between the groups ($F [3,24] = 12.051$, $p < 0.01$ for closed arms, $F [3,24] = 10.249$, $p = 0.01$ for opened arms, and $F [3,24] = 6.330$, $p < 0.05$ for the ends of the opened arms). **Both combined Ant + QRFP treatment and Ant + veh led to significant increase in time spent in the closed arms** ($p < 0.01$ for both groups), shorter periods of investigating the opened arms ($p = 0.01$ for both groups) and somewhat the most distal parts, i.e. the ends of the opened arms ($p < 0.05$ for both groups, Fig. 29D-F). In other words, **Ant treatment in LHA seems to induce an anxiogenic effect in EPM test**.

Fig. 29. Effects of QRFP and BIBP3226 microinjections into the LHA on the anxiety level in the Elevated plus maze



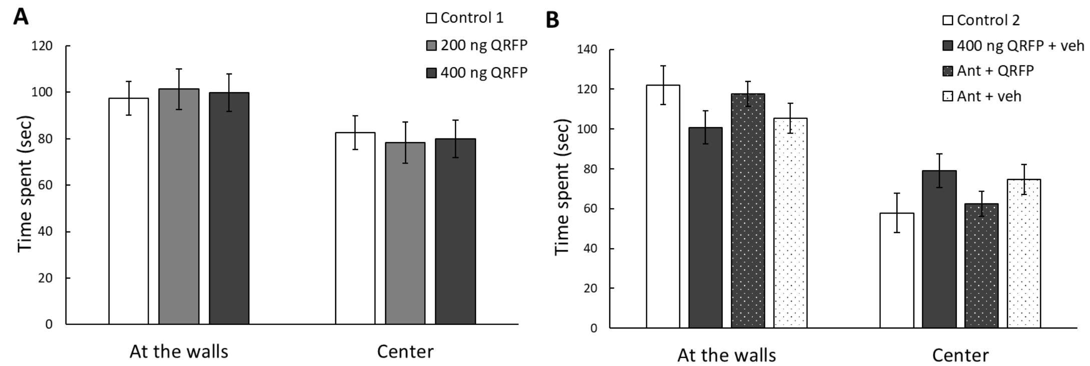
A, B, C: Effects of QRFP microinjections (0.4 µl): Vehicle treated rats (Control 1, n = 7), 200 ng QRFP (n = 8), 400 ng QRFP (n = 7).

*D, E, F: Effects of BIBP3226 (Ant) treatment (0.4 µl + 0.4 µl): Vehicle treated rats (Control 2, n = 6), 400 ng QRFP + veh (n = 7), Ant + QRFP (n = 7), Ant + veh (n = 8). Columns represent mean time spent in the closed arms, in the opened arms, and at the ends of the opened arms, respectively (±S.E.M.). Animals were tested 15 min after the corresponding treatment. * Symbols next to the graphs indicate significant difference (p < 0.05).*

4.5.4. Results of the MWM and OFT in LHA on anxiety

Animals' performance in other paradigms was analyzed for the signs of the anxiety deviations. **Following the LHA microinjections, in the MWM test the time spent by rats during the Probe trial in the outer area and in the central part of the pool did not differ between the treatment groups** (Fig. 30).

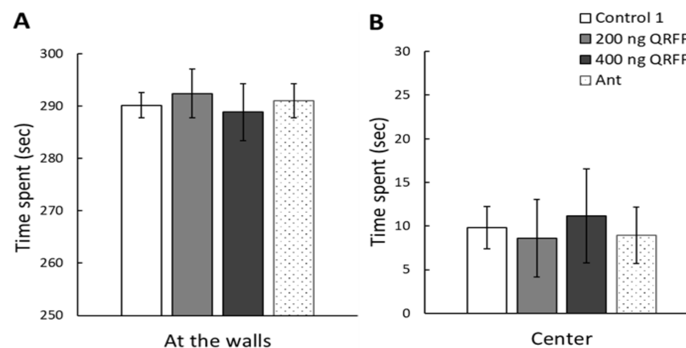
Fig. 30. Effects of QRFP and BIBP3226 microinjections into the LHA on the anxiety level during the Probe trial in Morris water maze



Columns represent the time spent at the walls, or time spent in the central area of the pool, respectively (±S.E.M.). The groups and the number of animals are identical to those present in the Fig.23. There was no significant difference recorded.

These findings correspond with the negative data received in the OFT (Fig. 31).

Fig.31. Effects of QRFP and BIBP 3226 microinjections into the LHA on the anxiety level in the Open field test



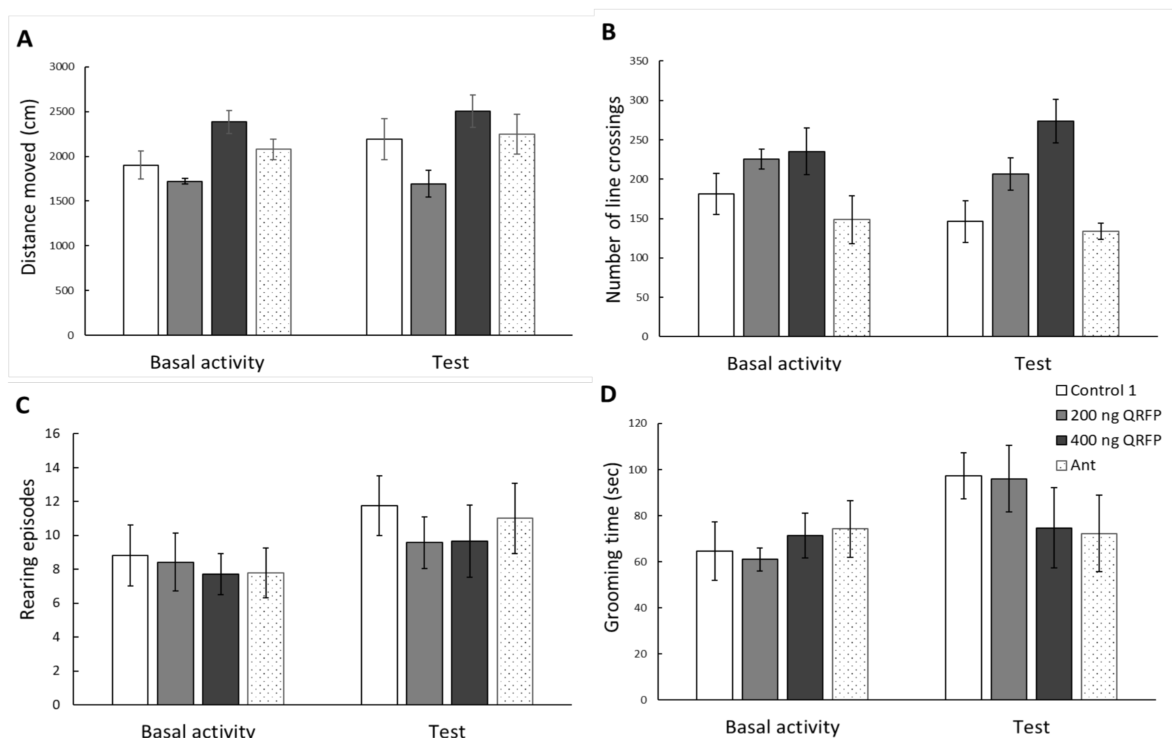
Columns represent the time spent at the walls, or time spent in the central area of the pool, respectively (±S.E.M.). The groups and the number of animals are identical to those present in the Fig. 33. There was no significant difference recorded.

4.6. Results of the experiments on general locomotion

4.6.1. Results of the Open field test in MHA

OFT was employed as a specific paradigm for observation of the treatment effects on the locomotion, horizontal and vertical explorative activity. The ANOVA evaluation of spontaneous motor activity parameters, such as total distance moved (Fig. 32A) and a number of crossings (Fig. 32B), **did not reveal a significant difference between the control, low and high doses of QRFP and Ant-treated groups following the MHA microinjections**. Similarly, no significant difference was detected in the other behavior patterns, such as rearing (Fig. 32C) and grooming (Fig. 32D).

Fig.32. Effects of QRFP and BIBP3226 microinjections into the MHA on general locomotion in the Open field test



Effects on general locomotion produced by Vehicle solution (0.4 µl, Control 1, n=8), 200 ng QRFP (n=8), 400 ng QRFP (n=7), Ant (n=8). Columns represent:

A: distance moved (cm) (±S.E.M.),

B: number of virtual lines crossings (±S.E.M.),

C: number of rearing episodes (±S.E.M.),

D: time spent with grooming (sec) (±S.E.M.). There was no significant difference.

4.6.2. Results of the EPM, CPP and MWM in MHA on locomotion

Besides the OFT, additional data reflecting the correlation between the treatments and locomotion, have been collected during the other experiments. Distances moved by the animals within the EPM following corresponding microinjections are presented in Table 4.

Table 4. Distances moved by the different treatment groups (MHA) during the experimental trial in the Elevated plus maze. Numbers represent the average values (±S.E.M.)

	Control 1	200 ng QRFP	400 ng QRFP	Control 2	400 ng QRFP + veh	Ant + QRFP	Ant + veh
Distance moved (cm)	**1689 (±180)**	**1493 (±94)**	**1354 (±107)**	**1804 (±186)**	**1596 (±203)**	**2121 (±176)**	**1938 (±96)**

Locomotive data have been recorded during the CPP and the MWM tests as well (Table 5). In both paradigms, the distance moved by the animals on the day of testing (i.e. Probe trial, or Test) was analyzed. Important to note, that according to the experimental protocols, these data represent long-lasting (24h) effects of the drugs.

Table 5. Distances moved by the different treatment groups (MHA) during the test trial in the CPP and Probe trial in the MWM. Numbers represent average values (±S.E.M.).

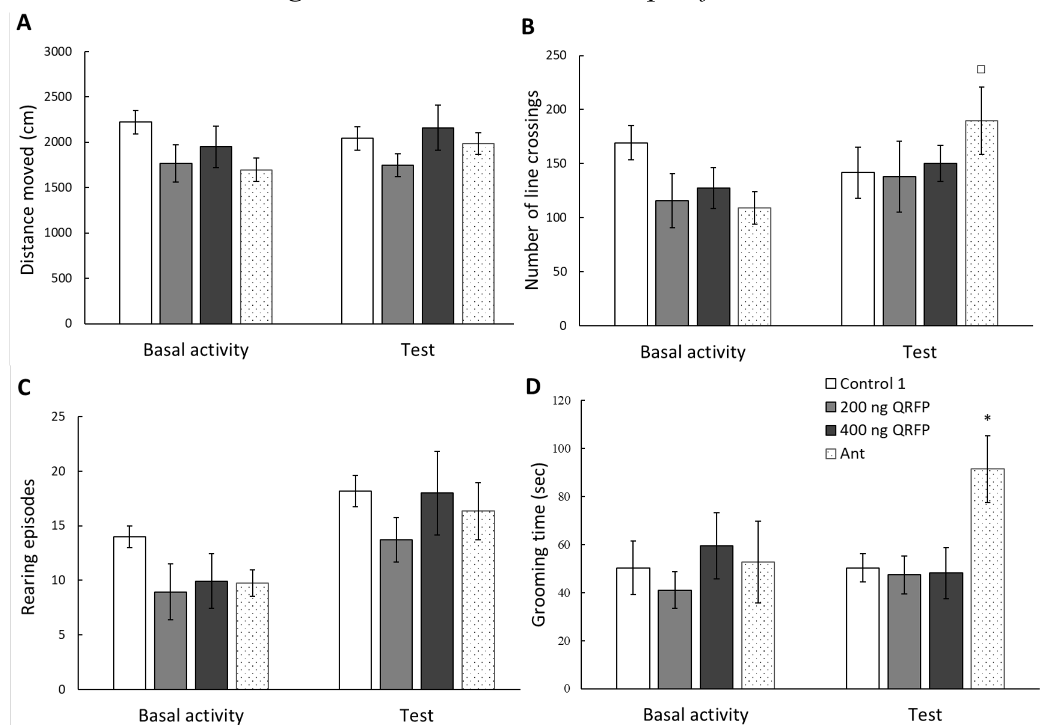
	Control 1	200 ng QRFP	400 ng QRFP	Control 2	400 ng QRFP + veh	Ant + QRFP	Ant + veh
Distance moved in CPP test (cm)	**5373 (±469)**	**5627 (±484)**	**5106 (±618)**	**5656 (±370)**	**5286 (±768)**	**5392 (±798)**	**5139 (±417)**
Distance moved in MWM test (cm)	**4182 (±283)**	**4657 (±254)**	**4807 (±207)**	**4383 (±395)**	**4745 (±247)**	**4452 (±190)**	**4400 (±315)**

There was no significant difference among the groups recorded neither in the EPM nor in the CPP or MWM. The moving abilities on the floor, as well as in the water, remained normal independently on applied treatment.

4.6.3. Results of the Open field test in LHA

Effects of the microinjections into the LHA on spontaneous locomotion are presented in Fig. 33. The ANOVA evaluation of the distance moved in the OFT (Fig. 33A) and rearing episodes (Fig. 33C) **did not reveal a significant difference between the groups following the LHA microinjections**. Altogether, all the treated groups performed somewhat higher rearing activity at the test trial, so this tendency is unlikely to be in direct connection with effects of injected drugs. Nevertheless, one-way ANOVA yielded a significant difference between the trials when analyzing **number of arena crossings** ($F[1,12] = 5.497$, $p < 0.04$, Fig. 33B) Similarly, difference was detected in grooming activity ($F[3,22] = 3.991$, $p < 0.03$, Fig. 33D). According to post hoc test, **Ant treated animals spent significantly more time with grooming comparing to control and QRFP- treated rats during test trial** ($p=0.05$, $p<0.04$, $p<0.04$, respectively). Also, a tendency for longer grooming latency during the test trial in comparison to basal activity data was noticed in Ant group (no significant difference).

Fig.33. Effects of QRFP and BIBP3226 microinjections into the LHA on general locomotion in the Open field test.



Effects on general locomotion produced by Vehicle solution (0.4 µl, Control 1, n=6), 200 ng QRFP (n=7), 400 ng QRFP (n=7), Ant (n=6). Columns represent:

A: distance moved (cm) (±S.E.M.),

B: number of virtual lines crossings (±S.E.M.),

C: number of rearing episodes (±S.E.M.),

*D: time spent with grooming (sec) (±S.E.M.). *Symbol next to the graph indicate significant difference (p < 0.05).*

4.6.4. Results of the EPM, CPP and MWM in LHA on locomotion

Supportive data from the EPM (Table 6)**, as well as the CPP and MWM tests** (Table 7) **confirm that applied treatments did not modify ability to move during the experiments**.

Table 6. Distances moved by the different treatment groups (LHA) during the experimental trial in EPM. Numbers represent the average values (±S.E.M.)

	Control 1	200 ng QRFP	400 ng QRFP	Control 2	400 ng QRFP + veh	Ant + QRFP	Ant + veh
Distance moved (cm)	**1830 (±216)**	**1599 (±154)**	**1608 (±247)**	**1904 (±74)**	**1829 (±167)**	**2014 (±92)**	**1969 (±141)**

Table 7. Distances moved by the different treatment groups (LHA) during the test trial in CPP and probe trial in MWM. Numbers represent average values (±S.E.M.).

	Control1	200 ng QRFP	400 ng QRFP	Control 2	400 ng QRFP + veh	Ant + QRFP	Ant + veh
Distance moved in CPP test (cm)	**5315 (±497)**	**5653 (±498)**	**5692 (±577)**	**6822 (±600)**	**6370 (±498)**	**5965 (±647)**	**6044 (±466)**
Distance moved in MWM test (cm)	**4794 (±218)**	**4303 (±202)**	**4850 (±265)**	**4975 (±526)**	**4518 (±356)**	**5043 (±283)**	**4534 (±309)**

5. Discussion

5.1. Discussion of the feeding experiments

Specific tissue distribution of the QRFP-expressing neurons within the hypothalamus led to intense research of the neuropeptide's role in feeding mechanisms. Initial studies in food-restricted and satiated mice discovered that i.c.v. administration of QRFP causes a dose-dependent increase in chow consumption [17, 18, 263]. In turn, studies in other species reported inconsistent results. First, experimental works in rats suggested that central administration of QRFP had no effects on feeding behavior and energy expenditure [267, 299]. Nevertheless, in food-restricted rats, i.c.v. administered QRFP increased food consumption [297]. QRFP was shown to promote food intake in birds (chick and zebra finch) as well [298, 354]. Interestingly, only those chicks consumed more food, which had been selected for meat production, while in lower-eating layer chicks QRFP did not induce such changes.

Analyzing diverse findings, we concluded several important factors that could drastically influence the outcomes of the experiments. The first factor is the drug itself. It has been proven that 43-aminoacid form of QRFP is slightly more potent in docking to QRFP receptor, as well as in inhibition of cAMP formation and stimulation of [Ca2+]i mobilization [261], comparing to a 26-aminoacid residue. According to frequent among the neuropeptides inversed U-shape dose/effect activity, too low, as well as too high concentrations of the neuropeptide could lead to negative results. The second point to consider in this matter is the method of drug delivery. The i.c.v. administration implies that the chemical substance is injected into the cerebral ventricle, gets mixed with the liquor, and following that, is diffused along the ventricle walls. With no doubt, this way of treatment has multiple advantages. Nevertheless, the diffuse action on multiple periventricular structures, and the impossibility to predict the exact drug concentration reaching the parenchyma of specific brain regions, may become a considerable limitation. In contrast, direct delivery of the chemicals into the brain structures can be a good alternative to i.c.v. allowing more precise monitoring of the induced changes. The experimental paradigm itself is another important point for discussion. Our results confirm that QRFP has rapid and short action on food intake [355]. Measurements in two-three or more hours may not register modification of the feeding behavior due to its temporary character. We also contemplate the liquid food drinking from the graduated tube as a more advantageous substrate in comparison to standard chow. This method [344, 345, 348] allows frequent and precise monitoring of food consumption without interrupting physiological eating acts. Finally, we would like to discuss experimental meals. Moriya et al. [296] first noticed increased body weight and adiposity in mice fed with a moderately fat diet in contrast

to ones fed with standard chow. Later, it was confirmed by multiple experiments of Primeaux et al., where rats were centrally injected with QRFP. Animals manifested hyperphagia exclusively on a food enriched by fats, while consumption of low-fat meals remained unchanged [14, 300, 356]. These data suggest that QRFP's orexigenic effect depends on the macronutrient profile of the diet as well.

5.1.1. Discussion of the experimental results

We have designed the feeding-related experiments in accordance with the aforementioned factors. Our observations suggest that QRFP administration into the medial hypothalamic nuclei VMN and DMN leads to hyperphagia. The orexigenic effect of QRFP was quite rapid, observed already 10 min after peptide administration (Fig. 14). Our data represent the cumulative value of food intake, so the difference remained significant during whole period, i.e. during 1 hour. The effective doses of QRFP delivered into the brain parenchyma (100 ng and 200 ng) have been established for the first time in our experiments. Herein, non-peptide antagonist BIBP3226 was applied in the equimolar amount (18 ng, 38 pmol) to the effective dose of QRFP (100 ng, 35 pmol). Treatment by Ant, as well as combined Ant and QRFP treatment, effectively suppressed the orexigenic effect caused by the neuropeptide and even transiently reduced food consumption about 5–25 min after injection (Fig. 15). By the end of the second hour appetite-modifying effects of the neuropeptide QRFP and Ant were compensated, so the food consumption of different treatment groups reached a similar level (data not shown).

In turn, the LHA administration of QRFP led to the opposite, anorexigenic effect. Both 100 ng and 200 ng rapidly and effectively decreased animals' food consumption (Fig. 16), but the lower dose had a stronger and more elongated effect (from 5th to 50th min). Ant treatment prior to QRFP and Ant treatment with the vehicle fully prevented deviations in consumed milk amount during all the observed period (Fig. 17) and for two more hours after (data not shown).

5.1.2. Discussion of possible mechanisms

According to the theory of hypothalamic "feeding and satiety centers", stimulation of the VMH should lead to lower food consumption and satiation, while stimulation of the LH would cause hyperphagia. However, our data suggest that QRFP administration induces opposite effects.

Presence of specific QRFP receptors in the hypothalamus in high density provides possibility for QRFP to modify feeding behavior via other neuropeptides. The role of the NPY system comes forward as possible mechanism of action. It was shown in genetically modified

mouse models (ob/ob and db/db), that leptin, known to regulate feeding behavior via the NPY system, also affects hypothalamic expression of preproQRFP mRNA [18]. Neuroanatomical observations in the Arc revealed a subpopulation of the NPY, but not POMC neurons, expressing QRFP receptors [297]. Several studies confirmed attenuating effects of NPYY1 receptor Ants, as well as Y5 subtype [18, 297, 356]. It was proposed that QRFP activates specific receptors in the Arc thus inducing NPY production, which in turn binds NPY1, and NPY5 receptors and blocks POMC synthesis leading to an increase in food intake. The main source of the NPY within the hypothalamus is exclusively the Arc, except for the special states of the negative energy balance when NPY synthesis transiently takes place in the DMN as well [357]. Robust prevention of QRFP-induced hyperphagia in MHA and decreased food consumption in LHA by non-peptide NPY/NPFF Ant BIBP3226 in our experiment supports a proposed concept (Fig. 15).

Specific QRFP receptors were shown to share nearly 50% of the amino acidic identity with cousin G protein-coupled receptor subtypes: NPFF1 and FF2, Orexin R1 and R2, the binding sights of the neuropeptides strongly involved in feeding regulation [260, 269, 270]. This way, an alternative explanation for the observed effects can be a cross-reactivity of the neuropeptide QRFP with NPFF and orexin systems. Orexin A study on OX knock-out mice suggests that QRFP-induced food consumption is independent of the OX signaling pathway since knock-out animals presented the same response on QRFP treatment as wild type [18]. Thus, this mechanism of action is unlikely to be involved.

Neuropeptide NPFF system is another possible candidate for mediation of feeding regulation. FF binding sites are widely present across the hypothalamus [325, 358] and veraciously have projections towards MeA, BMA, BLA, and AHip [359]. Moreover, the general anorexigenic effect of NPFF was proven to act via central μ and ƙ subtypes of opioid receptors with the mediation of NPY and β-endorphin [360]. These data are supported by other findings: concentration of ƙ receptors was higher, while μ lower in obese mice compared to lean ones [361]. Morphine exposure of the hypothalamus and pituitary in mice led to strong upregulation of NPY, AgRP, and some other neuropeptides involved in feeding [362]. Other multiple pieces of research suggest the possibility of the opioid system's role in NPY – induced regulation of feeding (for review see [363]).

Thus, **orexigenic effects produced by QRFP microinjections into the MHA and anorexigenic reaction after the LHA administration** are most likely to be linked to QRFP-NPY/POMC - regulating pathway. Possible involvement of the NPFF, opioid, and other neurotransmitter systems (NA, DA, 5-HT, etc.) may be a subject for further research.

5.2. Discussion of the Conditioned place preference test

The robust changes in feeding behavior, induced by QRFP administration into both "feeding centers" of the hypothalamus, put an idea into the head to investigate rewarding or aversive effects of the drug. The feeling of satiation is a positive emotional state, while hunger on the other side of the scale causes highly unpleasant sensations. When a stimulus (drug) induces a preference for the associated environment, it is considered conditioning learning. The CPP is a demonstrative paradigm to investigate rewarding or negative associations by coupling them with the surrounding environment, which turns into the conditioned stimulus.

By now there were no scientific data available regarding documented rewarding or aversive effects of QRFP. To avoid misleading results in CPP paradigm we employed a biased design, i.e. the preference of each individual subject within the apparatus was assessed prior to conditioning (during Habituation trial). Neither the least nor the most preferred quadrant was chosen for drug pairing to allow proper registration in case of aversion.

5.2.1. Discussion of the experimental results

To our surprise, microinjections of QRFP neither in lower nor in higher doses into the MHA (Fig. 18) or the LHA (Fig. 19) modified animals' place preference. The time spent within TQ during the test trial was similar to other quadrants. Ant treatment in MHA had no changes as well. In turn, LH administration of Ant led to longer time spent within the TQ comparing to all other treatment groups during the test trial and comparing to naïve state during habituation (Fig. 19). The effect was not observed in the case of QRFP microinjection following Ant administration (Ant + QRFP treatment group) indicating unstable, convertible nature of the changes. Our registrations suggest that the frequency of entries into the TQ remained at a basic level.

These data may indicate, on one side, that the Ant administration into the LHA evoked conditioned place preference. The other possibility is that animals spent more time in TQ because they experienced anxiety or debilitation of movements following Ant administration (also discussed later).

5.2.2. Discussion of possible mechanisms

QRFP administration neither into the MHA nor the LHA affected animals' performance in CPP paradigm. Only microinjections of Ant BIBP3226 applied into the LHA (but not MHA) led to significant changes in the CPP test.

Assuming the real conditioned learning, one of the explanations of the observed phenomenon might be the involvement of orexin. OX neurons are exclusively localized to the perifornical area, DMN, PHA, and LHA in the rat and in the human brain [364-367]. Due to different input/output connections, only LH OX neurons are primarily associated with reward-seeking function and abuse behavior [368-372]. Concurrent activation of the VTA and NAcc by dense projections from OX neurons could lead to expressed place preference in the CPP test. NPY was shown to inhibit orexin neurons by multiple presynaptic and postsynaptic mechanisms [373], including linkage to Y1 receptors. This way microinjections of BIBP3226 might have caused disinhibition of the LH orexin system and induced a cascade of a rewarding experience. Moreover, neuropeptide ghrelin was shown to enhance the rewarding value of a high-fat diet as it was recorded in CPP and operant conditioning with the involvement of OX1 receptors [374]. To the point, GHSRA1 receptors are widely abundant within the VTA, and ghrelin administration to this region causes DA release, as was confirmed by in vitro and in vivo experiments (for review see [375]). At the level of the VTA, opioid signaling (but not NPY) is required for ghrelin's effects on food motivation [376].

At the same time, place preference and the features of anxiety behavior occurred in the same animals treated by Ant.

Thus, in our experiments **QRFP did not evoke place preference** neither in the MHA nor in the LHA. Observed **Ant-induced place preference-like feature following LHA administration are likely to be linked to anxiogenic effect**.

5.3. Discussion of the Morris water maze

The MWM is a robust and reliable method developed decades ago to assess spatial learning abilities in rodents [377]. With the assistance of surrounding cues animals navigate within the opened pool towards a hidden platform. Repeated trials ensure the formation and consolidation of the spatial learning, and the result is evaluated at the probe trial based on several parameters of searching effectiveness of the removed platform.

Despite multiple physiological actions of QRFP, the neuropeptide's role in memory and learning processes was not a topic of research until our report.

5.3.1. Discussion of the experimental results

The two experiments in MWM revealed a similar „pattern" for the training trials in regard to control and QRFP-treated groups (Fig. 20). In the very first training trial, animals, habituated to the pool only, did not even know about the existence of a platform hidden in the water. Looking for an escape, rats eventually found it by themselves, or, if not, were guided towards by the researcher. Surrounding cues allowed memorizing the life-saving platform position. By trial 2, one minute later no significant learning was registered, though a tendency for shorter searching latency could be noticed. Supposedly, these new neuronal interconnections are positively affected and reinforced by QRFP microinjections. By the next day in the swimming trial 3, some difference between treatment groups could be observed: while the control animals required a longer time to find the platform, seeming to forget the route learned the day before, QRFP treated rats, especially the ones treated with 400 ng, reached the target area within the time similar to the previous trial, and even improved a little bit the time, thus reaching a significant level. The training trial 4 again showed the formation of short-term memory in those groups, which was reinforced by the second microinjection. By the test trial with the removed platform (probe trial), it became evident that control, as well QRFP- treated rats in both doses successfully learned where to search for the escape platform. The difference is that rats treated with 400 ng find the platform area faster comparing to the habituation trial and compared to the control and lower dose treated group during the same test. These findings have been confirmed by additional parameters, measured during the probe test: 400 ng QRFP animals took a shorter route to the place of platform and spent less time searching the target in the „wrong" place opposite to platform annulus (Fig. 21 and 22).

We suggest that the consolidation of short-term spatial memory was improved by 400 ng QRFP administration. We could notice that one microinjection (after trial 2) did not lead to the desired cognitive effects. The second microinjection (after trial 4) was required to reinforce that tendency and to decrease platform searching time to a significant level. Based on these data we suppose that QRFP microinjections lead to a positive learning effect, nevertheless, probably more than two administrations are required to make it stable and long-lasting. In contrast to vehicle and peptide treated groups, animals treated with antagonist did not show memory formation during the training trials (Fig. 20B). In turn, rats that received Ant and combined Ant + QRFP treatments seemingly lacked the ability to learn the location of the platform after four training trials. It took them much longer time (and distance) to reach the escape area (Fig. 20B, 21B, C).

LHA results in response to QRFP administration revealed that both 200 ng and 400 ng treated groups have learned the place of the platform, but higher dose made searching time significantly shorter compared to controls and the lower dose (Fig. 23). The pattern of the training

trials shows that the learning process in all the groups went in a parallel manner. Similar to the MHA, here the second microinjection (following trial 4) was of vital importance to induce improvements in memory. These findings are supported by the additional analytic data of the probe trial. Distance moved to the first platform crossing (Fig. 24) was much shorter for the 400 ng treated group. The Ant treated groups failed to show learning progress, it took them longer time and searching route to find the escape platform (Fig. 23, 24).

Previously we have already shown that in the case of direct intrahypothalamic microinjections 100 and 200 ng doses of QRFP effectively increased food intake in rats in MHA [378] and decreased when applied in LHA. Interestingly, in the present experiment lower dose was not so effective, but a 400 ng dose of QRFP significantly positively affected the memory consolidation in both MHA and LHA. We suppose, the reasons for this phenomenon lay in the anatomical and physiological features of the hypothalamus. QRFP administration directly into the place of action, i.e. "feeding centers" led to changes in feeding behavior by modulating NPY/POMC system [297]. At the same time, medial hypothalamic nuclei and LHA have rich interconnections between each other and to other brain structures, which can be one of the reasons for such a wide QRFP action spectrum. This way, probably, higher doses of peptide were required to reach the effect that was initiated in the hypothalamus but performed through the other brain areas, directly involved in the formation of spatial memory, such as the hippocampus, retrosplenial, entorhinal, or prefrontal cortex.

Since the rats treated with 400 ng QRFP in both experiments found the place of platform much faster than all the others, undoubtedly the neuropeptide has a memory-reinforcing effect. Another question is whether this improvement refers to the true spatial memory, or not. Indeed, as was noticed by Morris, the escape latency itself is not a sufficient parameter to measure spatial memory [379]. In this case, the probe trial provides a great opportunity to analyze whether the observed shortening of the latencies to finding platform happened due to place-specific learning and memory consolidation, or due to other factors. The most common way for such assessment is the comparison of time spent in the different quadrants of the maze, mainly target and opposite quadrants. An even better option is to use an imaginary annulus surrounding the platform and the mirroring annulus in the opposite quadrant since these areas are more representative in the meaning of place-specific learning. Another assessed parameter was the number of entries to the target and opposite annuli (Fig. 22, 25). To determine the change in place preference we have subtracted the time spent and the number of entries during the habituation swimming from those during the probe trial in the case of each animal. The remained difference reflected the place preference which is the result of learning [352].

The analysis of the probe trials in both MHA and LHA experiments revealed that all the treated groups demonstrated a similar preference to the target annulus, as the normalized time, and the normalized number of entries to the target annulus, were the same. The fact that the normalized time spent in the target annulus remained positive suggests that the incentive value of the platform was not changed by any of the drugs. In the case of the MHA, QRFP treatment leading to the shorter escape latency and distance, combined with the relatively short time searching around the target and higher number of entries, suggest that the animals initially searched for the platform in its original place, then not finding it they continued searching around involving wider area but consistently coming back and crossing the target annulus. These rats were quite specific in their searching strategy – even though not concentrating attention around the exact place of the platform, they did not go too far and demonstrated a lack of interest in the opposite annulus (spent there a significantly less time and had lower number of entries comparing to the target annulus, and compared to the other treatment groups, Fig. 22). The probe trial in the LH experiment showed that these animals also improved the memory-relevant parameters, since the escape latency and the distance moved to the platform were much shorter (Fig. 23, 24). Nevertheless, their searching strategy differed from those in MHA groups. As it can be seen at Figures 24C and 25, animals did not remember the specific place of the platform and searched for it with the same diligence in target and in opposite annuli. In contrast, antagonist-treated groups in both hypothalamic sights demonstrated another strategy. According to their latencies to finding platform, by the probe trial, these animals did not learn where the platform was situated and searched for escape randomly around the pool. Equally considering the target and the opposite annuli as possible escape areas, they spent there similar time and had a similar number of entries.

5.3.2. Discussion of possible mechanisms

According to the modern view, there are two distinct networks within the brain that ensure navigation in the MWM task. The first is the navigation based on distal cues of the surrounding environment, so-called allocentric navigation (based on object-to-object relations). The coordinating centers of this system are located in the hippocampus and the entorhinal cortex. The specific "place cells" found in these structures are responsible for the constant mapping of the surrounding cues as the animal moves, and thus allow spatial navigation. Moreover, the entorhinal cortex (along with the pre- and parasubiculum) contains edge-recognition cells, grid cells and participates in head-movement coordination also essential for platform search (for review see [380]). From the anatomical point of view, there were established direct pathways between VMN, DMN, and LHA with multiple hippocampal structures: CA1, CA2, subiculum, retrohippocampal

region [33, 34, 381]. Besides, there are several alternative paths considering the interconnections within the parts of the hippocampus, within the hypothalamic nuclei, and thalamic projections [33-35, 382-384]. Communication between the hypothalamus and the entorhinal cortex is possible indirectly across the hippocampus and thalamus.

Another brain network is responsible for egocentric navigation, based on subject-to-object relations, i.e. on internal and proximal cues. The brain regions involved in egocentric learning, are located within the medial and posterior parietal cortex. Head-direction cells essential for this type of coordination as well, are registered beside the cortical structures within the thalamus, mammillary nucleus, retrosplenial cortex, and striatum. The striatum was even named by Miyoshi et al. a "helmsman" of the hippocampus that navigates a ship to a safe port [385]. The anatomical basis for the connection of the MH and LHA with the egocentric learning network is given by multiple efferent pathways towards these brain regions.

It is suggested that allocentric neuronal network in rodents correlates with semantic (memory of facts and places) and episodic (order of events) memory in humans. The egocentric network seems to be responsible for episodic and procedural types of memory [386, 387]. It is important to note that beside we try to distinguish the two systems, they do not compete but rather cooperate and overlap one another.

In relation to our research, QRFP mRNA is mainly found in the medial hypothalamic cells [272], while specific and non-specific receptors are highly present in both allocentric and egocentric brain networks. The hippocampal subiculum and presubiculum, as well as dentate gyrus, indusium griseum, and fields 1-3 of CA, the entorhinal cortex, as well as thalamic nuclei, mammillary nuclei, basal ganglia, ventral pallidum, and striatum reveal moderate to the very high density of QRFP binding sites [267, 272]. Our results on QRFP administration remain contradictory in a matter of specificity of learning effects. On one hand, there are some features of spatial learning, as the animals treated with 400 ng dose spent less time in the opposite annulus. But still, there was no unequivocal preference towards the target annulus. When applied to LHA, QRFP did not narrow the searching area but improved total latency, suggesting other than „true" spatial learning mechanisms.

The role of the specific QRFP receptor (GPR103) has not been investigated yet in terms of involvement in cognitive processes. Nevertheless, previous reports, in accordance with the present data, suggest the potential role of NPY and FF receptors. Activation of Y1 and Y5 co-expressing neurons in knock-out mice enhanced spatial memory retention [388]. In rats with AD-like phenotype Y1 agonists prevented impairment of spatial memory, while BIBP3226 caused the opposite effect [389]. In addition, increased NPY gene expression was observed in hippocampal dentate interneurons of rats several hours after spatial learning performances in the MWM test

[390]. NPFF and its FF2 preferring agonist mildly impaired both short- and long-term memory tested in object location and MWM tasks [308]. The other study revealed a dual effect of NPFF administration: in low doses slightly improved, while in high doses significantly reduced spatial acquisition [307]. Despite diverse memory-associated effects, there is a reason to believe in the modulatory influence of QRFP via Y1, FF1, and/or FF2 receptors. Whether these effects are connected to QRFPRs as well, is a topic for further investigation.

This experiment provides the first data of such kind. Two important conclusions have been made according to the received data. First, the **QRFP administration into the MHA and the LHA improves short-term memory consolidation**. The effects in the LHA are not that pronounced, or possibly reflect the promotion of other than true spatial memory learning mechanisms. Another important finding was that antagonist **BIBP3226 effectively suppressed learning-promoting effects induced by neuropeptide QRFP in both brain regions**.

5.4. Discussion of the experiments on anxiety

Possible detection of the anxiety and stress reactions in response to treatment manipulations is an inevitable procedure. The anxiogenic effect may cause “freezing” of the animals and inappropriate interpretation of the results in other paradigms. EPM is well-known method for evaluation of anxiety based on natural rats’ preference towards dark and safe areas (closed arms of the apparatus) in contrast to enlighten, easily achievable by predators and thus potentially dangerous places (opened arms).

The implication of QRFP peptides in anxious behavior was suggested due to rich QRFPR1 and R2 mRNAs expression in rodent brain regions involved in anxiety and stress such as the bed nucleus of the stria terminalis, the lateral septum, and the PAG. The first studies in this matter suggested that centrally injected QRFP exerted neither anxiogenic nor anxiolytic effects. Nevertheless, neuropeptide increased the time of grooming, which can be considered as sign of stress [18]. Later reports revealed a reduction of anxious behavior in mice after i.c.v. administration of QRFP in EPM, and GABAergic and β-adrenergic transmissions were suggested. It is important to note that only one of four tested doses (nor the lowest neither the highest) caused anxiolysis [339]. Consistently, QRFP-deficient mice exhibited anxiety-like behavior [338].

5.4.1. Discussion of the results

In our experiments, following the QRFP applications, the behavior of animals did not differ from those of controls. Namely, no anxiogenic effect was recorded after different doses of QRFP (100, 200, 400 ng, respectively) injected to the MHA or LHA (Fig. 26A-C, 29A-C). These results were supported by data analysis of the OFT and MWM yielding similar time spent in the central areas of the apparatuses for different treatment groups (Fig. 27A, 28, 30A, 31).

Our interest was again attracted by animals' reactions to Ant administration. Treatment in both hypothalamic fields, in the MHA (Fig. 26D-F) and LHA (Fig. 29D-F), led to increase in anxiety levels. The anxiogenic effect was slightly more pronounced in the LHA (Fig. 29F). The time spent by the animals with grooming in OFT after LH, but not MH, Ant microinjections also points towards amplification of anxiety (Fig. 32D, 33D, discussed also in chapter 5.5). It is important to note, that other data coming from the OFT, as well as time-promoted effects from MWM, are negative. Taking into consideration that BIBP3226 performs affinity toward Y1, FF1 and FF2, one or more of these receptors may be responsible for the observed changes.

5.4.2. Discussion of possible mechanisms

QRFP and NPY are thought to act as linked system in particular regulation mechanisms. NPY is known for its central anxiolytic performance (for review see [250]), and Y1 and Y2 receptor subtypes' role was confirmed in this matter. It is supposed that Y1 receptors highly presented in the hypothalamus and Amy, induce inhibition of amygdaloid glutamatergic pyramidal neurons or antagonize calcineurin, which is implicated in synaptic plasticity and is highly colocalized with the Y1 receptor in the Amy [391-393]. Recently a pivotal role of PAG Y1 receptors was confirmed [394]. Y2 receptor activation seems to inhibit the release of glutamate, GABA, DA, and NA [395]. Y1 receptors distribution mapping within the rat CNS suggests low to moderate mRNA density in VMN and DMN, and high occurrence in LHA [396]. These data point towards NPYY1 receptors' involvement in anxiogenic reaction observed in our experiments after Ant administration into the MHA and LHA.

Ligands for FF receptors have been described to attenuate stress reactions as well. Dansyl-PQRamide, a putative antagonist of NPFF receptors, reduces the anxiety-like behavior of ethanol withdrawal in a plus-maze test in rats [337]. I.c.v. and i.p. administration of NPFF2 agonist activates the HPA axis and induces anxiogenic effects in rodents [336]. Low doses of RFRP-1 activating FF1 receptors in Amy cause the anxiolytic effect [397]. Despite the two receptors belong to the GPR family and share about 50% amino acid identities [270], the FF1 and FF2 have different

tissue distribution, affinity, and signaling pathways. NPFF and NPAF neuropeptides show a high affinity towards FF2 receptor subtype, while NPVF and NPSF (also referred to as RFRP-3 and RFRP-1) display a higher affinity to FF1 [325]. NPFF1 binding sites are rather presented in medial hypothalamic nuclei and absent in LHA, while the FF2 subtype has opposite distribution and mostly found in LHA [270]. Together these data rather exclude the possibility of FF receptors' participation in LHA due to the absence of FF1 and high concentration of anxiogenic FF2. As for the MHA, we can exclude the role of FF2 (not present) but not FF1 subtype, which is found here in high density and may induce anxiolytic reaction, similar to Y1.

The possible mechanisms underlying the observed effects may involve the opioid system. Both MHA and LHA are rich in ꞵ- opioid receptors and opioid peptides (POMC, Pro-Enc, Pro-Dyn) [398]. NPFF receptor agonist was shown to reduce anxiety caused by ethanol withdrawal [337], to attenuate morphine-induced antinociception [325].

The involvement of serotonin is another possible pathway. This monoamine was linked to depression and anxiety many decades ago (for review [399]). DMN and VMN have an extremely high abundance of 1a, 1c and 2 serotonin receptors. In LHA they are detected as well [400]. It was noted that Y1 receptors regulate aggressive behavior by modulating 5-HT pathways [401]. Also, it was reported, that cannabinoid CB1 and 5-HT 2c receptors play role in the expression of NPY1R mRNA in the hypothalamic area of rats [402].

Several studies have reported co-localization of NPY and NA in the brainstem and medulla [403, 404]. NPY inhibits NA production, as was confirmed by in vitro and in vivo experiments [405-408]. Parallel to this, NPY, presumably via Y1 receptors, stimulates the release of CRH [409, 410]. So, the alternative pathway implies mediation via NPFF2 and CRH [411]. As a matter of fact, it was shown that QRFP stimulates CRH mRNA expression in 4B hypothalamic cells [412]. Reciprocally, α and β noradrenergic receptors blockade in the dorsal raphe nucleus impairs the panic-like response elaborated by medial hypothalamus neurons [413]. A variety of stressful events was shown to increase NA release in several brain regions, including the Amy, LC, and hypothalamus. This pathway might be responsible for the provocation of anxiety [339, 414].

The same paper referred to the role of GABAa receptors in this process as the basic mechanism of anxiolytic drugs. Hypothalamic nuclei have a high abundance of both GABAa and GABAb receptor subtypes [415-417]. Consistently, the role of the MH nuclei and the PAG was shown in mediating the response of benzodiazepines in anxiety [418-421].

Thus, **QRFP had no effect on anxiety**, while **Ant microinjections lead to an expressed anxiogenic reactions in rats**. **Effects in the LHA are more pronounced**. According to the contradictory literature data, involvement of specific QRFP receptors remains unclear. Presumably, the Y1 and/or FF1 receptors in MHA, and Y1 receptors in LHA are involved in this

phenomenon to some extent. The possible role of other neurotransmitters (opioids, CRH, 5-HT, NA, GABA) is to be determined. The induction of anxiety is not strongly expressed since additional data from the OFT hardly could catch the deviations. Based on MWM data, the effect has temporary nature and could not be registered 24h after the treatment.

We consider the maintained normal anxiety level following QRFP administration as positive sign in terms of interpretation of the main effects and future drug development.

5.5. Discussion of the general locomotion

The OFT is a classic behavior evaluation paradigm. Parameters such as distance moved within the box during 5 min, a number of line crossings, rearing and grooming episodes describe general locomotion, exploration activity, and to some extent, the anxiety of the animals. The time spent in the central part is regarded as the indicator of exploratory behavior and anxiety. Rodents spontaneously prefer staying in a darker safer peripheral area than in the center [422]. Rearing, a behavioral pattern of standing on hind legs to sample the environment, is a measure for locomotion and emotionality, i.e. curiosity, vertical exploration. Grooming, a self-cleaning activity, is considered a replacement response, i.e., normal reaction to the novel surroundings. Increased grooming also may reflect anxiety level [423].

Previous data about the effects of QRFP on locomotion are inconsistent. Central administration of QRFP significantly stimulated locomotor activity during both the light and dark periods and increase grooming activity [18]. Dose-dependent stimulation of horizontal and vertical locomotor activity was also observed after administration of QRFP-26 in higher doses. It was shown that it is the N-terminal of the neuropeptide which is responsible for the observed effect [263]. Consistently, QRFP -/- mice were hypoactive in novel circumstances as compared with wild-type littermates [338]. At the same time, QRFP gene overexpression in zebrafish decreases daytime locomotor activity, without inducing sleep though [313]. Also several reports stay that locomotion is not altered by QRFP treatment in acute (i.c.v.) or chronic (i.c.v. and i.t.) paradigms [267, 296, 299, 424]. In contrast to cousin NPFF, it was indicated that QRFP effects on locomotion are not mediated via the opioid system [263]. Instead, it was proposed that QRFP-26 and its derivatives may behave as biased ligands inducing subtle conformational changes in a particular isoform of the specific QRFP receptor, which differently trigger downstream responses [268].

5.5.1. Discussion of the results

Our findings suggest that intrahypothalamic microinjections of QRFP did not affect general locomotor activity (Fig. 32, 33). The tendency for a higher number of line crossings caused by 400 ng QRFP treatment into the MHA (Fig. 32B) was the only detected deviation.

Consistent with our previous results, Ant administration did not modify most of the measured parameters of general locomotion, except for the promoting effect on number of line crossings and grooming activity in the LHA (Fig. 33D). Since the data of traveled distance in other experiments (Tab. 4-7) reinforce data from the OFT, we tend to interpret it rather as a sign of anxiety behavior than modification of the locomotion itself.

So, **neither QRFP, nor Ant affected general locomotion following the intrahypothalamic administration**.

6. Summary

Summarizing our data, the following results have been received:

- The MHA administration of QRFP in doses 100 and 200 ng led to a significant and rapid increase in food consumption. The orexigenic effect was attenuated, and for a short period even taken over into opposite direction, by an equimolar dose of receptor antagonist BIBP3226 (Ant).
- The LHA administration of QRFP in doses 100 and 200 ng led to a significant and rapid decrease in food consumption. The anorexigenic effect was abolished by an equimolar dose of the Ant.

Thus, QRFP microinjections into the MHA increase, while microinjections into the LHA decrease food intake. The Ant prevents these effects.

- The MHA administration of QRFP in doses 200 and 400 ng, as well as Ant in corresponding dose, did not induce place preference in the CPP paradigm.
- The LHA administration of QRFP in doses 200 and 400 ng, did not induce place preference in the CPP paradigm. Ant administration into the LHA led to significantly longer time spent in the TQ, which is considered as anxiogenic sign.

This way, neither QRFP, nor Ant induced place preference when applied into the MHA or the LHA.

- The MHA administration of QRFP in doses 200 and 400 ng improved consolidation of memory in the MWM test. The effect was abolished by an equimolar dose of the Ant.
- The LHA administration of QRFP in doses 200 and 400 ng promoted memory consolidation in the MWM test. The effect was abolished by an equimolar dose of the Ant.

These data suggest that QRFP administration into both the MHA and the LHA improve short-term memory. The Ant prevents these effects.

- The MHA administration of QRFP in doses 200 and 400 ng did not affect anxiety in the EPM test. Ant in corresponding dose caused an anxiogenic effect.
- The LHA administration of QRFP in doses 200 and 400 ng did not affect anxiety in the EPM test. Ant in corresponding dose caused an anxiogenic effect.

QRFP microinjections into the MHA and the LHA did not change anxiety level. These data prove that feeding- and learning-modifying effects of the QRFP were not affected by the changes in anxiety status.

- The MHA administration of QRFP in doses 200 and 400 ng, as well as Ant, did not affect general locomotor activity according to the OFT.
- The LHA administration of QRFP in doses 200 and 400 ng did not affect general locomotion according to the OFT. Ant did not change locomotor activity, except for the acceleration of the grooming activity, which we tend to account as a sign of anxiety.

QRFP administration into the MHA and the LHA did not affect general locomotion. These finding prove that feeding- and learning-modifying effects of QRFP were not induced by hyper- or hypo locomotion.

We have shown with this research that hypothalamic neuropeptide QRFP has a great potential as an application point for treatment development in multiple spheres. On one side, dual character of feeding regulation by QRFP depending on application sight, i.e., satiety or hunger center, opens doors for treatment of various feeding disorders from anorexia to binge eating and obesity. On the other hand, unique data received during our experiments suggest a new opportunity in the field of memory disturbances and dementia. Intact parameters of anxiety and general locomotion are very positive markers in the means of drug development. Nevertheless, by the moment we still do not have a clear picture about exact QRFP's action mechanisms. Application of other receptor antagonists, interaction with brain neuromediators, investigation of adjacent fields of actions - all are further research opportunities which are conscripted to shade light on multifaceted role of QRFP in the CNS and in the body in general.

7. Acknowledgements

I would like to express my deepest and sincere gratitude to my research supervisor Prof. Dr. Lénárd László. He is the Teacher with a capital letter. Sparing no time, with equal inspiration he introduced to me the versatile knowledge of Physiology and the specialties of Hungarian culture. His professional guidance and kind support were decisive in term of this work.

I would like to thank Prof. Dr. Karádi Zoltán, Prof. Dr. Ábrahám István and Dr. Buzás Péter for their valuable professional advises, multilateral support and confident belief in me over the years.

With special thanks I owe to several of my colleagues. Péliné Dr. Kovács Anita was one of my first advisers in the field of feeding experiments. Next to Dr. Ollmann Tamás and Dr. Péczely László I had a chance to master the skills in behavioral research. Dr. László Kristóf was my primary support as in experimental part of research, as in strategical thinking from the very beginning, and remains so nowadays. Their friendship and professional collaboration gave a big input to this work.

Heartfelt thanks are also extended to research colleagues: Dr. Gálosi Rita, Dr. Márosné Berta Beáta, Dr. Kállai Veronika, Dr. Kertes Erika for their helpful suggestions, inspiring discussions, and a great working atmosphere.

I express my deepest gratitude to Konyovitsné Schulteisz Anna, to Szabó Erika, to Fogasi Palma and to Takács Tiborné Irén for their outstanding laboratory performance during the experiments. I would like to acknowledge the valuable work of Kovácsné Korona Erzsébet and Géczi Fanni, whose skillful hands and kind words made the process of histological verification truly enjoyable. I also thank Wilhelm Ferenc, Klonga Ákos, Papp Gyüla and Dobszai Mihályné Júlia for their technical contribution. I am deeply grateful to all the colleagues of the Dept. of Physiology, who provided their kind support over the years. It is a great honor to be a part of this community.

My deepest appreciation belongs to my family. This manuscript is dedicated to my parents, the most caring people I knew, and the great doctors. I thank my parents-in-law and my husband Márk for their patience and endless support. The time and resources they dedicated for the sake of this work are priceless. Finally, a big thank goes to my children, who were the best time-management coaches, and the reminders of core life values. These people kept me moving despite anything.

This study was supported by SROP-4.2.2/B-10/1-2010-0029, SROP-4.2.1/B-10/2/KONV-2010-0002, by the ÚNKP-18-3-III (PTE-374) New National Excellence Program of the Ministry of Human Capacities, by 2/2015 PhD+1 Dean's Grant of the Pécs University Medical School, and by the Hungarian Academy of Sciences.

8. List of abbreviations

Abbreviation	Meaning
5-HT	Serotonin
AC	Adenylyl cyclase
AD	Alzheimers’ disease
A.D.	Anno Domini
AgRP	Agouti-related peptide
AHip	Amygdalo-hippocampal area
AHN	Anterior hypothalamic nucleus
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
Amy	Amygdaloid complex
ANOVA	Analysis of variants
Ant	BIBP3226
AP	Anterio-posterior
Arc	Arcuate nucleus
α-MSH	α-melanocyte-stimulating hormone
BDNF	Brain-derived neurotrophic factor
BIBP3226	(R)-N2-(diphenylacetyl)-N-[(4-hydroxyphenyl)-methyl]-argininamide)
BLA	Basolateral nucleus of amygdala
BMA	Basomedial nucleus of amygdala
BRF	Brainstem reticular formation
bw	Body weight
CA	Cornu ammonis field
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
CeA	Central nucleus of amygdala
CHO cells	Chinese hamster ovary cells, epithelial cell line
CNS	Central nervous system
CPP	Conditioned place preference test
CRH	Corticotropin-releasing hormone
DA	Dopamine
db/db	Diabetic mouse model
dlf	Dorsal longitudinal fasciculus
DMN, DMH	Dorsomedial nucleus
DV	Dorso-ventral
ERKs ½	Extracellular signal-regulated kinases
EPM	Elevated plus maze test
fasc.	Fasciculus
GABA	γ-aminobutyric acid
GAP-43	Growth associated protein 43
GIT	Gastrointestinal tract
GBA	Gut-brain axis
GHSR	Ghrelin receptors
GLP-1	Glucagon-like peptide 1
GPR	G protein-coupled receptor
GS	Glucose-sensitive neurons
h	Hours
HPA	Hypothalamic-pituitary-adrenal axis
IC	Internal capsule
i.c.v.	Intracerebroventricular
IH	Incertohypothalamic
i.p.	Intraperitoneal
LC	Locus ceruleus
LHA, LH	Lateral hypothalamic area
LHN	Lateral hypothalamic nucleus
LPN	Lateral preoptic nucleus
LPS	Lipopolysaccharide
LTP	Long term potentiation
MAPK	Mitogen-activated protein kinases

MC3/4R	Melanocortin 3 and 4 receptors
ME	Median eminence
MeA	Medial amygdala
mfb	Medial forebrain bundle
MH	Medial hypothalamus
MHA	Medial hypothalamic area
ML	Medio-lateral
MN	Mammillary nucleus
MPON	Medial preoptic nucleus
mRNA	Messenger Ribonucleic acid
mtt	Mammillothalamic tract
MW	Molecular weight
MWM	Morris water maze
NA	Noradrenaline
NAcc	Nucleus accumbens
NMDA	N-methyl-D-aspartate receptor
NPAF	Neuropeptide AF
NPFF	Neuropeptide FF
NPSF	Neuropeptide SF (= RFRP1)
NPVF	Neuropeptide VF (= RFRP3)
NPY	Neuropeptide Y
NT	Neurotensin
NTS	Nucleus of solitary tract
nucl.	Nucleus
ob/ob	Obesity mouse model
OFT	Open field test
OX	Orexin
Oxt	Oxytocin
PBN	Parabrachial nucleus
PE-10	Polyethylene tubing
PEG	Periaqueductal grey
PeVN	Periventricular nucleus
PHDA	Periventricular–hypophysial dopaminergic neurons
PHN	Posterior hypothalamic nucleus
PKC	Protein kinase C
PMN	Premammillary nucleus
POMC	Pro-opiomelanocortin
PrRP	Prolactin-releasing peptide
PVN, PaVN	Paraventricular nucleus
QRFP	QRF peptide, Pyroglutamylated RFamide peptide
RAS	Reticular activating system
REM	Rapid eye movements
RF	Reticular formation
RFamide	Amides with carboxy-terminal arginine (R) and amidated phenylalanine (F) residues
RFRPs	RFamide-related peptides
RT-PCR	Reverse transcription polymerase chain reaction
sec	Seconds
S.E.M.	Standard error of the mean
SCh	Suprachiasmatic nucleus
SN	Substantia nigra
SNAP-25	Synaptosomal associated protein 25
SON	Supraoptic nucleus
SP	Substance P
SST	Somatostatin
STP	Short term potentiation
TI	Tuberoinfundibular
tr.	Tract
TRH	Thyrotropin-releasing hormone
TQ	Treatment quadrant
VIP	Vasoactive intestinal peptide
VMN	Ventromedial nucleus
VP	Vasopressin
VTA	Ventral tegmental area
ZI	Zona incerta

9. References

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10. List of publications

10.1. Publications directly related to the topic of the thesis

1. Zagorácz, Olga; Ollmann, Tamás; Péczely, László; László, Kristóf; Kovács, Anita; Berta, Beáta; Kállai, Veronika; Kertes, Erika; Lénárd, László “QRFP administration into the medial hypothalamic nuclei improves memory in rats.” BRAIN RESEARCH 1727 Paper: 146563, 9 p. (2020) [IF: 3,0]
2. Zagoracz, O; Kovacs, A; Laszlo, K; Ollmann, T; Peczely, L; Lenard, L “Effects of direct QRFP-26 administration into the medial hypothalamic area on food intake in rats.” BRAIN RESEARCH BULLETIN 118 pp. 58-64., 7 p. (2015) [IF: 2,9]

10.2. Other publications

1. László, K; Péczely, L; Géczi, F; Kovács, A; Zagoracz, O; Ollmann, T; Kertes, E; Kállai, V; László, B; Berta, B et al. “The role of D2 dopamine receptors in oxytocin induced place preference and anxiolytic effect” HORMONES AND BEHAVIOR 124 Paper: 104777 , 7 p. (2020) [IF: 3.684]
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4. László, K; Ollmann, T; Kertes, E; Péczely, L; Gálosi, R; Kovács, A; Zagorácz, O; Petykó, Z; Tóth, A; Berta, B et al. “Neuropeptidek limbikus idegrendszeri hatásai: megerősítés és memória konszolidáció.” In: Hadjadj, Leila; Lajtai, Krisztina; Benkő, Rita; Ruisanchez, Éva; Sziva, Réka Eszter; Gerszi, Dóra; Péterffy, Borbála; Bányai, Bálint; Várbíró, Szabolcs “Vaszkuláris diszfunkció és policisztás petefészek szindróma: D-vitamin hiány és tesztoszteron hatása a nagyerek acetilkolin-függő relaxációjára és a nitratív stresszre fiatal nőstény patkányokban” Budapest, Magyarország : Printing-office, (2019) p. 50
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Total IF: 44,72

10.3. Conference abstracts

1. Dusa, DA; Kállai, V; Ollmann, T; László, K; Kertes, E; Marosné, BB; Gálosi, R; Zagoracz, O; Lénárd, L; Péczely, LZ “The effects of sulpirid on spatial learning in healthy and MAM E-17 schizophrenia model rats”. In: IBRO Workshop (2020) Paper: 46
2. László, K; Ollmann, T; Zagoracz, O; Péczely, L; Kertes, E; Kovács, A; Kállai, V; László, B; Berta, B; Karádi, Z et al. “Inhibition of dopamine D2 receptors can alter the positive reinforcing and anxiolytic effects of oxytocin”. In: 16th Meeting of the Hungarian Neuroscience Society (2019) p. 175
3. Ollmann, T; Péczely, L; Kállai, V; Dusa, D; László, K; Berta, B; Kovács, A; Kertes, E; Gálosi, R; Zagoracz, O et al. “Role of ventral pallidal dopamine-neurotensin interactions in the regulation of reward and anxiety”. In: Magyar Élettani Társaság 2018. évi Vándorgyűlése : előadás és poszter absztraktok (2018) Paper: PP1.52
4. Péczely, L; Ollmann, T; Kállai, V; Dusa, D; László, K; Berta, B; Kovács, A; Kertes, E; Gálosi, R; Zagoracz, O et al. “A ventralis pallidumba injektált szulpirid hatása a tanulási folyamatokra Morris-féle úsztatási tesztben egészséges és MAM-E17 skizofrénia modell állatokon”. In: Magyar Élettani Társaság 2018. évi Vándorgyűlése : előadás és poszter absztraktok (2018) Paper: P2.11
5. László, K; Ollmann, T; Kovács, A; Zagoracz, O; Péczely, L; Kertes, E; Csetényi, B; Karádi, Z; Lénárd, L “The role of intraamygdaloid oxytocin in novel object recognition memory”. In: Magyar Élettani Társaság 2018. évi Vándorgyűlése : előadás és poszter absztraktok (2018) Paper: P1.39
6. László K, Fittler K, Ollmann T, Kovács A, Zagoracz O, Péczely L, Kertes E, Karádi Z, Lénárd L “The role of oxytocin and dopamine interaction in amygdaloid reinforcing mechanisms.” In: FENS Regional Meeting (2017). 501 p. Paper P1-037. Pécs, Hungary.
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14. Lénárd L., Zagorácz O., Kovács A. "Latest findings on neuropeptides' effects on feeding behavior" In: Scientific Conference "Systemic mechanisms of body functions regulation in physiological and pathological states", devoted to 85th anniversary of prof. Danilov G.E., and 80th anniversary of the Physiology Department. Izhevsk, Russia.

15. Zagorácz O., Kovács A., László K., Lénárd L. "Feeding related effects of intrahypothalamic administration of neuropeptide QRFP-26 in rats" In: 5th Central European Congress on Obesity (CECON 2015), XXIII. Annual Congress of the Hungarian Society for the Study of Obesity. Budapest, Hungary. P: Obesitologia Hungarica, (14) Supplementum 2, S1-S92 p.45, 2015.

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17. Lénárd L., Zagorácz O., László K., Ollmann T., Péczely L., Gálosi R., Kovács A. "Effects of intraamygdaloid microinjections of RFamide-related peptides (RFRPs) on food intake in rats" In: 5th Central European Congress on Obesity (CECON 2015), XXIII. Annual Congress of the Hungarian Society for the Study of Obesity. Budapest, Hungary. P: Obesitologia Hungarica, (14) Supplementum 2, S1-S92 p.44, 2015.

18. Kovács A., László K., Ollmann T., Péczely L., Zagorácz O., Gálosi R., Lénárd L. "Effects of RFRP peptides on anxiety and passive avoidance learning in the amygdala". In: Meeting of the Hungarian Physiological society (MÉT 2015). Szeged, Hungary.

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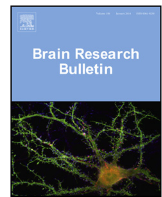
Publications directly related to the topic of the thesis

Brain Research Bulletin 118 (2015) 58–64

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull



Research report

Effects of direct QRFP-26 administration into the medial hypothalamic area on food intake in rats

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ARTICLE INFO

Article history:
Received 7 July 2015
Received in revised form 8 September 2015
Accepted 14 September 2015
Available online 15 September 2015

Keywords:
QRFP-26
Receptor antagonist BIBP3226
Hypothalamus
Food intake
Open-field test

ABSTRACT

The RFamide peptide family comprises a number of biologically active peptides sharing RF motif at their C-terminal end. These peptides are involved in the control of multiple physiological functions including regulation of metabolism and feeding behavior. QRFP-43 as well as its 26-aminoacid residue QRFP-26 are able to cause orexigenic effect when administered to the rodents' cerebral ventricles. QRFPs have been suggested as the endogenous ligands of the previously orphan GPR103 receptors. GPR103 receptors share amino acid identity with other receptors of neuropeptides involved in feeding (NPY, NPFF, galanin). QRFP-26 expressing neurons and binding sites are densely present in the rat medial hypothalamus (MHA), an area directly responsible for the regulation of feeding. QRFP-26 was delivered to the target area by direct intrahypothalamic microinjection, and the consumption of liquid food was measured over a 60 min period. Both doses (100 and 200 ng) significantly increased food intake. Non-specific receptor antagonist BIBP3226 eliminated the orexigenic effect caused by QRFP-26 administration. Effective doses of QRFP-26 did not modify general locomotor activity and behavioral patterns examined in the open-field test. This study is the first reporting feeding modulating effects following direct intrahypothalamic QRFP-26 administration.

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1. Introduction

The RFamide peptides represent one of the largest and most widespread groups of biologically active peptide families characterized by carboxy-terminal arginine (R) and amidated phenylalanine (F) residues (hence RFamide). Members of RFamide peptide family reveal a remarkable diversity in N-terminal sequence, which probably determines a wide range of biological activities. These peptides are involved in regulation of multiple functions such as control of locomotor activity, pain transmission, cardiovascular function, stress responses, regulation of sexual function, maintenance of water balance (Chartrel et al., 2011; Fukusumi et al., 2006; Oakley et al., 2009; Sun et al., 2005), and not least of all in the regulation of feeding (Bechtold and Luckman, 2007; Dockray, 2004). Experiments on rodents revealed that some of the RFamide peptides, such as NPFF (Murase et al., 1996; Sunter et al., 2001), PrRP (Bechtold and Luckman, 2006; Lawrence et al., 2002) and kisspeptin (Smith et al., 2006; Stengel et al., 2011), demonstrate anorexigenic effects after intracerebroventricular (i.c.v.) administration, while other members, e.g., RFRPs (Clarke et al., 2012; Johnson et al., 2007; Klingerman et al., 2011), enhance feeding.

QRFP (43RFa) and its 26-aminoacid residue (QRFP-26, also referred to as 26RFa or P518) are the most recently discovered members of RFamide neuropeptide family (Chartrel et al., 2003; Fukusumi et al., 2003; Jiang et al., 2003). Both forms exert similar effects, even though some studies suggest elongated form of peptide to be more potent (do Rego et al., 2006; Takayasu et al., 2006). QRFPs are thought to be involved in the regulation of feeding behavior as well. Acute i.c.v. administration of QRF peptides dose-dependently increases food consumption in mice (Chartrel et al., 2003; do Rego et al., 2006; Moriya et al., 2006; Takayasu et al., 2006), in rats (Lectez et al., 2009) and in birds (Ukena et al., 2010). But some of the previous studies indicated unsuccessful attempts to detect QRFP-induced effects on feeding behavior (Kampe et al., 2006; Patel et al., 2008). Another approach involving macronutrient selection criterion revealed an attenuating effect of

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http://dx.doi.org/10.1016/j.brainresbull.2015.09.004
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a fat-rich diet. Chronic injections of QRFP-43 in mice induced hyperphagic behavior associated with significant increase of body weight and fat mass with much more pronounced effects when offered moderately fat diet (Moriya et al., 2006). In agreement with the previous results acute i.c.v. injections of both elongated and short forms of the neuropeptide, QRFP-43 and QRFP-26, respectively, in rats led to significant augmentation of high-fat food consumption, while lack of appetite-modifying effects was observed when food with low fat content was introduced (Primeaux, 2011; Primeaux et al., 2008). Consistent with these observations, prepro-QRFP-26 mRNA levels have been found to be up-regulated in genetically obese ob/ob and db/db mice (Takayasu et al., 2006). QRFP-43 treated mice exhibited high plasma glucose, insulin, cholesterol and liver triglyceride suggesting obese phenotype (Moriya et al., 2006); QRFP-26 injections in rats inhibit insulin secretion in the pancreas (Egido et al., 2007).

In situ hybridization and immunocytochemical methods revealed that QRFP-expressing neurons within the rodents' CNS are localized in the hypothalamus, specifically in ventromedial nucleus (VMN), dorsomedial nucleus (DMN), arcuate nucleus (Arc), periventricular nucleus (PeVN), lateral hypothalamic area (LHA) and retrochiasmatic (RCh) area (Chartrel et al., 2003; Fukusumi et al., 2006; Kampe et al., 2006).

QRFPs have been suggested as the endogenous ligands of the previously orphan G protein-coupled receptor GPR103 (also referred to as AQ27 or SP9155) (Fukusumi et al., 2003; Jiang et al., 2003). Two distinct types of human GPR103 receptor were detected in mouse (Takayasu et al., 2006) and rat (Kampe et al., 2006) genomes (GPR103A, GPR103B and QRFP-r1, QRFP-r2, respectively). Studies regarding GPR103 mRNA expression in rodents' CNS suggest a broad receptor distribution with high concentration observed in the olfactory bulb, piriform cortex, amygdaloid and hippocampal areas, some thalamic nuclei, ventral pallidum, zona incerta, hypothalamic nuclei, namely the medial preoptic nucleus (MPON), RCh, VMN, LHA, anterior hypothalamic area (AHA), DMN, paraventricular nucleus (PaVN), Arc and posterior hypothalamic area (PHA), locus coeruleus, raphe nuclei, nucleus of the solitary tract and spinal cord (Bruzzone et al., 2007; Fukusumi et al., 2006; Kampe et al., 2006).

GPR103 receptors share amino acid identity with the other receptors—binding sites for neuropeptide Y (NPY), galanin (Gal), orexin, cholecystokinin (CCK) and neuropeptide FF (NPFF) (Fukusumi et al., 2003; Jiang et al., 2003; Lee et al., 2001). Consistent with these data, Bruzzone et al. (2007) reported that QRFP-26 binding sites in the rats CNS have much wider distribution than sites of GPR103 mRNA expression. Such findings suggest that QRFP-26 might be involved in activation of other than GPR103 receptors thus inducing multiple pathways of action.

In the present study, we have focused on the feeding-related effects of QRFP-26. Scientific data regarding the action of QRFPs' i.c.v. administration on feeding behavior remains contradictory. Herein, we have employed a unique experimental design with direct peptide microinjection into the brain parenchyma. So far as the location of receptors does not exactly follow the shapes of the hypothalamic nuclei, the medial hypothalamic area (MHA) including VMN and DMN, was chosen as the target area. Our paper is the first one to report QRFP-26 administration directly into the brain tissue, this way doses were determined analytically, based on the i.c.v. injections and on our previous experience from the cousin RFamide peptides (Kovacs et al., 2014; Kovács et al., 2012). To confirm the particular mechanism of action, the application of a receptor antagonist was performed. By the time of experiment no specific GPR103 receptor antagonist has been freely available on the market yet. We have applied a non-specific non-peptide NPY1/NPFF receptors antagonist BIBP3226 which previously had been shown to block orexigenic activity of QRFP-43 in i.c.v. experiments (Takayasu et al., 2006). Behavioral effects of the direct QRFP-26 injections have been studied as well.

2. Materials and methods

2.1. Subjects

In the present study, 82 adult male Wistar rats (LATI, Gödöllő, Hungary) were used weighing 270–320 g at the beginning of experiments. Animals were housed individually in a temperature- and light-controlled room ($22 \pm 2\,^{\circ}$C, 12–12 h light–dark cycle with lights on at 06:00 a.m.). Rats were cared for in accordance with institutional (Pécs University Medical School) and international standards (European Community Council Directive 86/609/EEC). Tap water and standard laboratory food chow (CRLT/N standard rodent food pellet, Charles River Laboratories, Budapest, Hungary) were available ad libitum before experiments. Body weight, food and water consumption were measured on a daily basis to the nearest grams and milliliters, respectively.

To overcome neophobia and to accustom rats to the palatable complex food, one week prior the operation animals were trained to consume the liquid diet. Liquid food with normal fat content (3%) was introduced to animals (milk, Isosource Standard Natur, Nestle). Graduated drinking cylinders with 1.0 ml divisions fitted with a glass sipper spout attached to a permanent point at the front of each home cage were used for measuring milk ingestion. Milk was available for three hours between 08:00 a.m. and 11:00 a.m., in the remaining time water and standard laboratory food pellets were available ad libitum. This feeding schedule was maintained until the end of the experiments. Rats, whose liquid food intake did not show stable baseline during habituation, were excluded from any experiments. Our method (Fekete et al., 2007; Kovács et al., 2012) allows frequent and precise monitoring of food consumption without interrupting physiological eating acts.

2.2. Surgery

Rats were anaesthetized i.p. with ketamine supplemented with diazepam (Calypsol, 80 mg/kg bw and Seduxen, 20 mg/kg bw; Richter, Hungary). Stainless steel guide tubes (22-gauge) were unilaterally implanted into the MHA of the right hemisphere (coordinates referring to the bregma: AP: −2.8 mm, ML: 1.0 mm and DV: 7.0–8.5 mm ventral from the surface of the dura mater) according to the stereotaxic rat brain atlas of Paxinos and Watson (1986). The tips of cannulae were positioned 0.5 mm above the intended injection site. Cannulae were fixed to the skull with acrylic cement (Duracryl) and stainless steel screws. When not used for injection, the guide tubes were occluded with stainless steel obturators (27-gauge). Following surgery, animals were allowed to have a minimum of 5 days for postoperative recovery before the testing began, during that time they were frequently handled.

2.3. Drug injections and liquid food intake measurements

In the first experiment we have studied the effects of different doses of QRFP-26 on food intake. QRFP-26 (Rat) (048-72, Phoenix Pharmaceuticals Inc., USA) was dissolved in 0.15 M sterile saline for intrahypothalamic microinjections in a volume of 0.4 μl. On test day rats received injection of peptide in the appropriate dose (100 ng, 35 pmol or 200 ng, 70 pmol) or vehicle injection (0.15 M sterile saline) for control measurement.

Second experiment has been performed to study effect on food intake of NPY/NPFF receptor antagonist BIBP3226 (B174, Sigma–Aldrich Kft., Hungary). Experimental procedure implicated double injection volume (0.4 μl + 0.4 μl) to each animal. For control values rats were treated with aforementioned vehicle solution

(Vehicle + Vehicle). Then animals were tested for double volume of treatment by 100 ng dose of QRFP-26 followed by vehicle injection (QRFP-26 + Vehicle). Antagonist treatment included administration of equimolar dose of BIBP3226 (18 ng, 38 pmol) 15 min prior to 100 ng QRFP-26 injection (Ant + QRFP-26), or BIBP3226 administration followed by vehicle injection (Ant + Vehicle).

In these two experiments animals served to their own control. Food consumption of the same rat was compared after either vehicle or drug (one dose of QRFP-26 or Ant) administration. Solutions were applied on counterbalanced manner, i.e. applications randomly started with vehicle or drugs within groups.

All substances were injected through stainless steel injection tube (27-gauge) extending 0.5 mm below the tips of the implanted guiding cannulae. The injection cannula was attached via polyethylene tubing (PE-10) to a Hamilton microsyringe (10 μl, Bonaduz, Switzerland). Drugs were injected during 1 min by automated syringe pumps (Cole Parmer, USA), and the injection cannula was left in place for an additional 1 min to allow diffusion into surrounding tissues. Drugs or vehicle injections were separated by at least 3-day period to prevent cumulative effects. Following microinjections liquid food intake was measured at milliliters accuracy every 5 min for the first half-an-hour and every 10 min for the following half-an-hour, so the 60-min measurement data were analyzed (Kovács et al., 2014, 2012).

2.4. Open-field test

Open-field test (OFT) was employed for measuring spontaneous motor activity and exploration behavior in response to QRFP-26 administration. The experimental arena presented itself a 50 × 50 × 50 cm gray painted box with floor virtually divided into 16 identical squares thus marking central and peripheral zones of the field. The apparatus was provided with homogenous illumination and performance in the open field was recorded by video camera. Naive rats were placed in the center of the arena and allowed to explore it for 5 min (Habituation), afterwards they were returned to their home cages. The next day (Test) animals received microinjection of QRFP-26 (100 ng) or vehicle and after 15 min experimental procedure was repeated. In OFT between subjects design was applied, i.e., activity of vehicle treated rats was compared to activity of the animals from peptide treated group. The arena was cleaned and deodorized with acetic acid after each animal. The number of lines crossed and the distance moved were analyzed by Noldus EthoVison System (Noldus Information Technology, The Netherlands). Other behavioral patterns such as grooming activity and rearing were analyzed on video recording.

2.5. Histology

In order to verify cannulae placements, animals were anaesthetized with urethane and perfused transcardially with 0.15 M saline followed by 10% formalin solution. Brains were sliced with a freezing microtome in 40 μm sections and stained with Cresyl-violet. Injection sites were reconstructed according to the stereotaxic atlas (Paxinos and Watson, 1986). The track of cannulae and the tips were determined on the basis of existence of debris and moderate glial proliferation. Only data from the rats with correctly placed cannulae were analyzed.

2.6. Statistical analysis

All results were expressed as a mean ± standard error of the mean (S.E.M.). Cumulative food intake per 100 g body weight (bw) in feeding-related experiments was evaluated by repeated measures analysis of variance (ANOVA, SPSS for Windows 11.0). When the analysis of main effect and/or the interaction showed significance, ANOVA was followed by paired-samples t test analysis. Choice of statistical methods was determined by the experimental design, implicating that each animal served as its own control (within subject design). Due to between subjects experimental design with two groups of animals, the data from OFT were evaluated by two-way ANOVA, and in case of significant effect ANOVA was followed by Tukey post-hoc test (Kovács et al., 2012). The statistical rejection criterion for all the experiments was established at $p < 0.05$ level.

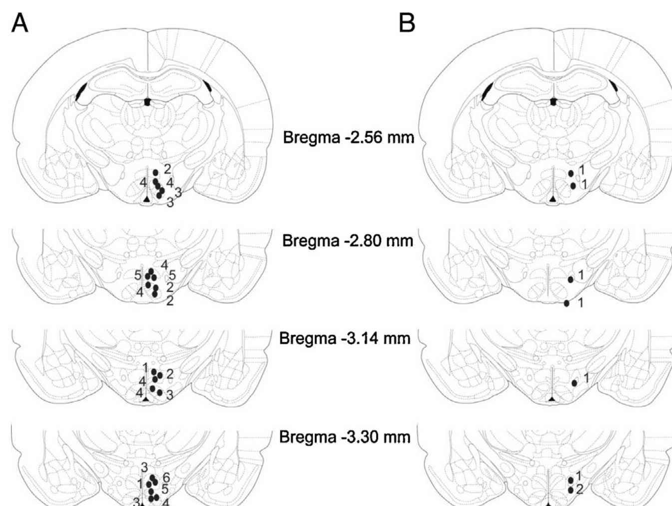

Fig. 1. Illustration of reconstructed injection sites from all experiments. Panel A: correct unilateral injection placements in the MHA (n = 74). Panel B: incorrect injection placements (n = 8). Brain structure diagrams of coronal sections are adapted from the stereotaxic atlas of Paxinos and Watson (1986). The numbers between the panels refer to anterior–posterior distance from bregma in mm. The numbers above circle symbols on Panels A and B indicate numbers of animals.

3. Results

3.1. Histology

After histological examination 8 of 82 operated animals were excluded from data analysis. Schematic illustration of cannulae placement is shown in Fig. 1. In 74 cases, the targeting of the cannulae was precisely tipped to the MHA, of which 33 injections reached the DMN and in 41 rats cannulae were placed to the VMN (Fig. 1A). Considering other 8 animals cannulae were not correctly positioned in the target area (Fig. 1B). Among them in 7 cases cannulae were led to the lateral hypothalamic area, in 1 rat cannula tip entered into the liquor space at the basis of the brain. Injections to these animals did not modify food intake, but such a few data are not enough to draw far-reaching inference.

3.2. Feeding related experiments

Food intake tests began from the fifth postoperative day, when all animals reached the preoperative level of body weight and food intake. Figures represent mean cumulative liquid food consumption in ml/100 g body weight (±S.E.M.) during 60 min period; "n" refers to a number of animals used in the experiments.

Effect of unilateral QRFP-26 microinjections into MHA on food intake is shown in Fig. 2. Administration of 100 ng dose induced significant orexigenic effect (Fig. 2A, n = 11). ANOVA analysis yielded significant effect of time (F [8,80] = 38.917, $p < 0.01$), treatment (F [1,10] = 12.833, $p < 0.01$) and significant effect of time × treatment (F [8,80] = 4.473, $p < 0.01$). Paired-samples t test analysis showed significant increase in liquid food intake at each time point from 10th to 60th min ($p < 0.03$). In case of 200 ng QRFP-26 treat-

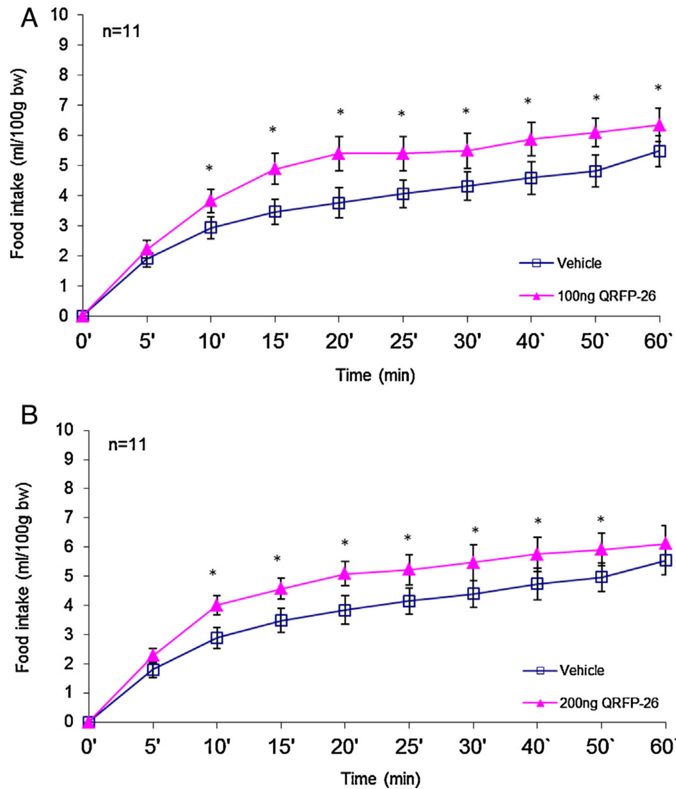

Fig. 2. Feeding-related effects of QRFP-26 microinjections into the MHA. Lines with symbols represent cumulative mean food intake in ml/100 g body weight (±S.E.M.) after application of the peptide in different doses or vehicle microinjections (0.4 μl). A: 100 ng QRFP-26 vs vehicle treatment ($n = 11$). B: 200 ng QRFP-26 vs vehicle treatment ($n = 11$). Symbol above lines indicates significant difference ($^*p < 0.05$).

ment, food consumption was markedly increased as well (Fig. 2B, $n = 11$), ANOVA indicated significant effect of time ($F[8,80] = 38.056$, $p < 0.01$) and treatment ($F[1,10] = 8.284$, $p < 0.02$), but not time × treatment interaction ($F[8,80] = 1.383$, $p > 0.05$). Paired-samples t test analysis showed significant raise in liquid food intake from 10 to 50 min ($p < 0.05$).

Data from the second experiment when antagonistic ability of BIBP3226 was examined are presented in Fig. 3. 100 ng QRFP-26 + Vehicle administration led to significant increase of food intake (Fig. 3A, $n = 12$) identical to the effects observed in the first experiment (see Fig. 2A). ANOVA analysis revealed significant effects of time ($F[8,88] = 50.466$, $p < 0.01$), treatment ($F[1,11] = 13.450$, $p < 0.01$) and time × treatment interaction ($F[8,88] = 2.225$, $p < 0.05$). According to paired-samples t test analysis, significant raise in food intake was detected at each time point from 10 to 50 min ($p < 0.03$). When animals received combined antagonist and peptide treatment with a 15 min interval (Ant + QRFP-26), food consumption was not affected, the results are presented at the Fig. 3B ($n = 10$). According to ANOVA analysis there was significant effect of time ($F[8,72] = 72.938$, $p < 0.01$), not significant effect of treatment ($F[1,9] = 1.636$, $p > 0.05$), nor time × treatment interaction ($F[8,72] = 1.062$, $p > 0.05$). As for treatment with antagonist alone, BIBP3226 injected into the MHA successfully inhibited orexigenic features of QRFP-26, and even transiently decreased liquid food consumption compared to vehicle treatment (Fig. 3C, Ant + Vehicle, $n = 12$). ANOVA analysis yielded significant effect of time ($F[8,88] = 79.139$, $p < 0.01$), not significant effect of treatment ($F[1,11] = 3.367$, $p > 0.05$) but significant effect of time × treatment ($F[8,88] = 2.597$, $p < 0.02$). Paired-samples t test analysis showed significant fall in food consumption at 20 and 25 min ($p < 0.03$).

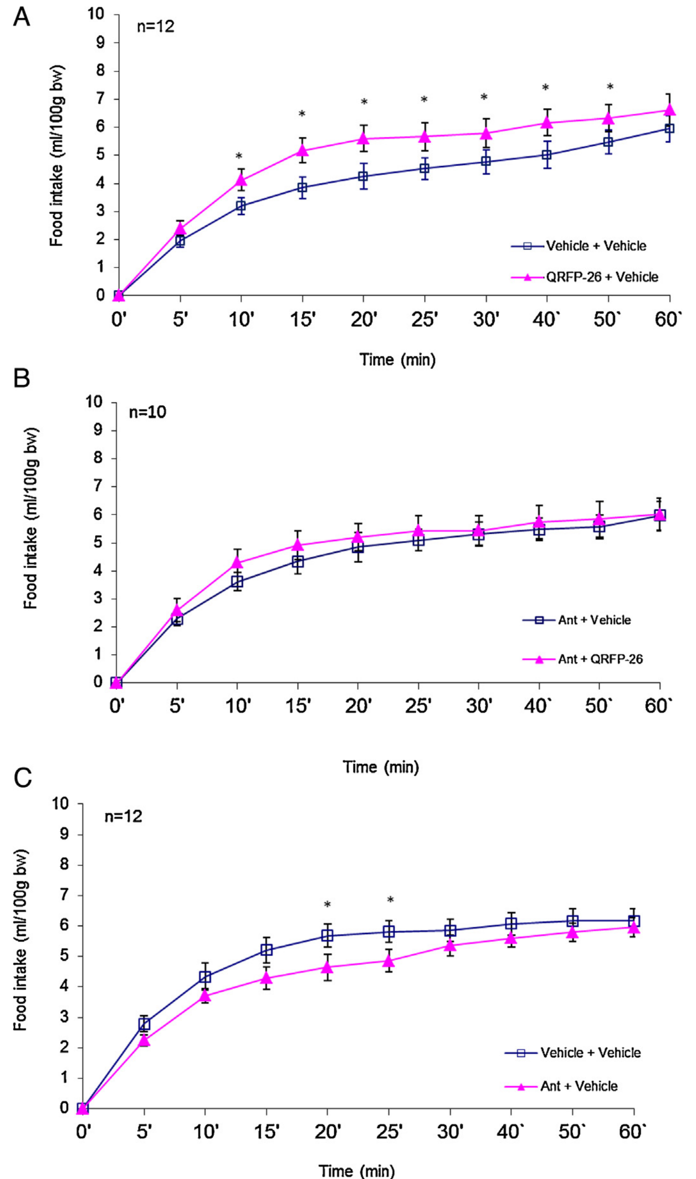

Fig. 3. Feeding-related effects of QRFP-26 and BIBP3226 (Ant) microinjections into the MHA. Line with symbols represent cumulative mean food intake in ml/100 g body weight (±S.E.M.) after application of the peptide/antagonist or vehicle microinjections (0.4 μl + 0.4 μl). A: 100 ng QRFP-26 (QRFP-26 + Vehicle) vs vehicle treatment (Vehicle + Vehicle, $n = 12$). B: Ant pretreatment followed by QRFP-26 microinjection (Ant + QRFP-26) vs only Ant (Ant + Vehicle, $n = 10$). C: Ant (Ant + Vehicle) vs vehicle (Vehicle + Vehicle, $n = 12$).

3.3. Open-field test

Effects of intrahypothalamic QRFP-26 microinjections on motor activity are presented in the Fig. 4. The ANOVA evaluation of spontaneous motor activity parameters, such as total distance moved ($F[1,32] = 0.829$, $p > 0.05$, Fig. 4A) and number of crossings ($F[1,32] = 0.01$, $p > 0.05$, Fig. 4B), did not reveal significant difference between control and QRFP-treated groups. Similarly, no significant difference was detected in the other behavior patterns, such as grooming ($F[1,32] = 0.171$, $p > 0.05$, Fig. 4C) and rearing ($F[1,32] = 0.181$, $p > 0.05$, Fig. 4D). The behavioral pattern was not modified by ant injections (data not shown).

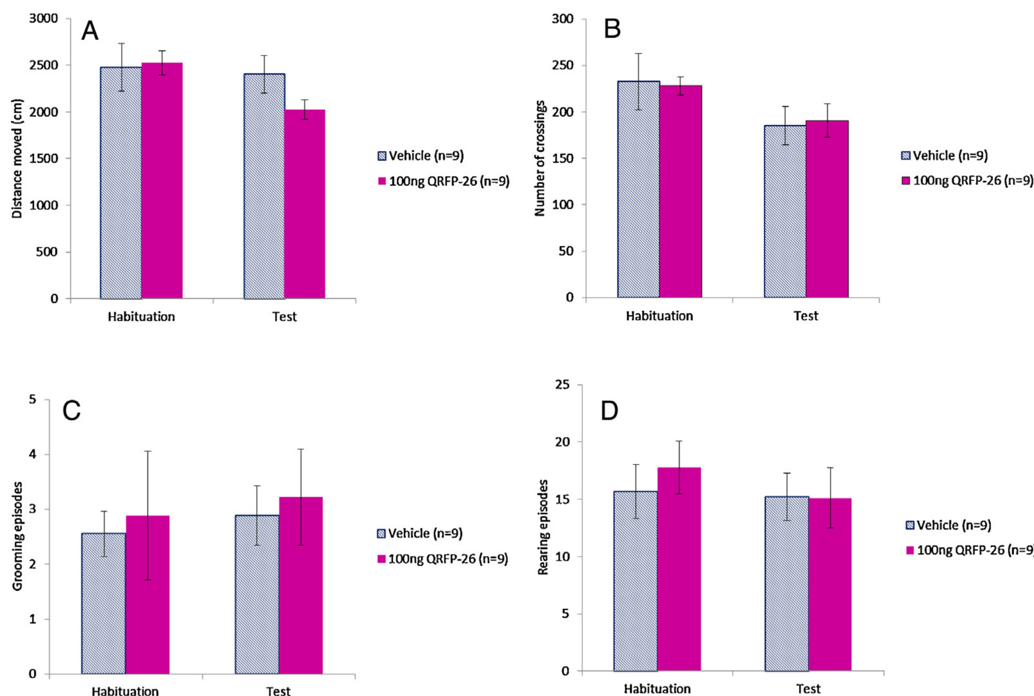

Fig. 4. Effects of QRFP-26 microinjections into the MHA in the OFT. A: Columns represent mean (±S.E.M.) distance moved in the open-field apparatus one day before (Habituation) and 10 min after (Test) QRFP-26 microinjections. B: Columns represent mean (±S.E.M.) number of crossings during Habituation and Test sessions, respectively. C: Columns represent mean (±S.E.M.) number of grooming episodes during Habituation and Test sessions, respectively. D: Columns represent mean (±S.E.M.) number of rearing episodes during Habituation and Test sessions, respectively. Vehicle: vehicle treated rats ($n = 9$); 100 ng QRFP-26: animals microinjected with 100 ng QRFP-26 ($n = 9$).

4. Discussion

The hypothalamus plays a crucial role in the central regulation of food intake as it was established decades ago by means of the electrolytic and chemical lesions and stimulations (Elmquist et al., 1999). The dual-center model of the hypothalamic regulation came into sight, where lateral hypothalamic area was accepted as a “feeding center” and the ventromedial hypothalamus as a “satiety center”. The modern view of the central regulation of feeding behavior rather considers the concept of neuronal circuits which involve hypothalamic nuclei and their interconnections with limbic system, lower brainstem and brain cortex. From anatomical point of view the Arc, VMN, DMN, PaVN nuclei, as well as the LHA, play crucial role in the regulation of feeding (Kalra et al., 1999).

There is a large body of scientific data that suggest involvement of the RFamides in the hypothalamic regulation of feeding behavior. Significant amounts of immunoreactive PrRP, kisspeptin, NPFF, NPAF and NPSF fibers are detected in the hypothalamic nuclei of different phyla. Besides, specific and non-specific receptors’ mRNA is widely expressed all along the hypothalamus. Prepro-QRFP mRNA in the rat CNS is localized in highest amount in the medial hypothalamus (VMN, Arc), the LHA and the RCh. QRFP-binding sites have much more broad distribution within the nervous system and on the periphery. Important to note that receptors have high density in the olfactory, hypothalamic and brainstem nuclei, also suggesting involvement of this neuropeptide in the control of feeding and energy balance (Chartrel et al., 2011). Data originating from behavioral experiments also confirm the assumption that members of the RFamide peptide family might be involved in the regulation of feeding. PrRP was shown to inhibit food intake in male rats and probably mediate satiety signaling (Lawrence et al., 2004; Seal et al., 2001). Food intake reduction following i.c.v. injections of NPFF was demonstrated in multiple studies (Cline and Mathews, 2008; Murase et al., 1996; Sunter et al., 2001). Chicks treated with i.c.v. NPAF and NPSF decreased both their food and water consumption (Cline et al., 2009; Newmyer and Cline, 2009). Centrally injected kisspeptin reduces food intake by increasing meal intervals in mice (Stengel et al., 2011). According to the earlier observations, the effects of QRFPs on appetite and food consumption are controversial and appear to be inconsistent across different dosages and phyla. However, most of the studies reveal a tendency of food intake enhancement in rats after i.c.v. QRFP-26 or QRFP-43 administration, particularly when food with high fat content is offered to the animals.

The purpose of the present experiments was to investigate feeding-related effects of QRFP-26 administration into the rat medial hypothalamus, the area with QRFP-synthetizing neurons and binding sites presented in high density. So far as receptors distribution is not strictly limited by the shape of a certain nuclei, medial hypothalamic area including VMN and DMN was chosen as a target for treatment (Fig. 1). Even if the target area within MHA seems to be wide, no significant difference in food consumption was detected when subgroups were compared (data not shown). Direct administration into the brain parenchyma allowed us to identify the exact effective concentration and the period of action without remarks on diffusion process from cerebral ventricles as it occurs in case of i.c.v. treatment. To our best knowledge, this is the first investigation regarding QRFP-26 administration directly into the hypothalamic parenchyma.

QRFP-26 microinjections (100 ng and 200 ng) into the MHA led to significant increase in liquid food consumption, however, slightly more pronounced effect was observed when animals were treated with the lower dose. We have also confirmed the result even if the same dose of the peptide was delivered in double volume as it was performed in the second experiment. The orexigenic effect of QRFP-26 was quite rapid, observed already 10 min after peptide administration (Fig. 2A). Since our data represent cumulative value of food intake, the difference remained significant during all the period of data recording, i.e., during 1 h. Until recently, there were no data available in the public domains regarding half-life

period of QRFPs. Fresh results were published by Jossart et al. (2014) who have detected that mRNA of QRFP rapidly decreases after the addition of the inhibitor of transcription, showing a half-life of approximately 90 min. This is highly valuable information in spite of the study was performed on murine macrophages and these data could not be directly applied to the present research.

One can infer that QRFP-26 feeding-related effects could be provoked by the changes in general locomotor activity. However, our observations in OFT clearly demonstrated the failure of such supposition (Fig. 4) since the locomotor activity and behavioral patterns were not modified by administration of QRFP-26. Present results are in agreement with previous reports regarding QRFP behavioral effects in rats (Kampe et al., 2006; Moriya et al., 2006; Patel et al., 2008; Yamamoto et al., 2011). However, one should not ignore that in mice acute i.c.v. administration of QRF neuropeptide is able to stimulate vertical and horizontal locomotor activity and grooming behavior (do Rego et al., 2006; Takayasu et al., 2006). This could be explained by a difference in receptor distribution between phyla. Another point in these studies is that relatively high doses of the neuropeptide were used for treatment probably involving activation of low affinity receptors as well.

To our best knowledge, at the time of experiment there was no specific GPR103 receptor antagonist available on the market. In the present study non-peptide NPYY1/NPFF receptors antagonist BIBP3226 (Fang et al., 2006; Mollereau et al., 2002; Rudolf et al., 1994) was applied to determine the mechanism of observed effects. Takayasu et al. (Takayasu et al., 2006) reported in their study that the effect of i.c.v. injected QRFP-43 on feeding behavior was suppressed by pretreatment of equimolar BIBP3226. Herein, BIBP3226 was applied in the equimolar amount (18 ng, 38 pmol) to the most effective dose of QRFP-26 (100 ng, 35 pmol). Combined Ant and QRFP-26 administration did not cause changes in food intake, which means that Ant had successfully blocked potent receptors and peptide could not display its' agonistic activity. Ant alone treatment effectively suppressed the orexigenic effect caused by QRFP-26 and even transiently reduced food consumption about 20–25 min after injection.

Recently a series of GPR103 antagonists containing pyrrolo [2,3-c] pyridine were developed and reported to mimic QRFP-26C-terminal motif (Georgsson et al., 2014). Antagonists were shown to have high potency to mouse and human GPR103 receptors, however, information related to rat receptors is not available yet. Obese female mice treated with pyrrolo[2,3-c]pyridine twice a day reduced their food intake in dose-dependent manner. Important to note that these drugs to be given orally, that would not fit our experimental design. Despite we could not employ pyrrolo[2,3-c]pyridines in our study, new substances seem to be promising findings though requiring further research.

It has been suggested that QRFP-26 i.c.v. administration induces NPY synthesis in the Arc and mediates inhibition of the POMC neurons via Y1 and Y5 receptors. Moreover, neuroanatomical observations revealed a subpopulation of the NPY, but not POMC, neurons expressing GPR103 in Arc (Lectez et al., 2009). So it was proposed that QRFP-26 activates specific receptors in the Arc thus inducing NPY production, which in turn binds NPY1, and NPY5 receptors and blocks POMC synthesis leading to increase in food intake. It is suggested that the main source of NPY within the hypothalamus is exclusively the Arc, except for the special states of the negative energy balance when NPY synthesis transiently takes place in the DMN as well (Mercer et al., 2011). This way an aforementioned hypothesis regarding the mechanism of QRFPs' orexigenic activity is not directly applicable to our research results. It has been shown that VMN has efferent projections towards Arc POMC neurons (Sternson et al., 2005). Since no specific receptors are identified there (Lectez et al., 2009), one of the possibilities is that QRFP-26 binds other, non-specific receptors inhibiting POMC production similarly to NPY. At the same time, there is no proof that it is the RFamide peptide signaling that courses from VMN to Arc, suggesting that other mechanisms might be involved. Another possibility is that QRFPs act within the MHA, enriched not only with specific but also other receptors responsive to feeding-modifying substances and to a certain level similar to GPR103 (NPYY2, NPFF1 and NPFF2, orexin, CCKa and CCKb). To the point, recent findings state that both NPFF1 and NPFF2 receptor subtypes display high affinity to the most of RFamide family members (Elhabazi et al., 2013; Engstrom, 2003). Considering multiple efferent projections from VMN and DMN (King, 2006), QRFPs may also potentially realize orexigenic action by activating/inhibiting other brain structures involved in feeding, such as PVN, LH, amygdala, thalamus. As for BIBP3226, it seems to act through NPY1 and/or NPFF receptors densely present in medial hypothalamus. Common opinion supports that it is Y1 receptor-linked mechanism, at the same time some studies suggest alternative mechanisms for BIBP3226 action (Iyengar et al., 1999; Morgan et al., 1998; Van et al., 2001). Even though we cannot exclude the possibility of cross-binding to GPR103 receptors, it seems unlikely considering our findings. Thus we may conclude that QRFP-26 performs its action in the medial hypothalamus not isolated but in close association with other neuroendocrine peptides.

Summary

This is the pioneer study regarding QRFP-26 administration directly into the brain parenchyma comparing to previously employed i.c.v. injections. In our research effective doses (100 and 200 ng) of QRFP-26 were established to modulate feeding. QRFP-26 revealed its orexigenic quality when microinjected into the MHA, causing a rapid increase in food consumption. At least partly, it is a NPYY1 and/or NPFF receptors linked effect so far as antagonist BIBP3226 has prevented the effect of the peptide. Further studies are required to determine the details of QRFP-26 action mechanism.

Conflict of interests

All authors declare that there is no any actual or potential conflict of interests including any financial, personal or other relationships with other people or organizations.

Acknowledgements

The authors express their thanks to Anna Schulteisz, Erzsébet Korona, Ferenc Wilhelm and András Belvárácz for their technical contribution to this work. This study was supported by SROP-4.2.2/B-10/1-2010-0029, SROP-4.2.1/B-10/2/KONV-2010-0002, by the Hungarian Academy of Sciences and by the Pécs University Medical School.

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Brain Research 1727 (2020) 146563

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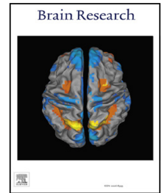

Research report

QRFP administration into the medial hypothalamic nuclei improves memory in rats

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HIGHLIGHTS

- QRFP was microinjected into the medial hypothalamic area of male Wistar rats.
- 400 ng QRFP improved consolidation of short-term memory in Morris water maze.
- The effective dose of QRFP did not affect nor general locomotion activity, neither anxiety level.
- Pretreatment with receptor antagonist BIBP3226 prevented memory associated effects of QRFP.

ARTICLE INFO

Keywords:
QRFP
Antagonist BIBP3226
Medial hypothalamus
Spatial memory
Morris water maze
Elevated plus maze

ABSTRACT

Even though several of RFamide peptides have been shown to modify memory and learning processes in different species, almost nothing is known regarding cognitive effects of recently discovered neuropeptide QRFP. Considering multiple physiological functions of QRFP, localization of QRFP-synthesizing neurons in the hypothalamus and its' widely spread binding sites within the CNS, the present study was designed to investigate the possible role of QRFP in the consolidation of spatial memory.

As target area for microinjection, the medial hypothalamic area, including dorsomedial (DMN) and ventromedial (VMN) nuclei, has been chosen. At first, the effects of two doses (200 ng and 400 ng) of QRFP were investigated in Morris water maze. After that receptor antagonist BIBP3226 (equimolar amount to the effective dose of neuropeptide) was applied to elucidate whether it can prevent effects of QRFP. To reveal possible changes in anxiety level, animals were tested in Elevated plus maze.

The higher dose of QRFP (400 ng) improved short-term memory consolidation in Morris water maze. Pretreatment with antagonist BIBP3226 abolished cognitive effects of QRFP. The neuropeptide did not affect anxiety level of rats.

This study provides unique evidence regarding the role of QRFP in the consolidation of memory and gives the basis for further investigations of neuropeptide's cognitive effects.

1. Introduction

Neuropeptide QRFP is one of the most recently discovered members of RFamide neuropeptide family next to the prolactin-releasing peptide (PrRP), neuropeptide FF (NPFF), RFamide-related peptides (RFRPs, GnIH), and kisspeptin (metastatin) (Chartrel et al., 2003; Fukusumi et al., 2003; Jiang et al., 2003). There are two molecular forms of QRFP recognized in rats: the longer and the shorter one (43RFa and 26RFa, respectively), and both of them reveal similar chemical and physiological characteristics.

In rodents QRFP gene is highly expressed over the body: in the eye, trachea, mammary gland, testis, also in thymus, salivary gland, duodenum, pancreas, uterus and in the CNS (Fukusumi et al., 2003; Jiang et al., 2003; Takayasu et al., 2006). As for the brain, QRFP-expressing neurons are almost exclusively localized in the hypothalamus, specifically in ventromedial nucleus (VMN), dorsomedial nucleus (DMN), arcuate nucleus (Arc), periventricular nucleus (PeVN), lateral hypothalamic area (LHA) and retrochiasmatic (RCh) area (Chartrel et al., 2003; Fukusumi et al., 2006; Kampe et al., 2006).

Recent studies discovered that the QRFP neuropeptide is involved in

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https://doi.org/10.1016/j.brainres.2019.146563
Received 4 July 2019; Received in revised form 31 October 2019; Accepted 19 November 2019
Available online 22 November 2019
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regulation of multiple physiological functions, such as feeding, energy balance, regulation of gonadotropic axis, production of adrenal steroid hormones, growth hormone (GH), control of nociceptive transmission, bone formation, cardiovascular effects, regulation of insulin secretion and glucose uptake (Leprince et al., 2017). Not much is known regarding the cognitive effects of this neuropeptide. Recent findings suggest a possible role of QRFP in the regulation of sleep (Chen et al., 2016). Another interesting research discovered a neuroprotective effect of QRFP in Alzheimer's disease (Davies et al., 2015). At the same time, cousin peptides from the RF peptide family (NPFF, NPAF, RFRP-1, kisspeptin) have been implicated to affect higher brain functions, i.e. to modulate learning and memory in different paradigms and mitigate the memory impairment caused by amyloid-β (Betourne et al., 2010; Jiang et al., 2015; Kavaliers and Colwell, 1993; Kovács et al., 2017; Palotai et al., 2016; Telegdy and Adamik, 2013).

Previously orphan receptor GPR103 (also known as AQ27 or SP9155) has been recognized as a specific binding site for QRFP, and recently it was renamed to QRFP receptor (Leprince et al., 2017). GPR103 mRNA is widely expressed within rodents' CNS, nevertheless localization and relative abundance of QRFP binding sites is much exceeding GPR103 localization and, besides the hypothalamus itself, includes regions directly involved in memory processing: the retrosplenial cortex, entorhinal cortex, hippocampal formation with especially high concentration in presubiculum (Bruzzone et al., 2007; Fukusumi et al., 2006; Kampe et al., 2006). GPR103 was found to share significant sequence identity with neuropeptide Y2, galanin GalR1, orexin OX1 and OX2, cholecystokinin CCKA, CCKB and neuropeptide FF NPFFR1, NPFFR2 receptors (Fukusumi et al., 2003; Jiang et al., 2003; Lee et al., 2001). These findings may give an explanation to the diverse functions of the neuropeptide and suggest alternative pathways of action.

Considering multiple physiological functions of QRFP and involvement of cousin RF peptides in learning and memory processes, with the knowledge of binding sites within the CNS, the present study was designed to investigate the possible role of QRFP in consolidation of spatial memory. Taking into account contradictory data regarding the effects of QRFP on locomotor activity (do Rego et al., 2006; Kampe et al., 2006; Moriya et al., 2006; Okamoto et al., 2016; Patel et al., 2008; Takayasu et al., 2006; Yamamoto et al., 2011; Zagoracz et al., 2015) and anxiety level (Okamoto et al., 2016; Takayasu et al., 2006), the aforementioned parameters have been taken into consideration in the present study as well.

So far as the location of receptors doesn't exactly follow the shapes of the hypothalamic nuclei, the medial hypothalamic area (MHA) including VMN and DMN, was chosen as the target area. Effects of different doses of the 26-aminoacid residue of QRFP on spatial memory have been studied in the Morris water maze paradigm, and effects on anxiety level have been investigated by the performance in the several paradigms: Elevated plus maze (EPM), Morris water maze (MWM), and Open field test (OFT). To confirm the particular mechanism of action, application of the receptor antagonist was performed. We have applied a non-peptide antagonist BIBP3226 which previously had already been shown to block the orexigenic activity of QRFP (Takayasu et al., 2006; Zagoracz et al., 2015).

2. Results

2.1. Histology

Following the histological examination 8 of 93 operated animals have been excluded from data analysis. Schematic illustration of cannulae placement is shown in Fig. 1. In 85 cases the targeting of the cannulae was precisely tipped to the MHA, of which 57 injections reached the DMN and in 28 rats cannulae were placed to the VMN (Fig. 1A). Considering other 8 animals cannulae were incorrectly positioned out of the target area (Fig. 1B). Among them, in 6 cases cannulae were led to the lateral hypothalamic area, in 1 rat cannula tip entered into the liquor space at the basis of the brain, and 1 ended in the arcuate nucleus. Injections to these animals did not modify learning abilities or behavior, but such a few data are not enough to draw a far-reaching inference.

2.2. MWM navigation task

In the first experiment the effect of neuropeptide QRFP on the escape latencies of rats was investigated (Fig. 2). Swimming trials without platform, i.e. Habituation and Probe trial, were evaluated separately from the training trials (1–4). In regard to the trials without platform (Fig. 2A), two-way ANOVA analysis revealed that there was a significant effect of trials ($F [1,42] = 40.110$, $p < 0.001$), but no significant effect of treatment or interaction between trials and treatment. According to one-way ANOVA, there were significant differences within each treatment group: control, 200 ng and 400 ng ($p = 0.01$, $p = 0.04$, $p < 0.001$, respectively), as well as between the groups during Probe trial ($p = 0.03$, $p = 0.01$). Concluding from these findings, by the day of testing (Probe trial) all the animals have learned that there was an escape platform in the pool, nevertheless, rats treated with 400 ng QRFP found the platform significantly faster. For better understanding of the learning dynamics, the training trials 1–4 were analyzed by two-way and one-way ANOVA (Fig. 2B). Significant difference have been found for the training trials: $F [3,84] = 7.651$, $p < 0.001$, but not treatment or their interaction. During the first two trials (1 and 2) animals did not show significant learning results. The trial 2 was followed by QRFP microinjections in corresponding doses. Twenty-four hours later, in the trial 3, mean latencies of control and 200 ng treated animals raised back, but 400 ng treated rats found the platform even faster than previous day, and their latencies became significantly shorter compared to the trial 1 ($p = 0.02$). Similarly to the previous experimental day, the next swimming trial (trial 4) followed one minute later. This time for all the animals time to finding platform was approximately the same, but due to minor differences in SEM, the analysis registered that 200 ng and 400 ng groups (but not control) have found the target significantly faster compared to the trial 1 ($p = 0.01$, $p < 0.02$, respectively). After the trial 4, all the animals received second microinjections.

Data from the second experiment, where the antagonistic ability of BIBP3226 was examined, are presented in Fig. 3. Experimental procedure implicated double volume injection to each animal with 15 min interval. The two-way ANOVA analysis of trials without platform (Fig. 3A) indicated a significant effect for trials ($F [1,60] = 35.799$, $p < 0.001$), however effect for treatment and the interaction between trials and treatment was not significant. According to one-way ANOVA, when comparing Habituation and Probe trials, there were significant differences registered within control and 400 ng treated animals ($p < 0.01$, $p < 0.001$, respectively), but not Ant or combined Ant + QRFP treated rats. Similarly to the first experiment, in the Probe trial, the one-way ANOVA indicated significant differences among the groups ($F [3,30] = 6.082$, $p = 0.002$). The Tukey's post hoc test confirmed that the mean latency of the QRFP + Vehicle group was significantly decreased compared to that of the control, Ant + Vehicle and Ant + QRFP treated groups ($p = 0.05$, $p = 0.01$, $p < 0.003$, respectively). Two-way ANOVA analysis was applied to compare means of four training trials to each other within each group (Fig. 3B), and revealed significant differences among trials ($F [3,120] = 7.284$, $p < 0.001$), but not treatment, or trial × treatment. The QRFP + Vehicle treated animals (400 ng QRFP), similarly to the first experiment, have shown shorter latencies, and by the trial 4 have learned to find the platform significantly faster than in the first trial (trial 1), $p = 0.05$. Control animals have shown good results at the trial 4 ($p < 0.03$) as well. By contrast, means of the Ant + QRFP and the Ant + Vehicle - treated groups remained similar to their latencies during the trials 1 and 3, so the progress is learning was not registered.

Considering mean swimming velocities of the animals, there were

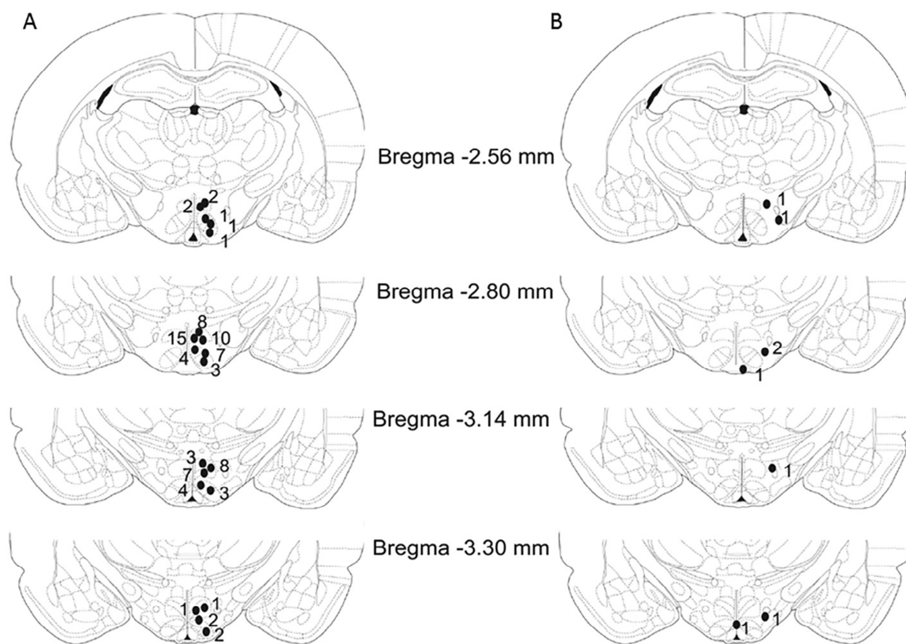

Fig. 1. Illustration of reconstructed injection sites from all experiments. Panel A: correct injection placements in the MHA (n = 85). Panel B: incorrect injection placements (n = 8). Brain structure diagrams of coronal sections are adapted from the stereotaxic atlas of Paxinos and Watson (Paxinos and Watson, 1986). The numbers between the panels refer to anterior-posterior distance from *bregma* in mm. The numbers above circle symbols on panels A and B indicate numbers of animals.

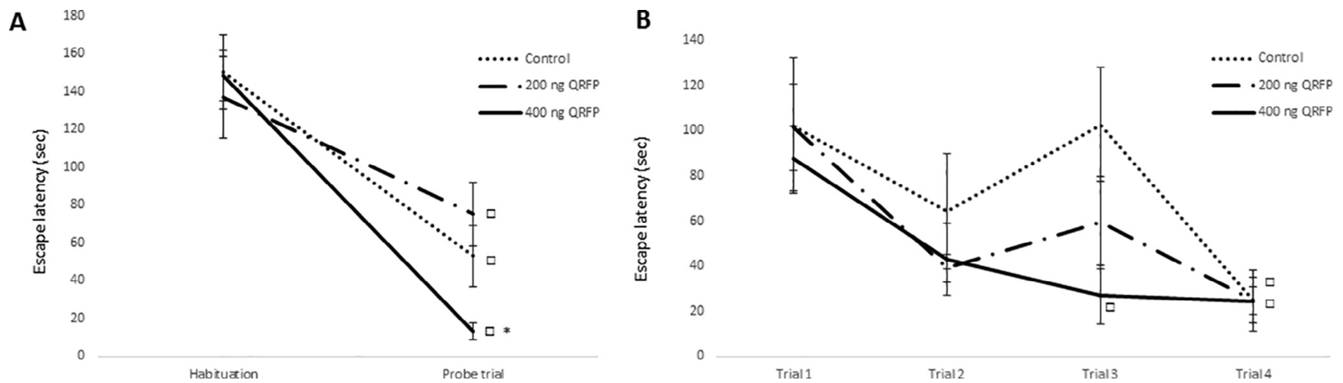

Fig. 2. Effects of QRFP microinjections into the MHA on the platform finding latency (escape latency) in Morris water maze. The drugs were microinjected immediately following the trials 2 and 4 (0.4 µl): 200 ng (n = 9), 400 ng QRFP (n = 7) or Vehicle solution (Control, n = 8). A: Trials without the platform, line graphs represent the mean latencies to finding the place of the removed platform (± S.E.M.), B: Training trials, line graphs represent the mean latencies to finding the hidden platform (± S.E.M.). Symbols next to the graphs indicate significant difference: □ refers to between-trial difference, * refers to between-group difference within one trial ($p < 0.05$).

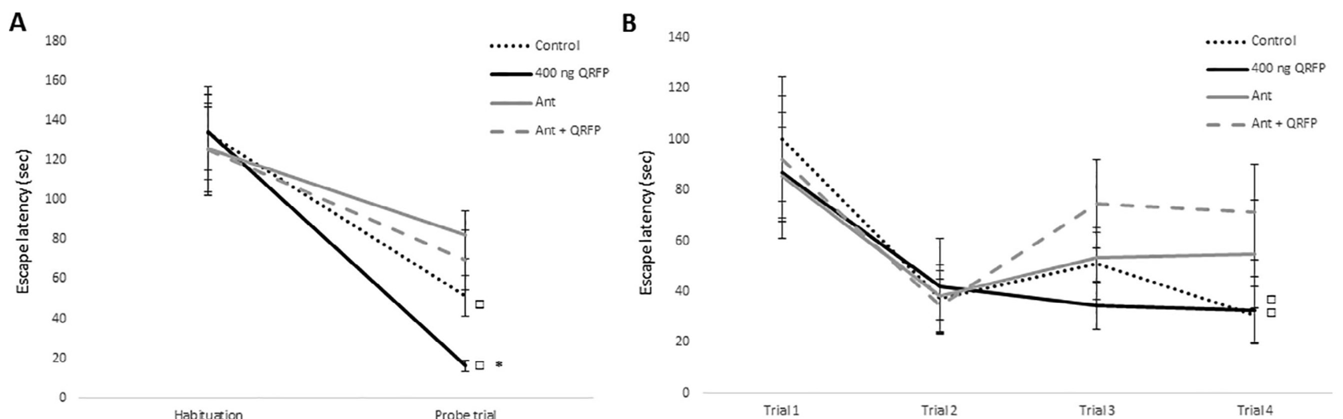

Fig. 3. Effects of QRFP and BIBP3226 (Antagonist) microinjections into the MHA on the platform finding (escape) latency in Morris water maze. Corresponding drugs (0.4 µl + 0.4 µl) were microinjected immediately following the swimming trials 2 and 4, with 15 min interval: 400 ng QRFP followed by Vehicle (400 ng QRFP, n = 8), BIBP3226 followed by Vehicle (Ant, n = 8) or BIBP3226 pretreatment followed by 400 ng QRFP (Ant + QRFP, n = 9) or double volume of saline treatment (Control, n = 9). A: Trials without the platform, line graphs represent the mean latencies to finding the place of the removed platform (± S.E.M.), B: Training trials, line graphs represent the mean latencies to finding the hidden platform (± S.E.M.). Symbols next to the graphs indicate significant difference: □ refers to between-trial difference, * refers to between-group difference within one trial ($p < 0.05$).

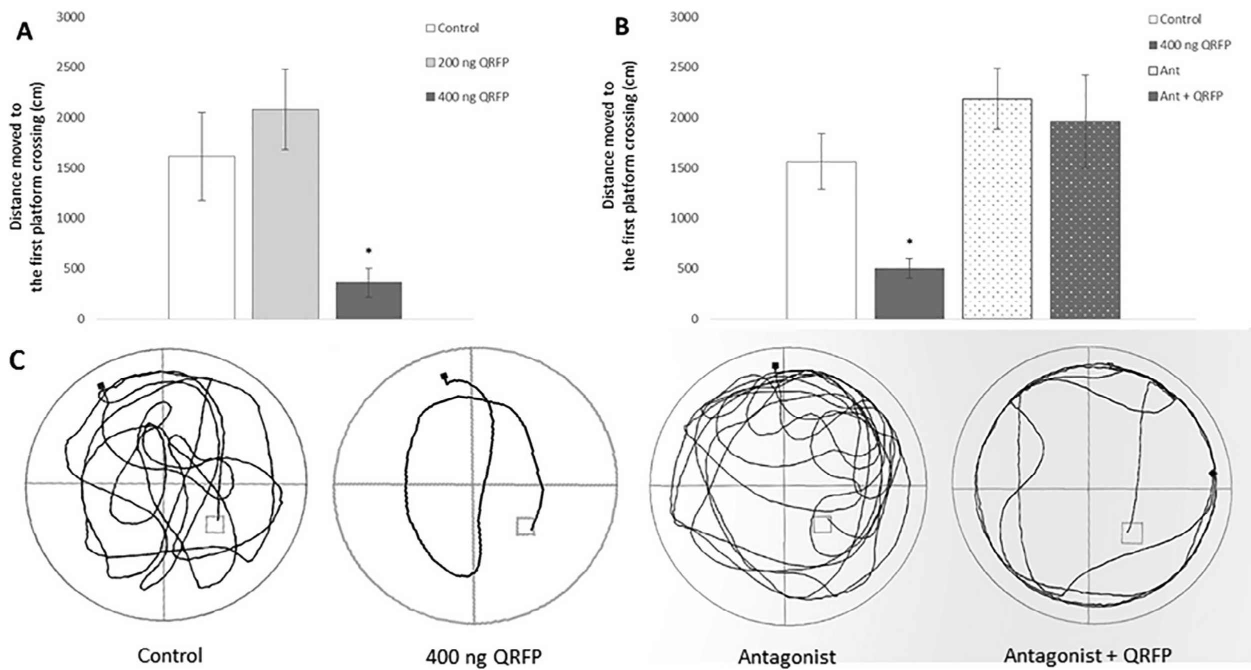

Fig. 4. Effects of QRFP and BIBP3226 microinjections on average distance to finding platform place during the Probe trial in Morris water maze. A: Columns represent distance moved to the first platform crossing during the first experiment (± S.E.M.), B: Columns represent distance moved to the first platform crossing during the second experiment (± S.E.M.), C: Illustrative images of rats' trajectories during the Probe trial in Morris water maze. The groups and the number of animals are identical to those present in the Figs. 2 and 3. Symbol * above columns indicate significant difference ($p < 0.05$).

no significant differences registered in any trial during both experiments (data not shown).

The distances that rats have covered during the Probe trial untill they crossed the place of removed platform are presented in the Fig. 4. Following the tendency, 400 ng QRFP treated rats made shorter routs to the target compared to all other groups, which was proved by ANOVA analysis ($F [2,19] = 5.673$, $p < 0.02$; $F [3,28] = 5.012$, $p < 0.01$, for the first and the second experiments, respectively). Images of representative trajectories for each group are present at the Fig. 4C.

Additional analysis of the probe trial (Fig. 5) revealed that according to the two-way ANOVA, normalised time spent in the target and in the opposite annuli had significant effect for annuli ($F [1,50] = 6.789$, $p < 0.02$), significant effect for treatment ($F [3,50] = 3.309$, $p < 0.03$), but not significant interaction between the treatment and annuli. Tukey's post hoc test demonstrated that the means of control group significantly differ from those of QRFP treated animals ($p < 0.03$). The one-way ANOVA indicated a significant difference among the groups only in the opposite annulus ($F [3,25] = 4.796$, $p < 0.01$). The Tukey's post hoc test proved that peptide treated animals spent there significantly less time comparing to control and Ant groups ($p = 0.01$, $p < 0.03$, respectively). The time spent in the target and opposite annuli within each group was compared applying one-way ANOVA as well. The analysis showed that the animals treated with 400 ng QRFP spent much more time searching in the target annulus comparing to the opposite one ($p < 0.01$). Another analyzed parameter, the normalised number of entries to the target and opposite annuli, showed a similar result. The two-way ANOVA revealed significant effect for annuli ($F [1,50] = 8.819$, $p = 0.005$), but not for treatment, and significant effect for treatment and annuli interaction ($F [3,50] = 3.374$, $p < 0.03$). According to one-way ANOVA the difference between the groups reached a significant level in the opposite annulus ($F [3,25] = 3.366$, $p < 0.04$). The Tukey's post hoc test

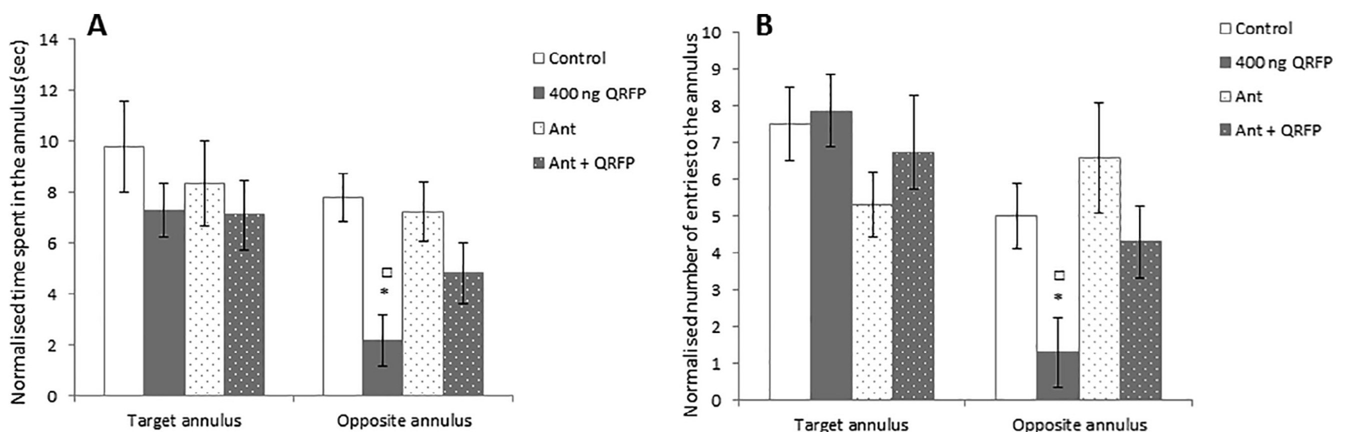

Fig.5. Effects of QRFP and BIBP3226 microinjections into the MHA on the normalised time and number of entries to the target and opposite annuli during the Probe trial in Morris water maze. A: Columns represent normalised time spent in the annuli (± S.E.M.), B: Columns represent normalised number of entries to the annuli (± S.E.M.). The groups and the number of animals are identical to those present in the Fig. 3. Symbols above columns indicate significant difference: □ refers to difference between two annuli, * refers to difference between the groups within one annulus ($p < 0.05$).

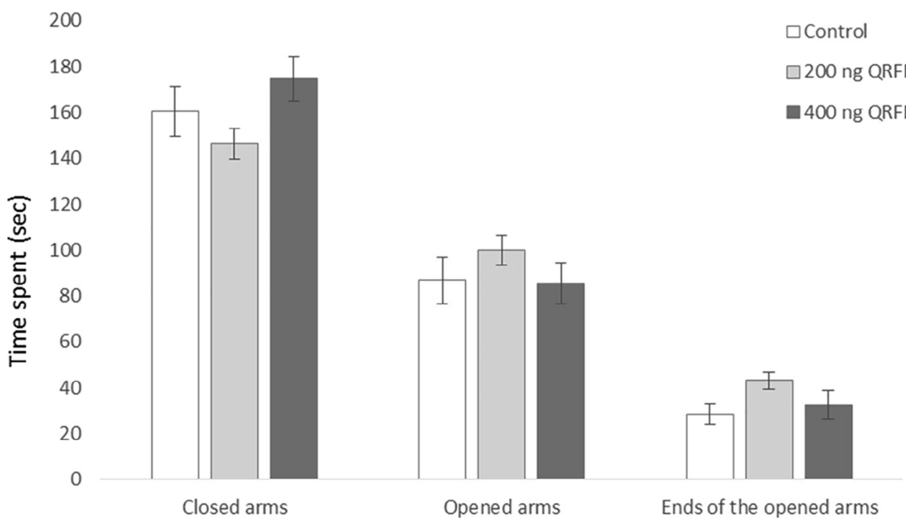

Fig. 6. Effects of QRFP microinjections into the MHA on the anxiety level in Elevated plus maze test. Columns represent mean time spent in the closed arms, time spent in the opened arms, and time spent at the ends of the opened arms, respectively (± S.E.M.). Animals were tested 15 min after the corresponding microinjection: 200 ng QRFP (n = 9), 400 ng QRFP (n = 9) or Vehicle treated rats (Control, n = 9). There was no significant difference recorded.

showed that QRFP treated animals had a lower number of entries compared to those treated with Ant ($p < 0.03$). Analysis by one-way ANOVA, of the number of entries to the target and opposite annuli within each group revealed that the animals treated with 400 ng QRFP appeared in the target annulus significantly more often comparing to the opposite one ($p = 0.001$).

2.3. Anxiety level

The third experiment, the effects of hypothalamic microinjection of QRFP have been investigated in EPM (Fig. 6). According to one-way ANOVA the time spent by the rats, treated with 200 and 400 ng QRFP, in opened and closed arms of the maze did not differ significantly from the data of control group or each other.

Besides, the performance in MWM was analyzed from the point of possible changes in the anxiety level. The time spent by rats during the Probe trial in the outer area and in the centrals part of the pool did not differ between the treatment groups (Fig. 7). These findings correspond the data recieved in the Open field test (Fig. 8), previously published by our research team (for details, see (Zagoracz et al., 2015)).

3. Discussion

Scientific data suggest the involvement of the RFamides in the regulation of higher brain functions. Significant amounts of immunoreactive PrRP, kisspeptin, NPFF, NPAF, and NPSF fibers are detected in the hypothalamic nuclei of different phyla. For QRFP, the hypothalamus, namely its' medial nuclei (VMN, DMN, Arc), is the major synthesizing area within the rat CNS. Data originating from behavioral experiments also confirm the assumption that members of the RFamide peptide family might be involved in the regulation of cognitive functions. NPFF was shown to modify short- and long-term memory depending on dose and paradigm (Betourne et al., 2010; Kavaliers and Colwell, 1993). Kisspeptin improved memory formation and revealed neuroprotective activity in passive avoidance paradigm, as well as novel object recognition and object location tasks (Jiang et al., 2015; Telegdy and Adamik, 2013). Similarly, NPAF and RFRP-1 peptides enhance learning processes and memory in aversive situations (Kovács et al., 2017; Palotai et al., 2016). But not much is known regarding the effects of QRFP on cognitive functions. There are results suggesting that QRFP signaling is involved in regulation of sleep in fish and may underlie some aspects of hypothalamic sleep control (Chen et al., 2016). Another data suggested the down-regulation of QRFP and orexin receptors in the hippocampal cells of Alzheimer's patients, and the neuroprotective role of QRFP and both orexins (Davies et al., 2015). It becomes even more intriguing taking into consideration that QRFP receptors share 48% and 47% homology with OX1 and OX2R, respectively (Jiang et al., 2003).

The purpose of the present study was to investigate memory-associated effects of QRFP administration into the rat medial hypothalamus, the area with QRFP-synthesizing neurons and binding sites presented in high density. The receptors' distribution here does not strictly follow

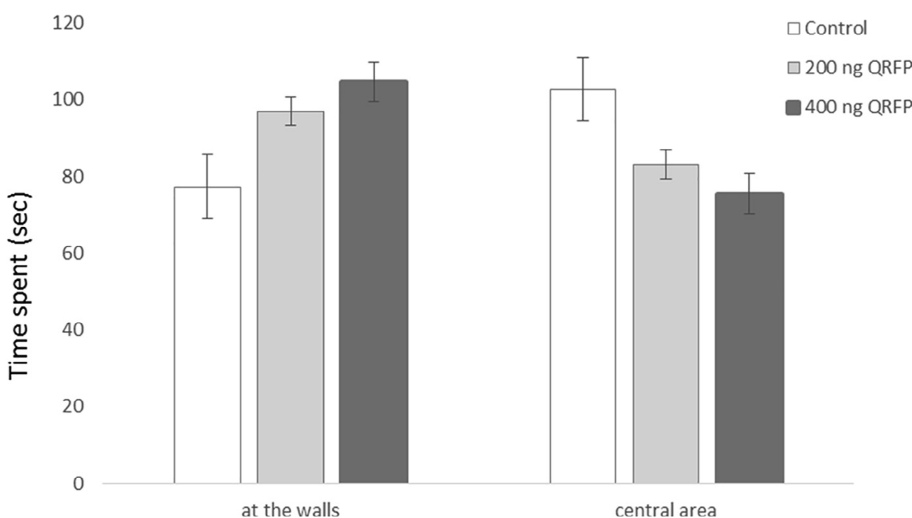

Fig. 7. Effects of QRFP microinjections into the MHA on the anxiety level during the Probe trial in Morris water maze. Columns represent the time spent at the walls, or time spent in the central area of the pool, respectively (± S.E.M.). The groups and the number of animals are identical to those present in the Fig. 2. There was no significant difference recorded.

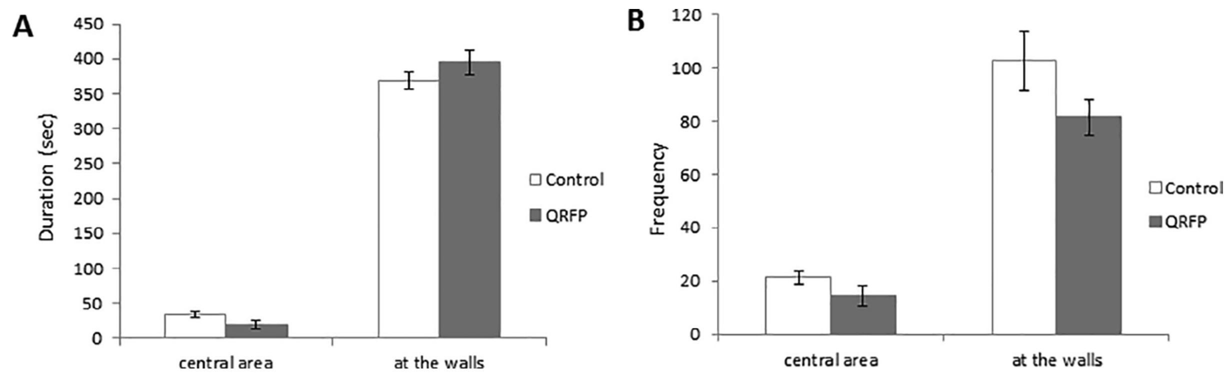

Fig. 8. Effects of QRFP microinjections into the MHA on the anxiety level in Open field test. Columns represent the time spent at the walls, or time spent in the central area of the apparatus, respectively (± S.E.M.). These are supporting data from our previous research, for details see (Zagoracz et al., 2015). There was no significant difference recorded.

the shape of certain nuclei. Thus, the medial hypothalamic area, including closely situated VMN and DMN, was chosen as a target for treatment (Fig. 1). Even if the target area within MHA seems to be wide, no significant difference in spatial task performance was detected when subgroups were compared (data not shown). For testing spatial memory consolidation the MWM paradigm was employed.

Two experiments in MWM revealed similar "pattern" for the training trials in regard to control and QRFP-treated groups. In the very first training trial, animals, habituated to the pool only, did not even know about the existence of platform hidden in the water. Looking for an escape, rats eventually found it by themselves, or, if not, were guided towards by the researcher. Surrounding cues allowed memorizing the life-saving platform position. By the trial 2, one minute later no significant learning was registered, though a tendency for shorter searching latency could be noticed, which suggests the formation of short-term memory in rats. Supposedly, these new neuronal interconnections are positively affected and reinforced by QRFP microinjections. By the next day in the swimming trial 3, some difference between treatment groups could be observed: while the control animals required a longer time to find the platform, seeming to forget the route learned the day before, QRFP treated rats, especially the ones treated with 400 ng, reach the target area within the time similar to the previous trial, and even improved a little bit the time, thus reaching a significant level in the first experiment. The training trial 4 again showed the formation of short-term memory in those groups, which was reinforced by the second microinjection. By the test trial with the removed platform (Probe trial), it became evident that control, as well QRFP- treated rats in both doses successfully learned where to search for the escape platform. The difference is that rats treated with 400 ng find the platform area faster comparing to Habituation trial, and comparing to the control and lower dose treated group during the same test. These findings have been confirmed by additional parameters, measured during the Probe test: 400 ng QRFP animals took a shorter rout to the place of platform, and spent less time searching the target in the "wrong" place opposite to platform annulus (Figs. 4 and 5). We suppose that the consolidation of short-term spatial memory was improved by 400 ng QRFP administration. We could notice that one microinjection (after the trial 2) did not lead to the desired cognitive effects. In the swimming trial 3, the tendency for memory consolidation could be registered, but no significant within-trial difference was detected. The second microinjection (after the trial 4) was necessary to reinforce that tendency and to decrease platform searching time to the significant level. Based on these data we suppose that QRFP microinjections lead to positive memorizing effect, nevertheless, probably more than two administrations are required to make it stable and long-lasting.

Previously we have already shown that in case of direct intrahypothalamic microinjections 100 and 200 ng doses of QRFP effectively increase food intake in rats (Zagoracz et al., 2015). Interestingly, in the present study lower dose turned to be ineffective, and only the 400 ng dose positively affected the consolidation of spatial memory. We suppose the reasons for this phenomenon lay in the anatomical and physiological features of the hypothalamus. It is widely accepted that VMH plays a role as satiety center, thus QRFP administration acted directly in place and exerted an orexigenic activity by modulating NPY/POMC system (Lectez et al., 2009). At the same time, medial hypothalamic nuclei have rich interconnections between each other and to other brain structures, which is one of the reasons for such a wide QRFP action spectrum. This way, probably, higher doses of peptide were required to reach the effect that was initiated in the hypothalamus but performed through the other brain areas, directly involved in the formation of spatial memory, such as the hippocampus, retrosplenial, entorhinal, or prefrontal cortex. Considering that hippocampus plays a crucial role in spatial memory and learning, it is likely, that this structure has been involved in memorial effects observed in Morris water maze. There have been shown direct pathways between CA1 and VMN (Cenquizca and Swanson, 2006), as well as between CA1, CA2 and DMN (Onat et al., 2002). Besides, there are several alternative paths considering the interconnections within the parts of hippocampus, within the hypothalamic nuclei, and thalamic projections (Cenquizca and Swanson, 2006; Cui et al., 2013; Goubillon et al., 2002; Hahn and Swanson, 2015; Onat et al., 2002; Zhou et al., 2019). Another point suggesting involvement of hippocampus is that QRFP mRNA is almost exclusively found in medial hypothalamic cells (Bruzzone et al., 2007), while specific and non-specific receptors are highly present all over the hippocampus: in subiculum and presubiculum, as well as in dentate gyrus, indusium griseum and fields 1–3 of CA (Bruzzone et al., 2007; Kampe et al., 2006).

Even though the role of QRFP receptor (GPR103) has not been investigated yet in terms of involvement in cognitive processes, previous reports also suggest the potential role of Y1 and FF receptors. Knockout mice with impaired Y1 receptors expression displayed enhanced spatial memory retention (Longo et al., 2014). In rats with Alzheimer's disease-like phenotype administration of NPY or Y1 preferring agonist prevented impairment of spatial memory, while BIBP3226 caused the opposite effect (Rangani et al., 2012). In addition, increased NPY gene expression was observed in hippocampal dentate interneurons of rats several hours after spatial learning performances in the MWM test (Hadad-Ophir et al., 2014). NPFF and its FF2 preferring agonist mildly impaired both short- and long-term memory tested in object location and MWM tasks (Betourne et al., 2010). The other study revealed a dual effect of NPFF administration: in low doses slightly improved, while in high doses significantly reduced spatial acquisition (Kavaliers and Colwell, 1993). Despite diverse memory associated effects, there is a reason to believe in the modulatory influence of Y1, FF1, and FF2 receptors. Whether these effects are connected to GPR103 receptors as well or involve only one receptor subtype, is a topic for further

investigation.

In the second experiment we intended to reveal whether observed cognitive effects are connected to specific receptors (Fig. 3). In the present study non-peptide NPYY1/NPFF receptor antagonist BIBP3226 was applied, which has already confirmed its efficiency against QRFP earlier (Takayasu et al., 2006; Zagoracz et al., 2015). In contrast to vehicle and peptide treated groups, animals treated with antagonist did not show memory formation during the training trials (Fig. 3B). After two microinjections, in the Probe trial rats treated with QRFP followed by vehicle (i.e. double volume of QRFP) found the place of the platform significantly faster comparing control and antagonist treated groups. Moreover, rats which received Ant and mixed Ant + QRFP treatments seemingly lacked the ability to learn the location of platform after four training trials, they required much longer time (and distance) to reach the escape area (Fig. 3B, B, C).

Two important conclusions have been done by the second experiment in MWM. Despite the double volume of injections and presumably larger physical damage caused by direct intraparenchymal administration, we succeeded to repeat the memory consolidating effect of 400 ng QRFP that was shown in the first experiment. Another important finding was that antagonist BIBP3226 effectively suppressed learning-promoting effects induced by neuropeptide QRFP.

Since the rats treated with 400 ng QRFP in both experiments found the place of platform much faster than all the others, undoubtedly the neuropeptide has a memory-reinforcing effect. Another question is whether this improvement refers to the true spatial memory, or not. Indeed, as it was noticed by Morris, the escape latency itself is not a sufficient parameter to measure a spatial memory (Morris, 1984). In this case, the Probe trial provides a great opportunity to analyze whether the observed shortening of the latencies to finding platform happened due to place-specific learning and memory consolidation, or due to other factors. The most common way for such assessment is the comparison of time spent in the different quadrants of the maze, mainly target and opposite quadrants. An even better option is to use an imaginary annulus surrounding the platform and mirroring annulus in the opposite quadrant, since these areas are more representative in the meaning of place-specific learning. Another assessed parameter was the number of entries to the target and opposite annuli (Fig. 5). To determine the change in place preference we have subtracted the time spent and the number of entries during the Habituation swimming from those during the probe trial in case of each animal. The remained difference reflected the place preference which is the result of learning (Péczely et al., 2016).

The additional analysis of the Probe trial revealed that all the treated groups demonstrated a similar preference to the target annulus. All the animals spent there similar time, and no significant difference could be registered in the normalised number of entries to the target annulus. The fact that the normalised time spent in the target annulus remained positive suggests that the incentive value of the platform was not changed by any of the drugs. In case of QRFP treatment the shorter escape latency and distance, combined with the relatively short time searching around the target and higher number of entries, suggest that the animals initially searched for the platform in its original place, then not finding it they continued searching around involving wider area but consistently coming back and crossing the target annulus. These rats were quite specific in their searching strategy – even though not concentrating attention around the exact place of platform, they did not go too far, and demonstrated lack of interest to the opposite annulus, i.e. spent there significantly less time and had lower number of entries comparing to the target annulus, and comparing to the other treatment groups. In contrast, antagonist treated groups demonstrated another strategy. According to their latencies to finding platform, by the Probe trial, these animals did not learn where the platform was situated and searched for it randomly around the pool. Equally considering the target and the opposite annuli as possible escape areas, they spent there similar time and had similar number of entries.

One can infer that QRFP memory-related effects could be provoked by the changes in general locomotor activity or anxiety. However, OFT data coming from our previous study (Zagoracz et al., 2015), in agreement with the swimming velocities in MWM, display that the neuropeptide has no impact on the locomotion. There are two research papers that examined anxiety effects of QRFP (Okamoto et al., 2016; Takayasu et al., 2006), and published the opposite results acquired in the EPM. Taking into account that both of them performed experiments on mice (and not rats), and the anxiety-like behavior was registered in genetically modified (not drug treated) animals, we considered it proper to conduct our experiments as well. Possible changes in the anxiety level have been inspected in three different paradigms: the time spent at the walls in OFT and in MWM, as well as the time spent in the opened arms in EPM (Figs. 6, 7, 8). Nor anxiogenic, neither anxiolytic signs could be indicated in rats' behavior. All the collected data support our hypothesis that the observed differences in the navigation task performance are due to memory consolidation effect of QRFP, and are not affected by changes in locomotor activity or anxiety level.

4. Summary

Our results suggest that neuropeptide QRFP is involved in the hypothalamic regulation of short-term spatial memory. At least to some extent, improvement in memory consolidation can be linked to NPYY1/NPFF receptor mechanism, so far as antagonist BIBP3226 appeared to prevent the observed effects. We have also confirmed that the effective dose of QRFP (400 ng) does not affect animals' locomotion or anxiety level, which might be an important point in further drug implementation. The present study opens a new chapter in QRFP research and gives the basis for further investigations of cognitive effects associated with this neuropeptide.

5. Materials and methods

5.1. Subjects

Ninety-three adult male Wistar rats (LATI, Gödöllő, Hungary) weighing 270–320 g at the beginning of experiments were housed individually in a temperature- and light-controlled room (22 ± 2 °C, 12–12 h light–dark cycle with lights on at 06:00 a.m.). Rats were cared for in accordance with institutional (Pécs University Medical School, BA02/2000–8/2012), national (Hungarian Government Decree, 40/2013 (II.14.)) and international standards (European Community Council Directive, 86/609/EEC, 1986, 2010). Tap water and standard laboratory food chow (CRLT/N standard rodent food pellet, Charles River Laboratories, Budapest, Hungary) were available *ad libitum*. Body weight, food, and water consumption were measured on a daily basis to the nearest grams and milliliters, respectively. All behavioral tests were performed during the rats' daylight period between 08:00 and 14:00 h.

5.2. Surgery

Rats were anaesthetized i.p. with ketamine supplemented with diazepam (Calypsol, 80 mg/kg bw and Seduxen, 20 mg/kg bw; Richter Gedeon Ltd., Hungary). Stainless steel guide tubes (22-gauge) were implanted into the MHA of the right hemisphere (coordinates referring to the *bregma*: AP: −2, 8 mm, ML: 0,6 mm and DV: 7.2–8,2 mm ventral from the surface of the *dura mater*) according to the stereotaxic rat brain atlas of Paxinos and Watson (Paxinos and Watson, 1986). The tips of cannulae were positioned 0.5 mm above the intended injection site. Cannulae were fixed to the skull with acrylic cement (Duracryl) and three stainless steel screws. When not used for injection, the guide tubes were occluded with stainless steel obturators (27-gauge). Following surgery, animals were allowed to have a minimum of 5 days for post-operative recovery before the testing began, during that time they were frequently handled.

5.3. Drugs and injection procedures

In the first experiment we have studied the effects of different doses of QRFP on spatial learning. QRFP-26 (Rat) (048–72, Phoenix Pharmaceuticals Inc., USA) was dissolved in 0.15 M sterile saline for intrahypothalamic microinjections in a volume of 0.4 μl. On the days of conditioning swimming, emergently after the daily second trials, rats received an injection of the peptide in appropriate dose: 200 ng (70 pM), or 400 ng (140 pM), respectively, or vehicle injection (0.15 M sterile saline) for control measurement.

The second experiment has been performed to verify the action of the receptor antagonist BIBP3226 (B174, Sigma-Aldrich Kft., Hungary) on spatial learning in rats. To unify procedure in different treatment groups, this experiment implicated double injection volume (0.4 μl + 0.4 μl) to each animal. For control values, rats were treated with the aforementioned vehicle solution (Control, Vehicle + Vehicle). Then animals were tested for treatment by 400 ng QRFP followed by saline injection (400 ng QRFP, QRFP + Vehicle). Antagonist treatment included administration of an equimolar dose of BIBP3226 (70 ng, 148 pM) 15 min prior to the 400 ng QRFP injection (Ant + QRFP), or BIBP3226 administration followed by vehicle injection (Ant, Ant + Vehicle).

All substances were injected through stainless steel injection tubes (27-gauge) extending 0.5 mm below the tips of the implanted guiding cannulae. The injection cannula was attached via polyethylene tubing (PE-10) to a Hamilton microsyringe (10 μl, Bonaduz, Switzerland). Drugs were injected over 60 sec interval by automated syringe pumps (Cole Parmer, IITC, Life Sci. Instruments, USA), and the injection cannula was left in place for an additional 60 sec to allow diffusion into surrounding tissues. During the microinjections, awake, well-handled rats were gently held by hand.

5.4. Morris water maze test (MWM)

MWM experiments were carried out in a circular pool (150 cm diameter, 60 cm height), virtually divided into four quadrants. One of the quadrants was chosen to place a square (10 cm × 10 cm) plexiglass target platform. The location of the platform was fixed during the experiments, except for the habituation and extinction trials. The water was kept at a constant temperature (23 ± 1 °C) and was colored with Potassium permanganate, so the platform was not visible for the animals. The surface of the water was kept 2 cm above the platform. Spatial reference cues around the pool were maintained in their fixed positions throughout the MWM experiments. The animals' behavior was recorded by a video camera and registered by special software (EthoVision; Noldus Information Technology, The Netherlands).

One day prior to the beginning of the experiments, rats were allowed to get acquainted with the surrounding environment and the pool (without platform) in a Habituating session lasting 180 sec. On the first day of conditioning two trials for spatial learning, separated by 60 sec interval, were performed (trials 1 and 2). This short interval ensured the possibility to observe the short term memory trace formed during the first trial. On the second day, 24 h later, training was continued on the same schedule (trials 3 and 4). In these four trials, the latency to finding the safe platform was measured. The four training trials were conducted as follows: rats were placed into the water maze at randomly assigned but predetermined locations to avoid the egocentric orientation. The task required the animal to search for the hidden platform guided by external spatial cues. Each trial lasted until the rat found the platform or for a maximum duration of 180 sec. Animals who failed to find the platform within the allocated time were gently guided to the platform. By finding the platform, the rat was allowed to stay there for 60 sec.

On the third day, 24 h following the last swimming training, a Probe trial was performed: the platform was removed, and the latency to the first crossing of the platform's place (escape latency) was measured. In addition to the latency to first occurrence, also distance and the rout trajectory were analyzed. The target annulus surrounding the platform and the opposite annulus in the opposite quadrant (in both cases the diameter was 37,5 cm, the quarter of the pool's diameter) were determined. The time spent in those annuli, as well as the number of entries, were analyzed (with the assistance of Noldus software) during the two swimming trials without platform (i.e. habituation and probe trials). The normalised data have been calculated, meaning that in case of each animal's data in the given annulus during Habituation trial have been subtracted from data achieved during the Probe trial. If the animal's preference for the given annulus increased, then the normalised time and the number of entries were positive, and if it decreased then parameters had negative values (Péczely et al., 2016). During the experiments in each trial the mean swimming velocities of the animals were analyzed as well. An additional parameter, indirectly indicating the the signs of anxiety, i.e. time spent by the animals at the walls, was evaluated during the Probe trial.

5.5. Elevated plus maze (EPM)

Anxiety was evaluated in the EPM test. The apparatus was constructed of grey colored wooden planks. The equipment consisted of two opposite open arms (50 cm × 10 cm) and two opposite closed arms (50 cm × 10 cm × 40 cm) with walls and opened roof. The maze was elevated to a height of 100 cm above the floor. Following the administration of the drugs, animals were placed into the center of the maze (central platform), facing one of the closed arms. Each rat was tested only once. Trials lasted for 5 min, and during this period the time spent on the opened and closed arms and at the ends of the opened arms was recorded.

5.6. Histology

In order to verify cannulae placements, animals received an overdose of urethane (20%) and were perfused transcardially with isotonic saline followed by 10% formaldehyde solution. Brains were sliced with a freezing microtome in 40 μm sections and stained with Cresyl-violet. Injection sites were reconstructed according to the stereotaxic atlas (Paxinos and Watson, 1986). The track of cannulae and the tips were determined on the basis of the existence of debris and moderate glial proliferation. Only data from the rats with correctly placed cannulae were analyzed.

5.7. Statistical analysis

All results are expressed as a mean ± standard error of the mean (S.E.M.). Data were evaluated by two-way and one-way ANOVA, followed by Tukey post-hoc test in case of significant effect (IBM SPSS Statistics 20 data analysis program). The statistical rejection criterion for all the experiments was established at $p < 0.05$ level.

Acknowledgements

The authors express their thanks to Anna Schulteisz, Erika Szabó, Erzsébet Korona, Fanni Géczi, Ferenc Wilhelm and András Belvárácz for their technical contribution to this work. This study was supported by SROP-4.2.2/B-10/1-2010-0029, SROP-4.2.1/B-10/2/KONV-2010-0002, by the ÚNKP-18-3-III (PTE-374) New National Excellence Program of the Ministry of Human Capacities, by 2/2015 PhD + 1 Dean's Grant of the Pécs University Medical School, and by the Hungarian Academy of Sciences.

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