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ROLE OF NEUROPEPTIDE QRFP IN THE HYPOTHALAMUS

PhD thesis

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1. Introduction

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 suffer from eating disorders, such as anorexia nervosa, bulimia nervosa or binge eating [1]. Often such patients reveal associated psychiatric symptoms: 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].

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 diseases combined [12]. For the patients with dementia, diagnosis attracts other health impacts and social isolation.

Precise coordination of the biological functions is required to maintain their fragile balance. The hypothalamus is one of such coordinating centers. The role of the hypothalamus in appetite and food intake was recognized long ago. Experiments with massive brain lesions and electrical stimulations of the hypothalamic nuclei contributed to the concept of distinct „feeding centers", considering the ventromedial hypothalamic nucleus (VMH) as satiety center and the lateral hypothalamic area (LHA) as hunger center [13-22]. Via the neurotransmitter signaling [noradrenaline (NA), dopamine (DA), serotonin (5-HT)] the hypothalamus 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 [23-30], the motivation and reward mechanisms in application to feeding and satiety regulation [31-33]. Also, the role of orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP)-expressing neurons on one side, and the anorexigenic pro-opiomelanocortin (POMC)-expressing neurons on the other, is recognized. Also, hypothalamus is an in integrating center of the „emotional and behavioral brain", i.e. the limbic system. Different parts of the hypothalamus have dense projections towards hippocampus, amygdala (Amy), septal nuclei, nucleum accumbens, etc. [34-38]. Multiple hypothalamic neuropeptides, such as corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), oxytocin (Oxt), vasopressin (VP), and many others are recognized to regulate behavior, feeding as well as memory and learning processes.

The neuropeptide QRFP is a recently discovered pyroglutamylated member of the RFamide peptide family. QRFP-expressing neurons are almost exclusively localized within the hypothalamus (VMN, DMN, LHA) [39-41]. The QRFP receptors, also known as GPR103, can be found in rodents in two isoforms (subtypes 1 and 2) [41, 42] . Within the brain, the QRFP receptors are primarily expressed in the cerebral cortex, the hypothalamus, thalamus, vestibular nucleus, and the trigeminal ganglion, also in the Amy, caudate nucleus, hippocampus, and the ventral tegmental area (VTA) [43, 44]. The biological effects of QRFP extend to regulation of feeding behavior, nutritional and metabolic state, locomotor activity, anxiety, nociception, cardiovascular activity, metabolism of skeletal muscles and adipose tissue, reproduction, adrenal gland activity, and even play role in some cancers [45]. Nevertheless, multiple data regarding central effects of QRFP remain inconsistent.

In this paper we explore properties of the novel pyroglutamylated RFamide neuropeptide QRFP when applied directly into the hypothalamic parenchyma, namely, the medial hypothalamic area (MHA, including ventromedial and dorsomedial nuclei, VMN and DMN) and lateral hypothalamic area (LHA). Despite the substance was identified in the early 2000s, its role and mechanisms of action are still under research. Besides investigations of QRFP's role in feeding, anxiety, and motivation regulation, here we present unique evidence regarding previously unknown aspect of neuropeptide's activity, i.e., in consolidation of spatial memory.

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 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 and 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. 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, 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 in CPP, MWM and EPM were analyzed as well.

5. Another strategically important goal of the present study was to examine whether neuropeptide QRFP alters the anxiety level. Elevated plus maze (EPM) test was recalled answering this question. Specific parameters collected during the OFT and MWM were analysed 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 intraperitoneally (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 [46]. 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 (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).

3.3. Drug injections

During the experiments rat 26-amino acid residue of neuropeptide 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 18ng, 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 and according to the findings in pilot experiments. 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, vehicle).

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.

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, 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.

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 [47-52].

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 were analyzed [53, 54].

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.

3.5. Conditioned place preference test (CPP)

The CPP test was employed to test the rewarding, positive reinforcing, or aversive effects of the drugs [55, 56]. The CPP apparatus consisted of a circular open field arena (85 cm in diameter and 40 cm in 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 [55]. 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 time spent 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.

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 swimming 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 cues. 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 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, a quarter of the pool's diameter) were determined [57]. 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 [58]. 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.

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.

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.

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 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 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 [46]. 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. 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. In 172 brains, the LHA was reached.

In the other 32 animals, cannulae were not correctly positioned in the target area. Among them in 4 cases cannulae were led to the zona incerta, in 7 animals to the entopeduncular nucl., in 3 cases towards arcuate nucleus, 3 other cannulae went out of the brain, in 15 rats' cannulae tips entered into the liquor space of the III 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.

4.2. Results of the feeding experiments

Feeding tests began from the fifth postoperative day when all animals reached the preoperative level of body weight and food intake. The mean cumulative liquid food consumption in ml/100 g body weight (±S.E.M.) was measured during 60 min period. **Administration of 100 ng QRFP into the MHA induced significant orexigenic effect.** The food consumption was elevated at each time point from 10^{th} to 60^{th} min ($p < 0.03$). In case of **200 ng QRFP treatment, food consumption was markedly increased as well**. There was significant raise in liquid food intake from 10^{th} to 30^{th} min ($p < 0.05$). Data from the experiments with antagonist (Ant), where the ability of BIBP3226 was examined confirmed that **vehicle + 100 ng QRFP administration led to a significant increase in food intake**. Significant raise in food consumption was detected at each time point from 10^{th} to 50^{th} min ($p < 0.03$). **When animals received combined antagonist and peptide treatment with a 15 min interval (Ant + QRFP), the food consumption did not only return to the control level but also was transiently depressed**. We 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** Significant fall in food consumption was noticed at 5^{th}, 20^{th} and 25^{th} min ($p < 0.03$ at all three time points).

Effects of **QRFP microinjections into the LH** on food intake showed that **100 ng dose of the peptide led to significant anorexigenic effect.** The food consumption was significantly lower at each time point during the first 50 minutes ($p < 0.04$). **Following the application of the QRFP in 200 ng dose, anorexigenic effects were registered as well.** The food consumption was significantly lower from 10^{th} till 25^{th} minutes ($p < 0.04$). **The effective dose of QRFP applied in double volume (veh + QRFP) confirmed the previous data by decreasing food intake**. A significant difference was shown at each time point during the first hour ($p < 0.03$). **Combined antagonist and neuropeptide** (Ant + QRFP), **as well as antagonist and vehicle treatments** (Ant + veh) **inhibited anorexigenic effects induced by QRFP**. The food consumption in control and Ant treated groups was identical ($p > 0.05$).

4.3. Results in Conditioned place preference paradigm

Results of the neuropeptide **QRFP microinjections into the MHA** on learning in the CPP test stay that rats, treated with **low and high doses of QRFP** 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**. **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.

The LHA QRFP microinjections had no effect on conditioned learning. Interestingly, **Ant treatment led to longer latency spent in TQ**. Post hoc test indicates that group of animals treated with Ant spent significantly more time within the TQ in comparison to Control, veh + QRFP and combined Ant + QRFP treated groups ($p<0.04$, $p <0.03$ and $p <0.01$, respectively). At the same time, there was a significant difference between the trials, indicating that Ant provoked rats to spend more time within the TQ during the Test comparing to the naïve state of the Habituation trial. Further experiments clarify whether the observed phenomenon is a result of real conditioned place preference learning or rather refers to increased anxiety.

4.4. Results in Morris water maze paradigm

At first, the effect of **MHA QRFP treatment** on the escape latencies of rats was investigated. 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, 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** comparing to other groups. Similar to the first experiment, in the Probe trial **the mean latency of the Vehicle + QRFP 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). **Meanwhile, treatment with Ant prevented this effect.** Following the tendency, **400 ng QRFP treated rats made shorter routes to the target compared to all other groups**. They **had lower number of entries into the opposite annulus comparing to target annulus** ($p = 0.02$) and **spent much more time searching the platform in the target annulus comparing to the opposite one** ($p < 0.01$).

The LHA administration of QRFP led to **significant latency decrease in 200 ng and 400 ng QRFP** ($p = 0.01$, $p = 0.002$, respectively). A significant difference between the groups during the Probe trial was not registered. 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 double-volume experiment, significant difference again was registered only in 400 ng treated animals ($p < 0.01$). **Rats treated with 400 ng QRFP made significantly shorter routes compared to the control and Ant + QRFP groups while searching the target platform**. Animals treated with the lower dose of the neuropeptide also revealed the tendency for faster search, not reaching significant values though. **The analysis of the 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**

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

Effects of any drugs on behavioral parameters might be altered by changes in the anxiety level. The effects of medial hypothalamic QRFP and BIBP3226 microinjection have been investigated in the EPM paradigm. T**he 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 the control group or each other**. **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), and shorter periods of investigating opened arms following the Ant treatments ($p < 0.04$ and $p < 0.03$, respectively). **These changes may be interpreted as signs of increased anxiety level induced by the Ant treatment**.

EPM-derived data with anxiety records following the **LHA microinjections** state that **control animals, as well as low and high- dose QRFP treated rats spent similar time in the closed and opened arms of the maze**. **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). In other words, **Ant treatment in LHA seems to induce an anxiogenic effect in EPM test**.

Animals' performance in other paradigms was analyzed for the signs of the anxiety deviations. **Following the MHA and 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. These data correspond **with negative results from OFT**.

4.6. Results of the experiments on general locomotion

OFT was employed as a specific paradigm for observation of the treatment effects on the locomotion, horizontal and vertical explorative activity. Following the **MHA treatment**, the test **did not reveal a significant difference** between the Control, low and high doses of QRFP and Ant-treated groups.

Effects of the microinjections into the LHA on spontaneous locomotion (distance moved and rearing episodes) **did not reveal a significant difference** between the groups. 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, **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 time during the test trial in comparison to basal activity data was noticed in Ant group (not significant difference).

Supportive data from the EPM, as well as the CPP and MWM tests confirm that applied treatments (both in the MHA and in the LHA) did not modify ability to move during the experiments.

5. Discussion

5.1. Discussion of the feeding experiments

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. 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. Treatment by non-peptide antagonist BIBP3226, as well as combined Ant and QRFP treatment, effectively suppressed the orexigenic effect and even transiently reduced food consumption about 5–25 min after injection. 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, but the lower dose had a stronger and more elongated effect (from 5^{th} to 50^{th} min). Ant treatment prior to QRFP and Ant treatment with the vehicle fully prevented deviations in consumed milk amount during all the observed periods.

The role of the NPY system comes forward as the most possible mechanism of action. It was shown that leptin, known to regulate feeding behavior via the NPY system, also affects hypothalamic expression of preproQRFP mRNA [42]. The Arc contains a subpopulation of the NPY, but not POMC neurons, expressing QRFP receptors [59]. 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. 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. An alternative explanation for the observed effects can be a cross-reactivity of the neuropeptide QRFP with NPFF and orexin systems, considering the amino acidic sequence identity between the cousin receptors [43, 44, 60].

5.2. Discussion of the Conditioned place preference test

The feeling of satiation is a positive emotional state, while hunger on the other side of the scale causes highly unpleasant sensations. Since no preliminary data regarding rewarding or aversive effects of QRFP was available, this aspect investigated in CPP paradigm.

Microinjections of QRFP neither into the MHA nor into the LHA provoked animals' place preference. 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 the habituation. 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).

5.3. Discussion of the Morris water maze

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

The rats microinjected with both doses of QRFP into the MHA and into the LHA showed positive learning tendency. Remarkably, 400 ng treated rats found the platform area faster comparing to the habituation trial and compared to the control and lower dose treated group during the same test. They also took a shorter route to the place of platform and spent less time searching the target in the „wrong" place opposite to platform annulus. We suggest that the consolidation of short-term spatial memory was improved by 400 ng QRFP administration. In contrast, animals treated with Ant did not show memory development during the training trials. 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. 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.

The potential role of NPY and FF systems may be considered in this matter. Activation of Y1 and Y5 co-expressing neurons in knock-out mice enhanced spatial memory retention [61] and prevented impairment of spatial memory in rats with Alzheimer-like phenotype [62]. Administration of NPFF revealed a dual effect: in low doses slightly improved, while in high doses significantly reduced spatial acquisition [63]. Despite diverse memory-associated effects, there is a reason to believe in the modulatory influence of QRFP via Y1, FF1, and/or FF2 receptors.

5.4. Discussion of the experiments on anxiety

Possible detection of the anxiety and stress reactions in response to treatment manipulations was an inevitable procedure. The anxiogenic effect may cause "freezing" of the animals and lead to inappropriate interpretation of the results in other paradigms.

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 (the bed nucleus of the stria terminalis, the lateral septum, and the PAG). Some studies suggest that QRFP does not affect anxiety level [42], while others reveal anxiolytic action of QRFP in the EPM [64, 65].

In our experiments, following the QRFP application, the behavior of animals did not differ from those of controls. Namely, no anxiogenic effect was recorded after different doses of QRFP (100, 200, and 400 ng/ rat, respectively) injected into the MHA or LHA. In turn, Ant treatment in both hypothalamic fields led to increase in anxiety levels. The anxiogenic effect was slightly more pronounced in LHA. The time spent by the animals with grooming in OFT after LH, but not MH, Ant microinjections also points towards amplification of anxiety. The induction of anxiety was 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.

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. 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 [66]). Y1 receptors, found within the VMN and DMN, and also in high concentration in the LHA and Amy [67], are thought to be key participant. Ligands for FF2 receptors have been described to attenuate stress reactions by activation of the HPA axis and inducing anxiogenic effects in rodents [68]. Activation of FF1 receptors in Amy led to the anxiolytic effect [69].

Presumably, the Y1 and/or FF1 receptors in MHA, and Y1 receptors in LHA are involved in this phenomenon, according to receptors' tissue distribution. The possible role of other neurotransmitters (opioids [70, 71], CRH [72], 5-HT [73, 74]), NA [75, 76], GABA [64, 77]) is to be determined.

Our results suggest that intrahypothalamic administration of QRFP itself does not evoke anxiety. According to the contradictory data of literature, the involvement of specific QRFP receptors in this area remains unclear. We consider the maintained normal anxiety level as positive sign in terms of interpretation of the main effects and future drug development.

5.5. Discussion of the general locomotion

Our findings suggest that intrahypothalamic microinjections of QRFP did not affect general locomotor activity. The tendency for a higher number of line crossings caused by 400 ng QRFP treatment into the MHA 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 when applied into the LHA. Since the data of traveled distance in other experiments reinforce data from the OFT, we tend to interpret it rather as a sign of anxiety behavior than modification of the locomotion itself.

Depending on applied paradigm and concentration, there are contradictory data in the literature about the effects of QRFP on locomotion. In several experiments QRFP stimulated locomotor activity during both the light and dark periods, increased grooming activity [42], as well as horizontal and vertical locomotor activity [78]. At the same time, QRFP gene overexpression in zebrafish decreases daytime locomotor activity, without inducing sleep though [79]. Also several reports stay that locomotion is not altered by QRFP treatment in acute or chronic paradigms [41, 80-82]. In contrast to cousin NPFF peptid, it was indicated that QRFP effects on locomotion are not mediated via the opioid system [78].

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.

7. References

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8. List of publications

8.1. 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]

8.2. 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]
2. Kállai, Veronika; Lénárd, László; Péczely, László; Gálosi, Rita; Dusa, Daniella; Tóth, Attila; László, Kristóf; Kertes, Erika; Kovács, Anita; Zagoracz, Olga et al. "Cognitive performance of the MAM-E17 schizophrenia model rats in different age-periods" BEHAVIOURAL BRAIN RESEARCH 379 Paper: 112345 , 8 p. (2020) [IF: 2.977]
3. Berta, Beáta; Kertes, Erika; Péczely, László; Ollmann, Tamás; László, Kristóf; Gálosi, Rita; Kállai, Veronika; Petykó, Zoltán; Zagorácz, Olga; Kovács, Anita et al. "Ventromedial prefrontal cortex is involved in preference and hedonic evaluation of tastes." BEHAVIOURAL BRAIN RESEARCH 367 pp. 149-157. , 9 p. (2019) [IF: 2.977]
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5. Berta Beáta, Péczely László, Kertes Erika, Petykó Zoltán, Ollmann Tamás, László Kristóf, Kállai Veronika, Kovács Anita, Zagorácz Olga, Gálosi Rita et al. "Iontophoretic microlesions with kainate or 6-hydroxidopamine in ventromedial prefrontal cortex result in deficit in conditioned taste avoidance to palatable tastants." BRAIN RESEARCH BULLETIN 143 pp. 106-115., 10 p. (2018) [IF: 3.103]
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