Theoretical Medical Sciences Ph.D. Program

Food intake and behavioural effects of RFamide-related peptides (RFRP-1 and RFRP-3) in the central nucleus of the amygdala

Ph.D.Thesis

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Pécs, 2014

1. Introduction

In our experiments we investigated the effects of two neuropeptides, namely the RFRP-1 (Neuropepride SF) and the RFRP-3 (Neuropeptide VF) on feeding and other behavioural processes in the central amygdaloid nuclei (CeA).

Numerous neurotransmitters and neuropeptides are involved in the central nervous system regulation of food intake and energy metabolism. According to our present knowledge, behind the body weight regulation disturbances, such as anorexia nervosa, bulimia or obesity (disregarding psychological factors, inactive lifestyle and others factors) the disturbances in the regulation of feeding behaviour and dysfunctions of neurochemical processes involved in energy metabolism can be revealed. Neuropeptides and peptide molecules play an important role in the regulation of these nervous system processes. Neuropeptides are produced in different brain structures of the central nervous system and in the peripheral tissues. These peptides represent peripheral neuronal and humoral signals and they are neurotransmitters and modulators in the hunger-satiety network of the central nervous system. Some peptides decrease food intake as they promote satiety (anorexigenic signals), while other peptides, increase food intake as they induce appetite (orexigenic signals).

The RFRP-1 and RFRP-3 are members of the RFamide peptide family. Characteristic feature of the RFamide peptides is that they contain a terminal arginine (R) and amidated phenylalanine (F), and the common name, i.e. RFamide peptide family is derived from this structure [18]. To date, five groups of the RFamide peptides have been documented: Neuropeptide FF (PQRFa) group [1,49], PrRP group[23], LPXRFamides (RFRPs) group [43,45], Kisspeptin group [40] and QFRP (26RFa) group [6,20]. Several members of the RFamide peptide family are known to have role in the regulation of feeding [9]. For example, i.c.v. application of neuropeptide FF and of prolactin-releasing peptide cause anorexigenic [29,33], while 26RFa results in orexigenic effects [37]. I.c.v. microinjection of RFamide-related peptide-3 (RFRP-3) facilitates feeding in rodents [25,32]. As far as the RFRP-1 is concerned, it was applied i.c.v. to chicken and exhibited anorexigenic properties [35]. RFRP peptides act on NPFF receptors, namely on NPFF1 and NPFF2 receptors (NPFF-R).

Subsequent studies have shown that RFRP-1 and RFRP-3 bind with high affinity to the NPFF-1 and with low affinity to NPFF2 [4,10]. RFRP-1 and RFRP-3 immunoreactive fibers were identified in the CeA. NPFF-1 receptors were also expressed in the CeA.

It is well known, that the amygdala, as a part of the limbic system, plays an important role in the regulation of food intake and body weight [12,27]. Lesion of the central part (CeA) of the amygdala causes hypophagia and body weight loss, while destruction of the basolateral amygdala (BLA) leads to hyperphagia and body weight gain [13-16]. On the basis of these results it has been accepted that a hunger center (CeA) and a satiety center (BLA) can be found the in the amygdala. In our experiments were focused on the CeA, because this intraamygalar subregion plays a role in the regulation of feeding behaviour, stress-related responses [21, 31, 47], reinforcement and reward-related processes [2,5,7].

2. The aim of our study

1) We examined, therefore, whether the RFamide-related peptides, which are found in the mammalian nervous system, namely the RFRP-1 and the RFRP-3 have any effect on feeding after their microinjection into the CeA.

a) Effects of RFRP-1 and RFRP-3 on food intake were investigated in ad libitum fed rats.

b) It has been shown by immunohistochemical and autoradiographic methods that the NPFF1 receptors in relatively high density can be found in the amygdala including the CeA. The RFRP-1 and RFRP-3 can also bind to NPFF2 receptors but these receptors were not identified in the CeA. It was studied whether the effect of RFRP-1 and RFRP-3 on feeding can be prevented by NPFF receptor antagonist pretreatment.

c) Effect of 24 h food deprivation induced hunger drive was investigated on the RFRP-1 and RFRP-3 induced changes in feeding behaviour.

d) Food intake modifying, nonspecific effects of RFRP-1 and RFRP-3 were also examined: effects of RFRP-1 and RFRP-3 on changes in body temperature (hypothermia or hyperthermia), on water intake and on behavioural activities (during feeding procedure, five behavioural categories were recorded: the time spent with feeding, locomotion, resting, grooming and scratching) were investigated.

2) After RFRP-1 and RFRP-3 microinjections into the CeA spontaneous motor activity and anxiety were examined in open field and elevated plus maze test in rats. It was important to investigate whether the peptide administration into the CeA influences locomotion and has anxiolytic or anxiogenic effects, because these factors can modify food intake. Futhermore, we attempted to clarify the role of NPFF1 receptors in mediating the anxiolytic or anxiogenic effect of RFRP-1 and RFRP-3 in the CeA by NPFF receptor antagonist pretreatment.

3) The possible rewarding-positive reinforcing or aversive effects of RFRP-1 and RFRP-3 were examined in conditioned place preference test. In the place preference test animals spend more time in a certain part of the apparatus associated with the rewarding effect of a drug. This could also be due to a nonspecific effect resulted from decreased motor activity (hypoactivity). We investigated the effects of RFRP-1 and RFRP-3 microinjections on anxiety in the elevated plus maze test, and in addition we analized data of specific parameters (distance moved, number of entries) obtained in place preference test.

3. Materials and Methods

3.1. Animals

Subjects were male Wistar rats weighing 280-320 g at the beginning of the experiments. Rats were housed individually and kept in a light-, temperature- and humidity-controlled room (12:12 h light-dark cycle with lights on at 06:00 a.m., 22 ± 1 °C, and 50-60 %, respectively). Tap water and standard laboratory food pellets (CRLT/N standard rodent food pellets, Charles River Laboratories, Budapest, Hungary) were available according to the experimental schedule. Animals were cared for in accordance with institutional (Pécs University, Medical School) and international standards (European Community Council Directive - 1986. November 24. 86-609-EEC).

3.2. Surgery

Animals were stereotaxically implanted bilaterally with stainless steel guide cannulae (22 gauge), directed toward and 1 mm above the dorsal border of the CeA. Coordinates according to the rats' stereotaxic atlas of Paxinos and Watson [36] were the following: coordinates referring to the bregma AP: 2,3 mm, ML: ± 4,1 mm and DV: -6,5 mm ventral from the surface of the dura.

3.3. Drugs, microinjections

In the experiments the RFRP-1 (048-48, Phoenix Pharmaceuticals, Inc., USA) was injected bilaterally in 25, 50, 100 or 200 ng (18,93; 37,8; 75,7 or 151,4 pmol) doses, the RFRP-3 (048-33, Phoenix Pharmaceuticals, Inc., USA) in 25, 50, 100 or 200 ng (25,25; 50,5; 100,1 or 201,9 pmol) doses and the NPFF receptor antagonist RF9 (R4282, Sigma Aldrich Co. trifluoracetate salt, henceforward ANT) in 20, 25 ng or 50 ng (41,4; 51,8 or 103,6 pmol) doses. Chemicals were dissolved in 0,15 M sterile staline. The volume of drug microinjections was 0.4 µl. Injection of vehicle (0,15 M steril NaCl) was used in the same volume. Since the injections were bilateral in all cases, the total doses were the twice of

the above mentioned doses. In this thesis all the doses mentioned are meant to be the dose per side value. Drugs or vehicle were bilaterally microinjected through 27 gauge stainless steel injection tubes extending 1 mm below the tips of the implanted guide cannulae. The injection cannula was attached to a 10 µl Hamilton microsyringe (Hamilton Co., Bonaduz, Switzerland) via a polyethylene tube (PE10). The Hamilton microsyringe was operated with a Cole Parmer automatic minipump (Cole-Parmer, IITC, Life Sci. Instruments, California). Drugs were injected for 1 min by the microsyringe pumps and the injection cannula was left in place for an additional 1 min to allow diffusion into the surrounding tissue. ANT was used alone or microinjected 15 min before the bilateral RFRP-1 or RFRP-3 application, respectively. During injections rats were gently hold in hands.

3.4. Food intake measurements

In experiments with ad libitum fed rats food pellets were available till 1h before liquid food consumption, while in food deprivation experiments rats were fasted for 24 h before microinjections and during tests only liquid food was available. From the 14th preoperative day on rats were trained for two weeks to consume the liquid diet (milk, 136.45 kJ/100 ml, Milk Quick, Debrecen, Hungary). Graduated drinking cylinders with 1.0 ml divisions were used for measuring milk ingestion. This feeding schedule was used to overcome neophobia and to accustom the rats to the palatable complex food [11,41,46]. Rats were excluded from any experiments if their liquid food intake did not show stable baseline. Our method made exact consumption measurement possible in 5 min intervals with ml accuracy without disturbing animals in their cages. Milk intake was measured at ml accuracy every 5 min for 30 min and at the 40th, 50th, 60th min, respectively. After the experiments rats were returned to ad libitum pellet feeding and tap water.

3.5. Behavioural examinations in food intake paradigm

Using the same feeding procedure, behavioural activities of rats were recorded for 25 min by a video camera (Panasonic SDR-H85). Individual rats were video-monitored in their home cages, beginning immediately after bilateral microinjections with 50 ng RFRP-1, 50 ng RFRP-3 or vehicle into the CeA. Five behavioural categories were identified and their

durations were determined. In the course of an off-line analysis the time spent with feeding, locomotion, resting, grooming and scratching was measured in 5 min periods for 25 min.

3.6. Water intake measurements

From the 7th preoperative day, rats were allowed to consume tap water from a calibrated (with 1 ml division) drinking tubes between 08:00 a.m. and 12:00 a.m. In these experiments the effects of bilateral microinjections of 50 ng RFRP-1, 50 ng RFRP-3 or 100 ng RFRP-3 were investigated on water intake. Water intake was measured every 5 min for 30 min and at the 40th, 50th, 60th min, respectively.

3.7. Body temperatures measurements

In a separate experiment the core temperature of animals was measured using a digital thermometer (digital clinical thermometer, Kruuse, Cat.no 291103). Temperature probe was inserted 25 mm into the colon. The temperature was measured 10 min before and 10 and 20 min after bilateral injection of 50 ng RFRP-1, 50 ng RFRP-3, 100 ng RFRP-3 or vehicle.

3.8. Behavioural tests

In these experiments, behavioural parameters of the animals were recorded and analyzed by means of the' Noldus EthoVision Basic' PC software (Noldus Information Technology b.v., Wageningen,The Netherlands). Digitally recorded and stored movement-related data of the rats in the delimited arena were analyzed on line and off line.

3.8.1. Open field test

Open field test was used to measure the spontaneous motor activity of the animals. Animals were placed into 60x60x60-cm grey painted cage for 10 minutes after bilateral intraamygdaloid microinjections. The ground of the box was divided into 16 identical squares. Behaviour of each rat was recorded for five min by means of CCD camcorder. During observation period the number of crossings and the distance moved were investigated.

Measurements of time spent in the central area of the open field apparatus (25% of the floor area of the box) were used for studying anxiety-like behavior.

3.8.2. Elevated plus-maze test

The elevated plus-maze test is an accurate method to investigate the anxiolytic or anxiogenic effect of a chemical substance [24]. The equipment consisted of two opposite open arms (50x12cm) and two opposite enclosed arms (50x12x40cm) with an open roof. The maze was elevated to a height of 1 meter above the floor. Ten minutes after intraamygdalar microinjections animals were placed into the center of the maze (central platform), facing to one of the enclosed arms. The trials lasted for 5 min and the time spent on, the distance moved on and the number of entries into the enclosed arms, the open arms and to the end of the open arms (end-arms) were measured. The total distance moved during 5 minutes was also measured. The total distance moved and the total number of entries were used for the characterization of the general activity of animals.

3.8.3. Conditioned place preference (CPP) test

The CPP test has been used to measure hedonic properties of drugs of abuse as well as of natural reinforcers [44]. Our apparatus consisted of a circular open field (85 cm in diameter, with 40 cm high wall) with black crossing lines that divided the floor into four quadrants of equal size. External visual cues in the surroundings helped the animals' spatial orientation inside the apparatus. The room was dimly lit by a 40 W bulb. The place preference procedure was carried out on four consecutive days and consisted of one habituation (day 1), two conditioning (days 2–3) and one test (day 4) trials. Each trial lasted for 900 s (15 min). The apparatus was cleaned and dried after each session. In habituation trial (day 1), animals were placed into the center of the open field and had free access to all parts of the apparatus for 15 min. The time that animals had spent in each of the four quadrants was measured. One of the quadrants in which the animal had spent neither the most, nor the least time during habituation was selected for conditioning (treatment quadrant). During conditioning trials (days 2–3), animals received the bilateral injections and subsequently rats were restricted to the treatment quadrant for 900 s by means of a

transparent plexiglass barrier. During test trial (day 4) the plexiglass barrier was removed and rats were placed again into the center of the apparatus. The time that rats had spent in each of the four quadrants was measured again. The number of entries into the four quadrants (locomotion) and total distance moved were also recorded during habituation and test trials, as a measure of gross locomotor activity. Place preference was defined as significant increase in time spent in the treatment quadrant during test trial compared to the habituation trial.

3.9. Data processing

3.9.1. Histology

At the end of the experiments, animals were anaesthetised with urethane and were perfused transcardially with isotonic saline followed by 10% formalin solution. After one week of postfixation 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 of Paxinos and Watson [36]. Animals with incorrect cannula placements were excluded from the statistical analysis.

3.9.2. Statistics

For the statistical evaluation of food intake measurements two-way analysis of variance with repeated-measures (ANOVA, SPSS Windows 18.0) were used. When the analysis of the main effect and/or the interaction showed significance, ANOVA was followed by paired-samples t test. This was an appropriate method because in these experiments each animal served as its own control. For data analysis of other feeding related experiments (water intake, feeding after food deprivation, body temperature measurements and spontaneous behaviour during feeding) similar statistical approach has been employed. In the behavioural tests data of different groups of animals were compared. Therefore, in these experiments data were analyzed by one-way or two-way ANOVA followed by Tukey post hoc test. Differences were considered significant when $p<0.05$.

4. Results

4.1. Food intake measurements

4.1.1. Effects of intraamygdalar microinjections of RFRP-1 on food intake

In these experiments the microinjection of the lowest and the highest doses of RFRP-1 (25 ng and 200 ng) did not cause any change in food intake compared to vehicle treatments. Application of the 50 ng RFRP-1 resulted in significant reduction in milk consumption from the beginning to the end of the measurement compared to that of vehicle treatments. The 100 ng dose of RFRP-1 had only a tendency to decrease food intake.

4.1.2. Effects of intraamygdalar microinjections of RFRP-3 on food intake

The 50 ng and 100 ng doses of RFRP-3 microinjections resulted in significant decrease of food intake. These doses of RFRP-3 caused significant reduction of liquid food consumption at every time points of the measurement compared to that of vehicle treatments. The application of 25 ng or 200 ng RFRP-3 was not effective to modify feeding.

4.1.3. Effects of intraamygdalar microinjections of NPFF-receptor antagonist RF9 on food intake

The substrate-specificity of the anorexigenic effect of RFRP-1 was studied by ANT pretreatments. First, the effects of bilateral microinjections of 20 ng and 50 ng ANT on food intake were investigated. Neither the lower nor the higher dose of ANT applied alone had influence on feeding. Then, the effects of combined treatments (ANT and RFRP-1 or ANT and RFRP-3) have been examined. According to data of literature and our previous observation the fifteen minutes interval between the ANT and the RFRPs injections is enough for the development of the antagonist effect. In these experiments the effects of equimolar amount of ANT (equimolar to the food intake decreasing consequences of the previously effective 50 ng RFRP-1, 50 ng RFRP-3 or 100 ng RFRP-3), namely 20 ng, 25 ng or 50 ng

ANT were investigated. The antagonist pretreatment blocked the food intake decreasing effect of both RFRP-1 and RFRP-3.

4.1.4. The effect of food deprivation on RFRP-1 or RFRP-3 induced food intake reduction

The aim of these experiments was to study whether bilateral microinjection of RFRP peptides could reduce food intake in 24 h food deprived rats. Application of 50 ng RFRP-1 had no effect on food intake after food deprivation. In contrast to this observation, the 50 ng RFRP-3 resulted in significant reduction of food intake from the 10th min to the end of the measurement period compared to that of vehicle treatment. The higher dose (100 ng) of RFRP-3 was not effective to modify feeding.

4.1.5. Effects of intraamygdalar microinjections of RFRP-1 or RFRP-3 on water intake

In our experiments the liquid food intake paradigm was used. Because in the execution of liquid food consumption and water intake similar motor mechanisms are involved, it was important to examine in separate experiments the possible effects of RFRP peptides on water intake. Our results showed that microinjection of 50 ng RFRP-1, 50 ng RFRP-3 or 100 ng RFRP-3 did not modify water intake.

4.2. Behavioural examinations in food intake paradigm

The RFRP-1 or the RFRP-3 injections did not cause changes in behaviour activities. Behavioural analysis showed a decreasing tendency in time spent for feeding during the first 10 min but this difference (compared to vehicle treatment) did not reach the level of significance. Thus, food intake decreasing effects of RFRP-1 and RFRP-3 could not be the consequences of changes in behavioural activities.

4.3. Results of body temperature measurements

It is well known that alteration of body temperature can influence feeding behaviour. In these experiments, therefore, effects of RFRP treatments on body temperature were investigated.

4.3.1. Effect of intraamygdalar microinjections of RFRP-1 on body temperature

Application of 50 ng RFRP-1 into the CeA did not change the body temperature at the 10th and at the 20th min after its injection. Thus, food intake decreasing effects of RFRP-1 could not be the consequences of temperature changes.

4.3.2. Effect of intraamygdalar microinjections of RFRP-3 on body temperature

The RFRP-3 injections into the CeA did not modify body temperature. Significant differences were not recorded in the core temperature 10 or 20 min after 50 ng RFRP-3 or 100 ng RFRP-3 microinjections. Thus, food intake decreasing effects of RFRP-3 could not be the consequences of temperature changes.

4.4. Behavioural tests

4.4.1. Effect of intraamygdalar microinjections of RFRP-1 in open field test

The microinjection of 50 ng RFRP-1 into the CeA did not modify spontaneous locomotor activity of the animals in the open field test. In both groups (50 ng RFRP-1 vs. vehicle treated animals) behavioral results after treatments were compared to data obtained one day before microinjections. The distance moved, the number of crossings and time spent in the central area of open field apparatus were evaluated. There were no any alterations in these parameters in the RFRP-1 treated animals compared to vehicle treated controls.

4.4.2. Effect of intraamygdalar microinjections of RFRP-3 in open field test

In these experiments, in open field test the spontaneous locomotor activity was investigated after bilateral intraamygdalar microinjections of 50 ng or 100 ng RFRP-3. In each group behavioral results after treatments were compared to data obtained one day before microinjections. The distance moved, the number of crossings and time spent in the central area of open field apparatus were evaluated. There were no significant differences between the measured parameters.

4.4.3. Effect of intraamygdalar microinjections of RFRP-1 in elevated plus-maze test

RFRP-1 injected into the CeA evoked anxiolytic effects. Time spent on and distance moved on the open arms and the end of the open arms significantly increased in the 50 ng RFRP-1 treated group while the general activity of animals did not change by the treatment. The higher dose (100 ng) of RFRP-1 did not influence any parameter in the elevated plus maze test. The anxiolytic effect of 50 ng RFRP-1 was specific because it could be eliminated by NPFF-R antagonist RF9 pretreatment.

4.4.4. Effect of intraamygdalar microinjections of RFRP-3 in elevated plus-maze test

The possible anxiogenic or anxiolytic effect of RFRP-3 was also investigated in the elevated plus maze test. There were no differences in the measured parameters. Our results showed that neither the 50 ng RFRP-3 nor the 100 ng RFRP-3 had effects on anxiety in the CeA.

4.4.5. Effect of intraamygdalar microinjections of RFRP-1 in conditioned place preference test

It has been shown in conditioned place preference test that RFRP-1 microinjection into the CeA has positive reinforcing effect. Time that animals had spent in the treatment quadrant during test session significantly increased after 50 ng microinjection. The 100 ng dose of RFRP-1 did not influence the behavior of animals. The ANT applied alone was also ineffective. The positive reinforcing effect of 50 ng RFRP-1 might be mediated through

NPFF receptors, because this effect could be blocked by prior application of NPFF receptor antagonist RF9.

4.4.6. Effect of intraamygdalar microinjections of RFRP-3 in conditioned place preference test

We could not found positive reinforcing effect of RFRP-3 in conditioned place preference test after its microinjection into the CeA. Time that animals had spent in the treatment quadrant did not change after low dose (50 ng) RFRP-3 injection. This parameter somewhat decreased after the injection of higher (100 ng) dose of RFRP-3 but this difference did not reach the level of significance.

5. Discussion

5.1. Food intake measurements

In the rat CeA both RFRP-1 and RFRP-3 immunoreactive fibers were detected and NPFF1 receptors were also identified there [4,30,50,51]. **Results of our food intake related experiments showed that 50 ng dose of RFRP-1 injected directly into the CeA resulted in significant decrease of liquid food consumption. Food intake reducing effect of RFRP-1 is specific since this effect could be eliminated by prior application of NPFF receptor antagonist RF9. In case of RFRP-3, our results showed that RFRP-3 injected directly into the CeA decreased liquid food consumption in the 50 ng-100 ng dose-range. Food intake decreasing effect of RFRP-3 is specific because it can be blocked by NPFF receptor antagonist RF9 pretreatment.** *These results are the first reporting that RFRP-1 and RFRP-3 can cause liquid food intake decrease in the CeA.* Our results are however, in contradiction to the data of literature. In fact, it has been shown that after i.c.v. application of RFRP-3 food intake increase was recorded [25,32]. But in these experiments RFRP-3 was applied in higher doses (505 pmol-1010 pmol/5µl, respectively) than in our experiments. It is important to know that RFRP-3 immunoreactive cell bodies were found in the dorsomedial hypothalamic nucleus and the dorsal tuberomammillary nucleus, as well as, within the periventricular nucleus [28,42,51]. RFRP-3 neurons project directly to appetite-regulating

cells within the lateral hypothalamic area, the ventromedial nucleus and the arcuate nucleus to POMC and NPY cells. An electrophysiological study showed that RFRP-3 inhibits POMC neurons and attenuates kisspeptin induced excitation of POMC neurons in mice [17]. On the basis of inhibition of anorexigenic neurons such as the POMC cells development of food intake enhancement could be predicted, which is consistent with the orexigenic actions [5]. Based on these results one may suppose that i.c.v. applied RFRP-3 increases food intake via the NPY/POMC systems, while RFRP-3 injected into the CeA influences a different mechanism. We also have to mention the important differences when a drug is used i.c.v., or microinjected into a distinct brain locus. In case of i.c.v. application, the diffusion speed is higher in the cerebrospinal fluid than in the brain parenchyma and RFRP-3 can spread to a relatively wide surface of the ventricular wall than it gets into the brain. The i.c.v. application, therefore, can cause a "more general" effect than the local microinjection and the effect depends on receptor density and the half-life time of the peptide. One may suppose, therefore, that i.c.v. injected RFRP-3 was bound mainly to brain NPFF receptors located closer to the ventricular wall, and that concentration of RFRP-3 might decrease gradually in the brain parenchyma. In case of direct CeA application of RFRP-3, the neuropeptide could be bound to local NPFF-1 receptors available in the structure modifying the activity of the local neuronal circuits of this limbic structure.

Our results showed that RFRP-3 decrease food intake. In chicken, similar effects were observed after i.c.v. administration of human RFRP-3 (amino acid sequence of human RFRP-3 is: VPNLPQRF-NH2) [8]. RFRP-3 caused a short-term reduction (lasted for 60 min) in food intake, and it did not affect water intake in chicken. I.c.v. applied neuropeptid FF (NPFF) in chicken also exerted anorexigenic properties (RFRPs share a similar C-terminal sequence with neuropeptide FF) than it was observed after application of RFRPs. NPFF, however, exhibited a longer lasting food intake reduction. It is supposed that anorexigenic effects of NPFF and RFRP-3 are mediated two different subtypes of NPFF receptors (NPFF1 and NPFF2, respectively). Anorexigenic effects of NPFF may be due to the greater affinity to NPFF2 rather than NPFF1 and most of the effects of NPFF are mediated through NPFF2 [38]. Additionally, RFRP-3 binds with greater affinity to NPFF1 than NPFF2 [4,30] and food intake decreasing effects of RFRP-3 are mediated through NPFF1. These results may indicate that feeding related effects associated with NPFF2 are stronger and last

longer effect than the similar effects associated with NPFF1. This is in good agreement with our results. According to our observation the anorexigenic effects of RFRP-1 and RFRP-3 are mediated through NPFF1, while anorexigenic effects of i.c.v. applied RFRP-1 and RFRP-3 might be mediated via both receptor subtypes.

In separate experiments the effect of 24 h food deprivation on RFRP-1 and RFRP-3 initiated food intake decrease was investigated in the CeA. In these experiments, differences were observed in the effects of RFRPs. We did not find any changes in food consumption in 24h food-deprived rats after RFRP-1 microinjection. Thus, **RFRP-1 induced food intake reduction depends on hunger-satiety condition. In contrast, the microinjection of 50 ng dose of RFRP-3 resulted in significant decrease of food intake even after 24h food deprivation. This result shows that the RFRP-3 exerts anorexigenic effect despite the increased hunger-drive.**

Furthetmore, we investigated possible non specific effects of RFRP-1 and RFRP-3 which might induce changes in feeding. The anorexigenic effect of RFRP-1 and RFRP-3 was observed from the 5th min to the 25th min after their injection. **In this time range the effects of previously effective dose of RFRP-1 and RFRP-3 on feeding was investigated on water intake and body temperature.** There were no differences in these parameters after RFRP treatments compared to vehicle microinjections. **The food intake reduction effect of RFRPs can not be explained by evoking alterations in body temperature or water intake.**

In food intake related behavioural experiments, when the activity of animals was video-monitored, similar food intake reduction was detected as in the previous experiments. **Our results showed that microinjection of RFRP-1 or RFRP-3 into the CeA inhibits food intake without influencing other behavioural actions (grooming, scratching, resting and locomotion). The food intake decreasing effect of RFRPs cannot be explained by altering stereotype behaviours.**

5.2. Behavioural experiments

1. Open field test is a widely used appropriate method to investigate general motor activity. Possible effects of RFRP-1 and RFRP-3 on motor activity may be important explaining our results. On the one hand, the alteration in motor activity may modify the food intake of animals. The food intake reducing property of RFRP-1 and RFRP-3 could be due to decreased motor activity. On the other hand, we have shown that RFRP-1 has positive reinforcing effect in conditioned place preference test. RFRP-1 treated animals spend significantly more time in the treatment quadrant. This effect could also be explained by hypoactivity or anxiety. Kaewwongse et al. observed that time spent in the central area of open field apparatus was significantly decreased following i.c.v. application of RFRP-1 or RFRP-3. Total locomotion was also decreased after i.c.v. administration of RFRP-3 [26]. **In our experiments 50 ng RFRP-1 or 50 ng or 100 ng RFRP-3 microinjected into the CeA did not influence spontaneous locomotor activity, and not modified the time spent in the central area of open field apparatus.**

2. Our results showed that RFRP-1 microinjected into the CeA in elevated plus maze test proved to be anxiolytic. **Time spent on and distance moved on the open arms and the end of the open arms significantly increased after the injection of the low dose (50 ng) of RFRP-1. NPFF-1 receptors may play a role in the mediation of these anxiolytic effects.** Our data indicated that RFRP-3 injected into the CeA in elevated plus maze test had neither anxiogenic nor anxiolytic effects. According to data of literature i.c.v. applied high dose of RFRP-3 had anxiogenic effect. Release of stress hormones (ACTH, CRH) may play a role in the mediation of anxiogenic effects. Furthermore, interaction of RFRP-1 with CRH, oxytocin, serotonin or benzodiazepines may play a role in the mediation of anxiolytic effect of RFRP-1 in the CeA.

3. **Our findigs indicate that lower dose of RFRP-1 microinjected into the CeA has positive reinforcing effects in place preference test. The NPFF-1 receptors may play a role in the mediation of rewarding-positive reinforcing effect of RFRP-1. The positive reinforcing effect of RFRP-1 is specific because pretreatment with NPFF receptor antagonist could block this action.** It is important to note that intraamygdalar injections of

RFRP-1 in place preference paradigm did not influence the total number of entries and distance moved: the gross locomotor activity of animals did not change by treatments. These results show that mediation of place preference could not be the consequences of any anxiogenic effect. Furthermore, our results showed that RFRP-1 microinjected into the CeA in elevated plus maze test exhibited anxiolytic effect. The CeA, part of the limbic system, plays an important role in memory [22,34] and reinforcement [2,5,7] and it has been shown that it is relatively rich in NPFF-1 receptors [3,10]. The RFRP-1 immunoreactive fibers and both NPFF receptor subtypes are localized in brain structures of central nervous system (including the AMY, the striatum, the hypothalamus, the VTA and the Nacc), which play important roles in reward processes and motivation [4,10,19, 30,39,48,51]. It could be supposed that RFRP-1 microinjected into the CeA has positive reinforcing properties through the modulation of the mesolimbic dopaminerg system. Further experiments with specific antagonists of different NPFF receptor subtypes and dopamine receptor agonists and antagonists are necessary to cast light on the detailed mechanisms of reinforcing properties of RFRP-1 in the AMY.

6. Summary

Our results show that the RFRPs injected into the CeA play important roles in the regulation of feeding and other behavioural processes. Application of both RFRP-1 and RFRP-3 into the CeA results in anorexigenic effect. Food intake decreasing effect of RFRP-3 was found more potent than that of RFRP-1 because it suppressed feeding even in 24 h food deprived rats. Furthermore, RFRP-3 was effective on food intake within a wider dose range. We demonstrated that RFRP-1 microinjected into the CeA proved to be anxiolytic in the elevated plus maze test. Application of RFRP-1 into the CeA exhibited reinforcing effects. RFRP-1 and RFRP-3 derived from the same preproprotein. The different C-terminal amino acid sequence of the peptides may explain their different effects. Further experiments with different antagonists of NPFF receptor subtypes and dopamine receptor antagonists are necessary to cast light on the detailed receptorial mechanisms and interactions of RFRP peptides. Although, these are the first experiments studying the functional role of RFRP peptides in the CeA, we hope that our results many contribute to the better understanding of

the central nervous system processing and mechanisms of human eating disturbances and other behavioural disorders.

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8. List of publications

I. Publications related to this thesis

Kovács, A., László, K., Gálosi, R., Tóth, K., Ollmann, T., Péczely, L., Lénárd, L.: Microinjections of RFRP-1 in the central nucleus of amygdala decreases food intake in the rat. BRAIN RESEARCH BULLETIN 88: (6) pp. 589-595. (2012) (**IF: 2.818**, Független idéző: 3).

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Lénárd, L., **Kovács, A.,** Ollmann, T., Péczely, L., Zagoracz, O., Gálosi, R., László, K.: Positive reinforcing effects of RFamide-related peptide-1 in the rat central nucleus of amygdala BEHAVIOURAL BRAIN RESEARCH 275: pp. 101-106. (2014) (**IF: 3,391**)

II. Further publications

Péczely, L., Ollmann, T., László, K., **Kovács, A.,** Gálosi, R., Szabó, Á., Karádi, Z., Lénárd, L.: Role of D1 Dopamine Receptors of the Ventral Pallidum in Inhibitory Avoidance Learning. BEHAVIOURAL BRAIN RESEARCH 270: pp. 131-136. (2014) (**IF: 3,391**)

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III. Presentations and abstracts

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