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Role of intraamygdaloid ghrelinergic mechanisms in the regulation of food intake and food intake related metabolic parameters and behaviours

Ph.D. Thesis

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1. Introduction

Because of their remarkable and continously increasing incidence the body weight regulation disturbances are considered to be endemic. The 1-1% of the adult population suffer from anorexia nervosa and bulimia nervosa. One of the most charachteristic features of these two psychiatric diseases can be the pathological leanness. Furthermore, the childhood incidence of these diseases are increasing, too. The other extremity of body weight disturbances is the obesity. According to the World Helth Organization's survey, approximately the 40% of the adult population is overweighted, while the ratio of obesity is over 20%. In the school-age population these rates are 25% and 11%, respectively. The obesity has negative influences on every organs of the human body resulting in consequent diseases such as diabetes mellitus, atherosclerosis, high blood pressure and stroke.

According to our present knowledge, behind the body weight regulation disturbances (disregarding social factors, improper feeding habits, the lack of physical exercises and so far unknown factors) the abnormalities in the regulation of feeding behaviour and/or the dysfunctions of the chemical processes involved in energy consumption and expenditure can be revealed.

Among others, peptide molecules, neuropeptides play essential roles in the regulation of the above mentioned chemical processes. The neuropeptides are produced by neurons in different brain areas as well as in different periferal tissues. The effects of these peptides are realized through the hunger-satiety systems of the perifery and/or of the cenral nervous system. Among these peptides, food intake decreasing (anorexigenic) and food intake increasing (orexigenic) peptides could be differentiated.

One representative of the above peptide-families is the ghrelin (Ghr). The Ghr is mainly produced by chromogranin A-immunoreactive X/A-like endocrine cells situated in the mucosal layer of the stomach fundus [15,18], but it is also produced in the CNS [12] and in other periferial tissues [15,30]. There are two main types of Ghr in the blood: the acylated (A-Ghr) and non-acylated, des-acyl Ghr (DA-Ghr) [31]. In case of A-Ghr during a posttranslational modification an 8 carbon long fattyacid chain joins to the serine aminoacid residue presented in the third position with ester bound, while in DA-Ghr the posttranslational modification is not present [31].

Intravenous (i.v.), intraperitoneal (i.p.) or intracerebroventricular (i.c.v.) injection of A-Ghr induces an immediate increase in food intake [35,48,64,65] and in human examinations its application enhances hunger. Repeated i.c.v. injections of A-Ghr increase body weight by the increase of food intake and adipogenesis and by the decrease of lipid oxidization processes [35,48,64,65]. I.v. injection of A-Ghr dose-dependently increases the gastric secretion and gastrointestinal motility [6,15,47], that is, it has a potent prokinetic effect [63].

I.c.v. applied A-Ghr induces anxiety and memory retention [9]. The memory retention enhancing effect of A-Ghr can be prevented by selective serotonin reuptake inhibitor, fluoxetine pretreatment [8]. Diano et al. demonstrated that either systemically or i.c.v. injected A-Ghr could pass the blood-brain barrier, got into the hippocampus and promoted dendritic spine synapse formation and generation of long-term potentiation [17].

Physiological effects of Ghr develop at least partly, through GH secretagogue receptors (GHS-R), which have two subtypes, GHS-R 1a and GHS-R 1b [32].

Although, several effects of A-Ghr are already known, there are undisclosed functions and unanswered questions concerning detailed A-Ghr mechanisms. 1) How do the brain areas invoved in the regulation of food intake (e.g. amygdala) participate in the A-Ghr mediated feeding effects? 2) Which are the exact receptorial mechanisms behind the effects of A-Ghr? 3) Have the different brain areas similar or different roles in the mediation of the feeding effects of A-Ghr and DA-Ghr? 4) Can the changes in feeding behaviour be explained either by the development of hunger-satiety or by the changes of other behaviours? Our present knowledge suggests that clarification of the complex role of Ghr may lead to the better understanding of energy-balance regulating mechanisms in humans, and it may result in new therapic strategies for the treatment of body weight regulation disturbances.

Results based on brain lesion studies and effects of electrical stimulation of the same brain areas revealed the distinct and different roles of these areas in the regulation of food intake and body weight. As a result of these experiments the hypothalamic (HT) dual-center hypothesis was widely accepted. Namely, the lesion of the lateral HT (LH) induces aphagia, adipsia and body weight loss, while the the lesion of the ventromedial HT (VMH) leads to hyperphagia and weight gain [4,29,43,44,60]. Further experiments showed that different extrahypothalamic brain areas play also important roles in the development and regulation of feeding behaviour. One of them is the amygdaloid body (AMY), which is an essential part of the limbic system [22,41,45].

The AMY, located in the temporal lobe, has an almond shaped structure, containing numerous distinct nuclei. The basolateral part of the amygdala-complex (BLA) is a distinguished

subdivision of the AMY and it is known as basolateral amygdaloid-complex or basolateral group of amygdaloid nuclei [11,27]. The BLA is connected bidirectionally with the temporal lobe, orbito-frontal lobe, medial- lateral-prefrontal lobe, hippocampus [2,27,57,58] and the dorsomedial part of HT [39,40]. We have to emphasize that the BLA is also innervated by the mesolimbic dopminergic system, which has an important role in learning and memory procresses.

The complexity and diverse connection system of the AMY may explain the functional heterogenity of this strucutre. For the first time Kluver and Bucy reported in 1939 that the leasion of the temporal lobe can change the eating habits of monkeys [37]. The similar effects were observed in cats after bilateral leasion of the AMY: the animals became hyperpgagic, their body weight gained for 6-8 weeks, moreover omniphagy and polyphagy were also detected [13]. These symptoms are known as the Kluver-Bucy syndrome.

Later, in the 1970s, experimental results of Fonberg verified that in dogs the role of the AMY is not uniform as far as the regulation of food intake is concerned. Namely, the lesions of the central amgdaloid nuclei cause hypophagia and decrease in body mass, while the destruction of the BLA leads to hyperphagia and an increas in body mass [21-26].

The AMY has important role in the regulation of memory processes, too. It is supposed to be a key structure of working memory. It takes part in the sreening and editing of the incoming information from the external world, and it enhances the effect of the unique and relevant stimuli [53]. After AMY lesion, the learning of new information is blocked [36]. Finally, the AMY participates in the regulation of spatial learning. It seems to be dependent, however, on the integrity of the amygdalo-hippocampal connection. Destruction of this neuronal pathway leads to impaired place learning capability of the animals [2,3].

2. The aim of our study

In former experiments it was demonstrated that i.c.v. or direct LH injection of A-Ghr enhanced food intake and memory processes. It has also been shown by immunohistochemical methodes that A-Ghr increased c-fos activity in the AMY [50,51]. No data are available, however, about feeding related or memory influencing effects of A-Ghr in the AMY, and the receptorial mechanisms of possible A-Ghr effects in the AMY are also unknown. Our experiments were focused on the BLA, which is a well-charcterised subregion of the AMY,

because there are evidences that the BLA participates in the central regulation of both hunger motivated behaviour and memory formation-retention.

Therefore:

1) In the BLA effects of A-Ghr microinjections on food intake were investigated in ad libitum fed rats.

a) It was studied whether the effect of A-Ghr on feeding can be prevented by specific antagonist pretreatment.

b) The effect of 24 h food deprivation on A-Ghr induced changes in feeding was examined.

c) After their microinjections into the BLA A-Ghr and DA-Ghr on food intake were compared.

d) The modulatory effect of i.c.v. injection of A-Ghr on feeding was also examined.

2) The acute modifying effects of A-Ghr were studied after BLA microinjections on concentration changes of feeding related metabolic parameters (blood glucose level, serum insulin, leptin, total-cholesterol, HDL-cholesterol, trigliceride, total-protein and uric acid concentration).

3) After A-Ghr microinjections into the BLA spontaneous motor activity and anxiety were examined in open field and elevated plus maze tests in rats.

4) The possible effects of A-Ghr in the BLA on learning, memory formation and retention were studied in passive avoidance and Morris water maze tests.

3. Methods

3.1. Animals

Subjects were male Wistar rats (LATI, Gödöllő, Hungary) weighing 280-320 g at the beginning of the experiments. Animals were housed individually and cared for in accordance with institutional (Pécs University Medical School) and international standards (European Community Council Directive 86/609/EEC). Rats were kept in a light- and temperature-controlled room (12:12 h light-dark cycle with lights on at 07:00 a.m., 22±2 °C). Tap water and standard laboratory food pellets (CRLT/N standard rodent food pellet, Charles River Laboratories, Budapest, Hungary) were available according to the experimental schedule.

3.2. Surgery

Stainless steel bilateral guide tubes (22 gauge) were stereotaxically implanted into the dorsal border of the ABL (coordinates referring to the bregma: AP: -2.3 mm, ML: 4.8 mm and DV: 6.1 mm ventral from the surface of the dura) or into the lateral ventricles (coordinates referring to the bregma: AP: -1 mm, ML: 1.5 mm and DV: 3 mm ventral from the surface of the dura) according to stereotaxic atlas of Paxinos and Watson [52]. The tips of cannulae were positioned 0.5 mm above the intended injection sites.

3.3. Drugs, microinjections

The BLA was injected with 25 ng, 50 ng, 100 ng, 250 ng or 500 ng (7,42; 14,83; 30,16; 74,16 or 148,32 pmol) A-Ghr (1465, Tocris), 15 ng or 30 ng (14,83; 32,25 pmol) GHS-R antagonist [D-Lys^3]-GHRP-6 (ANT) (1922, Tocris) or 25 ng, 50 ng or 100 ng (7,71; 15,41 or 30,82 pmol) DA-Ghr (2260, Tocris). Chemicals were dissolved in 0.15 M sterile saline. The volume of drug microinjections were 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.

Drugs or vehicle were microinjected through a 30 gauge stainless-steel injection tube extending 0.5 mm below the tips of the implanted guide cannulae.

In case of the i.c.v. application doses were 500 ng (148,32 pmol) or 1000 ng (296,64 pmol) A-Ghr. Preparation of the solutions and the procedure of microinjections were similar to those of BLA injections, but the injected volume was 1 µl.

ANT was used alone bilaterally or microinjected 15 min before the bilateral A-Ghr application, respectively. The ANT was injected into the BLA, too.

3.4. Food intake measurements

In examinations with ad libitum condition food was available throughout the experiments while in food deprivation experiment rats were fasted for 24 h before microinjections and during tests. From the 14th preoperative day rats were allowed to consume liquid diet on a limited access schedule (milk, 136,45kJ/100ml, Milk Quick, Debrecen, Hungary) for two weeks. The pretest exposure period was designed to overcome neophobia and to accustom the rats to the palatable complex food. Our method made exact consumption measurement possible in 5 min intervals with high accuracy without disturbing animals in their home-cages [20,62]. Furthermore, the constant taste and energy intake could be guaranteed. Milk intake was measured at ml accuracy every 5 min for 30 min and at the 40^{th}, 50^{th}, 60^{th} min, respectively.

3.5. Blood gucose level measurements

In blood glucose measurement experiments rats were fed ad libitum. The standard chow was removed just before the A-Ghr application and they got the pellets back at the end of the test. Tap water was available freely during the experiments. The injections into the BLA were made as described above in the food intake measurements section. The blood glucose levels were measured 10 min before and at 10^{th} , 20^{th} , 30^{th} , 50^{th} , 70^{th} , 90^{th} , 120^{th} min after the bilateral intraamygdalar application of A-Ghr by means of a calibrated glucometer (Glucometer Elite 2000, Bayer). The 3µl blood samples were collected by the cutting of the tails-ending of the rats.

3.6. Measurments of other metabolic parameters

The serum concentration of total-cholesterol, HDL-cholesterol, trigliceride, uric acid and concentration of total-protein were measured in ad libitum fed rats by ARKRAY, SPOTCHEM EZSP 4430 (Arkray Technology, Japan) instrument. In addition the serum insulin and leptin levels were detected by ELISA (ALPCO Diagnostics, United States). After decapitation, blood samples were taken by trunk-bleeding 20 min after bilateral BLA injections (the beginning of the food intake decreasing effect). Measurments were made from blood serum, 100 µl of serum samples was pipetted into the analyzer. In ELISA measurments the ALPCO made original solutions were used and the official guide was followed.

3.7. Behavioural tests

3.7.1. Open field test

Animals were put in 60x60x60- cm grey painted cage 10 minutes after bilateral 50 ng, 100 ng A-Ghr or vehicle microinjections. The ground of the box was divided into 16 identical squares by painted lines. Behaviour of each rat was recorded in five min intervals by means of CCD camcorder. During observation period the number of crossings and the distance moved were investigated. Results were analised by the help of Noldus Ethovison System (Noldus Information Technology, The Netherlands).

3.7.2. Elevated plus-maze test

The equipment consisted of two opposite open arms (50x10cm) and two opposite enclosed arms (50x10x40cm). The maze was elevated to a height of 100 cms above the floor. Experiments were carried out in a dimly-lit room, a 40W red bulb provided the illumination. Ten minutes after bilateral intraamygdalar A-Ghr administrations animals were placed into the center of the maze (central platform), facing to one of the enclosed arms. Trials lasted 5 min during wich the number of entries into and time spent on the open and enclosed arms and the end of the open arms (end-arms) were measured. After each trial, the maze was cleaned with 1% ethanol solution. Each rat was tested only once. Behaviour of the animals was recorded, data were stored and motion analysis was made by means of the Noldus EthoVision Basic software (Noldus Information Technology, The Netherlands).

3.7.3. Passive avoidance test

A one trial step-through avoidance paradigm was used in a two compartment passive avoidance apparatus. The experimental apparatus consisted of a large (60x60x60cm), well illuminated (Tungsraflex, 100W) compartment and a small box (15x15x15cm), painted black and having metal-grid floor for the delivery of electric shocks. Rats were habituated on the 1st day of the experiment when they were placed into the large compartment and were allowed a maximum time of 180 sec to enter the dark compartment. On the following day animals were *conditioned.* Subjects were placed again into the illuminated compartment and latency to enter the shock box through a guillotine trap door was measured. After rats had entered the dark box, they were given electric foot shock 3 times, each for 1 sec with weak (0.4 mA) electric current. Subsequently rats were removed from the apparatus and were microinjected bilaterally. Rats were *test*ed 24 h (Test 1) and one week after (Test 2) *conditioning* and the latency of entering the shock box was recorded. When the animal had not entered the shock box till the end of the trial the maximum value was given (180 sec). Data were recorded and evaluated by means of the Noldus Ethovison System.

3.7.4. Morris water maze test

Tests were made in a circular pool with a diameter of 1.5 meter. The height of the pool was 60 cm. The pool was filled with water (temperature: 23 ± 1 °C) and a square plexiglas platform with a diameter of 10 cm was placed in. The surface of the water was kept 2 cm above the platform and the water was painted to make the water opaque. The pool was surrounded with external cues. These cues were kept in constant position throughout the whole experiment. Behaviour of animals was recorded by a video camera and registered by a computer program (Noldus EthoVision System). The latency time to find the safe platform located in one of the quadrants of the maze was measured. One day before start of training, rats were habituated to the pool by allowing them to perform swimming for 90 s without platform. During conditioning for spatial learning, rats were placed into the water maze for two trials per day for two days (trial 1 and trial 2 were performed on the first day before the A-Ghr administration, trial 3 and trial 4 were made on the second day after the injection) at randomly assigned, but predetermined locations. The task required rats to swim to the hidden platform guided by external spatial cues. After finding the platform, rats were allowed to remain there for 10 s. Rats failing to find the platform in 180 s were placed on the platform and allowed to rest for 10 s.

3.8. Data processing

3.8.1. Histology

At the end of the experiments animals were anaesthetised with urethane and were perfused transcardially with saline (0.15 M) 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 a stereotaxic atlas [52]. The track of canulae and the tips were determined on the basis of existence of debris and moderate glial proliferation. Animals with incorrect cannula placements were excluded from the statistical evaluation.

3.8.2. Statisitics

For the statistical evaluation of food intake and blood glucose level measurments two-way analysis of variance with repeated-measures (ANOVA, SPSS Windows 15.0) were used and were followed by paired-samples t test analysis (SPSS for Windows 11.0) to compare the results of vehicle or A-Ghr/DA-Ghr/ANT treatments in each time points. This was an appropriate method because in all of the experiments each animal served as its own control. The design in our further experiments was not self-contolled. Therefore the statstical evaluation was made by one way ANOVA followed by Student-Newman-Keuls Multiple Comparisons post hoc test (ANOVA, GraphPad InStat for Windows 3.0). The statistical rejection criterion was set at $p<0.05$ level.

4. Results

4.1. Food intake measurements

4.1.1. Effect of bilateral intraamygdalar microinjection of A-Ghr on liquid food intake in ad libitum fed animals

In these experiments the microinjection of 50 ng, 100 ng and 250 ng A-Ghr significantly decreased food intake. The 50 ng A-Ghr caused significant reduction in food intake from the

40th min to the end of the measurement compared to that of vehicle treatment. The application of 100 ng A-Ghr at the 40th and at the 50th min while the microinfusion of 250 ng A-Ghr at the 25th, at the 30th and at the 40th min decreased milk consumption, respectively. The 25 ng or the 500 ng A-Ghr was not effective to modify feeding.

4.1.2. Effect of GHS-R antagonist D-Lys3-GHRP-6 microinjection on food intake in ad libitum fed animals

The substrate-specificity of the anorexigenic effect of A-Ghr was studied by ANT pretraetments. First, the effect of bilateral intraamygdalar 30 ng ANT microinjection alone was investigated. The ANT microinjection was ineffective to modify food intake. Than, combined ANT and A-Ghr treatments were made. According to data of literature and our observation the 15 min interval between the ANT and A-Ghr injections is enough for the development of the antagonist effect. The effects of equvimolar amount of ANT (equimolar to the previosly effective 50 ng and 100 ng A-Ghr, namely 15 ng or 30 ng ANT) were studied. The ANT pretreatment eliminated the food intake decreasing effect of A-Ghr in both cases.

4.1.3. Effect of bilateral intraamygdalar microinjection of A-Ghr on liquid food intake in food deprived rats

Effects of 50 ng or 100 ng A-Ghr on food intake were tested after 24 h food deprivation. Food deprivation increases hunger drive and the aim of the experiment was to study whether A-Ghr could reduce food intake in such condition. In these experiments neither the 50 ng, nor the 100 ng A-Ghr was effective to significantly modify food consumption, compared to controls.

4.1.4. Effect of bilateral intraamygdalar microinjection of DA-Ghr on liquid food intake in ad libitum fed animals

One may suppose that A-Ghr injected into the AMY can be hydrolised to DA-Ghr and this is the active agent reducing food intake. Therefore, in separate experiments, the effects of 25 ng, 50 ng or 100 ng bilateral DA-Ghr microinjections were investigated on food intake. These doses were comparable to the A-Ghr doses used in the previous experiments. Using the same

liquid food paradigm and food intake measurements it has been revealed that DA-Ghr did not modify food intake.

4.1.5. Effect of Intracerebroventricular injection of A-Ghr on food intake

Since in other experiments i.c.v. injection of A-Ghr enhanced the intake of solid food [35,48,64,65] , it was reasonable to examine the liquid food intake in our paradigm after i.c.v. application of A-Ghr. On the other hand, this experiment could serve as a control examination to compare its results with data obtained in the intraamygdaloid A-Ghr treatments.

The injection of 500 ng A-Ghr into the lateral ventricle did not change the milk consumption. However, the infusion of 1000 ng A-Ghr significantly increased the food intake from the 15th min to the end of the observation period.

4.2. Measurments of metabolic parameters

4.2.1. Effect of bilateral intraamygdalar microinjection of A-Ghr on blood glucose level in ad libitum fed animals

In these experiments the effects of vehicle or previously effective 50 ng or 100 ng A-Ghr on blood glucose level were studied. In case of both doses first the blood glucose level was measured 10 min before A-Ghr or vehicle microinjections and results were separately analyzed by paired-sample t test to verify that there was no difference in the initial glucose concentrationeither after vehicle or A-Ghr treatments. The 50 ng A-Ghr resulted in significant elevation of blood glucose concentration at the 20th and at the 30th min after its injection. The 100 ng A-Ghr elevated the blood glucose level, too. Values were significant at the 10th and at the 20th min, when compared to vehicle treatment.

4.2.2. Acute effect of bilateral intraamygdalar microinjection of A-Ghr on serum total-cholesterol, HDL-cholesterol, triglceride, protein, uric acid, insulin and leptin concentration in ad libitum fed animals

In these experiments the serum concentration of metabolites were measured that are involved in the regulation of food intake and/or their concentration characterize well the ongoing metabolic processes. Measurments were made from serum 20 min after vehicle, 50 ng or 100 ng A-Ghr microinjection into the BLA. The 20th min was choosen for taking sample because it was the time of the effective blood glucose level increase in the previous experiments. The statistical analysis did not show considerable alteration in total-protein, trigliceride, uric acid and leptin levels after A-Ghr treatment compared to controls.

The intraamygdalar application of 50 ng or 100 ng A-Ghr decreased the total-cholesterol and HDL-cholesterol levels. In serum insulin concentration significant reduction was detected after 50 ng A-Ghr injection compared to vehicle or 100 ng A-Ghr injected animals.

4.3. Behavioural tests

4.3.1. Effect of bilateral intraamygdalar microinjection of A-Ghr in open field test

In open field test the spontaneous locomotor activity was investigated 10 min after vehicle, 50 ng or 100 ng A-Ghr bilateral intraamygdalar injections. In each group behavioural results after treatments were compared to data obtained one day before microinjections (undrugged activity). The distance moved and the number of crossings were evaluated. There were no differences in the measured parameters.

4.3.2. Effect of bilateral intraamygdalar microinjection of A-Ghr in elevated plus maze test

The possible anxiogenic or anxiolytic effect of A-Ghr was tested in elevated plus maze test. The test was performed 10 min after bilateral intraamygdalar vehicle, 50 ng or 100 ng A-Ghr injections. There were no differences in the measured parameters. Our results suggest that neither the 50 ng nor the 100 ng A-Ghr has effects on anxiety.

4.3.3. Effect of bilateral intraamygdalar microinjection of A-Ghr in passive avoidance test

The effect of 50 ng, 100 ng A-Ghr, ANT or ANT pretreatment was studied on passive avoidance learning. The bilateral microinjection of 50 ng A-Ghr into the BLA significantly increased the latency time in passive avoidance learning 24 h after conditioning. One week after the foot shock the effect of 50 ng A-Ghr remained prominent but it did not reach the level of significance. Comparison of each group at 24 h after the application of A-Ghr yielded that 50 ng A-Ghr treated animals needed significantly more time to enter the dark box (the place of shock).

The 100 ng A-Ghr and the ANT alone were ineffective. Effect of 50 ng A-Ghr was eliminated by the bilateral intraamygdalar pretreatment of ANT.

4.3.4. Effect of bilateral intraamygdalar microinjection of A-Ghr in Morris water maze test

These experiments were designed to investigate possible modulatory effects of A-Ghr on spatial learning. Examinations were made in Morris water maze. Statistical evaluation of the experiments indicated that the intraamygdaloid microinjection of A-Ghr resulted in considerable alteration of learning in Morris water maze. The bilateral administration of 50 ng A-Ghr into the ABL significantly decreased the escape latency time in Morris water maze in post infusion trials. Furthermore, comparison of each group after the application of A-Ghr (post injection trial) yielded that 50 ng A-Ghr treated animals needed significantly less time to find the safe platform than the vehicle, ANT or ANT+ 50 ng A-Ghr treated rats.

The 100 ng A-Ghr and the ANT alone were ineffective. Effect of 50 ng A-Ghr was reversed by the bilateral intraamygdalar pretreatment of ANT (effect of 50 ng A-Ghr compared to effect of ANT+50 ng A-Ghr in post injection trial). During the trials all animals in each group showed a learning tendency. Rats injected with 50 ng A-Ghr needed only 21.5 % of time measured in preinfusion trial to get the safe platform. In case of the other groups this value varied between 53-74 %.

5. Summary and discussion

5.1. Food intake measurements

Results of our experiments showed that A-Ghr injected directly into the ABL decreased liquid food consumption in the 50 ng-250 ng dose-range. The lowest and the highest doses (25 ng and 500 ng, respectively) were ineffective corresponding to an inverted U shaped relationship commonly observed after application of different neuropeptides [19,33]. The effect of A-Ghr in the 50 ng-250 ng dose-range was specific because it could be eliminated by GHS-R antagonist.

Our results are, however, in contrast with those of others [7,50,61,64,65]. Namely, in experiments with i.c.v. or direct hypothalamic application of A-Ghr a significant increase of solid food intake was observed [7,61,64,65].

Therefore, a separate experiment was designed to study the feeding related effect of A-Ghr after its i.c.v. injection in the liquid food intake paradigm. As our results showed i.c.v. injection of A-Ghr (1000 ng/side) increased liquid food intake. This is in good agreement with previous results [7,61,65] obtained with consumption of solid food. On the other hand, it is in contrast with the results of our first experiments showing that direct microinjection of A-Ghr into the BLA inhibits feeding. This contradiction can be solved by the possible 'site of action' of i.c.v. applied A-Ghr. In case of i.c.v. application the diffusion speed is higher in the cerebrospinal fluid than in the brain parenchyma and A-Ghr 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 the receptor density and the half life time of the peptide (according to our best knowledge this is not known so far in the case of A-Ghr). Furthermore, according to the literature it is likely that Ghr exerts its feeding inducing effect by stimulation of NPY/AgRP neurons in the hypothalamus to promote the production and secretion of these orexigenic peptides after both i.c.v. application and direct microinjection to the arcuate or PVN [38]. In our experiment, location of i.c.v. cannulae were rather far from the amygdaloid body. Due to diffusion, the farther the specific brain area, the lesser is the concentration of the drug. Consequently, A-Ghr concentration in the distant BLA could have been very low (if there was any). Contrarily, in case of direct ABL application of A-Ghr, the neuropeptide could be bound to local GHS receptors available in the structure modifying the activity of the local neuronal circuits of this limbic structure.

In a separate experiment the effect of 24 h food deprivation on A-Ghr initiated food intake decrease was investigated in the BLA. It is well known that food deprivation increases the hunger-drive. In fasting state, at first the exploratory behaviour and locomotor activity of animals (quest for food) are increased. On the other hand, it is also known that serum concentration of Ghr (both A-Ghr and DA-Ghr) is increasing during fasting. In our experiment, it was detected that the effect of 50 ng or 100 ng A-Ghr which had been previously effective to decrease food intake was inhibited by the enhancment of hunger. In other words, the increased hunger-motivation could overcome the satiation effect of A-Ghr. It is suggested, therefore, that the A-Ghr injected into the BLA plays a modulatory role in feeding behaviour.

Since the octanoyl modification of Ghr (i.e. A-Ghr) is rapidly hydrolysed to desacyl DA-Ghr and the brain tissue is rich in nonspecific esterases, one may suppose that in our experiments relatively high quantity of A-Ghr could be desacylated and instead of A-Ghr actually the DA-Ghr decreased food intake. In our examinations, using the same liquid food intake paradigm, we demonstrated that bilaterally microinjected DA-Ghr into the BLA, in comparable doses to those used in the A-Ghr experiment (25 ng, 50 ng and 100 ng, respectively), did not modify food intake.

5.2. Measurments of metabolic parameters

In these experiments blood glucose level of ad libitum fed, normoglycaemic rats was measured after intraamygdaloid injection of 50 ng or 100 ng A-Ghr. It was found that both the 50 ng and 100 ng A-Ghr caused significant elevation in blood glucose concentration. Furthermore, the application of the lower (50 ng) A-Ghr dose decreased the insulin serum concentration, as well. The elevation of blood glucose level appeared just before the significant food intake reduction in case of both doses. These results suggest that in the BLA the A-Ghr initiated acute, transient increase in blood glucose level might act as a satiety signal and it could be responible for the transient decrease of food intake. The elevated glucose levels were, however, within the physiological range.

The increased blood glucose concentration definitely initiates insulin secretion. While the insulin secretion immediately starts at the beginning of food intake, the glucose absorption and its effect on blood glucose level can be observed only later [59]. The prerequisite of the insulin secretion of the ß cells is the membrane depolarization induced Ca^{2+} permeability increase. The increasing intracellular Ca^{2+} concentration initiates the release of the insulin containing vesicles.

The binding of A-Ghr to its GHS-R 1a receptor induces an increase in the intracellular Ca^{2+} concentration by the activation of the IP3 signal pathway [46]. It may suggest that the A-Ghr-GHS-R 1a receptor interaction would initiate insulin secretion by the increase of intracellular Ca^{2+}. Concerning this question, the experimental data are contradictory, however. Several authors showed that A-Ghr inhibits insulin secretion, while others reported that the insulin secretion is accelerated by A-Ghr [1,16,42,54]. It is possible that the A-Ghr has serum blood glucose level dependent effects on insulin and/or glucagon secretion [56].

The insulin participates in the regulation of lipoprotein- metabolism, too. It increases the cholesterol and HDL-cholesterol synthesis in the liver by the accelaration of the HMG CoA reductase enzime activity [49].

If we assume that A-Ghr decreases the insulin secretion in euglicaemic rats, it is likely that the lipoprotein synthesis in the liver decreases, too [55]. It could explain that A-Ghr applied into the BLA caused significant reduction in serum total-cholesterol and HDL-cholesterol levels. Since the AMY and the hypothalamus are interconnected, the possible neural mechanism of these metabolic changes induced by the intraamygdaloid A-Ghr injections could be the activation of the hypothalamic vegetative centers. Efferents from these centers innervate the pancreas and the liver to modify the hormone secretion and/or the metabolism of these organs, inducing the above mentioned metabolic changes.

5.3. Behavioural tests

Both passive avoidance test and Morris water maze test are paradigms investigating memory formation and memory retention. For the evaluation of data obtained in these tests, it is essential first to study the possible effect of A-Ghr on spontaneous motor activity and on anxiety.

5.3.1. Open field and elevated plus maze tests

The alteration in spontaneous motor activity may modify latency time measured in both passive avoidance and Morris water maze tests. On the one hand, the decreased motor activity can lead to prolonged latency time, and on the other, the increased motor activity can shorten the latency. Thus, the incrased or the decreased latency time can lead to untrue conclusions. Data of literature are diverse about the effect of A-Ghr in the open field, and several data are in

contradicion. Several authors observed that the i.c.v. applied A-Ghr increased the number of crossings and rearings [34], while others did not find any changes in spontaneous motor activity [9]. We have to emphasize that in these experiments the A-Ghr was administered i.c.v. wich method has more general effect than that of the direct inracerebral injection. In our experiments there were no changes in locomotion after 50 ng or 100 ng A-Ghr microionjections, however.

The elevated plus maze test is an accurate method to investigate the effect of a chemical substance on anxiety. The rats inherently spent more time on the enclosed arms of the apparatus [14]. The anxiogenic chemicals increase the normal tendency, i.e. the aversion against the open arms. This way the animal spend more time on the enclosed arms, its movement is limited to this area and the number of the entrance into the open arms is decreased. Under the effec of anxiolytic substances the animal frequently moves to the opened arms, goes to the end of the opened arms and spends more time there.

According to several data found in the literature, the i.p. or i.c.v. injected A-Ghr decreases the number of entrances to the opened arms and decreases the time spent on the opened arms, namely it is anxiogenic [5,9]. In our experiments, 50 ng or 100 ng A-Ghr injected into the BLA had no effect on anxiety.

5.3.2. Passive avoidance test

Carlini et al. reported first that A-Ghr is able to modify memory processes after its i.c.v. administration [9]. In their study the i.c.v. application of A-Ghr increased memory retention in step down test in a dose-dependent manner. In step down test the kernel of learning is that the animal does not step down from the small platform corresponding to its previous unpleasant experience. This situation in itself is more stressful than the step through paradigm used in our experiments even if in both situations weak (0.4 mA) electric shocks were used. In our experiments the aimed brain structure was the BLA. At this point we have to emphasize again the important differences between i.c.v. infusion and direct intracerebral injection [62]. As it was mentioned erlier, the i.c.v. application, can cause a “more general” effect than the local microinjection. Due to diffusion, the farther the specific brain area, the lesser is the concentration of the drug. In other experiments it was found that intra- hippocampal and –dorsal raphe administration of A-Ghr enhanced memory in step down task [10]. Our results provided evidence for that in aversive situations intraamygdaloid A-Ghr enhances learning processes and memory. We reported first that it is a specific effect because it was eliminated by pretreatment with

equimolar amount of ANT. Further experiments with specific antagonists of different GHS-R subgrups and investigations in other learning paradigms (active avoidance learning or rewarding situations) are necessary to cast light on the detailed learning mechanism of A-Ghr in the AMY.

5.3.3. Morris water maze test

The Morris water maze test is a widely used appropriate method to investigate spatial learning [28]. In the water maze task the learning mechanism is controlled by external cues and it requires the active contribution of animals. “Active” means that the animal in the experimental situation needs doing something to escape form the unpleasant situation to take a rest in the hidden platform. According to our best knowledge no data are available about the possible effect of A-Ghr on spatial learning in the AMY. The effective A-Ghr concentration used in our experiments was 50 ng (14.84 pmol). Our results suggest that intraamygdaloid A-Ghr enhances spatial learning processes and memory. It is a specific effect, because it was eliminated by equimolar amount of ANT pretreatment.

In our experiments in two different behavioural paradigms it was verified that intraamygdaloid ghrelinergic mechanisms are involved in the regulation of memory processes. This effect was eliminated by prior application of GHS-R specific ANT. According to the results of open field and elevated plus maze tests, the improvment of memory formation and retention induced by ghrelinergic mechanisms can not be explained by any alteration in spontaneous motor activity or anxiety.

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7. Publications

7.1. Publications related to the thesis

K. Tóth, K. László, É. E. Bagi, E. Lukács, L. Lénárd: Effect of intraamygdaloid microinjections of acylated-ghrelin on liquid food intake. Brain Research Bull. 77 (2008) 105-111. ***IF: 2,281***

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7.2. Further publications

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