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Investigating the effects of kisspeptin-13 and kisspeptin-8 on anxiety and locomotion in Wistar rats

Ph.D. Thesis

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Publications relevant to the thesis

I. Ibos, Katalin Eszter; Bodnár, Éva; Bagosi, Zsolt; Bozsó, Zsolt; Tóth, Gábor; Szabó, Gyula; Csabafi, Krisztina
Kisspeptin-8 Induces Anxiety-Like Behavior and Hypolocomotion by Activating the HPA Axis and Increasing GABA Release in the Nucleus Accumbens in Rats
BIOMEDICINES 9 : 2 Paper: 112 , 22 p. (2021)
Scopus - Medicine (miscellaneous) SJR: Q1
Scopus - Biochemistry, Genetics and Molecular Biology (miscellaneous) SJR: Q2
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II. Csabafi, Krisztina; Ibos, Katalin Eszter; Bodnár, Éva; Filkor, Kata; Szakács, Júlia; Bagosi, Zsolt
A Brain Region-Dependent Alteration in the Expression of Vasopressin, Corticotropin-Releasing Factor, and Their Receptors Might Be in the Background of Kisspeptin-13-Induced Hypothalamic-Pituitary-Adrenal Axis Activation and Anxiety in Rats
BIOMEDICINES 11 : 9 Paper: 2446 , 20 p. (2023)
Scopus - Medicine (miscellaneous) SJR: Q1
Scopus - Biochemistry, Genetics and Molecular Biology (miscellaneous) SJR: Q2
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Other publications

I. Pintér, Dávid; Balangó, Beáta; Simon, Balázs; Palotai, Miklós; Csabafi, Krisztina; Dobó, Éva; Ibos, Katalin Eszter; Bagosi, Zsolt
The effects of CRF and the urocortins on the hippocampal acetylcholine release in rats
NEUROPEPTIDES 88 Paper: 102147 , 5 p. (2021)
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II. Simon, Balázs; Buzás, András; Bokor, Péter; Csabafi, Krisztina; Ibos, Katalin Eszter; Bodnár, Éva; Török, László; Földesi, Imre; Siska, Andrea; Bagosi, Zsolt

The Effects of Alcohol Intoxication and Withdrawal on Hypothalamic Neurohormones and Extrahypothalamic Neurotransmitters

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The kisspeptin-1 receptor antagonist peptide-234 aggravates uremic cardiomyopathy in a rat model

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Chronic kidney disease may evoke anxiety by altering CRH expression in the amygdala and tryptophan metabolism in rats

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Abbreviations

ANOVA	analysis of variance
AVP or VP	(arginine) vasopressin
BNST	bed nucleus of the stria terminalis
CRH or CRF	corticotropin-releasing hormone or factor
ELISA	enzyme-linked immunosorbent assay
EPM	elevated plus maze
GABA	gamma-aminobutyric acid
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
icv.	intracerebroventricular
ip.	intraperitoneal
Kiss1R	kisspeptin receptor
KP	kisspeptin
LH	luteinizing hormone
MB	marble burying
MePD	posterodorsal medial amygdala
mRNA	messenger ribonucleic acid
NAc	nucleus accumbens
NPFFR	neuropeptide FF receptor
OF	open field
PCR	polymerase chain reaction
VTA	ventral tegmental area

1. Introduction

In 1996, a gene designated KiSS-1 was discovered in a human melanoma cell line, and was proven to act as a metastasis suppressor. In 2001, the C terminally amidated, 54-amino-acid-long peptide product of KiSS-1 was discovered and termed “metastin” based on its anti-metastatic activity. In the same year, three biologically active KiSS-1-derived peptides containing 54, 14, 13, and 10 amino acids were isolated in human placental samples and were designated kisspeptin-54, -14, -13, and 10 respectively. Kisspeptins (KPs) were identified as the natural ligands of GPR54, an orphan G-protein-coupled receptor showing structural similarity to the galanin receptors. After being deorphanized in 2001, the receptor was named kisspeptin-1 receptor (Kiss1R). Kisspeptins, as members of the RF amide family of neuropeptides, also bind and activate both neuropeptide FF receptors (NPFFR1 and NPFFR2).

The anti-metastatic activity of KP was discovered first, and later found in a variety of tumors including melanoma, bladder, ovarian, colorectal, pancreatic, pituitary, prostate, breast and thyroid cancers.

Later, KP was found to be the major regulator of the reproductive axis. Hypothalamic KP neurons are indispensable for puberty and are also responsible for mediating the positive and negative feedback of gonadal steroids on gonadotropin secretion, thereby governing the estrous cycle. Furthermore, KP not only orchestrates the HPG axis but has also been proven to be a key player in regulating sexual behavior and partner preference. Over the past few years, it has also become evident that kisspeptin signaling is involved in the modulation of metabolism, energy expenditure, thermoregulation, and cardiovascular function, as well as in their integration with reproductive functions.

As a part of the RF-amide family, an anti-opioid system of neuropeptides, a possible role of KP has been suggested in nociception by multiple studies. Recently our group has also demonstrated that KP-13 lowers the nociceptive threshold, reduces the analgesic effect of morphine, diminishes morphine tolerance, and evokes mechanical hypersensitivity in mice.

In rats, kisspeptins and Kiss1R are expressed in several brain regions involved in stress and anxiety, including the hypothalamus, the amygdala, hippocampus, lateral septum, the bed nucleus of the stria terminalis (BNST), striatum, nucleus accumbens (NAc), periaqueductal grey and locus coeruleus. Therefore, several studies have investigated the behavioral and emotional effects of KP.

An interplay between kisspeptin and the hypothalamic-pituitary-adrenal (HPA) axis was suggested in 2009 when Kinsey-Jones *et al.* discovered that stress-induced elevation of

plasma corticosterone suppresses hypothalamic kisspeptin signaling in rodents. Since that time, the literature has had conflicting results concerning the role of KP in anxiety.

In paraventricular nucleus-derived cell lines, KP-10 increased the gene expression of arginine vasopressin (AVP) and oxytocin, while suppressing the expression of corticotropin-releasing hormone (CRH). However, it failed to influence the activity of the HPA axis *in vivo*. Likewise, kisspeptin administration did not affect anxiety in human subjects.

In 2013, our group reported an anxiogenic effect of intracerebroventricularly (icv.) administered KP-13 in rats. KP-13 not only induced a significant increase in plasma corticosterone level but also decreased the number of entries into the open arms and the time spent in them in the elevated plus maze test. Moreover, it has stimulated spontaneous locomotion and it also had a hyperthermic effect lasting for several hours after treatment. An anxiogenic property of kisspeptin signaling has also been proposed by the experiments of Delmas *et al.*, in which Kiss1r KO mice have spent more time in the open arms in the elevated plus maze test, indicating a suppression of anxiety.

In zebrafish, however, the central administration of kisspeptin has been associated with an anxiolytic tendency. In a recent study, selective activation of MePD Kiss1 neurons in the posterodorsal medial amygdala (MePD) induced a significant increase in open-arm exploration in the elevated plus maze, suggesting an anxiolytic role of this neuron population.

There are several possible explanations for the ambiguous results reported in the literature. On one hand, the route of administration could be a determining factor, as peripheral administration of KP has failed to influence the activity of the HPA axis in rats and the activity of the limbic system in human subjects, but central KP-13 had a pronounced anxiogenic effect in rats. It is also likely that the doses applied peripherally by Rao *et al.* and Comninos *et al.* were too low to exert an anxiogenic effect. Likewise, the selective activation of MePD Kiss1 neurons points to the function of a distinct neuron population, whereas central kisspeptin treatment reflects a general central effect by activating neurons bearing Kiss1r throughout the brain. On the other hand, the differences could also be attributed to the variety of species involved in these experiments. The kisspeptin system of zebrafish is strikingly different from the mammalian one, thus the results of studies on zebrafish should be interpreted with caution.

Some studies have also reported that kisspeptin might play a role in the regulation of locomotor activity. Icv. KP-13 has induced an increase in not only spontaneous but also exploratory locomotion in male Sprague-Dawley rats. In line with these results, Tolson *et al.* have found that Kiss1r KO female mice exhibit decreased locomotor activity and energy

expenditure, leading to obesity. Since kisspeptin attenuates morphine effect, and is expressed in the NAc, it is possible involved in the regulation of mesocorticolimbic dopaminergic activity, which could in turn modulate locomotor activity.

2. Aims of the Study

Previously we reported that icv. KP-13 treatment evoked HPA axis activation and anxiety-like behavior in rats. In our study, we aimed to further investigate this effect by focusing on CRF and AVP, two hormones that are crucial for the neuroendocrine and behavioral response to stress. Following icv. treatment with KP-13, the gene expression of *Crf, Crf1r, Crf2r, Avp, Avpr1a,* and *Avpr1b,* as well as the protein expression of CRF and AVP were analyzed in the amygdala and hippocampus. These regions were selected based on their well-known role in the development of anxiety, and because the expression of KP and KISS1R, as well as CRF and AVP were detected in both the amygdala and the hippocampus. Therefore, we first investigated if KP-13 treatment modulates the CRF and AVP systems in these regions on the level of gene and protein expression. Next, we aimed to assess how AVP and CRF contribute to the HPA axis-stimulating and anxiogenic effect of KP-13. Following pre-treatment with non-selective CRF and AVP antagonists, computerized open-field test was performed to investigate the behavior of the animals, and corticosterone concentration was determined from trunk blood samples.

Nowadays an increasing number of studies focuses on kisspeptin analogs and antagonists due to their potential therapeutic use in gynecological conditions, such as infertility, polycystic ovary syndrome and precocious puberty. According to molecular docking studies, ASN4, SER5, GLY7, ARG9 and PHE10 of KP-10 are involved in the formation of hydrogen bonds with Kiss1r. Consequently, shorter fragments containing these amino acids might also be able to bind and activate the receptor. Therefore, we aimed to investigate the effects of kisspeptin-8 (KP-8), an 8-amino-acid-long kisspeptin fragment synthesized by the Department of Medical Chemistry. The goal of our experiments with KP-8 was to determine whether it elicits similar behavioral changes and HPA axis activation as KP-13, and whether it can also activate KISS1R, the canonical receptor of kisspeptins. Following icv. treatment with KP-8, the behavioral effects were assessed using the elevated plus maze, computerized open field, and marble burying tests. Serum corticosterone and luteinizing hormone levels were also determined. A rise in corticosterone corresponds to the the activation of the HPA axis, whereas the increase in LH levels can be interpreted as an indirect sign of KISS1R activation. The first behavioral results (as described later in this Thesis) suggested the possible involvement of the

ventral tegmental area (VTA) – nucleus accumbens (NAc) dopaminergic circuit in the behavioral effects of KP-8. This circuit is mostly known to play a role in reward and addiction, but it is also involved in the modulation of locomotor activity. Therefore, *ex vivo* superfusion was used to measure dopamine release from the VTA and NAc, as well as GABA release from the NAc in response to KP-8.

3. Materials and Methods

3.1. Animals and housing conditions

Adult male Wistar rats (Domaszék, Csongrád, Hungary) weighing 150–250 g were used for the experiments at the age of 6–8 weeks. The animals were housed under controlled conditions at constant room temperature, with a 12–12-h light-dark cycle (lights on from 6:00 a.m.). The rats were allowed free access to commercial food and tap water. The animals were kept and handled during the experiments in accordance with the instructions of the University of Szeged Ethical Committee for the Protection of Animals in Research, which approved these experiments. Permission for the experiments (number: X./1207/2018, date: 6 July 2018.) has been granted by the Government Office of Csongrád County Directorate of Food Chain Safety and Animal Health. Each animal was used for only one experimental procedure.

3.2. Intracerebroventricular cannulation

The animals were allowed 1 week to acclimatize before the surgery. A stainless steel Luer cannula (10 mm long) was implanted in the right lateral cerebral ventricle under sodium pentobarbital (Euthasol, Phylaxia-Sanofi, 35 mg/kg, ip.) anesthesia, according to the following stereotaxic coordinates: 0.2 mm posterior and 1.7 mm lateral to the bregma, and 3.7 mm deep from the dural surface. Subsequently, it was secured to the skull with dental cement and acrylate. The experiments started after a recovery period of 1 week. All experiments were carried out between 8:00 a.m. and 10:00 a.m. At the end of the experiments, the correct position and the permeability of the cannula were checked.

3.3. Peptide synthesis

KP-8 (WNSFGLRF-NH2) was synthesized on a Rink Amide MBHA resin (Bachem, Bubendorf, Switzerland, subst.: 0.52 mmol/g) using Nα-9-Fluorenylmethoxycarbonyl (Fmoc) protected amino acids (IRIS Biotech GmbH, Marktredwitz, Germany) by manual solid phase peptide synthesis by the Department of Medical Chemistry (University of Szeged).

3.4. Icv. treatment

Rats were injected with different doses of KP-13 (Bachem Ltd., Switzerland) or KP-8 (synthesized by the Department of Medical Chemistry) dissolved in 0.9% saline icv. in a volume

of 2 µl over 30 s with a Hamilton microsyringe, immobilization of the animals being avoided during handling. In our studies with KP-8, the peptide was injected in 0.1 or 1 µg doses. In the open field test with KP-13, doses of 0.5, 1, and 2 µg KP-13 were administered, in the case of experiments with antagonists, the most effective dose of KP-13 (1 µg) was applied that was chosen based on our previous experiments and that of the open field test. Antagonist treatment was performed 30 min prior to the peptide challenge. The following antagonists were applied: α-helical CRF(9-41) (Bachem Ltd., Switzerland), a non-selective CRFR blocker in a dose of 1 µg, and a V1R antagonist (Bachem Ltd., Switzerland) in a dose of 0.1 µg. The doses of the antagonists were selected based on previous dose-response studies, in which they had no effect *per se* on the investigated parameters. Control animals received saline alone. After KP-13 or KP-8 administration, the animals were sacrificed at different time points (15 min in case of LH measurement; 30 min in case of corticosterone measurement; 2 h in case of gene expression analysis; 4 h in case of protein measurements) or were subjected to behavioral testing.

3.5. Behavioral tests

3.5.1. Elevated plus maze (EPM) test

30 min after icv. treatment the rats were placed in the maze facing one of the open arms, then their behavior was recorded by a camera suspended above the maze for 5 min. The time spent in each arm, as well as the number of entries per arm were registered by an observer blind to the experimental groups. The percentage of entries into the open arms and the percentage of time spent in the open arms were also calculated.

3.5.2. Computerized open field (OF) test

The novelty-induced locomotor activity of rats was assessed using the Conducta 1.0 System (Experimetria Ltd., Budapest, Hungary). 30 min after icv. treatment the rats were placed in the center of the box and their behavior was recorded by the Conducta computer program for 5 minutes (in the case of KP-13) or 60 minutes (in the case of KP-8). Six behavioral parameters were measured during the experiment: total time and total distance of ambulation, immobility time, number of rearings (vertical locomotion), time spent in the central zone (central area of 24×24 cm), and distance traveled in the central zone. In addition, central ambulation time/total ambulation time% and central ambulation distance/total ambulation distance% were calculated from the raw data.

3.5.3. Marble burying (MB) test

MB is a regularly used paradigm for the assessment of anxiety-like and compulsive-like behavior. Our protocol was based on the method described by Schneider and Popik. The

animals were removed from home cages and the home cage was prepared for the experiment by increasing the depth of the bedding material to 5 cm. Following icv. treatment with KP-8 or saline, one animal was placed back into the home cage for 30 min in order to acclimatize. Then 9 glass marbles of 2.5 cm diameter were arranged in 3 rows along the shorter wall of the cage. The experiment was conducted for 10 min and recorded by a video camera above the cage. After the session, the animal was removed from the cage and the number of buried marbles (>50% covered by bedding material) was counted. The count and duration of two types of goal-oriented interactions with marbles (burying of marbles, and moving marbles without burying them) were assessed.

3.6. ELISA

The animals were decapitated 30 min and 15 min after icv. treatment with KP-8 or saline for the measurement of serum corticosterone and protein, as well as LH, respectively. Trunk blood was collected into test tubes and left at room temperature for 30 min to clot, then it was centrifuged for 10 min at 3500 rpm. The samples were stored at −80°C. To measure the AVP and CRF content of the amygdala and hippocampus, animals were decapitated 4 h after IBC treatment, regions were isolated as described in section 3.8, and samples were placed in Eppendorfs, frozen in liquid nitrogen and stored at -80°C. Serum corticosterone concentration was measured using a competitive corticosterone ELISA kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions. Serum LH concentration was determined using a sandwich LH ELISA kit (Wuhan Xinquidi Biological Technology Co., Wuhan, China), according to the manufacturer's instructions. CRF and AVP were determined using competitive ELISA kits (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA; EK-019-06, EK-065-07). The Pierce Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used, according to the manufacturer's instructions for the measurement of total serum protein concentration. The absorbance was measured at 595 nm with a NanoDrop OneC microvolume spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

3.7. *Ex vivo* superfusion

Before the *ex vivo* superfusion, the animals did not undergo icv. cannulation. The rats were rapidly decapitated, and their brains were removed from the skull. Dissection was performed with the help of a brain matrix, a tissue puncher, and razor blades, on a filter paper moistened with phosphate-buffered saline, on top of a Petri dish filled with ice. The NAc was removed from both sides, following the method of isolation described by Heffner. The VTA

was isolated as described by Salvatore *et al*. The tissue was cut into 300μm slices and incubated for 30 min in 5 mL of Krebs solution (Reanal, Hungary) bubbled with carbogen gas (5% CO2 and 95% O2). Then 5μL of [3H] GABA (PerkinElmer Inc., Waltham, MA, USA) was added to the NAc, and 5μL of [3H] Dopamine (PerkinElmer Inc., Waltham, MA, USA) was added to the VTA or the NAc. Afterwards, the slices were transferred evenly into the four cylindrical chambers of the superfusion system (Experimetria Ltd., Budapest, Hungary), and superfusion with carbogen-bubbled Krebs solution was started at body temperature (37°C). A constant flow rate of 227, 7 μL/min was maintained with a peristaltic pump (Minipuls 2, Gilson, Middleton, WI, USA). After 30 min of superfusion, the collection of superfusates into Eppendorf tubes was started with a multichannel fraction collector (FC 203B, Gilson, Middleton, WI, USA). Fractions were collected every two minutes for 32 min. At 6 min, 1 μg of KP-8 dissolved in 1 mL of Krebs solution was added directly into the chambers. From the 12th minute of fraction collection, electrical stimulation of square-wave impulses was delivered for two minutes (ST-02 electrical stimulator, Experimetria Ltd., Budapest, Hungary). Then, the tissue from each chamber was transferred into a beaker containing 600 μL of Krebs solution for ultrasonic homogenization (Branson Sonifier 250, Emerson Electric Co., St. Louis, MO, USA). Afterwards, 3 mL of Ultima Gold scintillation cocktail (Perkin-Elmer Inc., Waltham, MA, USA) was pipetted into 4 rows of 17 scintillation vials. Subsequently, 200 μL of the 16 fractions collected, and of the suspension of the tissue from the corresponding chamber were added to each row of vials. The samples were homogenized mechanically for 30 min. The radioactivity of samples was detected with a liquid scintillation spectrometer (Tri-carb 2100 TR, Hewlett-Packard Inc., Palo Alto, CA, USA). Fractional dopamine or GABA release (FR) was calculated from the counts per minute (CPM), according to the equation below, in which i stands for the number of fraction and n= 16. CPM_{17} refers to the CPM of the homogenized tissue sample corresponding to the fraction:

$$FR_i = 100 \cdot \frac{CPM_i}{4 \cdot CPM_{17} + \sum_{i+1}^{n} CPM_i}$$

3.8. Gene expression analysis

Two hours after icv. KP-13 administration animals were sacrificed by decapitation. After isolation of the brain, they were dissected with a pre-cooled adult rat brain matrix (Ted Pella Inc., Redding, CA, USA). Next, brains were manually sliced with pre-cooled razor blades in coronal sections (1 mm slots), then 1 mm in diameter tissue punches (Ted Pella Inc., Redding, CA, USA) were taken from the amygdala and hippocampus, and placed in Eppendorf tubes

filled with 1 ml TRIzol Reagent (Life Technologies, Carlsbad, CA, USA). The tissue samples were immediately frozen and stored at -80 ºC until gene expression analysis. The samples were homogenized with an ultrasonic homogenizer on ice, and then total RNA was extracted by using TRIzol extraction protocol and then using GeneJET RNA Purification Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The quality and quantity of extracted RNA were determined by NanoDrop OneC microvolume spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized from at least 100 ng of total RNA by using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. SybrGreen technology-based real-time quantitative PCR (CFX96 BioRad) was used to quantify the relative amount of the targeted mRNAs (*Crf, Crf1r, Crf2r, Avp, Avpr1a, Avpr1b* as well as housekeeping gene *Gapdh*). Specific exon-spanning gene expression assays were used (*Avp*: forward: CTG ACA TGG AGC TGA GAC AGT, reverse: CGC AGC TCT CGT CGC T; *Avpr1a:* forward: TGG ACC GAT TCA GAA AAC CCT, reverse: GTT GGG CTC CGG TTG TTA GA; *Avpr1b*: forward: CAG CAT AGG AGC CAA CCA TCA A, reverse: GAA AGC CCA GCT AAG CCG T; *Crf:* forward: TGG TGT GGA GAA ACT CAG AGC, reverse: CAT GTT AGG GGC GCT CTC TTC; *Crfr1:* forward: CGA AGA GAA GAA GAG CAA AGT ACA C, reverse: GCG TAG GAT GAA AGC CGA GA; *Crfr2:* forward: CCC GAA GGT CCC TAC TCC TA, reverse: CTG CTT GTC ATC CAA AAT GGG T; *Gapdh:* forward: CGG CCA AAT CTG AGG CAA GA, reverse: TTT TGT GAT GCG TGT GTA GCG). Cycling protocol: 1x UDG pretreatment (50°C, 2 min), 1x initial denaturation (95°C, 10 min), 40x denaturation (95°C, 15 s), annealing (60°C, 30s), extension (72°C, 30s). For controls, we used reaction mixtures without cDNA. Each sample was run in duplicates. The ratio of each mRNA relative to the housekeeping gene *Gapdh* was calculated using the 2-ΔΔCT method.

3.9. Statistical analysis

The statistical analyses and graph editing of experiments with KP-13 and KP-8 were carried out by SPSS and GraphPad Prism 8, respectively. A probability level of less than 0.05 was accepted as indicating a statistically significant difference.

4. Results

4.1. Studies with KP-13

4.1.1. Gene expression

The relative expression of *Avp, Avpr1a, Avpr1b, Crf, Crfr1,* and *Crfr2* genes was calculated compared to *Gapdh* expression and analyzed by the Mann-Whitney test.

In the amygdala, the mRNA expression of *Avp* and *Avpr1b* significantly increased, whereas the expression of *Crf* was reduced, compared to the control group. In the cases of *Avpr1a*, *Crfr1* and *Crfr2* no significant difference was detected. In the hippocampus, the relative gene expression of *Crf* was significantly higher in the KP-13-treated group. On the other hand, *Avpr1a* mRNA expression showed a marked decrease. In the cases of *Avpr1b*, *Avp*, *Crfr1* and *Crfr2*, no significant difference was detected.

4.1.2. Protein expression

A two-factor analysis of variance on AVP protein level revealed a significant main effect for the treatment factor and region factor. There was no significant interaction between the two factors, therefore, the effect of the different levels of treatment does not depend on which region is involved. Pairwise comparisons revealed that KP-13 treatment caused a significant increase in the AVP protein level in the amygdala, however, it had no effect in the hippocampus.

A two-way ANOVA on CRF protein content showed a significant main effect for the region factor, however no significant main effect was found for the treatment factor. KP-13 treatment did not affect CRF protein content.

4.1.3. Plasma corticosterone

Two-way ANOVA was conducted to assess the effect of KP-13 treatment and antagonist treatment on corticosterone concentration. Our result showed a statistically significant main effect for the KP-13 treatment and a statistically significant interaction between the two factors, thus, the effect of KP-13 depends on which antagonist pretreatment was applied. There was no significant main effect for the antagonist treatment factor. Pairwise comparisons revealed that KP-13 treatment caused a marked elevation in the corticosterone concentration compared to the saline-treated group. Furthermore, among the KP-13 treated animals both CRFR antagonist and V1R antagonist pretreated were significantly different.

4.1.4. The effect of KP-13 on open-field behavior

Univariate ANOVA was used to investigate the effect of KP-13 treatment on OF parameters total ambulation distance and time, immobility time, rearing activity, central ambulation distance and time, and finally central ambulation distance/total ambulation distance% and central ambulation time/total ambulation time%. Our result showed that KP-13 had no significant effect on total ambulation distance and total ambulation time. In the case of immobility time KP-13 showed a tendency to increase, the 2 μg dose of KP-13 significantly increased the immobility time of animals compared to the control. KP-13 had a significant effect on rearing activity. Again, the 2 μg dose of KP-13 was the most effective.

In the case of central ambulation distance, the test for homogeneity of variance was not satisfied, therefore a non-parametric ANOVA (Kruskal-Wallis) was performed followed by Dunn's test for multiple comparisons. Results showed that KP-13 treatment significantly decreased the central ambulation distance. Pairwise comparisons with Bonferroni correction revealed that both 1 μg and 2 μg of KP-13 significantly decreased the distance traveled in the center of the OF arena. KP-13 had a significant effect on central ambulation time. Pairwise comparisons revealed that both 1 μg and 2 μg of KP-13 significantly decreased the time spent in the center of the open field arena. KP-13 evoked a significant decrease in the central ambulation distance/total ambulation distance%. Pairwise comparisons showed that both 1 μg and 2 μg of KP-13 were significant compared to control. In the case of the central ambulation time/total ambulation time%, the result was similar. Again, both the 1 μg dose, as well as the 2 μg dose of KP-13 were found to be significant.

4.1.5. The effect of V1R and CRFR antagonists on KP-13-induced OF behavior

Two-way ANOVAs were conducted to investigate the effect of KP-13 treatment in the presence of CRFR and V1R antagonist pretreatment on OF parameters. There were no significant changes in ambulation distance and ambulation time. In the case of immobility time, there was a significant main effect for the treatment factor, but no significant difference for antagonist treatment or between the two factors. Pairwise comparison showed no significant difference between groups. There were no significant changes in the case of rearing activity.

However, in the case of central ambulation distance, our result showed a statistically significant main effect for the KP-13 treatment, but not for the antagonist treatment factor or the interaction between the two factors. Pairwise comparisons revealed that KP-13 treatment caused a marked decrease in the central ambulation distance 30 min after treatment compared to the saline-treated group. Among the KP-13 treated animals, CRFR antagonist pretreatment wasn't significantly different, however, V1R antagonist pretreatment showed a statistically significant difference. In the case of central ambulation time, our results showed a statistically significant main effect for the KP-13 treatment similar to central ambulation distance, but no statistically significant main effect for the antagonist treatment factor or the interaction between the two factors.

Pairwise comparisons revealed that the KP-13 treatment caused a significant decrease in the central ambulation time. Among the KP-13 treated animals, V1R antagonist pretreatment showed a statistically significant difference, however, CRFR antagonist pretreatment wasn't significant. In the case of central ambulation distance/total ambulation distance%, the main

effect for KP-13 treatment was found significant, however, no significant main effect was detected for the antagonist treatment and the interaction between the two factors. Pairwise comparison showed that KP-13 injection evoked a marked decrease in the central ambulation distance/total ambulation distance% and among the KP-13 treated groups, the V1R antagonist pretreatment was statistically significant, therefore, the V1R antagonist inhibited the KP-13-induced decrease in central ambulation distance/total ambulation distance%. CRFR antagonist, however, did not alleviate KP-13's effect. Our results on the central ambulation time/total ambulation time% were quite similar since a statistically significant main effect for KP-13 treatment was detected and no effect was found for the antagonist treatment or the interaction. Pairwise comparison revealed that KP-13 caused a significant decrease in central ambulation time/total ambulation time%. Furthermore, the V1R antagonist significantly decreased the KP-13-evoked fall in central ambulation time/total ambulation time%, whereas CRFR antagonist treatment among the KP-13 treated animals caused no statistically significant difference.

4.2. Studies with KP-8

4.2.1. Elevated plus maze test

The 0.1 μg dose of KP-8 significantly reduced the percentage of entries into the open arms of the EPM, as well as the percentage of time spent in the open arms of the maze. A decrease in the total number of entries into the arms was induced by both 0.1 μg and 1 μg of KP-8. There was no significant difference among the groups in the total time spent in the arms.

4.2.2. Computerized open-field test

The cumulative results obtained after 60 minutes of data collection did not show any significant change in behavior.

However, significant differences were found following the analysis of each 5-minute interval. The two-factor RM-ANOVA on the distance traveled in the arena revealed a significant main effect for the time factor. Following a peak in the first five minutes, the ambulation distance was steeply decreasing until a lower level of basal locomotor activity was reached around 30 minutes. The distance traveled at 50-55 and 55-60 minutes was lower in the 1 μg KP-8 group than in the control group. Regarding total ambulation time, there was a significant main effect for the time factor with a similar pattern of steep then mild decrease. The 1 μg KP-8 group spent less time with ambulation than the control group at 50-55 minutes and 55-60 minutes, as well. The two-way ANOVA on immobility yielded a significant main effect for the time factor and interaction. The time spent immobile was increasing during the experiment, showing a

tendency reciprocal to that of ambulation time and distance. Compared to control, the 1 μg dose of KP-8 significantly increased immobility at 50-55 and 55-60 minutes.

Considering the number of rearing sessions, a significant main effect for the time factor was detected, along with a statistically significant interaction between time and treatment. A pronounced difference started to appear among treatment groups after 30 minutes. There was a significant decrease in the number of rearings in the 1 μg KP-8 group at 30-35 minutes, 40-45 minutes, 50-55 minutes and 55-60 minutes.

Having calculated the average velocity for each timeframe, a significant main effect for the time factor and interaction could be seen. There was no significant difference between treatment groups until 55 minutes when the speed of the 1 μg KP-8 group dropped.

The percentage of central ambulation distance was calculated by dividing the distance traveled in the central zone of the arena by the total ambulation distance, multiplied by 100. Time factor, and interaction between time and treatment both significantly accounted for the variation, but there was no difference among the groups, except in the first 5 minutes, when the central ambulation distance of the 1 μg KP-8 group was higher than that of control. The percentage of central ambulation time was calculated by multiplying the ratio of central time and total ambulation time by 100. There was a significant main effect for the time factor, as well as for the interaction. In the first 5 minutes, the central ambulation time of the 1 μg KP-8 group significantly exceeded the central time of the control group, otherwise there was no difference among the groups.

4.2.3. Marble burying test

There was no significant difference in the number of buried marbles among the groups. Two types of goal-oriented interactions with the marbles were distinguished: marble burying and marble moving.

Marble burying is an interaction involving digging around the marbles, resulting in marbles covered with bedding material. Neither the number of marble burying sessions nor the duration of marble burying activity changed significantly with treatment, although a tendency of reduced burying activity was observable. Marble moving is an interaction that involves rolling, moving the marbles with the forelegs, without successfully covering them with bedding material. Similarly to marble burying, there was no significant difference in the number and duration of marble moving among the groups, although a tendency of suppressed marble moving could be seen in the groups treated with KP-8. However, taking the two types of interactions

together, the 1 μg KP-8 group interacted with the marbles fewer times than the control group and they also spent less time with goal-oriented interactions with the marbles.

4.2.4. Serum corticosterone, LH and total protein

One-way ANOVA showed a significant effect of KP-8 treatment both on corticosterone and LH concentration. A robust increase in serum corticosterone concentration was detected 30 minutes after icv. treatment with 1 μg of KP-8. The 0.1 μg dose tended to elevate corticosterone, but the change was not significant. The 1 μg dose of KP-8 also raised serum LH concentration 15 minutes after treatment, but the 0.1 μg dose had no effect. There was no difference in serum protein concentration among the groups.

4.2.5. Ex vivo superfusion

In the case of fractional dopamine release from the VTA, the p-value was not calculated for the time factor. There was no significant main effect neither for the treatment factor, nor for the interaction between treatment and time. There was no significant difference between the groups at any other time point. Likewise, KP-8 did not influence fractional dopamine release from the NAc. However, there was a significant main effect for the time factor. No significant main effect was found for the treatment factor, and for the interaction between treatment and time. No significant difference could be detected at any specific time point between the groups. However, KP-8 increased fractional GABA release from the NAc. There was a significant main effect for the time and interaction factors. In the seventh fraction, following electrical stimulation, fractional GABA release was significantly higher from the KP-8 treated brain slices than from the control tissue.

5. Summary and Conclusions

The objective of this dissertation was to present our newly published data on the possible effect of kisspeptins on the HPA axis, anxiety-related behavior, and locomotion. Kisspeptin is the product of the Kiss1 gene and is present in four biologically active forms consisting of 54, 14, 13, and 10 amino acids. It can bind with different affinity to their cognate receptor KISS1R or NPFF receptors. Kisspeptin's role as the main central regulator of the reproductive axis is well established, however, distribution data suggested a wider function. Therefore, our group previously investigated the possible effect of centrally injected KP-13 on HPA axis activity and anxiety-like behavior in rats. We have found that KP-13 increased the corticosterone level of rats and induced anxiety in the elevated plus maze test and open field test. Next, we wanted to explore how KP-13 might influence the HPA axis and stress-related behavior. We hypothesized that KP-13 might alter the expression of CRF, AVP, and their

receptor which are the main central regulators of the HPA axis and their role in mediating anxiety is well-established. We concentrated on two brain regions where kisspeptin and Kiss1r are present, which are the amygdala and the hippocampus. We also investigated how the open-field behavior and corticosterone response of kisspeptin-administered animals changed upon pretreatment with either CRF or V1 receptor antagonists. Furthermore, we also sought to explore whether an N-terminally truncated 8 amino acid long kisspeptin fragment, KP-8, retains its ability to affect the HPA axis and the behavior of rats similarly to KP-13.

Our results showed that KP-13 seems to alter the expression of *Avp*, *Crf*, and their receptors in a region-dependent manner. In the amygdala, KP-13 induced an upregulation of *Avp* and *Avpr1b* and downregulated the expression of *Crf*. In the hippocampus, KP-13 caused the mRNA level of *Crf* to increase and the mRNA level of *Avpr1a* to decrease. A significant rise in AVP protein content was also detected in the amygdala. Furthermore, KP-13 induced an anxiety-like behavior in the open-field test, that was antagonized by the V1R blocker. Furthermore, both CRFR and V1R antagonists reduced the KP-13-evoked rise in the plasma corticosterone level. All these data suggest that KP-13 could affect the AVP and CRF signaling pathways and that might be responsible for its effect on the HPA axis and anxiety-like behavior.

KP-8 also activated the HPA axis and evoked anxiety-like behavior in rats, similar to KP-13. However, it affected locomotion in the opposite direction, i.e. KP-8 suppressed locomotor activity, whereas KP-13 triggered an increase in spontaneous locomotion. In the background of this discrepancy, we postulated a different affinity to Kiss1R and NPFF receptors, a modulation of the VTA-NAc dopaminergic circuit, as well as a plausible effect on metabolism and thermoregulation. To investigate these possibilities, it is essential to conduct some experiments with antagonist pre-treatments, such as p234 (a Kiss1R antagonist) and GJ14 (an NPFF1R antagonist). Furthermore, telemetry should be performed to confirm the decrease in spontaneous locomotion and to gather body temperature data. In our previous studies, KP-13 induced a transient increase in body temperature, which could be a phenomenon linked to the increase in locomotor activity, or the result of another, activity-independent mechanism, such as the induction of non-shivering thermogenesis in the brown adipose tissue. It should be investigated, whether KP-8 also modulates body temperature, possibly along with locomotor activity. Finally, alterations in the VTA-NAc circuit should be further explored by gene and protein expression studies.

In conclusion, the activation of the KP signaling causes anxiety in rats accompanied by the elevation of corticosterone, which might be mediated by the altered expression of the CRF

and AVP systems in the amygdala and hippocampus. KP-8, a synthetic derivative of KP-13, retains the ability to activate the HPA axis and induce anxiety, but in contrast with KP-13, it suppresses locomotion, possibly via an NPFFR-mediated action on the VTA-NAc circuit. However, further are required to confirm the exact mechanism of action of kisspeptins.

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