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

Katalin Eszter Ibos M.D.

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Albert Szent-Györgyi Medical School
Doctoral School of Theoretical Medicine

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Katalin Eszter Ibos M.D.

Supervisor: Krisztina Csabafi M.D., Ph.D.

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List of Abbreviations

ACN	acetonitrile
ACTH	adrenocorticotropic hormone
AgRP	agouti-related peptide
ANOVA	analysis of variance
ARC	arcuate nucleus
AVP or VP or ADH	arginine vasopressin or vasopressin or antidiuretic hormone
AVPV	anteroventral paraventricular nucleus
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
CA1/2/3	Cornu Ammonis 1 or 2 or 3 (hippocampal regions)
cAMP	cyclic AMP
CART	cocaine- and amphetamine-regulated transcript
cDNA	complementary deoxyribonucleic acid
CeA	central amygdala
CeL	centrolateral amygdala
CeM	centromedial amygdala
CFA	complete Freund's adjuvant
CNS	central nervous system
COMT	catechol-o-methyltransferase
CPM	counts per minute
CRF or CRH	corticotropin-releasing factor or hormone
CRF-BP	CRF-binding protein
CRHR1	CRF receptor type 1
DCM	dichloromethane
DMF	N,N-dimethylformamide
DREADD	designer receptors exclusively activated by designer drugs
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, 5th edition
DYN	dynorphin
ELISA	enzyme-linked immunosorbent assay
EPM	elevated plus maze
ESI-MS	electrospray ionization mass spectrometry
Fmoc	Nα-9-Fluorenylmethoxycarbonyl

GnRH	gonadotropin-releasing hormone
GPCR	G-protein-coupled receptor
GPR54	G-protein-coupled receptor 54
ERK1/2	extracellular signal-regulated kinase 1/2
FR	fractional release
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
HPLC	high-performance liquid chromatography
ICD-11	International Classification of Diseases, 11th edition
icv.	intracerebroventricular
ip.	intraperitoneal
IP3	inositol 1,4,5-trisphosphate
KISS-1	kisspeptin gene
KNDy	kisspeptin – neurokinin B – dynorphin neurons
KO	knockout
KOR	kappa opioid receptor
KP	kisspeptin
LH	luteinizing hormone
MAPK	mitogen-activated protein kinase
MB	marble burying
MBHA	4-Methylbenzhydrylamine
Mdn	median
MeA	medial amygdala
MePD	medial posterodorsal amygdala
mPFC	medial prefrontal cortex
mRNA	messenger RNA
NAc	nucleus accumbens
NK3R	neurokinin receptor 3
NKB	neurokinin B
NO	nitric oxide
NPFF	neuropeptide FF
NPY	neuropeptide Y

OF	open field
PCR	polymerase chain reaction
PKC	protein kinase C
PLC	phospholipase C
POMC	pro-opiomelanocortin
PrRP	prolactin-releasing peptide
PVN	paraventricular nucleus
QRFP	pyroglutamylated RF-amide peptide
RF	arginine-phenylalanine
RFRP	RF-amide-related peptide
RM-ANOVA	repeated measures ANOVA
RNA	ribonucleic acid
RP3V	rostral periventricular area of the 3rd ventricle
rpm	revolutions per minute
SEM	standard error of the mean
SLC6A4	Solute Carrier Family 6 Member 4
SNP	single nucleotide polymorphism
TFA	trifluoroacetic acid
TNF-α	tumor necrosis factor α
Ucn	urocortin
UDG	uracil-DNA glycosylase
V1aR	vasopressin 1a receptor
vHPC	ventral hippocampus
VTA	ventral tegmental area

1. Introduction

1.1. The RF-amide family

The RF-amide peptide family encompasses a wide range of neuropeptides in all major animal phyla that share the C-terminal Arg-Phe-NH_2 sequence. In vertebrates, 5 major RF-amide neuropeptide families have been discovered, which are products of 5 distinct precursors and are natural ligands of 5 G-protein-coupled receptors (Table 1)[1]. Although NPFF1R and NPFF2R are the natural receptors of the RFRP and NPFF family, respectively, PrRP, QRFP and KP are also capable of binding and activating these receptors in the nanomolar range[2]. In contrast, PrRP-R, QRFR-R and Kiss1R are fairly selective to their respective peptide subgroups.

Family	Precursor	Peptides	Receptor
Neuropeptide FF (NPFF)	Pro-$NPFF_A$	NPFF, NPAF (neuropeptide AF), NPSF (neuropeptide SF)	NPFF2R (GPR74)
RF-amide-related peptide (RFRP)	Pro-$NPFF_B$	RFRP1, RFRP3	NPFF1R (GPR147)
Prolactin-releasing peptide (PrRP)	Prepro-PrRP	PrRP-20, PrRP-31	PrRP-R (GPR10)
Pyroglutamylated RF-amide peptide (QRFP)	Propro-QRFP	QRFP-26, QRFP-43	QRFP-R (GPR103)
Kisspeptin (KP)	KiSS-1 protein	KP-10, KP-13, KP-14, KP-52, KP-54	KiSS1R (GPR54)

Table 1 – Precursors, members and receptors of the 5 RF-amide neuropeptide families found in vertebrates. Based on Quillet et al., 2016

1.2. Kisspeptin

1.2.1. The KiSS-1 gene and its products

In 1996, a gene designated KiSS-1 was discovered in a human melanoma cell line (C8161), and was proven to act as a metastasis suppressor[3]. The human KiSS-1 gene, mapped to the long arm of chromosome 1, contains 4 exons, the third and fourth of which are translated[4]. 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[5]. 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[6] (Figure 1).

In rats, kisspeptins are expressed in several brain regions, including hypothalamic nuclei [e.g. arcuate nucleus, anteroventral paraventricular nucleus (AVPV)], thalamic nuclei, the amygdala, hippocampus, lateral septum, the bed nucleus of the stria terminalis, striatum, nucleus accumbens (NAc), periaqueductal grey and locus coeruleus [7,8]. The major peripheral sites of expression include the gonads, uterus, placenta, liver, pancreas, adipose tissue, and intestines[5].

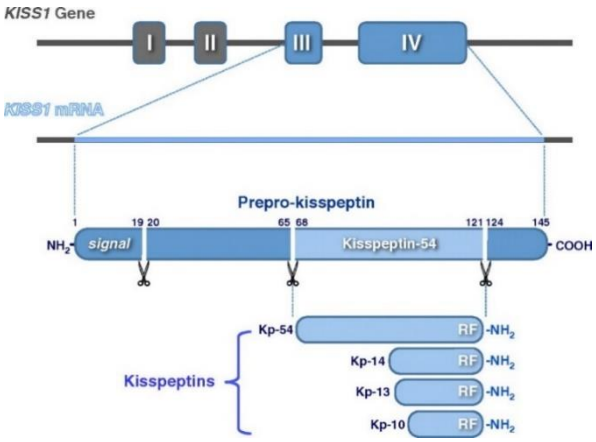

Figure 1- Products of the Kiss1 gene. Pinilla et al., 2012

1.2.2. Kisspeptin receptors

Kisspeptins were identified as the natural ligands of GPR54, an orphan G-protein-coupled receptor showing structural similarity to the galanin receptors, which is also known as hOT7T175 or AXOR12 in humans[9,6,10]. After being deorphanized in 2001, the receptor was named kisspeptin-1 receptor (Kiss1R)[5].

Upon activation of the Gαq/11-coupled Kiss1r, phospholipase C (PLC) is activated, triggering inositol 1,4,5-trisphosphate (IP3)-mediated intracellular Ca^{2+} mobilization. Moreover, several mitogen-activated protein kinases (MAPKs) are activated via protein kinase C (PKC) and the Gαq-independent recruitment of β-arrestins including extracellular signal-regulated kinases 1/2 (ERK1/2) and p38 [11]. The activation of MAPKs modulates gene expression and induces long-term biological effects[12].

Kiss1r has been mapped in rats to the hypothalamus (e.g. paraventricular, arcuate and supraoptic nuclei), thalamus, hippocampus, amygdala, septum, striatum, raphe nuclei and cortex [9,13].

Kisspeptins also bind and activate both neuropeptide FF receptors (NPFFR1 and NPFFR2)[14]. As NPFF receptors are coupled with Gαi/o, their activation inhibits cAMP production. The Gβγ heterodimer released from Gi/o proteins was found to inhibit voltage-gated Ca2+ channels. Moreover, it is capable of potentiating Gq signaling via physical interaction with PLC [1].

The expression of NPFF1 receptor mRNA has been detected in the lateral septum, in thalamic and brainstem nuclei, as well as in the ventral tegmental area (VTA), NAc, the bed nucleus of the stria terminalis, the amygdala, and hippocampus. NPFF2 receptor mRNA

expression has been reported in thalamic nuclei, in the hypothalamus, hippocampus, VTA, the A5 noradrenergic cell group, and also in the dorsal horn of the spinal cord [15,16].

1.2.3. Major roles of kisspeptins

- *Anti-metastatic effect*

Following the discovery of kisspeptin's anti-metastatic activity in malignant melanoma[3], the loss of KISS1 expression has been associated with increased metastasis formation in a variety of tumors, including bladder, ovarian, colorectal, pancreatic, pituitary, prostate, breast and thyroid cancers [17,18]. The anti-metastatic effect could be achieved via reduced chemotaxis, increased apoptosis mediated by ERK, Rho kinases, and TNF-α, as well as impaired autophagy[19–21]. Kisspeptins also trigger a metabolic shift in tumor cells by reversing the Warburg effect (the tumor cells' preference for anaerobic glycolysis), and supporting mitochondrial biogenesis[22]. It should be noted though that kisspeptin signaling has also been associated with increased angiogenesis, tumor growth, and invasiveness by some groups, the background of which has not been elucidated yet[23–25].

- *Regulation of the reproductive axis*

The involvement of kisspeptin in the regulation of the hypothalamic-pituitary-gonadal (HPG) axis has been the focus of research since it was discovered in 2003 that loss-of-function mutations of GPR54 are associated with hypogonadotropic hypogonadism[26]. In the hypothalamus, neurons expressing *Kiss1* are mainly localized to the arcuate nucleus (ARC) and anteroventral periventricular area (AVPV) in rodents. The majority of GnRH neurons in the preoptic area (POA) co-express Kiss1R[27] and get activated in response to KP, leading to the stimulation of the HPG axis[28]. 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[29]. The sexually dimorphic Kiss1 neuron population of the AVPV expresses ERα and mediates the positive feedback of estrogen, thus it contributes to the rapid increase in GnRH secretion and the consequent LH surge[30]. In the ARC, KNDy neurons (coexpressing KP, neurokinin B [NKB], and dynorphin [DYN]) are responsible for pulsatile GnRH secretion and mediate the negative feedback of estrogen[30]. The pulses are generated as a result of the interplay between NKB (and supposedly other tachykinins) and the endogenous opioid DYN, the former stimulating KP secretion via neurokinin 3 receptors (NK3R) and the latter acting as a "brake" via kappa opioid receptors (KOR)[31]. Recent studies have proposed that KNDy neurons function as an integrative hub of various modifiers of the reproductive axis, including metabolic signals, olfactory clues, and

circadian rhythm[32]. The anorexigenic POMC/CART neurons and the orexigenic NPY/AgRP neurons in the hypothalamus are in physical contact with the KP neuron population and elicit a stimulatory or inhibitory effect on KP secretion, respectively[31,33].

- *Effects on metabolism, thermoregulation and cardiovascular functions*

Over the past few years, it has 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[34]. Tolson *et al.* have described in adult *Kiss1r* KO female mice a change in body composition with increased adiposity and lower lean body mass, resulting in higher body weight, elevated leptin levels and glucose intolerance[35,36]. In the background of these alterations a reduction in energy expenditure has been implicated, which couldn't be explained solely with the drop in circulating estrogen levels[35,37], since the same metabolic changes have been observed in *Kiss1r* KO ovariectomized female mice compared to ovariectomized wild type females[35]. Interestingly, *Kiss1r* KO has not increased food intake[36], although KP treatment has had an anorexigenic effect in multiple studies[38,39], possibly due to the modulation of hypothalamic NPY/AgRP and POMC/CART neurons[40].

KP and Kiss1R are both expressed in the adipose tissue[41], therefore KP might also directly modulate adipocyte function, but *in vitro* studies in 3T3-L1 cells and rat adipocytes have yielded conflicting results. KP-10 has inhibited proliferation, viability, and adipogenesis, and promoted lipolysis post-differentiation[42], whereas it has had the opposite effect on lipogenesis when applied during differentiation[43], therefore its effect might be time-dependent.

- *Modulation of nociception*

Several members of the RF-amide family have been implicated in the modulation of nociception, both under basal conditions and in chronic pain disorders. It has been postulated that RF amides are part of an anti-opioid system, consequently, they could antagonize the effects of morphine[44]. The effects of KP on nociception have also been investigated, albeit the literature on this topic is scarce. In 2008, the expression of Kiss1 and GPR54 was detected in the L4/5 dorsal root ganglia in rats, both of which were upregulated in response to the Complete Freund Adjuvant (CFA)-induced arthritis, suggesting a possible role of KP in chronic inflammatory pain[45]. Similarly, an upregulation of Kiss1 and/or Kiss1R has been observed in the trigeminal ganglia, trigeminal nucleus caudalis, and the peripheral blood mononuclear cells in CFA-induced orofacial inflammation in rats[46]. In a study by Spampinato *et al.*, intraplantar KP has lowered the pain threshold in the hot plate test in mice and both peripheral (intraplantar) and central (intrathecal) KP have induced hyperalgesia in the formalin test, which was

antagonized by p234 (a Kiss1R antagonist)[47]. 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[48].

- *Modulation of the hypothalamic-pituitary-adrenal (HPA) axis and anxiety*

Based on the expression of Kiss1 and Kiss1R in limbic and paralimbic structures, including the medial amygdala, thalamus, and hippocampus[49,50], several studies have investigated the behavioral and emotional effects of KP.

An interplay between kisspeptin and the 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[51]. Since that time, several studies have been conducted with controversial results.

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*, as intraperitoneally (ip.) administered KP-10 did not affect plasma corticosterone and adrenocorticotropic hormone (ACTH) levels in rats[52]. Likewise, kisspeptin administration did not affect anxiety in human subjects[53].

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[54].

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. The most pronounced anxiolytic effect was observed when kisspeptin signaling in GnRH neurons was selectively rescued in Kiss1r KO animals, suggesting a modulatory role of gonadal steroids. Interestingly, no significant effect of Kiss1r KO was detected on the behavioral parameters of the open field test[55].

In zebrafish, however, the central administration of kisspeptin has been associated with an anxiolytic tendency in the novel-tank diving test and a significantly reduced fear response to alarm substance[56].

In a recent study, a Cre-dependent, stimulatory DREADD (Designer Receptors Exclusively Activated by Designer Drugs) viral construct has been targeted to the Kiss1 neurons of the posterodorsal medial amygdala (MePD) in mice. Upon selective activation of

MePD Kiss1 neurons by clozapine-N-oxide, a significant increase in open-arm exploration has been observed in the elevated plus maze, suggesting an anxiolytic role of this neuron population[57].

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. Peripheral administration of KP has failed to influence the activity of the HPA axis in rats (0.13 μg/μL KP-54 ip.)[52] and the activity of the limbic system in human subjects (1 nmol/kg/h KP-54 iv. over 75 minutes)[53]. In contrast, central KP-13 (1 or 2 μg icv.) had a pronounced anxiogenic effect in rats[54]. It is likely that the doses applied by Rao *et al.*[52] and Comninos *et al.*[53] were too low to exert an anxiogenic effect. In their investigation into the effect of peripheral or central KP administration on the reproductive axis in rats, Thomson *et al.* have found that 1 nmol of icv. KP-10 was sufficient to significantly raise plasma luteinizing hormone (LH) concentration, but a 100-fold dose was required for the same effect in the case of ip. treatment[58]. Likewise, the selective activation of MePD Kiss1 neurons[57] points to the function of a distinct neuron population, whereas central kisspeptin treatment[54] 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, both in terms of anatomy and function[56,59], thus the results of studies on zebrafish should be interpreted with caution.

- *Effect on locomotor activity*

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[54]. 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[35].

It has been discovered that kisspeptin attenuates morphine effect[48], and is expressed in the NAc[60], pointing to its possible involvement in the regulation of mesocorticolimbic dopaminergic activity. Interestingly, the centers of reward and addiction have also been implicated in the regulation of locomotion. First, quinpirole (a D2 receptor agonist) injected into the NAc has suppressed exploratory locomotion in rats[61], whereas bicuculline (a $GABA_A$ receptor antagonist) administration into the nucleus induced hyperactivity with prolonged exploratory behavior in rats[62]. Second, the selective activation of dopaminergic neurons in the VTA by DREADD has induced a pronounced and sustained hyperactivity in rats, which effect

could be reproduced by selectively activating the dopaminergic pathway between the VTA and NAc[63]. Thus, it is possible that kisspeptin might influence locomotion by modulating the activity of the VTA or NAc.

- *Other behavioral effects*

Regarding the regulation of mood, icv kisspeptin has had an antidepressant-like effect in the forced swim test in rats [64], and intravenous kisspeptin could also reduce negative mood in human subjects[53].

Kisspeptin not only orchestrates the HPG axis but has also been proven to be a key player in regulating sexual behavior and partner preference. Using retro- and anterograde tracers, reciprocal connections have been detected between the accessory olfactory bulb and the amygdalar kisspeptin neurons in rodents, moreover, the latter population also sends projections to the hypothalamic GnRH neurons[65]. In agreement with these findings, exposure to female urine has activated KP neurons in the MeA and induced an LH surge in male mice[66], suggesting that amygdalar KP neurons could indeed transmit olfactory signals to the reproductive axis.

Kisspeptin signaling also seems to modulate sexual behavior, since KP neurons in the rostral periventricular area of the 3rd ventricle (RP3V) were activated by male urinary odors and facilitated copulatory behavior (lordosis) in a NO-dependent manner[67,68]. In a study by Kauffman *et al.*, Kiss1R KO male and female mice have failed to display sexual behavior, but it could be rescued by testosterone, and estradiol with progesterone, respectively, suggesting that KP might influence copulatory behavior indirectly, via gonadal steroids[69]. The male Kiss1R KO mice have also lost the olfactory partner preference for estrous females, but it was not reinstated by testosterone. underlining the essential role of kisspeptin signaling in the olfactory control of reproduction[69].

1.3. The neurobiology of anxiety

1.3.1. The definition of anxiety

Fear and anxiety are defensive behavioral responses observed in many animal species, which help the organism avoid harmful stimuli and survive in a potentially dangerous environment. The key difference is that fear is evoked by actual fearful sensory stimuli, whereas anxiety is triggered by potential, anticipated threats[70]. Although both fear and anxiety can be adaptive, they are considered pathological when they are disproportionate to the threat, and so severe and permanent that they interfere with normal functioning. According to epidemiological studies, anxiety disorders are the most common mental disorders, peaking in adolescence and young adulthood with an estimated cumulative prevalence of 20-30%[71,72]. Anxiety disorders

include separation anxiety, selective mutism, specific phobias, social anxiety disorder, generalized anxiety disorder, panic disorder, and agoraphobia, which are defined in the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) and the 11th edition of International Classification of Diseases (ICD-11)[73,74].

The heritability of anxiety disorders has been estimated to be 30-40%[75], and recent studies have uncovered several anxiety-associated candidate genes (e.g. *CRHR1* [corticotropin-releasing factor receptor 1], *COMT* [catechol-o-methyltransferase] and *SLC6A4* [serotonin transporter]) and single nucleotide polymorphisms (SNPs), but the reproducibility of these studies have been limited[76,77].

However, some common mechanisms have been identified in the background of anxiety disorders, including the dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, and neural circuitry involving limbic brain regions[76], which will be discussed in detail in the following sections.

1.3.2. The hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis is the major endocrine mediator of the stress response (Figure 2). In response to stressful stimuli, the parvocellular subdivision of the hypothalamic paraventricular nucleus (PVN) secretes corticotropin-releasing factor (CRF) and – to a lesser extent – arginine vasopressin (AVP), also known as vasopressin (VP) or antidiuretic hormone (ADH). The PVN consists of three major functional subdivisions[78]. Firstly, the parvocellular neurons in the dorsomedial and anterior PVN are the primary drivers of HPA axis activation, releasing CRF and AVP to the portal circulation, which trigger the secretion of adrenocorticotropic hormone (ACTH) in the anterior pituitary, which in turn stimulates the release of glucocorticoids in the adrenal cortex[79]. Secondly, the lateral, dorsal and ventromedial parvocellular neurons also secrete CRF and are in reciprocal connection with the brainstem nuclei, governing the autonomic response to stress[78,80]. Thirdly, the magnocellular neurons of

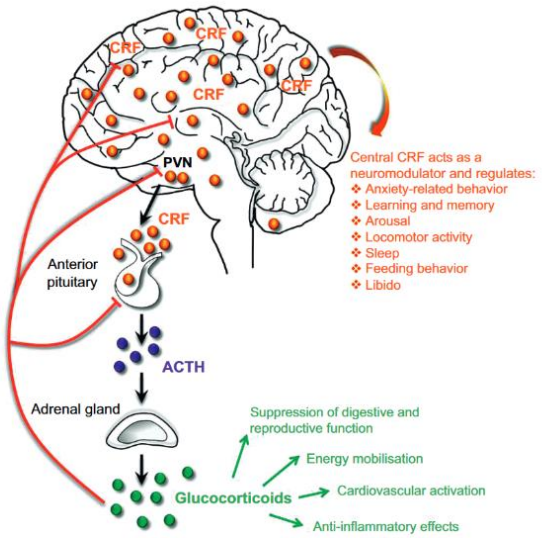

Figure 2 – Schematic figure of the HPA axis. ACTH: Adrenocorticotropic hormone; CRF: corticotropin-releasing factor, PVN: hypothalamic paraventricular nucleus. Dedic et al., 2017.

the anteromedial and dorsolateral PVN secrete vasopressin and oxytocin and project to the posterior pituitary[81].

CRF is a 41-amino-acid-long neuropeptide mostly expressed in the PVN and limbic brain regions (e.g. amygdala, BNST, locus coeruleus)[82], which exerts its effects on target cells via two types of GPCRs: type 1 and type 2 CRF receptors (CRFR1 and CRFR2, respectively)[83]. CRFR1 is abundantly expressed in the CNS (in the pituitary, cortex, cerebellum, hippocampus, amygdala, olfactory bulb, and medial septum), whereas CRFR2 expression is mostly limited to the hypothalamus and lateral septum in the CNS, but it is more widespread in peripheral tissues (e.g. cardiovascular, gastrointestinal system)[83]. The biological activity of CRF is further modulated by a non-receptor CRF-binding protein (CRF-BP)[83].

The CRF family of neuropeptides also includes urocortin 1, 2, and 3 (Ucn1, Ucn2, Ucn3), of which Ucn1 binds to both CRFR1 and CRFR2 with equal affinities, while Ucn2 and Ucn3 are considered quite selective for CRFR2[84].

The majority of the nonapeptide AVP is secreted from the magnocellular neurons of the PVN and mediates vasoconstriction and water reabsorption as a neurohypophyseal hormone. On the other hand, parvocellular VP is co-released with CRH into the pituitary portal circulation in response to stress and potentiates the effect of CRH[79]. Three different G-protein coupled VP receptors have been identified: V1a, V1b, and V2. V1a receptors are expressed on various peripheral tissues (e.g. vascular smooth muscle, hepatocytes, myometrium, thrombocytes)[85], but are also found centrally, especially in the hippocampus, cerebellum, and olfactory nucleus[86], contributing to the effects of vasopressin on behavior, circadian rhythm, and thermoregulation, among others[85]. V1b receptors are primarily expressed in pituitary corticotropes, therefore they are responsible for the modulation of ACTH release. V2 receptors are mostly localized to the renal collecting duct and loop of Henle, inducing water reabsorption[85].

At the level of the anterior pituitary, CRF acts as a potent stimulator of ACTH secretion, partially via increasing the transcription of its precursor, pro-opiomelanocortin (POMC), which also gives rise to β-lipotropin and β -endorphin in the pituitary corticotroph[87]. CRFR1 is required for ACTH secretion, as evidenced by studies showing that the genetic ablation of these receptors prevents the ACTH response in stress[83]. VP, on the other hand, is only a weak stimulator of ACTH secretion on its own, but potentiates the effect of CRH on ACTH release via V1b receptors and also has a trophic effect on the pituitary, similarly to CRH[79,88].

The end target of HPA axis activation is the adrenal cortex, which is composed of three zones: the zona glomerulosa, fasciculata, and reticularis[89]. The intermediate layer, zona fasciculata is responsible for the secretion of glucocorticoids[89].

In acute stress, the rapid activation of CRH neurons is accompanied by an increase in VP production which is delayed by 60-120 minutes, according to stress models[90]. In chronic stress, the characteristics of the CRH and ACTH response depend on the stressor, i.e. certain repeated stimuli (e.g. restraint, cold exposure) lead to a desensitization of ACTH response, whereas others (e.g. foot shock, hypertonic saline injection) trigger a sustained response. VP expression, however, is increased in all chronic stress paradigms, suggesting that VP might be a major regulator of HPA axis activation in chronic stress[79].

1.3.3. The pathomechanism of anxiety beyond the HPA axis

Anxiety develops as a result of complex, circuit-level interactions among key brain regions involved in emotional processing, such as the amygdala, the bed nucleus of the stria terminalis (BNST), the ventral hippocampus (vHPC), and the prefrontal cortex (PFC)[91].

The basolateral amygdala (BLA) is the main input nucleus of the amygdala, receiving sensory input from the thalamus and sensory cortices. These stimuli are assigned a negative or positive valence, i.e. the cues are deemed either threatening or rewarding, and then fear or reward pathways are recruited, respectively[92]. In response to anxiogenic stimuli, projections to the central amygdala (CeA) and BNST are activated[93].

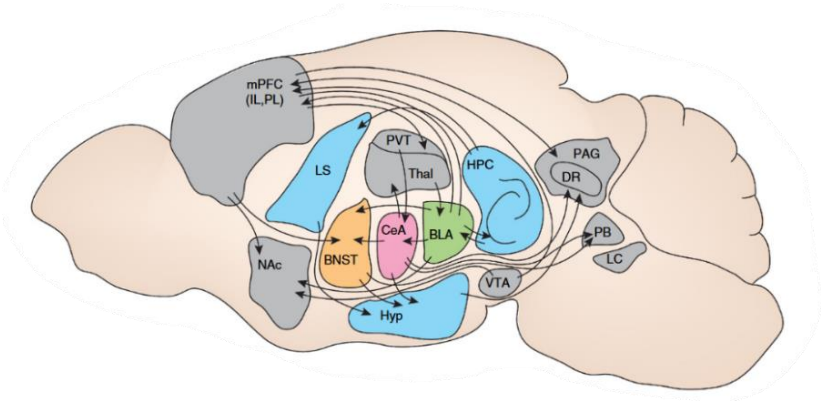

Figure 3 - Sagittal view of rodent brain regions involved in the neurocircuitry of anxiety. BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; CeA, central amygdala; DR, dorsal raphe nucleus; HPC, hippocampus; Hyp, hypothalamus; IL, infralimbic division of the mPFC; LC, locus coeruleus; LS, lateral septum; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; PAG, periaqueductal gray; PB, parabrachial nucleus; PL, prelimbic division of the mPFC; PVT, paraventricular thalamus; Thal, thalamus; VTA, ventral tegmental area. Calhoon & Tye, 2015

The CeA consists of lateral (CeL) and medial (CeM) subdivisions, the former receives input from the BLA. In the CeL, two mutually inhibitory subpopulations of GABAergic interneurons are responsible for the gating of fear responses: the inhibition of CeL_{OFF} cells by CeL_{ON} cells leads to the disinhibition of CeM and, consequently, to the recruitment of downstream fear pathways[94].

The BNST is a key region in sustained anxiety, activated by BLA afferents, as well as by inputs from the hippocampus and mPFC, among others[91]. Different subregions of the BNST modulate anxiety in different ways. For instance, the activation of the anterodorsal BNST is

anxiolytic *per se*, whereas the ventral and ventrolateral BNST are both associated with anxiety, partially via projections to the ventral tegmental area (VTA)[91].

The BLA also maintains reciprocal connections with the vHPC, thereby promoting anxiety-like behavior and fear learning. The activation of vHPC evokes anxiety-like behavior via connections to the lateral septum, and consequent activation of the hypothalamic paraventricular nucleus and the periaqueductal gray[95].

Finally, the mPFC plays an evaluative role in anxiety, determining the behavioral response to a threatening stimulus, largely influenced by previous experience (fear learning, habituation) and the presence of motivational signals[91,96]. The major circuits of anxiety are shown in Figure 3.

2. Aims of the Study

Previously we reported that icv. KP-13 treatment evoked HPA axis activation and anxiety-like behavior in rats[54]. 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[79,97]. 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[91,98], and because the expression of KP and KISS1R[7–9,13], as well as CRF and AVP were detected in both the amygdala and the hippocampus[83,99]. 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[100]. According to molecular docking studies, ASN4, SER5, GLY7, ARG9 and PHE10 of KP-10 are involved in the formation of hydrogen bonds with Kiss1r[101]. 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[79], whereas the increase in LH levels can be interpreted as an indirect sign of KISS1R activation[102]. 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[103,104], but it is also involved in the modulation of locomotor activity[62,63,105]. 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. Experimental design

This thesis encompasses two sets of rodent experiments, involving KP-13 and KP-8, respectively.

The experimental design of our studies involving KP-13 can be seen in Figure 4. Male Wistar rats were implanted with an icv cannula, through which they were treated with KP-13 and/or antagonists. Firstly, the effect of KP-13 on the gene expression of *Crf*, *Crf1r*, *Crf2r*, *Avp*, *Avpr1a*, and *Avpr1b* was determined in the amygdala and hippocampus, compared to *Gapdh* expression. Moreover, the expression of AVP and CRF proteins was determined in the amygdala and hippocampus. The contribution of AVP and CRF to KP-13-induced HPA axis activation was determined by pre-treatment with either α-helical CRF(9-41) or a V1R antagonist before the icv treatment with KP-13. Likewise, a computerized open field test was carried out after icv treatment with various doses of KP-13, which could be preceded by a pre-treatment with one of the above-mentioned antagonists.

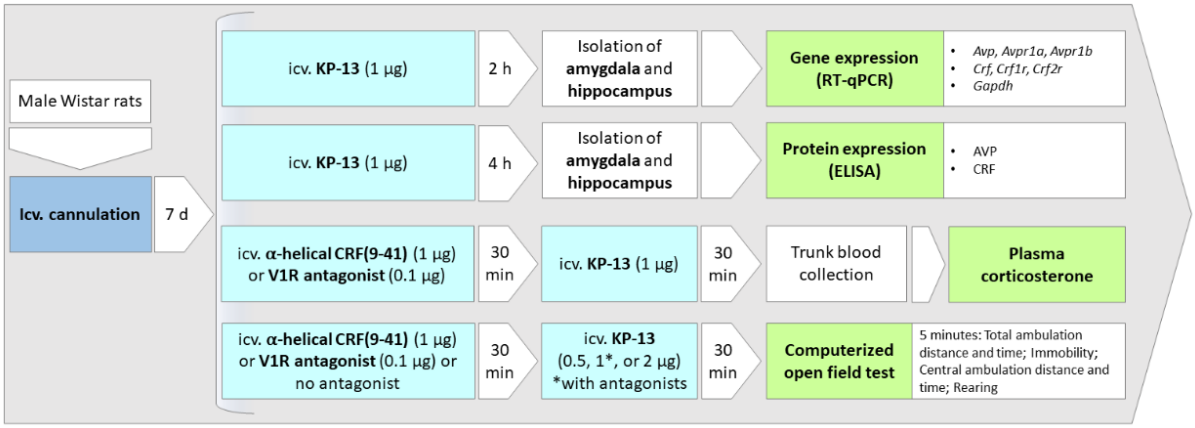

Figure 4 - Experimental design of our studies with KP-13.

Figure 5 illustrates the experimental design of our studies with KP-8. Male Wistar rats underwent intracerebroventricular cannulation then they were treated via the cannula with different doses of KP-8. Anxiety-like behavior and locomotor activity were analyzed using the elevated plus maze, computerized open field, and marble burying tests. Trunk blood was collected for LH and corticosterone measurement. For ex vivo superfusion, the rats were not cannulated. Following decapitation, their brains were dissected and fractional GABA and dopamine release in response to KP-8 were quantified in the nucleus accumbens and ventral tegmental area. The experimental methods are detailed in the following sections.

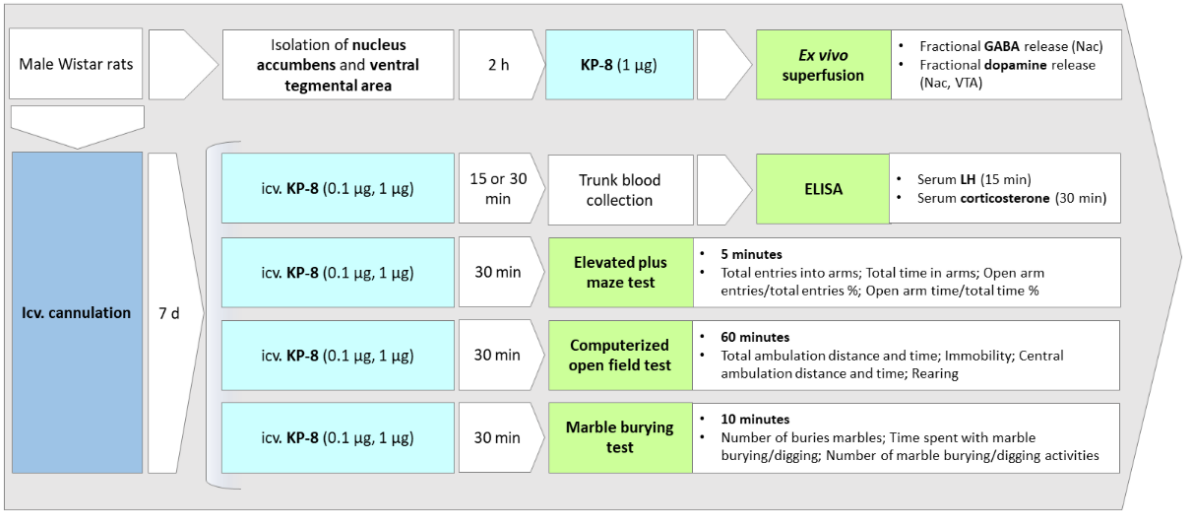

Figure 5 - Experimental design of our studies with KP-8.

3.2. 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.

3.3. 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 for icv. administration. The cannula was inserted 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[54]. 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. After the behavioral tests, each rat was sacrificed, after which methylene blue was injected via the implanted cannula, and the brains were then dissected. Only data from

animals exhibiting the diffusion of methylene blue in all the ventricles were included in the statistical evaluation.

3.4. 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). The resin was swollen in dichloromethane (DCM). The Fmoc group was removed by treating the peptide-resin with 20% piperidine/N,N-dimethylformamide (DMF) solution twice (5+15 min). Solvents were purchased from VWR (Radnor, PA, USA). The amino acids were activated with N,N′-dicyclohexylcarbodiimide, and 1-hydroxybenzotriazole in 50% DCM/DMF. The peptide-resin was incubated with this mixture for 3 h. The resin was washed with DMF (3×) and DCM (3×) after the deprotection and coupling steps. The assembled peptides were cleaved from the resin by treating it with the following cleavage cocktail for 3 h: 90% trifluoroacetic acid (TFA) (Pierce, Rockford, IL, USA), 5% water, 2% dithiotreitol, 2% triisopropylsilane. The peptides were precipitated with diethyl ether, dissolved in a mixture of acetonitrile (ACN) and water, and lyophilized. The crude peptides were analyzed by HPLC (Hewlett-Packard Agilent 1100 system, column: Luna, c18 (2), 250×4.6 mm, 5µm, 100 Å, Phenomenex, Aschaffenburg, Germany) and ESI-MS. The peptides were purified on a preparative HPLC column (Phenomenex Luna, c18 (2), 250×21.2 mm, 10µm, 100 Å) using a Shimadzu 20-LC system. The fractions were analyzed on the above-mentioned analytical HPLC system and measured by electrospray ionization mass spectrometry (ESI-MS) (Figures 6 and 7). The pure fractions were pooled and freeze-dried.

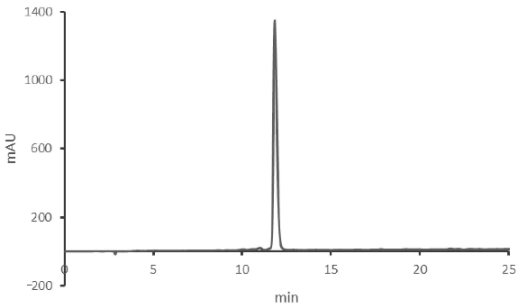

Figure 6 - HPLC trace of Kisspeptin-8. Column: Phenomenex Luna C18, 5 µ, 100 Å, 4.6 mm ×250 mm, flow rate: 1 mL/min, wavelength: 220 nm, A eluent: 0.1% TFA in water, B eluent: 0.1% TFA/80% ACN/water, gradient: 30–55% eluent B in eluent A over 25 min.

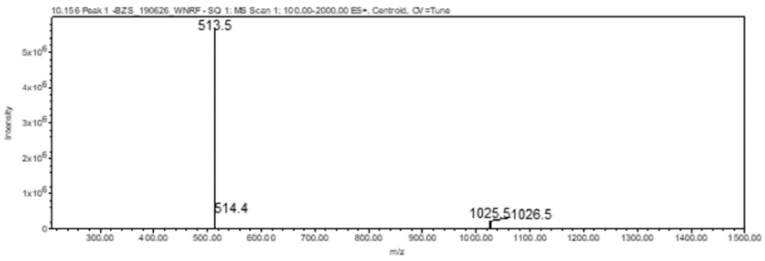

Figure 7 - The ESI-MS trace of KP-8 peptide

3.5. 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[54] 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[106–108]. 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.

3.6. Behavioral tests

3.6.1. Elevated plus maze test

The EPM apparatus is a plus-shaped platform 50 cm above the ground (Figure 8). The maze consists of four arms (50 cm×10 cm each): two opposing open arms and two closed arms enclosed by a 10 cm high wall. The test is based on two conflicting motivations of rodents: to avoid open, brightly lit spaces and to explore novel environments. The avoidance of open arms reflects anxiety-like behavior[6]. 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. The experiments were conducted between 8 a.m. and 10 a.m. and the apparatus was cleaned with 96% ethyl-alcohol after each session.

3.6.2. Computerized open field test

The novelty-induced locomotor activity of rats was assessed using the Conducta 1.0 System (Experimetria Ltd., Budapest, Hungary). The system consists of black plastic OF arenas (inside dimensions: 48×48 cm, height: 40 cm) with 5 horizontal rows of infrared diodes on the walls to register both horizontal and vertical locomotion. The center of each box is illuminated by a LED lightbulb (230 lumens) from above the box. The central zone of the arena is defined as a 24×24 cm area in the center of the box (Figure 8). 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. The OF experiments were conducted between 8 a.m. and 10 a.m. and the apparatus was cleaned with 96% ethyl-alcohol after each session.

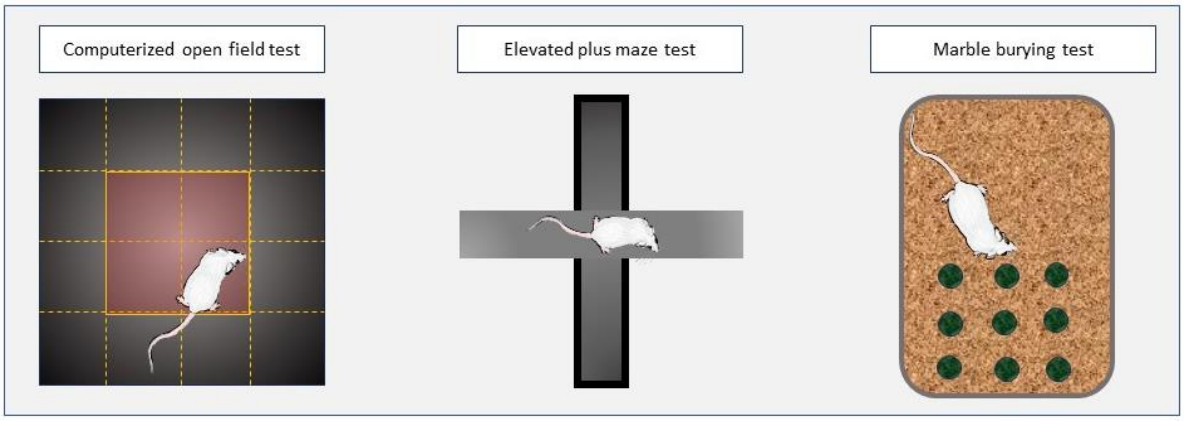

Figure 8 - Schematic figure showing the behavioral tests (computerized open field, elevated plus maze, marble burying) performed in our study.

3.6.3. Marble burying test

MB is a regularly used paradigm for the assessment of anxiety-like and compulsive-like behavior[7]. Our protocol was based on the method described by Schneider and Popik[109]. The animals were removed from their plexiglass home cages (420×275×180 mm) and temporarily moved into another cage before the experiment. Meanwhile, 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 to the thick bedding. Then 9 glass marbles of 2.5 cm diameter were arranged in 3 rows along the shorter wall of the cage (Figure 8). 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% marble covered by bedding material) was counted. The marbles were cleaned with 96% ethyl alcohol after each session. After the experiment, the video recording was evaluated. The count and duration of two types of goal-oriented interactions with marbles (burying of marbles and moving marbles without burying them) were assessed.

3.7. ELISA

For the measurement of serum corticosterone and protein concentration, the animals were decapitated 30 min after icv. treatment with KP-8 or saline. For the assessment of serum LH, decapitation was performed 15 min after icv. treatment. 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 until the assays were performed.

For the measurement of CRF and AVP content in the amygdala and hippocampus, animals were decapitated 4 h after icv. treatment. The amygdala and hippocampus were dissected with the help of a pre-cooled adult rat brain matrix (Ted Pella Inc., Redding, CA, USA), and were manually sliced with razor blades into coronal sections (1 mm slots), after which the brain regions were dissected on ice with the guidance of a rat brain atlas[110]. 1 mm in diameter tissue punches (Ted Pella Inc., Redding, CA, USA) were taken from the amygdala and hippocampus, then placed in Eppendorf tubes and snap frozen in liquid nitrogen. The samples were 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 a competitive CRF and AVP ELISA kit (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA; EK-019-06, EK-065-07), according to the manufacturer's instructions. 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 NanoDropOneC microvolume spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

3.8. *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[111]. The VTA was isolated as described by Salvatore *et al.*[112]. 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:

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

3.9. 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), after of which the brain regions were dissected on ice with the guidance of a rat brain atlas [110]. 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. To purify and isolate RNA from the samples obtained from the amygdala and hippocampus, 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; primer sets are listed in Table 2, respectively. The cycling protocol is listed in Table 3. 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.

Genes	**Forward (5' → 3')**	**Reverse (5' → 3')**
Avp	CTG ACA TGG AGC TGA GAC AGT	CGC AGC TCT CGT CGC T
Avpr1a	TGG ACC GAT TCA GAA AAC CCT	GTT GGG CTC CGG TTG TTA GA
Avpr1b	CAG CAT AGG AGC CAA CCA TCA A	GAA AGC CCA GCT AAG CCG T
Crf	TGG TGT GGA GAA ACT CAG AGC	CAT GTT AGG GGC GCT CTC TTC
Crfr1	CGA AGA GAA GAA GAG CAA AGT ACA C	GCG TAG GAT GAA AGC CGA GA
Crfr2	CCC GAA GGT CCC TAC TCC TA	CTG CTT GTC ATC CAA AAT GGG T
Gapdh	CGG CCA AAT CTG AGG CAA GA	TTT TGT GAT GCG TGT GTA GCG

Table 2 - Custom primers

Steps	Temperature °C	Time	Number of cycles
UDG pretreatment	50	2 min	1
Initial denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing	60	30 s	
Extension	72	30 s	

Table 3 – qPCR cycling protocol

3.10. Statistical analysis

Data are presented as means ± SEM. The prerequisites of ANOVA were assessed via histograms, skewness, and kurtosis, the Kolmogorov–Smirnov and Levene's tests. 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.

For KP-13, statistical analysis of the PCR results was performed by Mann–Whitney's test. For all other data with KP-13, estimated marginal means were calculated and analyzed by analysis of variance (ANOVA). For the effect of different doses of KP-13 on open-field test parameters, one-way ANOVA was employed, followed by the Bonferroni post hoc test for multiple comparisons when the test prerequisites were fulfilled. When the test of the homogeneity of variances was not satisfied, nonparametric ANOVA on ranks (Kruskal–Wallis) was performed, followed by Dunn's test for multiple comparisons. For the evaluation of ELISA results and all tests with combined treatments two-way ANOVA was performed followed by the Bonferroni post hoc test for multiple comparisons.

For KP-8, one-way ANOVA with Holm-Sidak's post-hoc test was applied for the analysis of EPM results. One-way ANOVA with Dunnett's post-hoc test was used for the analysis of cumulative OF results, as well as for the evaluation of serum corticosterone, LH, and total protein measurements. Two-way RM-ANOVA with Holm–Sidak's post-hoc test was performed for the evaluation of 5-min intervals in the OF test as well as for the interpretation of dopamine and GABA release from the NAc. Mixed-effects analysis with Holm–Sidak's multiple comparison test was performed for the evaluation of fractional dopamine release from the VTA. Kruskal–Wallis test with Dunn's post-hoc test was performed for the analysis of MB results. Curve fitting for ELISA tests was performed according to the manufacturers' instructions.

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* ($Mdn_{control}$=1, Mdn_{KP-13}=3.646, U=8, p=0.0057) and *Avpr1b* ($Mdn_{control}$=1, Mdn_{KP-13}=1.359, U=20, p=0.0135) significantly increased, whereas the expression of *Crf* ($Mdn_{control}$=1, Mdn_{KP-13}=0.6726, U=0, p=0.0002) was reduced, compared to the control group. In case of *Avpr1a* ($Mdn_{control}$=1, Mdn_{KP-13}=0.9272, U=36, p=0.709), *Crfr1* ($Mdn_{control}$=1, Mdn_{KP-13}=0.8754, U=32, p=0.9999) and *Crfr2* ($Mdn_{control}$=1, Mdn_{KP-13}=1.244, U=36, p=0.709) no significant difference was detected (Figure 9).

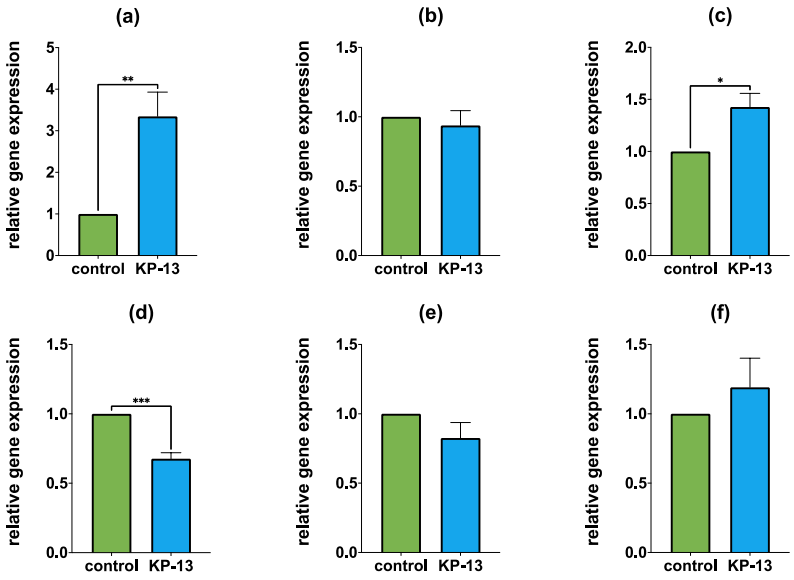

*Figure 9 – Relative gene expression in the amygdala: (a) Avp; (b) Avpr1a; (c) Avpr1b; (d) Crf; (e) Crfr1; (f) Crfr2; mean+SEM, n=8-9; *p<0.05, **p<0.01; ***p<0.001*

In the hippocampus, the relative gene expression of *Crf* ($Mdn_{control}$=1, Mdn_{KP-13}=1.504, U=6, p=0.0476) was significantly higher in the KP-13-treated group. On the other hand, *Avpr1a* ($Mdn_{control}$=1, Mdn_{KP-13}=0.7788, U=0, p=0.0002) mRNA expression showed a marked decrease. In case of *Avpr1b* ($Mdn_{control}$=1, Mdn_{KP-13}=1.644, U=5, p=0.127), *Avp* ($Mdn_{control}$=1, Mdn_{KP-13}=0.7133, U=12, p=0.3636), *Crfr1* ($Mdn_{control}$=1, Mdn_{KP-13}=0.8939, U=21, p=0.69) and

Crfr2 ($Mdn_{control}$=1, Mdn_{KP-13}=0.6008, U=21, p=0.69), no significant difference was detected (Figure 10).

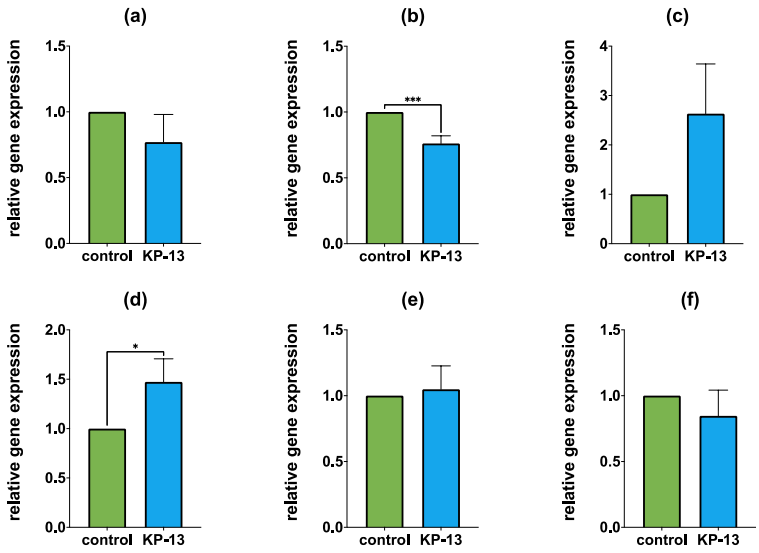

*Figure 10 – Relative gene expression in the hippocampus: (a) Avp; (b) Avpr1a; (c) Avpr1b; (d) Crf; (e) Crfr1; (f) Crfr2; mean+SEM, n=5-8; *p<0.05, ***p<0.001*

4.1.2. Protein expression

A two-factor analysis of variance on AVP protein level revealed a significant main effect for the treatment factor [F(1, 18)=13.416, p=0.002], region factor [F(1, 18)=22.869, p < 0.001]. There was no significant interaction between the two factors [F(1,18)=1.432, p=0.250), 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 (p=0.002), however, it had no effect in the hippocampus (p=0.125) (Figure 11).

A two-way ANOVA on CRF protein content showed a significant main effect for the region factor [F(1,17)=13.235, p=0.003], however no significant main effect for the treatment factor [F(1,17)=0.018, p=0.896]. KP-13 treatment did not affect CRF protein content (Figure 11).

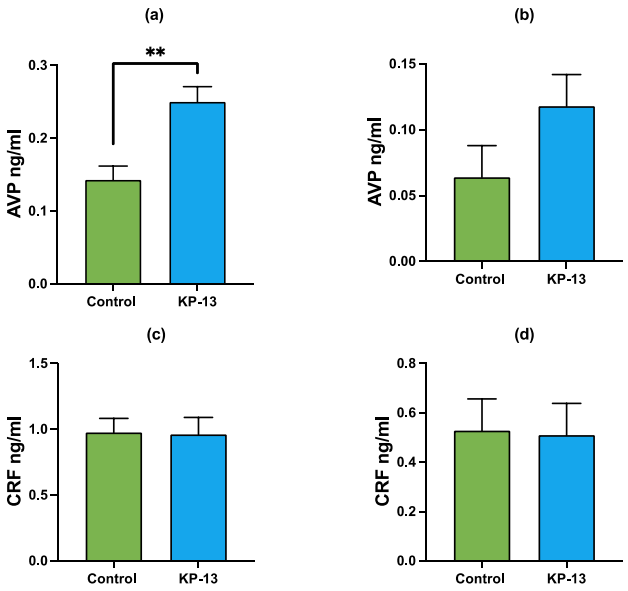

*Figure 11 – AVP and CRF protein expression in the amygdala and hippocampus: (a) AVP in the amygdala; (b) AVP in the hippocampus; (c) CRF in the amygdala; (d) CRF in the hippocampus; mean+SEM, n=4-6; **p<0.01*

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 [$F(1, 54)=19.997$; $p < 0.001$] and a statistically significant interaction between the two factors [$F(2, 54)=9.058$; $p<0.001$], thus, the effect of KP-13 depends on which antagonist pretreatment was applied. There was no significant main effect for the antagonist treatment factor [$F(2, 54)=2.752$; $p=0.074$]. Pairwise comparisons revealed that KP-13 treatment caused a marked elevation in the corticosterone concentration ($p < 0.001$) compared to the saline-treated group. Furthermore, among the KP-13 treated animals both CRFR antagonist ($p=0.025$) and V1R antagonist ($p<0.001$) pretreated were significantly different (Figure 12).

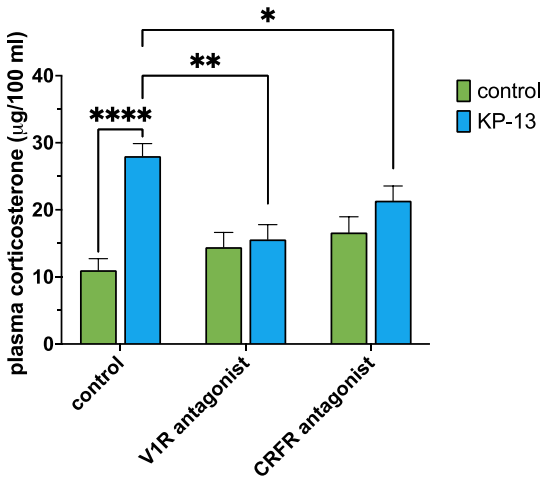

*Figure 12 – Plasma corticosterone results: mean +SEM, n=7-13; *p<0.05, **p<0.01, ***p<0.001*

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 open field 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 [$F(3,39)=0.888$; $p=0.457$] and total ambulation time [$F(3,39)=1.611$; $p=0.204$]. In the case of immobility time [$F(3, 39)=2.831$; $p=0.052$] 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 (Tukey HSD revealed $p=0.048$). KP-13 had a significant effect on rearing activity [$F(3, 39)=4.368$; $p=0.01$]. Again, the 2 µg dose of KP-13 was the most effective ($p=0.007$). 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 [Kruskal-Wallis $H(3)=15.831$; $p=0.001$].

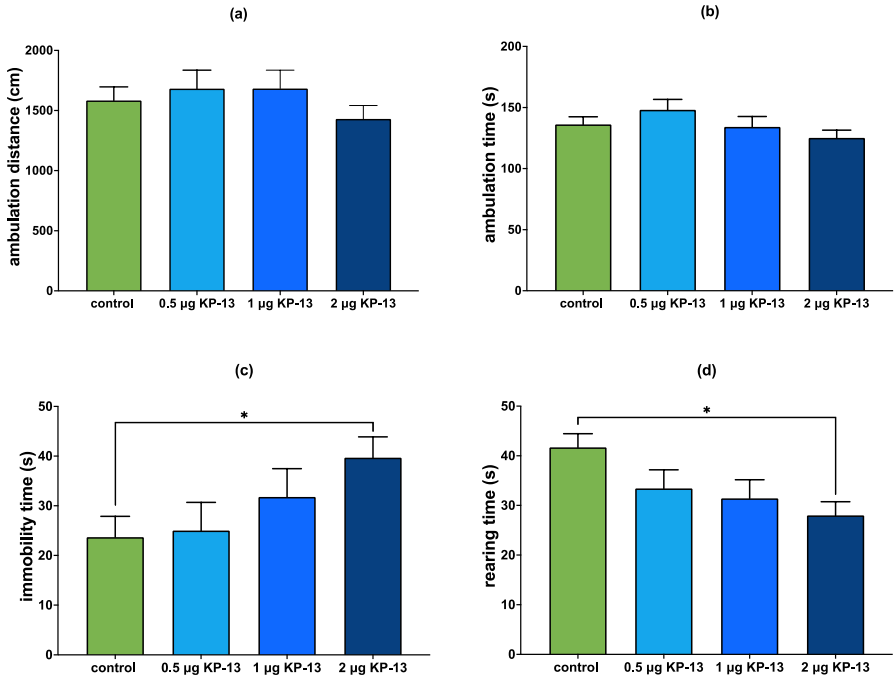

*Figure 13 – Ambulation in the open field test: (a) total distance traveled; (b) total time of ambulation; (c) immobility time; (d) number or rearings; mean+SEM, n=4-6; *p<0.05*

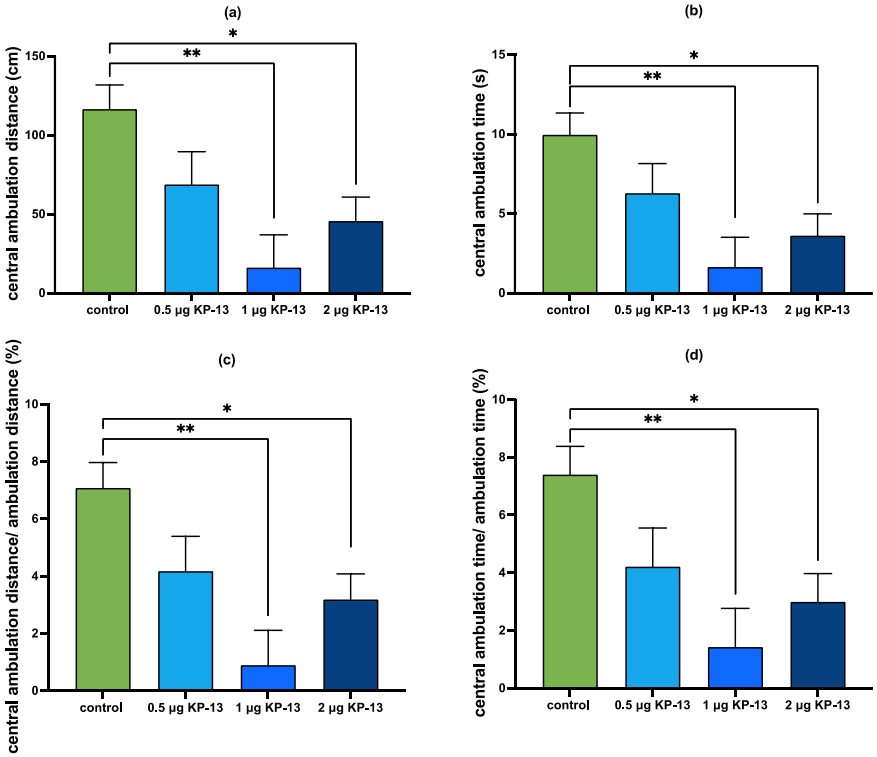

*Figure 14 – Central ambulation in the open field test: (a) distance traveled in the center of the arena; (b) time spent in the center of the arena; (c) central ambulation distance/total ambulation distance %; (d central ambulation time/total ambulation time %; mean+SEM, n=4-6; *p<0.05, **p<0.01*

Pairwise comparisons with Bonferroni correction revealed that both 1 µg (p=0.001) and 2 µg (p=0.036) of KP-13 significantly decreased the distance traveled in the center of the open field arena. KP-13 had a significant effect on central ambulation time [F(3,39)=5.6; p=0.003].

Pairwise comparisons revealed that both 1 μg (p=0.006) and 2 μg (p=0.014) 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% [F(3,39)=6.367; p=0.001]. Pairwise comparisons showed that both 1 μg (p=0.001) and 2 μg (p=0.023) of KP-13 were significant compared to control. In the case of the central ambulation time/total ambulation time%, the result was similar [F(3,39)=5.439; p=0.003]. Again, both the 1 μg dose (p=0.006), as well as the 2 μg dose (p=0.019) of KP-13 were found to be significant (Figure 13 and 14).

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

Two-way ANOVAs were conducted to investigate the effect of KP-13 treatment in the presence of CRFR and V1R antagonist pretreatment on open-field parameters. There were no significant changes in ambulation distance [KP-13 treatment: F(1,59)=0.688; p=0.410, antagonist treatment: F(2,59)=0.360; p=0.699, interaction: F(2,59)=0.354; p=0.704], and ambulation time [KP-13 treatment: F(1,59)=2.725; p=0.105, antagonist treatment: F(2,59)=0.490; p=0.615, interaction: F(2,59)=0.598; p=0.553].

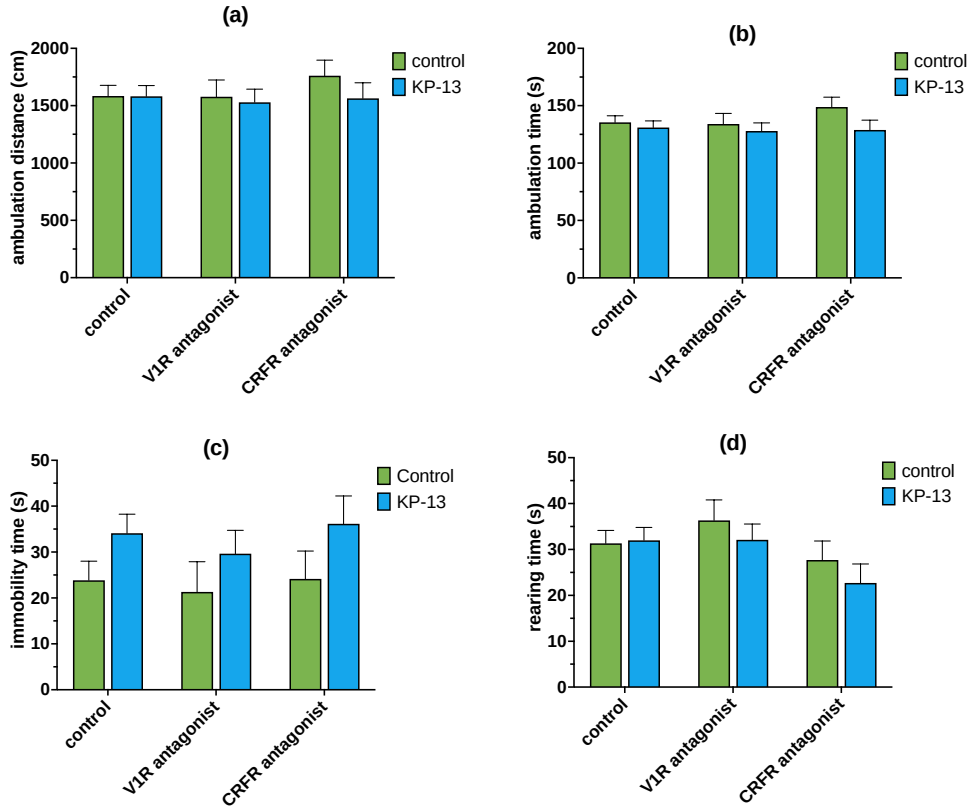

*Figure 15 – Ambulation in the open field test in the presence of V1R and CRFR antagonists in the open field test:: (a) total distance traveled; (b) total time of ambulation; (c) immobility time; (d) number or rearings; mean+SEM, n=6-15; *p<0.05*

In the case of immobility time, there was a significant main effect for the treatment factor (F(1,59)=5.272; p=0.026), but no significant difference for antagonist treatment or between the two factors [antagonist treatment: F(2,59)=0.348; p=0.708, interaction: F(2,59)=0.048; p=0.953]. Pairwise comparison showed no significant difference between groups. There were no significant changes in the case of rearing activity [KP-13 treatment: F(1,59)=0.897; p=0.348, antagonist treatment: F(2,59)=2.660; p=0.079, interaction: F(2,59)=0.434; p=0.560] (Figure 15).

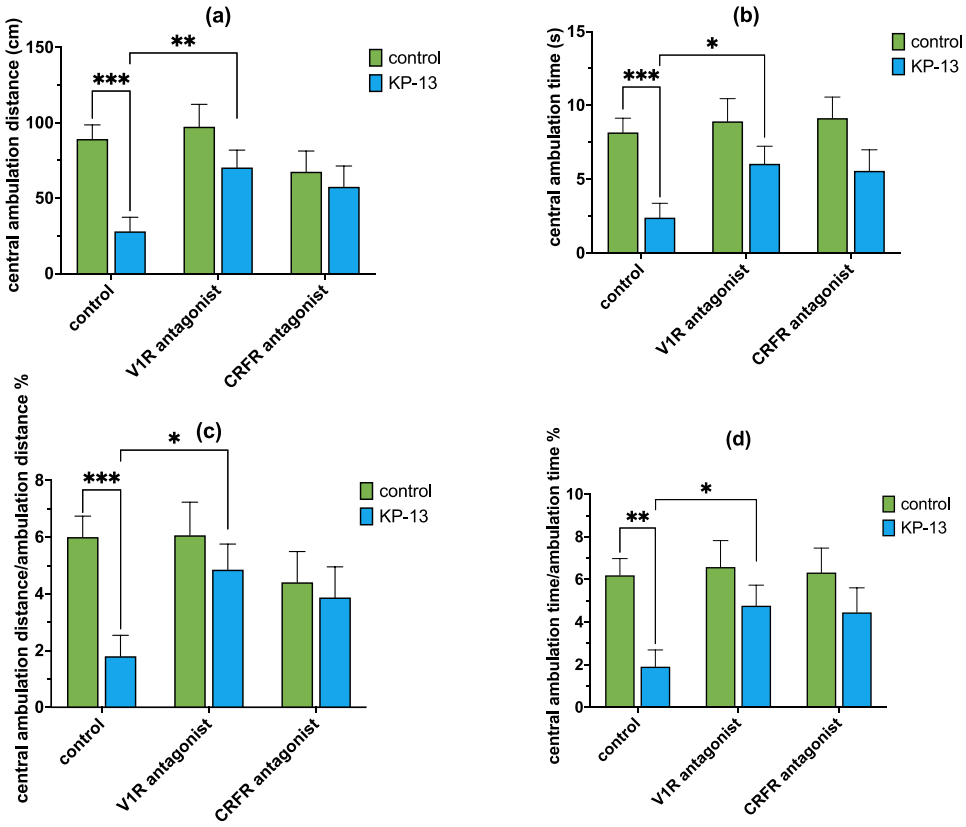

*Figure 16 – Central ambulation in the presence of V1R and CRFR antagonists in the open field test: (a) distance traveled in the center of the arena; (b) time spent in the center of the arena; (c) central ambulation distance/total ambulation distance %; (d) central ambulation time/total ambulation time %; mean+SEM, n=6-15; *p<0.05*

However, in the case of central ambulation distance, our result showed a statistically significant main effect for the KP-13 treatment [F(1, 59)=10.665; p =0.002], but not for the antagonist treatment factor [F(2, 59)=2.501; p=0.091] or the interaction between the two factors [F(2, 59)=2.724; p=0.075]. Pairwise comparisons revealed that KP-13 treatment caused a marked decrease in the central ambulation distance 30 min after treatment (p<0.001) compared to the saline-treated group. Among the KP-13 treated animals, CRFR antagonist (p=0.08) pretreatment wasn't significantly different, however, V1R antagonist (p=0.006) pretreatment showed a statistically significant difference (Figure 16). In the case of central ambulation time,

our results showed a statistically significant main effect for the KP-13 treatment [$F(1,59)=15.355$; $p<0.001$] similar to central ambulation distance, but no statistically significant main effect for the antagonist treatment factor [$F(2, 59)=2.389$; $p=0.101$] or the interaction between the two factors [$F(2,59)=0.885$; $p=0.418$].

Pairwise comparisons revealed that the KP-13 treatment caused a significant decrease in the central ambulation time ($p<0.001$). Among the KP-13 treated animals, V1R antagonist ($p=0.021$) pretreatment showed a statistically significant difference, however, CRFR antagonist ($p=0.071$) pretreatment wasn't significant. In the case of central ambulation distance/total ambulation distance%, the main effect for KP-13 treatment was found significant [$F(1,59)=6.339$; $p=0.015$], however, no significant main effect was detected for the antagonist treatment [$F(2,59)=1.540$; $p=0.224$] and the interaction between the two factors [$F(2,59)=2.531$; $p=0.089$]. Pairwise comparison showed that KP-13 injection evoked a marked decrease in the central ambulation distance/total ambulation distance% ($p<0.001$) and among the KP-13 treated groups, the V1R antagonist pretreatment was statistically significant ($p=0.011$), 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 ($p=0.119$). 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 [$F(1, 59) = 10.019$; $p = 0.003$] and no effect was found for the antagonist treatment [$F(2,59)=1.797$; $p=0.175$] or the interaction [$F(2,59)=1.200$; $p=0.309$]. Pairwise comparison revealed that KP-13 caused a significant decrease in central ambulation time/total ambulation time% ($p=0.003$). Furthermore, the V1R antagonist significantly decreased the KP-13-evoked fall in central ambulation time/total ambulation time% ($p=0.025$), whereas CRFR antagonist treatment among the KP-13 treated animals caused no statistically significant difference ($p=0.072$) (Figure 16).

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 plus maze [Figure 17(a), F(2,20)=9.196, p=0.0007], as well as the percentage of time spent in the open arms of the maze [Figure 17(b), F(2,20)=4.431, p=0.0202]. A decrease in the total number of entries into the arms was induced by both 0.1 µg and 1 µg of KP-8 [Figure 17(c), F(2,20)=5.927, p=0.0153]. There was no significant difference among the groups in the total time spent in the arms [Figure 17(d), F(2,20)=1.932, p=0.1710].

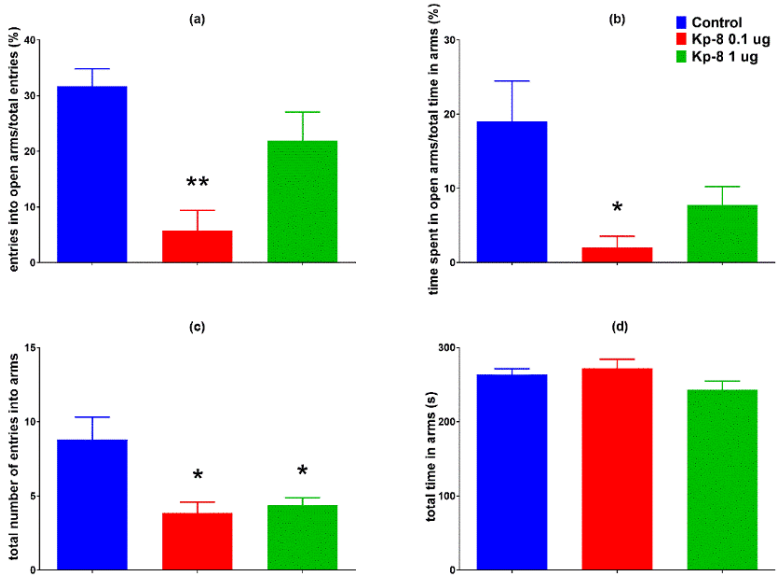

*Figure 17 – Elevated plus maze results: (a) percentage of entries into open arms, (b) percentage of time spent in the open arms, (c) total number of entries into arms, (d) total time spent in the arms of the maze, n=7-9, *p<0.05 vs control, **p<0.01 vs control.*

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 [Ambulation distance: F(2,34)=1.691; p=0.1994; ambulation time: F(2,34)=1.728; p=0.1928; immobility: F(2,34)=1.731; p=0.1919; rearing: F(2,34)=1.522; p=0.2328; central distance: F(2,34)=0.2424; p=0.7861; central time: F(2,34)=0.3141; p=0.7325] (see Figure 18).

However, significant differences were found following the analysis of each 5-minute interval. As seen in Figure 19(a), the two-factor RM-ANOVA on the distance traveled in the arena revealed a significant main effect for the time factor [F(5.389, 183.2)=113.8, p<0.0001]. 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 (p=0.0334 and p=0.0410, respectively).

Regarding total ambulation time, there was a significant main effect for the time factor [F(6.138, 208.7)=98.03, p<0.0001) with a similar pattern of steep then mild decrease [Figure

19(b)]. The 1 µg KP-8 group spent less time with ambulation than the control group at 50-55 minutes (p= 0.0090) and 55-60 minutes (p= 0.0326), as well.

The two-way ANOVA on immobility yielded a significant main effect for the time factor [F(5.396,183.5)=34.51, p<0.0001] and interaction [F(22, 374)=2.249, p=0.0012]. The time spent immobile was increasing during the experiment, showing a tendency reciprocal to that of ambulation time and distance [Figure 20(a)]. Compared to control, the 1 µg dose of KP-8 significantly increased immobility at 50-55 and 55-60 minutes (p= 0.0202 and p= 0.0186, respectively).

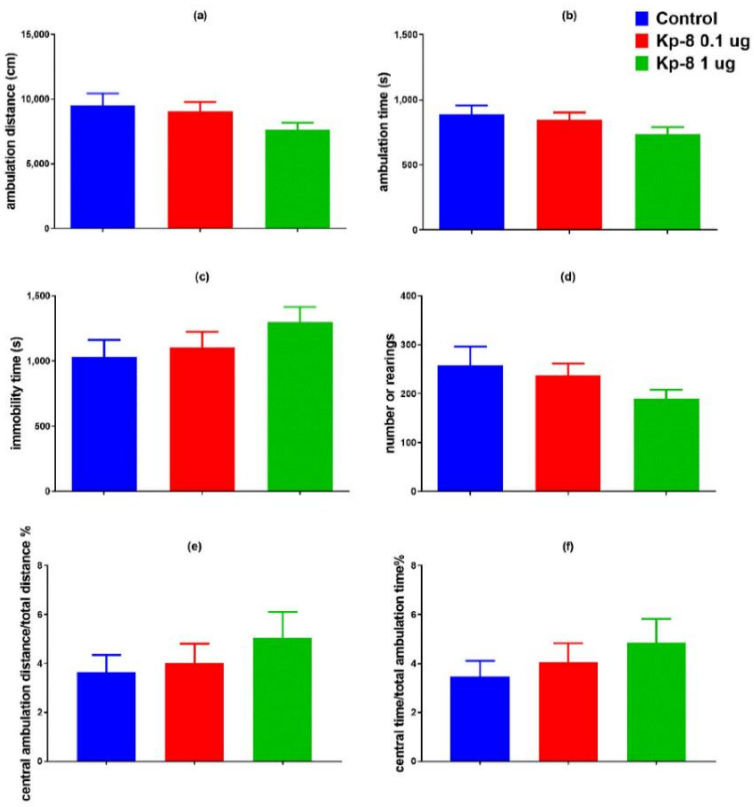

Figure 18 - Cumulative data of 60-min open field test: (a) total distance of ambulation, (b) total time of ambulation, (c) total time spent immobile, (d) number of rearings, (e) percentage of distance travelled in the central zone, (f) percentage of time spent in the center

Considering the number of rearing sessions, a significant main effect for the time factor was detected [F(6.756, 229.7)=7.52, p<0.0001], along with a statistically significant interaction between time and treatment [F(22, 374) = 3.095, p<0.0001]. As seen in Figure 20(b), a pronounced difference started to appear among treatment groups after 30 minutes. There was a

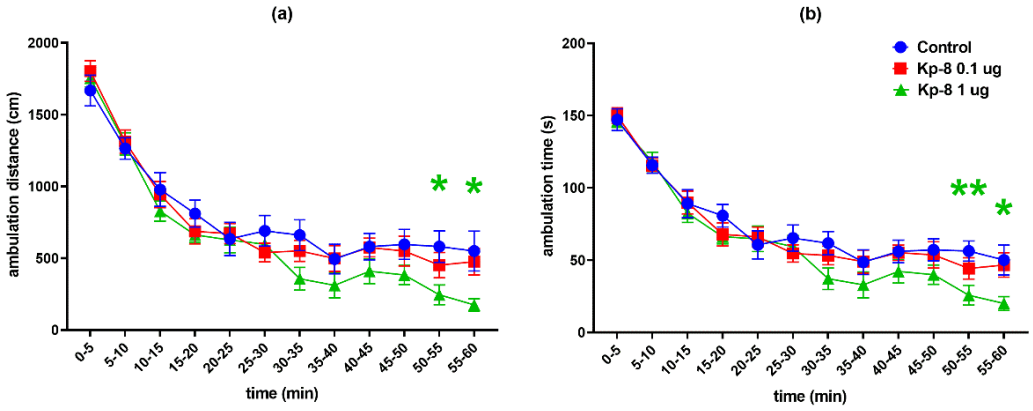

*Figure 19 – Open field test results in 5-minute intervals: **(a)** total distance travelled in the arena, **(b)** total ambulation time. The color of * refers to the treatment group which significantly differs from the control group. n=12-13, *p<0.05 vs control*

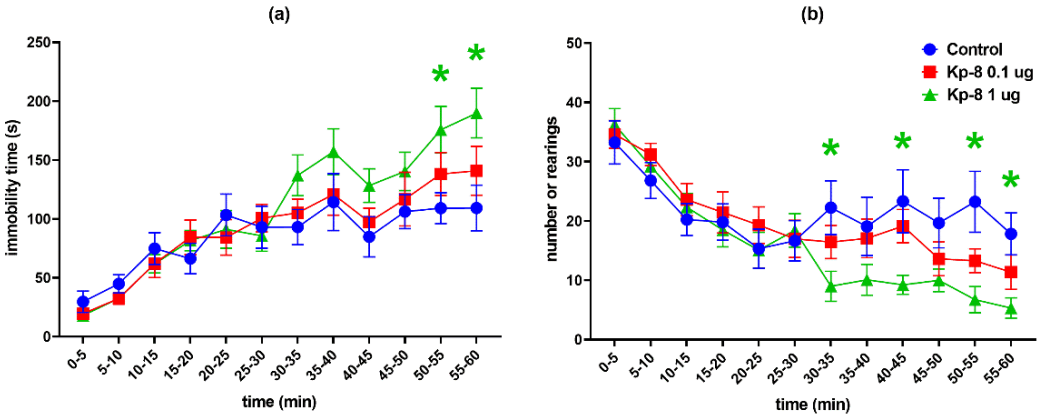

*Figure 20 - Open field test results in 5-minute intervals: **(a)** total time spent immobile, **(b)** total number of rearings. The color of * refers to the treatment group which significantly differs from the control group. n=12-13, *p<0.05 vs control.*

significant decrease in the number of rearings in the 1 µg KP-8 group at 30-35 minutes (p= 0.0369), 40-45 minutes (p= 0.0445), 50-55 minutes (p= 0.0182) and 55-60 minutes (p= 0.0108).

Having calculated the average velocity for each timeframe, a significant main effect for the time factor [F (4.044, 129.4) = 12.17, p<0.0001] and interaction [F (22, 352) = 1.940,

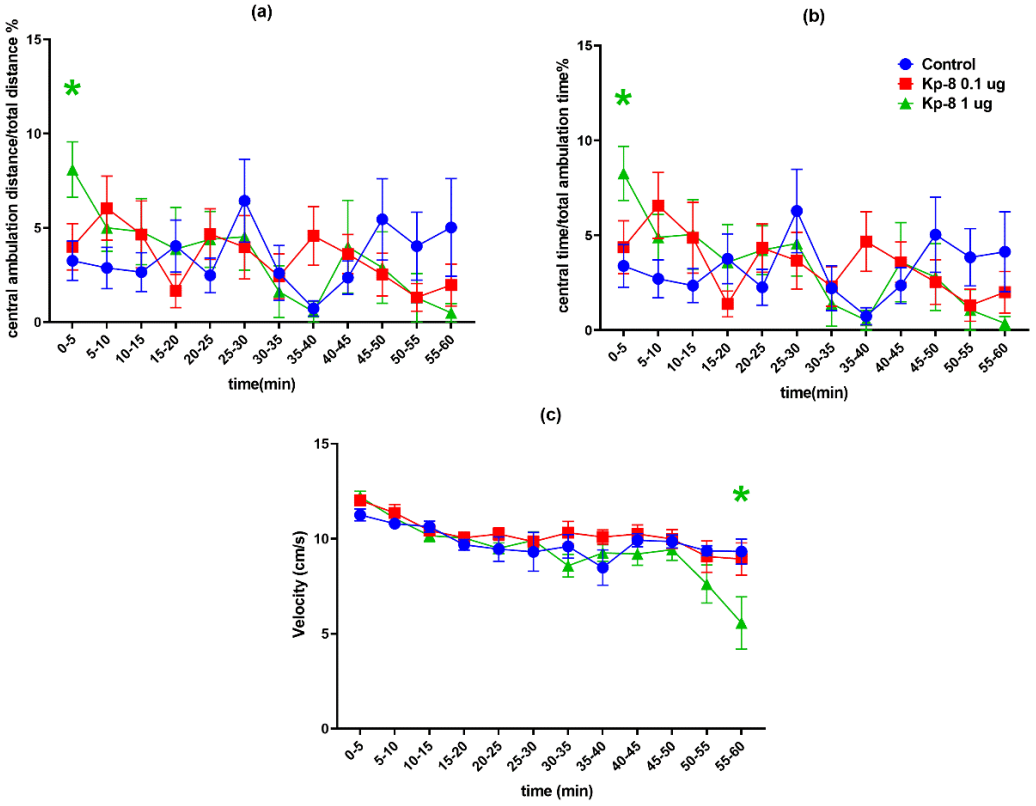

*Figure 21- Open field test results in 5-minute intervals: **(a)** percentage of distance travelled in the central zone of the arena, **(b)** percentage of time spent in the central zone of the arena, **(c)** average velocity of ambulation. The color of * refers to the treatment group which significantly differs from the control group. n=12-13, *p<0.05 vs control.*

p=0.0073] could be seen, as shown in Figure 21(c). There was no significant difference between treatment groups until 55 minutes when the speed of the 1 µg KP-8 group dropped (p=0.0479).

Figure 21(a) shows the percentage of central ambulation distance, calculated by dividing the distance traveled in the central zone of the arena by the total ambulation distance, multiplied by 100. Time factor [F (6.920, 235.3) = 2.207, p=0.0351) and interaction between time and treatment [F (22, 374) = 1.767, p=0.0185] 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 (p=0.0429).

The percentage of central ambulation time was calculated by multiplying the ratio of central time and total ambulation time by 100, as shown in Figure 21(b). There was a significant main effect for the time factor [F (6.981, 237.3) = 2,931, p= 0.0059], as well as for the interaction [F(22, 374)=1.945, p=0.0070]. 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 (p=0.0409), otherwise there was no difference among the groups.

4.2.1. Marble burying test

There was no significant difference in the number of buried marbles among the groups [Figure 22(a)]. Two types of goal-oriented interactions with the marbles were distinguished: marble burying and marble moving.

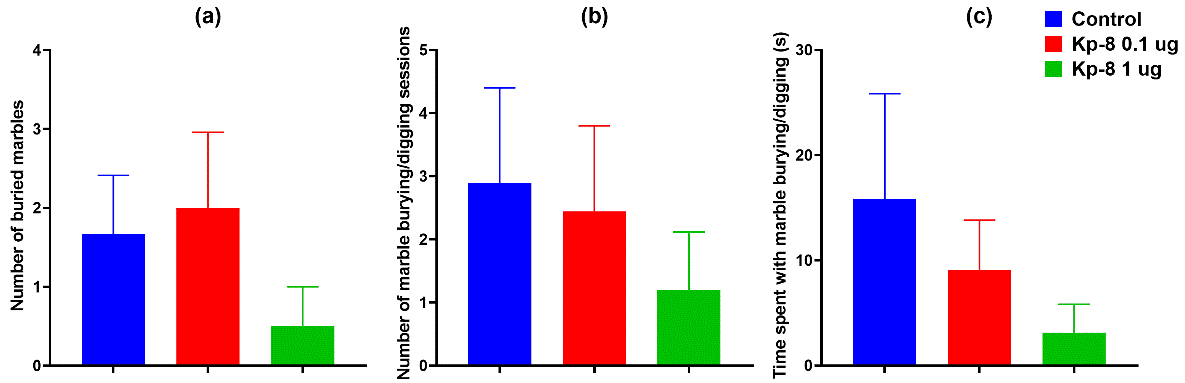

Figure 22 - Results of marble burying test: (a) number of buried marbles (at least 50% covered with bedding material), (b) number of marble burying sessions, (c) duration of marble burying activity, n=9-10.

Marble burying is an interaction involving digging around the marbles, resulting in marbles covered with bedding material. As seen in Figures 22(b) and 22(c), 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 [Figure 23(a) and 23(b)], although a tendency of suppressed marble moving could be seen in the groups treated with KP-8.

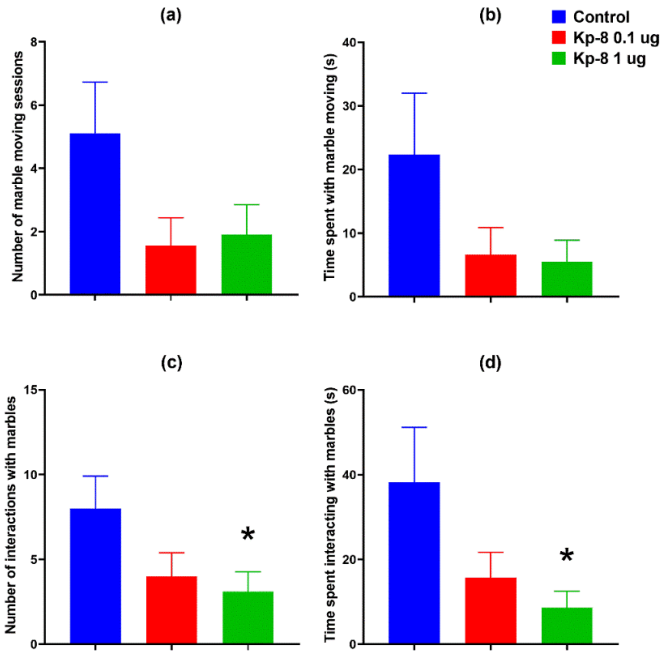

*Figure 23 - Results of marble burying test: (a) number of marble moving sessions, (b) duration of marble moving activity, (c) total number of interactions with marbles, (d) total duration of interactions with marbles, *p<0.05 vs control. n=9-10.*

However, taking the two types of interactions together, the 1 µg KP-8 group interacted with the marbles fewer times than the control group [Figure 23(c), p=0.0499] and they also spent less time with goal-oriented interactions with the marbles [Figure 23(d), p=0.0274].

4.2.2. Serum corticosterone, LH and total protein

The results of serum corticosterone and LH measurement can be seen in Figure 24. One-way ANOVA showed a significant effect of KP-8 treatment both on corticosterone [F (2, 10) = 12.02, p=0.0022] and LH concentration [F (2, 15) = 41.31, p<0.0001].

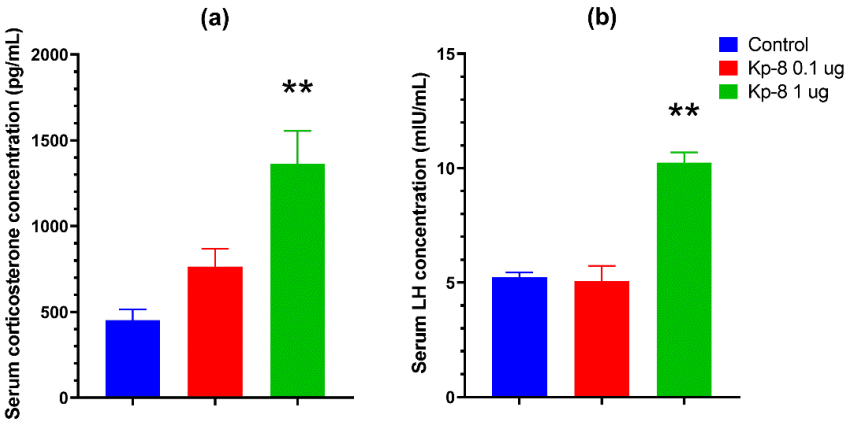

*Figure 24 - ELISA results: (a) serum corticosterone concentration (pg/mL), n=4-5, **p<0.01 vs control, (b) serum LH concentration (mIU/mL), n=4-9, **p<0.01 vs control.*

A robust increase in serum corticosterone concentration was detected 30 minutes after icv. treatment with 1 µg of KP-8 (p=0.001 vs control). The 0.1 µg dose tended to elevate corticosterone concentration, but the change was not significant (p=0.306 vs control). The 1 µg dose of KP-8 also raised serum LH concentration 15 minutes after icv. treatment (p=0.0001 vs control), but the 0.1 µg dose had no effect on LH (p=0.961 vs control). There was no difference in serum protein concentration among the groups [F (2, 17) = 2.365, p=0.124, n=5-8]: The mean serum protein concentrations with SD were 45.74±4.898, 40.44±3.115 and 45.13±7.045 g/L in the control, 0.1 µg and 1 µg groups, respectively.

4.2.3. Ex vivo superfusion

Figure 25 shows fractional dopamine release from the VTA. A p-value was not calculated for the time factor [F (15.00, 104.0) = 16.41]. There was no significant main effect neither for the treatment factor [F (1, 7) = 0.0008258, p=0.9779], nor for the interaction between treatment and time [F (15, 104) = 0.5151, p=0.9273]. There was no significant difference between the groups at any other time point.

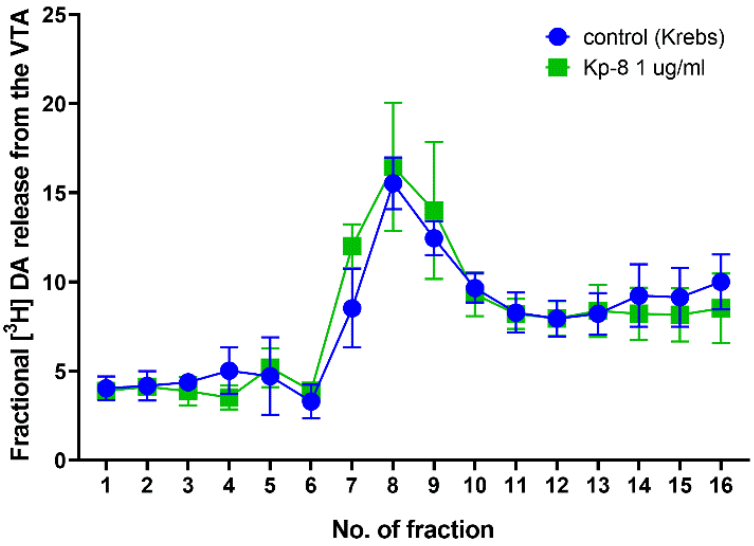

Figure 25– Fractional dopamine release from the ventral tegmental area. n=4-5.

Likewise, KP-8 did not influence fractional dopamine release from the NAc (Figure 26). However, there was a significant main effect for the time factor [F (3.134, 40.75) = 22.48, p<0.0001]. No significant main effect was found for the treatment factor [F (1, 13) = 0.0007717, p = 0.9783], and for the interaction between treatment and time [F (15, 195) = 0.4387, p = 0.9658]. No significant difference could be detected at any specific time point between the groups.

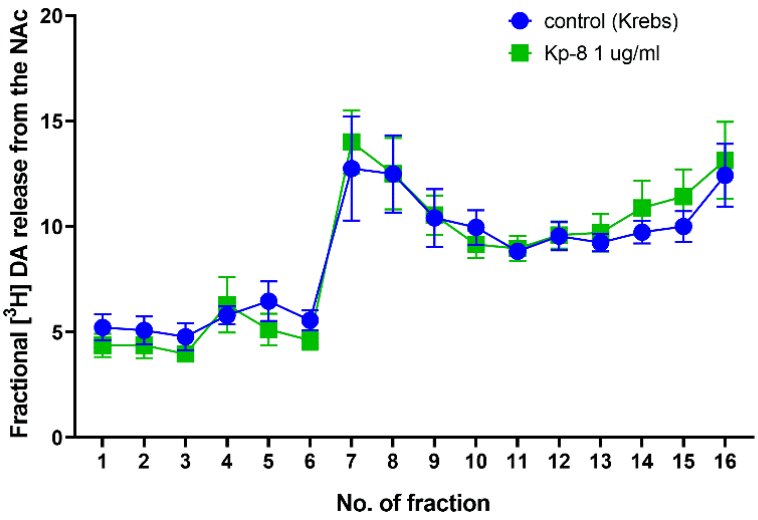

Figure 26 – Fractional dopamine release from the nucleus accumbens. n=7-8

As shown in Figure 27, KP-8 increased fractional GABA release from the NAc. There was a significant main effect for the time [F (2.227, 17.82) = 60.49, p<0.0001] and interaction [F (15, 120) = 7.395, p<0.0001] 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 (p=0.0039).

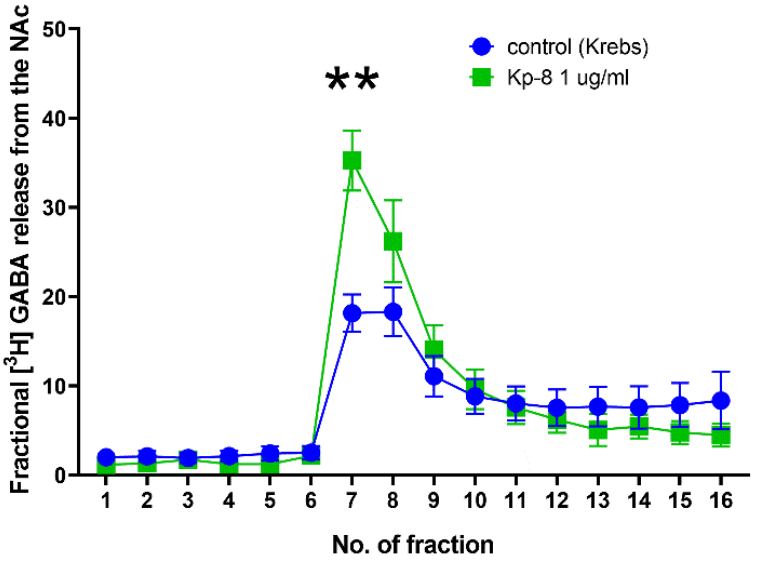

*Figure 27 – Fractional GABA release from the nucleus accumbens. **p<0.01 vs control, n=5.*

5. Discussion

Previously, our group reported that icv KP-13 injection activated the HPA axis, induced anxiety-like behavior in the elevated plus maze and open field tests, increased the locomotor activity, and elicited a transient hyperthermia in rats[54].

Firstly, we aimed to further characterize the anxiogenic action of KP-13 and investigate its effect on the expression of CRF, AVP, and their receptors in the amygdala and hippocampus. Both KP and Kiss1R expression has been detected in the amygdala and hippocampus[7–9,13], but literature data on the effects of kisspeptin in these regions in connection with anxiety have been lacking. It should be noted that both of these regions are involved in the pathomechanism of anxiety[91], furthermore, they are expression sites of AVP and CRF[85,97,113,114]. Therefore, it can be postulated that CRF and AVP signaling in these regions may be involved in the effect of KP on anxiety.

In our study, we found a brain region-specific change in the gene expression of the AVP and CRF systems in response to KP-13.

In the amygdala, KP-13 induced a significant upregulation of AVP expression, both at mRNA and protein levels. Although the majority of KP neurons are found in the hypothalamus, a significant population is also present in the amygdala[65]. In rodents, KP expression is confined to the medial amygdala (MeA), most prominently to the posterodorsal subnucleus of MeA (MePD). KP neurons in the MeA maintain reciprocal connections with the accessory olfactory bulb and project to the hypothalamic GnRH neurons[65]. In recent studies, the role of these KP neurons in the regulation of the reproductive axis has been investigated. The stimulation of KP neurons in the MePD has been found to increase the LH pulse frequency, possibly mediated by both GABAergic and glutamatergic signaling[115], and it also promotes sexual behavior[116]. Nevertheless, the MeA also plays a role in the processing of emotional signals, therefore, it might be involved in the mediation of anxiety[117]. A sexually dimorphic population of AVP neurons has been detected in the MeA, which sends direct and indirect projections to the hypothalamic PVN and elicits a greater recruitment of AVP neurons in the PVN in response to stressful stimuli[118], consequently leading to increased stress responsiveness. Since vasopressin fibers have been found in close apposition with KP neurons in the MePD[65], KP might contribute directly to the activation of amygdalar AVP neurons. In addition, AVP-expressing neurons in the amygdala are under the control of circulating gonadal steroids, especially in male rodents, suggesting a possible indirect effect of KP[119]. In fact, treatment with exogenous testosterone in castrated male mice has induced hypomethylation of the AVP promoter in the MePD and the

bed nucleus of the stria terminalis (BNST), thereby increasing AVP expression[119]. Furthermore, AVP neurons in the MePD could play a role in the integration of pheromonal and hormonal information, as well as in the regulation of sexual behavior. Following exposure to an inaccessible female, AVP neurons in the MePD of male Wistar rats were activated[120]. Since the activation of KP neurons induces HPG axis activation, and consequently the elevation in circulating gonadal steroid levels[26,31], KP-13 may exert its effect on the AVP expression in the amygdala indirectly, via an increase in testosterone. Circulating gonadal steroids, in turn, positively modulate KP expression in the amygdala[121,122].

Moreover, KP-13 elicited an upregulation of *Avpr1b* in the amygdala, which, based on literature data, could contribute to the development of anxiety-like behavior. In mice, a selective nonpeptide V1b receptor antagonist (SSR149415) has exerted an anxiolytic-like and antidepressive-like effect in a battery of behavioral tests[123]. Likewise, microinfusion of SSR149415 into the basolateral amygdala has reduced anxiety-like behavior in male rats[124]. Taken together, the upregulation of *Avp* and *Avpr1b* expression in the amygdala is in accordance with the anxiogenic effect of KP-13 that we detected previously in rats[54].

Interestingly, downregulation of *Crf* gene expression was detected in the amygdala after KP-13 treatment. According to the literature, amygdalar CRF has mostly been associated with anxiety. For instance, the activation of CRF-expressing neurons in the central amygdala using cre-dependent AAV-DREADD in CRF-cre mice has induced anxiety-like behavior, whereas the inhibition of these neurons has reduced anxiety[125]. Recently, however, a novel population of GABAergic CRF neurons has: been described in the lateral central amygdala (CEA) which sends projections to the ventral tegmental area (VTA) and exerts an anxiolytic-like effect, possibly via CRFR1 receptors[126,127]. Since whole amygdala samples were used in our gene expression study, it cannot be determined which amygdalar neuron population was *Crf* downregulated.

Nevertheless, it must be noted that no significant change was detected in the protein level of CRF in response to KP-13 in the amygdala. Protein levels vary on a high dynamic range, regulated by the rate of translation and degradation, the former being the determining factor [128]. The rate of translation is regulated by multiple mechanisms, only one of which is transcription. Other mechanisms include the activity of eukaryotic initiating factors (EIFs), structural features (e.g., internal ribosome-entry sequences, upstream open reading frames, and secondary or tertiary RNA structures), RNA-binding proteins, as well as microRNAs and small interfering RNAs that are involved in the regulation of translation [129]. Any of these mechanisms might be in play, and thus, gene expression results might not translate to the protein levels.

In the hippocampus, KP and KISS1R are expressed in high densities in the granule cell layer of the dentate gyrus and have also been detected in a low density in the pyramidal cells of CA1 and CA3[9,130]. It was thus suggested that KP signaling might be involved in hippocampal functions such as learning and memory. Central administration of KP-13 has facilitated passive avoidance consolidation[131] and it could alleviate amyloid-β neurotoxicity in the hippocampus[132,133]. Additionally, KP has been implicated in the regulation of BDNF expression and neurogenesis in the hippocampus[134,135], but its connection to CRF, AVP, and their receptors has not been explored in this region yet.

In our study, a significant upregulation of *Crf* and downregulation of *Avpr1a* was found following KP-13 treatment in the hippocampus. CRF is expressed on basket-type GABAergic interneurons of the pyramidal cell layer[113,136] and the hippocampal upregulation of *Crf* has been linked to the development of anxiety-like behavior. In juvenile rats, moderate psychological stress has induced the activation of pyramidal cells, which could be significantly dampened by a CRFR1 antagonist[136]. In another study involving young rats, restraint stress on postnatal day 18 has induced hippocampal activation, demonstrated by upregulation of *Fos* in CA3 and *pCREB* in CA1, CA3, and the dentate gyrus, which could be prevented by treatment with a CRFR1 antagonist[137]. Furthermore, the upregulation of CRF and CRFR1 in the CA1 and CA3 regions has also been observed in rats displaying extreme behavioral response (i.e., strong anxiety-like behavior) 7 days after exposure to predator scent[138]. To conclude, the upregulation of *Crf* in the hippocampus seems to agree with the development of anxiety-like behavior.

We also demonstrated a downregulation of *Avpr1a* in the hippocampus in response to KP-13. V1aRs have been detected on the GABAergic interneurons of the dentate gyrus, CA3, CA2, and CA1 regions[86,139]. In the pyramidal cells of the CA1 region, a dose-dependent increase in the frequency of IPSCs has been found in response to AVP, mediated by V1aR activation[140]. Several studies assign an anxiogenic effect for V1aR signaling. Bielsky *et al.* found a reduction in anxiety-like behavior in V1aR KO mice[141]. V1aR antagonist treatment was also associated with an anxiolytic effect[142], but the role of hippocampal V1aRs has not been investigated in anxiety yet.

Overall, our gene expression results suggest that KP-13 does, indeed, affect the expression of *Crf, Avp,* and their receptors in a brain region-dependent manner since, for instance, *Crf* expression decreased in the amygdala, whereas it increased in the hippocampus. Similarly, *Avp* expression showed an elevation in the amygdala, but no change was detected in the hippocampus.

To further establish the connection between CRF and AVP signaling pathways and KP-13's anxiety- and HPA axis-stimulating effect, we conducted a set of experiments in which, before the administration of KP-13, pretreatment with either a non-selective CRFR antagonist or with a V1R antagonist was performed.

First, a computerized open field test was performed to confirm KP-13's anxiety-inducing effect. Male Wistar rats were injected icv with different doses of KP-13, then their behavior was recorded in a non-familiar environment in an open field box. Our results showed that KP-13 dose-dependently reduced the distance traveled and the time spent in the center of the arena, which corresponds to anxiety-like behavior.

The 1 μg dose was the most effective in reducing central ambulation, thus the dose–response curve showed a U-shape that is characteristic of peptides[143,144]. This phenomenon, when a lower dose is stimulatory, whereas a higher dose is inhibitory or ineffective, is called hormesis[145]. A review by Calabrese has reported a wide range of explanations for hormetic responses, including receptorial and intracellular mechanisms[143]. Homologous desensitization may develop by G-protein coupled receptor kinases that phosphorylate already activated receptors, lowering the responsiveness of the cell specifically to ligands of those receptors, or inducing receptor downregulation[143,144]. Furthermore, higher doses of KP-13 could bind to and activate less specific receptors that might oppose the KISS1R- and/or NPFF receptor-mediated response[143,144].

In the open field test, the 2 μg dose of KP-13 also increased immobility time and decreased rearing activity. These changes support the anxiogenic effect of KP-13 since they can be interpreted as increased freezing and decreased exploratory behavior that are characteristic features of anxiety in rodents[146]. It should also be mentioned that there was a tendency of KP-13 to increase the total ambulation time and distance, although these changes were not significant. However, this tendency is in accordance with our previous findings with KP-13, in which KP-13 evoked an increase in square crossings in the open field test, and stimulated spontaneous locomotion[54].

After KP-13's effect on open-field behavior was established, the animals were pretreated with either α-helical CRF(9-41) or a V1R blocker before the administration of KP-13. Our results showed that the V1R antagonist reduced the KP-13-evoked decline in central ambulation distance and central ambulation time, whereas the non-selective CRFR antagonist had no effect. Consequently, AVP signaling pathways might mediate KP-13's anxiety-inducing effect.

Taking these results together with that of the qPCR and the ELISA, the upregulation of AVP signaling in the amygdala and the downregulation of V1aR in the hippocampus might be involved in the anxiety-like behavior induced by the icv administration of KP-13.

It must be mentioned though that KP's effect on anxiety-like behavior is somewhat contradictory in the literature. In fact, some studies found KP to exert an anxiolytic effect [56,57], some reported no effect [52,53], and some indicated an anxiety-like effect [54,55]. The reason for these discrepancies might lie in the differences in the experimental setup. For instance, in the experiments of Rao *et al.* and Comninos *et al.*, KP was injected peripherally [52,53], therefore, KP concentration in stress-related brain areas may not have reached sufficient levels to induce stress-related behaviors. In addition, it is not surprising that systemic administration of the peptide and local activation of a selected population of neurons elicit different effects[57].
Another confounding factor is that KP's effect on stress-related behavior was investigated in different species. Ogawa *et al.* showed an anxiolytic effect in zebrafish, whereas studies that ascribed an anxiogenic effect to KP were performed in rodents[54]. An additional explanation for the contradictory results could be that different forms of KP analogs were administered. Since the affinity of KP to the NPFF receptors is determined by the length of the peptide[147], it is also plausible that the different KP analogs might exert different actions. Nevertheless, our present results are in accordance with our previous results[54] as well as with the findings of Delmas *et al.*[55], who reported an anxiolytic phenotype in Kiss1r KO animals.

In our previous study, we demonstrated that corticosterone levels of rats rose 30 min after icv KP-13 treatment[54]. In our present study, we wanted to determine if CRF and/or AVP signaling might be involved in this effect. It is well established in the literature that both CRF and AVP are crucial for the activation of the HPA axis and consequently for the corticosterone response. In fact, CRF and AVP are released from the PVN and they synergistically induce the release of ACTH in the pituitary[79]. Since, in our experiments, KP 13 altered the expression of both AVP and CRF, it is plausible that it also activates the HPA axis directly or indirectly via these two hormones. Therefore, a set of animals was pretreated with either α-helical CRF(9-41) or a V1R antagonist 30 minutes before the KP-13 challenge. Our results showed that KP-13 induced a robust increase in the plasma corticosterone level, which was inhibited by both antagonists, suggesting that both CRF and AVP, which are the master regulators of the HPA axis[79], contribute to KP-13's stimulatory effect on the axis.

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.

After we established the anxiogenic property of KP-13 in rodents[54], a new direction of research emerged. Short kisspeptin analogs have been discovered as promising candidates in the treatment of infertility and other gynecological conditions[148]. In our studies, we aimed to investigate the behavioral and biological effects of icv. KP-8, an 8 amino acid long fragment of kisspeptin, that was synthesized by the Department of Medical Chemistry. In male rats, a battery of behavioral tests (EPM, OF, MB) was performed, serum corticosterone and LH levels were determined, and dopamine release from the VTA and NAc, and GABA release from the NAc were measured.

In the EPM, the 0.1 μg dose of KP-8 (but not the 1 μg dose) decreased the percentage of open arm entries and open-arm time, which is characteristic of anxiety-like behavior[149]. It is in accordance with our previous experiments in which a preference for closed arms has been observed following icv. treatment with KP-13[54]. Still, it must be noted that only a higher dose of KP-13 has exerted an anxiogenic action, whereas in the case of KP-8, an approximately 10 times lower dose was effective. The dose–response curve of KP-8, similar to KP-13 (see above), shows a bell-shape (or inverted U-shape), that has been reported in several studies involving neuropeptides[150–152].

In the computerized OF test, however, the animals treated with 1 μg of KP-8 spent more time in the central zone of the arena in the first 5 minutes, which is considered a sign of anxiolysis [153]. As there was no difference in central locomotor activity at any other time point or in the case of the 0.1 μg dose, this result should be interpreted with caution. At the beginning of the OF, the animals are placed in the center of the arena, so the increase in central time may reflect an initial latency in approaching the periphery rather than a real anxiolytic effect. In addition, it is not uncommon to have discrepancies between the EPM and OF results. For example, chlordiazepoxide has reduced anxiety-like behavior in the EPM but has had no significant effect in the OF in Lewis rats[154]. Although the principles of OF and EPM are similar, the two tests seem to load on different factors of anxiety[155]. Moreover, the approach of open arms in the EPM and central locomotion in the OF seem to be independently inherited in rats[156]. It should also be mentioned that the cumulative 60-minute results of OF showed a decreasing tendency in central ambulation, which was more pronounced with the 1 μg dose, but these changes did not reach the level of significance.

KP-8 also induced an elevation in serum corticosterone concentration: the 0.1 μg dose showed only a tendency to increase it, whereas the 1 μg dose elicited a significant change. This result ties well with our previous study in which icv. KP-13 has also caused an elevation in corticosterone concentration in a higher dose[54]. Corticosterone elevation is indicative of HPA

axis activation: the parvocellular neurons in the hypothalamic paraventricular nucleus (PVN) might have released CRH and AVP, followed by the secretion of ACTH from the pituitary, which consequently triggered the secretion of glucocorticoids from the adrenal cortex[79]. In a study by Rao *et al.*, KP induced an increase in AVP mRNA expression in PVN-derived cell lines[52], and thus it is also possible that KP-8 activated the axis by increasing AVP release. Moreover, the activity of the HPA axis is modulated by limbic brain regions, including the amygdala [79]—an expression site of both Kp and Kiss1r [157]—which stimulates the HPA axis and regulates the behavioral response to stress[79,158]. The increase in glucocorticoid signaling in itself is also associated with anxiety-like behavior[159]. Looking back at our experiments with KP-13, it is possible that both CRF and AVP are involved in the KP-8-induced activation of the HPA axis, although it should be confirmed with the use of antagonists.

Taken together, KP-8 seemed to increase anxiety-like behavior and activate the HPA axis. These results are in accordance with the anxiogenic effect of icv. KP-13 in rats[54] and the anxiolysis observed in Kiss1r KO mice[55].

The behavioral tests that we performed are not only suitable for the assessment of anxiety but also give information about other behavioral parameters, such as locomotor activity and repetitive behaviors.

For instance, the total number of arm entries in the EPM reflects the general locomotor activity of the animals[160]. Both the 0.1 and 1 μg doses of KP-8 reduced the number of arm entries, suggesting that KP-8 might cause hypolocomotion. In our previous studies, KP-13 did not cause any change in the total number of entries in the EPM, however, it showed a tendency to increase square crossings in the OF test, which suggests an increase in locomotor activity[54].

To further investigate the effects of KP-8 on anxiety-like behavior and locomotion, a 60-minute OF test was also performed, which yielded some remarkable results. When placed in a novel environment, all groups exhibited a pronounced exploratory activity with intense ambulation and a high number of rearings. Following a gradual decline until approximately 30 min, the activity returned to a basal level. From that point, differences started to develop among the groups, as there was a decrease in ambulation and rearing activity, as well as an increase in immobility in the group treated with 1 μg of KP-8. Taken together, these results point to a decrease in spontaneous locomotion.

KP-8 has also significantly reduced the number and time of goal-oriented interactions with marbles in the MB test. MB has been considered a test for anxiety-like behavior, however, several authors have expressed doubts about it[161]. According to Thomas *et al.*, the number of buried marbles does not correlate with other anxiety-like traits, namely central time in the open

field test and light-dark transitions in the light-dark box test[162]. The utility of the test as a screening tool for anxiety has also been questioned based on the findings that most anxiolytics and antidepressant drugs reduce marble burying behavior secondary to drug-induced hypolocomotion[162]. It has been suggested that digging and burying are species-specific, innate behavioral patterns that are likely triggered by an exploratory drive[163] or by exposure to the bedding itself[164]. Nowadays marble burying is regarded as a sign of repetitive, compulsive-like behavior, which is highly dependent on general locomotor activity[165]. Consequently, the reduction in goal-oriented interactions with the marbles is most likely a sign of suppressed locomotion in our study.

To conclude, icv KP-8 seemed to suppress locomotor activity in our studies. These findings contrast with the results reported by our group on the effects of icv. KP-13, as it has increased exploratory and spontaneous locomotion in rats[54]. In a study by Tolson *et al.*, Kiss1r KO female mice have exhibited a decrease in spontaneous locomotion and energy expenditure, but the mutation has had no such effect in male animals[35], pointing to a possibly gender-dependent effect.

The opposing effect of KP-8 and KP-13 on locomotion raises the question, which receptors might mediate KP-8's behavioral effects.

Based on a review on RF-amides and their receptors, kisspeptins, in general, can bind to their cognate receptor, Kiss1r, and to NPFF receptors with different affinity[1,14], the latter of which depends on the length of the peptide: the full-length KP-54 (in rats KP-52) has a lower affinity to NPFF receptors, whereas the shorter endogenous derivatives' binding affinity to NPFF receptors is higher[1]. Furthermore, Rouméas *et al.* have performed systemic N-terminus deletions and benzoylations of KP-10, which has revealed a progressive loss of affinity of the shorter fragments to Kiss1r and a conserved high affinity to NPFF receptors. In contrast, these shorter benzoylated fragments could still act as full agonists on Kiss1r, whereas on NPFF receptors a partial agonistic action has been observed[166]. In fact, the N-terminally benzoylated form of KP-8 has been shown to fully preserve the affinity of Kp-10 to NPFF1 and NPFF2 receptors[166], which are universally activated by all members of the RF-amide family[1]. How the benzoylation affects the affinity profile of these Kp-10 fragments is not exactly known, yet it is possible that the unmodified KP-8 also has an altered binding profile. According to Lyubimov et al., KP-8 could activate human NPFF2 receptors in Xenopus oocytes[147], which further supports the plausibility of NPFF receptor activation in the background of our findings. In point of fact, several members of the RF-amide family have been reported to modulate locomotor activity, pointing to the possible role of NPFF receptors in the regulation of locomotion.

Similarly to KP-8, icv. treatment with RF-amide-related peptide 1 (RFRP-1) has reduced total locomotor activity and has also induced anxiety-like behavior and HPA axis activation[167]. Likewise, intra-VTA injection of NPFF has reduced spontaneous locomotion in rats[168]. Interestingly, icv. NPFF has inhibited morphine-induced hyperlocomotion but has failed to affect the locomotor activity of naïve rats[169]. Although icv. neuropeptide AF (NPAF) has also had an anxiogenic effect, contrary to RFRP-1 and NPFF, it has stimulated spontaneous and exploratory locomotion[150].

Nevertheless, the cognate receptor of kisspeptins is Kiss1R, which is responsible for the activation of GnRH neurons[170], therefore we aimed to obtain an indirect sign of Kiss1R activation by measuring LH concentration in response to icv KP-8. Kiss1R is expressed on hypothalamic GnRH neurons[11], and upon its activation, repetitive LH pulses are generated[171]. In our study, the 1 μg dose of KP-8 elicited a significant increase in LH concentration, which is a sign of reproductive axis activation, secondary to hypothalamic Kiss1R binding and activation. This is in accordance with literature data, since icv. injection of a similar dose of KP-10 has also been reported to induce an LH surge[58]. It must be noted though, that 0.1 μg of KP-8 did not affect LH release. This is not surprising since Thomson *et al.* obtained a similar result when KP-10 was administered icv in a similarly low dose[58]. In a study by Pheng *et al.*, icv. administered KP-10 at a similarly low dose was unable to stimulate LH release in male rats, only the full-length KP-52 did. The authors have postulated that slower degradation of KP-52 might explain their results, but it is also possible that the different binding profiles of KP-52 and KP-10 are in the background[172]. Since kisspeptins' affinity to NPFF receptors is in inverse correlation with their length[1], it is plausible that KP-8, similarly to KP-10, might bind with higher affinity to the NPFF receptors than KP-13. One of the ligands of the NPFF1 receptor is the RF-amide-related peptide 3 (RFRP-3), which has an inhibitory effect on the reproductive axis in adult male rats[173,174]. Thus, it is possible that at a lower dose, the opposing actions of Kiss1R and NPFF1R activation in response to KP-8 result in no net change in the LH concentration. Nevertheless, further studies are required to determine the affinity of KP-8 to its receptors and the degree of calcium mobilization upon receptor activation.

Another possible reason for the development of hypolocomotion is the modulation of the mesocorticolimbic dopaminergic system. Based on the expression of kisspeptin in the NAc, as well as the expression of NPFF1 and NPFF2 receptors in the NAc and VTA[16,60], it was reasonable to investigate whether KP-8 has a direct effect on the VTA-NAc circuitry. The dopaminergic pathway connecting the VTA and NAc has long been implicated in the regulation of locomotion. As a matter of fact, VTA dopaminergic neurons are responsible for the

locomotor-enhancing effect of cocaine[175]. We hypothesized that KP-8 might suppress locomotion by directly modulating the activity of VTA dopaminergic neurons. However, in our *ex vivo* superfusion study, KP-8 has not affected dopamine release from slices obtained from the VTA and NAc.

As the interaction between Kp and GABA is known from the literature[176,177], it also seemed possible that KP-8 might directly affect GABA release in the NAc. GABAergic neurons in the NAc have been shown to inhibit dopaminergic projections from the VTA[178]. In fact, GABAergic activity can also be connected with the suppression of locomotion, as locomotor activity has increased when $GABA_A$ receptor antagonists were injected into the NAc core[62]. In our study, KP-8 significantly increased GABA release from NAc slices. This result suggests that KP-8 might directly modulate the activity of GABAergic neurons in the NAc, which could contribute to the suppression of locomotor activity. It must be mentioned, however, that *ex vivo* superfusion measures only the direct effect of KP-8 on live tissue slices obtained from the NAc, but the complex assessment of the whole VTA-NAc circuitry is beyond the scope of this method. Consequently, further studies (e.g., in vivo microdialysis) are required to confirm these findings on the circuit level.

When considering the receptors involved, it is possible that KP-8 alters GABA release via NPFF1 or NPFF2 receptors, which are abundantly expressed in the VTA and the NAc, and likely involved in the modulation of both dopaminergic and GABAergic neuronal activity[16]. The role of NPFF receptors is further supported by the results of Cador *et al.*, who have reported a decrease in novelty-induced locomotion upon intra-VTA NPFF treatment. Kiss1r expression, however, has only been detected in the NAc of humans, but not in rodents[179], so it is unlikely that KP-8 could modulate NAc activity via Kiss1r.

Furthermore, the contribution of altered metabolism and thermoregulation should not be ruled out in the background of altered locomotor activity. Kisspeptin's stimulatory effect on locomotion seemed to be coupled with metabolic effects in the literature. Icv. KP-13 has induced hyperthermia[54], and Kiss1r KO has resulted in obesity, increased adiposity, and impaired glucose tolerance in female mice[35]. KP could also be involved in hypothalamic appetite regulation by exciting proopiomelanocortin (POMC) neurons and inhibiting neuropeptide Y/Agouti-related peptide (NPY/AgRP) neurons, resulting in an anorexigenic effect[180]. Although only a few studies have addressed the metabolic effects of other RF-amides, they have usually revealed significant results. Icv. NPFF has reduced food intake in food-deprived rats[181] and also had a hypothermic effect in mice[182]. Moreover, the stimulation of

central NPFF1 and NPFF2 receptors has evoked hypothermia and hyperthermia, respectively[183].

Alternatively, KP-8 might modulate the activity of other, locomotion-related systems differently than the naturally occurring kisspeptins, resulting in an opposing effect of locomotion. For example, central KP-10 treatment has stimulated vasopressin release in rats[184], and vasopressin has been shown to induce hyperlocomotion by acting on V1a receptors on hypothalamic orexin/hypocretin neurons in mice[185]. Furthermore, kisspeptin has been shown to induce BDNF expression in the hippocampus[134] and the lack of active BDNF in tissue plasminogen activator deficient mice has been associated with a decrease in nocturnal wheel running activity[186]. It is a question of future research to investigate whether KP-8 could modulate vasopressin release and BDNF secretion similarly or differently as other kisspeptins.

It is somewhat difficult to assess the clinical significance of our findings in rodents. Our results taken together with those of others indicate that kisspeptins influence the regulation of the HPA axis and stress-associated behaviors. Thus, it is also possible that changes in KP signaling might be involved in the development of HPA axis-related pathologies (e.g., major depression, post-traumatic stress disorder, and anxiety disorders)[187,188].

Several clinical trials based on the modulation of KP signaling are already on the way, aiming for new diagnostic and therapeutic options for infertility, hypogonadism, and hormone-dependent tumors (such as prostate cancer), among others[100]. In these trials, not only various endogenous KPs (most commonly KP-10 and KP-54) but also several artificial analogs have been used, some of which are agonists, whereas others are antagonists of the KISS1R[100,189]. Therefore it is crucial to establish the full range of the biological actions of endogenous KP variants and KP analogs, and it must be taken into account that they might influence mood[189]. Nevertheless, further extensive research is needed to fully establish the mechanism of KP's action on the HPA axis and stress-related behaviors.

It should be noted that the exclusive use of male Wistar rats is a significant limitation of our studies. We aimed to avoid the sex differences and the modulatory effect of hormonal changes during the estrous cycle. However, most KP-related clinical studies involve women with reproductive disorders[189], therefore the effects of KP should be investigated in female rodents, as well. In fact, the HPA axis responsiveness shows marked sex differences in rodents, as females exhibit a more notable corticosterone response in the presence of any stressors, owing to the circulating estradiol levels[190]. Furthermore, when compared to males, female rats exhibited less anxiety-like behavior in the EPM and OF[191]. The behavior of females is also modulated by the hormonal changes of the estrous cycle, especially in tests based on

unconditioned threatening stimuli, in which responsiveness markedly increases in the late diestrus[192]. Therefore, results with more translational value are yielded when animals of both sexes are utilized, and stages of the estrous cycle are taken into consideration. To address this issue, we're planning to investigate the effects of KP-13 and KP-8 in female rats, as well.

Secondly, in the present study, we have investigated the effect of KP-13 on the gene expression of AVP and its receptors, as well as CRF and its receptors. However, only AVP and CRF were investigated at the level of protein expression, the protein expression of CRF and AVP receptors hasn't been investigated yet by our group.

Thirdly, it must be noted that other brain regions may also be involved in mediating KP-13's effect on the HPA axis and anxiety-like behavior. The amygdala and hippocampus were chosen due to the distribution data of KP and KISS1R and the crucial role these regions play in the regulation of the neuroendocrine stress system [13,193]. KP and KISS1R are expressed in other brain regions as well, albeit at a much lower expression level[7,13]. Still, it is plausible that other brain regions and circuits are also involved in the HPA axis-stimulating and anxiogenic action of KP-13, but further studies are required to investigate this possibility.

6. 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[194]. It can bind with different affinity to their cognate receptor KISS1R or NPFF receptors[147]. Kisspeptin's role as the main central regulator of the reproductive axis is well established[30], 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[54]. 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[79]. We concentrated on two brain regions where kisspeptin and Kiss1r are present, which are the amygdala and the hippocampus[9,13,49]. 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[54]. 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[195]) and GJ14 (an NPFF1R antagonist[196]). 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[54], which could be a phenomenon linked to the increase in locomotor activity[54], or the result of another, activity-independent mechanism, such as the induction of non-shivering thermogenesis in the brown adipose tissue[197]. 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.

7. Acknowledgment

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Appendix

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

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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

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I.

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Article

Kisspeptin-8 Induces Anxiety-Like Behavior and Hypolocomotion by Activating the HPA Axis and Increasing GABA Release in the Nucleus Accumbens in Rats

Katalin Eszter Ibos [1,*], Éva Bodnár [1], Zsolt Bagosi [1], Zsolt Bozsó [2], Gábor Tóth [2], Gyula Szabó [3] and Krisztina Csabafi [1]

[1] Department of Pathophysiology, Faculty of Medicine, University of Szeged, H-6720 Szeged, Hungary; dobo.eva@med.u-szeged.hu (É.B.); bagosi.zsolt@med.u-szeged.hu (Z.B.); csabafi.krisztina@med.u-szeged.hu (K.C.)
[2] Department of Medical Chemistry, Faculty of Medicine, University of Szeged, H-6720 Szeged, Hungary; bozso.zsolt@med.u-szeged.hu (Z.B.); toth.gabor@med.u-szeged.hu (G.T.)
[3] Office of International Affairs, Budapest Campus, McDaniel College, H-1071 Budapest, Hungary; gyula.szabo.prf@mcdaniel.hu
* Correspondence: ibos.katalin.eszter@med.u-szeged.hu

Abstract: Kisspeptins (Kp) are RF-amide neuropeptide regulators of the reproductive axis that also influence anxiety, locomotion, and metabolism. We aimed to investigate the effects of intracerebroventricular Kp-8 (an N-terminally truncated octapeptide) treatment in Wistar rats. Elevated plus maze (EPM), computerized open field (OF), and marble burying (MB) tests were performed for the assessment of behavior. Serum LH and corticosterone levels were determined to assess kisspeptin1 receptor (Kiss1r) activation and hypothalamic-pituitary-adrenal axis (HPA) stimulation, respectively. GABA release from the nucleus accumbens (NAc) and dopamine release from the ventral tegmental area (VTA) and NAc were measured via ex vivo superfusion. Kp-8 decreased open arm time and entries in EPM, and also raised corticosterone concentration, pointing to an anxiogenic effect. Moreover, the decrease in arm entries in EPM, the delayed increase in immobility accompanied by reduced ambulatory activity in OF, and the reduction in interactions with marbles show that Kp-8 suppressed exploratory and spontaneous locomotion. The increase in GABA release from the NAc might be in the background of hypolocomotion by inhibiting the VTA-NAc dopaminergic circuitry. As Kp-8 raised LH concentration, it could activate Kiss1r and stimulate the reproductive axis. As Kiss1r is associated with hyperlocomotion, it is more likely that neuropeptide FF receptor activation is involved in the suppression of locomotor activity.

Keywords: kisspeptin; anxiety; locomotion; Kiss1 receptor; HPA axis; HPG axis; nucleus accumbens

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1. Introduction

The *KISS1* gene was discovered as a novel metastasis-suppressor in human melanoma cells in 1996 in Hershey, named after the famous chocolate of the city, Hershey's Kisses [1].

KISS1 encodes a 145-amino-acid propeptide, from which kisspeptin-54 (Kp-54) is cleaved. The proteolytical cleavage of this 54-amino-acid peptide results in shorter biologically active products, designated kisspeptin-14 (Kp-14), kisspeptin-13 (Kp-13) and kisspeptin-10 (Kp-10) [2]. Mammalian kisspeptins belong to the family of RF-amide peptides, as they carry the characteristic, conserved carboxyl-terminal Arg–Phe–NH_2 sequence [3].

The canonical receptor of kisspeptins is a G protein-coupled receptor, Gpr54, that is fully activated by all biologically active products of the *Kiss1* gene [4]. Although Gpr54 was initially described in 1999 as an orphan receptor similar to galanin receptors [5], after being deorphanized in 2001, it was designated kisspeptin-1 receptor (Kiss1r) [6].

Upon activation of the $G\alpha_{q/11}$-coupled Kiss1r, phospholipase C (PLC) is activated, leading to inositol 1,4,5-trisphosphate (IP3)-mediated intracellular Ca^{2+} mobilization. Moreover, the activation of protein kinase C (PKC) and the $G\alpha_q$-independent recruitment of β-arrestins result in the phosphorylation of several mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinases 1/2 (ERK1/2) and p38 [4]. MAPKs in turn regulate gene expression and induce long-term alterations in a wide range of biological processes [7], including progesterone secretion [8], trophoblast adhesion [9], and glucose-induced insulin secretion [10].

Kisspeptins also bind and activate both neuropeptide FF receptors (NPFFR1 and NPFFR2) [11]. As NPFF receptors are coupled with $G\alpha_{i/o}$, their activation inhibits cAMP production. The $G\beta\gamma$ heterodimer released from $G_{i/o}$ proteins was found to inhibit voltage-gated Ca^{2+} channels. Moreover, it is capable of potentiating G_q signaling via physical interaction with PLC [3].

Kisspeptin is expressed in several regions of the rat central nervous system, including hypothalamic nuclei [e.g., arcuate nucleus, anteroventral paraventricular nucleus (AVPV)], thalamic nuclei, the amygdala, hippocampus, lateral septum, the bed nucleus of stria terminalis, striatum, nucleus accumbens (NAc), periaqueductal grey, and locus coeruleus [12,13]. Likewise, Kiss1r has been localized in rats in the hypothalamus (e.g., paraventricular, arcuate and supraoptic nucleus), thalamus, hippocampus, amygdala, septum, striatum, raphe nuclei, and cortex [5,14].

The expression of NPFF1 receptor mRNA has been detected in the lateral septum, in thalamic and brainstem nuclei, as well as in the ventral tegmental area (VTA), NAc, the bed nucleus of the stria terminalis, the amygdala and hippocampus. NPFF2 receptor mRNA expression has been reported in thalamic nuclei, in the hypothalamus, hippocampus, VTA, the A5 noradrenergic cell group and also in the dorsal horn of the spinal cord [15,16].

Following the original discovery of its metastasis suppressor role in melanoma [1], the anti-metastatic activity of kisspeptin has been found in a variety of tumors, including bladder, ovary, colorectal, pancreas, pituitary, prostate and thyroid cancer [17].

The involvement of kisspeptin in reproduction has been a topic of extensive research since it was discovered in 2003 that kisspeptin is a potent stimulator of gonadotropin secretion [18]. The role of kisspeptin in the regulation of puberty is underlined by the finding that various loss-of-function mutations of *KISS1R* and *KISS1* are associated with isolated hypogonadotropic hypogonadism, whereas activating mutations result in central precocious puberty [19]. Hypothalamic *Kiss1* neuron populations are responsible for the regulation of the estrous cycle by mediating positive and negative feedback of gonadal steroids on gonadotropin secretion [20]. The sexually dimorphic *Kiss1* neuron population of the AVPV is responsible for the positive feedback of estrogen, thus it contributes to the surge-like secretion of GnRH. However, pulsatile GnRH secretion is regulated by the KNDy neurons (coexpressing kisspeptin, neurokinin B, and dynorphin) of the arcuate nucleus that mediate the negative feedback of estrogen [21]. Compelling evidence has suggested that KNDy neurons in the arcuate nucleus function as a major integrator of various modifiers of the reproductive axis, including metabolic signals, olfactory clues, and circadian rhythm [22–25].

Similarly to other members of the RF-amide family [26], kisspeptin has also been implicated in the regulation of nociception [27]. In a recent study, Kp-13 lowered the nociceptive threshold in mice, decreased the analgesic effect of morphine, diminished morphine tolerance and caused mechanical hypersensitivity [28].

Based on the expression of *Kiss1* and *Kiss1r* in limbic brain structures [29,30], several studies have investigated the behavioral effects of kisspeptin.

An antidepressant-like effect of kisspeptin has been reported in rats [31], and intravenous kisspeptin has also decreased negative mood in human subjects [32].

Kisspeptin neurons in the rostral periventricular area of the 3rd ventricle (RP3V) seem to regulate sexual behavior in rodents, as they are activated by male urinary odors in female mice and facilitate copulatory behavior in a NO-dependent pathway [33,34].

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 [35]. Since that time, several studies have been conducted with controversial results.

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, as intraperitoneally (ip.) administered Kp-10 had no effect on plasma corticosterone and adrenocorticotropic hormone (ACTH) levels in rats [36]. Likewise, kisspeptin administration had no effect on anxiety in human subjects [32].

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 [37].

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. The most pronounced anxiolytic effect was observed when kisspeptin signaling in GnRH neurons was selectively rescued in *Kiss1r* KO animals, suggesting a modulatory role of gonadal steroids. Interestingly, no significant effect of *Kiss1r* KO was detected on the behavioral parameters of the open field test [38].

In zebrafish, however, the central administration of kisspeptin has been associated with an anxiolytic tendency in the novel-tank diving test and a significantly reduced fear response to alarm substance [39].

In a recent study, a Cre-dependent, stimulatory DREADD (Designer Receptors Exclusively Activated by Designer Drugs) viral construct has been targeted to the *Kiss1* neurons of the posterodorsal medial amygdala (MePD) in mice. Upon selective activation of MePD *Kiss1* neurons by clozapine-N-oxide, a significant increase in open arm exploration has been observed in the elevated plus maze, suggesting an anxiolytic role of this neuron population [40].

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. Peripheral administration of Kp has failed to influence the activity of the HPA axis in rats (0.13 µg/µL Kp-54 ip.) [36] and the activity of the limbic system in human subjects (1 nmol/kg/h Kp-54 iv. over 75 min) [32]. In contrast, central Kp-13 (1 or 2 µg icv.) had a pronounced anxiogenic effect in rats [37]. It is likely that the doses applied by Rao et al. [36] and Comninos et al. [32] were too low to exert an anxiogenic effect. In their investigation into the effect of peripheral or central Kp administration on the reproductive axis in rats, Thomson et al. have found that 1 nmol of icv. Kp-10 was sufficient to significantly raise plasma luteinizing hormone (LH) concentration, but a 100-fold dose was required for the same effect in case of ip. treatment [41]. Likewise, the selective activation of MePD *Kiss1* neurons [40] points to the function of a distinct neuron population, whereas central kisspeptin treatment [37] 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, both in terms of anatomy and function [39,42], 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 in exploratory locomotion in male Sprague-Dawley rats [37]. 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 [43].

It has been discovered that kisspeptin attenuates morphine effect [28], and is expressed in the NAc [44], pointing to its possible involvement in the regulation of mesocorticolimbic dopaminergic activity. Interestingly, the centers of reward and addiction have also been implicated in the regulation of locomotion. First, quinpirole (a D2 receptor agonist) injected into the NAc has suppressed exploratory locomotion in rats [45], whereas bicuculline (a $GABA_A$ receptor antagonist) administration into the nucleus induced hyperactivity with prolonged exploratory behavior in rats [46]. Second, the selective activation of dopaminergic neurons in the VTA by DREADD has induced a pronounced and sustained hyperactivity in rats, which effect could be reproduced by activating selectively activating the dopaminergic pathway between the VTA and NAc [47]. Thus, it is possible that kisspeptin might influence locomotion by modulating the activity of the VTA or NAc.

Nowadays kisspeptin analogs and antagonists are attracting considerable attention due to their potential therapeutic use in various gynecological conditions, including infertility, polycystic ovary syndrome and precocious puberty [48]. The shortest natural bioactive form of kisspeptin is the 10 amino acid long Kp-10, which has higher affinity to Kiss1r than Kp-54 [49]. According to molecular docking studies, ASN4, SER5, GLY7, ARG9 and PHE10 of Kp-10 are involved in the formation of hydrogen bonds between the peptide and Kiss1r [50]. Consequently, shorter kisspeptin fragments containing these amino acids might be able to bind and possibly activate the receptor.

The aim of the current study was to investigate whether the 8 amino acid long fragment of kisspeptin is capable of influencing the behavior of rats similarly to kisspeptin. Following icv. treatment with Kp-8, elevated plus maze (EPM), computerized open field (OF), and marble burying (MB) tests were performed. Serum corticosterone and luteinizing hormone concentrations were measured to assess the activation of the HPA axis and Kiss1 receptors, respectively. Moreover, dopamine release from the VTA and NAc and GABA release from the NAc were measured using ex vivo superfusion to further characterize the mechanism of action.

2. Materials and Methods

2.1. 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.

2.2. Intracerebroventricular Cannulation

A stainless steel Luer cannula (10 mm long) was implanted in the right lateral cerebral ventricle for icv. administration. The cannula was inserted under sodium pentobarbital (Euthasol, Phylaxia-Sanofi, 35 mg/kg, ip.) anaesthesia, 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 [37]. 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.

2.3. Peptide Synthesis

Kisspeptin-8 (WNSFGLRF-NH2) was synthesized on a Rink Amide MBHA resin (Bachem, Bubendorf, Switzerland, subst.: 0.52 mmol/g) using N^{α}-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). The resin was swollen in dichloromethane (DCM). The Fmoc group was removed by treating the peptide-resin with 20% piperidine/*N*,*N*-dimethylformamide (DMF) solution twice (5 + 15 min). Solvents were purchased from VWR (Radnor, PA, USA).

The amino acids were activated with *N*,*N*′-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole in 50% DCM/DMF. The peptide-resin was incubated with this mixture for 3 h. The resin was washed with DMF (3×) and DCM (3×) after the deprotection and coupling steps.

The assembled peptides were cleaved from the resin by treating it with the following cleavage cocktail for 3 h: 90% trifluoroacetic acid (TFA) (Pierce, Rockford, IL, USA), 5% water, 2% dithiotreitol, 2% triisopropylsilane.

The peptides were precipitated with diethyl ether, dissolved in a mixture of acetonitrile (ACN) and water and lyophilized. The crude peptides were analyzed by HPLC (Hewlett-Packard Agilent 1100 system, column: Luna, c18 (2), 250 × 4.6 mm, 5 µm, 100 Å, Phenomenex, Aschaffenburg, Germany) and ESI-MS. The peptides were purified on a preparative HPLC column (Phenomenex Luna, c18 (2), 250 × 21.2 mm, 10 µm, 100 Å) using a Shimadzu 20-LC system. The fractions were analyzed on the above mentioned analytical HPLC system and measured by electrospray ionization mass spectrometry (ESI-MS) (see Appendix A Figures A1 and A2 for the results). The pure fractions were pooled and freeze-dried.

2.4. Treatment

The rats were treated icv. in a volume of 2 µL over 30 s using a Hamilton microsyringe (Merck KGaA, Darmstadt, Germany). The doses applied were 0.1 or 1 µg of Kp-8 dissolved in 0.9% saline. Control animals were injected with 2 µL of 0.9% saline alone. The animals were treated 30 min prior to the behavioral tests. Collection of trunk blood for LH ELISA, corticosterone ELISA and serum corticosterone measurement were carried out 15 min and 30 min after icv. treatment, respectively.

2.5. Behavioral Tests

2.5.1. Elevated Plus Maze Test

The EPM apparatus is a plus-shaped platform 50 cm above the ground. The maze consists of four arms (50 cm × 10 cm each): two opposing open arms and two closed arms enclosed by a 10 cm high wall. The test is based on two conflicting motivations of rodents: to avoid open, brightly lit spaces and to explore novel environment. The avoidance of open arms reflects anxiety-like behavior [51]. 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. The experiments were conducted between 8 a.m. and 10 a.m. and the apparatus was cleaned with 96% ethyl-alcohol after each session.

2.5.2. Computerized Open Field Test

The novelty-induced locomotor activity of rats was assessed using the Conducta 1.0 System (Experimetria Ltd., Budapest, Hungary). The system consists of black plastic OF arenas (inside dimensions: 48 × 48 cm, height: 40 cm) with 5 horizontal rows of infrared diodes on the walls to register both horizontal and vertical locomotion. The center of each box is illuminated by a LED lightbulb (230 lumen) from above the box. The central zone of the arena is defined as a 24 × 24 cm area in the center of the box. 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 60 min. 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 travelled in the central zone. The OF experiments were conducted between 8 a.m. and 10 a.m. and the apparatus was cleaned with 96% ethyl-alcohol after each session.

2.5.3. Marble Burying Test

MB is a regularly used paradigm for the assessment of anxiety-like and compulsive-like behavior [52]. Our protocol was based on the method described by Schneider and Popik [53]. The animals were removed from their plexiglass home cages (420 × 275 × 180 mm) and temporarily moved into another cage before the experiment. Meanwhile the home cage was prepared for the experiment by increasing the depth of bedding material to 5 cm. Following icv. treatment one animal was placed back into the home cage for 30 min in order to acclimatize to the thick bedding. 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% marble covered by bedding material) was counted. The marbles were cleaned with 96% ethyl-alcohol after each session. After the experiment, the video recording was evaluated. The count and duration of two types of goal-oriented interactions with marbles (burying of marbles and moving marbles without burying them) were assessed.

2.6. Serum Corticosterone, Luteinizing Hormone and Total Protein Concentration Measurement

For the measurement of serum corticosterone and protein concentration, the animals were decapitated 30 min after icv. treatment. For the assessment of serum LH, decapitation was performed 15 min after icv. treatment. 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 until the assays were performed. Serum corticosterone concentration was measured using a competitive corticosterone ELISA kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instruction. Serum LH concentration was determined using a sandwich LH ELISA kit (Wuhan Xinquidi Biological Technology Co., Wuhan, China), according to the manufacturer's instructions. 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 One^{C} microvolume spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.7. 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 [54]. The VTA was isolated as described by Salvatore et al. [55]. The tissue was cut to 300 μm slices and incubated for 30 min in 5 mL of Krebs solution (Reanal, Hungary) bubbled with carbogen gas (5% CO_2 and 95% O_2). Then 5 μL of [3H]GABA (PerkinElmer Inc., Waltham, MA, USA) was added to the NAc and 5 μL 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 into 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:

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

2.8. Statistical Analysis

Data are presented as mean + SEM. Statistical analysis and graph editing were performed using the GraphPad Prism 8 software. One-way ANOVA with Holm-Sidak's post-hoc test was applied for the analysis of EPM results. One-way ANOVA with Dunnett's post-hoc test was used for the analysis of cumulative OF results, as well as for the evaluation of serum corticosterone, LH and total protein measurements. Two-way RM ANOVA with Holm–Sidak's post-hoc test was performed for the evaluation of 5-min intervals in the OF test as well as for the interpretation of dopamine and GABA release from the NAc. Mixed-effects analysis with Holm–Sidak's multiple comparison test was performed for the evaluation of fractional dopamine release from the VTA. Kruskal–Wallis test with Dunn's post-hoc test was performed for the analysis of MB results. Curve fitting for ELISA tests was performed according to the manufacturers' instructions.

3. Results

3.1. Behavioral Tests

3.1.1. Elevated Plus Maze

The 0.1 µg dose of Kp-8 significantly reduced the percentage of entries into the open arms of the plus maze (Figure 1a, F (2, 20) = 9.196, $p = 0.0007$), as well as the percentage of time spent in the open arms of the maze (Figure 1b, F (2, 20) = 4.431, $p = 0.0202$). A decrease in the total number of entries into the arms was induced by both 0.1 µg and 1 µg of Kp-8 (Figure 1c, F (2, 20) = 5.927, $p = 0.0153$). There was no significant difference among the groups in the total time spent in the arms (Figure 1d, F (2, 20) = 1.932, $p = 0.1710$).

3.1.2. Computerized Open Field Test

The cumulative results obtained after 60 min of data collection did not show any significant change in behavior (see Appendix B Figure A3).

However, significant differences were found following the analysis of each 5-min interval. As seen in Figure 2a, the two-factor RM-ANOVA on the distance travelled in the arena revealed a significant main effect for the time factor (F (5.389, 183.2) = 113.8, $p < 0.0001$). 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 min. The distance travelled at 50–55 and 55–60 min was lower in the 1 µg Kp-8 group than in the control group ($p = 0.0334$ and $p = 0.0410$, respectively).

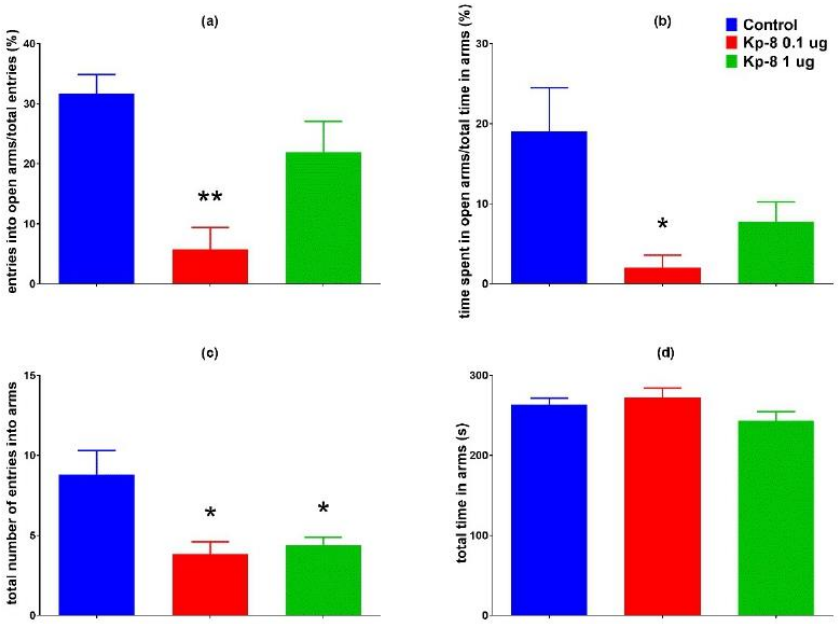

Figure 1. Elevated plus maze results: (**a**) percentage of entries into open arms, (**b**) percentage of time spent in the open arms, (**c**) total number of entries into arms, (**d**) total time spent in the arms of the maze, $n = 7–9$, * $p < 0.05$ vs. control, ** $p < 0.01$ vs. control.

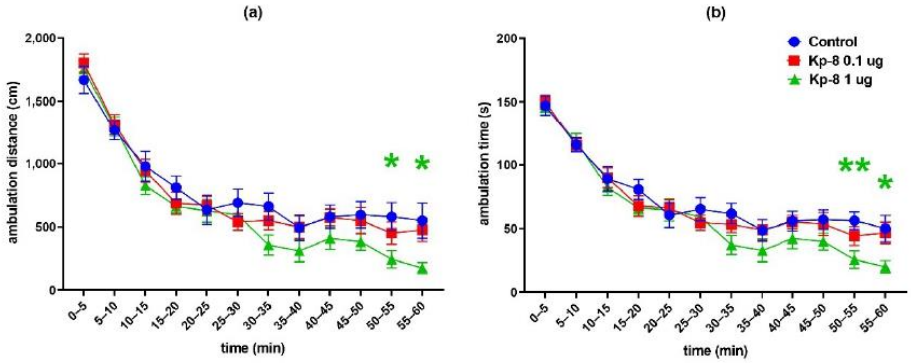

Figure 2. Open field test results in 5-min intervals: (**a**) total distance travelled in the arena, (**b**) total ambulation time. The color of * refers to the treatment group which significantly differs from the control group. $n = 12–13$, * $p < 0.05$ vs. control, ** $p < 0.01$ vs. control.

Regarding total ambulation time, there was a significant main effect for the time factor ($F(6.138, 208.7) = 98.03$, $p < 0.0001$) with a similar pattern of steep then mild decrease (Figure 2b). The 1 µg Kp-8 group spent less time with ambulation than the control group at 50–55 min ($p = 0.0090$) and 55–60 min ($p = 0.0326$), as well.

The two-way ANOVA on immobility yielded a significant main effect for the time factor ($F(5.396, 183.5) = 34.51$, $p < 0.0001$) and interaction ($F(22, 374) = 2.249$, $p = 0.0012$). The time spent immobile was increasing during the experiment, showing a tendency reciprocal to that of ambulation time and distance (Figure 3a). Compared to control, the 1 µg dose of Kp-8 significantly increased immobility at 50–55 and 55–60 min ($p = 0.0202$ and $p = 0.0186$, respectively).

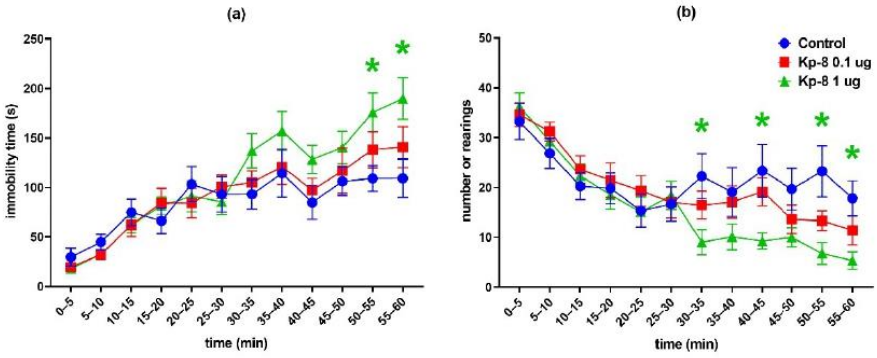

Figure 3. Open field test results in 5-min intervals: (**a**) total time spent immobile, (**b**) total number of rearings. The color of * refers to the treatment group which significantly differs from the control group. n = 12–13, * $p < 0.05$ vs. control.

Considering the number of rearing sessions, a significant main effect for the time factor was detected (F (6.756, 229.7) = 7.52, $p < 0.0001$), along with a statistically significant interaction between time and treatment (F (22, 374) = 3.095, $p < 0.0001$). As seen in Figure 3b, a pronounced difference started to appear among treatment groups from 30 min. There was a significant decrease in the number of rearings in the 1 µg Kp-8 group at 30–35 min ($p = 0.0369$), 40–45 min ($p = 0.0445$), 50–55 min ($p = 0.0182$) and 55–60 min ($p = 0.0108$).

Having calculated the average velocity for each timeframe, a significant main effect for the time factor (F (4.044, 129.4) = 12.17, $p < 0.0001$) and interaction (F (22, 352) = 1.940, $p = 0.0073$) could be seen, as shown in Figure 4c. There was no significant difference between treatment groups until 55 min, when the speed of the 1 µg Kp-8 group dropped ($p = 0.0479$).

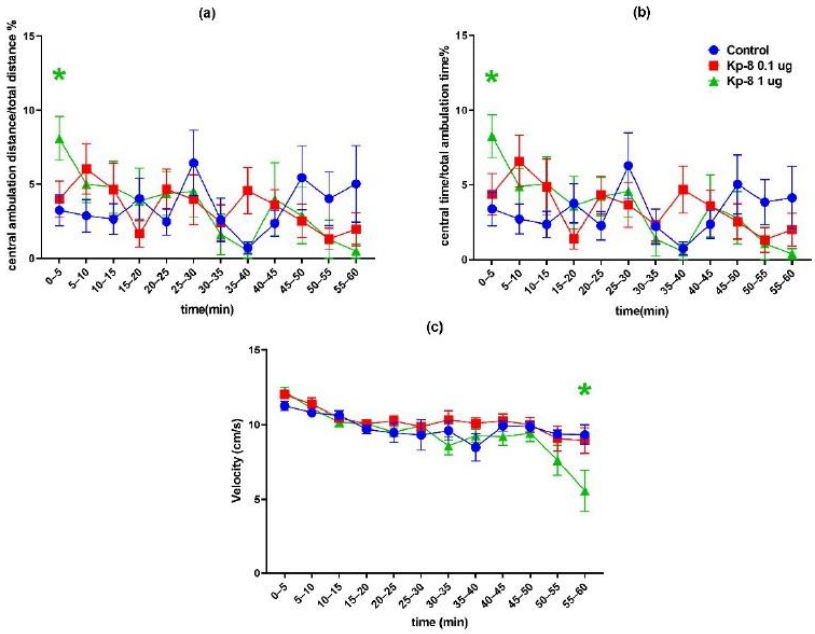

Figure 4. Open field test results in 5-min intervals: (**a**) percentage of distance travelled in the central zone of the arena, (**b**) percentage of time spent in the central zone of the arena, (**c**) average velocity of ambulation. The color of * refers to the treatment group which significantly differs from the control group. n = 12–13, * $p < 0.05$ vs. control.

Figure 4a shows the percentage of central ambulation distance, calculated by dividing the distance travelled in the central zone of the arena by the total ambulation distance, multiplied by 100. Time factor (F (6.920, 235.3) = 2.207, $p = 0.0351$) and interaction between time and treatment (F (22, 374) = 1.767, $p = 0.0185$) both significantly accounted for the variation, but there was no difference among the groups, except in the first 5 min, when the central ambulation distance of the 1 µg Kp-8 group was higher than that of control ($p = 0.0429$).

The percentage of central ambulation time was calculated by multiplying the ratio of central time and total ambulation time by 100, as shown in Figure 4b. There was a significant main effect for the time factor (F (6.981, 237.3) = 2931, $p = 0.0059$), as well as for the interaction (F (22, 374) = 1.945, $p = 0.0070$). In the first 5 min, the central ambulation time of the 1 µg Kp-8 group significantly exceeded the central time of the control group ($p = 0.0409$), otherwise there was no difference among the groups.

3.1.3. Marble Burying Test

There was no significant difference in the number of buried marbles among the groups (Figure 5a). Two types of goal-oriented interactions with the marbles were distinguished: marble burying and marble moving.

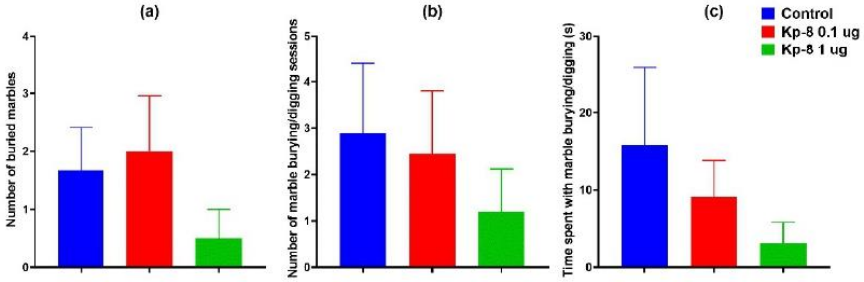

Figure 5. Results of marble burying test: (**a**) number of buried marbles (at least 50% covered with bedding material), (**b**) number of marble burying sessions, (**c**) duration of marble burying activity, $n = 9–10$.

Marble burying is an interaction involving digging around the marbles, resulting in marbles covered with bedding material. As seen in Figure 5b,c, 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 it with bedding material. Similarly to marble burying, there was no significant difference in the number and duration of marble moving among the groups (Figure 6a,b), although a tendency of suppressed marble moving could be seen in the groups treated with Kp-8.

However, taken the two types of interactions together, the 1 µg Kp-8 group interacted with the marbles fewer times than the control group (Figure 6c, $p = 0.0499$) and they also spent less time with goal-oriented interactions with the marbles (Figure 6d, $p = 0.0274$).

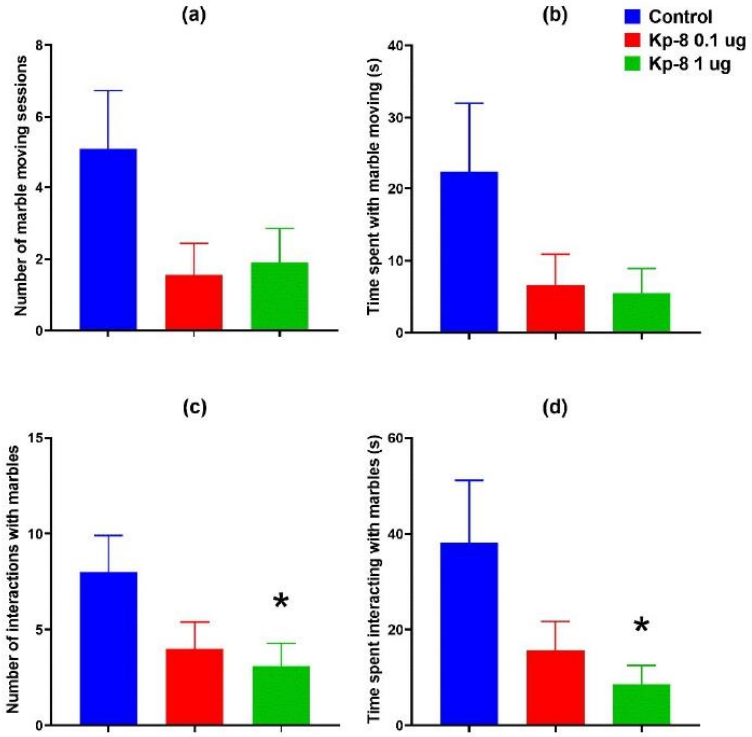

Figure 6. Results of marble burying test: (**a**) number of marble moving sessions, (**b**) duration of marble moving activity, (**c**) total number of interactions with marbles, (**d**) total duration of interactions with marbles, * $p < 0.05$ vs. control. $n = 9$–10.

3.2. Serum Corticosterone, LH and Total Protein

The results of serum corticosterone and LH measurement can be seen in Figure 7. One-way ANOVA showed a significant effect of Kp-8 treatment both on corticosterone ($F(2, 10) = 12.02$, $p = 0.0022$) and LH concentration ($F(2, 15) = 41.31$, $p < 0.0001$). A robust increase in serum corticosterone concentration was detected 30 min after icv. treatment with 1 μg of Kp-8 ($p = 0.001$ vs. control). The 0.1 μg dose had a tendency to elevate corticosterone concentration, but the change was not significant ($p = 0.306$ vs. control). The 1 μg dose of Kp-8 also raised serum LH concentration 15 min after icv. treatment ($p = 0.0001$ vs. control), but the 0.1 μg dose had no effect on LH ($p = 0.961$ vs. control). There was no difference in serum protein concentration among the groups ($F(2, 17) = 2.365$, $p = 0.124$, $n = 5$–8): The mean serum protein concentrations with SD were 45.74 ± 4.898, 40.44 ± 3.115 and 45.13 ± 7.045 g/L in the control, 0.1 μg and 1 μg groups, respectively.

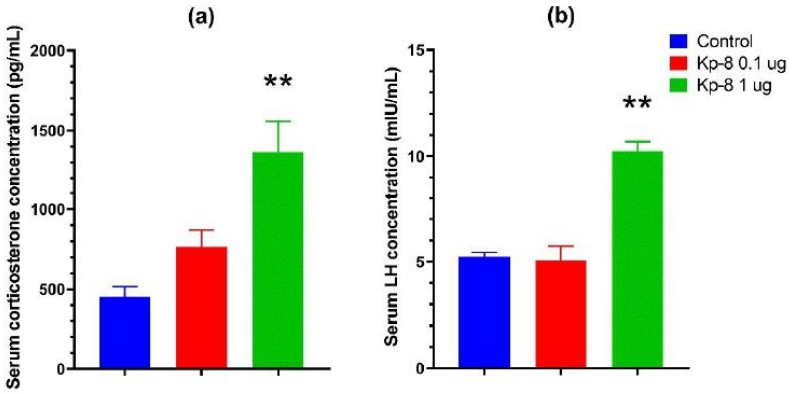

Figure 7. ELISA results: (**a**) serum corticosterone concentration (pg/mL), n = 4–5, ** $p < 0.01$ vs. control, (**b**) serum LH concentration (mIU/mL), n = 4–9, ** $p < 0.01$ vs. control.

3.3. Ex Vivo Superfusion

Figure 8 shows fractional dopamine release from the VTA. A p value was not calculated for the time factor (F (15.00, 104.0) = 16.41). There was no significant main effect neither for the treatment factor (F (1, 7) = 0.0008258, p = 0.9779), nor for the interaction between treatment and time (F (15, 104) = 0.5151, p = 0.9273). There was no significant difference between the groups at any other time point.

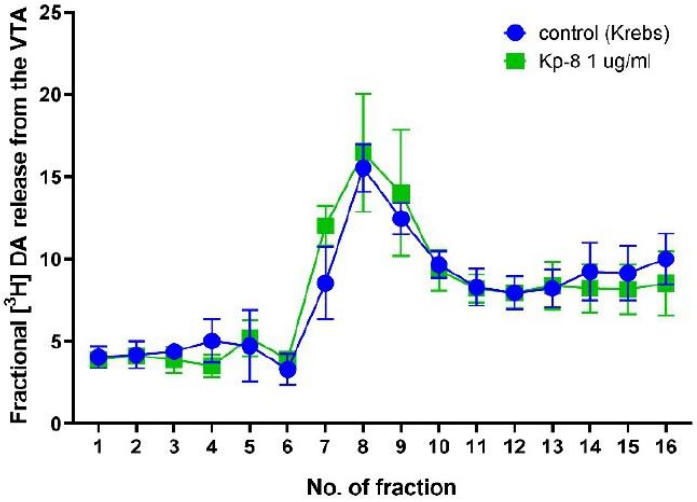

Figure 8. Fractional dopamine release from the ventral tegmental area. n = 4–5.

Likewise, Kp-8 did not influence fractional dopamine release from the NAc (Figure 9). However, there was a significant main effect for the time factor (F (3.134, 40.75) = 22.48, $p < 0.0001$). No significant main effect was found for the treatment factor (F (1, 13) = 0.0007717, p = 0.9783), and for the interaction between treatment and time (F (15, 195) = 0.4387, p = 0.9658). No significant difference could be detected at any specific time point between the groups.

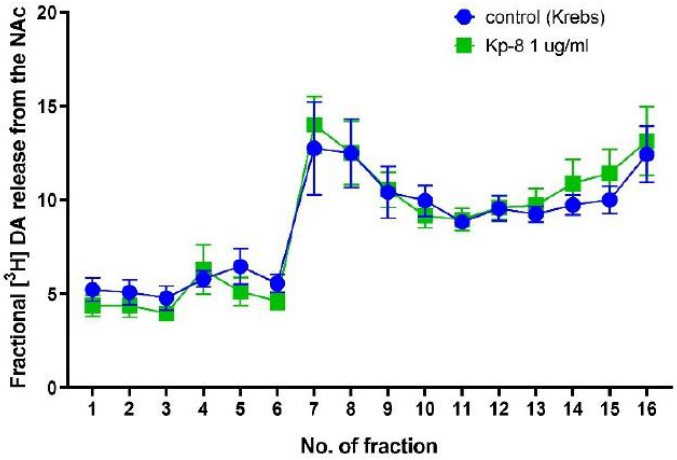

Figure 9. Fractional dopamine release from the nucleus accumbens. n = 7–8.

As shown in Figure 10, Kp-8 increased fractional GABA release from the NAc. There was a significant main effect for the time (F (2.227, 17.82) = 60.49, $p < 0.0001$) and interaction (F (15, 120) = 7.395, $p < 0.0001$) 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 ($p = 0.0039$).

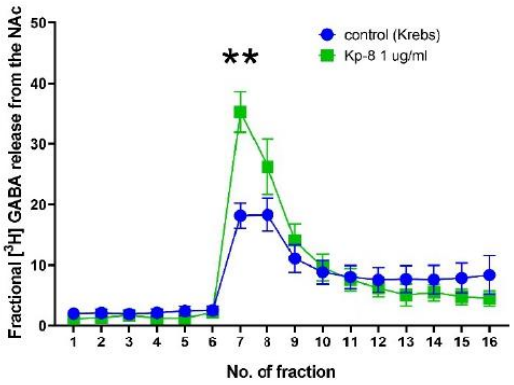

Figure 10. Fractional GABA release from the nucleus accumbens. ** $p < 0.01$ vs. control, n = 5.

4. Discussion

Short kisspeptin analogs are promising candidates in the treatment of infertility and other gynecological conditions [48]. Kisspeptins exert their effect on the reproductive axis via Kiss1r [4], but they also bind to and activate NPFF1 and NPFF2 receptors with lower affinity [11]. In our study, we investigated the behavioral and biological effects of icv. Kp-8 in male rats via performing a battery of behavioral tests (EPM, OF, MB), determining serum corticosterone and LH levels, as well as measuring dopamine release from the VTA and NAc, and GABA release from the NAc.

The 0.1 µg dose of Kp-8 (but not the 1 µg dose) decreased the percentage of open arm entries and open arm time in the EPM, which is characteristic of anxiety-like behavior [51]. It is in accordance with our previous experiments in which a preference for closed arms has been observed following icv. treatment with Kp-13 [37]. Still, it must be noted that only a higher dose of Kp-13 has exerted an anxiogenic action, whereas in the case of Kp-8 an approximately 10-times lower dose was effective. The dose–response curve of Kp-8 shows

a bell-shape (or inverted U-shape), that has been reported in several studies involving neuropeptides [56–58]. This phenomenon, when a lower dose is stimulatory, whereas a higher dose is inhibitory or ineffective, is called hormesis [59]. A review by Calabrese has reported a wide range of explanations for hormetic responses, including receptorial and intracellular mechanisms. For example, the same substance might have a stimulatory effect in a low dose, but an inhibitory effect in a high dose either via the same receptor (often mediated by a so-called 'molecular switch'), or via different receptors to which it has higher and lower affinity, respectively [60]. Based on a review on RF-amides and their receptors, kisspeptins in general can bind to their cognant receptor, Kiss1r, and to NPFF receptors with different affinity [3], the latter of which depends on the length of the peptide: the full length Kp (in rats Kp-52) has a lower affinity to NPFF receptors, whereas the shorter endogenous derivatives' binding affinity to NPFF receptors is higher. Furthermore, Rouméas et al. have performed systemic N-terminus deletions and benzoylations of Kp-10, which has revealed a progressive loss of affinity of the shorter fragments to Kiss1r and a conserved high affinity to NPFF receptors. In contrast, these shorter benzoylated fragments could still act as full agonists on Kiss1r, whereas on NPFF receptors a partial agonistic action has been observed [61]. How the benzoylation affects the affinity profile of these Kp-10 fragments is not known, yet it is possible that the unmodified Kp-8 also has an altered binding profile. In point of fact, agonists of NPFF1 and NPFF2 receptors have been implicated in anxiety [62,63]. Indeed, both Kiss1r and NPFF receptors could mediate the anxiety-like action of Kp-8, and we cannot rule out the possible activation of other receptors, as well.

Kp-8 also induced an elevation in serum corticosterone concentration: the 0.1 µg dose showed a tendency to increase it, whereas the 1 µg dose was significant. Corticosterone elevation is indicative of the activation of the HPA axis, the parvocellular neurons in the hypothalamic paraventricular nucleus (PVN) might have released CRH and AVP, followed by the secretion of ACTH from the pituitary, which consequently triggered the secretion of glucocorticoids from the adrenal cortex. In a study by Rao et al., Kp induced an increase in AVP mRNA expression in PVN-derived cell lines [36], and thus it is possible that Kp-8 activated the axis by increasing AVP release. Moreover, the activity of the HPA axis is modulated by limbic brain regions, including the amygdala [64]—an expression site of both Kp and Kiss1r [65]—which stimulates the HPA axis and regulates the behavioral response to stress [64,66]. The increase in glucocorticoid signaling in itself is also associated with anxiety-like behavior [67]. This result ties well with our previous study in which icv. Kp-13 has also caused an elevation in corticosterone concentration in a higher dose [37].

However, in the first 5 min of computerized open field test, the animals treated with 1 µg of Kp-8 spent more time in the central zone of the arena, which is considered a sign of anxiolysis [68]. As there was no difference in central locomotor activity at any other time point, this result should be interpreted with caution. At the beginning of the OF, the animals are placed in the center of the arena, so it is possible that the increase in central time reflects an initial latency in approaching the periphery rather than a real anxiolytic effect.

In addition, it is not uncommon to have discrepancies between the EPM and OF results. For example, chlordiazepoxide has reduced anxiety-like behavior in the EPM, but has had no significant effect in the OF in Lewis rats [69]. Although the principles of OF and EPM are similar, the two tests seem to load on different factors of anxiety [70]. Moreover, the approach of open arms in the EPM and central locomotion in the OF seem to be independently inherited in rats [71].

Altogether Kp-8 seemed to increase anxiety-like behavior and activate the HPA axis. These results are in accordance with the anxiogenic effect of icv. Kp-13 in rats [37] and the anxiolysis observed in *Kiss1r* KO mice [38]. However, Kp has not influenced anxiety in rats in the study by Rao et al. [36], which might be attributed to the peripheral route of administration and the relatively low dose of Kp used in the experiment. Likewise, intravenous Kp has had no effect on anxiety in human subjects [32]. Apart from the route of administration, another important factor to consider is the species: the Kp system of

zebrafish greatly differs from that of mammals, which might explain the anxiolytic property of kisspeptin observed in the study of Ogawa et al. [39]. Moreover, when compared to a systemic treatment, the regional modulation of neuronal activity can have strikingly different consequences. For example, the selective activation of kisspeptin neurons in the medial posterodorsal amygdala has decreased anxiety [40].

The number of total arm entries in the EPM reflects the general locomotor activity of the animals [72]. Both the 0.1 and 1 μg doses of Kp-8 reduced the number of arm entries, suggesting that Kp-8 might cause hypolocomotion.

The 60-min OF also yielded some remarkable results. When placed in a novel environment, all groups exhibited a pronounced exploratory activity with intense ambulation and a high number of rearings. Following a gradual decline until approximately 30 min, activity returned to a basal level. From that point, differences have started to appear among the groups, as there was a decrease in ambulation and rearing activity, as well as an increase in immobility in the group treated with 1 μg of Kp-8. These results point to a decrease in spontaneous locomotion.

Kp-8 has also significantly reduced the number and time of goal-oriented interaction with marbles in the MB. Although MB has long been considered a test for anxiety-like behavior, now several authors have expressed doubts about it [73]. According to Thomas, the number of buried marbles does not correlate with other anxiety-like traits, namely central time in the open field test and light-dark transitions in the light-dark box test [74]. The utility of the test as a screening tool for anxiety has also been questioned based on the findings that most anxiolytics and antidepressant drugs reduce marble burying behavior secondary to drug-induced hypolocomotion [73]. It has been suggested that digging and burying are species-specific, innate behavioral patterns that are likely triggered by an exploratory drive [75] or by the bedding itself [76]. Nowadays marble burying is regarded as a sign of repetitive, compulsive-like behavior, which is highly dependent on general locomotor activity [52]. Consequently, the reduction in goal-oriented interactions with the marbles is most likely a sign of suppressed locomotion in our study.

These findings contrast with the results reported by our group on the effects of icv. Kp-13, as it has increased exploratory and spontaneous locomotion in rats [37]. In a study by Tolson et al., *Kiss1r* KO female mice have exhibited a decrease in spontaneous locomotion and energy expenditure, but the mutation has had no such effect in male animals [43], pointing to a possibly gender-dependent effect.

Icv. Kp-8 has stimulated LH release in our study, which is a sign of reproductive axis activation, secondary to Kiss1R binding and activation in the hypothalamus. Kiss1R is expressed on hypothalamic GnRH neurons [4], and upon its activation repetitive LH pulses are generated [77]. In our study, the 1 μg dose of Kp-8 caused a significant increase in LH concentration. This is in accordance with literature data as icv. injection of a similar dose of Kp-10 has exerted an LH surge [41]. It must be noted though, that 0.1 μg of Kp-8 did not affect LH release. This is not surprising since Thomson et al. have obtained a similar result when Kp-10 was administered icv in a similarly low dose [41]. Furthermore, in a study by Pheng et el., icv. administered Kp-10 at a similarly low dose was unable to stimulate LH release in male rats, only the full length Kp-52 did. The authors have postulated that slower degradation of Kp-52 might explain their results, but it is also possible that the different binding profiles of Kp-52 and Kp-10 are in the background [78]. This also might explain our result, since Kp-8 similarly to Kp-10 might bind with higher affinity to the NPFF receptors, more specifically to NPFF1 receptor. One of the ligands of NPFF1 receptor is the RF-amide-related peptide 3 (RFRP-3), which has an inhibitory effect on the reproductive axis in adult male rats [79,80]. Thus, it is possible that at a lower dose, the two opposing actions of Kp-8 result in no change in LH concentration. Nevertheless, further studies are required to determine the affinity of Kp-8 to its receptors and the degree of calcium mobilization upon receptor activation. As the hypolocomotor effect of Kp-8 seems to be in contrast with previous studies on kisspeptin and locomotion [37,43], it is likely that this effect is mediated by other mechanisms.

One possible explanation is the activation of NPFF receptors. Kp-8 has activated human NPFF2 receptors in *Xenopus* oocytes [81], and its N-terminally benzoylated form has shown to fully preserve the affinity of Kp-10 to NPFF1 and NPFF2 receptors [61], which are universally activated by all members of the RF-amide family [3].

It is noteworthy that several members of the RF-amide family have been reported to modulate locomotor activity, pointing to the possible role of NPFF receptors in the regulation of locomotion. Similarly to Kp-8, icv. treatment with RF-amide related peptide 1 (RFRP-1) has reduced total locomotor activity and has also induced anxiety-like behavior and HPA axis activation [63]. Likewise, intra-VTA injection of NPFF has reduced spontaneous locomotion in rats [82]. Interestingly, icv. NPFF has inhibited morphine-induced hyperlocomotion, but has failed to affect the locomotor activity of naïve rats [83]. Although icv. neuropeptide AF (NPAF) has also had an anxiogenic effect, contrary to RFRP-1 and NPFF, it has stimulated spontaneous and exploratory locomotion [56].

Another possible reason for the development of hypolocomotion is the modulation of the mesocorticolimbic dopaminergic system. Based on the expression of kisspeptin in the NAc, as well as the expression of NPFF1 and NPFF2 receptors in the NAc and VTA [16,44], it was reasonable to investigate whether Kp-8 has a direct effect on the VTA-NAc circuitry. The dopaminergic pathway connecting the VTA and NAc has long been implicated in the regulation of locomotion. As a matter of fact, VTA dopaminergic neurons are responsible for the locomotor-enhancing effect of cocaine [84]. Our hypothesis was that Kp-8 might suppress locomotion by directly modulating the activity of VTA dopaminergic neurons. However, in our ex vivo superfusion study, Kp-8 has not affected dopamine release from slices obtained from the VTA and NAc.

As the interaction between Kp and GABA is known from the literature [85,86], it also seemed possible that Kp-8 might directly affect GABA release in NAc. GABAergic neurons in the NAc have been shown to inhibit dopaminergic projections from the VTA [87]. In fact, GABAergic activity can also be connected with the suppression of locomotion, as locomotor activity has increased when $GABA_A$ receptor antagonists were injected into the NAc core [46]. In our study, Kp-8 significantly increased GABA release from NAc slices. This result suggests that Kp-8 might directly modulate the activity of GABAergic neurons in NAc, which could contribute to the suppression of locomotor activity. It must be mentioned, however, that ex vivo superfusion measures only the direct effect of Kp-8 on live tissue slices obtained from the NAc, but the complex assessment of the whole VTA-NAc circuitry is beyond the scope of this method. Consequently, further studies (e.g., in vivo microdialysis) are required to confirm these findings on the circuit level.

When considering the receptors involved, it is possible that Kp-8 alters GABA release via NPFF1 or NPFF2 receptors, which are abundantly expressed in the VTA and the NAc, and likely involved in the modulation of both dopaminergic and GABAergic neuronal activity [16]. The role of NPFF receptors is further supported by the results of Cador et al., who have reported a decrease in novelty-induced locomotion upon intra-VTA NPFF treatment. Kiss1r expression, however, has only been detected in the NAc of humans, but not in rodents [88], so it is unlikely that Kp-8 could modulate NAc activity via Kiss1r.

Furthermore, the contribution of altered metabolism and thermoregulation should not be ruled out in the background of altered locomotor activity. Kisspeptin's stimulatory effect on locomotion seemed to be coupled with metabolic effects in the literature. Icv. Kp-13 has induced hyperthermia [37], and *Kiss1r* KO has resulted in obesity, increased adiposity and impaired glucose tolerance in female mice [43]. Kp could also be involved in hypothalamic appetite regulation by exciting proopiomelanocortin (POMC) neurons and inhibiting neuropeptide Y/Agouti-related peptide (NPY/AgRP) neurons, resulting in an anorexigenic effect [89]. Although only a few studies have addressed the metabolic effects of other RF-amides, they have usually revealed significant results. Icv. NPFF has reduced food intake in food-deprived rats [90] and also had a hypothermic effect in mice [91]. Moreover, the stimulation of central NPFF1 and NPFF2 receptors have evoked hypothermia and hyperthermia, respectively [92].

Alternatively, Kp-8 might modulate the activity of other, locomotion-related systems differently than the naturally occurring kisspeptins, resulting in an opposing effect of locomotion. For example, central Kp-10 treatment has stimulated vasopressin release in rats [93], and vasopressin has induced hyperlocomotion by acting on V1a receptors on hypothalamic orexin/hypocretin neurons in mice [94]. Furthermore, kisspeptin has been shown to induce BDNF expression in the hippocampus [95] and the lack of active BDNF in tissue plasminogen activator deficient mice has been associated with a decrease in nocturnal wheel running activity [96]. It is a question of future research to investigate whether Kp-8 could modulate vasopressin release and BDNF secretion in a similar or different fashion as other kisspeptins.

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Appendix A

Following solid-phase peptide synthesis, Kp-8 was analyzed using HPLC and ESI-MS, the results of which are shown in Figures A1 and A2, respectively.

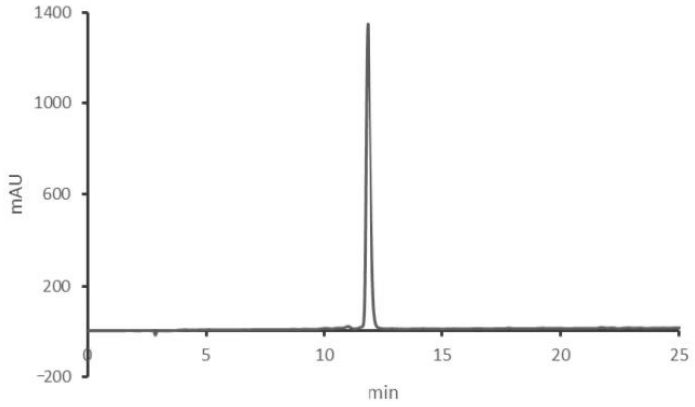

Figure A1. HPLC trace of Kisspeptin-8. Column: Phenomenex Luna C18, 5 µ, 100 Å, 4.6 mm × 250 mm, flow rate: 1 mL/min, wavelength: 220 nm, A eluent: 0.1% TFA in water, B eluent: 0.1% TFA/80% ACN/water, gradient: 30–55% eluent B in eluent A over 25 min.

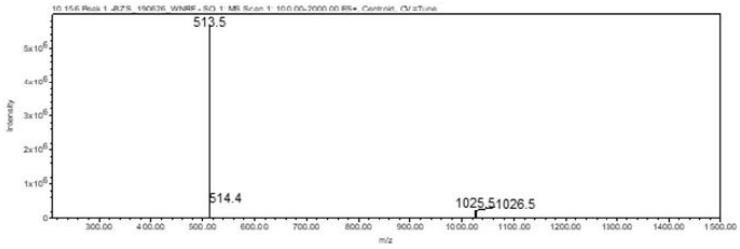

Figure A2. The ESI-MS trace of Kp-8 peptide.

Appendix B

During the computerized open field test, data were collected for 60 min, in 5-min timeframes. The cumulative results obtained after 60 min of data collection can be seen in Figure A3. There was no significant difference in any of the parameters measured: total distance of ambulation (Figure A3a, F (2, 34) = 1.691, $p = 0.1994$), total time of ambulation (Figure A3b, F (2, 34) = 1.728, $p = 0.1928$), time spent immobile (Figure A3c, F (2, 34) = 1.274, $p = 0.2927$), number of rearings (Figure A3d, F (2, 34) = 1.522, $p = 0.2328$), percentage of central ambulation distance (Figure A3e, F (2, 34) = 0.6885, $p = 0.5092$), and percentage of central ambulation time (Figure A3f, F (2, 34) = 0.7265, $p = 0.4910$).

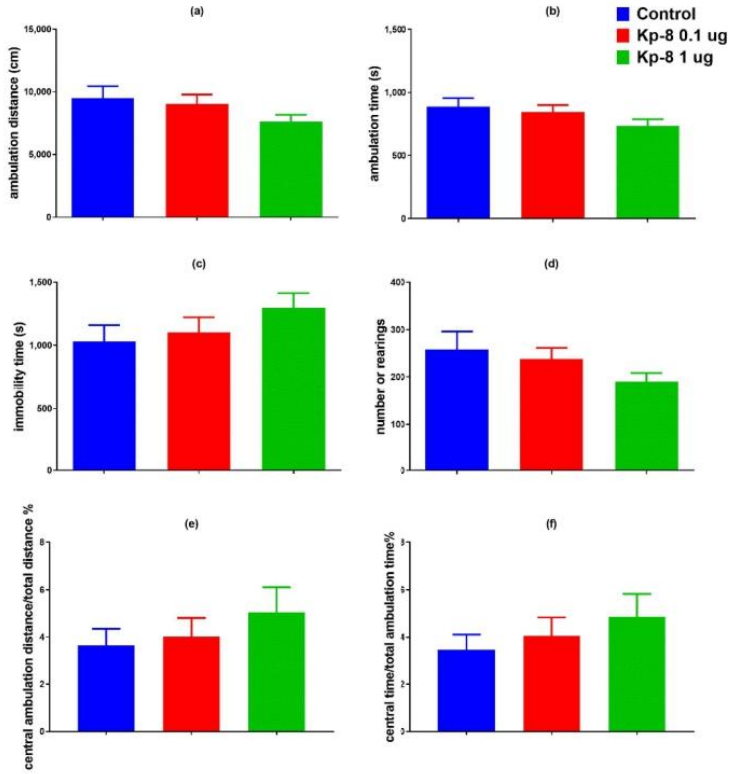

Figure A3. Cumulative data of 60-min open field test: (**a**) total distance of ambulation, (**b**) total time of ambulation, (**c**) total time spent immobile, (**d**) number of rearings, (**e**) percentage of distance travelled in the central zone, (**f**) percentage of time spent in the central zone, $n = 12–13$.

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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

Krisztina Csabafi *, Katalin Eszter Ibos, Éva Bodnár, Kata Filkor, Júlia Szakács and Zsolt Bagosi

Department of Pathophysiology, University of Szeged, P.O. Box 427, H-6701 Szeged, Hungary; filkor.kata@gmail.com (K.F.)
* Correspondence: csabafi.krisztina@med.u-szeged.hu; Tel.: +36-62-545993

Abstract: Previously, we reported that intracerebroventricularly administered kisspeptin-13 (KP-13) induces anxiety-like behavior and activates the hypothalamic-pituitary-adrenal (HPA) axis in rats. In the present study, we aimed to shed light on the mediation of KP-13's stress-evoking actions. The relative gene expressions of the corticotropin-releasing factor (*Crf*, *Crfr1*, and *Crfr2*) and arginine vasopressin (*Avp*, *Avpr1a*, and *Avpr1b*) systems were measured in the amygdala and hippocampus of male Wistar rats after icv KP-13 treatment. CRF and AVP protein content were also determined. A different set of animals received CRF or V1 receptor antagonist pretreatment before the KP-13 challenge, after which either an open-field test or plasma corticosterone levels measurement was performed. In the amygdala, KP-13 induced an upregulation of *Avp* and *Avpr1b* expression, and a downregulation of *Crf*. In the hippocampus, the mRNA level of *Crf* increased and the level of *Avpr1a* decreased. A significant rise in AVP protein content was also detected in the amygdala. KP-13 also evoked anxiety-like behavior in the open field test, which the V1 receptor blocker antagonized. Both CRF and V1 receptor blockers reduced the KP-13-evoked rise in the plasma corticosterone level. This suggests that KP-13 alters the AVP and CRF signaling and that might be responsible for its effect on the HPA axis and anxiety-like behavior.

Keywords: kisspeptin; anxiety; corticosterone; hypothalamic-pituitary-adrenal axis; corticotropin-releasing factor; arginine vasopressin; amygdala; hippocampus; stress

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1. Introduction

Kisspeptins (KPs) are amidated neurohormones part of the Arg-Phe (RF)-amide family with a pivotal role in the central organization of the hypothalamic-pituitary-gonadal axis [1,2]. KP was first isolated from the human placenta as the endogenous ligand of the orphan G-protein coupled receptor GPR54, now referred to as kisspeptin receptor 1 (KISS1R) [3,4]. KP is a 54-amino-acid-long product of the KiSS-1 gene that, via alternative splicing, generates shorter biologically active derivatives containing 14, 13, or 10 amino acids termed KP-14, KP-13, and KP-10, respectively [3,5]. KP and KISS1R are present in a wide range of tissues/organs such as the central nervous system, cardiovascular system, liver, and placenta. Most abundantly, they are expressed in the hypothalamus, more specifically in the arcuate nucleus and the anteroventral paraventricular nucleus (AVPV) in rodents. However, among others, two distinct brain regions also show a moderate-high expression of KP and KISS1R: the amygdala and the hippocampus [6–10], and so far, not much is known about the possible role of KP neurons in these brain regions. Next to KISS1R, KPs can bind to neuropeptide FF receptors (NPFFRs: NPFFR1 and NPFFR2) as well, although with lower affinity than to KISS1R [11,12]. Neuropeptide FF receptor 1

(Npffr1) mRNA is strongly expressed in several brain areas, among them the hypothalamus (e.g., paraventricular nucleus (PVN), periventricular nucleus) as well as in the central amygdaloid nucleus and medial amygdala [13–15]. Npffr2-expressing neurons were detected mainly in thalamic and brainstem nuclei, as well as in the hypothalamus [15].

KPs were first investigated in cancer biology as a metastasis suppressor [8], but later multiple studies demonstrated the pivotal role of the KP system in the central regulation of the reproductive axis [2,16]. Furthermore, an increasing body of research suggests that KP signaling might be involved in other neuroendocrine functions, as well as in the modulation of nociception, energy homeostasis, and behavior [17–19].

Previously we have reported that acute intracerebroventricular (icv) administration of KP-13 evokes an elevation of corticosterone 30 min after treatment and induced anxiety-like behavior in the elevated plus maze test and the traditional open field test, well-known behavioral tests for anxiety [19]. Furthermore, a shorter derivative of KP, KP-8 exerted a similar effect on the hypothalamus-pituitary-adrenal (HPA) axis and anxiety-like behavior [20]. Therefore, the objective of the present study was to further characterize the anxiety-inducing action of KP-13 and investigate its effect on two hormones well-known for the regulation of the neuroendocrine response to stress as well as stress-related behavior.

Corticotropin-releasing factor (CRF; also referred to as CRH) and arginine vasopressin (AVP) are both crucial regulators of the stress response [21–23]. It is well established that stressful stimuli activate the parvocellular neurosecretory cells of the PVN that express CRF as well as AVP, which in turn, via synergistic action on the corticotrophs of the anterior pituitary, cause the release of adrenocorticotropic hormone (ACTH) and, consequently, corticosterone in the adrenal gland of rodents [21,22]. Furthermore, the extra-hypothalamic release of CRF and AVP is involved in coordinating the endocrine and behavioral responses to stress.

Target cells discern hormonal stimuli by CRF using two distinct receptors: CRF 1 receptor (CRFR1) and CRF 2 receptor (CRFR2), out of which CRF has a high affinity to CRFR1 [22,24]. A plethora of data is available in the literature that attests to the important role of the CRF system in stress responsivity, anxiety, and depression. In fact, *Crfr1* knockout mice show an anxiolytic phenotype [24] that indicates that CRFR1 mediates an anxiety-like action; however, region-specific knockdown of *Crfr1* in globus pallidus externa caused an increase in anxiety-like behavior that highlighted that the effect of CRFR1 activation is brain region-dependent [25]. CRFR2 has been implicated in stress coping and overall CRFR2 activation mediates an anxiolytic effect [26,27]. Nevertheless, it seems that similarly to CRFR1, the effect of CRFR2 activation depends on the brain region. In the medial amygdala, for instance, it mediates anxiety-like behavior [28], whereas in the ventromedial hypothalamus, CRFR2 signaling mediates an anxiolytic effect [29].

AVP is also involved in the regulation of stress and associated behavior. AVP can bind to its three distinct receptor subtypes: V1a receptor (V1aR), V1b receptor (V1bR), and V2 receptor [30]. V2 is mainly found in the periphery, more specifically in the renal distal tubules, and is responsible for the antidiuretic action of AVP, whereas V1aR and V1bR next to peripheral expression are also present in the central nervous system and might mediate AVP's effect on stress, behavior, and mood [21,30,31]. AVP, released in the PVN, has a synergistic effect with CRF on the pituitary ACTH secretion [21]. Furthermore, several literature data point to AVP in the brain mediating an anxiogenic effect. Both the AVP-deficient Brattleboro rats and *V1aR* knockout mice show an anxiolytic phenotype [32,33]. Furthermore, icv administration of AVP induces anxiety-like behavior [34].

CRF and AVP are expressed in abundance in the amygdala and hippocampus. In fact, the highest expression of CRF outside of the hypothalamus is found in the amygdala [35,36]. Furthermore, CRF is coexpressed in subpopulations of hippocampal interneurons throughout the hippocampal layers [37]. These CRF-neurons activate upon stress and mediate stress-induced effects of the hippocampus [37,38]. Hypothalamic AVP-expressing fibers project to the amygdala, which expresses both V1aR and V1bR to exert its stress-inducing effect [39,40]. Also, AVP-producing neurons are found in the amygdala [39]. AVP signaling

is also involved in the regulation of hippocampal processes and consequently stress-related behaviors [41,42].

All the above-mentioned data highlight the important role these neuropeptides play in stress response and stress-related behavior. Based on this and our previous experiments [19,20], we hypothesized that KP-13 might alter the CRF and AVP signaling in the amygdala and hippocampus, two brain areas that are involved in the regulation of anxiety-like behavior and express KP and its receptors, as well as CRF and AVP and their receptors [36]. Therefore, the purpose of the present study was to assess if KP influences the CRF and AVP expression in the amygdala and hippocampus and if these two stress hormones might mediate KP's anxiety- and HPA axis-inducing effects. First, the expressions of *Crf*, *Crfr1*, *Crfr2*, *Avp*, *Avpr1a*, and *Avpr1b* were measured after KP-13 treatment to assess if KP-13 influences the expression of these genes in the amygdala and hippocampus. Next, we also determined CRF and AVP protein contents in these brain regions. To see if they might mediate KP-13's anxiety-inducing and HPA-activating effect, animals were pretreated with a non-selective CRF or VP antagonist, after which the behavior of the animals was recorded in a computerized open field test or trunk blood was collected to measure the plasma corticosterone level.

2. Materials and Methods

2.1. Animals and Housing Conditions

Adult male Wistar rats (Domaszék, Csongrád, Hungary) that weighed 160–250 g were used at the age of 7–8 weeks. They were housed under controlled conditions (12/12 h light/dark cycle, lights on from 6:00 a.m., at constant room temperature) and 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 (X./1207/2018). Approximately 180 animals in total were used in our experiments. Every experiment was carried out separately; the same animal was never used for different experimental procedures.

2.2. Surgery

The animals were allowed 1 week to acclimatize before surgery. Then, they were implanted with a stainless steel Luer cannula (10 mm long) aimed at the right lateral cerebral ventricle under pentobarbital (35 mg/kg, intraperitoneally) anesthesia. The stereotaxic coordinates were 0.2 mm posterior and 1.7 mm lateral to the bregma, and 3.7 mm deep from the dural surface, according to the atlas of Paxinos et al. [43]. The cannula was secured to the skull with dental cement and acrylate. Further experiments were conducted after a recovery period of 7 days. All experiments were carried out between 8:00 and 10:00 a.m.

At the end of the experiments, the correct position and the permeability of the cannula were checked. In the behavioral studies, each rat was sacrificed under pentobarbital anesthesia, and in the endocrinological experiments, the head was collected after decapitation. Methylene blue was injected via the implanted cannula and the brains were then dissected. Only data from animals exhibiting the diffusion of methylene blue in all the ventricles were included in the statistical evaluation.

2.3. Treatment

Rats were injected with different doses of KP-13 (Bachem Ltd., Bubendorf, Switzerland) 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 the open field test, 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 [19] 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., Bubendorf, Switzerland), a non-selective CRFR blocker in a dose

of 1 µg, and a V1R antagonist (Bachem Ltd., Bubendorf, 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 [44–46]. Control animals received saline alone. After KP-13 administration, animals were sacrificed at different time points (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. The experimental setup can be viewed in Figure 1.

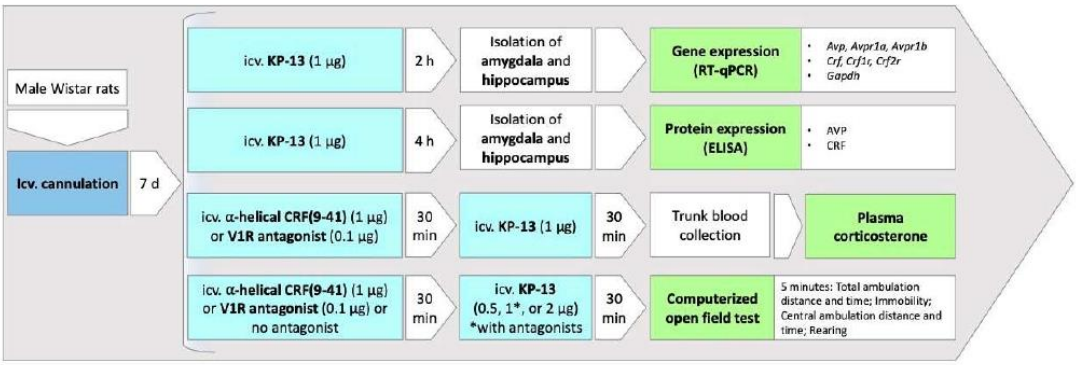

Figure 1. Experimental setup. Abbreviations: icv.: intracerebroventricular; KP-13: kisspeptin-13; V1R: V1 receptor; Avp: Arginine vasopressin, Avpr1a: arginine vasopressin receptor 1A, Avpr1b: arginine vasopressin 1B, Crf: corticotropin-releasing factor, Crfr1: corticotropin-releasing factor receptor 1, Crfr2: corticotropin-releasing factor receptor 2, Gapdh: glyceraldehyde 3-phosphate dehydrogenase.

2.4. mRNA Extraction and Quantitative Real-Time PCR

Two hours after icv. KP-13 administration, the animals were sacrificed by decapitation. After isolation of the brain, they were dissected with a pre-cooled adult mouse brain matrix (Ted Pella Inc., Redding, CA, USA). Next, the brains were manually sliced with pre-cooled razor blades in coronal sections (1 mm slots), after which the brain regions were dissected on ice with the guidance of a rat brain atlas [43]. An amount of 1 mm in diameter tissue punches (Ted Pella Inc., Redding, CA, USA) was 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 °C until gene expression analysis. To purify and isolate RNA from the samples obtained from the amygdala and hippocampus, samples were homogenized with an ultrasonic homogenizer on ice, and then the 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; primer sets are listed in Table 1, respectively. The cycling protocol is listed in Table 2. For controls, we used reaction mixtures without cDNA. Each sample was run in duplicates. The ratio of each mRNA relative to the housekeeping gene was calculated using the $2^{-\Delta\Delta CT}$ method, and the relative gene expressions were determined for data presentation.

Table 1. Custom primers.

Genes	Forward (5′ → 3′)	Reverse (5′ → 3′)
Avp	CTG ACA TGG AGC TGA GAC AGT	CGC AGC TCT CGT CGC T
Avpr1a	TGG ACC GAT TCA GAA AAC CCT	GTT GGG CTC CGG TTG TTA GA
Avpr1b	CAG CAT AGG AGC CAA CCA TCA A	GAA AGC CCA GCT AAG CCG T
Crf	TGG TGT GGA GAA ACT CAG AGC	CAT GTT AGG GGC GCT CTC TTC
Crfr1	CGA AGA GAA GAA GAG CAA AGT ACA C	GCG TAG GAT GAA AGC CGA GA
Crfr2	CCC GAA GGT CCC TAC TCC TA	CTG CTT GTC ATC CAA AAT GGG T
Gapdh	CGG CCA AAT CTG AGG CAA GA	TTT TGT GAT GCG TGT GTA GCG

Abbreviations: Avp: Arginine vasopressin, Avpr1a: arginine vasopressin receptor 1A, Avpr1b: arginine vasopressin 1B, Crf: corticotropin-releasing factor, Crfr1: corticotropin-releasing factor receptor 1, Crfr2: corticotropin-releasing factor receptor 2, Gapdh: glyceraldehyde 3-phosphate dehydrogenase.

Table 2. Real-time polymerase chain reaction cycling protocol.

Steps	Temperature °C	Time	Number of Cycles
Uracil DNA glycosylase pretreatment	50	2 min	1
Initial denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing	60	30 s	
Extension	72	30 s	

2.5. Enzyme-Linked Immunosorbent Assay (ELISA)

CRF and AVP content was measured in brain tissue extracts obtained from the amygdala and hippocampus. Animals were decapitated 4 h after icv. treatment. After isolation of the brain, the amygdala and hippocampus were immediately dissected with a pre-cooled adult mouse brain matrix (Ted Pella Inc., Redding, CA, USA). Next, brains were manually sliced with pre-cooled razor blades in coronal sections (1 mm slots), after which the brain regions were dissected on ice with the guidance of a rat brain atlas [43]. An amount of 1 mm in diameter tissue punches (Ted Pella Inc., Redding, CA, USA) was taken from the amygdala and hippocampus, then placed in Eppendorf tubes and immersed in liquid nitrogen to snap freeze. The samples were stored at −80 °C until the assays were performed. 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). CRF and AVP were then measured using a competitive CRF and AVP ELISA kit (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA; EK-019-06, EK-065-07), according to the manufacturer's instruction.

2.6. Open Field Test

In the open field test, novelty-induced locomotor activity was assessed via the Conducta 1.0 System (Experimetria Ltd., Budapest, Hungary). The system consists of black plastic open field arenas (inside dimensions: 48 × 48 cm, height: 40 cm) with five horizontal rows of infrared diodes on the walls to register both horizontal and vertical locomotion. The center of each box is illuminated by an LED lightbulb (230 lumens) from above the box. The central zone of the arena is defined as a 24 × 24 cm area in the center of the box (Figure 2). The rats were removed from their home cages and placed at the center of the box 30 min after icv. peptide treatment and their behavior was recorded by the Conducta computer program for 5 min. Six behavioral parameters were measured during the experiment: total ambulation distance, total ambulation time, 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 (Figure 2). In addition, central ambulation dis-

tance/total ambulation distance% and central ambulation time/total ambulation time% were calculated from raw data. The open field experiments were conducted between 8 a.m. and 10 a.m. and the apparatus was cleaned with 96% ethyl-alcohol after each session.

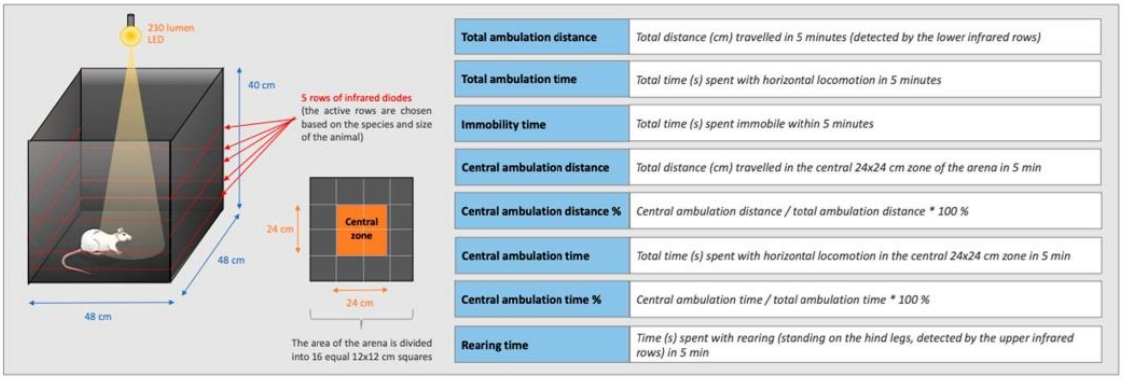

Figure 2. Schematic presentation of the open field box and the open-field parameters.

2.7. Plasma Corticosterone Measurement

To determine plasma corticosterone concentrations, trunk blood was collected in heparinized tubes. The plasma corticosterone concentration was measured by the fluorescence assay described by Zenker and Bernstein [47] as modified by Purves and Sirett [48].

2.8. Statistical Analysis

Data are presented as means ± SEM. The prerequisites of ANOVA were assessed via histograms, skewness, and kurtosis, the Kolmogorov–Smirnov and Levene's tests. Statistical analysis of the PCR results was performed by Mann–Whitney's test. For all other data, estimated marginal means were calculated and analyzed by analysis of variance (ANOVA). For the effect of different doses of KP-13 on open field test parameters, one-way ANOVA was employed, followed by the Bonferroni post hoc test for multiple comparisons when the test prerequisites were fulfilled. When the test of the homogeneity of variances was not satisfied, nonparametric ANOVA on ranks (Kruskal–Wallis) was performed, followed by Dunn's test for multiple comparisons. For the evaluation of ELISA results and all tests with combined treatments two-way ANOVA was performed followed by Bonferroni post hoc test for multiple comparisons. A probability level of less than 0.05 was accepted as indicating a statistically significant difference. The statistical analysis was carried out by SPSS.

3. Results

3.1. qPCR

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 since the normality test and histograms showed a non-normal distribution of the data.

3.1.1. Gene Expression in the Amygdala

In the amygdala, the mRNA expression of *Avp* ($Mdn_{control} = 1$, $Mdn_{KP\text{-}13} = 3.646$, $U = 8$, and $p = 0.0057$) and *Avpr1b* ($Mdn_{control} = 1$, $Mdn_{KP\text{-}13} = 1.359$, $U = 20$, and $p = 0.0135$) significantly increased, whereas the expression of *Crf* ($Mdn_{control} = 1$, $Mdn_{KP\text{-}13} = 0.6726$, $U = 0$, and $p = 0.0002$) was reduced compared to the control group. In the case of *Avpr1a* ($Mdn_{control} = 1$, $Mdn_{KP\text{-}13} = 0.9272$, $U = 36$, and $p = 0.709$), *Crfr1* ($Mdn_{SHAM} = 1$, $Mdn_{CKD} = 0.8754$, $U = 32$, and $p = 0.9999$) and *Crfr2* ($Mdn_{SHAM} = 1$, $Mdn_{CKD} = 1.244$,

U = 36, and $p = 0.709$), no significant difference was detected between the two groups (Figure 3).

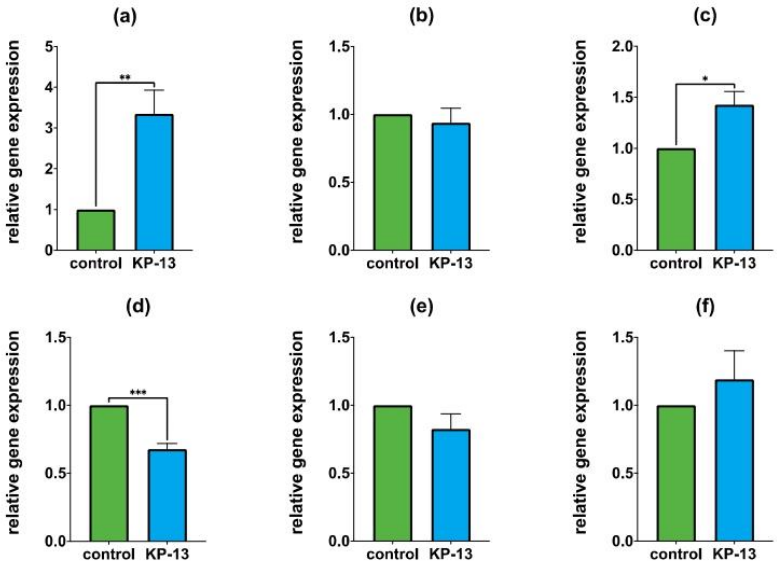

Figure 3. Relative gene expression in the amygdala: (**a**) *Avp*; (**b**) *Avpr1a;* (**c**) *Avpr1b;* (**d**) *Crf;* (**e**) *Crfr1;* and (**f**) *Crfr2*; mean + SEM, $n = 8–9$; * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$; Abbreviations: Avp: arginine vasopressin, Avpr1a: arginine vasopressin receptor 1A, Avpr1b: arginine vasopressin receptor 1B, Crf: corticotropin-releasing factor, Crfr1: corticotropin-releasing factor receptor 1, Crfr2: corticotropin-releasing factor receptor 2, KP-13: kisspeptin-13.

3.1.2. Gene Expression in the Hippocampus

In the hippocampus, the relative gene expression of *Crf* ($Mdn_{control} = 1$, $Mdn_{KP-13} = 1.504$, U = 6, and $p = 0.0476$) was significantly higher in the KP-13-treated group. On the other hand, *Avpr1a* ($Mdn_{control} = 1$, $Mdn_{KP-13} = 0.7788$, U = 0, $p = 0.0002$) mRNA expression showed a marked decrease. In the case of *Avpr1b* ($Mdn_{control} = 1$, $Mdn_{KP-13} = 1.644$, U = 5, and $p = 0.127$), *Avp* ($Mdn_{control} = 1$, $Mdn_{KP-13} = 0.7133$, U = 12, and $p = 0.3636$), *Crfr1* ($Mdn_{SHAM} = 1$, $Mdn_{CKD} = 0.8939$, U = 21, and $p = 0.69$) and *Crfr2* ($Mdn_{SHAM} = 1$, $Mdn_{CKD} = 0.6008$, U = 21, and $p = 0.69$), no significant difference was detected (Figure 4).

3.2. Enzyme-Linked Immunosorbent Assay

A two-factor analysis of variance on AVP protein level revealed a significant main effect for the treatment factor [$F(1,18) = 13.416$, $p = 0.002$], region factor [$F(1,18) = 22.869$, $p < 0.001$]. There was no significant interaction between the two factors [$F(1,18) = 1.432$, $p = 0.250$), 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 ($p = 0.002$); however, it had no effect in the hippocampus ($p = 0.125$) (Figure 5).

A two-way ANOVA on CRF protein content showed a significant main effect for the region factor [$F(1,17) = 13.235$, $p = 0.003$]. However, no significant main effect for the treatment factor [$F(1,17) = 0.018$, $p = 0.896$]. KP-13 treatment did not affect CRF protein content (Figure 5).

3.3. Plasma Corticosterone Level

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 [F(1,54) = 19.997; $p < 0.001$] and a statistically significant interaction between the two factors [F(2,54) = 9.058; $p < 0.001$], thus, the effect of KP-13 depends on which antagonist pretreatment was applied. There was no significant main effect for the antagonist treatment factor [F(2,54) = 2.752; $p = 0.074$]. Pairwise comparisons revealed that KP-13 treatment caused a marked elevation in the corticosterone concentration ($p < 0.001$) compared to the saline-treated group. Furthermore, among the KP-13-treated animals, both CRFR antagonists ($p = 0.025$) and V1R antagonists ($p < 0.001$) pretreated were significantly different (Figure 6).

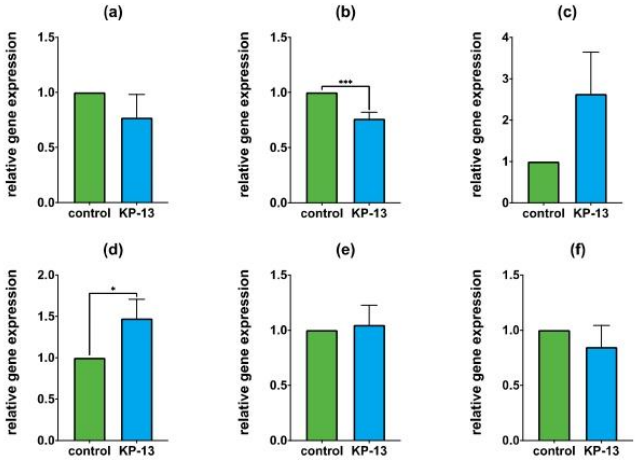

Figure 4. Relative gene expression in the hippocampus: (**a**) *Avp*; (**b**) *Avpr1a*; (**c**) *Avpr1b*; (**d**) *Crf*; (**e**) *Crfr1*; (**f**) *Crfr2*; mean + SEM, $n = 5–8$; * $p < 0.05$, *** $p < 0.001$; Abbreviations: Avp: arginine vasopressin, Avpr1a: arginine vasopressin receptor 1A, Avpr1b: arginine vasopressin receptor 1B, Crf: corticotropin-releasing factor, Crfr1: corticotropin-releasing factor receptor 1, Crfr2: corticotropin-releasing factor receptor 2, KP-13: kisspeptin-13.

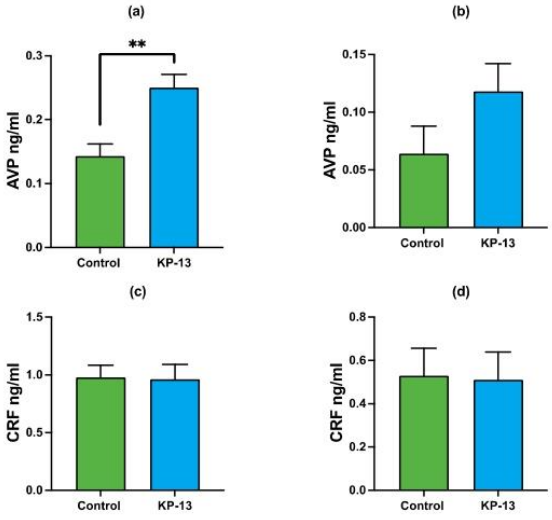

Figure 5. Arginine vasopressin and corticotropin-releasing factor protein expression in the amygdala and hippocampus: (**a**) AVP in the amygdala; (**b**) AVP in the hippocampus; (**c**) CRF in the amygdala; (**d**) CRF in the hippocampus; mean + SEM, $n = 4–6$; ** $p < 0.01$; Abbreviations: AVP: arginine vasopressin, CRF: corticotropin-releasing factor, KP-13: kisspeptin-13.

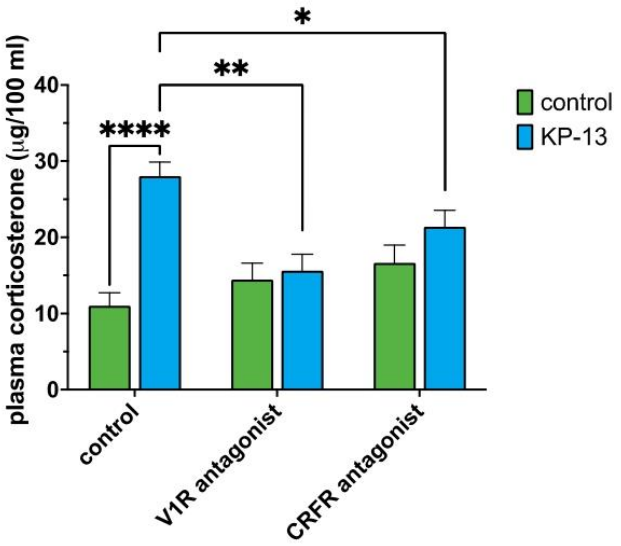

Figure 6. Plasma corticosterone results: mean + SEM, n = 7–13; * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$; Abbreviations: V1R: V1 receptor, CRFR: corticotropin-releasing factor receptor, KP-13: kisspeptin-13.

3.4. Open Field Test

3.4.1. The Effect of KP-13 on Open-Field Behavior

Univariate ANOVA was used to investigate the effect of KP-13 treatment on the following open-field parameters: total ambulation distance and time, immobility time, rearing activity, central ambulation distance and time, central ambulation distance/total ambulation distance%, and central ambulation time/total ambulation time%. Our result showed that KP-13 has no significant on total ambulation distance [$F(3,39) = 0.888$; $p = 0.457$] and total ambulation time [$F(3,39) = 1.611$; $p = 0.204$]. In the case of immobility time [$F(3,39) = 2.831$; $p = 0.052$], 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 (Tukey HSD revealed $p = 0.048$). KP-13 has a significant effect on rearing activity [$F(3,39) = 4.368$; $p = 0.01$]. Again, the 2 μg dose of KP-13 was the most effective ($p = 0.007$). 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 [Kruskal–Wallis $H(3) = 15.831$; $p = 0.001$]. Pairwise comparisons with Bonferroni correction revealed that both 1 μg ($p = 0.001$) and 2 μg ($p = 0.036$) of KP-13 significantly decreased the distance traveled in the center of the open field arena. KP-13 had a significant effect on central ambulation time [$F(3,39) = 5.6$; $p = 0.003$]. Pairwise comparisons revealed that both 1 μg ($p = 0.006$) and 2 μg ($p = 0.014$) 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% [$F(3,39) = 6.367$; $p = 0.001$]. Pairwise comparisons showed that both 1 μg ($p = 0.001$) and 2 μg ($p = 0.023$) of KP-13 were significant compared to the control. In the case of the central ambulation time/total ambulation time%, the result was similar [$F(3,39) = 5.439$; $p = 0.003$]. Again, both the 1 μg dose ($p = 0.006$) as well as the 2 μg dose ($p = 0.019$) of KP-13 was found to be significant (Figures 7 and 8).

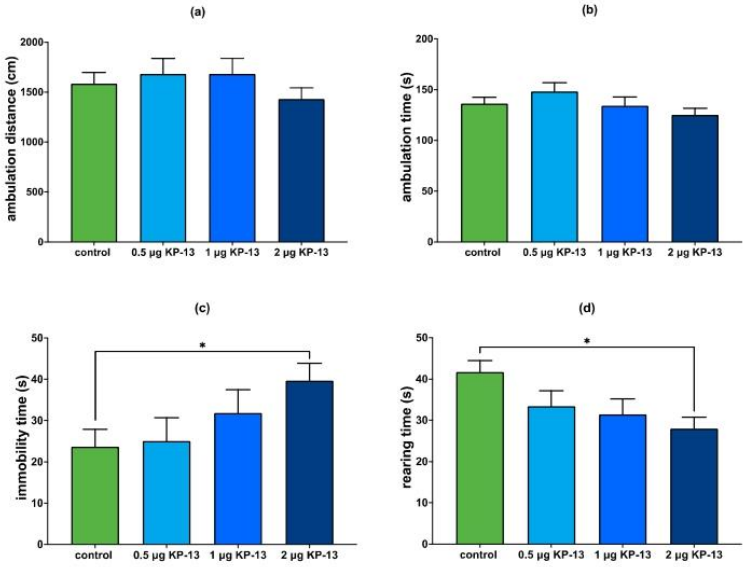

Figure 7. Ambulation in the open field test: (**a**) total distance traveled; (**b**) total time of ambulation; (**c**) immobility time; (**d**) number or rearings; mean + SEM, $n = 4–6$; * $p < 0.05$; Abbreviation: KP-13: kisspeptin-13.

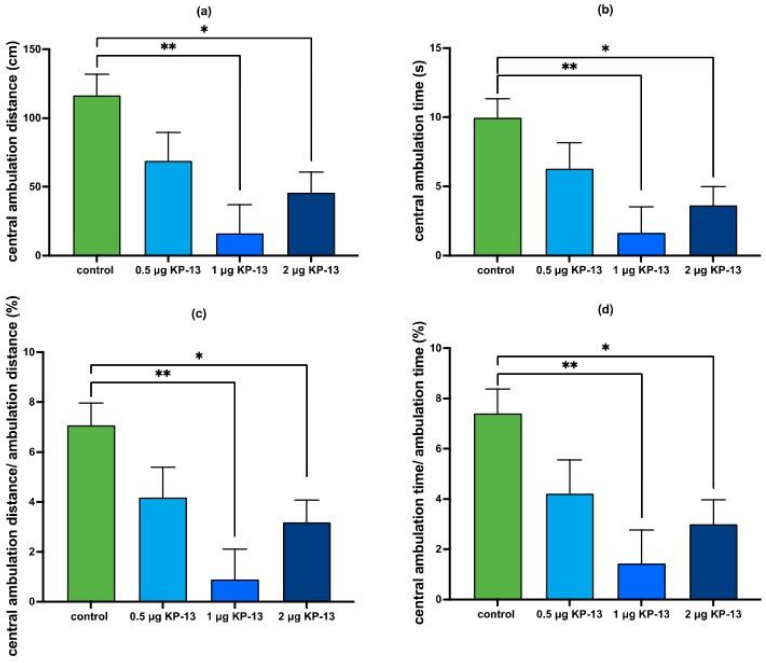

Figure 8. Central ambulation in the open field test: (**a**) distance traveled in the center of the arena; (**b**) time spent in the center of the arena; (**c**) central ambulation distance/total ambulation distance %; (**d**) central ambulation time/total ambulation time %; mean + SEM, $n = 4–6$; * $p < 0.05$, ** $p < 0.01$; Abbreviation: KP-13: kisspeptin-13.

3.4.2. The Effect of V1R and CRFR Antagonists on KP-13-Induced Open-Field Behavior

Two-way ANOVAs were conducted to investigate the effect of KP-13 treatment in the presence of CRFR and V1R antagonist pretreatment on open-field parameters. There were no significant changes in the case of ambulation distance [KP-13 treatment: $F(1,59) = 0.688$; $p = 0.410$, antagonist treatment: $F(2,59) = 0.360$; $p = 0.699$, interaction: $F(2,59) = 0.354$; $p = 0.704$] and ambulation time [KP-13 treatment: $F(1,59) = 2.725$; $p = 0.105$, antagonist treatment: $F(2,59) = 0.490$; $p = 0.615$, interaction: $F(2,59) = 0.598$; $p = 0.553$]. In the case of immobility time, there was a significant main effect for the treatment factor ($F(1,59) = 5.272$; $p = 0.026$), but no significant difference for antagonist treatment or between the two factors [antagonist treatment: $F(2,59) = 0.348$; $p = 0.708$, interaction: $F(2,59) = 0.048$; $p = 0.953$]. Pairwise comparisons showed no significant difference between groups. There were no significant changes in the case of rearing activity [KP-13 treatment: $F(1,59) = 0.897$; $p = 0.348$, antagonist treatment: $F(2,59) = 2.660$; $p = 0.079$, interaction: $F(2,59) = 0.434$; $p = 0.560$] (Figure 9).

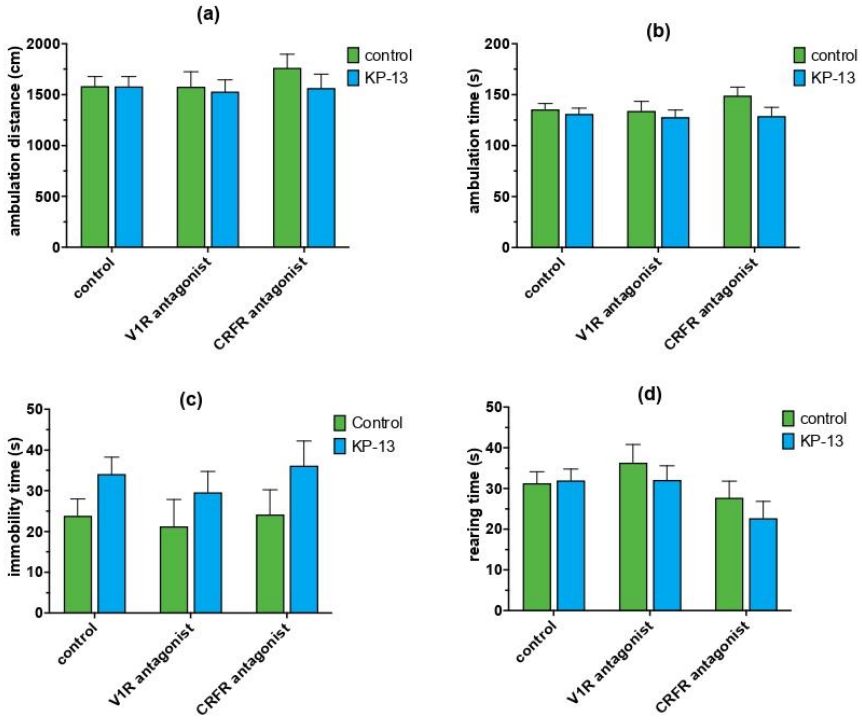

Figure 9. Ambulation in the open field test in the presence of V1R and CRFR antagonists in the open field test: (**a**) total distance traveled; (**b**) total time of ambulation; (**c**) immobility time; (**d**) number or rearings; mean + SEM, $n = 6$–15; Abbreviations: V1R: V1 receptor, CRFR: corticotropin-releasing factor receptor, KP-13: kisspeptin-13.

However, in the case of central ambulation distance, our result showed a statistically significant main effect for the KP-13 treatment [$F(1,59) = 10.665$; $p = 0.002$], but no statistically significant main effect for the antagonist treatment factor [$F(2,59) = 2.501$; $p = 0.091$] or the interaction between the two factors [$F(2,59) = 2.724$; $p = 0.075$]. Pairwise comparisons revealed that KP-13 treatment caused a marked decrease in the central ambulation distance 30 min after treatment ($p < 0.001$) compared to the saline-treated group. Among the KP-13-treated animals, CRFR antagonist ($p = 0.08$) pretreatment was not significantly differ-

ent. However, V1R antagonist ($p = 0.006$) pretreatment showed a statistically significant difference (Figure 10).

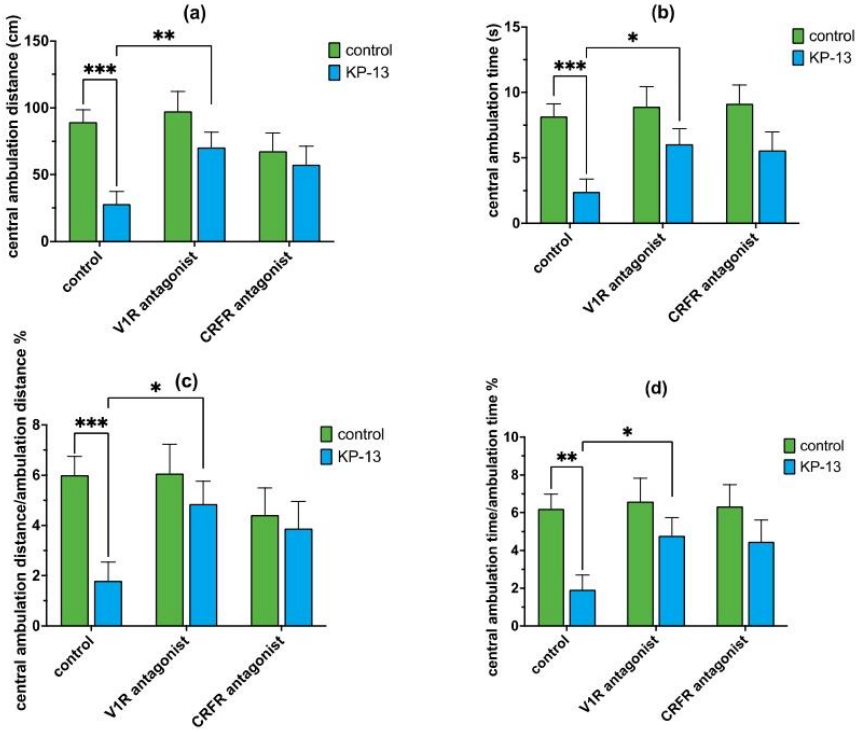

Figure 10. Central ambulation in the presence of V1R and CRFR antagonists in the open field test: (**a**) distance traveled in the center of the arena; (**b**) time spent in the center of the arena; (**c**) central ambulation distance/total ambulation distance %; (**d**) central ambulation time/total ambulation time %; mean + SEM, $n = 6$–15; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Abbreviations: V1R: V1 receptor, CRFR: corticotropin-releasing factor receptor, KP-13: kisspeptin-13.

In the case of central ambulation time, our results showed a statistically significant main effect for the KP-13 treatment [$F(1,59) = 15.355$; $p < 0.001$] similar to central ambulation distance, but no statistically significant main effect for the antagonist treatment factor [$F(2,59) = 2.389$; $p = 0.101$] or the interaction between the two factors [$F(2,59) = 0.885$; $p = 0.418$]. Pairwise comparisons revealed that the KP-13 treatment caused a significant decrease in the central ambulation time ($p < 0.001$) compared to the saline-treated group. Among the KP-13-treated animals V1R antagonist ($p = 0.021$) pretreatment showed a statistically significant difference, however, CRFR antagonist ($p = 0.071$) pretreatment was not significant. In the case of central ambulation distance/total ambulation distance%, again the main effect for KP-13 treatment was found significant [$F(1,59) = 6.339$; $p = 0.015$], however, no significant main effect was detected for the antagonist treatment [$F(2,59) = 1.540$; $p = 0.224$] and the interaction between the two factors [$F(2,59) = 2.531$; $p = 0.089$]. Pairwise comparison showed that KP-13 injection evoked a marked decrease in the central ambulation distance/total ambulation distance% of animals ($p < 0.001$) and among the KP-13 treated groups, the V1R antagonist pretreatment was statistically significant ($p = 0.011$), 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 ($p = 0.119$). 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 [F(1,59) = 10.019; $p = 0.003$] and there was no effect for the antagonist treatment [F(2,59) = 1.797; $p = 0.175$] or the interaction [F(2,59) = 1.200; $p = 0.309$]. A pairwise comparison found that KP-13 caused a significant decrease in central ambulation time/total ambulation time% ($p = 0.003$). Furthermore, the V1R antagonist significantly decreased the KP-13-evoked fall in central ambulation time/total ambulation time% ($p = 0.025$), whereas CRFR antagonist treatment among the KP-13-treated animals showed no statistically significant difference ($p = 0.072$) (Figure 10).

4. Discussion

Previously, we have reported that KP-13 injection into the lateral ventricle of rats activated the HPA axis and induced anxiety-like behavior in the elevated plus maze and open field tests [19]. In the present study, we aimed to further characterize the anxiety-inducing action of KP-13 and investigate its effect on the expression of two hormones and their receptors, well-known for the regulation of the endocrine, behavioral, and autonomic response to stress: CRF and AVP in the amygdala and hippocampus.

In our experiments, KP-13 caused brain region-specific changes in the gene expression of the AVP and CRF systems. In the amygdala, KP-13 induced a significant upregulation of AVP expression, both at mRNA and protein levels. Although the majority of KP neurons are found in the hypothalamus, a significant population is also present in the amygdala [49]. In rodents, amygdalar KP expression seems to be confined to the medial amygdala (MeA), most prominently to the posterodorsal subnucleus of MeA (MePD). KP neurons in the MeA have been found to maintain reciprocal connections with the accessory olfactory bulb, and project to the hypothalamic GnRH neurons [49]. So far, studies have focused on investigating the role of these KP neurons in the regulation of the reproductive axis. Literature data suggest that the stimulation of KP neurons in the MePD increases the LH pulse frequency possibly mediated by both GABAergic and glutamatergic signaling [50], and sexual behavior [17]. Nevertheless, the MeA also plays a role in the processing of emotional signals, therefore, it might be involved in the mediation of anxiety [51]. In the MeA, a sexually dimorphic population of AVP neurons has been detected, which sends direct and indirect projections to the hypothalamic PVN and triggers greater recruitment of AVP neurons in the PVN following stressful stimuli [40], consequently leading to increased stress responsiveness. Since vasopressin fibers have been found in close apposition with KP neurons in the MePD [49], KP might play a direct role in the activation of AVP neurons in the amygdala. In addition, AVP-expressing neurons in the amygdala are under the control of circulating gonadal steroids, especially in male rodents [52]. In fact, in castrated male mice, treatment with exogenous testosterone has induced hypomethylation of the AVP promoter in the MePD and the bed nucleus of the stria terminalis (BNST), thereby increasing AVP expression [52]. Furthermore, AVP neurons in the MePD have also been involved in integrating pheromonal and hormonal information and regulating sexual behavior. In male Wistar rats, AVP neurons in the MePD were activated following exposure to an inaccessible female [53]. Since the activation of KP neurons leads to the activation of the reproductive axis and consequently the elevation in circulating gonadal steroids [2,16], it is also possible that KP-13′s action on the amygdalar AVP expression is mediated indirectly by testosterone. Furthermore, circulating gonadal steroids, in turn, positively modulate KP expression in the amygdala [54,55].

Kp-13 also induced upregulation of *Avpr1b* in the amygdala. V1bRs have been implicated in anxiety since treatment with a selective nonpeptide V1b antagonist (SSR149415) has exerted an anxiolytic-like and antidepressive-like effect in a battery of behavioral tests in mice [56]. Likewise, microinfusion of SSR149415 into the basolateral amygdala has reduced anxiety-like behavior in male rats [57]. Overall, an increased *Avp* and *Avpr1b* expression is in accordance with our previous result that KP-13 induces anxiety-like behavior in rats [19,20].

Interestingly, the expression of amygdalar *Crf* decreased in response to KP-13. According to the literature, amygdalar CRF has mostly been associated with anxiety. For instance,

the activation of CRF-expressing neurons in the central amygdala using cre-dependent AAV-DREADD in CRF-cre mice has induced anxiety-like behavior, whereas the inhibition of these neurons has reduced anxiety [36]. It should be noted that although recently a novel population of GABAergic CRF neurons has: been described in the lateral central amygdala (CEA) which sends projections to the ventral tegmental area (VTA) and exerts an anxiolytic-like effect, likely mediated by CRFR1 receptors [58,59]. Since whole amygdala samples were used in our gene expression study, it cannot be determined which amygdalar neuron population was *Crf* downregulated. Nevertheless, it must be noted that whereas *Crf* gene expression was downregulated in the case of KP-13 icv injection, no significant change was detected in the protein level of CRF in the amygdala.

In the hippocampus, KP and KISS1R are expressed in high densities in the granule cell layer of the dentate gyrus and have also been detected in a low density in the pyramidal cells of CA1 and CA3 [38,60]. It was thus suggested that KP signaling might be involved in hippocampal functions such as learning and memory. Central administration of KP-13 facilitate passive avoidance consolidation [61] and it seems to alleviate amyloid-beta neurotoxicity in the hippocampus [62,63]. Additionally, KP has been implicated in the regulation of BDNF expression and neurogenesis in the hippocampus [64,65], but its connection to CRF, AVP, and their receptors has not been explored in this region yet. In our study, a significant upregulation of *Crf* and downregulation of *Avpr1a* was found following KP-13 treatment in the hippocampus. CRF is expressed in basket-type GABAergic interneurons of the pyramidal cell layer [38,60] and the upregulation of *Crf* in the hippocampus has been linked to the development of anxiety-like behavior. In juvenile rats, moderate psychological stress has evoked the activation of pyramidal cells, which could be significantly dampened by the administration of a CRFR1 antagonist [38]. In another study involving young rats, restraint stress on post-natal day 18 has induced hippocampal activation, demonstrated by upregulation of Fos in CA3 and pCREB in CA1, CA3, and the dentate gyrus, which could be prevented by treatment with a CRFR1 antagonist [66]. Furthermore, the upregulation of CRF and CRFR1 in the CA1 and CA3 regions has also been observed in rats displaying extreme behavioral response (i.e., strong anxiety-like behavior) 7 days after exposure to predator scent [67].

We also demonstrated a downregulation of *Avpr1a* in the hippocampus. V1aRs have been detected on the GABAergic interneurons of the dentate gyrus, CA3, CA2, and CA1 regions [68,69]. In the pyramidal cells of the CA1 region, a dose-dependent increase in the frequency of IPSCs has been found in response to AVP, mediated by V1aR activation [70]. There are several studies that assign an anxiety-inducing effect for V1aR signaling. Bielsky et al. found a reduction in anxiety-like behavior in V1aR KO mice [71]. V1aR antagonist treatment also resulted in an anxiolytic effect [72], but the role of hippocampal V1aRs has not been investigated in anxiety yet.

Overall, our gene expression results suggest that KP-13 does, indeed, affect the expression of *Crf, Avp*, and their receptors in a brain region-dependent manner since, for instance, *Crf* expression in the amygdala decreased, whereas in the hippocampus, it increased. Similarly, *Avp* expression showed a definite elevation in the amygdala and no change in the hippocampus.

To further establish the connection between CRF and AVP signaling pathways and KP-13's anxiety- and HPA axis-stimulating effect, we conducted a set of experiments in which, before the administration of KP-13, a pretreatment with non-selective CRFR or a V1R antagonist was performed.

First, we performed a computerized open field test to underlie KP-13's anxiety-inducing effect. Different doses of KP-13 were injected icv into male Wistar rats, the behavior of which were then recorded in a non-familiar environment in an open field box. Our results showed that KP-13 dose-dependently reduced the distance traveled and time spent in the center of the arena, which corresponds to anxiety-like behavior. The dose–response curve showed a U-shape that is often seen in the case of peptides [73,74]. The possible mechanism could be homologous desensitization by G-protein coupled receptor

kinases that phosphorylate already activated receptors thus lowering the responsiveness of the cell specifically to ligands of those receptors or receptor downregulation [73,74]. Furthermore, higher doses of KP-13 could bind to and activate less specific receptors that might oppose the KISS1R- and/or NPFF receptor-mediated response [73,74]. KP-13 also caused increased immobility time and decreased rearing activity. These underlie the anxiety-inducing effect of KP-13 since they suggest increased freezing and decreased exploratory behavior that is characteristic of anxiety in rodents [75].

After we demonstrated KP-13's effect in this computerized open field test, we pretreated the animals with either α-helical CRF(9-41) or V1R blocker. Our results showed that the V1R antagonist reduced the KP-13-evoked decline in central ambulation distance and central ambulation time, whereas the non-selective CRFR antagonist had no effect. This indicates that AVP signaling pathways might mediate KP-13's anxiety-inducing effect. Taking these results together with that of the qPCR and the ELISA, the upregulation of AVP signaling in the amygdala and the downregulation of V1aR in the hippocampus might be involved in the anxiety-like behavior induced by the icv administration of KP-13. It must be mentioned that KP's effect on anxiety-like behavior is somewhat contradictory in the literature. In fact, some studies found KP to exert an anxiolytic effect [76,77], some reported no effect [78,79], and some indicated an anxiety-like effect [19,20,80]. The reason for these discrepancies might lie in the differences in the experimental setup. For instance, in the experiments of Rao et al. and Comninos et al., KP injection was peripheral [78,79], therefore, it is possible that the KP concentration in stress-related brain areas did not reach sufficient levels to induce stress-related behaviors. In addition, it is not so surprising to see different effects in the case of systemic administration and local selective activation of neurons [77]. Also, a confounding factor is that KP's effect on stress-related behavior was investigated in different species. Ogawa et al. showed an anxiolytic effect in zebrafish, whereas studies that ascribed an anxiogenic effect to KP were performed in rodents [12]. An additional explanation for the contradictory results in KP's effect on stress-related behavior could be that different forms of KP analogs were administered, and since the affinity of KP to the NPFF receptors is determined by the length of the peptide [12], it is also plausible that the different KP analogs might exert different actions. Nevertheless, our present results are in accordance with our previous results [19,20] as well as the findings of Delmas et al. [80], who reported an anxiolytic phenotype in Kiss1r KO animals.

In our previous study, we demonstrated that corticosterone levels of rats elevated 30 min after icv KP-13 treatment [19]. In our present study, we wanted to determine if CRF and/or AVP signaling might be mediating KP-13's effect. It is well established in the literature that both CRF and AVP are crucial for the activation of the HPA axis and consequently for the corticosterone response. In fact, CRF and AVP are released from the PVN and they synergistically induce the release of ACTH in the pituitary [21]. Since, in our experiments, KP-13 altered the expression of both AVP and CRF, it is plausible that it also activates the HPA axis directly or indirectly via these two hormones. Therefore, a set of animals was pretreated with either α-helical CRF(9-41) or V1R antagonist 30 min before the KP-13 challenge. Our results showed that KP-13 induced a robust increase in the plasma corticosterone level, the effect of which was inhibited by both antagonists suggesting that both CRF and AVP are involved in mediating KP-13's effect on the HPA axis. This is in harmony with the literature data mentioned before that both CRF and AVP are involved in the regulation of the HPA axis [21].

It is somewhat difficult to assess the clinical significance of our findings in rodents. Our results taken together with those of others indicate that kisspeptins have an influence on the regulation of the HPA axis and stress-associated behaviors. Thus, it is also possible that changes in KP signaling might be involved in the development of HPA axis-related pathologies (e.g., major depression, post-traumatic stress disorder, and anxiety disorders) [81,82]. Several clinical trials are already on the way that are based on the modulation of KP signaling, therefore it is crucial to establish the full range of KP's biological action and must be taken into account that it might influence mood [83]. Nevertheless, further extensive

research is needed to fully establish the mechanism of KP's action on the HPA axis and stress-related behaviors.

Our results must be interpreted in the context of several limitations of our study. First, in our study, only male Wistar rats were used to avoid the sex differences and the effect of the hormonal changes associated with the estrous cycle. Notably, HPA axis responsiveness shows marked sex differences [84]. In fact, in rodents, females exhibit a more notable corticosterone response in the presence of any stressors, owing to the circulating estradiol levels [84]. Therefore, animal models of both sexes would have yielded more translatable results, and in the future, KP's effect on the HPA axis and stress-related behaviors in females must be addressed as well.

Furthermore, in the present study, we have investigated the effect of KP-13 on the gene expression of AVP and its receptors as well as CRF and its receptors. However, only AVP and CRF were investigated at the protein levels.

Protein levels vary on a high dynamic range, regulated by the rate of translation and degradation, the former being the determining factor [85]. The rate of translation is regulated by multiple mechanisms, only one of which is transcription. Other mechanisms include the activity of eukaryotic initiating factors (EIFs), structural features (e.g., internal ribosome-entry sequences, upstream open reading frames, and secondary or tertiary RNA structures), RNA-binding proteins, as well as microRNAs and small interfering RNAs that are involved in the regulation of translation [86]. Any of these mechanisms might be in play, and thus, our gene expression results on the receptors might not translate to the protein levels.

Also, it must be noted that other brain regions may also be involved in mediating KP-13′s effect on the HPA axis and anxiety-like behavior. The amygdala and hippocampus were chosen due to the distribution data of KP and KISS1R and the crucial role these regions play in the regulation of the neuroendocrine stress system [10,24]. KP and KISS1R are expressed in other brain regions as well, however, at a much lower expression level [6,10]. Still, it is possible that KP exerts its effect via another route.

5. Conclusions

In conclusion, KP-13 seems to alter the expression of *Avp, Crf,* and their receptors in a region-dependent manner. In the amygdala, the KP-13 treatment upregulated the expression of Avp and *Avpr1b* and downregulated the expression of *Crf*, whereas 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 evoked an anxiety-like behavior in the open field test, that was antagonized by the V1R blocker. In the case of the HPA axis, 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.

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III.

3. számú melléklet: Társszerzői lemondó nyilatkozat

Co-author certification

I, myself as a corresponding author of the following publication(s) declare that the authors have no conflict of interest, and Dr. Katalin Eszter Ibos Ph.D. candidate had significant contribution to the jointly published research(es). The results discussed in her thesis were not used and not intended to be used in any other qualification process for obtaining a PhD degree.

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date

Éva Bodnár
author

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