Effects of kisspeptin-13 on the hypothalamic-pituitary-adrenal axis, thermoregulation, anxiety and locomotor activity in rats

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Abstract

Kisspeptin is a mammalian amidated neurohormone, which belongs to the RF-amide peptide family and is known for its key role in reproduction. However, in contrast with the related members of the RF-amide family, little information is available regarding its role in the stress-response. With regard to the recent data suggesting kisspeptin neuronal projections to the paraventricular nucleus, in the present experiments we investigated the effect of kisspeptin-13 (KP-13), an endogenous derivative of kisspeptin, on the hypothalamus-pituitary-adrenal (HPA) axis, motor behavior and thermoregulatory function. The peptide was administered intracerebroventricularly (icv.) in different doses (0.5-2 μg) to adult male Sprague-Dawley rats, the behavior of which was then observed by means of telemetry, open field and elevated plus maze tests. Additionally, plasma concentrations of corticosterone were measured in order to assess the influence of KP-13 on the HPA system. The effects on core temperature were monitored continuously via telemetry. The results demonstrated that KP-13 stimulated the horizontal locomotion (square crossing) in the open field test and decreased the number of entries into and the time spent in the open arms during the elevated plus maze tests. The peptide also caused marked elevations in the spontaneous locomotor activity and the core temperature recorded by the telemetric system, and significantly increased the basal corticosterone level. In conclusion, our data indicate that icv. administered KP-13 stimulates the HPA axis, induces hyperthermia, activates motor behavior and causes anxiety in rats.

Keywords: kisspeptin; locomotor activity; hyperthermia; hypothalamic-pituitary-adrenal axis.

1. Introduction

Kisspeptin, classified as a member of the Arg-Phe (RF)-amide family [1], is a C-terminally amidated neurohormone and is a key regulator of the hypothalamic-pituitary-gonadal (HPG) axis [2-4]. The kisspeptin related peptides are neuropeptide FF and AF, prolactin releasing peptide (PrRP), RFamide-related peptides, and the most recently found, pyroglutamylated RFamide peptide [1]. They all share an N-terminal sequence homology and are widely distributed in the CNS, but they vary in their structure and receptor preference [1] binding to either one or several G-protein coupled receptors [5]. Literature shows that the effects of RF-amide peptides partially overlap, but in case of some physiological parameters they exert opposite actions. For example, PrRP activates the hypothalamic-pituitary-adrenal (HPA) axis [6], increases stereotyped locomotion [7] and pressor response [8]. Neuropepide AF (NPAF) also induces the HPA axis and locomotor activity, however, it causes a decrease in heart rate and core temperature [9]. Thus, in light of the above-mentioned data, kisspeptin might also have a wider range of function then so far assumed and may influence the same biological parameters as other RF-amide peptides.

Kisspeptin, itself, was first isolated from the human placenta as the endogenous ligand of the orphan G-protein coupled receptor GPR54, later designated as KISS1R [10, 11]. Kisspeptin is the product of the *KiSS-1* gene; the peptide consists of 54 aminoacids (KP-54), but its cleavage can give rise to biologically active derivatives containing 14, 13 or 10 aminoacids, christened kisspeptin-14 (KP-14), kisspeptin-13 (KP-13) and kisspeptin-10 (KP-10), respectively [2, 10]. Kisspeptin and its receptor are abundant in the central nervous system (CNS), especially in the limbic system, the striatum, the pituitary and the hypothalamus, including the paraventricular nucleus (PVN) [10, 12-14]. Recent evidence

suggests that kisspeptin, beside the KISS1R, also activates the neuropeptide FF2 receptor [15], which mediates autonomic, endocrine, behavioral and nociceptive processes [9, 16].

The first biological action associated with kisspeptin was the suppression of metastasis in melanoma [17], but recently a number of publications [4, 18, 19] has demonstrated the pivotal role of the kisspeptin system in the regulation of the reproductive axis. Kisspeptin is necessary for the normal secretion of gonadotropin releasing hormone (GnRH) [20, 21] and subsequently luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [21], meanwhile, it may also control the onset of puberty [20, 22] through its activity on the biological clock of the CNS [23, 24]. These seemingly disparate activities can be attributed to the ability of the peptide to stimulate diverse intracellular signal transduction cascades involving the activation of phospholipase C (PLC), mitogen activated protein kinase (MAPK), calcineurin and NFκB [25]. These pathways can influence hormone secretion, chemotaxis, and the organization of the cytoskeleton, neuronal activity and plasticity [24-26].

Taking the special importance of kisspeptin in the regulation of the HPG axis into account, and the fact that recent data suggests kisspeptin neuronal projections to the PVN [13, 27], it seems plausible that kisspeptin may take part in the control of the HPA axis, the interaction between the two systems and may exert further integrative activities in autonomic and endocrine control.

Therefore, in the present study, we investigated the central action of KP-13 on the stress response, behavior and thermoregulation, processes controlled by the hypothalamus and the limbic system, where kisspeptin and its receptors are found in abundance [13]. As an index of the activation of the HPA system the corticosterone response was used. The spontaneous locomotion and core temperature were monitored continuously with a telemetric system, while the exploratory and anxiety-associated behavior was observed in open field and elevated plus maze tests.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats (Domaszék, Hungary) weighing 150-250 g were used at the age of 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. Approximately 160 animals in total were used in our experiments. Every experiment was carried out separately; the same animal has never been used for different experimental procedure.

2.2. Surgery

The animals were allowed 1 week to acclimatize before surgery. Subsequently, 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 Pellegrino et al. [28]. The cannula was secured to the skull with dental cement and acrylate. The rats were used after a recovery period of 5 days. All experiments were carried out between 8:00 and 10:00 a.m.

For implantation of the telemetric radio transmitter (E-Mitter: a temperature-activity transponder), the rats were anesthetized with pentobarbital (35 mg/kg, intraperitoneally). The abdomen was opened by making a 2-cm midline incision along the linea alba. The E-Mitter was placed in the abdominal cavity, along the sagittal plane, in front of the caudal arteries and veins, but dorsal to the digestive organs. The abdominal wound was then closed with

absorbable suture material, while the skin was closed with stainless steel suture clips. After a recovery period of 5 days, the rats were implanted with the stainless steel Luer cannula for intracerebroventricular (icv.) administration.

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., Switzerland) icv. in a volume of 2 µl over 30 s with a Hamilton microsyringe, immobilization of the animals being avoided during handling. The doses applied were 0.5, 1, 2 or 5 µg dissolved in 0.9% saline. Control animals received saline alone. Thirty minutes after peptide administration, the rats were decapitated to obtain trunk blood for corticosterone measurement or were subjected to behavioral testing.

2.4. Plasma corticosterone measurement

In order 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 [29] as modified by Purves and Sirett [30].

2.5. Telemetry

Different doses of KP-13 (1, 2 μg) or saline alone were injected icv. into conscious rats, between 8:20 and 8:35 a.m. The animals had previously been implanted with an E-mitter (Mini Mitter, USA), which recieves power from the radiofrequency field generated by an energizer-reciever placed below the home cage. The system recorded the motor activity and core temperature every 10 min, the output of which then was processed by the VitalView program provided by the manufacturer.

2.6. Open field test

In the open field test novelty-induced locomotor activity was assessed. The rats were removed from their home cages and placed at the center of a white wooden open field box, the floor area of which measured 60 x 60 cm, marked into 36 10 x 10 cm squares. The standard source of illumination was a 60 W bulb at a height of 80 cm. The observed parameters were horizontal locomotion, vertical locomotion, grooming and the number of defecations. The horizontal locomotor activity was characterized by the total number of squares crossed during a 5-min test session (square crossing), the vertical locomotion was determined by the number of rearings (standing on the hind legs), and the grooming activity was established by observing face washing, forepaw licking and head stroking. Every episode of face washing, forepaw licking and head stroking was counted as a separate grooming session, independently of how long it actually lasted.

2.7. Elevated plus maze test

The elevated plus maze apparatus is a plus-shaped platform elevated 50 cm above the floor. It consists of two opposing arms (50 cm x 10 cm each) with 10 cm high enclosing walls (closed arms) and two arms with no walls (open arms). A 60 W light bulb at a height of 80 cm

provided the illumination. The maze was cleaned between each session with 96% ethyl-alcohol and all experiments were conducted between 8:00 a.m. and 10 a.m. Naive rats were placed in the center of the maze facing toward an open arm, and the number of entries per arm and the times spent in the various arms were recorded for a 5-min period by an observer who was blind to the experimental groups, sitting approximately 1.5 m away from the apparatus. The test is designed to assess anxiety based on the concept that the open arms are more aversive, and anxious rats therefore spend less time in them [31]. In the figures the ratio of time spent in open arms to total time spent in all arms, the ratio of entries to open arms to total number of entries and the total number of entries into all arms are presented.

2.8. Statistical analysis

Data are presented as means ± SEM. Statistical analysis of the results was performed by analysis of variance (ANOVA). For the corticosterone measurements, open field and elevated plus maze tests, one-way ANOVA was employed, followed by the Holm-Sidak *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 the telemetric recordings, repeated measure ANOVA was performed; only the means were plotted and the pooled standard deviation (PSD) is provided in the Figure captions. A probability level of less then 0.05 was accepted as indicating a statistically significant difference.

3. Results

3.1. Effects of KP-13 on corticosterone secretion

The icv. injection of KP-13 induced a dose-dependent elevation in basal plasma corticosterone level. The corticosterone level following the 2 μg dose proved to be statistically different from the control [$F(3,31)=3.955$, $p<0.02$; Holm-Sidak *post hoc* test: $p<0.01$ vs control; Fig. 1].

3.2. Effects of KP-13 on spontaneous locomotion and core temperature

After the KP-13 treatments between 8:20 and 8:35 a.m., increases in both locomotor activity [$F(2,30)=5.842$, $p<0.01$; Holm-Sidak *post hoc* test: $p<0.05$ for 1 μg and 2 μg KP-13 vs control; Fig. 2] and core temperature [$F(2,30)=4.988$, $p<0.02$; Holm-Sidak *post hoc* test: $p<0.01$ for 2 μg KP-13 vs control; Fig. 3] were observed in the home cages of the animals. In the case of locomotion, this effect was present only for approximately 1 hour after peptide injection and the activity of the rats then returned to the level of the control animals, whereas in the case of the core temperature the hyperthermic action of KP-13 persisted for several hours after peptide administration.

3.3. Effects of KP-13 on open field behavior

KP-13 evoked a marked increase in the number of square crossings in the open field test [$F(4,41)=3.001$, $p<0.05$; Holm-Sidak *post hoc* test: $p<0.01$ vs control; Fig. 4], but did not affect the other recorded parameters: rearing activity [$F(4, 41)=0.518$, $p<0.723$], grooming [$H=6.079$, $p=0.193$] or defecation [$F(4, 41)=1.225$, $p=0.315$] (not shown in Figures). The effect of KP-13 administered in a 1 μg dose on the number of square crossings proved to be statistically significant.

3.4. Effects of KP-13 on elevated plus maze behavior

KP-13 reduced dose-dependently the number of entries into [$F(3, 36)=7.095$, $p<0.001$; Holm-Sidak *post hoc* test: $p<0.05$ for 1 μg and $p<0.001$ for 2 μg KP-13 vs control] and the time spent [$F(3, 36)=3.298$, $p<0.05$; Holm-Sidak *post hoc* test: $p<0.01$ vs control] in the open arms (Fig. 5). A statistically significant change in the time spent in the open arms was caused by the 2 μg dose of KP-13, while as concerns the number of entries into open arms, both the 1 and 2 μg doses induced significant reductions. There was no difference in the number of total entries between the treatments groups [$F(3, 36)=0.555$, $p=0.648$].

4. Discussion

In our experiments, KP-13 evoked an elevation of the corticosterone concentration. The most important activators of the HPA axis are corticotropin releasing factor (CRF) and arginine vasopressin (AVP) [32], secreted by the parvocellular part of the PVN. Rao et al. [33] recently reported that in PVN-derived cell lines KP-10 generated significant increases in AVP and oxytocin mRNA expression, whereas the CRF mRNA level was affected only at a high dose [33]. Thus, a possible explanation for our result is that KP-13 may stimulate the AVP-expressing neurons in the PVN, leading to activation of the HPA axis. Furthermore, a recent study found that kisspeptins can bind to the NPFF2R [15]. Accordingly, in our previous experiments, NPAF, a potent NPFF2R ligand, also stimulated the HPA axis [9]. NPAF was most effective at the dose of 0.5 μg, whereas it was the 2 μg dose of KP-13 that elicited the greatest response. This might be explained by the differences in the affinity and the efficacy of the two peptides. There is also evidence pointing to the direct action of kisspeptin at the level of the pituitary. Kisspeptin has been detected in ovine hypophyseal portal blood [34] and KISS1R has also been found in the pituitary by RT-PCR [14], here co-

localized with ACTH expressing cells [35]. However, it must be noted that Rao et al. [33] found that ip. administered KP-10 had no effect on corticosterone secretion in mice. Recent work has also revealed that the activity of KP-10 is strongly dependent on the route of administration: central injection of KP-10 inhibited food intake, whereas ip. Administration in mice did not influence it [36]. Additionally, Scott and Brown [37] found KP-10 to be effective in increasing the firing rate of oxytocin neurons on intravenous injection, but not on icv administration. It is possible that kisspeptin, like the vast majority of neuropeptides, cannot cross the blood-brain barrier or do not reach the neuroendocrine regions relevant to the HPA axis, in sufficient concentration, due to enzymatic degradation [38]. These pharmacokinetic problems can clearly be circumvented by properly designed analogs [38]. Another explanation for the discrepancy between our results and that of Rao et al. [33] might be the use of KP-13 in our experiments instead of KP-10. Lyubimov et al. [15] reported that the NPFF2R binding of kisspeptins depends on the length of the peptide and the presence of the amidated C-terminal dipeptide. KP-13, therefore, proved to be a more potent activator of NPFF2R then KP-10 [15].

Our results demonstrate that kisspeptin can influence the behavior of rats. Open field and telemetric observations revealed that the icv. injection of KP-13 caused a marked activation of novelty-induced and spontaneous locomotion. Increasing doses of KP-13 exhibited a bell-shaped dose-response curve. This type of response is well-known in the literature and has been described in case of other neuropeptides [39, 40]. Since KISS1R has been found abundantly in locomotor centers of key importance such as the striatum and amygdala [10, 12, 41], it is plausible that KP-13 stimulated these regions directly.

Furthermore, KP-13 evoked a preference for the closed arms in the elevated plus maze test, which is indicative of the anxiogenic action of KP-13 in rats. This reinforces our finding that KP-13 activated the HPA axis, as both CRF [42] and AVP [43, 44] are potent activators

of stress-related behavior. In fact, the central amygdala and the bed nucleus of stria terminalis, both of which have a pivotal role in generating negative emotional responses [45], receive input from kisspeptin neurons [41]

KP-13 induced a significant elevation of core temperature that persisted for several hours. An increased locomotor activity was also observed in these experiments, however, this lasted only an hour suggesting that it is not the cause of the detected changes in temperature. As kisspeptin is a well-known stimulator of GnRH [2], GnRH might mediate the hyperthermic action of KP-13, which would be in accord with the possible role of GnRH in thermoregulation, suggesting GnRH as a causative factor in hot flashes [46, 47]. Other possible explanation could involve the activation of hypothalamic prostaglandin synthesis, increased basal metabolic activity or the stimulation of the hypothalamus-pituitary-thyroid axis.

Our findings are in complete harmony with the growing body of evidence suggesting that kisspeptin may play a more general role in autonomic, neuroendocrine and behavioral regulation. The peptide takes part in cardiovascular [48] and metabolic [49] functions, pregnancy [50] and cognitive processes [51]. Obviously, the control of the aforementioned processes necessitates integration with gonadal activities. The gender-dependent nature of the stress response, stress tolerance and longevity, the interactions between the HPG axis and the hypothalamic-pituitary-adrenal (HPA) system have been well described in the literature [52-56]. Sexual steroids influence the expression of CRF and AVP in the hypothalamus [57, 58], whereas chronic stress suppresses the reproductive function [53, 54]. However, a series of experiments demonstrate that glucocorticoid release from the adrenal gland, actually, preserves the HPG activity during stress [59, 60]. Taking these phenomena and the versatile physiological functions of kisspeptin into account, it is apparent that, besides the well-

characterised PrRP [61-63], further members of the RF-amide family may play integrative roles in the harmonization of the HPG and HPA activity.

Similarly, the dense expression of kisspeptin in the arcuate nucleus and the innervations of the suprachiasmatic nucleus [27] underlines our findings and argues for the role of the peptide in the circadian regulation of metabolic processes, core body temperature and hormone production. Indeed, the basal HPA activity shows a circadian rhythm that is provided by input from the suprachiasmatic nucleus, leading to the pulsatile secretion of CRF [64, 65]. The role of kisspeptin in circadian control is further supported by recent work establishing the kisspeptin system as an important relay center for the integration of environmental cues and the precise timing of puberty [22], the preovulatory LH surge [19, 24], and structural plasticity in seasonal reproduction [26]. Similarly, the observed effect on motor paradigms can also be attributed to a plausible regulatory role in circadian activity and sleep-wake cycle suggested by the expression of kisspeptin neurons in the suprachiasmatic nucleus [27] and the preoptic nucleus of the hypothalamus [66].

In conclusion, our results indicate that centrally injected KP-13 activates the HPA axis, induces hyperthermia and stimulates spontaneous and novelty-induced locomotion. Furthermore, KP-13 seems to generate anxiety-associated behavior in adult rats. Our data confirm that RF-amide peptides belong to those neuropeptide families that have especially important role in neuroendocrine control. Notwithstanding, further investigations are necessary to clarify the mediation and signal transduction of the presented physiological phenomena, with special emphasis on the separation of the unique and overlapping features in the activity profile of the different RF-amides.

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Figures

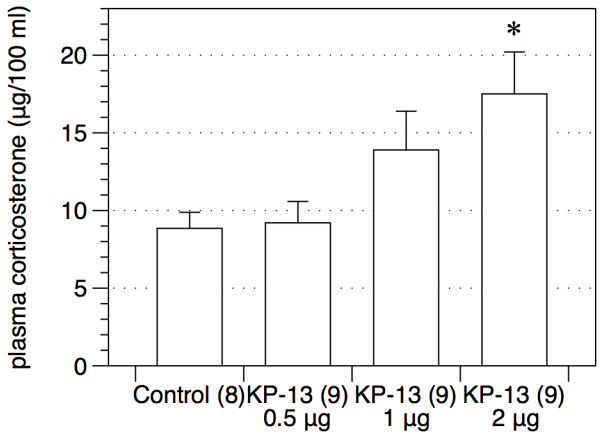

Fig. 1. The effect of KP-13 on the hypothalamus-pituitary-adrenal system. Mean and SEM are expressed. Numbers in parenthesis denote the number of animals used. * $p < 0.05$ vs. control.

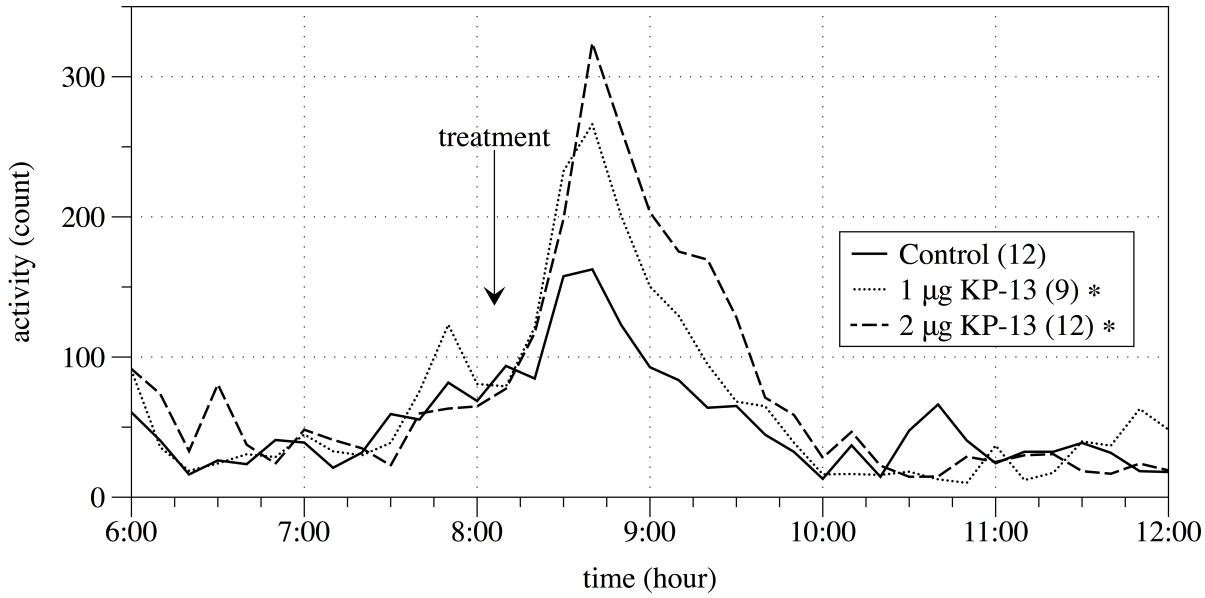

Fig. 2. The effect of KP-13 on the spontaneous motor activity. Data are expressed as means. The pooled standard deviations (PSDs): 62.34 for the control, 69.34 for the 1 µg KP-13 treated group, 72.81 for the 2 µg KP-13 treated group. Numbers in parenthesis denote the number of animals used. * $p < 0.05$ vs. control.

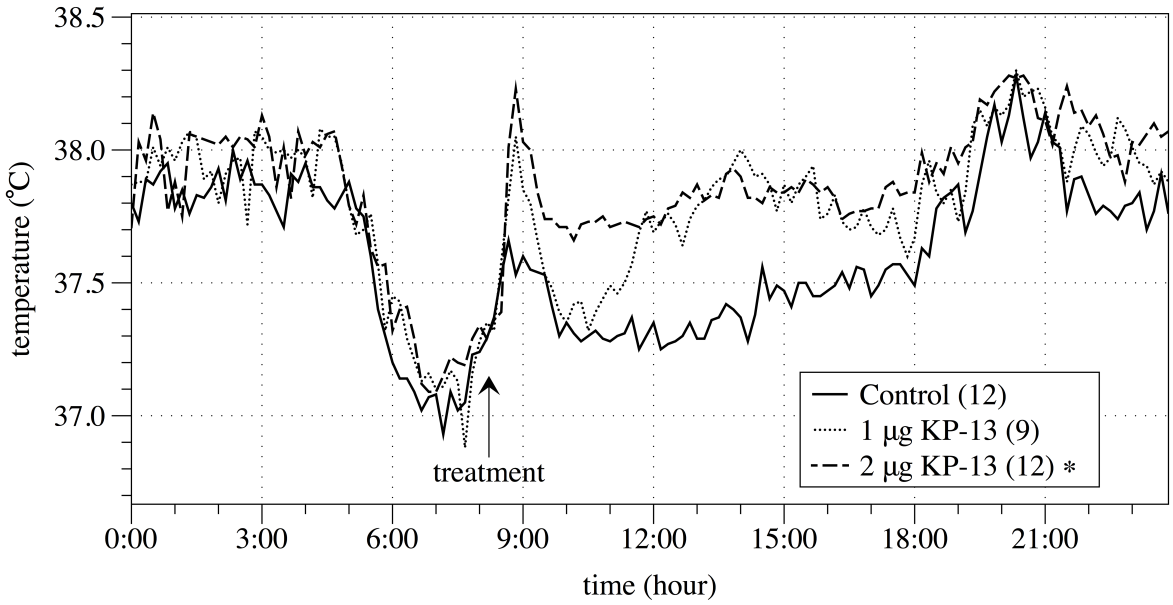

Fig. 3. The effect of KP-13 on the core temperature. Data are expressed as means. The pooled standard deviations (PSDs): 0.40 for the control, 0.49 for the 1 µg KP-13 treated group, 0.52 for the 2 µg KP-13 treated group. Numbers in parenthesis denote the number of animals used. * $p < 0.05$ vs. control.

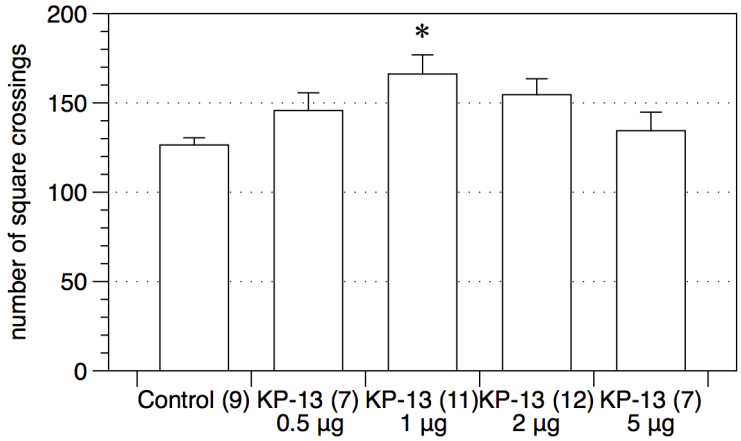

Fig. 4. The effect of KP-13 on exploratory locomotor activity. Mean and SEM are expressed. Numbers in parenthesis denote the number of animals used. * $p < 0.05$ vs. control.

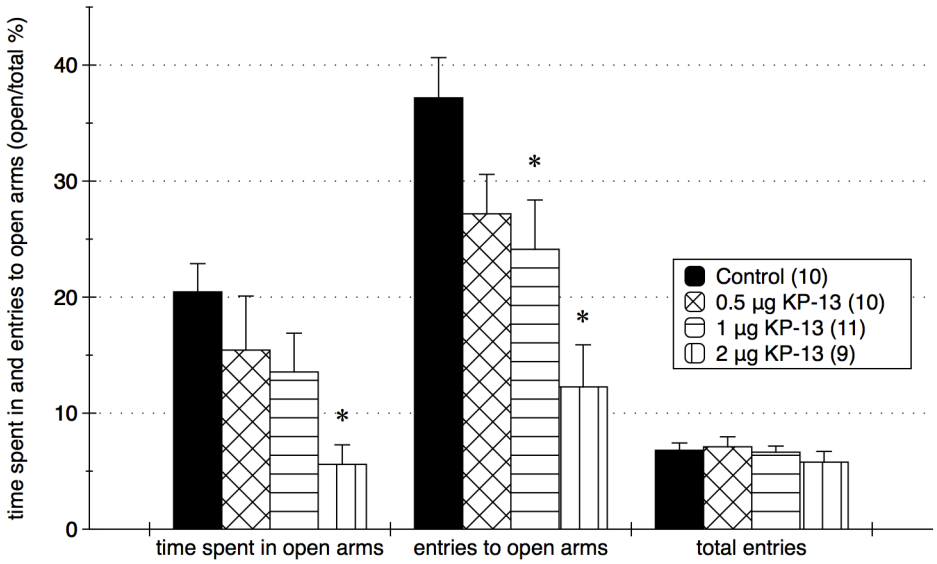

Fig. 5. The effect of KP-13 on elevated plus maze behavior. Mean and SEM are expressed. Numbers in parenthesis denote the number of animals used. * $p < 0.05$ vs. control.