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Kisspeptin modulates pain sensitivity of CFLP mice

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ABSTRACT

Kisspeptin, a hypothalamic neuropeptide, is a member of the RF-amide family, which have been known to modify pain sensitivity in rodents. The aim of the present study was to investigate the effect of kisspeptin-13 (KP-13), an endogenous derivative of kisspeptin, on nociception in adult male and female CFLP mice and the possible interaction of KP-13 with morphine on nociception.

Mice were injected with different doses of KP-13, 30, 60 and 120 min after of which the nociceptive sensitivity were assessed via the tail-flick test. To investigate the receptor involved in the mediation a kisspeptin receptor antagonist (KP-234) pretreatment was applied before KP-13 administration. Furthermore, we investigated the effect of KP-13 on the acute antinociceptive effect of morphine, on acute morphine tolerance and on naloxone-precipitated withdrawal. Last, the Von Frey test was used in order to assess KP-13′s effect on mechanical nociception.

Our results showed that KP-13 decreased the nociceptive threshold of both males and females independent of sex, which was prevented by KP-234. Furthermore, KP-13 treatment depressed the acute antinociceptive effect of morphine and attenuated the development of morphine tolerance. KP-13 also induced a mechanical hypersensitivity. These data underlie kisspeptin's hyperalgesic action and argues for the role of kisspeptin receptor 1 in the mediation of its action. Furthermore, our results suggest that central KP-13 administration can modify the acute effects of morphine.

1. Introduction

Kisspeptin, a member of the Arg-Phe (RF)-amide family [38], is a C-terminally amidated neurohormone and is one of the key regulators of the hypothalamic-pituitary-gonadal (HPG) axis [43]. The other members of the RF-amide family are neuropeptide FF (NPFF) and AF (NPAF), prolactin releasing peptide (PrRP), RFamide-related peptides, and the most recently found, pyroglutamylated RFamide peptide [38]. They all share an N-terminal sequence homology and are widely distributed throughout the central nervous system (CNS); however, they vary in their structure and receptor preference binding to either one or several G-protein coupled receptors [2,38].

Kisspeptin, itself, was first isolated from the human placenta as the endogenous ligand of the orphan G-protein coupled receptor GPR54, later designated as Kisspeptin receptor 1 (KISS1R) [16,34]. Kisspeptin is a 54 amino acid long product of the KiSS-1 gene; however, alternative splicing can give rise to biologically active derivatives containing 14, 13 or 10 aminoacids, named kisspeptin-14 (KP-14), kisspeptin-13 (KP-13) and kisspeptin-10 (KP-10), respectively [16]. Kisspeptin and its receptor are abundant in the CNS, especially in the limbic system, the striatum, the pituitary gland and the hypothalamus [15,34].

The first biological action of kisspeptin was the suppression of metastasis in melanoma [20], but later, multiple studies demonstrated the pivotal role of the kisspeptin system in the regulation of the reproductive axis [43]. Kisspeptin is necessary for the normal secretion of the gonadotropin releasing hormone (GnRH) [9,42] as well as the luteinizing hormone (LH) and the follicle-stimulating hormone (FSH) [42,43]. Meanwhile, it may also control the onset of puberty [9] through its activity on the biological clock of the CNS [45].

Kisspeptin is a member of the RF-amide family that has been previously implicated in nociception control [38]. Accumulating evidence strongly suggests a role for the RF-amide family in nociceptive mechanism. NPFF and analogues were found to have analgesic, pronociceptive, and morphine modulating activities [38]. Although initially described as a solely anti-opioid system, evidence proved otherwise and now it is believed that the nature of the pharmacology depends on the subtype targeted, route of administration, and opioid activity [18]. In point of fact, intracerebroventricular (icv.) administration of NPFF inhibits opioid-induced antinociception [11], whereas intrathecal injection results in the opposite [37]. Both in vitro and in vivo

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pharmacological data suggest that these effects are mediated by the activation of NPFF receptor 1 and 2 (NPFF1R and NPFF2R) [18], respectively. Distribution data indicates that NPFF2R is found most abundantly in the spinal cord, whereas in the brain, both receptors are highly expressed, especially NPFF1R [2]. Recent evidence suggests that all endogenous RF-amide peptides not only target their cognate receptors, but also the NPFF1R and NPFF2R receptors, which raises the idea that all may take a part in pain modulation [10]. It is proposed that the Arg-Phe-NH2 motif is sufficient for binding with high affinity to both NPFF receptors [10].

Although much evidence has accumulated in the past couple of years that ascribes a critical role for the NPFF1R and NPFF2R receptors for the pain-modulating effects of RF-amide peptides; however, recent distribution data draws attention to other receptors, such as KISS1R and GPR10, and their endogenous ligands that are expressed in several brain areas involved in the control of pain [19]. For instance, both KISS1R and kisspeptin mRNAs and proteins have been detected in the dorsal horn of the spinal cord and in the dorsal root ganglia in rats [29]. They have also been detected in brain regions associated with pain processing (paraventricular thalamic nucleus, periaqueductal gray, amygdala and locus coeruleus) [6]. Furthermore, kisspeptin has already been implicated in nociception: intraplantar and intrathecal injection of kisspeptin-54 evoked hyperalgesia in the formalin test [46], and intracerebroventricular (icv.) kisspeptin-10 also caused a decrease in nociceptive threshold in the tail immersion test [10]. All of this data point towards the kisspeptin system's involvement in nociceptive control.

Moreover, numerous articles have reported sex differences in pain sensitivity, responsiveness to pharmacological and non-pharmacological pain interventions, the cause of which is unclear as of yet [32]. Emerging evidence indicates the reproductive system and the role of sex hormones in the regulation of pain sensitivity [1]. Therefore, in the present study, we first investigated the effect of kisspeptin-13 (KP-13), an endogenous derivative of kisspeptin, on nociception in adult male and female CFLP mice to address if KP-13 retains pronociceptive effect in the tail-flick test and if there's a difference in KP-13′s effect between sexes. Secondly, after establishing KP-13′s pronociceptive action, we set out to assess the possible interaction of KP-13 with the acute effects of morphine on nociception and the potential involvement of KP-13 in acute morphine tolerance and withdrawal. Furthermore, with KISS1R antagonist pretreatment, we wished to illuminate the receptor responsible for kisspeptin's actions. Finally, to further characterize KP-13′s pronociceptive effect we investigated if KP-13 influences mechanical sensitivity in the Von Frey test.

2. Materials and methods

2.1. Animals

Female and male adult CFLP white mice (30 ± 5 g of weight) of an outbred strain (Domaszék, Hungary) were used. They were kept under a standard light–dark cycle (lights on between 07.00 and 19.00 h) with food and water available ad libitum. The animals were kept and treated according to the rules of the Ethical Committee for the Protection of Animals in Research (Faculty of Medicine, University of Szeged, Hungary).

2.2. Surgery

For icv. cannulation, the mice were anesthetized with an intraperitoneal (ip.) injection of Sodium Pentobarbital (Euthasol, Phylaxia-Sanofi, Budapest, Hungary; 50 mg/kg). A polyethylene cannula was inserted stereotaxically into the right lateral cerebral ventricle at the coordinates of 0.2 mm posterior, 0.2 mm lateral to the bregma and 2 mm deep from dura surface and then cemented to the skull with cyanoacrylate-containing instant glue. The experiments were started 4 days after icv. cannulation. Upon conclusion of the experiments, 10 µl of methylene blue were injected into the cerebral ventricle of the decapitated animals and the position of the cannula was inspected visually. The spread of methylene blue throughout the ventricular space indicated that the whole amount of KP-13 got into the ventricles. Mice with improper cannula placement were excluded from the final statistical analysis.

2.3. Treatments

Mice were injected with different doses of KP-13 (human, 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, or 2 µg (0.307, 0.615, and 1.229 nmol, respectively) dissolved in 0.9% saline. For experiments testing the effect of morphine, subcutaneous (sc.) morphine–HCl (Sigma-Aldrich Co., USA) and naloxone–HCl (Sigma-Aldrich Co., USA) injections were used. Kisspeptin-234 (KISS1R antagonist; KP-234) (Sigma-Aldrich Co., USA) was injected in 0.1 µg (0.077 nmol) dose icv. The doses selected were based on dose-response studies conducted in our lab previously that did not affect nociceptive sensitivity per se. Control animals received saline alone.

2.4. Tail-flick test

The nociceptive sensitivity was tested by the heat-radiant tail-flick system (IITC Life Science, California, USA) described by [8]. All experiments were started with an initial tail-flick latency measurement, then nociceptive threshold was measured 30, 60, and 120 min after peptide challenge. For tail-flick measurement, animals were habituated to the experimental room at least 30 min prior to testing. During the measurement, they were loosely restrained and the tail was positioned so that the light beam focused on the tail approximately 1–2 cm from the base. Tail stimulation was delivered after the tail skin of the mice was preheated to 36 °C and was performed at different sites in consecutive measures to prevent tissue damage. The antinociceptive effect was expressed according to the following equation:

$$\text{analgesic effect (\%)} = (\text{TFn} - \text{TF0})/\text{TFmax} - \text{TF0}) \times 100$$

where TF0 is the tail-flick latency in the preliminary test mentioned above or (in tolerance studies) before morphine injection. TFn is the value of a repeated corresponding measurement n after KP-13 or/and morphine injection, and TFmax indicates the cutoff (20 s).

2.5. Manual Von Frey test

The manual Von Frey test was performed to assess the paw withdrawal thresholds to tactile stimulation [5]. For the manual Von Frey measurement, animals were habituated to the experimental room at least 60 min prior to testing. Paw withdrawal thresholds (PWTs) of the right hind paws of the mice were determined in response to probing with eight calibrated von Frey filaments (Somedic Sales AB, Hörby, Sweden). The logarithmically spaced increments range from 0.27 to 6.2 g. Each filament was applied perpendicularly to the plantar surface of the paw of CFLP mice kept in suspended wire-mesh cages. PWT was determined in grams by the up-down Von Frey method. Mean 50% paw withdrawal thresholds were calculated by the following equation: PWT (g) = $10^{(X+kd)}/10000$, where X is the final Von Frey filament used (in log units), k is the tabular value of the response pattern, d mean difference between stimuli (in log units) (d = 0.269). After the preliminary measurement, the effect of KP-13 on nociceptive sensitivity was recorded 30, 60 and 120 min after peptide administration in male mice.

The following experiments were carried out:

First, the effect of KP-13 on nociceptive sensitivity was measured

30, 60 and 120 min after peptide administration in both female and male mice.

Second, the effect of KP-234 on KP-13 induced pronociception was investigated, in which animals received 0.1 μg dose of KP-234 icv. followed by KP-13 treatment 30 min later. 30, 60 and 120 min later tail-flick latency was assessed.

Third, in experiments with KP-13 on the acute antinociceptive effect of a single dose of morphine, the peptide was administered 30 min prior to the test dose of morphine (2.4 mg/kg sc.) and the nociceptive threshold was measured 30 and 60 min later.

Fourth, in acute tolerance studies, animals were pretreated with KP-13 and 60 min later, a tolerance-inducing dose of morphine (60 mg/kg sc.) was administered, 24 h after of which a test dose of morphine (4 mg/kg sc.) was injected to assess the antinociceptive effect.

Fifth, in acute withdrawal studies, 30 min after KP-13 pretreatment a tolerance-inducing dose of morphine (60 mg/kg) was administered, 3 h after morphine injection animals received naloxone to precipitate withdrawal signs. The precipitated abstinence syndrome was assessed by scoring the latency of the stereotyped jumping from a circular platform with a diameter of 35 cm placed 70 cm high. A cut-off time of 900 s was applied. Meanwhile, body temperature and weight of the animals were measured before naloxone treatment, 15 min, 30 min and 60 min after.

Finally, the effect of KP-13 on mechanical sensitivity was measured 30, 60 and 120 min after peptide administration in male mice.

All responses were recorded by an investigator blinded to treatments.

2.6. Statistical analysis

Data is presented as means ± SEM. Statistical analysis of the results was performed by analysis of variance (ANOVA). For the effect of KP-13 on thermal nociceptive sensitivity in male and female mice, a three-way mixed ANOVA was performed using between-subjects factors treatment and sex and within-subject factor time. For multiple comparisons, the Bonferoni post hoc test was used. In the case of the tail-flick tests with antagonist and morphine treatments, two-factor ANOVA was done (between-subject factors: KP-13 treatment and antagonist or morphine treatment), followed by the Bonferoni post hoc test. For the analysis of jumping latency in the acute withdrawal study, one-way ANOVA was employed, followed by the Holm-Sidak post hoc test for multiple comparisons. For the analysis of the PWTs two-way mixed ANOVA was done, followed by the Bonferoni post hoc test. 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 tail-flick, weight and temperature recordings with combined treatments, two-way ANOVA was performed, followed by the Bonferoni test for multiple comparisons. A probability level of less then 0.05 was accepted as indicating a statistically significant difference.

3. Results

3.1. Effect of KP-13 on tail-flick latency

A three-way mixed ANOVA was conducted to examine the effect of sex, time, and KP-13 treatment on nociceptive threshold of the animals (Fig. 1). There was a statistically significant main effect for the treatment factor [$F(3,77) = 10.603$, $p < 0.001$]. However, no significant main effect for the sex factor was observed [$F(1,77) = 0.179$, $p = 0.674$] and no statistically significant interaction between the effect of KP-13 treatment and sexes [$F(3,77) = 0.304$, $p = 0.823$]. In case of the within-subject factor time, Mauchly's test indicated that the assumption of sphericity had been violated; therefore degrees of freedom were corrected using the Huynh-Feldt estimates of sphericity. The results show that there was a significant main effect for time [$F(1.94,77) = 3.292$, $p = 0.042$]. No significant interaction was found between the factors time and the treatment factor [$F(5.81,77) = 1.798$, $p = 0.106$]. Overall, kisspeptin induced a significant decrease in nociceptive threshold, and the effect of the different levels of KP-13 treatment did not depend on what level of sex or time is present (Fig. 1). As kisspeptin's effect on nociception did not differ among sexes, the rest of the experiments were preformed with male mice.

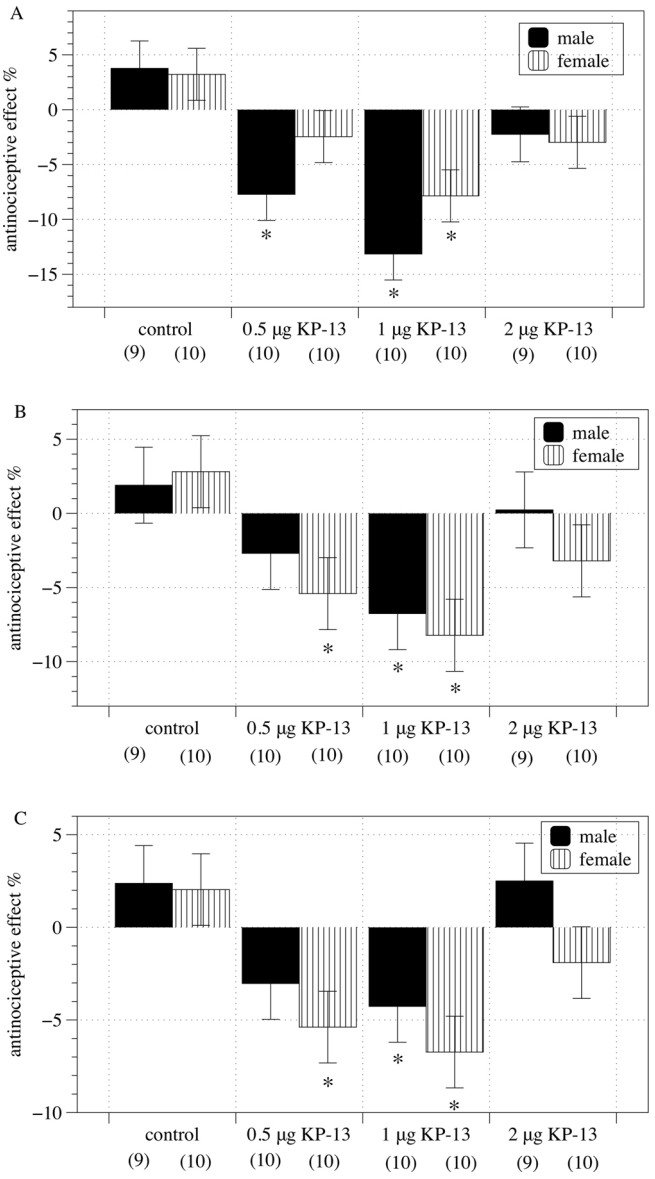

Fig. 1. The effect of KP-13 on pain sensitivity in the tail-flick test 30 min after treatment (A.), 60 min after treatment (B.) and 120 min after treatment (C.). Mean and SEM are expressed. Numbers in parenthesis denote the number of animals used. * $p < 0.05$ vs. control.

3.2. Effect of KP-234 on KP-13 induced pronociception

The two-factor ANOVA revealed a statistically significant interaction between the effect of KP-13 treatment and the effect of antagonist treatment at 30 min [$F(1,27) = 12.586$, $p < 0.01$], 60 min [$F(1,27) = 22.860$, $p < 0.001$] and 120 min [$F(1,27) = 22.955$, $p < 0.001$] (Fig. 2). No significant main effect was detected for the antagonist factor [$F(1,27) = 2.415$, $p = 0.133$ at 30 min; $F(1,27) = 1.306$, $p = 0.264$ at 60 min; $F(1,27) = 0.554$, $p = 0.465$ at 120 min]; however, a statistically significant main effect was found for

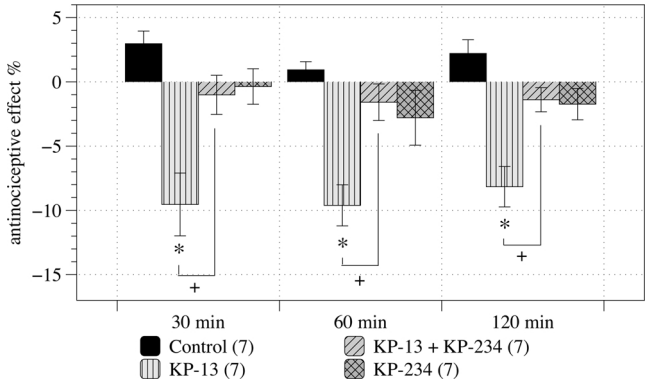

Fig. 2. The effect of KP-234 on KP-13-induced pronociceptive action. Mean and SEM are expressed. Numbers in parenthesis denote the number of animals used. * $p < 0.05$ vs. control, + $p < 0.05$.

the KP-13 treatment factor at all time points [$F(1,27) = 15.498$, $p < 0.01$ at 30 min; $F(1,27) = 9.029$, $p < 0.01$ at 60 min; $F(2,27) = 13.611$, $p < 0.01$ at 120 min]. Based on these results, the effect of KP-13 treatment depends on the presence of the antagonist. KP-234 was able to prevent KP-13's effect on nociception. The 0.1 μg of KP-234 per se did not affect tail-flick latency (Fig. 2).

3.3. Effect of KP-13 on challenge dose of morphine

A two-factor ANOVA was conducted to analyze the effect of morphine and KP-13 treatments on nociceptive threshold of the animals 30, 60 min after morphine injections (Fig. 3). There was a statistically significant main effect for KP-13 treatment factor at 30 min [$F(1,25) = 51.025$, $p < 0.0001$] and 60 min [$F(1,25) = 10.458$, $p = 0.004$]. Significant main effect for the morphine treatment factor was also observed [$F(1,25) = 217.226$, $p < 0.0001$ for 30 min; $F(1,25) = 28.630$, $p < 0.0001$ for 60 min) and a statistically significant interaction between the two factors [$F(1,25) = 26.210$, $p < 0.0001$ for 30 min; $F(1,25) = 5.930$, $p = 0.023$ for 60 min]. A single dose of 2.4 mg/kg sc morphine induced an appr. 80% antinociception; 1 μg dose of KP-13 significantly lowered the antinociceptive effect of morphine 30 and 60 min after the narcotic challenge (Fig. 3).

3.4. Effect of KP-13 on acute morphine tolerance

The two-way ANOVA on acute morphine tolerance showed a significant main effect for the KP-13 treatment factor [$F(1,24) = 25.563$, $p < 0.001$] 30 min after the morphine challenge, a significant main effect for the morphine tolerant factor [$F(1,24) = 268.053$, $p < 0.001$] and a significant interaction between the two factors [$F(1,24) = 42.735$, $p < 0.001$]. Thus, the effect of different levels of KP-13 treatment does depend on which level of morphine tolerance is present (Fig. 4). Acute tolerance was observed 24 h after a tolerance inducing dose of morphine was applied sc. Our results showed that KP-13 treatment 30 min before tolerance induction prevents the development of acute morphine tolerance. The KP-13-treated animals that received the tolerance inducing dose of morphine showed a significantly higher antinociceptive effect than tolerant animals both 30 min [$F(3,22) = 78.333$, $p < 0.05$], 60 min [$F(3,22) = 28.853$, $p < 0.05$] and 120 min [$F(3,22) = 13.188$, $p < 0.05$] after injection of the 4 mg/kg sc test dose of morphine (Fig. 4).

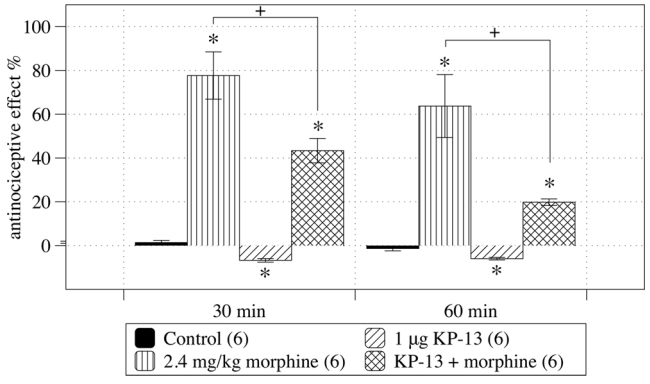

Fig. 3. The effect of KP-13 on morphine-induced antinociception in the tail-flick test. Mean and SEM are expressed. Numbers in parenthesis denote the number of animals used. * $p < 0.05$ vs. control. + $p < 0.05$.

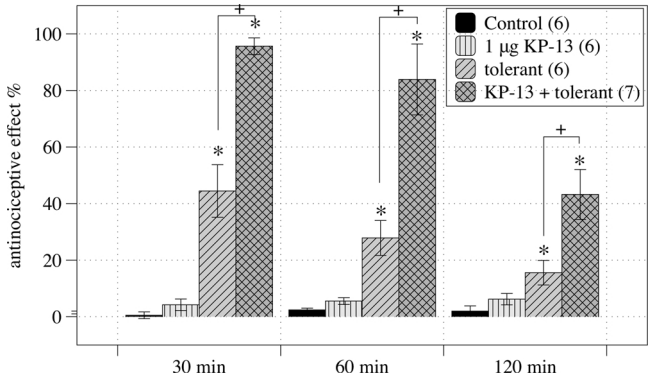

Fig. 4. The effect of KP-13 on acute morphine tolerance in the tail-flick test. Mean and SEM are expressed. Numbers in parenthesis denote the number of animals used. * $p < 0.05$ vs. control. + $p < 0.05$.

3.5. Effect of KP-13 on naloxone-precipitated acute morphine withdrawal

In the naloxone-precipitated withdrawal studies KP-13 caused a marked decrease in the jumping latency [$F(3,39) = 19.995$, $p = 0.008$] (Fig. 5) of animals from the platform.

The two-factor analysis of variance on weight changes revealed a significant main effect for the treatments factor [$F(3,118) = 36.746$, $p < 0.001$] and a significant main effect for the time factor [$F(2,118) = 7.445$, $p < 0.001$]; however, the interaction between treatments and time was not significant [$F(6,118) = 0.245$, $p = 0.96$], so the effect of different levels of treatments does not depend on which level of time is present (Fig. 6). The two-way ANOVA on body temperature changes yielded a significant main effect for the treatments factor [$F(3,118) = 140.576$, $p < 0.001$] and a significant main effect for the time factor [$F(2,118) = 45.568$, $p < 0.001$]; however, the interaction between treatments and time was not significant [$F(6,118) = 1.431$, $p = 0.209$]. The effect of different levels of treatments does not depend on what level of time is present. Morphine tolerant

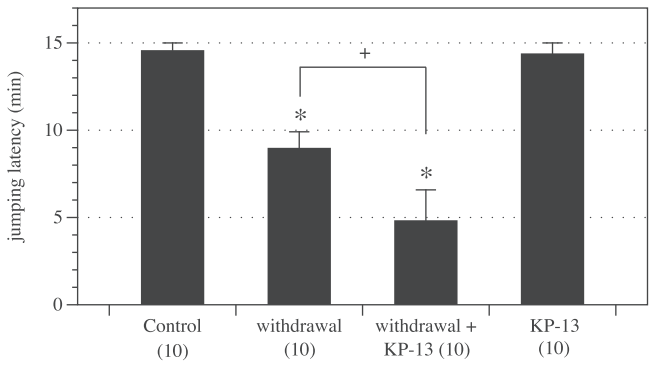

Fig. 5. The effect of KP-13 on jumping behavior in naloxone-precipitated morphine withdrawal. Mean and SEM are expressed. Numbers in parenthesis denote the number of animals used. * $p < 0.05$ vs. control. + $p < 0.05$.

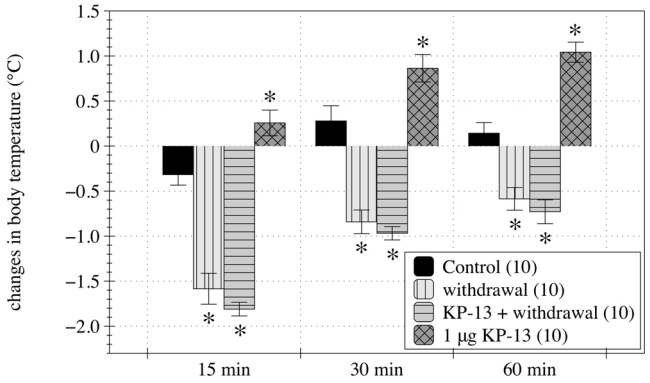

Fig. 6. The effect of KP-13 on the hypothermia induced by naloxone-precipitated morphine withdrawal. Mean and SEM are expressed. Numbers in parenthesis denote the number of animals used. * $p < 0.05$ vs. control.

mice showed a significant reduction in both weight (data not shown) and temperature (Fig. 6) within the different levels of time (15 min, 30 min and 60 min) [$p < 0.001$]. The KP-13-treated morphine tolerant group did not differ significantly from the morphine tolerant group. Only a slight tendency for KP-13 to further reduce the weight loss and hypothermia was observed. Of note is the result that KP-13 alone caused a marked elevation in body temperature compared to the control animals within all levels of time [$p < 0.001$] (Fig. 6).

3.6. Effect of KP-13 on PWTs

A two-way mixed ANOVA was conducted to analyze the effect of KP-13 (1 μg) on PWTs 30, 60 and 120 min after peptide injections (Fig. 7). In case of the within-subject factor time, Mauchly's test indicated that the assumption of sphericity had been violated; therefore degrees of freedom were corrected using the Huynh-Feldt estimates of sphericity. There was a statistically significant main effect for the KP-13 treatment factor [$F(1,9) = 21.27$, $p = 0.0013$]. On the other hand, the time factor and the interaction between factors treatment and time were not significant [$F(3,27) = 1.561$, $p = 0.221$; $F(3,27) = 0.626$, $p = 0.604$]. 1 μg dose of KP-13 significantly lowered the mechanical sensitivity 60 min after the peptide treatment (Fig. 7).

4. Discussion

Central administration of KP-13 to CFLP mice causes a marked pronociception in the tail-flick test. This is in accordance with previous findings in hot plate and formalin tests conducted by Spampinato et al. [46] and more recent results that of Elhabazi et al. [10]. In our experiments, no linear dose-response curve, but rather an inverted bell-shaped response was observed, which is well known in the literature,

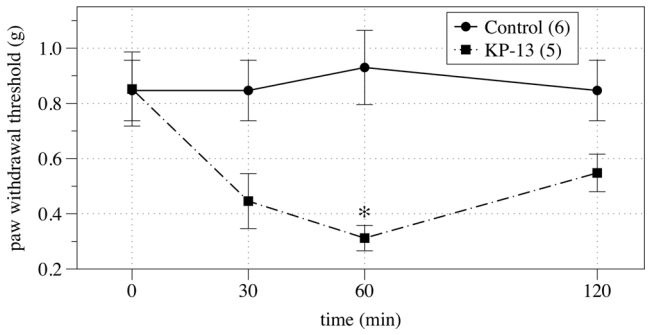

Fig. 7. The effect of KP-13 on the paw withdrawal thresholds. Mean and SEM are expressed. Numbers in parenthesis denote the number of animals used. * $p < 0.05$ vs. control.

and has already been described in the case of other neuropeptides [3].

Based on our results, there was no significant difference in KP-13's action on nociceptive sensitivity among the sexes. Both males and females exhibited similar pronociceptive phenotype in any given time point. Sex differences in pain sensitivity as well as the over-representation of characteristic pain syndromes in women point toward the involvement of the reproductive system in pain modulation [1,35]. Clearly, there is a connection between the HPG axis and the regulation of pain sensation; however, the underlying mechanism is not well understood and many of the published studies reported contradictory results [32]. Kisspeptin might provide a link between the reproductive and pain modulatory systems; however, our results suggest that kisspeptin is not involved in mediating the sex differences in the pain system. At the same time, pain threshold and tolerance varies over the menstrual cycle, therefore it is possible that the reason for not detecting any differences in KP-13's action between the sexes is due to not taking into consideration the cycle stage of the animals. As responsiveness to KP-13 does not seem to be sex-dependent, the experiments performed after the dose-response study were conducted in male mice only.

The effect of KP-13 on nociceptive sensitivity may be mediated through its cognate receptor, KISS1R, or the NPFFRs receptors. In support of KISS1R mediation stand several results. First, kisspeptin and KISS1R are expressed in neuronal structures involved in nociception signaling such as dorsal root ganglia and the dorsal horn lamina I and II of the spinal cord as well as brain regions such as hippocampus, amygdala, and periaqueductal grey [15,29]. This pattern of expression suggests a function in pain modulation. Second, in the chronic inflammatory pain model, kisspeptin and KISS1R up-regulation was detected at the level of the dorsal root ganglia and dorsal horn neurons [29]. Third, Spampinato et al. reported that the KISS1R antagonist, KP-234 exhibits a clearly analgesic effect in formalin test [46]. Fourth, the NPFFR agonist, NPFF in numerous studies caused analgesia when the route of administration was intrathecal instead of icv. [36]. Based on the fact that kisspeptin also lowered nociceptive sensitivity in the case of intrathecal and intraplantar injections, it is plausible that the effect of kisspeptin is mediated by the KISS1R and not by the NPFFRs. In point of fact, in our experiments KP-234, a KISS1R antagonist, blocked the pronociceptive action of KP-13 suggesting that indeed KISS1R mediation is involved in KP-13's effects on nociception. However, in the face of the above mentioned data, Elhabazi et al. in a publication reported that RF9 suppressed the hyperalgesic, and anti-morphine activity of icv. administered kisspeptin [10]. In this study, the route of administration compared to the study conducted by Spampinato et al. differed as animals received kisspeptin icv. and not peripherally. Additionally, receptor binding data and two different functional assays provide further support, in which all RF-amide peptides were found to target NPFF1R and NPFF2R in a nanomolar concentration range [10]. Furthermore, both NPFF1R and NPFF2R have been previously indicated in the modulation of pain [18]. Hence, these results indicate NPFFR involvement, at least, in the central kisspeptin actions rather then KISS1R mediation. It must be noted that recent publications raise the possibility that RF9 might not be a true selective antagonist of NPFF receptors as it was unable to reverse the anorectic effect of a NPFF analogue and it alone displayed an anorectic effect as well [25]. Plus, it might be a KISS1R agonist in vitro and in vivo as well [30]. Furthermore, it must be noted that Elhabazi et al. administered 3 nmol of kisspeptin-10 icv, whereas in our experiment as well as that of Spampinato et al. no more then 1 nmol was injected. It is possible that smaller concentrations of kisspeptin act via KISS1R, while higher concentrations might also bind NPFFRs. Undoubtably, results from these studies have been inconclusive. Still, in our experiments KP-234 prevented KP-13's pronociceptive action which supports KISS1R mediation.

The activation of KISS1R results in calcium mobilization and recruitment of Mitogen-activated protein kinases (MAPKs) such as the extracellular signal-regulated kinases (ERK) and p38 [4]. Both ERK and p38 contribute to central pain sensitization via post-translational and

transcriptional mechanisms [14]. As kisspeptins are potent stimulators of ERK1/2 and p38, it is plausible that they induce increased nociceptive sensitivity by affecting glutamate transmission[44], opioid [48] and/or other neuropeptide signaling [22] in the spinal cord dorsal horn neurons or upper brain regions implicated in nociceptive regulation.

The present results revealed an interaction between centrally injected kisspeptin and morphine. KP-13 showed a clear anti-morphine activity as it blunted the antinociceptive effect of a single injection of morphine. This result is in concert with previous publications in which KP-10 reversed morphine antinociception as well, the effect of which have been blocked by RF9 [10]. Furthermore, in our experiments KP-13 inhibited the development of acute tolerance to morphine. Short-term tolerance, an exposure to morphine up to 1 day, can be due to multiple adaptive mechanisms that regulate μ opioid receptor (MOR) trafficking to the cell surface [49] and/or counter adaptive mechanisms engaging opposing or compensatory regulatory systems. One proposed is the RF-amide family that might also contribute to the development of tolerance [12]. As kisspeptin has been previously demonstrated to bind and activate NPFF receptors [23], it is plausible that its effect on acute morphine tolerance is mediated by the NPFF system. Results of several experiments suggest that NPFF might play a role in morphine tolerance and dependence. First, antibodies against the peptide can reverse morphine tolerance [17]. Second, NPFF was found to induce withdrawal-like symptoms [26]. Last, more recent publications have reported that the tolerance inducing effect of NPFF might be mediated by GRK2-dependent phosphorylation of MOR induced by NPFF receptor activation in cell cultures, which will ultimately lead to a decrease in the number of functional MOR with no associated internalization of the receptors [33]. Based on the above mentioned data, NPFF receptors induce tolerance rather then inhibit it, which is in contrast with our results with KP-13. This discrepancy might be explained by the difference in treatment protocols used in previous publications with NPFF compared to our experiments. In other studies, NPFF was co-administered with morphine; however, in our study, the animals received kisspeptin 60 min before morphine treatment. It is possible that KP-13 already reduced the number of functional MORs on the cell surface by the time of morphine treatment, thus blunting the tolerance-inducing effect of morphine [40]. Another possible reason behind our results might be that the tolerance-inhibiting action of KP-13 was mediated not by NPFFRs but KISS1R. This is supported by the distribution data of KISS1R expression and the diverse signal transduction cascades mobilized in case of receptor activation [4,15].

Our results also demonstrate that KP-13 aggravates naloxone-precipitated abstinence syndrome. It must be noted that a significant change was only detected in escape behavior (jumping latency). Significant change in temperature and weight between the morphine withdrawal and the KP-13 treated morphine withdrawal groups was not found, rather, only a tendency was observed. As previously mentioned, the NPFF system as an opioid-modulating system involved in maintaining homeostasis counteracts the action of opioids contributing possibly to the development of tolerance [12]. In withdrawal studies, NPFF was able to produce some signs of a withdrawal syndrome in morphine-dependent rats [26]. Furthermore, the immunoneutralization of NPFF or injection of antisense oligonucleotides to the precursor proNPFFA decreased the intensity of withdrawal signs in morphine-tolerant animals [11,17,27]. This suggests that NPFF system might play a role in the mediation of the withdrawal syndrome. Taking these data into consideration, it is possible that the effect of KP-13 on withdrawal is mediated by the NPFF system.

Another interesting observation of our experiments was that KP-13 induced a hyperthermia in the animals revealed by the temperature recordings in the acute withdrawal study. 1 μg of KP-13 caused a marked elevation of body temperature 15, 30 and 60 min after peptide treatment compared to control mice. This underlies our previous results in rats, in which both 1 μg and 2 μg of KP-13 induced a lasting (appr. 6 h long) hyperthermia [7]. Thus, it seems that the hyperthermic action of KP-13 is shared among the two species. This might be due to KP-13's direct effect on the thermoregulatory center in the hypothalamus and/or on cardiovascular control and/or on metabolic rate. It has been previously implicated by Rance et al. that the KNDy (kisspeptin, neurokinin B and dynorphin) neurons of the arcuate nucleus send projections to the preoptic area where the thermoregulatory center is found [39]. They propose that these neurons acting via the neurokinin 3 receptor (NK3R) might mediate a peripheral vasodilation [39]. This is also supported by an article by Mittelman-Smith, which shows that the ablation of neurokinin-3 expressing neurons (KNDy neurons) results in a decreased tail skin temperature [31]. These results suggest that these neurons mediate peripheral vasodilation; however, via neurokinin-3/NK3R, not via kisspeptin/KISS1R signaling. Still, it is possible that KP-13 increases the set-point of the thermoregulatory center. Literature data also shows that kisspeptins might have a role in cardiovascular control, namely a positive inotropic and vasoconstrictor effect. However, it must be noted that these seem to be peripheral rather then central effects [24,28,41]. Considering the fact that we administered KP-13 icv, it is possible, but rather unlikely, that it would reach a plasma concentration high enough to act on the heart and vasculature. Finally, kisspeptin might influence basic metabolism. In point of fact, Tolson and colleagues found that in Kiss1r knock-out mice the body weight, leptin level and adiposity increased. Furthermore, these mice developed glucose intolerance and a decrease in appetite [47]. Overall, it is not clear how KP-13 exerts its hyperthermic action. Nevertheless, it must be noted that due to the increased core temperature, it is possible that tail skin temperature might increase as well, which could influence thermal nociception. Hole and Tjolsen proposed that an increased tail skin temperature might lower the threshold in heat-radiant tail-flick measurements [13]. Therefore, it is possible that the pronociceptive effect of KP-13 is due to an increased tail temperature rather than to altered nociception. However, in our experiments the tail skin of the mice was preheated to 36 °C, and only after that was the noxious thermal stimulus applied and the tail-flick latency measured. Thus, the differences in tail skin temperatures were accounted for in the experiment. Second, Lichtman et al. found that a 2 °C increase in tail temperature did not cause a significant change in nociceptive threshold. In our experiments the rise in core temperature did not exceed the 2 °C [21]. Third, the skin-tail temperature might not have increased with the core temperature in case of KP-13 acting via the thermoregulatory center or the circulation. These, taken together, all suggest that KP-13 exerts a pronociceptive effect that is not due to any change in tail skin temperature. To further characterize the pronociceptive effect of KP-13, we also investigated KP-13's effect on mechanical nociception and found that icv. administration of KP-13 produced a marked increase in sensitivity to non-noxious mechanical stimuli. This further supports the contention that KP-13 indeed has an effect on nociception.

In conclusion, our results indicate that centrally injected KP-13 reduces the nociceptive threshold independent of the sex of CFLP mice in the tail-flick test possibly through KISS1R activation, reverses morphine antinociception, and reduces acute morphine tolerance. Furthermore, KP-13 seems to worsen the withdrawal signs precipitated by naloxone in adult mice. Our data confirms and provides further support to the concept that all RF-amide peptides might play a modulatory role in nociception and raises the idea that with their diverse neuroendocrine functions might be important for the integration of neuroendocrine and nociceptive processes. In point of fact, several lines of evidence suggest an interaction between the reproductive axis and pain sensitivity, therefore kisspeptin might be an additional factor relaying information about reproductive state to nociceptive centers.

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