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Hyperalgesic activity of kisspeptin in mice

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Abstract

Background: Kisspeptin is a neuropeptide known for its role in the hypothalamic regulation of the reproductive axis. Following the recent description of kisspeptin and its 7-TM receptor, GPR54, in the dorsal root ganglia and dorsal horns of the spinal cord, we examined the role of kisspeptin in the regulation of pain sensitivity in mice.

Results: Immunofluorescent staining in the mouse skin showed the presence of GPR54 receptors in PGP9.5-positive sensory fibers. Intraplantar injection of kisspeptin (1 or 3 nmol/5 μ l) induced a small nocifensive response in naive mice, and lowered thermal pain threshold in the hot plate test. Both intraplantar and intrathecal (0.5 or 1 nmol/3 μ l) injection of kisspeptin caused hyperalgesia in the first and second phases of the formalin test, whereas the GPR54 antagonist, p234 (0.1 or 1 nmol), caused a robust analgesia. Intraplantar injection of kisspeptin combined with formalin enhanced TRPV1 phosphorylation at Ser800 at the injection site, and increased ERK1/2 phosphorylation in the ipsilateral dorsal horn as compared to naive mice and mice treated with formalin alone.

Conclusion: These data demonstrate for the first time that kisspeptin regulates pain sensitivity in rodents and suggest that peripheral GPR54 receptors could be targeted by novel drugs in the treatment of inflammatory pain.

Keywords: Kisspeptin, GPR54, inflammatory pain, nociceptive sensitization

Background

Kisspeptin is a 54-amino acid peptide originally discovered for its activity as metastasis-suppressor [1]. It is encoded by the *Kiss1* gene as a 145-amino acid precursor protein and cleaved to a 54-amino acid protein as well as into shorter products (kisspeptin-10,-13,-14) known to play a critical role in the neuroendocrine regulation of reproduction [2-5].

In the brain, kisspeptin is localized not only in areas involved in gonadotropin secretion, but also in other regions such as the amygdala, hippocampus, and the periaqueductal gray [6,7].

Its action is mediated by a 7-TM receptor named GPR54, also known as KISS1R, which is coupled to polyphosphoinositide hydrolysis *via* a $G_{q/11}$ GTP binding protein [2,8].

Loss-of-function mutations of GPR54 cause a non-Kallman variant of hypogonadotropic/hypogonadism in humans (i.e. hypogonadotropic/hypogonadism without anosmia) [2,9]. Interestingly, the expression of kisspeptin

and GPR54 is not restricted to the hypothalamus. Relatively high levels of kisspeptin and GPR54 are found in forebrain regions, such as the hippocampus and amygdala, as well as in the periaqueductal grey [10]. The investigation of the extrahypothalamic functions of kisspeptin is still at its infancy. Treatment with kainic acid increases kisspeptin mRNA levels in the hippocampus, and kisspeptin enhances the amplitude of excitatory postsynaptic currents in granule cells of the hippocampal dentate gyrus [6,7]. This suggests a potential role for kisspeptin in the regulation of synaptic plasticity in the CNS. Recent findings have shown an intense kisspeptin and GPR54 immunostaining in dorsal root ganglia (DRG) neurons and in lamina I and II of the dorsal horns of the spinal cord [11,12]. The transcripts of kisspeptin and GPR54 are up-regulated in DRG and dorsal horn neurons in the complete Freund adjuvant (CFA) model of chronic inflammatory pain [12], suggesting that kisspeptin may play a role in mechanisms of nociceptive sensitization. However, how precisely kisspeptin regulates pain sensitivity is obscure at present.

We now report that peripheral or intrathecal injection of kisspeptin causes hyperalgesia and induces biochemical

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changes that are consistent with mechanisms of peripheral and central nociceptive sensitization.

Methods

Animals

Adult male CD1 mice (Charles River, Calco, CO, Italy), 129S6/Sv/Ev wild-type, and 129S6/Sv/Ev *Gpr54*- knock-out mice [13] aged between 8 and 9 weeks were used in these experiments. Mice were housed 10 animals per cage with food and water *ad libitum* in standard 12/12 h light/dark cycle, for a period of 2 weeks before testing.

All experiments were carried out according to the recommendations of Institutional Animal Care and Use Committee (IACUC). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Drug administration

Kisspeptin (Calbiochem Merck KGaA, Darmstadt, Germany) was dissolved in 5% DMSO and injected intrathecally (3 μ l) or subcutaneously (5 μ l) into the plantar surface of the right hind paw using a 10 μ l luertip-syringe (Hamilton) fitted with a 30-gauge needle. p234 (Sigma-Aldrich, St. Louis, MO) was dissolved in phosphate buffered saline (PBS) and injected in a volume of 3 μ l for intrathecal administration or 5 μ l for intraplantar administration.

Behavioral experiments

Hot plate test

The hot plate test (Ugo Basile, Italy) was used to assess thermal sensitivity. CD1 mice were placed onto the hot plate at the temperature of $55 \pm 0.1^\circ\text{C}$. Paw withdrawal thresholds were determined in the hind paws of ipsilateral hind limb. Animals were kept on the plate until the first sign of ipsilateral paw lift or lick was recorded as basal withdrawal latency (pre-drug latency). A maximum cut-off paw withdrawal latency of 20 seconds was chosen to prevent tissue damage (cut-off time). Post-dose thresholds were taken at 5, 15, 30, and 60 minutes after drug administration (post-drug latency). For each animal, results were expressed as the percentage maximum possible effect (%MPE) calculated using the following formula: $[(\text{post-drug latency} - \text{pre-drug latency}) / (\text{cut-off time} - \text{pre-drug latency})] \times 100$.

Formalin test

Inflammatory pain was assessed using the formalin test. Ten μ l of a 5% formalin solution was injected subcutaneously into the plantar surface of the right hind paw of CD1 mice. After the injection, mice were immediately placed in a plexiglas box (20 \times 15 \times 15 cm) surrounded by mirrors to allow the observation of nociceptive responses that include licking, lifting and shaking of the injected paw. Tests were performed between 08:00 h and 12:00 h to minimize variability. Mice were observed for

1 hour. Formalin scores were separated into two phases, phase I (0-10 min) and phase II (15-45 min). The mean behavioural score was calculated in blocks of 5 min for each of the two phases. A mean response was then calculated for each phase.

Spontaneous pain

CD1 mice that received intraplantar injection of kisspeptin or vehicle were placed in a cage immediately after the injection, and the duration of hind paw lifting and licking during the first 5 minutes were measured.

All behavioral tests were analyzed by observers blind to the treatment of the animals.

Immunohistochemistry

Skin biopsies

Animals were euthanized with chloral hydrate (320 mg/kg i.p.). 2.5-mm punch skin biopsies from the plantar surface of the hind paws were performed and fixed in Zamboni fixative (2% paraformaldehyde, 15% picric acid saturated aqueous solution, 0.1 M phosphate buffer pH 7.4) for 24 hours. Biopsies were cryoprotected with 20% sucrose in PBS overnight at 4°C . Sections of 10 μ m were cut at the cryostat and mounted on glass slides for immunohistochemical analysis.

Immunohistochemistry procedures were performed as previously described [14]. Double immunofluorescence was performed in skin biopsies from CD1 male mice incubating sections overnight with polyclonal rabbit anti-human PGP 9.5 (1:1000; AbD Serotec, Kidlington, UK) and goat polyclonal anti-GPR54 (1:20; Santa Cruz Biotechnology, Santa Cruz, CA) and then for 1 h with secondary fluorescein anti-rabbit (1:100; Vector Laboratories, Burlingame, CA) and Cy3 anti-goat (1:400; Chemicon, Billerica, MA) antibodies. Control staining was performed without the primary antibodies.

Immunostaining was performed in skin biopsies from male 129S6/Sv/Ev wild-type and 129S6/Sv/Ev *Gpr54*-knock-out mice [13] to test the specificity of the anti-GPR54 antibody. Tissue sections were incubated overnight with goat polyclonal anti-GPR54 (1:20; Santa Cruz Biotechnology, Santa Cruz, CA) and then for 1 h with secondary biotin-coupled anti-goat (1:100; Vector Laboratories, Burlingame, CA). SG (SG substrate kit; Vector Laboratories, Burlingame, CA, USA) chromogen was used for detection.

Spinal cord

CD1 mice ($n = 5$ per group) were used. 3 min after kisspeptin (3 nmol) or vehicle (DMSO) were co-injected with formalin in the right hind paw and lumbar spinal cords were removed and fixed in formalin (4%) overnight, transferred in 70% ethanol and included in paraffin. Ten serial sections were cut and used for immunohistochemical analysis. Deparaffinized sections were treated with 10 mmol/L citrate buffer, pH 6.0, and heated by

microwave for 10 minutes for antigen unmasking. Sections were soaked in 3% hydrogen peroxide to block endogenous peroxidase activity. Tissue sections were incubated overnight with monoclonal rabbit antibody anti-phospho-p44/42 (Erk1/2) (Thr202/Tyr204) (D13.14.4E)XP™ (1:200; Cell Signaling Technology, Denver, MA, USA) and then for 1 h with secondary biotin-coupled anti-rabbit (1:200; Vector Laboratories). 3,3-Diaminobenzidine tetrachloride was used for detection. Control staining was performed without the primary antibodies.

Densitometric analysis of p-ERK immunoreactivity

Intensity of p-ERK immunoreactivity was quantified by measuring the optical densities of the outer laminae of the dorsal horn in the stained sections relative to the background (ventral horn). Images were acquired using a computer-based microdensitometer (NIH Image Software, Bethesda, MD, USA). Values were the mean of measurements made on ten sections (10 μ m) sampled 1 into a 3 series spanning the extent of the L4-L5 spinal cord.

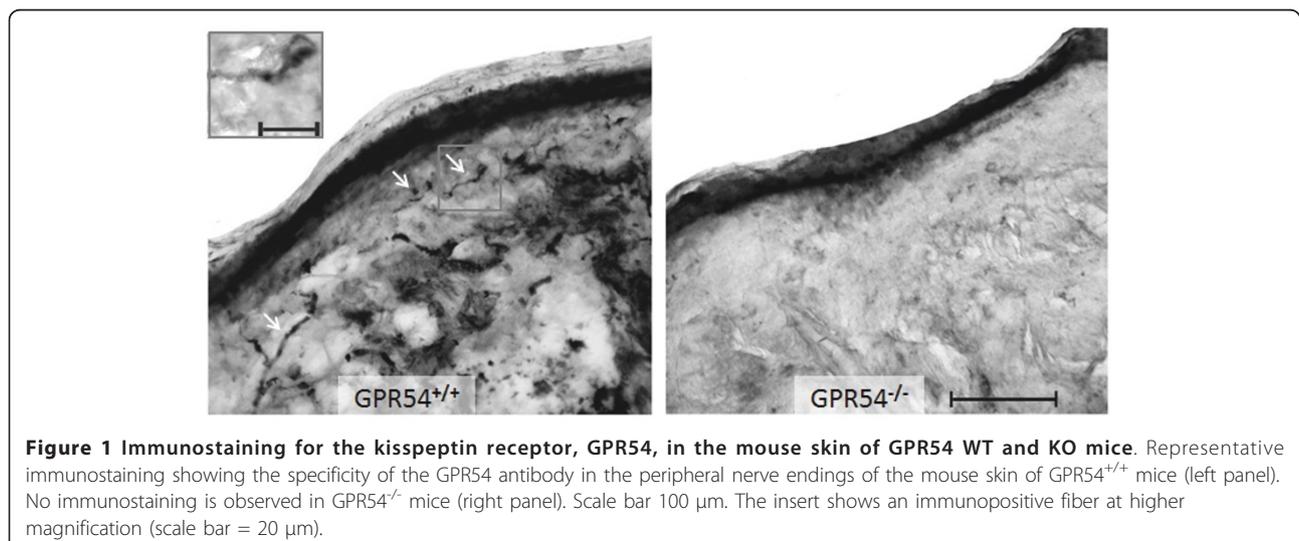
Western blot analysis

CD1 Mice were sacrificed 3 min following treatment and skin lysates of all groups were processed in western blot. Skin homogenates were obtained as previously described [15]. Ten μ g of total protein were separated by 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred onto protein-sensitive nitrocellulose membranes (Criterion blotter; Bio-Rad Laboratories, Hercules, CA). The membranes were blocked in Odyssey blocker (LI-COR Biosciences, Lincoln, NE) for 1 h, and the following primary antibodies were used: anti-TRPV1 (phospho S800) polyclonal antibody (1:400, Abnova, Aachen, Germany); anti-actin monoclonal antibody (1:1000, Sigma). Secondary antibodies were: goat anti-rabbit (IRD800CW)

and goat anti-mouse (Alexa 680, LI-COR, Bioscience) antibodies. Proteins were detected with the Odyssey Infrared Fluorescence Imaging System (LI-COR).

Results

Knowing that the kisspeptin receptor, GPR54 (KISS1R), is present in DRG neurons [12], we performed immunofluorescent analysis to examine whether the receptor was also present in peripheral nociceptors. We focused on the peripheral role of kisspeptin in the modulation of acute and inflammatory pain. First we examined the specificity of the GPR54 antibody in skin biopsies from GPR54 KO mice. No immunostaining was seen in sensory nerve terminals of GPR54 KO mice (Figure 1). The nature of the nonspecific staining seen in the outer portion of the skin of GPR54 KO mice is unknown. In punch skin biopsies from the mouse hind paw, sensory fibers ascending vertically between the keratinocytes to reach the stratum corneum of the epidermis were identified by fluorescent immunostaining for the neuron-specific ubiquitin hydrolase, PGP9.5 [14] (Figure 2A). These fibers also expressed GPR54, as shown by double fluorescence immunostaining (Figure 2B, C). Behavioral experiments were performed after peripheral (intraplantar) and central (intrathecal) administration of kisspeptin at doses ranging from 0.1 to 3 nmol [16]. We first examined the effect of intraplantar injection of kisspeptin on nocifensive behavior in naïve mice. Nocifensive behavior consisting of licking, flinching and shaking of the injected paw was evaluated after a single injection of kisspeptin (3 nmol/5 μ l) or vehicle into the plantar surface of the right hind paw. The time spent in nocifensive behavior was recorded for 5 min after the injection. Intraplantar injection of kisspeptin (3 nmol/5 μ l) induced brief nocifensive behavior that lasted for about 5-15 seconds, whereas no signs of pain were seen in vehicle-



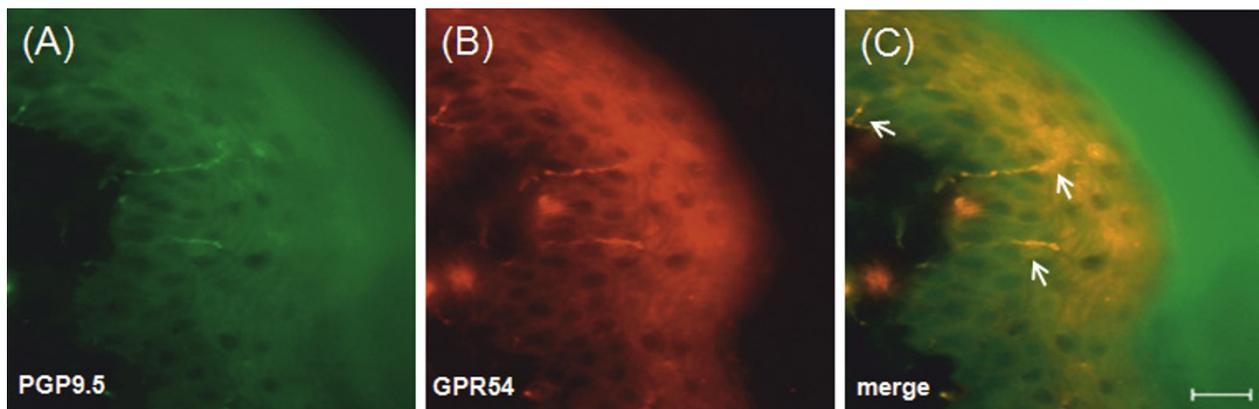


Figure 2 Double immunofluorescent staining for the kisspeptin receptor, GPR54, and PGP9.5 in the mouse skin. Immunofluorescent staining of PGP9.5 and GPR54 is shown in (A) and (B), respectively. Co-immunolocalization is shown in (C) (see arrowheads). Scale bar 20 μ m.

injected mice (Figure 3A). We then assessed the effect of kisspeptin on acute thermal pain using the hot plate test. Intraplantar injection of kisspeptin (3 nmol/5 μ l) significantly reduced paw withdrawal latency in response to heat as compared to intraplantar injection of vehicle (Figure 3B), whereas no differences were observed after p234 injection (0.1 nmol/5 μ l) (Figure 3C).

For the assessment of inflammatory pain, mice were subjected to the formalin test, 15 min after intraplantar (0.1, 1 and 3 nmol/5 μ l) or intrathecal (0.1, 0.5 and 1 nmol/3 μ l) injection of kisspeptin. Intraplantar injection of formalin elicits a biphasic nocifensive response characterized by licking, lifting and shaking of the injected paw. The first phase of the formalin test, starting immediately after formalin injection and lasting for about 10 min, represents a form of acute pain elicited by direct activation of nociceptors. The second phase of the test (occurring

approximately 15-45 min after formalin injection) reflects the development of nociceptive sensitization in the dorsal horns of the spinal cord [17,18]. Intraplantar injection of both 1 and 3 nmol/5 μ l of kisspeptin (15 min prior to formalin injection) caused hyperalgesia in the first and second phases of the formalin test whereas no effects were observed at the lower dose of 0.1 nmol/5 μ l (Figure 4). We also assessed the effect of the selective GPR54 antagonist, peptide 234 (p234) [19] in the formalin test. As opposed to kisspeptin, intraplantar injection of p234 (1 nmol/5 μ l; 15 min prior to formalin) significantly reduced nocifensive behavior (Figure 4B). A lower dose of p234 (0.1 nmol/5 μ l) induced a trend to an analgesic effect, which was not statistically significant (Figure 4B). We also examined whether intrathecal injection of kisspeptin or p234 could affect nocifensive behavior in the formalin test. Kisspeptin injected intrathecally at the dose of 1 nmol/3 μ l, 10 min prior to

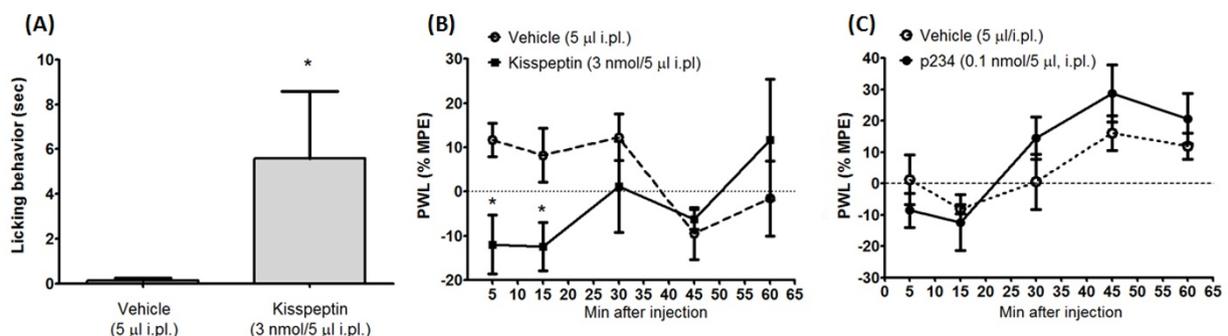
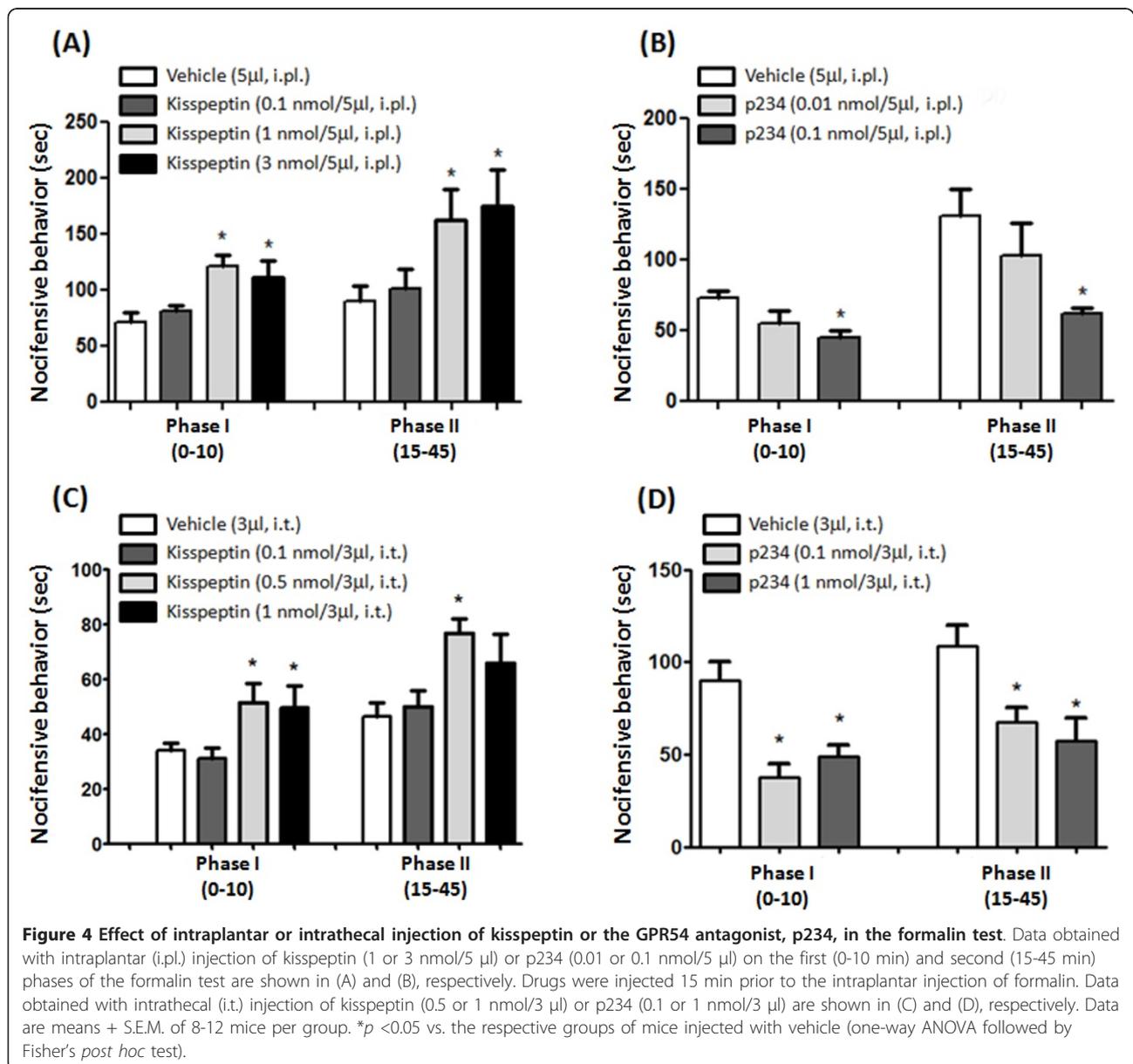


Figure 3 Intraplantar injection of kisspeptin lowers pain threshold in the hot plate. The nocifensive response to intraplantar injection of kisspeptin (3 nmol/5 μ l) in naïve mice is shown in (A). Data are means \pm S.E.M. of 6 mice, and refer to the number of sec spent in licking behavior in the first 5 min following injection. * p < 0.05 (Student's *t* test) vs. mice injected with vehicle. Data obtained in the hot plate test are shown in (B). For each animal, the percentage maximum possible effect (%MPE) was calculated using the following formula: [(post-drug latency) - (pre-drug latency)] / (cutoff time - (pre-drug latency)) \times 100. Data are means \pm S.E.M. of 6 to 8 mice. * p < 0.05, two-way ANOVA followed by Fisher's *post hoc* test. PWL, Paw-withdrawal latency.

intraplantar injection of formalin, significantly increased nocifensive behavior in the first and second phases of the formalin test. A lower dose of kisspeptin (1 nmol/3 μ l) caused hyperalgesia in the first phase, and a non-significant trend to hyperalgesia in the second phase of the test (Figure 4C). When injected intrathecally, compound p234 was analgesic at doses of 0.1 and 1 nmol/3 μ l in both phases of the formalin test (Figure 4D). The hyperalgesic activity of kisspeptin in both phases of the formalin test led us to investigate whether the peptide could induce biochemical changes that were consistent with mechanisms of peripheral and central sensitization. We therefore examined TRPV1 channel phosphorylation in the skin of the hind paw, and activation of ERK1/2 in the dorsal horns of the

spinal cord in mice subjected to intraplantar injection of formalin preceded by kisspeptin or vehicle. Immunoblot analysis with anti-phosphorylated TRPV1 antibodies showed a single band at the expected molecular size of 95 kDa. We observed that in mice pretreated with vehicle, intraplantar injection of formalin slightly increased the levels of phosphorylated TRPV1 in the ipsilateral hind paw as compared to naïve mice. This effect was largely amplified in mice pretreated with kisspeptin (3 nmol/5 μ l, 15 min prior to formalin injection) (Figure 5). Activation of the mitogen activated protein kinase (MAPK) pathway was examined by immunohistochemical analysis of phosphorylated ERK1/2 in the dorsal horns of the spinal cord after intraplantar injection of formalin preceded by vehicle



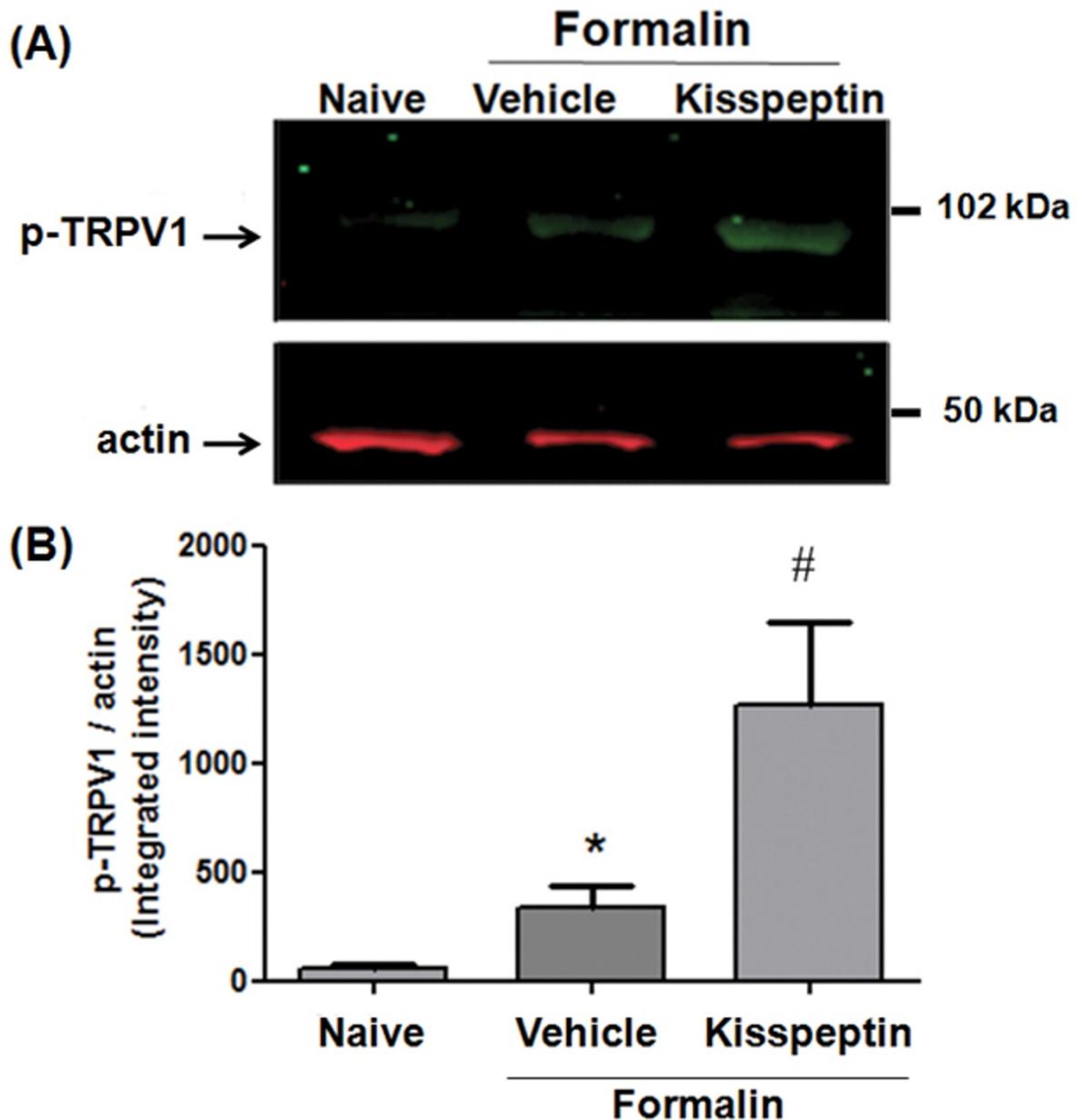


Figure 5 Intraplantar injection of kisspeptin amplified the increase in TRPV1 phosphorylation in the skin of mice treated with formalin. A representative immunoblot of (Ser800)-phosphorylated TRPV1 in the skin of naïve mice and mice injected with formalin in the absence or presence of kisspeptin (3 nmol/5 μ l) is shown in (A). Densitometric analysis is shown in (B), where values are means + S.E.M. of 4 determinations. * $p < 0.05$ vs. naïve mice, # $p < 0.05$ or vs. mice treated with formalin alone (one-way ANOVA followed by Fisher's *post hoc* test).

or kisspeptin. Formalin injection preceded by vehicle slightly enhanced phosphorylated ERK1/2 immunostaining in the dorsal horn ipsilateral to the injection side as compared to the contralateral dorsal horn or the dorsal horns of naïve mice (Figure 6). Pretreatment with kisspeptin (3 nmol/ μ l) dramatically enhanced the expression of phosphorylated ERK1/2 in the ipsilateral dorsal horn (Figure 6).

Discussion

These data offer the first demonstration that kisspeptin, a peptide known for its role in the regulation of the hypothalamic-pituitary-gonadal axis, lowers pain threshold and enhances nocifensive behavior in mice. Immunohistochemical analysis showed the presence of the kisspeptin receptor, GPR54, in peripheral sensory fibers, a finding

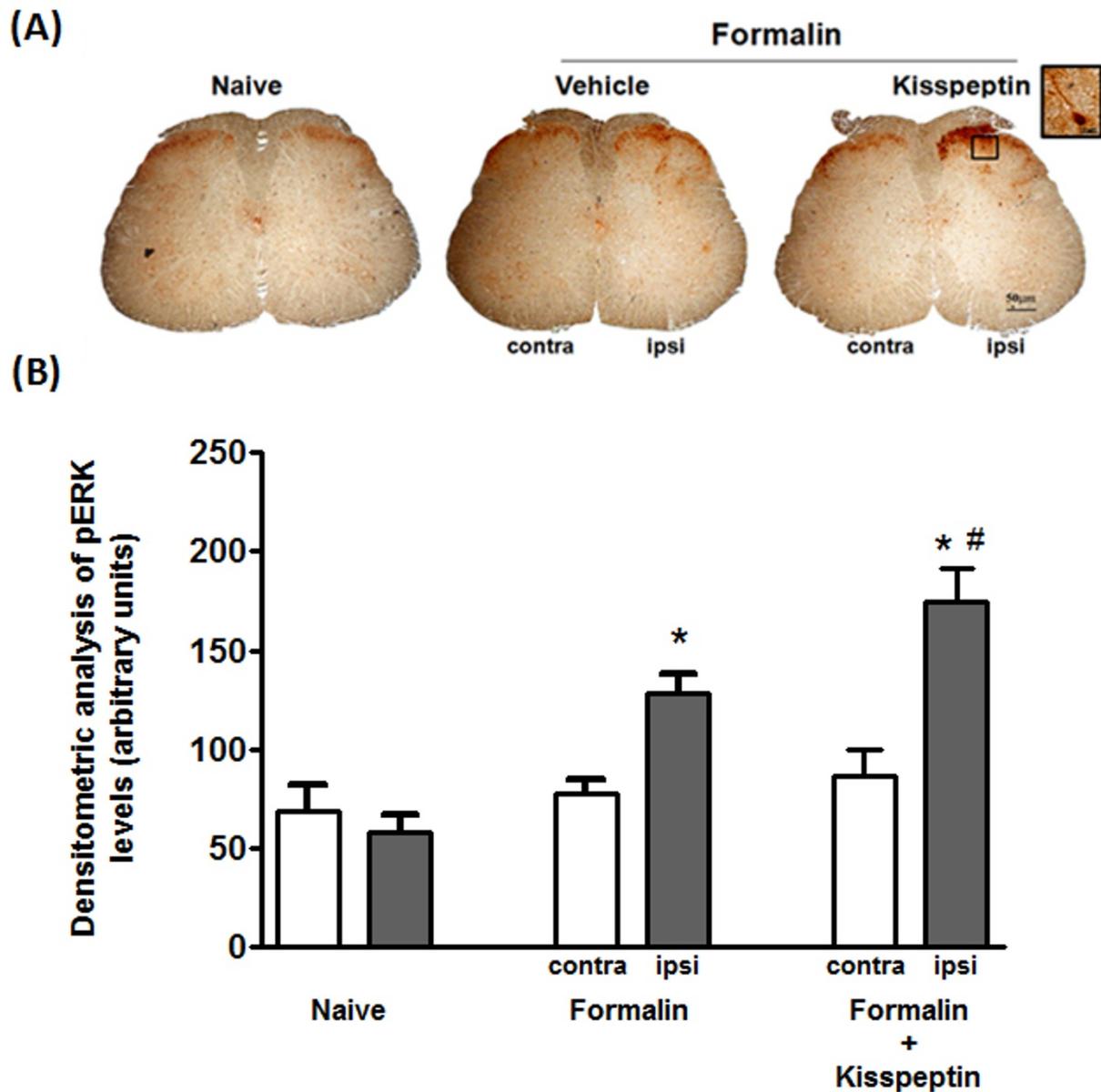


Figure 6 Intraplantar injection of kisspeptin increased ERK phosphorylation in the ipsilateral dorsal horn of the spinal cord. (A) Immunohistochemical analysis of phosphorylated-ERK1/2 in the dorsal horns of the spinal cords of naive mice and mice treated with formalin in the absence or presence of kisspeptin (3 nmol/5 μ l) is shown. Contra = contralateral; ipsi = ipsilateral. Scale bar = 50 μ m. The insert shows an immunopositive neuron at higher magnification (scale bar = 10 μ m). (B) Densitometric analysis of p-ERK immunoreactivity in the superficial laminae of the dorsal horn. * p < 0.05 vs. contralateral values; # p < 0.05 vs. formalin alone values (one-way ANOVA + Dunnett's Multiple Comparison Test).

that is consistent with the detection of GPR54 mRNA and protein in DRG neurons [11,12]. The lack of staining in GPR54 KO mice indicates that GPR54 is present in peripheral nociceptors explaining the hyperalgesia caused by intraplantar injection of kisspeptin in the hot plate and formalin test. We wish to highlight that intraplantar kisspeptin induced only a small nociceptive response on its

own, suggesting that a main action of kisspeptin is to amplify pain sensitivity in response to noxious stimuli. Intraplantar injection of the GPR54 antagonist, p234, caused a robust analgesia in the formalin test, suggesting that endogenous kisspeptin acts extracellularly to activate GPR54 receptors during inflammatory pain. Kisspeptin is present in DRG neurons, where it co-localizes with

isolectin B4 and calcitonin gene-related peptide, and its expression is up-regulated by chronic inflammatory pain [12]. It is likely that kisspeptin is released from peripheral nociceptors in response to noxious stimuli, therefore behaving as an autocrine/paracrine factor to promote peripheral nociceptive sensitization. Whether other cells can produce and secrete kisspeptin during inflammation is unknown at present. Phosphorylation of the TRPV1 ion channel is a key event in mechanisms of peripheral nociceptive sensitization [20-22]. The TRPV1 receptor can be phosphorylated by multiple protein kinases, including protein kinase A, protein kinase C (PKC), calcium/calmodulin-dependent protein kinase II, and SRC [23-34]. In particular, PKC phosphorylates TRPV1 at Ser-502 and Ser-800, thus amplifying ion channel activity [31,35-37]. Intraplantar kisspeptin caused a robust increase in (Ser800)-TRPV1 phosphorylation, an effect that was likely mediated by the activation of the GPR54 receptor, with ensuing stimulation of inositol phospholipid hydrolysis, diacylglycerol formation, and PKC activation [2,8]. Thus, kisspeptin might act similarly to other hyperalgesic molecules that activate Gq-coupled receptors and phosphorylate TRPV1 channels in peripheral nociceptors, such as bradykinin, group-I mGlu receptor agonists, P2Y2 receptor agonists, EP1 receptor, and prokineticin [28,38-48].

Hyperalgesia by kisspeptin and analgesia by p234 were also seen in the second phase of the formalin test, which reflects the development of central nociceptive sensitization in the dorsal horns of the spinal cord [17,18]. Central nociceptive sensitization is mediated by a series of mechanisms that ultimately lead to an enhancement of excitatory transmission at the synapses between primary afferent fibers and second order sensory neurons in the dorsal horns of the spinal cord [24]. The relevance of the MAPK pathway in the development of central sensitization has been highlighted in a recent review [48]. Intraplantar injection of formalin is known to induce a rapid phosphorylation of ERK1/2 in the spinal cord, which has been causally related to the increase in nocifensive behavior seen in the second phase of the formalin test [49]. Pharmacological activation of mGlu1 and mGlu5 receptors, which also couple to the Gq protein just like GPR54 [50], can also enhance ERK1/2 phosphorylation in the spinal cord [51]. Activation of GPR54 by kisspeptin has been shown to stimulate the ERK/MAPK pathway both in recombinant expression systems and hypothalamic explants [52,53]. Intraplantar injection of kisspeptin markedly amplified ERK1/2 phosphorylation induced by formalin in the ipsilateral dorsal horn, evidence that nicely supports the behavioral data obtained with kisspeptin in the second phase of the formalin test. Interestingly, kisspeptin retained the hyperalgesic activity (and p234 the analgesic activity) when injected by the intrathecal route. Thus, it is likely that the modulation of pain sensitivity by

GPR54 extends beyond peripheral nociceptors. Effects of kisspeptin on different receptors cannot be excluded. In particular it has been reported that kisspeptin can also bind neuropeptide FF (NPFF) receptors [54]. However in our hands intrathecal injection of kisspeptin lowers pain threshold, whereas intrathecal injection of NPFF is known to cause analgesia [55], thus the effect of kisspeptin in the spinal cord is likely mediated by the activation of the GPR54 receptor excluding an interaction of kisspeptin with NPFF receptors.

The presence of GPR54 receptor in the amygdala [56] may suggest that kisspeptin acts also at higher brain centers that control the affective components of pain and contributes to the top-down regulation of pain threshold.

Conclusions

In conclusion, our data disclose a new aspect in the physiology of kisspeptin and suggest that peripheral GPR54 receptor antagonists (lacking potential hypothalamic side effects) can be developed as new drugs for the treatment of inflammatory pain. In addition, it will be interesting to explore whether individuals with hypogonadotropic hypogonadism due to inactivating mutations of GPR54 show alterations in the sensitivity to pain.

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Authors' contributions

The study was conceived and the experiments were designed by FN, SC, MAS, AC GC and WHC. SC and SS performed behavioral experiments and western blot analysis. AT, AB and FB, performed immunohistochemical analysis. All authors contributed to writing the manuscript, and all read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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