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Hemopressin, an inverse agonist of cannabinoid receptors, inhibits neuropathic pain in rats

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Abstract

Direct-acting cannabinoid receptor ligands are well known to reduce hyperalgesic responses after nerve injury, although their psychoactive side effects have damped enthusiasm for their therapeutic development. Hemopressin (Hp) is a nonapeptide that selectively binds CB1 cannabinoid receptors (CB_1 receptors) and exerts antinociceptive action in inflammatory pain models. We investigated the effect of Hp on neuropathic pain in rats subjected to chronic constriction injury (CCI) of the sciatic nerve, and explored the mechanisms involved. Oral administration of Hp inhibits mechanical hyperalgesia of CCI-rats up to 6h. Hp treatment also decreases Egr-1 immunoreactivity (Egr-1Ir) in the superficial layer of the dorsal horn of the spinal cord of CCI rats. The antinociceptive effect of Hp seems to be independent of inhibitory descending pain pathway since methysergide ($5HT_{1A}$ receptor antagonist) and yohimbine (α-2 adrenergic receptor antagonist) were unable to prevent Hp antinociceptive effect. Hp decreased calcium flux on DRG neurons from CCI rats, similarly to that observed for AM251, a CB_1 receptor antagonist. We also investigated the effect of Hp on potassium channels of CCI rats using UCL 1684 (a blocker of Ca^{2+}-activated K^+ channels) which reversed Hp-induced antinociception. Furthermore, concomitant administration of URB-584 (FAAH inhibitor) but not JZL-184 (MAGL inhibitor) potentiates antinociceptive effect of Hp in CCI rats indicating an involvement of anadamide on HP-induced antinociception. Together, these data demonstrate that Hp displays antinociception in pain from neuropathic etiology through local effects. The release of anandamide and the opening of peripheral K^+ channels are involved in the antinociceptive effect.

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Keywords

CB1 cannabinoid receptors; Hemopressin; Chronic constriction injury model; Hyperalgesia; Dorsal root ganglion

1. Introduction

Neuropathic pain is defined as a chronic or persistent pain state that results from an injury or dysfunction of the nervous system, and includes clinical symptoms of hyperalgesia, allodynia and spontaneous ongoing pain [13]. Due to its persistent condition, neuropathic pain represents a major public health problem. A variety of therapeutic approaches, including opioid analgesics, tricyclic antidepressants, anticonvulsants, and local anesthetics have been used to treat neuropathic pain. But due to the complexity of the mechanisms involved, the treatment is often ineffective [13]. The use of Δ9-THC (derived from *Cannabis sativa)* for the treatment of various neurological disorders, including chronic pain, is supported by experimental and clinical data [6,10,23]. Although they are seen as promising target for the development of medications, clinical and preclinical studies have shown that Δ9-THC and other CB1 ligands generally produce undesirable effect in the central nervous system. CB1 agonists are generally at risk for psychoactive effects and dependence, limiting the optimization of doses in clinical trials and preclinical studies [28]. Thus, development of drugs capable of binding to the cannabinoid receptors without psychoactive effects provide therapeutic potential without the risk of adverse effects, making it a valuable tool for the treatment of several disorders related to the cannabinoid system [28]. Hemopressin (Hp), a nonapeptide (PVNFKFLSH) derived from the hemoglobin α1 chain was previously shown to target CB_1 receptor, and to modulate its signaling [19]. Hp exhibits antinociceptive effects in inflammatory pain models [18,19]. In this sense, it was demonstrated that Hp inhibits carrageenan-induced hyperalgesia only at the injured paw; without antinociceptive effect observed in the contralateral, uninflamed paw, indicating that the effect of Hp is limited to tissue injury-induced pain [19]. Also, intrathecal administration of Hp induces significant antinociception in the first and second phases of the formalin test [18]. The effects of Hp on carrageenan-induced hyperalgesia are independent of route of administration (oral, local, or intrathecal) [19]. More interesting is the fact that neurological side effects that are typically associated with antinociceptive doses of CB_1 receptor ligands, including hypothermia, catalepsy and hypoactivity, were not reported with antinociceptive doses of Hp [19]. This, taken with the fact that the effects of Hp on carrageenan-induced hyperalgesia were found to be independent of route of administration, raises the possibility that Hp could be developed as a novel class of drug that modulates CB_1 receptor for the treatment of pain.

Since the majority of the previous studies focused on inflammatory pain and relatively little information is available regarding the role of Hp in alleviating chronic pain, in this study the effects of Hp on neuropathic pain using chronic constriction injury model (CCI) were examined.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 160-180 g, age-matched, were used throughout this study. Animals were maintained under controlled light cycle (12/12h) and temperature (22 ± 2 °C) with free access to food and water. Throughout the experiments, animals were managed using the principles and guidelines for the care of laboratory animals in studies involving pain and were approved by the Ethics Committee on the Use of Animals of Hospital Sírio-Libanês (CEUA, protocol number 2008/07).

2.2. Induction of neuropathic pain

Rats were anesthetized with halothane (2.5%) (Cristália) and subjected to chronic constriction injury (CCI) of the sciatic nerve according to the method of Bennett and Xie [3]. In the procedure, the sciatic nerve of the right paw was exposed at the middle of the thigh by blunt dissection through the biceps femoris. Proximal to the sciatic nerve's trifurcation (about 7 mm), the nerve was freed of adhering tissue and four ligatures (4.0 chromic gut) were tied loosely around it with about 1 mm spacing. Great care was taken to tie the ligatures, so that the diameter of the nerve was seen to be just barely constricted. The incisions were sutured in layers using silk suture wire (5-0) (Ethicon). As a control, rats were sham-operated by exposing the sciatic nerve without nerve compression ligation or constriction. Experiments were conducted on the 14th day after CCI induction.

2.3. Behavioral analysis

Pain threshold was measured using a paw pressure apparatus (Ugo Basile®, Italy), essentially as described [31]. Briefly, a force with increasing magnitude (16g/s) was applied to the right hind paw of rats. When animals reacted by withdrawing the paw, the force (in grams) needed to induce this response represented the pain threshold. Antinociceptive activity was expressed as the increase in the force needed to induce the withdrawal response in treated versus control animals.

2.4. Pharmacological treatments

2.4.1. Hemopressin—Hemopressin (Proteimax Biotechnology) was administered orally at the doses of 0.5 or 0.25 mg/kg as described [19].

2.4.2. Methysergide and yohimbine—Methysergide (Met) was administered intraplantar at the dose of 5 mg/kg; 100μl, and yohimbine (Yoh) was administered intrathecally (30 μg/animal; 50 μl). Both were administered 30 min before Hp (0.25 mg/kg, orally). Mechanical hyperalgesia was evaluated in sham-operated and CCI rats by the paw pressure 1 h after Hp administration. Both groups (Sham and CCI) were treated with only Hp, Met or Yoh, or the combinations Hp + Met and Hp + Yoh. Met and Yoh were purchased from Sigma–Aldrich®.

2.4.3. UCL1684—Intraplantar injection of UCL1684 (10 μg/paw; 100 μl) was administered concomitantly with oral Hp (0.25 mg/kg). Mechanical hyperalgesia was evaluated in sham-

operated and CCI rats by the paw pressure test. Both groups (Hp and Hp + UCL1684) were tested 1 h after Hp administration. UCL1684 was purchased from Sigma–Aldrich®.

2.4.4. URB597 andJZL184—Intraplantar injection of URB597 (100 μg/paw) and JZL184 (100 μg/paw) was administered concomitantly with oral Hp (0.25 mg/kg). Mechanical hyperalgesia was evaluated in sham-operated and CCI rats by the paw pressure test. The animals were treated with Hp, JZL184, URB597, Hp + JZL184 or Hp + URB597 and were evaluated 1 h after treatments. JZL184 and URB597 were purchased from Sigma®.

2.5. Immunohistochemistry

One hour after the hemopressin administration (0.25 mg/kg) rats were transcardially perfused with phosphate-buffered saline and 4% paraformaldehyde (Sigma–Aldrich®) in 0.1 M phosphate buffer, pH 7.4 (PB). The spinal cords (L4 and L5) were removed, left in the same fixative for 5–8 h and then cryoprotected overnight in 30% sucrose. Thirty μm frozen sections were immunostained for Egr-1 expression. The spinal cord sections were incubated free floating with a rabbit polyclonal antibody against the nuclear protein which is the product of the early response genes *egr-1* (also known as Zif268) (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:500 in PB containing 0.3% Triton X-100 plus 5% of normal goat serum. Incubation with the primary antibody was conducted overnight at 24 °C. After three washes (10 min each) in PB, the sections were incubated with biotinylated goat anti-rabbit sera (Vector Labs, Burlingame, CA) diluted 1:200 in PB for 2h at 24 °C. The sections were washed again in PB and incubated with the avidin–biotin–peroxidase complex (ABC Elite; Vector Labs). After the reaction with 0.05% 3,3′-diaminobenzidine and a 0.01% solution of hydrogen peroxide in PB and intensification with 0.05% osmium tetroxide in water, the sections were mounted on gelatin-and chromoalumen-coated slides, dehydrated, cleared, and coversliped. The material was then analyzed on a light microscope, and digital images were collected. A quantitative analysis was performed on the density of nuclei representative of the immunore-activity for Egr-1 (Egr1-IR) in the dorsal horn of the spinal cord (DHSC; laminae I–VI of the L4–L5 dorsal horn). Measurements were taken from 10 different sections for each animal analyzed, by using a 10× objective for the DHSC (680,800 lm2). A quantitative analysis of the immunolabeled material was performed with NIH Image J. The number of Egr-1 immunoreactive neurons of the right dorsal horn (treated side) was compared with the left side, to obtain the difference between treated and non-treated sides. The results were compared and subjected to statistical analysis.

2.6. Dorsal root ganglia (DRG) neuron cultures and calcium imaging

DRG from lumbar spinal cord (L4/L5) of CCI rats were minced in cold Hank's balanced salt solution (HBSS; Sigma–Aldrich®) and incubated for 90min at 37 °C in DMEM (low glucose; Sigma–Aldrich®) containing 0.5 trypsin, 1.0 collagenase type I and 0.1 DNase type A (Sigma–Aldrich®). Soybean trypsin inhibitor (SBTI; Sigma–Aldrich®) was added to neutralize trypsin. Neurons were pelleted, suspended in DMEM containing 10% fetal bovine serum, 10% horse serum, 1% PSS, glutamine (2 mM ml^{-1}) and 2.5 μg ml^{-1} DNAse type IV and plated on glass coverslips in petri dishes (35 mm diameter; MatTek Corporation, Ashland, MA) coated with Matrigel (BD Biosciences, Belford, MA). Cells were cultured for a minimum of 72 h. The petri dishes were then washed twice with a Hank's buffered salt

solution, pH 7.4 and incubated for 60min at 37 °C with Hank's buffered salt solution supplemented with 0.1% bovine serum albumin in the same solution to which 3–5 μM of Fluo3-AM (Molecular Probes, Eugene, OR) was added. After the incubation period, the petri dishes were washed twice again with the assay buffer (the same as described above) of which 2 ml were left in each Petri dish.

Cells on the coverslips were observed using a wide-field fluorescence Olympus IX-70 microscope and an LCPlan FL40× objective. A series of 30 pictures were taken within 90s; the five initial pictures were used to determine the baseline. Before the fifth picture, neurons were treated by an acute administration of KCl (5mM), or hemopressin (1,3 and 5 μM). The experiment was repeated 3 times per group. Fluorescence measurements reflecting elevations of intracellular calcium were taken at 460–490 nm excitation and 515 nm emission in individual cells using the acquisition program OpenLab software. Approximately 15–20 neurons were imaged per group.

2.7. Statistical methods

Results are presented as the mean ± standard error of the mean (SEM). Statistical analyses of data were generated using GraphPad Prism, version 4.02 (GraphPad Software Inc., San Diego, CA, USA). Statistical comparison of more than two groups was performed using analysis of variance (ANOVA), followed by Bonferroni's test. Statistical comparison for treatment over time was performed using two way ANOVA followed by Bonferroni's test. In all cases, $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Effect ofHp on CCI-induced hyperalgesia

The anti-nociceptive effects of Hp were investigated in an experimental model of chronic pain. Groups of rats were submitted to the constriction of the sciatic nerve (CCI); that caused a significant decrease of the pain threshold, inducing mechanical hyperalgesia in the right hind paw (Fig. 1), observed on day 14 after surgery. Sham-operated rats did not present alterations in threshold measurements, as compared to basal values (Fig. 1, dotted line). Oral administration of Hp (0.25 mg/kg) was able to reverse the hyperalgesic response in animals with neuropathic pain, when compared to the initial measurements of the animals after the chronic constriction of the sciatic nerve (Fig. 1A). Same results were observed with a higher dose of Hp (0.5 mg/kg; Fig. 1B). The inhibitory effect of Hp was observed up to 6 h after one single administration of the peptide and was reestablished for another 6 h after a second administration (Fig. 1C). These data show analgesic properties for oral administration of Hp peptide, which decreased mechanical hyperalgesia.

3.2. Activation of spinal nociceptors

It is generally accepted that the expression of immediate early genes such as *c-fos* and *egr-1* is enhanced following nociceptive stimulation in the superficial laminae of the dorsal horn of the spinal cord of rats [22]. Herein, the effects of oral administration of Hp on Egr-1 immunoreactivity (Egr-1-IR), a marker of neural activation, were evaluated. The immunohistochemical tests were performed on the sections obtained from animals which

had previously been evaluated for nociception and we examined only tissues from those animals that exhibited absence of nociception under sham-operation condition, the presence of hyperalgesia in animals with peripheral neuropathy and the reversal of this phenomenon after Hp treatment. Results presented herein correspond to the mean of the density of nuclei labeled for Egr1-IR in the superficial layer of the dorsal horn of the spinal cord of rats (DHSC; Table 1). The immunohistochemistry assays were performed throughout the L4-L5 spinal cord segments, which receive the majority of sciatic nerve afferents [33]. A significant increase of Egr-1-IR occurred bilaterally in the DHSC, in the animals with CCI (Table 1). Oral administration of Hp (0.25 mg/kg) significantly inhibited the number of Egr-1 immunolabeled nuclei in the superficial laminae of the dorsal horn (Table 1), while Hp on sham animals had no effect on the number of immunolabeled nuclei in basal conditions (Table 1).

3.3. Hp-induced analgesia is independent on the activation of the descending pain pathway

Next it was investigated whether the inhibitory effects induced by Hp on hyperalgesia involved the activation of the inhibitory descending pain pathway. For this, $5HT_{1A}$ receptors and α_2 adrenergic receptors were targeted. Methysergide treatment (Met; 30 μg per paw, intraplantar) that antagonizes $5HT_{1A}$ receptors and yohimbine treatment (Yoh; 30 μg per animal, intrathecal) that antagonizes α_2 adrenergic receptors did not affect the analgesic effect of Hp on CCI-induced mechanical hyperalgesia (Fig. 2A and B). Furthermore, treatment with either Met or Yoh, per se, did not modify CCI-induced hyperalgesia (Fig. 2A and B). These results suggest that neither serotonin nor α_2 adrenergic receptors activation are involved in the antinociceptive effect of Hp during CCI-induced hyperalgesia.

3.4. Hp-induced analgesia occurs directly on primary afferent neurons

Next it was investigated whether Hp was able to act directly on primary afferents leading to the inhibition of signaling in sensory neurons. Hp (1, 3 and 5 μM) significantly inhibited KCl-induced calcium mobilization (5 mM) on DRG from normal rats (Fig. 3A). To test whether the potency/efficacy of Hp on DRG neurons could be greatly increased in the inflamed state compared to the normal, uninjured state, the effect of Hp was evaluated on calcium flux of DRG cells from CCI rats. Hp was able to decrease KCl-induced calcium flux on DRG neurons, similar to the observed for normal DRG neurons (Fig. 3A). The inhibitory effect induced by Hp was similar to that observed for AM251, a CB_1 receptor antagonist (Fig. 3B). Also, concomitant treatment of DRG with Hp and AM251 induced similar decrease on DRG cell response.

3.5. Hp-induced analgesia depends on peripheral activation of apamin-sensitive Ca^{2+}-activated K^+ channels

We investigated whether the inhibitory effects induced by Hp on hyperalgesia could involve the participation of peripheral K^+ channels. 6,10-Diaza-3(1,3)8,(1,4)-dibenzena-1,5(1,4)-diquinolinacy clodecaphane (UCL1684) that blocks the calcium-activated K^+ channels was injected (10 μg/paw, i.pl.) immediately before the oral administration of Hp. We found that this treatment reversed the analgesic effect of Hp on CCI-induced hyperalgesia (Fig. 4).

UCL1684 by itself did not interfere with nociceptive threshold of CCI animals. These results support a role for peripheral potassium channels in mediating Hp-induced analgesia.

3.6. Involvement of the anandamide on Hp-induced antinociception

Finally, we evaluated whether endocannabinoids could be mediating Hp-induced antinociception. Inhibitors of fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase, responsible for the degradation of anandamide and 2-arachidonoylglycerol (2-AG), respectively, were evaluated on nociceptive behavior of CCI rats. Results demonstrate that JZL-184, an inhibitor of monoacylglycerol lipase, enzyme responsible for 2-AG degradation, did not interfere with Hp-induced antinociception (Fig. 5A). However, treatment of rats with URB-584, an inhibitor of FAAH, enzyme responsible for anandamide degradation, potentiated Hp-induced antinociception (1h CCI 28.33 ± 1.667; Hp 90.83 ± 8; URB597 93.33 ± 7.2; Hp + URB 146.7± 4.2; $P < 0.05$; $n = 5$; (Fig. 5B). These results suggest that the endocannabinoid anandamide might be involved on Hp-induced antinociception.

4. Discussion

In this study, it was evaluated the antinociceptive effects of the recently identified CB_1 receptor ligand Hp in a rat model of neuropathic pain using chronic constrictive injury of the sciatic nerve [3]. The nerve damage causes the paw of the injured limb to become hypersensitive to mechanical and thermal stimulus inducing behavioral changes on animals which are manifested by hyperalgesia, cutaneous hypersensitivity (allodynia) and spontaneous pain [3,30]. These symptoms begin at 2–5 days after injury induction with maximum responses at the end of 14 days and lasting up to 28 days [3]. Structural changes include the degeneration of A-fibers and most of the C-fibers [1,8] as well as to central sensitization [37].

We found that oral treatment with Hp could completely block signs of pain 14 days after CCI, supporting the idea that Hp induces true antinociception in this neuropathic pain model. Also, it was observed that Hp has a long lasting effect in blocking hind paw hypersensitivity; it lasts up to 6 h after peptide administration and it is reestablished for another 6 h upon a second Hp administration. The antihyperalgesic effect of Hp was noticed to be of the same intensity, regardless the dose tested on CCI model. These results are consistent with data published in previous reports using carrageenan-induced hyperalgesia test and on the writhing test where different doses of Hp were found to produce the same inhibitory effect on pain sensitivity [9,22]. Moreover, oral administration of Hp blocked the signs of neuropathic pain, demonstrating that Hp has a systemic action which is not influenced by gastric digestion. The tendency of Hp to self assemble to form nanostructure aggregates [5] might protect it from rapid degradation in an in vivo environment [17] what could explain the duration of its oral effect. Also, it was previously shown that administration of Hp by oral or intraperitoneal or intraplantar route achieve similar level of analgesia [19] supporting its oral bioavailability. These properties make Hp an attractive candidate scaffold for the development of novel therapeutics for the treatment of pain.

The mechanism of Hp-mediated inhibition of CCI-induced hyperalgesia in rats was examined using the expression of immediate early genes. The expressions of proto-oncogenes from the *c-fos, c-jun* and *egr-1* family are widely used as tools for assessing the degree of nociceptive activation [7,20]. These factors are induced in neurons in response to extracellular stimuli, including depolarization, neurotransmitters, and growth factors [38]. Results showed here demonstrate that CCI induces a significant increase of Egr-1 expression, which is characteristic of neuronal activation. This increase was observed specifically in the superficial laminae of the spinal cord (I–IV), areas that are associated with exteroceptive sensitivity, and that receive the terminals of A-delta and C noxious fibers [35]. Hp-treatment was able to reverse CCI-induced Egr-1 increase, thus suggesting that Hp is able to interfere with the transmission of CCI-induced pain message to the central nervous system, reducing nociceptor activation at a central level. Immunohistochemical findings revealed a highly concentrated accumulation of monoacylglycerol lipase (MGL) in the dorsal horn, especially in superficial layers, in pre-synaptic terminals colocalizing with neurochemical markers of peptidergic and non-peptidergic nociceptive terminals, and also with markers of local excitatory or inhibitory interneurons [21]. MGL is the enzyme responsible to inactivate the endocannabinoid 2-arachidonoylglycerol (2AG) [2,11,25,26] and its inhibition reduces mechanical and cold allodynia in neuropathic and inflammatory chronic pain [15,27,39]. Thus it is possible to suggest that the presence of MGL in the dorsal horn maybe involved in synaptic endocannabinoid signaling in the dorsal horn pain circuitry [21]. One hypothesis is that Hp could inhibit MGL in the dorsal horn and this in turn would lead to an increase of 2-AG inducing analgesia.

More interesting is the fact that Hp is able to inhibit calcium mobilization in DRG neurons from CCI animals reinforcing the idea that Hp modulate primary afferent nociceptive signal by inhibiting sensory neurons. This effect could be explained by the presence of multiple distinct voltage-gated potassium (Kv) channels in the rat DRG neurons [14,16,36]. These Kv play an important role in setting resting membrane potentials and in controlling action potential firing frequency and repolarization [26,32]. It was recently demonstrated that over 90% of small DRG neurons co-express Kv1.4 (an A-type potassium channel) and the cannabinoid receptor CB1, suggesting a functional synergistic action between Kv1.4 and CB1 [4]. Activation of Kv1.4 is regulated by its degree of phosphorylation [30,34,40]. The balance between phosphorylated and dephosphorylated Kv1.4 channels is regulated by changes in the intracellular Ca^{2+} concentrations [34]. Our results show that the blockade of calcium-activated K^+ channel by UCL1684 inhibited the antinociception induced by Hp, reinforcing the involvement of peripheral K^+ channels on Hp-induced analgesic effect on CCI-induced hyperalgesia. Also, as mentioned above Hp reduces Ca^{2+} on DRG neurons. So one hypothesis is that Hp could act directly on Kv channels thus reducing its activation state. Such as the neuroprotective compound riluzole that prolongs the activation of Kv1.4 by slowing dramatically its inactivation, by a direct oxidation of a cysteine residue in the N-terminal inactivation domain of the channel thus leading to a cAMP-independent inhibition of glutamate release in the nerve terminals by a reduced Ca^{2+} influx-dependent depolarization [41].

Data presented herein demonstrates that the blockade of calcium-activated K^+ channel by UCL1684 inhibited the antinociception induced by Hp, reinforcing the idea of the involvement of peripheral K^+ channels on Hp-induced analgesic effect on CCI-induced hyperalgesia. Taken together these data reveal sensory neurons as important cellular target for the effects of Hp in the context of pain.

Several mechanisms have been proposed to underlie the antinociceptive effect of CB_1 receptor block. The literature demonstrates a considerable increase of anandamide and 2-AG in areas known to be involved in nociceptive transmission during noxious stimulation of different origins [12]. These authors also correlated increases in endocannabinoid levels with an increase in activation of the inhibitory descending pain pathway. It is known that serotonergic pathways participate the antinociceptive processes, and that 5-HT receptors in the modulation of nociception. 5-HT_{1A} receptors are enriched in (superficial) dorsal horn laminae involved in the processing of primary afferent information [29], and that norepinephrine can act via α2-adrenoceptors in spinal dorsal horn neurons to reduce their hyperexcitability following chronic nerve injury [24]. However, our results demonstrate that the antinociception induced by Hp does not involve a direct effect of the peptide on descending pain pathway, once the inhibition of serotonin and norepinephrine receptors did not interfere with the antinociceptive effect. On the other hand, Hp treatment increased levels of endocannabinoids once the concomitant treatment of HP with URB 597 potentiated Hp-induced antinociceptive effect. Thus suggesting that Hp might induce an increase of endocannabinoid levels and this in turn would act by activating the descending inhibitory pain pathway inducing analgesia.

5. Conclusion

Our data demonstrates that Hp exhibits antinociceptive properties under chronic conditions. This effect is independent of the inhibition of the descending pain pathway and involves the activation of peripheral potassium channels and endocannabinoids. We also show that Hp can directly signal to sensory neurons reinforcing the potential prominent role that Hp plays in the control of neuropathic pain. Development of Hp-based therapeutics represents an exciting new avenue for the development of drugs for the treatment of chronic, neuropathic pain.

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Abbreviations

CB1 receptors	type 1 cannabinoid receptors
Hp	hemopressin
CCI	chronic constriction injury
PB	phosphate buffer
DRG	dorsal root ganglia
Egr1-IR	EGR-1 immunoreactivity
Met	methysergide
Yoh	yohimbine

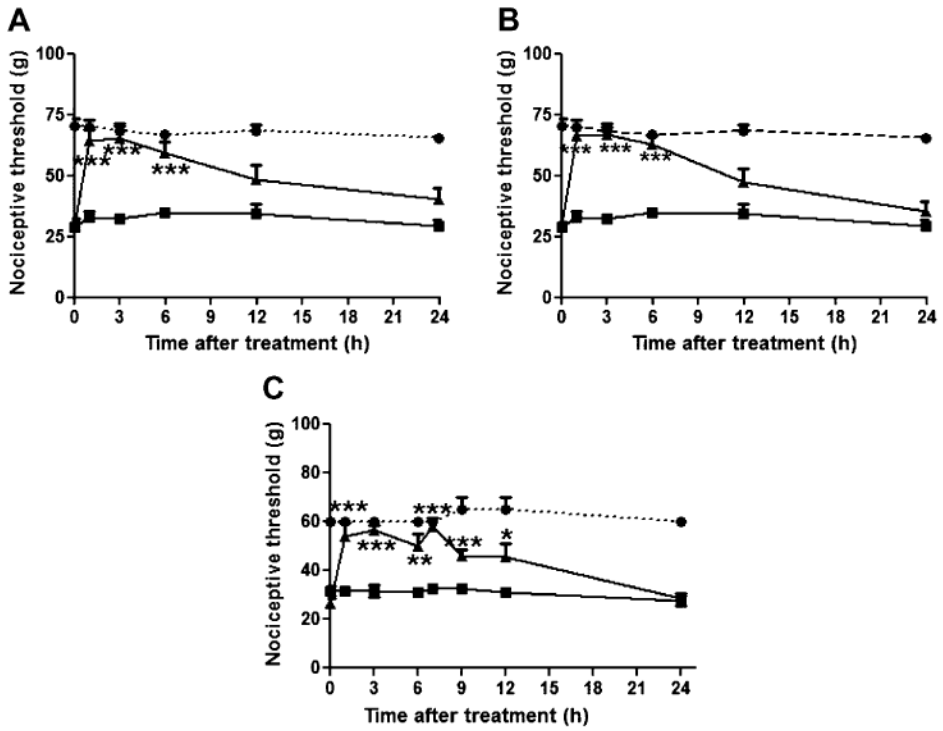

Fig. 1.

Hp inhibits mechanical hyperalgesia in rats submitted to the chronic constriction of the sciatic nerve. Mechanical hyperalgesia evaluated by the rat paw pressure test (A–C), in sham-operated rats (Sham, ●), animals submitted to chronic constriction injury of the sciatic nerve (CCI, ■) and animals with nerve injury treated with hemopressin (Hp, ▲). Hp was administered orally at the dose of 0.25 (A) or 0.5 (B) mg/kg. The nociception was tested before the surgeries (initial measure), 14 days after the CCI (time 0) and after different times following Hp treatment (panels A and B). On panel (C), Hp was administered on time 0 and again on the 6th hour. Values represent the mean ± SEM of seven animals from each group. (*) Significantly different from CCI group; *$P < 0.05$, ***$P < 0.001$ two-way ANOVA followed by Bonferroni's test multiple comparison post test.

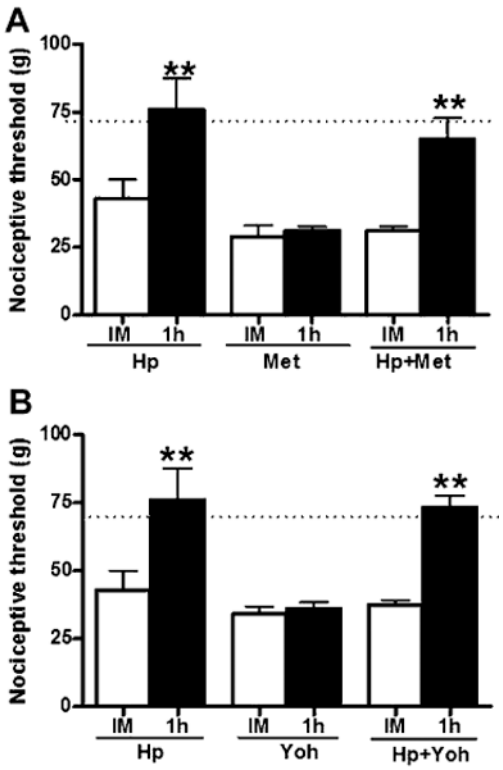

Fig. 2.

Hp-induced antinociception does not occurs through a direct effect on descending pain pathways. Mechanical hyperalgesia evaluated by the rat paw pressure test. In panel A hyperalgesia was evaluated in sham-operated rats (dotted line), animals with nerve injury treated with Hp, animals with nerve injury treated with methysergide (Met) or animals with nerve injury treated with both (Hp + Met). In panel B hyperalgesia was evaluated in sham-operated rats (dotted line), animals with nerve injury treated with Hp, animals with nerve injury treated with yohimbine (Yoh) or animals with nerve injury treated with both (Hp + Yoh). Met was administered (5 mg/kg, i.p.) 30 min before Hp (0.25 mg/kg, i.pl.). Yoh was administered (30 μg, i.t.) 30 min before Hp (0.25 mg/kg, oral). The nociceptive test was applied before the surgeries (basal measure), on day 14 after the CCI (initial measurement, IM; white bars), and following 1 h after Hp treatment (1 h, black bars). Values represent the mean ± SEM of five animals from each group. (*) Significantly different from CCI group *P<0.05, **P<0.01, ***P<0.01 one-way ANOVA followed by Bonferroni's multiple comparison post test.

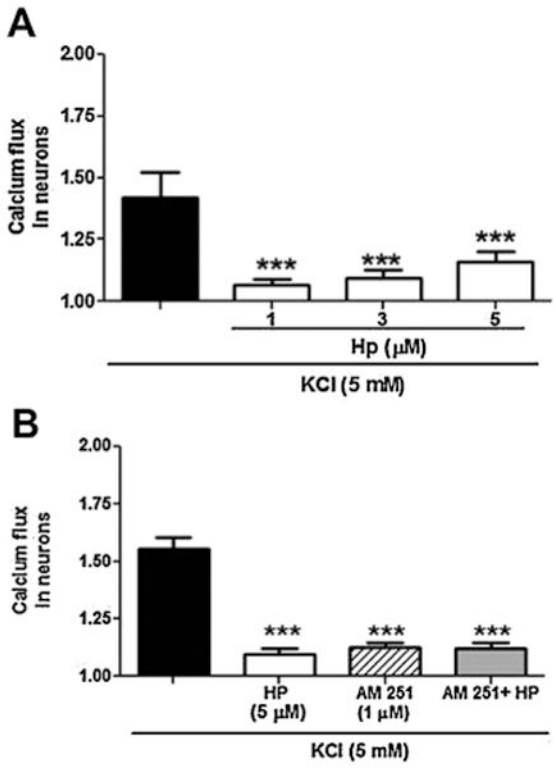

Fig. 3.

Effect of Hp on calcium flux in DRG neurons (L4/L5), from CCI rats. Neurons loaded with Fluo-3 (as described in Section 2) were treated with KCl (5 mM) concomitantly to Hp (1, 3 or 5 μM – panel A). The calcium flux measurements were performed by fluorescence at 460–490 nm excitations and 515 nm emissions in individual cells using a wide-field fluorescence microscope. A kinetic of 30 pictures in 90 s was performed. Neurons treated only with KCl were considered as control group. In panel B, neurons were treated with KCl (5 mM) concomitantly to Hp (5 μM) or AM251 (1 μM) or a combination of Hp + AM251. Values represent mean ± SEM of 22 neurons per group. (*) Significantly different from KCl *P<0.05, **P<0.01, ***P<0.001 one-way ANOVA followed by Bonferroni's multiple comparison post test.

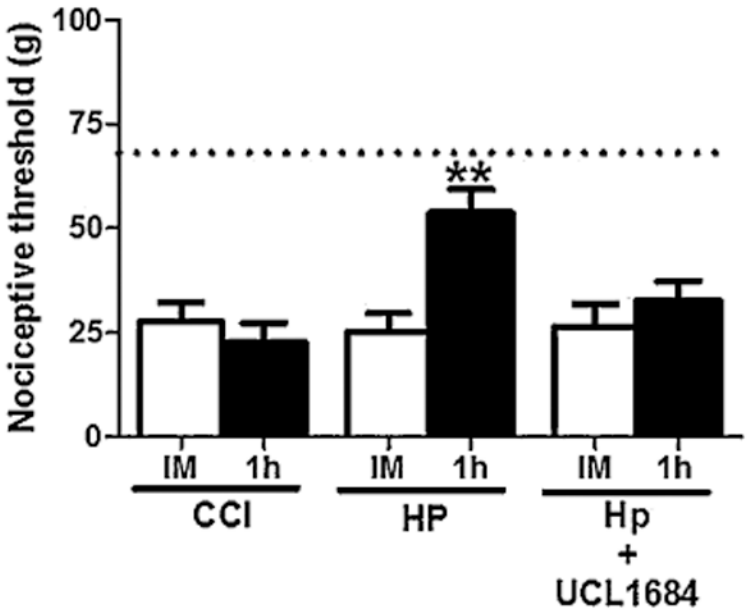

Fig. 4.

Hp-induced analgesia depends on peripheral activation of apamin-sensitive Ca^{2+}-activated K^+ channels in CCI rats. Mechanical hyperalgesia evaluated by the rat paw pressure test, in sham-operated rats (Sham, dotted line), animals submitted to chronic constriction injury of the sciatic nerve (CCI), animals with nerve injury treated with Hp and animals with nerve injury treated with Hp concomitantly with UCL1684 (Hp + UCL1684). Hp was administered orally at the dose of 0.25 mg/kg concomitantly with an intraplantar injection of UCL1684 (10 μg/100 μl). The nociception was measured 14 days after the CCI (white bars) and 1 h after Hp and/or UCL1684 treatments. Values represent the mean ± SEM of seven animals from each group. (**) Significantly different from CCI group, $P < 0.01$ two-way ANOVA followed by Bonferroni's multiple comparison post test.

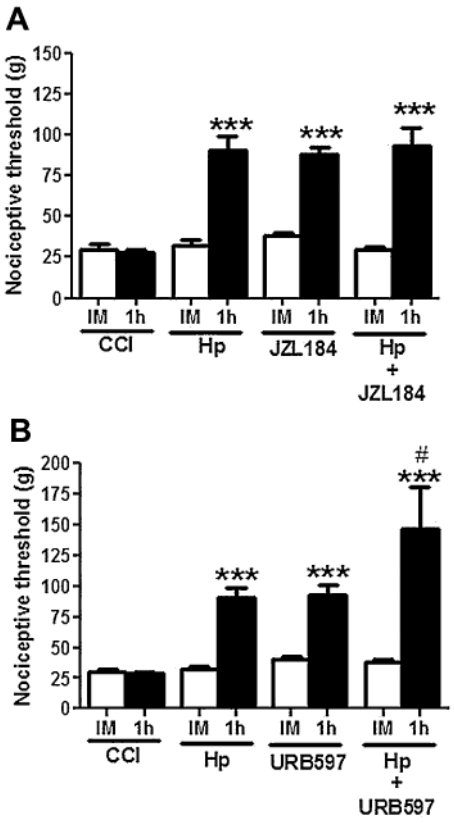

Fig. 5.

Anandamide is involved on Hp-induced antinociception. Mechanical hyperalgesia evaluated by the rat paw pressure test. In panel A, hyperalgesia was evaluated in animals submitted to chronic constriction injury of the sciatic nerve (CCI, Control), animals with CCI treated with Hp, animals with CCI treated with JZL 184 (JZL 184) or animals with CCI treated with Hp concomitantly with JZL 184 (Hp + JZL184). In panel B, hyperalgesia was evaluated in animals submitted to chronic constriction injury of the sciatic nerve (CCI, Control), animals with CCI treated with Hp, animals with CCI treated with URB 597 or animals with CCI treated with Hp concomitantly with URB 597 (Hp + URB 597). Hp was administered orally at the dose of 0.25 mg/kg concomitantly with an intraplantar injection of either JZL 184 or URB 597 (100 μl). The nociception was measured 14 days after the CCI (IM) and following 1 after Hp treatments. Values represent the mean ± SEM of five animals from each group. (***) Significantly different from CCI group, $P<0.001$ and (#) significantly different from Hp group, $P<0.05$ two-way ANOVA followed by Bonferroni's multiple comparison post test.

Table 1

Egr-1 immunoreactivities in the DHSC. Mean of the density of nuclei labeled for Egr1-IR in the superficial layer of the dorsal horn of the spinal cord of rats.

	Right side	Left side
Sham	10.41 ± 1.1	10.63 ± 1.1
CCI	20.67 ± 1.9[a]	16.93 ± 2.4[b]
CCI + Hp	16.03 ± 0.6	12 ± 0.5
Hp	13.71 ± 0.76	11.45 ± 0.43

Immunolabel of the right side and left side of the dorsal horn of the spinal cord (DHSC) in sham-operated rats (Sham), sham-operated rats treated with Hp, animals with nerve injury (CCI) and animals with nerve injury and treated with Hp (CCI + Hp). CCI surgery was performed on the right side.

[a] $P < 0.001$ as compared to sham group.

[b] $P < 0.05$.