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The endogenous fatty acid amide, palmitoylethanolamide, has anti-allodynic and anti-hyperalgesic effects in a murine model of neuropathic pain: involvement of CB_1, TRPV1 and PPARγ receptors and neurotrophic factors

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Abstract

Palmitoylethanolamide (PEA) is an endogenous lipid that is thought to be involved in endogenous protective mechanisms activated as a result of stimulation of inflammatory response. In spite of the well demonstrated anti-inflammatory properties of PEA, its involvement in controlling pain pathways still remains poorly characterized. On this basis, we tested the efficacy of PEA *in vivo* against a peculiar persistent pain, such as neuropathic one. PEA was administered i.p. to mice with chronic constriction injury of sciatic nerve (CCI) once a day for one week starting the day after the lesion. This therapeutic regimen evoked a relief of both thermal hyperalgesia and mechanical allodynia in neuropathic mice. Various selective receptor antagonists were used in order to clarify the relative contribution of cannabinoid, vanilloid and peroxisome proliferator-activated receptor to PEA-induced effects. The results indicated that CB_1, PPARγ and TRPV1 receptors mediated the antinociception induced by PEA, suggesting that the most likely mechanism might be the so-called "entourage effect" due to the PEA-induced inhibition of the enzyme catalyzing the endocannabinoid anandamide (AEA) degradation that leads to an enhancement of its tissue levels thus increasing its analgesic action. In addition, the hypothesis that PEA might act through the modulation of local mast cells degranulation is sustained by our findings showing that PEA significantly reduced the production of many mediators such as TNFα and neurotrophic factors, like NGF. The findings presented here, in addition to prove the beneficial effects of PEA in chronic pain, identify new potential targets for analgesic medicine.

Keywords: Palmitoylethanolamide; Cannabinoid; Neuropathic pain; Neurotrophic factors; Vanilloid

1. Introduction

The endogenous fatty acid palmitoylethanolamide (PEA) is a congener of endocannabinoid anandamide (AEA) which belongs to superfamily of *N*-acylethanolamines (NAE), a class of lipid mediators. Yet, recent reports suggest that AEA and PEA likely belong to independent signalling pathways with distinct synthesis, receptors and inactivation [34]. Particularly, PEA is produced on-demand within the lipid bilayer via NAPE-PLD [41] and its signal is rapidly terminated through its degradation catalysed by at least two enzymes: the fatty acid amide hydrolase (FAAH) [51] and the *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) [61]. In addition to its known anti-inflammatory activity [8,9,37], PEA may elicit analgesia in acute and inflam-

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matory pain [6,8,22], inhibition of food intake [47], reduction of gastrointestinal motility [7], inhibition of cancer cell proliferation [10] and neuroprotection [15,26,52]. Moreover, it has been recently reported that pain hypersensitivity that follows sciatic nerve constriction (CCI) in rats is associated with a significant decrease in the level of endogenous PEA in spinal cord and in brain areas directly or indirectly involved in nociception [44], so suggesting that this lipid might be involved in pain response. Accordingly, the single administration of PEA [18] or its analogue palmitoylallylamide (L29) [62], that supplies the lack of endogenous PEA, significantly relieves neuropathic pain in the partial sciatic nerve injury model in the rat.

Despite its potential clinical significance, the molecular mechanism responsible for the actions of PEA is a rebus and is still debated. PEA exhibits poor affinity for cannabinoid CB_1 or CB_2 receptors even if the CB_2 antagonist SR144528 reversed many of the pharmacological action of PEA, including analgesia [6,13,18]. Thus, different hypotheses of the mechanism of PEA action have been advanced including an interaction of PEA with uncharacterised CB_2-like receptors at which SR144528 is also a functional antagonist [12], an activation of the nuclear receptor peroxisome proliferator-activated receptor-α (PPAR α) which mediated the anti-inflammatory effect of PEA [32], the so-called "entourage effect" [3,25,32], due to the PEA-induced inhibition of FAAH that leads to an increase of tissue levels of AEA strengthening its analgesic action through different molecular mechanisms including the stimulation of cannabinoid receptor CB_1, the desensitization of noxious transient receptor potential channel of the vanilloid type 1 (TRPV1) and the activation of PPARγ.

On these bases this study aimed to investigate the therapeutic potential of a prolonged treatment with PEA in relieving neuropathic pain in mice with sciatic nerve constriction (CCI); further aim has been to explore the mechanism underlying such an action, focusing the attention on receptor hypothesis and on entourage hypothesis, trying to propose a unified mechanism.

2. Methods

2.1. Animals and surgical procedure

All experiments performed were in accordance with Italian State and European regulations governing the care and treatment of laboratory animals (permit no. 101/2004B), and conformed to the guidelines for the study of pain in awake animals established by the International Association for the Study of Pain [65]. Experiments were conducted using male C57BL/6J mice weighing 25–30 g (Harlan, Italy). Animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and submitted to surgical procedure to induce neuropathic pain according to the method of Bennet and Xie [2], with some modifications. Briefly, the common sciatic nerve was exposed at the level of the mid thigh and, proximal to the sciatic nerve trifurcation, three ligatures were tied around it until a brief twitch was seen in the respective hind limb. Sham animals (sciatic nerve exposure without ligature) were used as controls.

2.2. Drugs and treatments

PEA was purchased from Cayman Chemical (Ann Arbor, MI, USA), dissolved in ethanol:saline (1:9), and used at a dose of 10 mg/kg. CCI mice received i.p. the compound or its vehicle once a day for seven days, starting the day after the surgical technique. To study the involvement of different receptors in the PEA-induced effect, the ability of specific CB_1, CB_2, TRPV1, PPARα and PPARγ antagonists to reverse the anti-hyperalgesic effect of PEA was tested. Particularly, on the last day of PEA administration, the cannabinoid CB_1 receptor specific antagonist SR141716 (1 mg/kg i.p.), the cannabinoid CB_2 receptor selective antagonist SR144528 (1 mg/kg i.p.), the vanilloid TRPV1 specific antagonist capsazepine (10 mg/kg i.p.), the PPARα receptor antagonist GW6471 (1 mg/kg i.p.) or the PPARγ selective antagonist GW9662 (1 mg/kg i.p.) was administered i.p. 10 min before PEA (10 mg/kg i.p.) or its vehicle. Sham animals received the vehicles of drugs. SR141716 and SR144528 were kindly supplied by Sanofi-Aventis (Montpellier, France) and were dissolved in a mixture of Tween80:DMSO:distilled water (1:2:7). Capsazepine, GW6471 and GW9662 were purchased from Sigma–Aldrich (Milano, Italy) and dissolved in a 1:1:8 mixture of ethanol:Tween80:saline.

2.3. Nociceptive tests

Animal pain response was monitored before surgery, on day 1st (before starting the treatment), 4th and 8th (24 h after the last administration). In the antagonism studies, the pain behaviour was tested on day 7th (90 min after the administration of compounds). In the experiments aimed to evaluate the effect of acute administration of PEA, a time-course study was performed with the nociceptive behaviour evaluated 30, 90, 120 and 180 min after the single administration. Heat hypersensitivity was tested according to the Hargreaves procedure [17] using the plantar test (Ugo Basile, Varese, Italy). Briefly, animals were placed in a clear plexiglass box and allowed to acclimatize. A constant intensity radiant heat source was aimed at the midplantar area of the hind paw. The time, in seconds, from initial heat source activation until paw withdrawal was recorded. Mechanical allodynia was assessed using the Dynamic Plantar Aesthesiometer (Ugo Basile, Varese, Italy). Particularly, animals were placed in a test cage with a wire

mesh floor, and the tip of von Frey-type filament was applied to the middle of the plantar surface of the hind paw. The filament exerted an increasing force starting below the threshold of detection, and increased until the animal removed its paw. Withdrawal threshold was expressed as tolerance level in g.

2.4. Biochemical evaluations

Eight days following surgery, 24 h after the last administration, pain assessment was recorded and animals were sacrificed. The spinal cord (L4–L6 tract) was removed; part was frozen in liquid nitrogen and stored at −80 °C until the determination of TNFα content and neurotrophic factor level, part was mixed with Trizol reagent in order to perform total RNA extraction and RT-PCR for TNFα and part was submitted to the procedure for the nuclear extract preparation that was then stored at −80 °C until the transcription factor NF-kB assay. At least 1 cm of sciatic nerve proximal to the ligature was removed and immediately frozen in liquid nitrogen to evaluate the production of neurotrophic factors.

2.4.1. TNFα content assay

The spinal cord was homogenized in phosphate buffered saline (PBS), pH 7.4, containing a mix of protease inhibitors (Roche Diagnostics, Monza, Italy), in a ratio of 20 μl of PBS/mg tissue, using an ultra-sonic homogenizer (Branson Sonifier®W-250). The homogenates were centrifuged at 1500*g*, at 4 °C for 10 min and the supernatant was immediately used for the assay. The concentration of TNFα was measured with commercially available sandwich enzyme-linked immunosorbent assay (ELISA) (Biosource Int., Camarillo, CA, USA) according to the procedures recommended by the manufacturer. Briefly, samples, including standards of known mouse cytokine, were added into the wells of microtiter strips coated with an antibody specific for mouse TNFα, followed by the addition of a biotinylated secondary antibody. During the first incubation, the mouse antigen binds simultaneously to the immobilized antibody on one site and to the solution phase biotinylated antibody on the second site. After the removal of excess secondary antibody, streptavidin-peroxidase is added that binds to the biotinylated antibody to complete the four-member sandwich. After a wash to remove all the entire unbound enzyme, a substrate solution is added. The intensity of the coloured product (recorded at 450 nm with a spectrophotometer Multiskan® EX, ThermolabSystem) is directly proportional to the concentration of cytokine.

2.4.2. Transcription factor NF-kB assay

Transcription factor analysis was performed with an ELISA kit (Active Motif, Rixensart, Belgium) that allowed for the detection of NF-kB activation by a combination of NF-kB-specific oligonucleotide binding and subsequent detection of the p65 subunit of NF-kB with specific antibody. Spinal cord was homogenized in 100 μl ice-cold hypotonic lysis buffer (supplied with the nuclear extract kit, Active Motif, Rixensart, Belgium)/mg tissue. After centrifugation at 850*g* for 10 min, 500 μl of hypotonic buffer supplemented with 25 μl of Nonidet P-40 was added to the pellet and the mixture was centrifuged at 14,000*g* for 2 min at 4 °C. Pellets were suspended in 50 μl of hypertonic lysis buffer and incubated with shaking for 30 min at 4 °C. Samples were then centrifuged at 14,000*g* for 10 min at 4 °C, and the supernatant containing nuclear extracts was stored at −80 °C until use. Nuclear protein extract (10 μg) was added onto the oligonucleotide-coated ELISA plate and then incubated for 1 h at room temperature. Primary antibody recognizing an epitope on p65, which is accessible only when NF-kB is activated and bound to its target DNA, was added to wells and incubated for 1 h. This is followed by the addition of an HRP-conjugated secondary antibody and, after 1 h, the HRP substrate was added. The reaction was stopped after 5–10 min, and the absorbance was measured on a spectrophotometer (Multiskan® EX, ThermolabSystem) at 450 nm. Jurkat cell nuclear extracts were used as an activated NF-kB positive control. NF-kB wild-type and mutated consensus oligonucleotides were used in order to monitor the specificity of the assay: a wild-type oligonucleotide should compete with NF-kB for binding, whilst the mutated consensus oligonucleotide should have no effect on NF-kB binding.

2.4.3. RNA extraction and RT-PCR

Total RNA was extracted from homogenized lumbar spinal cord using TRIzol Reagent (Invitrogen, Milano, Italy) according to the manufacturer's protocol. The extracted RNA was subjected to *DNase* I (Invitrogen, Milano, Italy) treatment at 25 °C for 15 min. The total RNA concentration was determined by UV spectrophotometer. Reverse transcription of total RNA (1 μg) was performed with oligo dT primer and SuperScript III RT (Invitrogen, Milano, Italy) according to the manufacturer's specifications. RT-PCR was performed with primers specific for TNFα and GAPDH, which was used as a reference gene. Primer sequences for the PCR were as follows: TNFα: 5′ GGCAGGTCTACTTTGGAGTC 3′ and 5′ ACATTCGAGGCTCCAGTGAATTCGG 3′; GAPDH 5′ C GGAGTCAACGGATTTGGTCGTAT 3′ and 5' AGCCTT CTCCATGGTGGTGAAGAC 3′. The number of cycles were 38 at 54 °C. PCR amplification products were separated on ethidium bromide-stained 1.5% agarose gel, visualized by ultraviolet light. The amplified bands showed their predicted sizes: TNFα 301 bp and GAPDH 306 bp.

2.4.4. NGF-, GDNF- and NT-3-like immunoreactivity (LI)

Tissues were homogenized in a cold lysis buffer (250 μl). The homogenates were centrifuged at 4500*g* at 4 °C for 10 min, and the resulting supernatants were then diluted 5-fold with Dulbecco's PBS buffer. Samples were acidified to pH <3.0 by adding 1 N HCl and then neutralized with 1 N NaOH to pH 7.6. NGF-LI, GDNF-LI and NT-3-LI were determined by enzyme-linked immunosorbent assay (ELISA) using ELISA kit according to the manufacturer's instructions (Promega, USA). The absorbance at 450 nm was recorded on a microplate reader (Multiskan® EX, ThermolabSystem). Neurotrophins were determined by interpolation with standard curves assayed on individual plates and normalize to protein content in each tissue sample.

2.5. Statistical analysis

Data were expressed as the mean ± SEM and analysed using one-way repeated measures analysis of variance (ANOVA) followed by Tukey's test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effect of PEA on thermal hyperalgesia and mechanical allodynia

As expected, the day after the nerve injury mice developed a significant decrease in thermal withdrawal latency of the paw ipsilateral to the injury, as compared to sham operated animals (Fig. 1, panel A). After CCI, mice also developed mechanical allodynia to normally innocuous mechanical stimulation with a von Frey filament (Fig. 1, panel B). Treatment of CCI mice with a single dose of PEA (10 mg/kg, i.p.) resulted in a significant relief of both thermal hyperalgesia and mechanical allodynia. The time course of the effect elicited by acute PEA is shown in Fig. 1, panels A and B, for thermal hyperalgesia and mechanical allodynia, respectively. PEA produced a significant anti-hyperalgesia that peaked 30 min after the administration, persisted at 90 min and decreased during the following time points, even if the withdrawal latency did not reach the physiological value at any time. The anti-allodynic effect was maximum 90 min after the acute administration of PEA and disappeared at 2 h time point. Thus PEA evoked a significant but short-lasting relief of pain in CCI mice after single systemic administration. To determine whether the PEA antinociceptive effect could be enhanced by repetitive administration, we subjected neuropathic mice to a 7-day regimen with the same dose of PEA (10 mg/kg i.p., once daily) or vehicle and we monitored the nociceptive responses 24 h after the last PEA administration. The results of this repeated treatment are shown in Fig. 2 (panel A: thermal hyperalgesia, panel B: mechanical allodynia). As expected, nociceptive behaviour is still present in CCI mice treated with vehicle for 7 days; on the contrary the repeated administration of PEA abolished thermal hyperalgesia and significantly attenuated mechanical allodynia in a time-dependent manner. These results indicate that the repeated treatment with PEA led to a more potent and long-lasting relief of neuropathic pain. The same prolonged treatment did not affect the response to thermal and mechanical stimuli of the paw contralateral to the injury (data not shown).

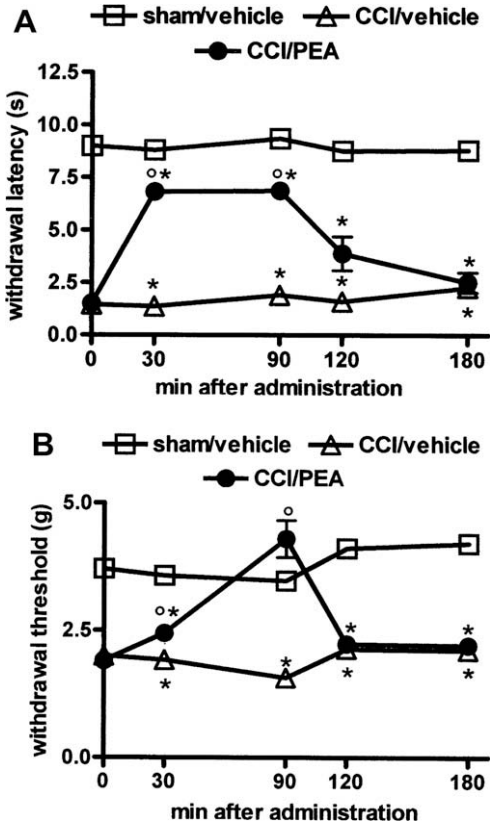

Fig. 1. Effect of palmitoylethanolamide (PEA) 10 mg/kg i.p. administered to neuropathic (CCI) mice the day after the injury, on thermal hyperalgesia (A) and mechanical allodynia (B) at different time points after the treatment. Withdrawal latency to heat and mechanical allodynia of the injured paws are expressed as s and g, respectively. Data represent mean ± SEM of 12 mice. $^{*}P < 0.01$ vs sham/vehicle;°$P < 0.01$ vs CCI/vehicle.

3.2. Effect of CB_1, CB_2, TRPV1, PPARα and PPARγ receptor antagonists on PEA-induced relief of neuropathic pain

The ability of specific CB_1, CB_2, PPARα, PPARγ and TRPV1 antagonists to reverse the effect of PEA was tested only on thermal hyperalgesia since the partial efficacy on mechanical allodynia. These studies were performed on the last day of PEA administration, and the

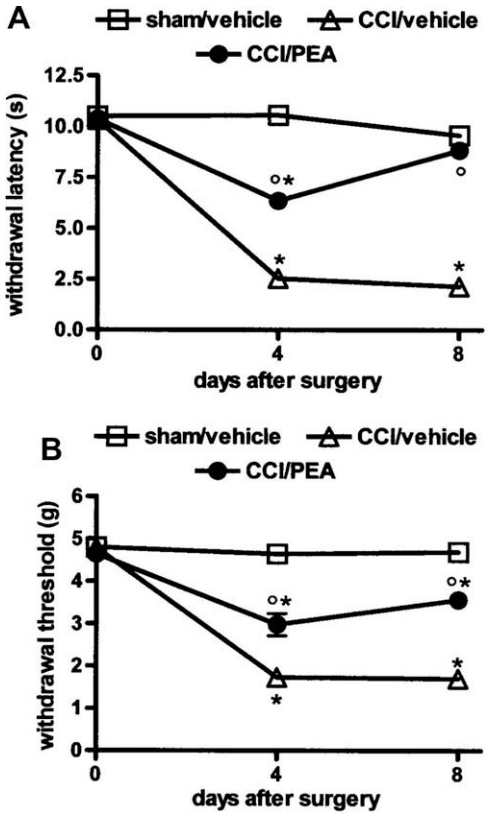

Fig. 2. Effect of palmitoylethanolamide (PEA) 10 mg/kg i.p., daily administered to neuropathic (CCI) mice for one week from the day after the surgery on thermal hyperalgesia (A) and mechanical allodynia (B). Withdrawal latency to heat and mechanical allodynia of the injured paws are expressed as s and g, respectively. Data represent mean ± SEM of 15 mice. $^{*}P < 0.001$ vs sham/vehicle;$^{\circ}P < 0.001$ vs CCI/vehicle.

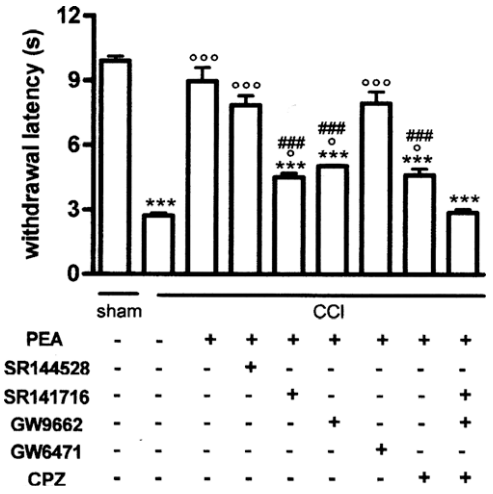

Fig. 3. Effect of SR144528 (1 mg/kg i.p.), SR141716 (1 mg/kg i.p.), GW9662 (1 mg/kg i.p.), GW6471 (1 mg/kg i.p.) and capsazepine (CPZ, 10 mg/kg i.p.) on PEA (10 mg/kg i.p.)-induced anti-hyperalgesia in neuropathic (CCI) mice, 90 min after the last PEA administration (on day 7th). Withdrawal latency to heat of the injured paws is expressed as s. Data represent mean ± SEM of 8 mice. $^{***}P < 0.001$ vs sham/vehicle; $^{\circ\circ\circ}P < 0.001$,$^{\circ}P < 0.05$ vs CCI/vehicle; $^{\#\#\#}P < 0.001$ vs CCI/PEA.

results are shown in Fig. 3. Neither the pretreatment with SR144528, the specific CB_2 antagonist, nor the pretreatment with GW6471, the selective PPARα receptor antagonist, did reverse PEA-induced anti-hyperalgesia; on the contrary, the PPARγ receptor antagonist GW9662, the CB_1 receptor antagonist, SR141716, and the TRPV1 receptor antagonist, capsazepine, given with PEA, were able to partially counteract its anti-hyperalgesic effect. Fig. 3 shows that a combination of all three antagonists completely reversed the anti-hyperalgesic property of PEA without altering the hypersensitivity of the model. All the antagonists employed, when administered alone to CCI mice, did not affect their nociceptive response (data not shown).

3.3. Effect of PEA on NF-kB activation and TNFα level in the spinal cord

The results of the assay for activated NF-kB are shown in Fig. 4 and revealed that the DNA-binding activity of NF-kB subunit p65 was increased in the spinal cord (L4–L6 tract) of neuropathic mice on day 8th after the injury. The repeated treatment with PEA resulted in a significant inhibition of the upregulation of NF-kB DNA-binding activity. Determination of TNFα level by ELISA revealed higher levels (33%) in the spinal cord (L4-L6 tract) of CCI mice 8 days post lesion as compared to sham animals (Fig. 5, panel A). Repeated administration of PEA restored the physiological TNFα level (Fig. 5, panel A). The enhanced level of TNFα found in the spinal cord of CCI mice is accompanied by an increase in mRNA for this cytokine, as revealed by RT-PCR analysis performed on cDNA obtained after RNA extraction from spinal cord and employing specific primers for mouse TNFα (Fig. 5, panel B). RT-PCR demonstrated that there was a

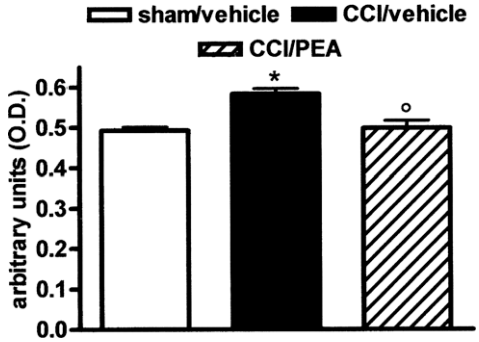

Fig. 4. Effect of repeated treatment with palmitoylethanolamide (PEA) (10 mg/kg i.p., once daily for one week) to neuropathic (CCI) mice on NF-kB activation in the nuclear fraction of lumbar spinal cord (L4-L6 segment) expressed as arbitrary units (optical density (O.D.)) at 450 nm. Data represent mean ± SEM of 5 mice. $^{*}P < 0.05$ vs sham/vehicle;$^{\circ}P < 0.05$ vs CCI/vehicle.

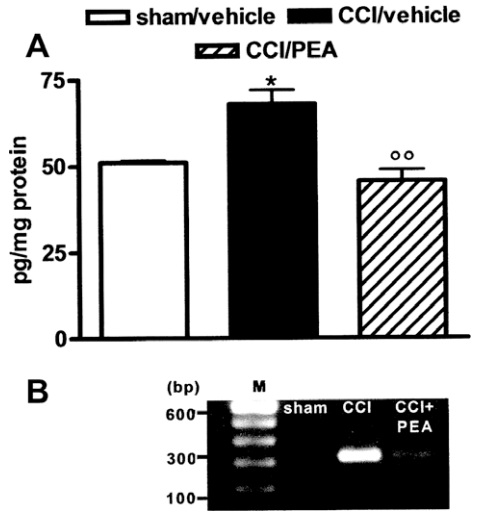

Fig. 5. Effect of repeated treatment with palmitoylethanolamide (PEA) (10 mg/kg i.p., once daily for one week) to neuropathic (CCI) mice on TNFα level (A) in the lumbar spinal cord (L4-L6 segment). Data represent mean ± SEM of 5 mice. $^{*}P < 0.05$ vs sham/vehicle; $^{\circ\circ}P < 0.01$ vs CCI/vehicle. Panel B represents the agarose gel analysis of the RT-PCR products of the expression of mouse TNFα. cDNA fragments of mouse TNFα (301 bp) were amplified after reverse transcription of total RNA extracted from the lumbar spinal cord (L4–L6 segment).

decrease in mRNA level for TNFα protein after repeated PEA administration (Fig. 5, panel B).

3.4. Effect of PEA on neurotrophic factors

We examined three neurotrophic factors (NGF, GDNF and NT-3) in both lumbar spinal cord (L4-L6) (Fig. 6) and sciatic nerve (Fig. 7), at the end of the repeated pharmacological treatment. In the spinal cord of CCI mice the level of all three neurotrophic factors was significantly higher than that found in sham animals. Particularly, the increase in NGF-LI was about 58%, whereas that of both GDNF-LI and NT-3-LI was 35% (Fig. 6). The repeated treatment with PEA brought the content of both GDNF-LI and NT-3-LI down to that of uninjured mice (Fig. 6, panels B and C) and induced a remarkable reduction of NGF-LI (67%) as compared to sham animals (Fig. 6, panel A). A significant increase of all the neurotrophic factors has been found also in the sciatic nerve of CCI mice, as shown in Fig. 7. The augment of NGF-LI, GDNF-LI and NT-3-LI in the site of the lesion was much more higher than that found in the spinal cord; in fact, the relative increases were 175% for NGF, 162% for GDNF and 637% for NT-3. After PEA repeated treatment NGF-LI was reduced to the same level of sham mice (Fig. 7, panel A), whereas both GDNF-LI and NT-3-LI remained higher than uninjured animals but reduced as compared to CCI mice (Fig. 7, panels B and C).

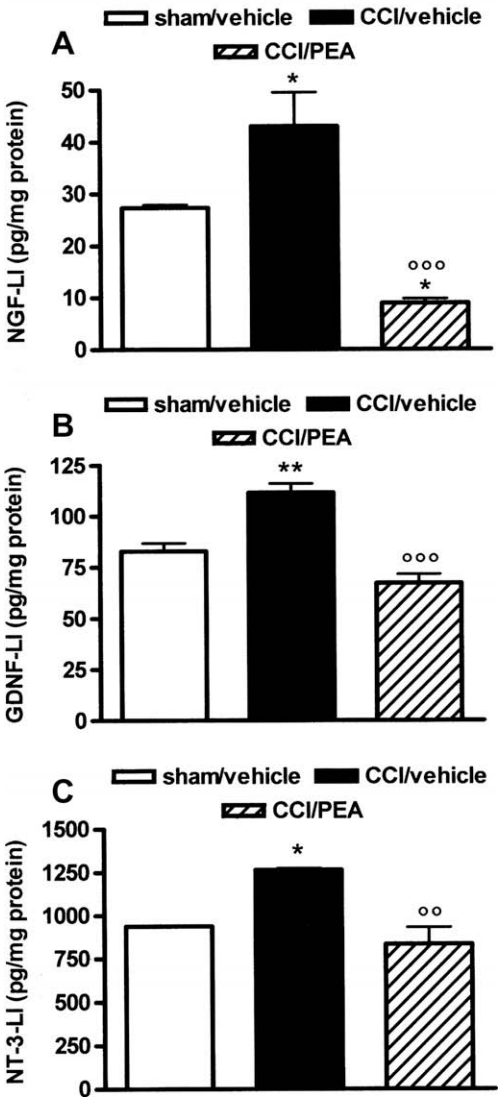

Fig. 6. Effect of repeated treatment with palmitoylethanolamide (PEA) (10 mg/kg i.p., once daily for one week) to neuropathic (CCI) mice on NGF (A), GDNF (B) and NT-3 (C) level in the lumbar spinal cord (L4-L6 segment). Data represent mean ± SEM of 5 mice. $^{*}P < 0.05$, $^{**}P < 0.01$ vs sham/vehicle; $^{\circ\circ}P < 0.01$, $^{\circ\circ\circ}P < 0.001$ vs CCI/vehicle.

4. Discussion

We have shown here that the thermal hyperalgesia and the mechanical allodynia that follow sciatic nerve constriction in mice are relieved by repeated administration of the fatty acid amide PEA. Particularly, we have characterized the time course of PEA effect when it was administered acutely, showing that the anti-hyperalgesic and anti-allodynic action was already present 30 min after PEA injection and disappeared 3 h later, indicative for a short-lasting efficacy of PEA. Daily injection of PEA for one week evoked a complete relief of pain hypersensitivity in CCI mice that was still present 24 h after the last administration of the compound. The effi-

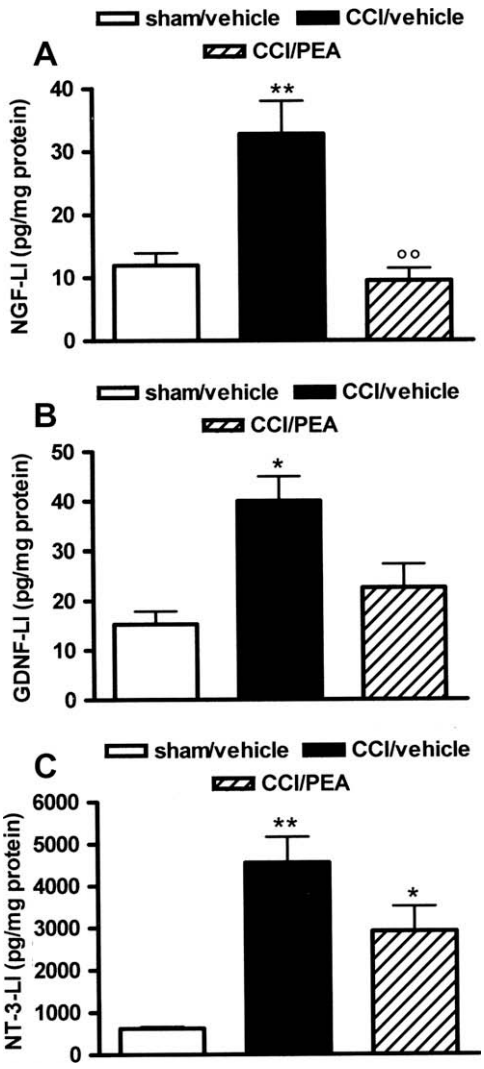

Fig. 7. Effect of repeated treatment with palmitoylethanolamide (PEA) (10 mg/kg i.p., once daily for one week) to neuropathic (CCI) mice on NGF (A), GDNF (B) and NT-3 (C) level in the injured sciatic nerve. Data represent mean ± SEM of 15 mice. $^{*}P < 0.05$, $^{**}P < 0.01$ vs sham/vehicle; $^{\circ\circ}P < 0.01$ vs CCI/vehicle.

cacy of PEA in attenuating neuropathic pain is consistent with the recent report by Petrosino et al. [44], who found in CCI rats decreased level of endogenous PEA in spinal cord and in brain areas directly or indirectly involved in nociception. We can suggest that PEA down-regulation plays a role in the development and maintenance of pain hypersensitivity after nerve lesion, and that the administration of exogenous PEA for the whole time period (one week after the injury) leads to a restore of its physiological level and consequently to the relief of pain. In the work by Petrosino et al. [44] it was also reported that the levels of the two major endocannabinoids, AEA and 2-arachidonoylglycerol, were significantly increased, probably as an adaptive response to the pathology, aimed to counteract pain transmission. On the basis of the demonstrated ability of PEA to strengthen the antinociceptive effect of AEA [3,25,38,55], we cannot exclude that the administration of PEA performed by us also leads to a further increase of AEA in the spinal and supraspinal areas of CCI animals.

Despite its various described pharmacological properties, the cellular/receptor mechanism responsible for the actions of PEA is still debated. The structural similarity between PEA and AEA first suggested that PEA shared the ability of AEA to target cannabinoid receptors. The demonstration that PEA does not bind to cannabinoid receptors [25,27,53,59] opened a new scenario in the pharmacology of PEA. It is now debated whether PEA can interact with the so-called CB_2-like receptor [12] or whether it can activate CB_2 receptor indirectly, augmenting the level of AEA that binds to CB_2 receptors causing anti-inflammation and analgesia (entourage hypothesis) [3,25,32]. If this is the case, we cannot exclude that also the other receptors for which AEA shows more potency than CB_2, such as CB_1, TRPV1 [68] and PPARγ [4] can be involved in PEA-induced effects. In addition, recent data demonstrated that some anti-inflammatory actions of PEA are mediated by a direct activation of PPARα receptor [32] An unified scheme representing all these different hypotheses is shown in Fig. 8. The "entourage hypothesis" proposes that PEA may act as an enhancer of the anti-inflammatory and antinociceptive activity exerted by another endogenous substance, AEA, via the inhibition of its metabolic degradation due to the ability of PEA to compete with AEA for FAAH catalytic activity [23, 61]. Thus, we tested all the above quoted hypothesis by evaluating the ability of different receptor antagonists to reverse PEA-induced anti-hyperalgesia. Our findings showed that neither CB_2 nor PPARα antagonists affected the PEA-elicited anti-hyperalgesia, suggesting that such receptors are not involved in its antinocicep-

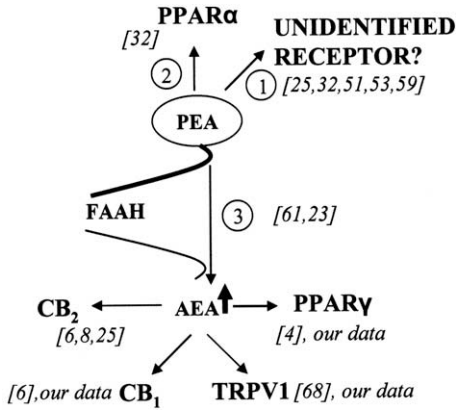

Fig. 8. PEA may exert its effects directly through an unknown receptor (1) or engaging PPARα receptors (2). Moreover, through an "entourage" mechanism (3), exogenous PEA may compete with AEA for FAAH-mediated degradation causing an increase in the level of AEA (3), which in turn activates CB_1, CB_2, TRPV1 and PPARγ receptors.

tive effect. On the contrary, the anti-hyperalgesia elicited by PEA was antagonized by the administration of the antagonists for CB_1, TRPV1 and PPARγ receptors, highlighting the involvement of such receptors in PEA-induced relief of neuropathic pain. This result seems to support the so-defined "entourage hypothesis" as mechanism underlying the anti-hyperalgesia evoked by repeated PEA administration. The implication of CB_1 receptors in nociceptive pathway, as well as its upregulation in neuropathic pain condition has been well established [31], thus CB_1 involvement in PEA-induced antinociception was expected. Furthermore, AEA at high doses may activate TRPV1 receptors whose role in neuropathic pain is still unclear, even if it is substantially proved that its desensitization following repeated agonist stimulation evoked analgesia [24]. Since AEA is a weak ligand for CB_2 receptors, the lack of CB_2 involvement in PEA-evoked relief of neuropathic pain represents further data in favour of an "entourage effect" (Fig. 8). In the light of recent evidence showing that PPARγ agonists prevents neuronal damage and myelin loss other than pain behaviour in a model of spinal cord injury [43], we suggest that the role of these receptors in neuropahic pain is linked to a neuroprotective effect. In the CNS, PPARγ agonists were shown to be potent inhibitors of microglia-mediated production of inflammatory molecules [33,58], including TNFα. [16] which was reported to play a pivotal role in the generation and maintenance of neuropathic pain [56,57]. This effect is related to the ability of PPARγ agonists to interfere with inflammatory transcription pathways, such as AP-1 and NF-kB [14]. A wealth of data suggests that the NF-kB signalling pathway responds to a variety of inflammatory stimuli controlling the expression of dozens of inflammatory mediators such as cytokines, chemokines, adhesion molecules, growth factors. On this basis, PPARγ activation can lead to an inhibition of NF-kB-dependent gene expression through at least two proposed mechanisms: upon ligand binding, PPARγ can interact with p65 subunit of the transcription factor NF-kB blocking its ability to bind with the promoters of proinflammatory and proalgogen genes (a process termed "cross-coupling") or it can squelch the expression of these genes sequestering the coactivators of NF-kB [14]. The inhibition of NF-kB activation and the consequent decrease in the level of mRNA encoding for TNFα as well as the decrease in the protein level demonstrated by us in the spinal cord of CCI mice repeatedly treated with PEA strongly support this idea and account for the PPARγ involvement in the PEA-induced relief of neuropathic pain.

The possibility of a substantial adjunctive mechanism arises from the data obtained herein showing an important modulator effect induced by repeated PEA on three neurotrophic factors. Considerable evidence has accumulated from both humans and animals that they are involved as pain mediators. Particularly, NGF can promote the sensitization and activation of nociceptors [45,48]. It has been shown that, after nerve injury, NGF is up-regulated by Schwann cells at the nerve injury site for several weeks and this up-regulation is involved in the onset of neuropathic pain behaviours in rodent models [19,30,46]. Moreover, single endoneurial injection of NGF is sufficient to produce transient histological and behavioural effects like those seen in neuropathic pain models [49]. The role of NT-3 in neuropathic pain has not been fully worked out. Although it has been shown to be up-regulated in models of neuropathic pain contributing to mechanical hyperalgesia [64], NT-3 has been recently reported to suppress thermal hyperalgesia associated with neuropathic pain [63]. Similarly, the effect of glial-derived neurotrophic factor (GDNF) on nociception is still a matter of debate. A recent report highlights that GDNF produces thermal hyperalgesia *in vivo* [35], whereas other studies report the opposite effect [5,11,50]. Here, we demonstrated for the first time that there is a significant enhancement of NGF-LI, GDNF-LI and NT-3-LI during neuropathic pain both at central (spinal cord) and at peripheral (sciatic nerve) level. The repeated treatment with PEA abolished such an increase, differently affecting neurotrophins. In fact, in the spinal cord the PEA administration prevented the increase in GDNF-LI and NT-3-LI and strongly decreased NGF-LI under physiological value, whereas at peripheral level, close to the injury, the effect was significant only upon NGF-LI. We can suggest that this scenario can be related to another important target of PEA, namely the mast cells. There is a resident population of mast cells in the peripheral nerve [42], which are degranulated at the site of nerve lesion [67] releasing mediators such as histamine and TNFα, which sensitize nociceptors and contribute to the recruitment of neutrophils and macrophages [39,60]. Notably, mast cells also release NGF [28] and express trkA receptors [20], and thus NGF binding may cause mast cell degranulation leading to a further release of NGF and many other proinflammatory and pronociceptive mediators, finally leading to peripheral sensitization and hyperalgesia. Unlike NGF, NT-3 does not seem to be stored in endoneurial mast cells following nerve transaction [66] nor NT-3 is able to induce mast cell degranulation [54]. To our knowledge no data have been published to date on the synthesis or effects of GDNF on mast cells. Further studies are requested to characterize the effect of PEA on neurotrophins, especially NGF; particularly, immunolocalisation and *in situ* hybridisation studies, as performed by others [36,40] will allow us to understand the source of NGF (mast cells, Schwann cells, macrophages, DRG neurons, etc.) and to find whether the changes are related a blockade of retrograde transport or whether a *de novo* synthesis occurs. The findings presented herein strongly

suggest that PEA may prevent mast cell degranulation through the already described ALIA (Autacoid Local Injury Antagonism) mechanism [1,29] that was originally disclosed in the mid nineties to indicate that some endogenous *N*-acyl-ethanolamines, like PEA, exerted a local antagonism on inflammation. Later the acronym was designed to explain the local antagonism on inflammation and pain exerted by PEA through the down-modulation of mast cell hyperactivity [21,37].

In conclusion, PEA induces relief of neuropathic pain probably through both an action upon receptors located on the nociceptive pathway (CB_1, TRPV1) via an "entourage effect" and a more direct action on an exclusive target, namely the mast cells, via an ALIA mechanism.

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