Non-Neuronal Cell Modulation Relieves Neuropathic Pain: Efficacy of the Endogenous Lipid Palmitoylethanolamide

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Abstract: We have previously shown that the endogenous lipid palmitoylethanolamide (PEA) induced relief of neuropathic pain through an action upon receptors located on the nociceptive pathway. Recently, it has been proposed that immune cells, in particular mast cells, and microglia, by releasing algogen mediators interact with neurons to alter pain sensitivity thereby contributing to the development and maintenance of chronic pain states. The aim of this work was to explore whether the anti-nociceptive properties of PEA might be accompanied by modulation of these non-neuronal cells. Mice were subjected to a chronic constriction injury model of neuropathic pain and treated with PEA. The data show that at the earlier (3 days) time-point after nerve injury there was a substantial recruitment of mast cells whose activation was not yet pronounced. In contrast, at the later time point (8 days) there was no further increase in mast cell number, but rather a marked activation of these cells. An up-regulation of activated microglia was found in the spinal cord of neuropathic pain mice. PEA delayed mast cell recruitment, protected mast cells against degranulation and abolished the nerve growth factor increase in sciatic nerve concomitantly preserving the nerve from degeneration, while reducing microglia activation in the spinal cord. These findings support the idea that non-neuronal cells may be a valuable pharmacological target to treat neuropathic pain since the current neuronal-direct drugs are still unsatisfactory. In this context PEA could represent an innovative molecule, combining a dual analgesic activity, both on neurons and on non-neuronal cells.

Keywords: Cannabinoid, mast cell, microglia, nerve growth factor, neuropathic pain, palmitoylethanolamide, tumor necrosis factor α.

INTRODUCTION

Palmitoylethanolamide (PEA), a congener of the endocannabinoid anandamide, is a bioactive endogenous *N*-acylethanolamine, first identified half a century ago in lipid extracts of various tissues [1]. PEA shows a pleiotropic pattern of bioactivities that includes neuroprotection [2, 3], anti-inflammation [4-6] and analgesia [7]. Despite its potential clinical significance, the molecular target(s) responsible for the analgesic and anti-inflammatory properties of PEA remains to be elucidated. PEA behaves as a local autacoid capable of downregulating mast cell activation [8], suggesting a key role of these cells in the anti-inflammatory effect of exogenous administered PEA. Alterations in the levels of PEA occur during pathological conditions, including pain states. PEA levels significantly decrease in spinal cord and in brain areas involved in nociception following sciatic nerve constriction in rats [9], and in the spinal cord of rats subjected to spinal nerve ligation [10], suggesting decreased PEA to be associated with pain responses. Accordingly, under experimental painful conditions, exogenous PEA has been reported to exert anti-nociceptive effects in a model of murine spinal cord injury [11], in the mouse carrageenan-induced paw model of hyperalgesia [12], and in the complete Freund's adjuvant model of inflammatory pain [13]. In this context, we have recently extended knowledge about the analgesic property of PEA showing that this compound induces relief of neuropathic pain [14]. We hypothesized that the reduction of pain behaviour elicited by PEA in neuropathic mice might be ascribed to an action upon receptors located on the nociceptive pathway. The "receptor mechanism" is based on the capability of PEA to directly stimulate either an as-yet uncharacterized cannabinoid CB_2 receptor-like target [15] or the nuclear peroxisome proliferator-activated receptor-α [16]. Moreover, PEA may act *via* an "entourage effect" by enhancing the anti-nociceptive effects exerted by anandamide, so indirectly activating cannabinoid CB_1 and CB_2 receptors or transient receptor potential vanilloid type 1 channels [17, 18]. Beside such mechanisms, PEA analgesic activity may also ascribed to a more direct action on mast cells, *via* an ALIA (Autacoid Local Injury Antagonism) mechanism [14].

Increasing evidence is now emerging suggesting a pivotal role of mast cells in the onset of chronic pain [19, 20], especially neuropathic pain, where activated mast cells contribute directly by releasing algogenic mediators after degranulation. Mast cell mediators which can sensitise nociceptors and contribute to neutrophil recruitment include histamine [21], tumor necrosis factor α (TNFα) [22] and nerve growth factor (NGF) [23, 24]. Zou and collaborators [25] demonstrated that, in rats where the sciatic nerve was

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partially ligated, stabilisation of mast cells with sodium cromoglycate protected mast cells from degranulation, reduced the recruitment of neutrophils and monocytes to the injured nerve and suppressed the development of hyperalgesia. Moreover, treatment with histamine receptor antagonists suppressed the development of hyperalgesia following nerve injury and alleviated hyperalgesia once established. Mast cells also express the high-affinity NGF receptor trkA [26]. NGF binding to mast cells may thus cause their degranulation leading to a further release of NGF and other pro-inflammatory and pro-nociceptive mediators, finally leading to peripheral sensitization and hyperalgesia. Interestingly, in our previous work we demonstrated that PEA-induced reduction of neuropathic pain was accompanied by a significant decrease in TNFα and NGF production within the spinal cord and sciatic nerve of neuropathic mice [14]. One aim of the present work was to better characterize the role of mast cells during neuropathic pain and to relate the analgesic effect of PEA to its capability to inhibit mast cell degranulation. For these studies we employed the same animal model (chronic constriction injury of the sciatic nerve, CCI) and the same pharmacological treatment as described previously [14].

PEA can exert an action on microglia cells, as well. Esposito and collaborators [27] recently demonstrated that PEA treatment significantly reduced the activation of microglia and astrocytes expressing cannabinoid CB_2 receptors after spinal cord injury. This is of particular interest since also spinal microglial cells contribute to chronic pain states. Microglia are resident macrophage-related cells in the central nervous system (CNS) normally in a “resting” state in order to perform a constant immune surveillance. Upon activation (after trauma or nerve injury), microglia are characterized by an increased expression of cell surface markers and receptors, and by a release of algesic substances, such as cytokines, prostaglandins, and excitatory amino acids that contribute to neuronal excitability and pain generation [28, 29]. Chronic treatment with minocycline, an inhibitor of activated microglia, significantly reduced the development of mechanical allodynia in spinal nerve ligated rats concomitantly with an attenuation of spinal microglia activation [10]. As well described in a recent review by Skaper *et al*. [30], a communication/interaction between microglia and mast cells may occur during CNS pathologies. Particularly, microglia can respond to pro-inflammatory signals released from other non-neuronal cells, like mast cells. The crosstalk between mast cells and microglia may contribute to the exacerbation and acceleration of chronic pain symptoms. A second aim of the present work was therefore to investigate whether the analgesic effect of PEA [14] also related to its capability to inhibit spinal microglia activation.

It is therefore clear that neurons are not the only players that drive the establishment and maintenance of common clinical pain states. The recognition that immune cells (particularly mast cells), microglia and neurons form an integrated network in which the activation of an immune response modulates the excitability of pain pathways, offers a completely new treatment approach. This is certainly needed because current therapeutic strategies for chronic pain, aimed to reduce the excitability of neurons in the peripheral nervous system or the CNS by modulating the activity of ion channels (i.e., gabapentin, pregabalin, carbamazepine, lidocaine, capsaicin) or by mimicking and enhancing endogenous inhibitory mechanisms (i.e., tricyclic antidepressants, duloxetine, opioids), lack satisfactory efficacy for neuropathic pain and produce undesirable side effects. In contrast, the design of therapies targeting chronic pain by differentially modulating the activation of non-neuronal cells, both peripherally and centrally, may provide the opportunity not only to reduce symptoms but especially to modify the disease progression by aborting neurobiological alterations that support the development of persistent pain.

In this context, PEA, combining a dual analgesic activity, as previously described, both on neurons of the nociceptive pathway and on the modulation of non-neuronal cells, offers more advantages than classical anti-nociceptive drugs and consequently could represent an innovative molecule for the treatment of chronic pain states, especially of a neuropathic nature.

MATERIALS AND METHODS

Animals

All experiments were conducted using male C57BL/6J mice weighing 25-30 g (Harlan, Italy) in accordance with Italian State and European regulations governing the care and treatment of laboratory animals (permit n° 41/2007B) and conformed to the guidelines for the study of pain in awake animals established by the International Association for the Study of Pain [31]. Animals were housed three per cage under controlled illumination (12:12 h light/dark cycle) and environmental conditions (room temperature 22±1°C, humidity 60%) for at least one week before testing. Mouse chow and tap water were available *ad libitum*. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Chronic Constriction Injury of the Sciatic Nerve (CCI)

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 [32], 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.

Drug and Treatment

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 surgery. In our hands this drug treatment evoked a complete relief of neuropathic pain [14]; however, since rapid mast cell activation after injury was proposed, some mice were submitted to a shorter treatment period (two days). Thus, after three or eight days after the surgery, 24 h after the 2nd or 7th PEA administration, respectively, mice were sacrificed after pain behaviour assessment. Sciatic nerves were

harvested and processed to evaluate mast cell and axon morphology and to perform immunohistochemical detection of mast cell protease I and trkA receptor. One part of each sciatic nerve was immediately frozen in liquid nitrogen to evaluate NGF content.

Assessment of Thermal Hyperalgesia and Mechanical Allodynia

Animal pain response was monitored before surgery and on days 3 and 8 after neuropathy induction. Heat hypersensitivity was tested according to the Hargreaves procedure [33] 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). 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 increasing until the animal removed its paw. Withdrawal threshold was expressed as tolerance level in g.

Mast Cell Determination

Some sciatic nerves were harvested and processed to evaluate mast cell morphology. Briefly, tissues were fixed in fresh 4% paraformaldehyde, dehydrated and embedded in paraffin wax. Longitudinal sections (5 µm) of sciatic nerves were cut and stained with 0.5% toluidine blue [34] in order to characterize mast cell morphology [35]. Mast cells with heavily stained granules scattered outside the cell membrane were defined as degranulated, and cells with a clearly outlined cell membrane and dense granules were defined as not active. Positively stained cells in 30 fields of the entire sciatic nerve per section were counted to calculate the mast cell density (number/mm^2) and mast cell ratio (degranulated/non-active).

Immunohistochemical Localization of Mast Cell Protease I and trkA Receptor

Some longitudinal sections were deparaffinized and rehydrated through graded concentrations of ethanol. To block endogenous peroxidase, sections were incubated with 1% H_2O_2 for 10 min at room temperature. Afterwards, sections were incubated in 2% bovine serum albumin (BSA) and 0.1% Triton X-100 in phosphate buffered saline (PBS) (pH 7.4) for 30 min in order to block non-specific staining. Longitudinal sections were incubated overnight at 4°C with polyclonal sheep anti-mouse mast cell protease 1 (MMCP-1) (Morendum, diluted 1:100 in 1% BSA-0.1% Triton X-100 in PBS pH 7.2). Sections were then incubated with biotinylated anti-sheep IgG (Vector, diluted 1:300 in 0.05% Tween 20 in PBS pH 7.8) for 1 h at room temperature. They were then incubated for 2 h with avidin-biotin peroxidase (Vectastain Elite ABC kit, Vector). The complex was detected for 2 min with diaminobenzidine (ImmPACT DAB peroxidase substrate, Vector), which produced a dark brown end product. Some other longitudinal sections were used to perform immunofluorescence. Deparaffinized sections were at first incubated overnight at 4°C with 1%BSA in PBS pH 7.4 and then were incubated in a mixture containing sheep MMCP-1 polyclonal antisera (Morendum, diluted 1:100 in 1% BSA-PBS) and with rabbit trkA monoclonal antibody (Abcam, diluted 1:200 in 1% BSA-PBS) for 3 h at room temperature. After washing with 1% BSA-PBS for 45 min sections were incubated for 2 h at room temperature in a mixture of two fluorescent conjugated secondary antibodies: fluorescein anti-sheep and rhodamine anti-rabbit antibody (Millipore, both diluted 1:200 in 1% BSA-PBS). For negative control the primary antibodies were replaced by 1% BSA-PBS. The labelled tissues were observed using a Zeiss Axioplan MC 100 microscope. Images were taken using a colour digital camera (Image ProPlus version 4.5.1, Media Cybernetics).

Activated Microglia Assessment

Mice were injected with the anticoagulant heparin (100 µl, 5000 U/mL, i.p.) and then anaesthetized with sodium pentobarbital (60 mg/kg, i.p.). Intracardiac perfusion was carried out with fresh 4% paraformaldehyde. The lumbar (L4-L5) spinal cord was removed, fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin wax to obtain serial cross sections (6 µm) and used for immunohistochemistry with F4/80 antibody in order to identify activated microglia. F4/80 is an immunohistological epitope expressed on microglia [36] that stains both activated and non-activated microglia: its expression increases after activation. Sections were then incubated with 1% BSA at room temperature for 6 h then with primary antibody, followed by incubation with monoclonal rat anti-mouse F4/80 at 4°C overnight (dilution 1:50). After several washes with phosphate buffer (pH 7.4) and a further incubation with 1% BSA for 45 min at room temperature, sections were incubated at room temperature for 2 h with anti-rat IgG alkaline phosphatise-conjugated antibody (dilution 1:50). Sections were then incubated with a tablet of FastRed TR/ NaphtolAs-MX (4-chloro-2-methylbenzenediazonium/3-hydroxy-2-naphtoic acid 2, 4-dimethylanilide phosphate) dissolved in distilled water for 7 min. Interaction between substrate and alkaline phosphate produces a pink colour. Slides were washed with distilled water to stop the reaction and then counterstained with Mayer's hematoxylin for 10 sec in order to stain nuclei.

Histology of Mouse Sciatic Nerve

Sciatic nerves were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.4), rinsed with this buffer, post-fixed with 1% osmium tetroxide, dehydrated in a graded series of alcohol solutions and embedded in Epon resin. Transverse semi-thin sections (1 µm) were obtained using a LKB ultramicrotome and stained with an aqueous solution of 0.2% toluidine blue to study axon morphology under a Zeiss Axioplan MC 100 light microscope. Images were taken with a colour digital camera (Image ProPlus version 4.5.1, Media Cybernetics). About 300 sections proximal to the injury were taken for each animal and 10 sections were randomly selected for intact myelinated fiber quantification performed by an

experimenter blind to pharmacological treatment. The data were expressed as fiber density (number/mm^2). Myelin thickness was also determined in the same sections and expressed in µm.

NGF Content Assay

Tissues were homogenized in 250 µl cold lysis buffer (137mM NaCl, 20mM Tris HCl (pH 8.0), 1% NP40, 10% glycerol, 1mM PMSF, 10µg/ml aprotinin, 1µg/ml leupeptin, 0.5mM sodium vanadate). The homogenates were centrifuged at 4500 *g* at 4°C for 10 min, and the resulting supernatants diluted 5-fold with Dulbecco's PBS. Samples were acidified to pH < 3.0 by adding 1 N HCl and then neutralized with 1 N NaOH to pH 7.6. NGF content was determined by enzyme-linked immunosorbent assay using a kit according to the manufacturer's instructions (Promega, USA). The absorbance at 450 nm was recorded on a microplate reader (Multiskan® EX, ThermolabSystem). NGF was determined by interpolation with a standard curve assayed on individual plates and normalized to protein content in each tissue sample.

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 $P<0.05$. All statistical analyses were performed by using PRISM 4.01 (GraphPAD Software, San Diego, CA, USA).

RESULTS

Effect of PEA on Thermal Hyperalgesia and Mechanical Allodynia

As expected from our previous work [14], treatment of CCI mice with a repeated administration of PEA (10 mg/kg, i.p.) resulted in a significant, time-dependent relief of both thermal hyperalgesia and mechanical allodynia (Fig. **1A**, **B**). PEA significantly attenuated both painful symptoms already after two administrations whereas abolishing thermal hyperalgesia while concomitantly attenuating mechanical allodynia required one week of treatment (Fig. **1A**, **B**).

Effect of PEA on Mast Cells

Fig. (**2**) shows sciatic nerve longitudinal sections stained with toluidine blue. Mast cells with heavily stained granules scattered outside the cell membrane were defined as degranulated, and cells with a clearly outlined cell membrane and dense granules were defined as non-active. In the normal sciatic nerve (Fig. **2A**), resident mast cells were uniformly distributed. Nerve injury caused a significant recruitment of mast cells that was confined to the side region of the sciatic nerve at 3 days after injury (Fig. **2B**). There was a subsequent infiltration towards the inner part of the nerve at day 8 (Fig. **2D**), when mast cell morphology was clearly changed with many degranulated cells. Treatment of mice with PEA appeared to delay mast cell recruitment (Fig. **2C**) and protect against their degranulation (Fig. **2E**). The results obtained with toluidine blue staining were substantially

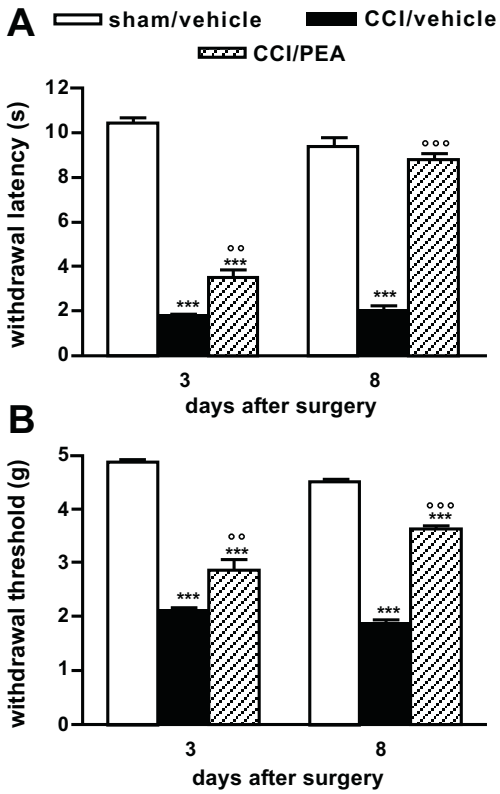

Fig. (1). Effect of palmitoylethanolamide (PEA) in neuropathic (CCI) mice on thermal hyperalgesia (**A**) and mechanical allodynia (**B**). PEA (10 mg/kg i.p.) treatment consisted of two or seven daily administrations. Pain behaviour was assessed 24 h after the last administration (on day 3 or 8 after surgery). Withdrawal latency to heat and withdrawal threshold to mechanical stimulus of the injured paws are expressed as s and g, respectively. Data represent mean ± SEM of 8 mice. ***$P<0.001$ *vs* sham/vehicle; °°$P<0.01$,°°°$P<0.001$ *vs* CCI/vehicle.

confirmed through immunostaining of the longitudinal sections with anti-MMCP-1 antibody, as illustrated in Fig. (**3**): panels **A** and **B** show the same sciatic nerve longitudinal section stained with toluidine blue and anti-MMCP-1, respectively. The density of mast cells (mast cell number/area) was quantified by an experimenter blind to the treatment conditions. The average density of mast cells in the control (sham/vehicle) group was about 330 both 3 and 8 days after surgery (Fig. **4A**). Mast cell density significantly increased (about 270%) in mice subjected to sciatic nerve injury already 3 days after lesion, and such an increase remained 8 days after injury (Fig. **4A**). Mice treated with two doses of PEA showed a density significantly lower than that found in the corresponding CCI mice, whereas after one week of treatment no significant difference was found with respect to CCI mice treated with vehicle (Fig. **4A**). The average ratio of degranulated mast cells to non-active cells was significantly higher in the sciatic nerve of CCI mice 8 days after the injury compared to control group (8-fold increase), whereas no significant differences were found between CCI and control mice 3 days after injury (Fig. **4B**). After seven PEA administrations the average ratio of degranulated mast cells over non-active cells was the same as in non-pathological mice (Fig. **4B**).

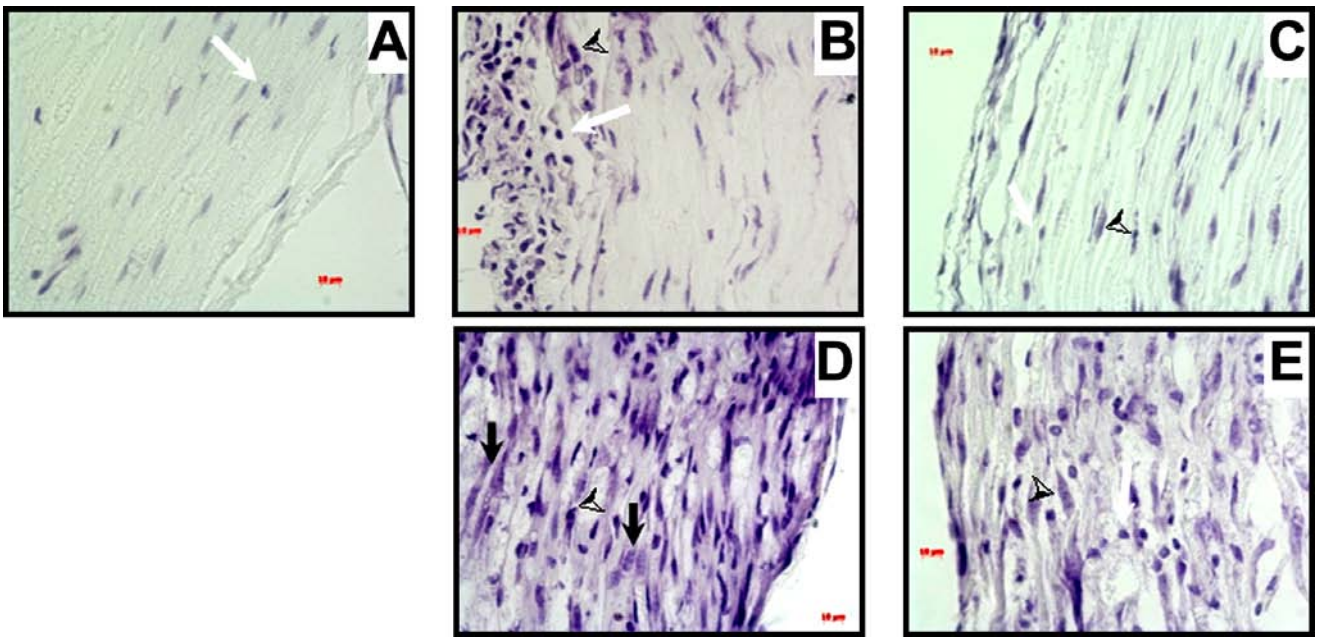

Fig. (2). Light micrographs (40x) showing mast cell morphology in the sciatic nerve in the following experimental groups: sham/vehicle (**A**), CCI/vehicle 3 days after surgery (**B**), CCI/palmitoylethanolamide (PEA) 3 days after surgery (**C**), CCI/vehicle 8 days after surgery (**D**), CCI/PEA 8 days after surgery (**E**). Non-active mast cells were intact and stained intensely with toluidine blue (white arrow). Degranulated mast cells were recognized by the extensive loss of dye and for the presence of residual cytoplasmic granules (black arrow), while degranulating mast cells had an oblong shape and intense blue staining (black arrowhead).

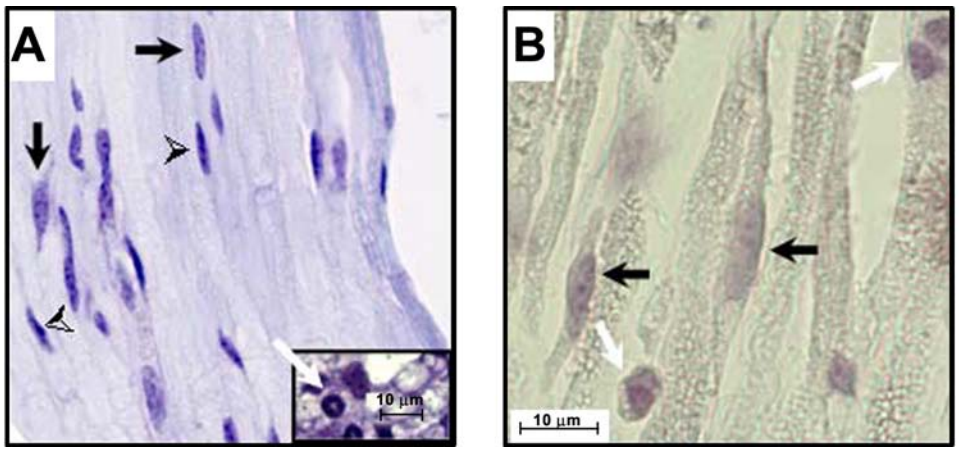

Fig. (3). Representative light micrograph of activated mast cells in the sciatic nerve longitudinal section stained with toluidine blue (40x). Inset shows an intact mast cell (**A**). Higher magnification of mast cells and their granules immunostained with anti-mouse mast cell protease I (100x) (**B**). White arrows, black arrows and black arrowhead highlight intact, degranulated and degranulating mast cells, respectively.

Effect of PEA on NGF Levels

The level of NGF was evaluated in the sciatic nerve from sham and CCI mice. Nerve injury caused an increase in NGF content only eight days after surgery, without affecting endogenous production of this growth factor earlier (Fig. **5A**). Repeated administration of PEA to CCI mice avoided such an increase, and the level of NGF was superimposable with that of control mice (Fig. **5A**). The biological activity of NGF is mediated *via* receptors of the trk family, particularly trkA. Mast cells express trkA receptors, as previously demonstrated in human mast cells [37] and as confirmed by the immunofluorescence analysis performed on sciatic nerve sections showing that MMCP-1 positive cells (mast cells) co-expressed trkA receptors (Fig. **5A-F**).

Effect of PEA on Sciatic Nerve Demyelination

Qualitative examination of the sciatic nerve sections was performed and representative images are shown in Fig. (**6**). Histological appearance was essentially normal for all sciatic nerves from sham/vehicle group with similar distribution of small and large diameter myelinated nerve fibers, Schwann cells and blood vessels and with a regular proportion

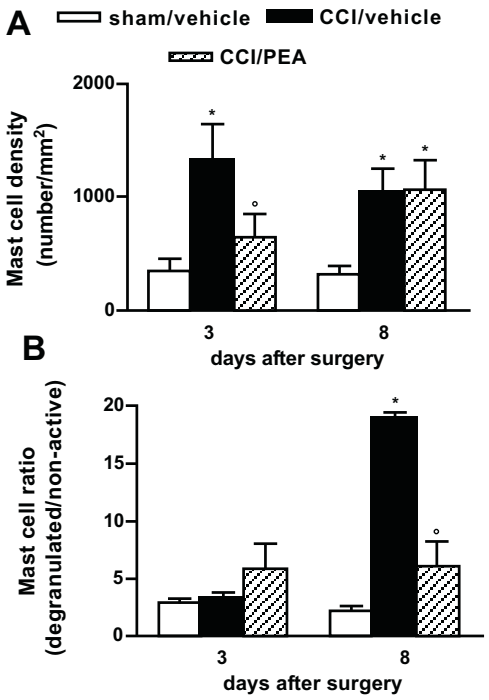

Fig. (4). Effect of palmitoylethanolamide (PEA) 10 mg/kg i.p., following two or seven daily administrations to neuropathic (CCI) mice on mast cell density (**A**) and on the ratio of degranulated to non-active mast cells (**B**). Animals were sacrificed 24 h after the last administration (on day 3 or 8 after surgery). Data represent mean ± SEM. **P*<0.05 *vs* sham/vehicle; °*P*<0.05 *vs* CCI/vehicle.

between myelin sheath thickness and fiber diameter (Fig. **6A**). Already 3 days after nerve lesion, many signs of Wallerian degeneration were present. Endoneural edema was evident, indicated by the separation between nerve fibers, many fibers with an irregular myelin sheath and axon atrophy, damaged fibers and myelin balls released in the endonerium (Fig. **6B**). The same degree of nerve degeneration was observed at day 8 after injury (Fig. **6D**). After two administrations of PEA no evident amelioration was detectable (Fig. **6C**); on the contrary, after seven doses of PEA, we found evidence of axonal regeneration, with the appearance of the nerve that is more normal, although some degenerating fibers were still present; groups of small thinly myelinated fibers were also observed (Fig. **6E**).

An examiner blind to the treatment next counted the intact myelinated fibers in different sections from at least three animals for each group (Fig. **7A**). The average density of normal nerve fibers in the control nerve (sham/vehicle) group at 3 days post-surgery was 11656 fibers/mm^2, as expected for a mouse sciatic nerve, with no differences as compared to that of control mice at day 8 after surgery (Fig. **7A**) or to sciatic nerve harvested from the paw contralateral to the injury site (data not shown). The analysis of sciatic nerves from CCI mice highlighted, as expected, a significant reduction of the average density of normal fibers, that decreased by about 75% at both 3 and 8 days after injury (Fig. **7A**). A reduction of normal fiber density was still present in mice treated with two administrations of PEA, even if the degree of reduction was significantly lesser than in untreated animals (60%) (Fig. **7A**). Treatment with PEA

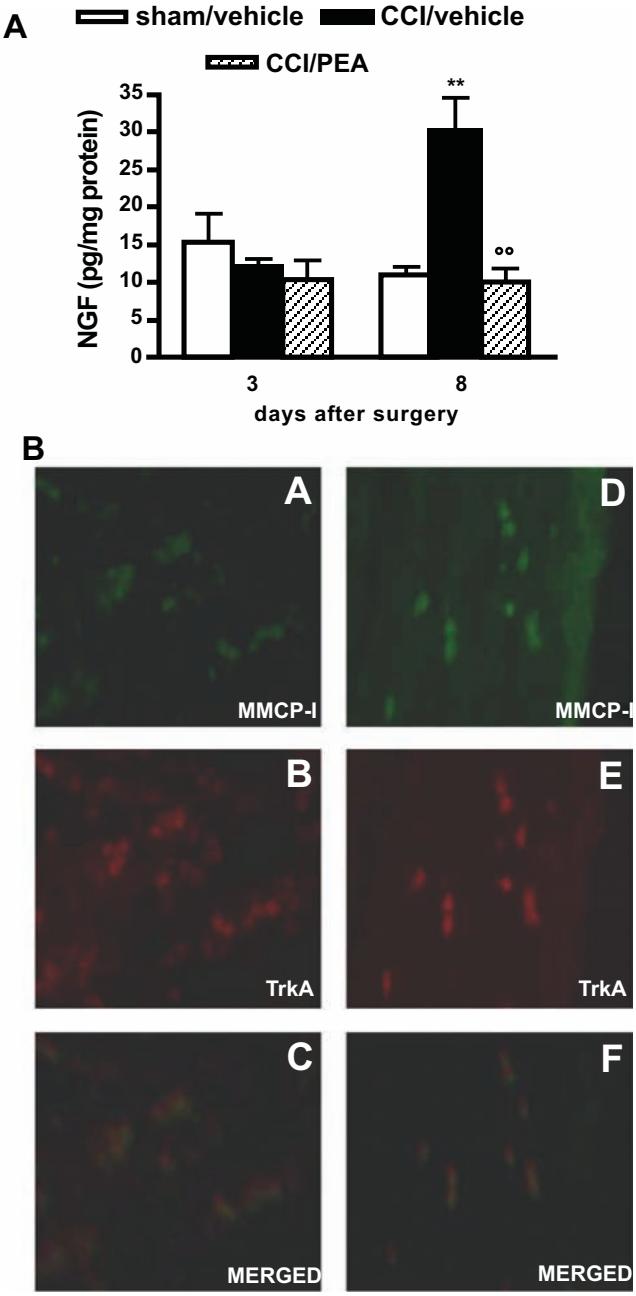

Fig. (5). Effect of palmitoylethanolamide (PEA) 10 mg/kg i.p. following two or seven daily administrations to neuropathic (CCI) mice on nerve growth factor (NGF) levels in the sciatic nerve (**A**). Animals were sacrificed 24 h after the last administration (on day 3 or 8 after surgery). Data represent mean ± SEM of 5 mice. ***P*<0.01 *vs* sham/vehicle; °°*P*<0.01 *vs* CCI/vehicle. Panel **B** shows immunofluorescence staining of longitudinal sections of injured sciatic nerves on day 8 after surgery in CCI/vehicle (**A-B-C**) and CCI/PEA (10 mg/kg, i.p.) (**D-E-F**) mice. Longitudinal sections were immunolabeled with anti-MMCP-1 (**A-D**, green) and with anti-trkA (**E-F**, red). Panel **C** and **F** represent the co-localization of MMCP-1 and trkA. Original magnification 40x.

for a whole week promoted a nerve regeneration process, since the fiber density revealed a significant increase as compared to vehicle-treated mice (Fig. **7A**). In the CCI group myelin thickness was significantly lower than the

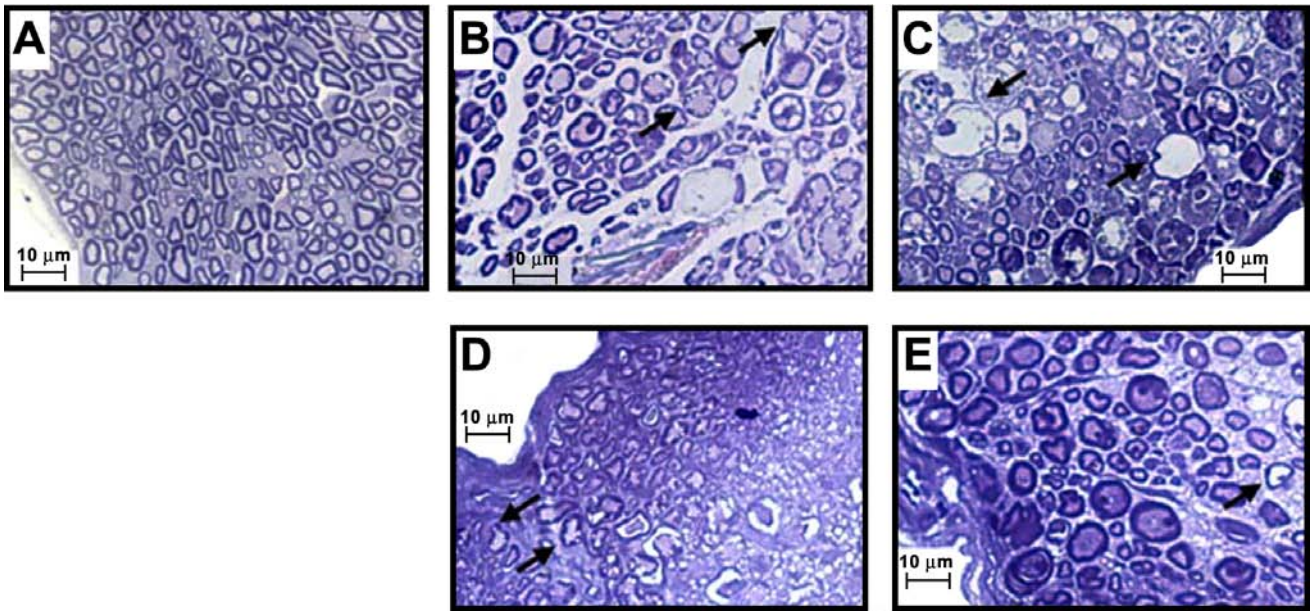

Fig. (6). Transversal semithin sections of sciatic nerve stained with toluidine blue from sham mice treated with vehicle (**A**), from neuropathic mice (CCI) treated with vehicle on day 3 (**B**) or 8 (**D**) after surgery and from neuropathic mice (CCI) treated with palmitoylethanolamide (PEA, 10 mg/kg/day, i.p.) on day 3 (**C**) or 8 (**E**) after surgery. Normal appearance of myelinated fibers (**A**), demyelinated fibers or fibers undergoing demyelination interspersed with normal myelinated fibers (black arrow).

measure estimated in the sham/vehicle group both 3 and 8 days after nerve injury (Fig. **7B**). Following PEA treatment, the average myelin thickness was that expected for non-pathological mice (Fig. **7B**).

Effect of PEA on Microglia Activation

Activation of microglia in the spinal cord and the consequent release of pro-inflammatory and pro-algogen mediators are believed to contribute to central sensitization, a crucial step for neuropathic pain establishment. Spinal cord sections were exposed to anti-mouse F4/80 primary antibody and to the anti-rat IgG alkaline phosphatase conjugated antibody to stain activated microglia deep pink (Fig. **8**). The sections were also counterstained with Mayer's hematoxylin to visualize cell nuclei (Fig. **8**). We found an increased expression of F4/80 positive cells, indicative of microglia activation, in the spinal cord of CCI mice treated with vehicle already three days after sciatic nerve injury (Fig. **8B**), that increased at the later time point (Fig. **8D**) as compared to healthy mice (Fig. **8A**). PEA treatment strongly reduced microglia activation as demonstrated in the representative sections (Fig. **8C**, **E**).

DISCUSSION

We now more fully appreciate that immune cells, in particular mast cells and microglia, by releasing algogen mediators, interact with neurons to alter pain sensitivity and thus contribute to the development and maintenance of chronic pain states [29]. The bioactive endogenous PEA was able to reduce pain behaviour in neuropathic mice through an action upon receptors located on the nociceptive pathway, as described previously by our research group [14]. Moreover, this lipid molecule significantly reduced mast cell

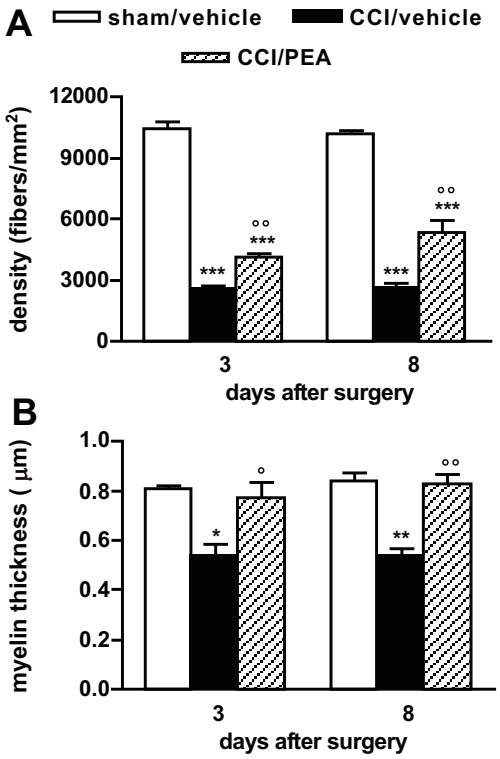

Fig. (7). Morphometric analysis of sciatic nerves stained with toluidine blue evaluating fiber density (**A**) and myelin thickness (**B**) in the experimental groups, three and eight days after surgery. Data represent mean ± SEM of 3 mice. About 300 sections proximal to the injury were taken for each animal and 10 sections were randomly selected. A mean from the 10 sections was obtained as the value corresponding to that mouse and used for the statistical analysis. ****P*<0.001,***P*<0.01,**P*<0.05 *vs* sham/vehicle; °°*P*<0.01, °*P*<0.05 *vs* CCI/vehicle.

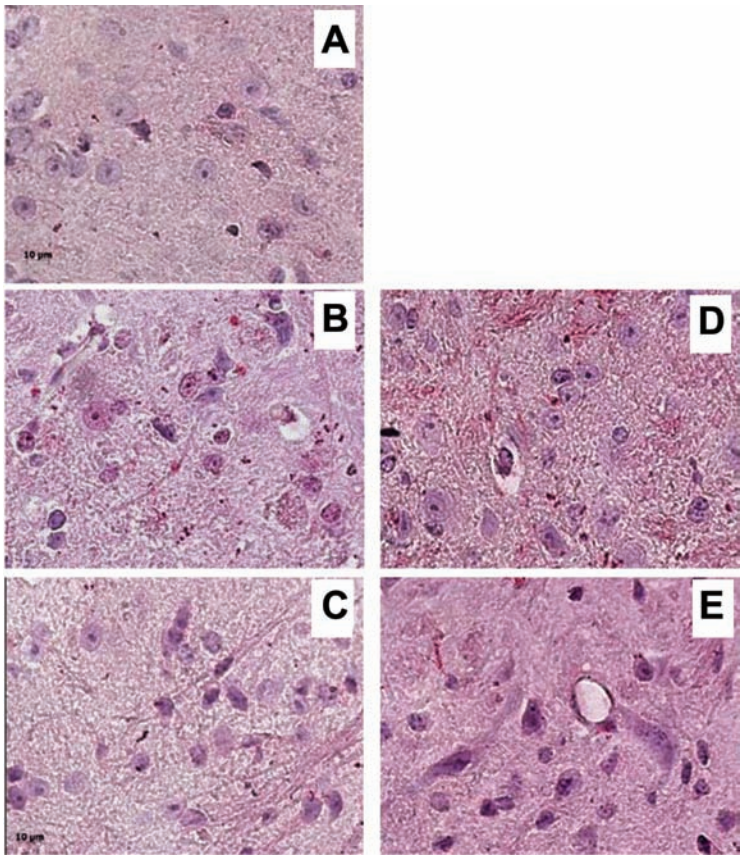

Fig. (8). Representative images of transverse sections of dorsal horn of the lumbar (L4-L5) spinal cord at the ipsilateral site of the injury on day 3 and 8 after surgery immunostained with F4/80 antibody and counterstained with hematoxylin. Experimental group: sham/vehicle (**A**), CCI/vehicle 3 days after surgery (**B**), CCI/palmitoylethanolamide (PEA) 3 days after surgery (**C**), CCI/vehicle 8 days after surgery (**D**), CCI/PEA 8 days after surgery (**E**). Activated microglia are stained deep pink. Original magnification 40x.

degranulation in λ-carrageenan-induced granuloma tissue [38] and reduced microglia activation after spinal cord injury, as recently demonstrated [27]. For these reasons, PEA, combining this dual activity both on neurons of the nociceptive pathway and on the modulation of non-neuronal cells, offers more advantages than classical anti-nociceptive drugs, so representing an ideal molecule for the treatment of chronic pain, like neuropathic one. The CCI-induced mechanical allodynia and thermal hyperalgesia were significantly attenuated, in a time-dependent manner, in mice repeatedly treated with PEA [14]. We have previously suggested that PEA induces relief of neuropathic pain probably through both an action upon receptors located on the nociceptive pathway (cannabinoid receptor CB_1, transient receptor potential vanilloid type 1) *via* an "entourage effect" [14] and through a more direct action on an exclusive target, namely the mast cells, *via* an ALIA mechanism.

To explore this latter hypothesis, we evaluated whether the anti-nociceptive properties of PEA might be accompanied by a modulation of non-neuronal cells such as mast cells in the periphery (sciatic nerve) and microglia in the spinal cord, with a consequent down-regulation of non-neuronal mediators of pain response. In spite of the well-established role of many mediators released by mast cells in sensitizing nociceptors (TNFα, prostaglandins, ATP, NGF), we know of only one report concerning mast cell activity within the sciatic nerve in neuropathic pain [25]. Thus, until now direct evidence relating mast cell recruitment and degranulation in the peripheral nerve and the establishment of neuropathic pain is lacking. In this context our data now show that at the earlier time-point after nerve injury there was a substantial recruitment of mast cells whose activation was not yet pronounced. In contrast at the later time point (8 days post-injury), there was no further increase in mast cell number, but rather a marked activation of these cells, with a high ratio of degranulated to non-active cells. Conceivably the early steps triggering hyperalgesia during neuropathy involve mediators initially produced independently from mast cells, with subsequent activation of mast cells leading to release of many inflammatory mediators, thereby sensitizing nociceptors and consequently resulting in hyperalgesia. The evaluation of mast cell behaviours in the sciatic nerve of CCI mice treated with PEA clearly indicates that this compound is able to significantly delay their recruitment in the early phase of nerve injury-induced neuropathic pain and inhibit their degranulation during the subsequent phase. Importantly, there is a good correlation between the time-course of PEA effect upon neuropathic pain and its action upon mast cell activity. Particularly, our data suggest that the partial reversal of allodynia and hyperalgesia exerted by PEA at the earlier time point could be ascribed to its action upon receptors located on the nociceptive pathway (CB_1, transient receptor potential

vanilloid type 1) *via* the "entourage effect" (the enhancement of the anti-nociceptive activity exerted by anandamide, *via* inhibition of its metabolic degradation due to the ability of PEA to compete with anandamide for its catabolism). At the later time point, the effect on neurons of the nociceptive pathway persists but, concomitantly, PEA exerts its action upon mast cell degradation with a consequent complete relief of pain.

Our experimental conditions do not allow one to explore the mechanism by which PEA inhibits mast cell recruitment. In addition, how mast cells migrate *in vivo* is poorly understood compared to other cell types (granulocytic leukocytes, neutrophils, eosinophils and basophils). Numerous chemoattractants are able to induce chemotaxis in mast cells, including stem cell factors, eicosanoids, chemokines, and adenosine [39]. The mechanisms underlying localisation are complex and, as the cells proliferate and mature their surface expression of chemoattractant and adhesion molecules changes to effect their migration into the tissue. The nature of these expressed molecules differs depending on the tissue, as do the cytokines and growth factors generated in the local microenvironment that determine the phenotype of the mature mast cell [39]. On the basis of the functional contribution by these candidate pathways to mast cell recruitment and considering their production in the inflamed microenvironment of the lesioned nerve, we can suggest that PEA-evoked inhibition of mast cell recruitment in our experimental conditions could be ascribed to the well known capability of PEA to exert anti-inflammatory effects, decreasing the local production of those factors (eicosanoids, chemokines) responsible for mast cell recruitment. This hypothesis awaits further experimental clarification thorough *in vitro* assays.

The factors causing mast cell activation following nerve injury are unclear and may hypothetically include many mediators. Based on recent studies the most plausible candidates are myelin and NGF. In fact, mast cells can be activated by membrane-derived structures such as myelin [40]. Wallerian degeneration occurs following nerve injury, characterized by myelin instability also during the early steps of neuropathy development. Concerning NGF, this neurotrophin is up-regulated by Schwann cells at the injury site and this up-regulation is involved in the onset of neuropathic pain behaviours in rodent models [41-43]. Moreover, a single endoneurial injection of NGF is sufficient to produce transient histological and behavioural effects like those seen in neuropathic pain models [44, 45]. Other than directly sensitizing nociceptors, NGF has been investigated for its possible role in regulating mast cell function, and its capability to promote *in vitro* growth and differentiation of murine and human mast cells has been demonstrated [46, 47]. Finally, mast cells respond to NGF up-regulation since they express trkA [37] which acts as a high-affinity receptor for NGF. Immunostaining of sciatic nerve of CCI mice revealed that MMCP-1 positive cells (mast cells) also expressed trkA receptors, supporting the previous results obtained in human mast cells [37] and suggesting that also in our animals mast cells recruited in the sciatic nerve can respond to NGF. In addition, we demonstrated a significant enhancement of NGF during neuropathic pain at the peripheral (sciatic nerve) level only at 8 days post-injury, whereas NGF content of CCI mice was superimposable to that of healthy animals at 3 days. This result is strongly related to our data showing that mast cell activation occurred only on the 8th day after nerve lesion, highlighting NGF as a key mediator in activating mast cells. Although NGF is synthesized by a variety of peripheral cell types other than mast cells, including lymphocytes, keratinocytes and vascular endothelium, a recent study evaluating the time-course of NGF production demonstrated that 1 week after application of CCI to the sciatic nerve, mast cells represent a principal source of rapidly released NGF [48]. Based on the above, we suggest the following scenario: during the early phase of nerve injury, pain hypersensitivity is sustained by mediators probably released from Schwann cells or neutrophils with a simultaneous recruitment of mast cells; during the later phase NGF, released from the same Schwann cells undergoing degeneration, sustains both the later hyperalgesia and the activation of mast cells. The latter, in turn, release many pro-algogen mediators, including NGF itself, initiating a positive loop able to maintain hyperalgesia. Repeated treatment with PEA reduced mast cell activation, and consequently NGF increase. Therefore, the relief of neuropathic pain elicited by PEA can be closely associated with its ability to negatively modulate mast cell activation and the consequent release of NGF. Further studies are needed to characterize the mechanism of PEA-induced mast cell modulation. We propose two actions: indirect, through the "entourage effect", based on the recently reported ability of anandamide to inhibit the degranulation-promoting effects of key endogenous and exogenous mast cell activators *via* the CB_1 receptor [49]; a direct action of PEA *via* activation of the CB_2 receptor, as originally described by Facci *et al*. [50], or through other receptors for which PEA exhibits some affinity, such as GPR55.

PEA may prevent mast cell degranulation through the already-described ALIA mechanism [8] originally proposed in the mid nineties to indicate that some endogenous *N*-acyl-ethanolamines, like PEA, exert a local antagonism on inflammation. Later the acronym was modified to explain the local antagonism on inflammation and pain exerted by PEA through the down-modulation of mast cell hyperactivity [51, 54]. Since many mediators released by mast cells strongly contribute to the degeneration of myelinated fibers (particularly TNFα), we performed a histological evaluation of the sciatic nerve to verify whether the inhibition of mast cell activity induced by PEA might be accompanied by an improvement in the pattern of Wallerian degeneration. Histological analysis, as well as quantification of fiber density and of myelin thickness indicated an amelioration in sciatic nerve morphology following repeated treatment of PEA, confirming that inhibiting the release of mast cell-derived mediators leads not only to the relief of painful symptoms due to a reduction of peripheral sensitization, but also to a reduction of nerve degeneration consequently to the lesion.

Numerous findings suggest that also spinal cord glia play an important role in pain facilitation [52, 53]. Of interest, a report shows that a proliferative burst of glial cells occurs in the ipsilateral dorsal horn following peripheral nerve injury and that the time-course of microglia proliferation closely correlated with the development of neuropathic pain, suggesting an important link between microglia activation

and pathogenesis of pain hypersensitivity [54]. Microglia activation within the spinal cord leads to hyperalgesia and allodynia *via* release of algesic soluble factors, such as pro-inflammatory cytokines, nitric oxide, prostaglandins, excitatory amino acids and NGF, which act on neurons in the pain pathways [53]. Our findings confirm an up-regulation of activated microglia in the spinal cord of neuropathic mice that contributes to aberrant pain behaviour and plays an important role in central sensitization, and demonstrate that PEA treatment reduced microglia activation within the spinal cord. It is noteworthy that microglia produce, release and respond to PEA [55] suggesting that this lipid can behave as an important endogenous microglia modulator. Furthermore, in neuropathic rats levels of PEA were significantly decreased in the ipsilateral spinal cord, compared to the contralateral spinal cord. Following chronic treatment with a dose of minocycline (a microglia inhibitor) efficacious against neuropathic pain, levels of PEA were increased in the ipsilateral spinal cord, suggesting that microglia play a major role in modulating levels of PEA [10]. Exogenous supply of PEA, as in our experiments, probably enhances the endogenous neuroprotective effect of PEA within the spinal cord through the modulation of microglia activation.

In vitro studies are in progress in our laboratory to verify whether PEA directly modulates microglia activity *via* an interaction with specific receptors. Besides this possibility we cannot exclude that the effect observed on spinal microglia is due to peripheral effects on mast cells; in fact, it has been proposed that there are potential avenues of microglia-mast cell communication that could serve to propagate some diseases [30].

Therefore, PEA modulates both types of non-neuronal cells strongly involved in triggering neuropathic pain. The employment of mast cells stabilizers, such as cromoglycate, or microglia inhibitors, such as minocycline, have been shown to attenuate neuropathic pain in animal models [25, 10]; however, since mast cells and microglia express both beneficial and detrimental phenotypes, the use of modulators instead of classical blockers should be of considerable advantage and our findings demonstrate that PEA possesses such characteristics.

CONCLUSION

The findings support our hypothesis that PEA-induced relief of neuropathic pain might be, at least in part, ascribed to the ability of this compound to modulate mast cell recruitment and activation in the sciatic nerve and microglia activation in the spinal cord, strengthening the idea that such non-neuronal cells may be an important and valuable pharmacological target to treat neuropathic pain given that current pain therapeutic strategies directed to neurons are unsatisfactory for many patients. PEA could represent an innovative molecule, combining a dual analgesic activity, both on neurons of the nociceptive pathway and on non-neuronal cells.

ABBREVIATIONS

BSA = Bovine serum albumin

CCI = Chronic constriction injury

CNS = Central nervous system

MMCP-1 = Mouse mast cell protease 1

NGF = Nerve growth factor

PBS = Phosphate buffered saline

PEA = Palmitoylethanolamide

TNFα = Tumor necrosis factor α

trkA = Tyrosine kinase A

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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