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Morphological characterization of anti-nociceptive effect of endogenous lipid Palmitoylethanolamide in two murine models: peripheral mononeuropathy and diabetic polyneuropathy

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Abstract

Recent evidences suggest that mast cell activation and degranulation have a key role in the beginning and maintenance of a persistent pain, such as neuropathic one. Particularly mast cells are known to release NGF (Leon et al. 1994) and to express trkA (Horigome et al. 1993) receptors able to bind NGF. NGF loop may cause mast cell degranulation, leading to a further release of the neurotrophic factor NGF and many other pro-nociceptive and pro-inflammatory mediators. The release of NGF and other pro-inflammatory cytokines leads to a degeneration of nerve fibers. Therefore inhibition of mast cell degranulation could represent a good strategy in the cure of neuropathic pain. A class of molecules able to negatively modulate mast cell degranulation is represented by ALIAmides (from the acronym Autacoid Local Injury Antagonist), naturally-occurring lipid amide deriving from membrane fatty amides and structurally releted to endocannabinoids (Facci et al.1993, Mazzari et al., 1996). Palmitoylethanolamide (PEA), an endogenous lipid, is the most studied, in addition Petrosino and colleagues (2007) demonstrated that endogenous palmitoylethanolamide level decreased in the spinal cord of neuropathic mice.

In our previous study (Costa et al., 2008) we demonstrated that palmitoylethanolamide (PEA), administered i.p. at the dose of 10 mg/kg for 7 days from the chronic constriction injury, a well established model of peripheral mononeuropathy, evoked a relief of both thermal hyperalgesia (increased sensitivity to thermal stimulus) and mechanical allodynia (pain due to a mechanical stimulus which does not normally provoke pain) in neuropathic mice (chronic constriction injury model) and significantly reduced the NGF levels in

the sciatic nerve of neuropathic mice. In addition Costa and colleagues demonstrated PEA efficacy on allodynia which develops in diabetic mice and to partially increase NGF level. Starting by this assumption we wanted to characterize mast cell degranulation in the same animal model and after the same PEA treatment.

In the chronic constriction injury model in addition to prolonged treatment (7 days), animals received PEA for a short period (2 days) because rapid mast cell activation was supposed. On 3rd and 8th day, 24h after the last administration, mice were sacrificed and the sciatic nerve and the spinal cord were removed from 3d and 8d group in each experimental group (sham/vehicle, CCI/vehicle and CCI/PEA). Sciatic nerves were processed in paraffin wax or Epon-Araldite resin to obtain respectively longitudinal (6 µm) or semi thin transversal (1 µm) sections, while spinal cord was in paraffin wax to obtain transversal (6 µm) sections. Another experiment was performed to collect spinal cords for a biochemical analysis. Spinal cord was removed because recent evidences suggest that microglia cells, in particular activated microglia, play a key role in the induction and maintenance of neuropathic pain.

In the sciatic nerve longitudinal sections of control mice (3d and 8d group) arranged and consistent nerve morphology with a homogeneous localization of nuclei Schwann cells was observed, and limited number of intact and degranulated mast cells was present, indicative of physiological resident mast cells. Sciatic nerve of CCI mice (both groups) appeared oedematous without compactness of the fibers. In particular, in CCI mice of 3d group, the mast cells were localized in the surrounding tissue with a strong recruitment of intact

mast cells, while in CCI mice of 8d group mast cells infiltrated in the inner central part of the sciatic nerve. In neuropathic mice treated with PEA of 3d and 8d group a mild inflammation and mast cell recruitment was observed. The evaluation of mast cell density (expressed as total mast cell/mm^2) confirmed a time-dependent mast cell recruitmant after 3 and 8 days from the injury, indicating that mast cells degranulation plays a key role in the beginning and maintenance of neuropathic pain. The 2 days PEA treatment acted on mast cell recruitment; in fact mast cell density was comparable to that observed in sham mice. The 7 days PEA treatment modulated mast cell degranulation; in fact a significant reduction of average ratio of degranulated mast cells over intact mast cell compared to CCI mice was observed. The presence and implication of mast cell was confirmed by double immunostaining images obtained from longitudinal sections showing a co-expression of mast cell proteases I (MMCP-I) and trkA receptor.

In the tranversal sections of sham mice of both groups no histological abnormalities were present: typically myelinated axons are packed in the sciatic nerve in an orderly, parallel arrangement with minimal interaxonal space. In transversal sections of CCI nerves of 3d and 8d group a lot of myelinated fibers underwent a Wallerian-like degeneration, suggested by a dense and flocculent axoplasmic matrix. In mice treated with PEA for 2 and 7 days a mild degeneration of fibers and compact arrangement like sham transversal section was observed. The quantitative analysis confirmed that the intact axons density (expressed as intact axons/mm^2) and the myelin thickness (expressed as μm^2) in CCI mice (both groups) significantly decreased

respect to sham mice, and PEA treatment partially reduced fiber degeneration at both times, emphasizing its role role to prevent the reduction of myelin thickness at both 3 and 7 days after the injury.

In order to verify the presence of activated microglia in neuropathic mice treated with PEA, the expression of F4/80 protein in transversal sections of the dorsal horn of the lumbar (L4-L5) spinal cord was investigated. This region was considered because the afferent fibers from the periphery (sciatic nerve) arrive here. Representative images show that in CCI mice a time-dependent increase of activated microglia was observed in the dorsal horn of L4 and L5 spinal cord compared to sham mice. In the contralateral horn of the spinal cord a milder activation of microglia was detected in L4 and L5 only in the 8d group. These results were confirmed by the densitometric analysis of F4/80 expression obtained by western blotting. The treatment with PEA for two consecutive days attenuated the limited microglia activation, restoring to physiological level the expression of F4/80 protein.

In the third part of this project we evaluated the involvement of mast cell in the anti-allodinic effect of PEA in diabetic and neuropathic mice. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, 120 mg/kg). Mice were divided into two groups: 17d and 14d group. In the 17d group mice developed allodynia 14 days after STZ injection. Before the beginning of the treatment and at the end of experiment, hyperglycaemia was evaluated. On 14^{th} day, PEA treatment started and three experimental groups were present: non diabetic, diabetic mice treated with vehicle and diabetic mice treated with PEA. PEA was administered i.p. at the dose 10 mg/kg for

three consecutive days. 24 hours after the last administration PEA had an anti-allodinic effect and the following organs were removed: sciatic nerves (right and left), kidneys and pancreas. In 14d group PEA treatment started on 7^{th} day, when mice were diabetic but non allodinic, and 24 hours from the last administration pancreas was removed. Since Costa and colleagues demonstrated that a mild insulin level in the blood was present in PEA treated mice of 14d group but non that of 17d group, morphological analysis and a subsequant quantative analysis was performed on pancreas tissue.

Longitudinal and transversal sections of sciatic nerve were stained with toluidine blue in order to examine morphology of mast cell and axons. In longitudinal sections intact mast cell, degranulating and degranulated mast cells were uniformely distributed throughout the nerve of the three experimental groups and the count of total mast cell (total number of mast cell/mm^2) didn't show any difference among the three experimental groups. In transversal sections axons undergoing degeneration were present in diabetic mice treated with vehicle or PEA respect to non diabetic mice. The count of intact axons revealed that at the end of treatment diabetic mice treated with vehicle showed a significant decrease of intact axons and that this decrease remained constant in diabetic mice treated for three consecutive days with PEA. Nephropathy is another consequence of diabetes; therefore renal transversal sections were stained with periodic acid Shiff (PAS) reagent to investigate the presence of an expansion of mesangium in the glomeruli. Diabetic mice showed an increase of mesangial matrix and subsequently an increase of the glomeruli area compared to non-diabetic mice. In mice treated with three consecutive injections of

PEA appeared similar to the images observed in diabetic mice treated with vehicle. The evaluation of the glomeruli area (expressed in μm^2) confirmed the increase of mesangial area. In fact in diabetic mice treated with vehicle a significant increase of the area was recorded compared to non diabetic mice and the same increase was measured in diabetic mice treated with PEA for three consecutive days.

In pancreas sections of diabetic mice treated with vehicle of 17d group, the islet of Langerhans appeared smaller and less numerous, while in diabetic mice treated with PEA could be observed a mild improvement. A quantitative analysis (the density expressed as number/mm^2 and the area expressed as μm^2) of islet of Langerhans demonstrated a significantly decreased in diabetic mice treated with vehicle and PEA had a protective partial effect on density and area of islets of Langerhans.

In the group 14d pancreas sections of non diabetic mice appeared well organized with large and uniformely distributed islet of Langerhans. In diabetic mice treated with vehicle pancreas appeared with few islets which were small. In PEA treated mice an improvement was present: in fact islets were numerous and they appeared bigger than in diabetic mice. The quantitative analysis confirmed the previously considerations: in fact the density and the area of islets of Langerhans significantly decreased in diabetic mice treated with vehicle compared to non diabetic mice and 7 days PEA treatment highlighted the protective effect on the islets of Langerhans.

The findings presented herein strongly suggest that PEA, without any side effects, is a promising compound in the cure of neuropathy. It may prevent mast cell degranulation through the already described

Abstract

ALIA (Autacoid Local Inflammation Antagonism) mechanism, modulates microglia activation in the spinal cord and this effect accounts for the antinociceptive property of PEA. In addition, PEA administered to diabetic and neuropathic mice elicited allodynia and exerts a time-dependent protective effect on islets of Langerhans.

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Introduction

Mast cells

Paul Ehrlich first described mast cells (MCs) or ‘mastzellen’ in 1878 based on their unique staining characteristics and their large granule content.

Like other myeloid cells, mast cells derive from the bone marrow (Chen et al., 2005), recent findings have shown that mast cell is derived from a common myeloid progenitor (CMp), but not from a granulocyte/macrophage progenitor (GMp), as reported previously (Franco et al., 2010) (Fig.1). Despite mast cells all deriving from a common lineage and having a granulated morphology, they are extremely heterogeneous and ‘plastic’ in phenotype and function (Stevens and Adachi, 2007).

The morphological shape of mast cells varies in according to their habitat. In loose connective tissue they appear rounded, in close apposition to blood vessels they are elongated or ovoid, while in derma fibres they may be spindle shaped, stellate or filiform. Mast cells which are freely suspended in peritoneal fluid in the rat are round. They have a mean diameter of 12.6-13.5 μm and their cytoplasm is occupied by granules (50-55%) (reviewed in Yong, 1997).

Each granule (0.5-0.7 μm in diameter) is surrounded by a membrane. In addition to granules the mast cell possesses mitochondria, Golgi apparatus, centrioles, rough endoplasmic reticula, free ribosomes, vesicles and fibrils.

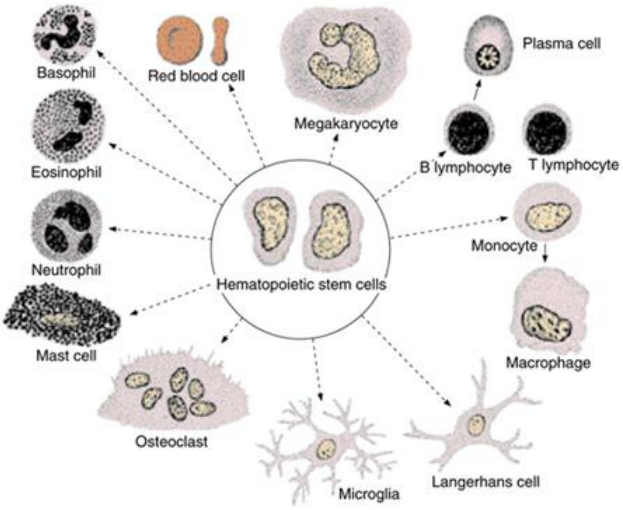


Fig.1*: Simplified representation of the connective tissue cell lineage derived from the multipotential embryonic mesenchyme cell. Dotted arrows indicate that intermediate cell types exist between the examples illustrated. Note that the cells are not drawn in proportion to actual sizes, eg, adipocyte, megakaryocyte, and osteoclast cells are significantly larger than the other cells illustrated. (Basic Histology, text and atlas. Junqueira and Carneiro, Mc-Graw Hill)*

The surface topography of mast cell has been examined by scanning electron microscope and mast cells have numerous short and long microvilli, numerous bulges corresponding to granules lying beneath the plasma membrane and "opening" or caveolae where granular discharge has taken place (Fig. 2). Mast cells on their surface express integrin (LFA-1 and VLA4), which bind Inter-cellular Adhesion molecule (ICAM) and Vascular cellular Adhesion molecule (VCAM), *c-kit* receptor for stem cell factor (Galli et al., 1993) a range of pattern recognition receptors (e.g.toll like receptors), which are activated by pathogens. In addition mast cell surface express the high-affinity IgE receptor FcεRI, bradykinin receptor (B2), corticotropin-releasing hormone receptor (CRH-receptor). Receptors for chemokines (e.g.

TNF recepotr) and growth factor receptor (e.g. trkA receptor for nerve growth factor) (reviewed in Rao and Brown, 2008) are present on mast cell surface.

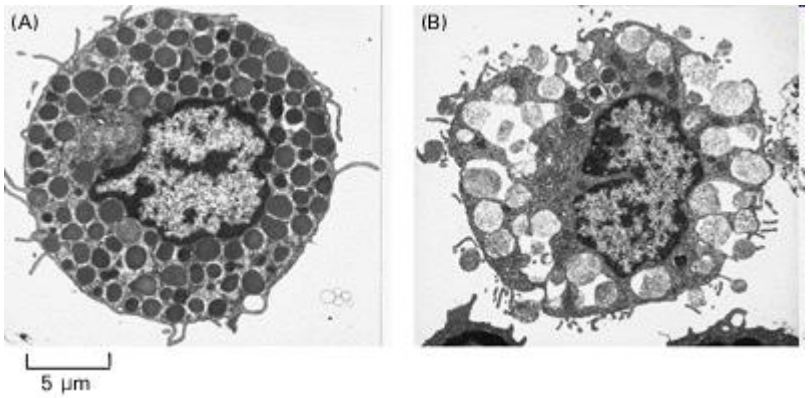


Fig.2*: A transmission electron micrograph showing no active mast cell (A) and activated mast cell (B). Note cytoplasmic electron dense granules (black spheres, panel A) while those that have released their histamine are light (panel B) (From D. Lawson, C. Fewtrell, B. Gomperts, and M. Raff, J. Exp. Med. 142:391-402, 1975, by copyright permission of the Rockefeller University Press.)*

Compound 48/80 is one of the first substances used to study the degranulation of mast cells. When exposed to this compound the granules lose their usual electron density within a second. The granules then become empty vacuoles which may fuse with other empty vacuoles to form larger ones. These vacuoles may bridge with the cell membrane to form openings with the outside of the cell so the granular contents can then discharged directly to the outside of the cell (Fig2). This change can be also observed with the light microscope. In fact Bloom and Hangermark (1967, reviewed in Yong, 1997) reported the change in staining characteristics from dark blue or purple to red or pink with the toluidine blue stain after the discharge form the cell.

This change is due by a tissue staining phenomen: the metachormasia. This phenomenon may be related to the granule content of histamine and heparin (reviewed in Yong, 1997).

Acidified toluidine blue staining permits to distinguish between intact (no active), in degranulation and degranulated mast cells. No active cells present a round/ oval form stained intensively with toluidine blue, the granules sometimes mask the nucleus sometimes it is appreciable and is light-coloured. In degranulation mast cell appeared flattened or irregular and cytoplasm appeared granular. Many cells showed spreading granules and in some cases granules were found dispersed in the surrounding tissue. The cell borders were not defined and the nucleus was only partially appreciable. Degranulated mast cells appeared paler as the staining had changed from metachromatic violet to light pink. This staining change is caused by the release of granules in the surrounding tissue. The nucleus when visible is well defined (Fig.3).

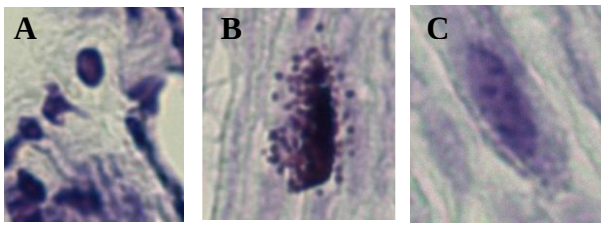


Fig.3: Representative images of mast cells stained with acidified toluidine blue in the sciatic nerve of mice. Intact (no active) mast cell (A), in degranulation (B) and degranulated mast cells (C) are represents.

Rodent mast cells can be broadly divided into two phenotypes, connective tissue mast cells (CTMCs), preferentially localised in the mucosa of the airways and gastrointestinal tract, and mucosal mast cells (MMCs), are typically found in the skin, synovium, peritoneum

and perivascular tissues. Mouse mast cells have been characterized based on their heterogeneous expression of proteases, including the chymases (mMCP-1, -2, -4, -5 and -9), tryptases (mMCP-6, -7. -11 and mTMT, a transmembrane tryptase) and carboxypeptidase (mMC-CPA) (Metcalfe et al., 1997). Connective tissue mast cells contain chymases and tryptases, bound to heparin and the mucosal mast cells contain just chymases, bound to chondroitin sulphate (Welle, 1997). Human mast cells can also be distinguished by these two types of protease, the mast cells containing tryptase (MC_T), predominantly found in the alveoli of the lung and in the intestinal mucosa, or mast cells containing tryptase chymase (MC_{TC}), predominantly found in the skin, the conjuntiva and in the intestinal submucosa (Ghildyal et al., 1992; Irani. et al., 1990). Protease expression can also be altered with different stimulation. For example, in mouse, mast cell protease content can be altered by IL-10 (Toru et al., 1998), and treatment of human mast cells with IL-4 increased the level of chymase in their granules (Abraham and St John, 2010).

Mast cell localization and function

Mast cells are found at the interface between the host and the external environment near blood vessels, lymphatic vessels, nerve fibres and a range of immune cells, including dendritic cells (reviewed in Kunder et al., 2009). This strategic positioning allows them to act as sentinels of invading microbes, but also to respond rapidly to any change in environment by communicating with different cells involved both in physiological and immunological responses. Mast cells are important to maintain physiological homoeostasis in fact they maintain homeostasis of organs that undergo continuous growth and

remodelling such as hair follicles and bones. $MC^{-/-}$ mice have normal hair growth; however, hair follicle cycling is severely impaired (Maurer et al., 1995). MC-derived histamine, tumor necrosis factor (TNF) and substance P are implicated in regulating growth and regression of hair follicles between periods of hair growth and rest (Maurer et al., 2003). Mast cells also contribute to bone remodelling. In $MC^{-/-}$ mice, femurs are lighter, thinner and more fragile (Silberstein et al., 1991). It has been speculated that mast cell interleukin-1 (IL-1), tranforming growth factor- beta (TGF-β), IL-6 and histamine could influence osteoclast recruitment and development. Recently, mast cells were found to produce osteopontin (OPN), a secreted glycoprotein that controls bone metabolism and also has a role in immune responses (Nagasaka et al., 2008). They can also maintain homoeostasis by degrading toxins such as the endogenous peptide endothelin-1 (ET-1) and snake venom (Maurer et al 2004).

In the gut, mast cells and neurons maintain homoeostasis by regulating ion transport, secretory activity of mucous epithelial cells, vascular permeability and intestinal motility (Van et al., 2007). They are known to be involved in wound healing from the initial inflammatory response followed by re-epithelialization and revascularization of the damaged tissue and finally in deposition of collagen and re-modelling of the matrix (Noli and Miolo, 2001). Recently, vitamin D_3 has been reported to reduce skin pathology at sites of chronic UVB irradiation, by inducing IL-10 production from cutaneous mast cells via engagement of the vitamin D receptor (Biggs et al., 2010). In addition Zuo and colleagues (2003) demonstrated the key role of mast cells in the maintenance of hyperalgesia in a well

established model of neuropathic pain in the rat, the chronic constriction injury (CCI model) (Zuo et al., 2003).

Mast cell activation

Mast cells are recruited in the injury site through the blood vessels by pro-inflammatory cytokines (e.g. tumor necrosis factor-alfa, interleukin-1β, nerve growth factor), reactive oxygen species (ROS) and other mediators like prostaglandins or adenosine triphosphate (ATP) produced in the early step from the injury. Mast cells, when degranulated, release many different growth factors including nerve growth factor (NGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2), histamine and tryptase, which are involved in proliferation of epithelial cells and fibroblasts (Abe et al., 2000) and IL-10, TNF-α, prostaglandin E_2 (PGE_2), serotonin, platelet-activating factor (PAF) and IL-4.

IgE-dependent activation

The best studied mechanism of MC activation is through the high-affinity IgE receptor FcεRI (Fig.4).

These receptors on the surface of MCs can bind to both IgE as well as IgG and become sensitized to antigens that the host has previously contacted. Cross-linking of IgE or IgG on the surface of mast cells by specific antigens leads to activation and the subsequent degranulation and *de novo* synthesis of mediators (Galli and Tsai, 2008).

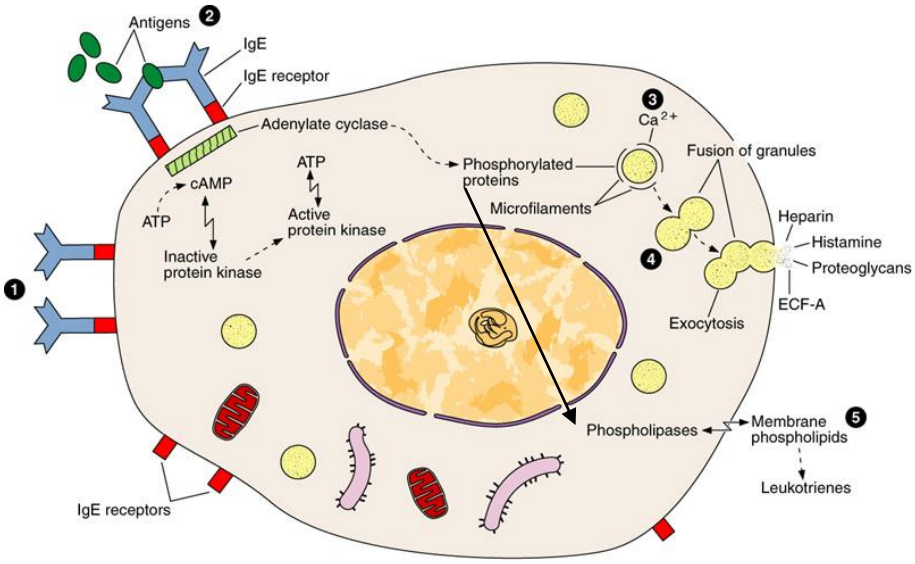


Fig.4: *Mast-cell secretion. 1: IgE molecules are bound to the surface receptors. 2: After a second exposure to an antigen (eg, bee venom), IgE molecules bound to surface receptors are cross-linked by the antigen. This activates adenylate cyclase and results in the phosphorylation of certain proteins. 3: At the same time, Ca2+ enters the cell. 4: These events lead to intracellular fusion of specific granules and exocytosis of their contents. 5: In addition, phospholipases act on membrane phospholipids to produce leukotrienes. The process of extrusion does not damage the cell, which remains viable and synthesizes new granules. ECF-A, eosinophil chemotactic factor of anaphylaxis (Basic Histology, text and atlas. Junqueira and Carneiro, Mc-Graw Hill).*

Non-IgE-dependent activation

Mast cells can also be activated through a variety of non-IgE-dependent mechanisms including proteases, cytokines, complement, adenosine, TLRs (Toll-like receptors), neuropeptides and hyperosmolarity (Rivera and Gilfillan, 2006). Activation can be positively or negatively regulated by ligands to many receptors expressed on the surface of mast cells (el-Lati et al., 1994). For example, mast cells are activated by complement products C3a and C5a (Ali et al., 2000) to induce chemokine gene expression in mast cells (Oskeiritzian et al., 2005). Complement receptors are

differentially expressed on mast cells from various tissue locations (Ali et al., 2000); in the lung, mast cell containing tryptase do not express C5aR, whereas mast cells containing tryptase and chymase do, and this is correlated with substantial C5a-induced degranulation in mast cells containing tryptase anc chymase (Fritz et al., 2006).

The initial recognition of microbes is mediated by a set of PRR (pattern recognition receptors) including TLRs and the newly defined nucleotide-binding domain, NLRP (leucine-rich repeat proteins) or NLR (NOD-like receptors) which are present on many different cells of the immune system, including mast cells (Haidl et al., 2010; Franchi et al., 2008). The TLRs and NRLPs are a family of cell-surface and cytosolic receptors which collectively recognize PAMPs (pathogen-associated molecular patterns) and 'danger' signals (Applequist et al., 2002). Human and mouse mast cells express TLR1–7 and -9 (Matsushima et al., 2004; Supajatura et al., 2002) and specific TLR stimulation (by different pathogens) is able to induce different responses from mast cells. For example, LPS (lipopolysaccharide) stimulation of mast cells through TLR4 leads to cytokine production without degranulation; however, stimulation of TLR2 by peptidoglycan induced both degranulation and cytokine production in some but not all studies (Ikeda and Funaba, 2003; Gurish et al 2001). Mast cells can also detect a range of pathogens specifically through binding of antibodies specific for pathogen-associated epitopes and CD48, which can detect the presence of pathogens including *Escherichia coli* (Malaviya et al., 1999). Exogenous components associated with pathogens such as wasp venom can also activate mast cells. Although mechanisms are not

completely clear, certain venom constituents may directly activate trimeric G proteins and other signalling molecules (Ferry et al., 2002; Chahdi et al., 2004).

Mast cells and Nerve Growth Factor

Nerve growth factor (NGF) is a member of the family of proteins known as neurotrophins, including brain- derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3),and NT-4/5, which is essential for the development and survival of neuronal cells (Barde, 1990; Levi-Montalcini, 1987). NGF, one of the most thoroughly studied neurotrophic factors, plays a crucial role in the survival and development of specific peripheral and brain neurons (Levi-Montalcini, 1987; Thoenen et al, 1987). In recent years, however, numerous studies have provided evidence that NGF acts not only on cells of the peripheral and central nervous system (CNS) but also in the immune system (Aloe et al., 1994a; Levi-Montalcini et al., 1990). Numerous observations have demonstrated that NGF is produced by fibroblasts, keratinocytes, mast cells, thymus cells, and lymphocytes (Aloe et al., 1997; Levi-Montalcini et al., 1968; Thoenen et al., 1987). The purified form of 2.5S NGF is a dimer of two identical subunits linked together by noncovalent bonds and with a molecular weight of about 26 kD (Bocchini and Angeletti, 1966). The amino acid sequence and primary structure of this neurotrophin has been well characterized (Hogue-Angeletti and Bradshaw, 1971) and indicates that NGF is a well-conserved molecule sharing strong homology within different species (Gotz and Schartl, 1994). The biological effects of NGF are mediated by two distinct classes of specific cell-surface receptors with different affinities: low-affinity (p75) receptor, a transmembrane

glycoprotein which can also bind the other neurotrophins, and a high-affinity (trkA) receptor, a molecule with a transmembrane tyrosine kinase domain that is coded by the trk-proto-oncogene (for details, see Barbacid, 1993; Green et al., 1986; Meakin and Shooter, 1992).

Under normal conditions, NGF is present in the bloodstream of rodents and humans but increases during stressful events and other neuroendocrine alterations (Aloe et al., 1986, 1994b). Numerous studies have shown that the circulating level of NGF is affected not only by these homeostatic disregulations, but also by changes in age, following neurological insults, and during autoimmune and allergic diseases (Aloe et al., 1994a; Bonini et al., 1996; Gasser et al., 1986; Thoenen et al., 1987). Moreover, studies carried out not only *in vitro* indicate that the synthesis and release of NGF is often associated with an increase of proinflammatory cytokines, particularly interleukine-1 beta (IL-1b) and tumor necrosis factor-alpha (TNF-a) (Friedman et al., 1990; Gadient et al., 1990; Gasser et al., 1986; Hattori et al., 1993).

Activated mast cells *in vitro* can synthesize, store, and release biologically active NGF (Leon et al., 1994), it has been suggested that these cells represent a potential source of NGF. Mast cells were the first non-neuronal cell types identified as a target for NGF. It has been shown that neonatal rats receiving daily injections of purified NGF display an increase in mast cell number and size in the central nervous system and in areas close to peripheral sensory and sympathetic nerve fibers (Aloe and Levi-Montalcini 1977).

Several findings reported in the last few years clearly indicate that the basal level of NGF is significantly altered in autoimmune inflammatory diseases such as multiple sclerosis (Bracci-Laudiero et

al., 1992), systemic lupus erythematosus (Bracci-Laudiero et al., 1993), rheumatoid arthritis (Aloe et al., 1992) and injured tissues (Costa et al., 2008). The increase of NGF basal level causes the onset of painful stimuli: in inflammatory hyperalgesia NGF binds trkA receptor expressed on primary afferent neurons (Marshall and Waserman, 1995) and on mast cell surface. This binding causes a mast cell degranulation and releasing pro-inflammatory mediators including more NGF. Biochemical studies have also indicated that the altered local and systemic level of NGF is associated with the distribution of mast cells within the inflamed tissue (Aloe et al., 1994b, 1995; Chaldakov et al., 1995).

Nerve morphology

Peripheral nerves are organs distributed throughout the body reaching all tissue and organs to provide motor and /or sensory innervation. In a histological image a parenchyma (the nerve fibers, i.e., axons and Schwann cells) and a stroma (various connective elements) can be recognized.

It is possible to distinguish two subgroups of fibers: myelinated and unmyelinated fibers (Fig.5).

The first subgroup consists of a single axon that is enveloped individually by a single Schwann cell. The Schwann cell wraps around the nerve fiber to form a multilaminated myelin sheath. A-δ fibers are thinly myelinated fibers with a moderate conduction of velocity and they are associated with sensation of temperature and pressure. A-δ nerve fibers can be divided into type I and II. The both of types are activated by thermal and mechanical stimuli; the type I is

more sensible towards intense stimuli (> 52°C) than the type II (43°C).

The second one consists of several nerve axons enveloped as a group by a single Schwann cell to form a monolaminate myelin sheath. Among the unmyelinated fibers there are the C fibres. This lack of myelination is the cause of their slow conduction velocity.

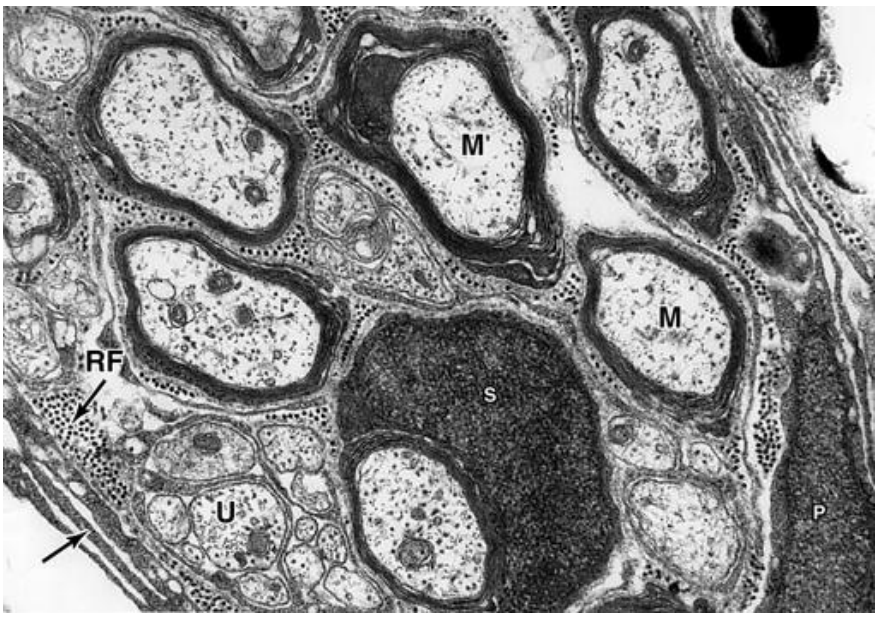


Fig.5: *Electron micrograph of a peripheral nerve containing both myelinated (M) and unmyelinated (U) nerve fibers. The reticular fibers (RF) seen in cross section belong to the endoneurium. Near the center of the figure is a Schwann cell nucleus (S). The perineurial cells (P [over a nucleus], arrows) form a barrier that controls access of materials to nerve tissue. x30,000. (Basic Histology, text and atlas. Junqueira and Carneiro, Mc-Graw Hill)*

Nerve degeneration

Wallerian degeneration (WD) is one of the most elementary reactions of the peripheral nervous system. It occurs when nerve fibre continuity is interrupted through traumatic, toxic, ischemic or metabolic events. Axonal injury triggers a cascade of events

including, for example, breakdown of the blood–nerve barrier (Mellick and Cavanagh, 1968; Olsson, 1966), proliferation of Schwann cells (Salonen et al., 1988; Siironen et al., 1994), recruitment of circulating macrophages (Perry and Brown, 1992; Taskinen and Roytta, 1997), reorganization of the endoneurial space (Roytta et al., 1987; Tona et al., 1993) and changes in the endoneurial extracellular matrix (ECM) components (Siironen et al., 1992a; Siironen et al., 1992b; Tona et al., 1993), as well as elevation of neurotrophin and cytokine production (Camara-Lemarroy et al., 2010; Ebadi et al., 1997; Fu and Gordon, 1997, Liefner et al., 2000; Terenghi, 1999). Both cellular and molecular events distal to nerve injury induce the development of a microenvironment that stimulates axonal regrowth from proximal stumps and supports axonal regeneration (Fu and Gordon, 1997; Raivich and Makwana, 2007; Webber and Zochodne, 2010).

The sequence of WD following axotomy begins with prompt degradation of axoplasm, axolemma and myelin sheath distal to the site of nerve lesion. Axonal degeneration is an active, intrinsic process of self-destruction, which, when initiated, proceeds independently of non-neuronal cells surrounding the axon (Saxena and Caroni, 2007). Impaired axonal transport probably triggers axonal degeneration (Mack et al., 2001). Next steps of axonal degeneration involve an increase in free intracellular Ca^{2+} and the activation of calpains leading to a massive decrease of microtubular and neurofilament protein levels (Coleman, 2005; Glass et al., 2002) and to axonal fragmentation within 48 h (Beirowski et al., 2005). This suggests that axonal fragmentation and the degradation of neurofilament proteins is

in progress before significant recruitment of macrophages, which finally clear the distal stump of axonal and myelin debris.

Schwann cells, having lost axonal contact, are able to isolate small whorls of myelin and generate lipid droplets (Stoll et al., 1989). Fragmentation of the myelin sheath into early ovoids before the arrival of macrophages indicates that Schwann cells can initiate degeneration of their own myelin sheaths after axotomy (Stoll and Hartung, 1992). Schwann cells carry out the first step in the myelin sheath evacuation by myelin fragmentation. Then, the degraded myelin is phagocytized by the recruited macrophages to effect full myelin clearance (Bruck, 1997; Stoll et al., 1989; Stoll and Muller, 1999).

Rapid clearance of myelin appears to be the most important precondition for axonal regeneration after peripheral nerve injury because of the presence of axon growth inhibitors in peripheral nerve myelin and myelinated Schwann cells (Barrette et al., 2008; David et al., 1995; Schafer et al., 1996; Shen et al., 1998). Nerve injury triggers infiltration and activation of immune cells not only in the distal nerve stump but also in the dorsal root ganglion (DRG) and spinal cord (Moalem and Tracey, 2006; Pineau and Lacroix, 2009; Ren and Dubner, 2010; Thacker et al., 2007). This immune cell reaction after focal nerve injury illustrates the extension of the neuroinflammatory reaction from the nerve fibres undergoing WD to other compartments of the nervous system.

Mast cells have role during inflammatory conditions. They are recruited from blood vessels accumulate and degranulate within the endoneurium of injured peripheral nerves (Monk et al., 2007; Olsson, 1968; Zuo et al., 2003). Mast cells, when activated, release pro-

inflammatory mediators, NGF, TNF-α, proteases in the sourrounding tissue. Mast cell mediators such as histamine and chemokines are released within hours of nerve injury and contribute to the recruitment of neutrophils and monocytes/macrophages, initiation of inflammatory reaction, and development of hyperalgesia (Moalem and Tracey, 2006; Zuo et al., 2003). NGF has a role in the development and neuronal survival. Its elevated levels are associated to a nociceptor sensitization and participate in the development of nociceptive pain (Costa et al., 2008; Malin et al. 2006; Pertens et al. 1999; Andreev et al. 1995; Lewin et al. 1993).

Neutrophils are essential cells to the innate immune system that are generally implicated in the pathogenesis of tissue injury and inflammatory reaction. They release a wide range of inflammatory factors (e.g., cytokines, chemokines and proteases). The recruitment of leukocytes from the circulation into injured nerve, as true of other tissues, involves rolling along the endothelium (mediated by selectins), adhesion to the surface of the vascular endothelium (mediated by integrins), and diapedesis in that they pass through the endothelium. It seems that migration of neutrophils into the injured nerve is induced by resident mast cells (Moalem and Tracey, 2006) and chemoattractants such as cytokines (Casale and Carolan, 1999). Neutrophils invade endoneurium distal to partial sciatic nerve lesion for the first 7 d and contribute to peripheral mechanisms of neuropathic pain (Perkins and Tracey, 2000).

Lymphocytes infiltrate endoneurium of traumatic injured nerve in various neuropathic pain models (Cui et al., 2000; Moalem et al., 2004). In addition, lymphocyte infiltration has been observed after

experimental neuritis to induce neuropathic pain without apparent structural damage to axons and glia (Eliav et al., 1999). A considerable number of local macrophages reside within the endoneurium of peripheral nerves (Griffin et al., 1993). Resident endoneurial macrophages of intact nerves are elongated cells with small ramifications (Griffin et al., 1993; Monaco et al., 1992).

Resident macrophages are recognized in rat nerves by ED-2 antibody, which is in contrast to haematogenous monocytes/macrophages that display immunoreactivity to ED-1 antibody (Dijkstra and Damoiseaux, 1993). As early as 2–3 d after sciatic nerve crush and before robust influx of haematogenous macrophages, resident endoneurial macrophages undergo morphological and immunophenotypic signs of activation. Cytokines and chemokines play a key role in chemotaxis of haematogenous macrophages. In addition, pro-inflammatory cytokines break the blood-nerve barrier and increase vascular permeability to promote the recruitment of immune cells into injured nerve. It is generally accepted that TNF-α and IL-1β play a crucial role in the initiation of degenerative processes after peripheral nerve injury, including in macrophage recruitment (Gillen et al., 1998; Liefner et al., 2000; Perrin et al., 2005; Shamash et al., 2002). Simultaneously to the induction of TNF-α and IL1, other cytokines and chemokines can be implicated in recruiting haematogenous macrophages during early stages of WD. For example, the upregulation of the macrophage-specific chemokine-1 (MCP-1) is detected very early after nerve injury (Taskinen and Roytta, 2000) and is essential to macrophage recruitment and myelin debris clearance (Perrin et al., 2005). In addition, neuropoietic cytokines like

leukaemia inhibitory factor (LIF) and interleukin-6 (IL-6) are also involved in macrophages recruitment (Tofaris et al., 2002). The mechanisms of early upregulation of cytokines and chemokines in Schwann cells and their involvement in macrophage recruitment are still largely unknown.Macrophage recruitment leads to rapid clearance of myelin debris, which contains axon growth inhibitors, and thereby facilitates nerve regeneration. Macrophages not only remove axon and myelin debris, but also participate in the production of mitogenic factors for Schwann cells and fibroblasts (Perry and Brown, 1992) and induce the synthesis of NGF by IL-1β (Lindholm et al., 1987) (NGF is synthesized by Schwann cells and fibroblasts).

Macrophages proliferate and express ED-1 immunoreaction, rendering them morphologically indistinguishable from haematogenous macrophages (Mueller et al., 2001). Although early studies using nerve explants in Millipore chambers into the peritoneal cavity had suggested that not resident endoneurial but haematogenous macrophages are required for myelin removal (Bonnekoh et al., 1989), experiments with bone marrow chimeric rats demonstrate a notable proportion of resident endoneurial macrophages phagocytosing myelin (Mueller et al., 2003). Haematogenous macrophages infiltrate epineurium of the lesion site at day 3 and the endoneurial space at days 5 and 7 (Taskinen and Roytta, 1997), then spread into the entire distal nerve stump and reach maximal numbers at 14 d (Mueller et al., 2003; Omura et al., 2005). Phagocytosis of degenerating myelin by macrophages is receptor dependent. Complement receptor 3 (CR3), an Fc-receptor, and a macrophage scavenger receptor are involved in the process (Bruck and Friede, 1991; da Costa et al., 1997). It is important

that Schwann cells and macrophages cooperatively achieve myelin phagocytosis, although the molecular mechanisms of myelin phagocytosis by Schwann cells and macrophages are thought to be different. The first step in myelin phagocytosis by Schwann cells is lectin-mediated (i.e., opsonin-independent), whereas that of macrophages is mainly opsonin-dependent (Hirata and Kawabuchi, 2002). Macrophage numbers and phagocytic activity peak 2 weeks after nerve injury and then decline toward normal levels (Mueller et al., 2003; Taskinen and Roytta, 1997). In contrast to crushed nerve, cut or ligation injury results in more severe and prolonged invasion of macrophages (Fry et al., 2007). Macrophages in the distal stump of injured nerve are eliminated by local apoptosis and circulation into regional lymph nodes (Kuhlmann et al., 2001). Accompanying macrophage recruitment and their involvement in clearing cell and myelin debris, the blood–nerve barrier (BNB) is broken in the entire distal nerve stump and its restoration is followed by axonal regeneration in a proximal–distal direction (Liu et al., 2008; Omura et al., 2004). Invasion of haematogenous macrophages distal to nerve injury is related to breakdown of the blood–nerve barrier; it is decreased with blood–nerve barrier restoration following axonal regeneration (Omura et al., 2004). In contrast to reports by Taskinen and Roytta (1997) and Omura et al. (2005) observed that the number of ED1-positive macrophages did not return to control levels even 56 d after nerve crush. They concluded that a type of nerve lesion with rapid initiation of axonal regeneration, like nerve crush, may result in early restoration of the blood–nerve barrier, thereby preventing systemic elimination of macrophages via circulation to the local

lymph nodes (Omura et al., 2005). Since activated macrophages secrete a wide range of cytokines and induce expression of neurotrophins, the macrophages remaining within the regenerated nerve segment for a longer time could play a role not only in promoting axonal regeneration but also in modulating regenerated axonal activity (Leskovar et al., 2000; Ley et al., 2010; Mendell et al., 1999). Schwann cells upregulate cytokines and chemokines, when they lose axonal contacts. It seems that early cytokine expression in Schwann cells can be mediated by calpain produced by injured axons (Uceyler et al., 2007) or by toll-like receptors (TLRs). TLRs are able to recognize both exogenous as well as endogenous stresses, such as infections, injury, dying cells or extracellular matrix degradation (Takeda et al., 2003). Recently, it has been reported that cell debris can induce the expression of TLRs in Schwann cells (Deng and Zhou, 2008; Lee et al., 2006), and especially TLR3, TLR4, and TLR7, thus pointing to their possible role in immune surveillance (Goethals et al., 2010). Axonal injuries strongly induce TLR1, while most other TLR expression levels remain unaffected (Goethals et al., 2010). Furthermore, Karanth et al. (2006) have demonstrated that molecules derived from nerve homogenate induce MCP-1 expression in Schwann cells and that activity of nerve homogenate is partially inhibited by TLR4 function-blocking antibodies. It was also found that macrophage recruitment/activation, myelin debris clearance, and axonal regeneration are significantly delayed in mice deficient in TLR signalling (Boivin et al., 2007).

Haematogenous macrophages invade not only the distal stump of injured nerve but also DRG (Dubovy et al., 2007; Hu and McLachlan,

2002), where they are located close to the surface of neuronal bodies. Activated macrophages secrete many cytokines which are potential mediators of hyperalgesia, such as TNF, IL-1β and IL-6 (Sommer and Kress, 2004), and are thought to be involved in inducing neuropathic pain (Austin and Moalem-Taylor, 2010; Liu et al., 2000; Moalem and Tracey, 2006, Myers et al., 1996; Thacker et al., 2007). Other experiments based on systemic inhibition or depletion however, suggest a limited role for activated macrophages in generating nerve injury-induced mechanical allodynia (Rutkowski et al., 2000). Coincident to axonal degeneration, quiescent Schwann cells proliferate and dedifferentiate. This event is critically important for promoting axonal regeneration. The dedifferentiated Schwann cells line up inside the basal lamina forming the so-called bands of Büngner (Fawcett and Keynes, 1990; Nadim et al., 1990; Pellegrino et al., 1986). Loss of axonal contact induces Schwann cell proliferation and a change in their phenotype from that of myelinating to a dedifferentiated, growth-supportive phenotype. The change of Schwann cell phenotype is associated with downregulation of the myelin-associated genes (e.g., P0, MAG) and upregulation of the regeneration-associated genes (e.g., neurotrophins and adhesion molecules) (Leblanc and Poduslo, 1990). The immature Schwann cells aligned in columns known as bands of Büngner comprise the main source of neurotrophic factors (Funakoshi et al., 1993; Gordon, 2010; Terenghi, 1999) and cell adhesion molecules (Kirsch et al., 2009; Lefcort et al., 1992; Shen et al., 2008) to stimulate and guide axonal regeneration.

The immature Schwann cells of the distal nerve stump upregulate the synthesis of NGF, BDNF and NT-4, but not NT-3 (Funakoshi et al., 1993). Neurotrophins are able to promote axonal regrowth from proximal nerve stump alone as well as in synergistic combination (Cao and Shoichet, 2003; Gordon, 2010; Terenghi, 1999). The axon growth-supportive environment progressively deteriorates when nerve repair is delayed or axons must regenerate over long distances. These situations often reduce the amount of regenerated axons (Fu and Gordon, 1995; Sulaiman and Gordon, 2000).

Microglia

Central nervous system (CNS) is constituted by nervous cells (neurons) and by non nervous cells (glia cell) with trophic function. Glia cells (protoplasmic and fibrous astrocyte, microglia and oligodendrocyte) provide support and protection for neurons and represent 70% of the cells in the central nervous system under normal conditions, and microglia represent 5-10% of glia (Watkins et al., 2001) (Fig. 6).

Microglial cells have a small soma bearing thin and branched processes under normal conditions (Watkins et al., 2001). Microglia are located in close vicinity to neurons in the grey matter and between fibre tracts in the white matter of the CNS (Lawson et al., 1990). A specialized type of microglia, the perivascular microglia, are in close contact to blood vessels (Graeber et al., 1989). Microglia share many phenotypic markers and effector molecules with hematogenous macrophages making a distinction between these two cell types in pathological settings difficult.

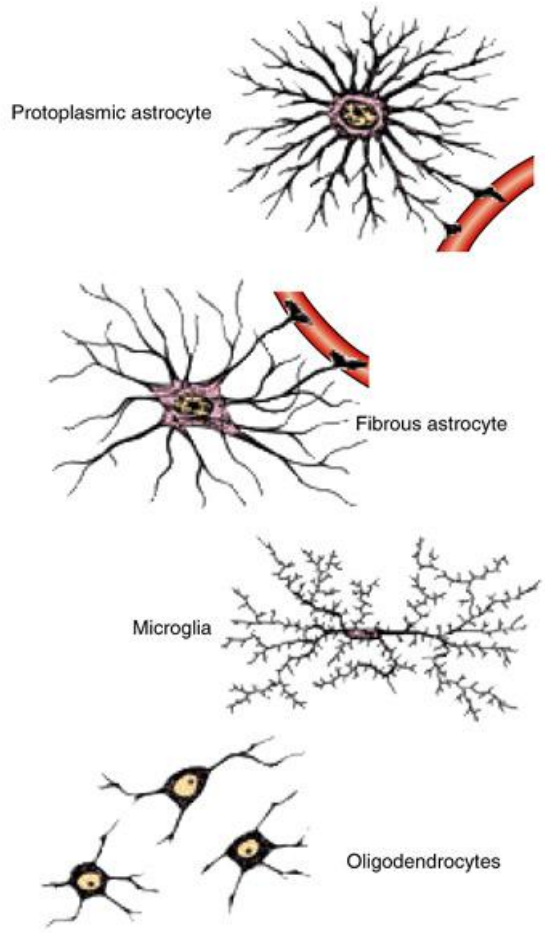


Fig. 6: *Drawings of neuroglial (protoplasmic and fibrous astrocyte, microglia and oligodendrocytes) cells as seen in slides stained by metallic impregnation. Note that only astrocytes exhibit vascular end-feet, which cover the walls of blood capillaries. (Basic Histology, text and atlas. Junqueira and Carneiro, Mc-Graw Hill)*

In some CNS lesion paradigms microglia are selectively activated, in others additional macrophages are recruited from the circulation. Del Rio Hortega (1932) was the first to describe microglia in the CNS. By silver impregnation techniques he characterized a unique cell type in the CNS with an elongated soma bearing processes extending from

both poles of the cell, the ramified microglia. The existence of ramified microglia was further confirmed by electron microscopy in the 1960 s (Fig.7). Nowadays microglia can unequivocally be distinguished from other resident, nonneuronal cells in the CNS by immunocytochemistry. Resting microglia constitutively express the complement type-3 receptor (CR3; CD11b/ CD18 complex) which is further upregulated upon activation, and galactose-containing glycoconjugates that bind isolectin B4 from *Griffonia simplicifolia* seeds (Streit and Kreutzberg, 1987). However, both CR3 and the lectin binding site are also present on the surface of hematogenous macrophages. Resting microglia express a keratan sulfate proteoglycan (KSPG) that is not present on macrophages in other tissues (Bertolotto et al., 1993; Jander and Stoll, 1996a) However, KSPG is expressed only on a subpopulation of microglia and underlies modification during immune-mediated processes in the CNS (Jander and Stoll, 1996a) Thus, it cannot be used as a distinctive and reliable marker for microglia in all CNS lesion paradigms. In the normal CNS of different rat strains the activation status of microglia already differs fundamentally (Sedgwick et al., 1993; Jander and Stoll., 1996b): high microglial KSPG expression in Lewis and Fischer 344 rats, but low expression in Brown Norway and Dark August rats (Jander and Stoll, 1996b). Interestingly, constitutive major histocompatibility complex (MHC) class II expression was inversely related to KSPG expression. These findings suggest a significant impact of genetic factors on the molecular di€erentiation of resident microglia in the normal CNS.

The origin of ramified microglia has been a longstanding controversial issue (reviewed in Ling and Wong, 1993; Fedoroff, 1995). Bone

marrow-derived cells, that is mesodermal elements or alternatively, neuroectodermal matrix cells which also serve as precursor cells for astrocytes and oligodendrocytes, have been suggested as developmental sources of microglia. The observation that the decline of blood-derived amoeboid cells in the CNS during the first postnatal weeks was accompanied by a dramatic increase in the number of ramified microglia was suggestive for a transition of amoeboid cells into resident ramified microglia (Ling and Wong, 1993). However, transitional forms between brain macrophages and resting microglia could not be detected in the developing brain in mice receiving bone marrow transplants from transgenic mice and only 10% of parenchymal microglia displayed the transgenic signal (De Groot et al., 1992). In adult animals attempts to directly demonstrate the replacement of ramified parenchymal microglia from bone-marrow-derived precursors so far yielded inconclusive results. It turned out that ramified microglia in the adult CNS are an extremely sessile cell population exhibiting virtually no turnover from circulating monocytic precursor cells (Hickey et al., 1992; Lassmann et al., 1993). Based on their findings that astroglial cultures initiated from newborn mouse neopallium contained bipotential progenitor cells, that could give rise to both astrocytes and microglia, Fedoroff and colleagues put forward the idea that parenchymal microglia are of neuroectodermal origin as all other glia (Richardson et al., 1993; Fedoroff et al., 1997). This is in accordance with findings of De Groot et al. (1992) showing that the majority of microglia lacked the transgenic signal after bone marrow transplantation. Despite the uncertainty about their origin, microglia share most surface molecules with bone-marrow derived macrophages

(Flaris et al., 1993). A subset of microglia, the perivascular microglia, which have also been termed perivascular cells, can be distinguished by their unique association with vessels and by immunocytochemistry using monoclonal antibody ED2 in the rat (Graeber et al., 1989; Graeber and Streit, 1990). By use of chimeric rats Hickey and Kimura (Hickey and Kimura, 1988; Hickey et al., 1992) demonstrated that this population of perivascular microglia is regularly replaced from the bone marrow. Perivascular microglia constitutively express immune activation markers such as CD4 and MHC class II molecules.

Microglia and neuropathic pain

The most characteristic feature of microglia is their rapid activation in the CNS in response to pathological events, including trauma, ischemia, inflammation, hypoxia, neurodegeneration and viral or bacterial infection. Activated microglia have dual regulatory functions in the maintenance and facilitation of tissue homeostasis in the CNS. They remove dead cells or dangerous debris by releasing toxic factors and phagocytosis, but they also repair injured cells by releasing neurotrophic factors (Franke et al., 1999; Nakajima and Kohsaka, 1993; Okimura et al., 1996; Quattrini et al., 1996). After activation, microglia cells change morphology from a resting, ramified shape into an active, amoeboid shape (Kreutzberg, 1996; Nakajima and Kohsaka, 2001; Perry, 1994; Stoll and Jander, 1999) (Fig.7).

In contrast to neuronal processes, the phenomenon of microglial cell activation is multidirectional, and these cells dynamically modulate neuronal function under both normal and pathological conditions.

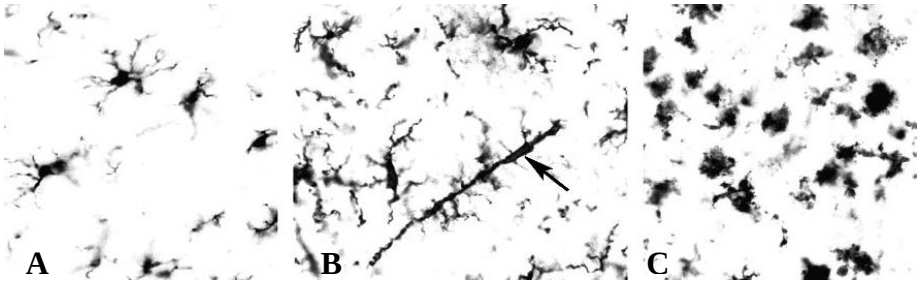


Fig. 7.*: Microglial activation in tissue sections from human brain. Resting microgli a in normal brain (A) extend thin processes. Activated microglia in diseased cerebral cortex (B) have thicker processes, and some may have a rodlike shape (arrow). In regions of frank pathology (C), microglia transform into phagocytic macrophages, which can also develop from monocytes that enter the brain from the circulation. Scale bar = 40 um. (From Hof P, Trapp BD, de Vellis J, et al: The cellular components of nervous tissue. In Zigmond M, Bloom FE, Landis SC, et al. Neurological Surgerg\eds]: Fundamental Neuroscience. San Diego, Calif, Academic Press, 1999, pp 41-69.).*

Clinical neuropathic pain syndrome can develop as a result of damage to nerves due to tumors, diabetic neuropathy, herpes zoster, complex regional pain syndrome, AIDS, sclerosis multiplex, hypoxia, or stroke (Coyle, 1998; McMahon, 2001). The role of glia in the cellular mechanisms underlying the symptoms of neuropathic pain, such as hyperalgesia or allodynia, is not clear (Fu et al., 2000; Weihe et al., 1991; Woolf and Mannion, 1999). Microglial cells secrete a large variety of substances, including growth factors, cytokines, complement components, lipid mediators, extracellular matrix components, enzymes, free radicals, neurotoxins, nitric oxide and prostaglandins (Minghetti and Levi, 1998). Indeed, it seems that activation of glia in the CNS is a driving force behind pain (Coyle, 1998; Fu et al., 2000; Hanish and Guerra, 2002; Kreutzberg, 1996; Nakajima and Kohsaka, 2001; Stoll and Jander, 1999; Watkins et al., 2001). Some proinflammatory cytokines derived from microglia are

already known to be common mediators of allodynia and hyperalgesia (Boddeke, 2001; Colburn et al., 1999; Coyle, 1998; DeLeo et al., 1987; Raghavendra et al., 2004; Watkins at la., 2001; Watkins et al., 2003). Glial activation enhances neuronal nociceptive transmission, but the mechanism of this phenomenon is poorly understood. Production of various immune factors, including cytokines interleukin (IL)-1α, IL-1β, IL-10, IL-6 and tumor necrosis factor (TNF)-α as well as complement components C1q, and C5a, is believed to play an important role in nervous system inflammation and may lead to abnormal processing of pain signals (Colburn et al., 1999; Coyle, 1998; DeLeo et al., 1987; Mika et al 2008; Watkins at la., 2001).

Palmitoylethanolamide (PEA)

Palmitoylethanolamide is an endogenous fatty acid amide and it belongs to the family of the N-acyl ethanolamines (NAEs). It is an analogue of *N*-arachidonoyl-ethanolamine (anandamide, AEA).

Palmitoyletahnolamide is produced on demand within the lipid bilayer (Cadas et al, 1996) and it is involved in endogenous protective mechanisms. In mammalian tissue PEA is synthesized by two independent biochemical reactions. The first is the transfer of a fatty acid from membrane-bound phospholipids to phosphatidylethanolamine (PE), catalyzed by a calcium ion and cyclic-AMP regulated *N*-acyltransferase (NAT), to form the fatty acid ethanolamides (FAE) precursor *N*-acyl phosphatidylethanolamine (NAPE). Different FAE precursors are generated according to which fatty acid is initially transferred to PE (i.e., the initial transfer of palmitic acid will yield a PEA precursor, while that of arachidonic

acid will yield an anandamide precursor). The second step in FAE synthesis is the cleavage of membrane-bound NAPE to release free PEA, which is mediated by a NAPE-specific phospholipase D (PLD). This lipid hydrolase shares little sequence homology to other members of the PLD family and recognizes multiple NAPE species, producing PEA along with other FAEs (Okamoto et al., 2004).

Alternatively, a separate mechanism of synthesis has been proposed involving a similar two-step reaction: (1) the hydrolysis of NAPE to *N*-palmitoyl-lysoPE (lyso-NAPE) by soluble phospholipase A_2 ($sPLA_2$) and (2) the subsequent cleavage of lyso-NAPE by a lysophospholipase D (lyso-PLD) (Natarajan et al., 1984). The activities of these two enzymes are highest in the stomach, brain, and testis (Sun et al., 2004). The relative contribution of each of these synthetic pathways is unknown at present (Fig.8).

Palmitoylethanolamide inactivation occurs through an intracellular hydrolysis by fatty acid amide hydrolase (FAAH) (Schmid et al, 1985). Mice lacking by *faah* gene have dramatically reduced PEA hydrolysis and increased PEA levels in brain and liver, where FAAH is particularly abundant. In contrast with FAAH, the PEA-preferring acid amidase (PAA) is particularly abundant in the rodent intestine, spleen and lung. PAA plays a broad role in the deactivation of all FAEs in intact cells, but in presence of detergent, it prefers PEA substrate (Ueda et al., 2001).

Palmitoylethanolamide has an anti-inflammatory activity: the administration of palmitoylethanolamide prevents the formation of oedema in a murine model of acute inflammation (Conti et al 2002; Costa et al 2002).

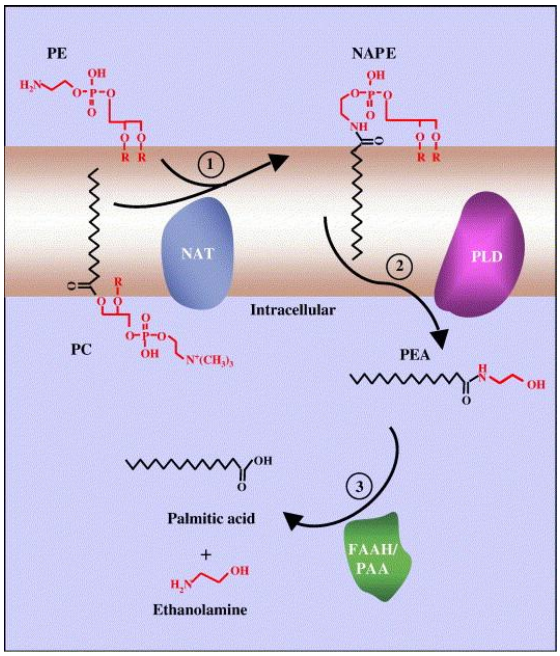


Fig.8*: *PEA biosynthesis and inactivation.
The biosynthesis of PEA occurs within tha lipid bilayer and through two different and independent steps. (1 and 2).
PEA is hydrolyzed by fatty acid amide amidohydrolase (FAAH) or PEA-preferring acid amidase (PAA) to form palmitic acid and ethanolamine (3).
(Lo Verme et al, 2005).

Palmitoylethanolamide also attenuates pain behaviour in a model of acute pain (Calignano et al 1998, Conti et al 2002), inhibition of food intake (Rodriguez et al, 2001), reduction of gastrointestinal motility (Capasso et al, 2001), inhibition of cancer cell proliferation (De Petrocellis et al, 2002), and neuroprotection (Franklin et al, 2003; Lambert et al, 2001; Sheerin et al, 2004). In the mid- 1990's reaserchers demonstrated that PEA also inhibits mast cell degranulation (Aloe et al., 1993; Mazzari et al., 1996) and in the late 1990's pulmary inflammation in mice (Berdyshev et al., 1998). Petrosino and colleagues demonstrated that in neuropathic rats there was a decrease of palmitoylethanolamide levels in three brain areas involved in nociception (dorsal raphe (DR), periaqueductal grey (PAG) and rostral ventral medulla (RVM)), as well in the spinal cord (Petrosino et al., 2007). Recently, it was demonstrated the therapeutic

efficacy of exogenous palmitoylethanolamide: the repeated treatment with PEA was able to abolish hyperalgesia and to attenuate allodynia in a murine model of mononeuropathy, the chronic constriction injury (Costa et al 2008).

Despite its potential clinical significance, the mechanism of action of PEA remains to understand. There are at least three hypothesis concerning PEA mechanism of action: the ALIA, the receptor and entourage hypothesis.

Mechanism of action of PEA

ALIA hypothesis

This hypothesis was proposed by Aloe and colleagues (1993), who introduced the ALIA acronym (Autacoid Local Inflammation Antagonism). This acronym indicated the local antagonism on inlaflammation of some endogenous N-acylethanolamines, like PEA. This effect was attributed to the control of mast cell activity (Jack, 1996), over this year Mazzari and colleagues demonstrated that the *in vivo* anti-inflammatory effects of PEA (10 mg/kg) were due to down-regulation of mast cell degranulation. Similar results were obtained in cats and dogs. Densitometric analisys performed on skin biopsies from cats with eosinophilic granuloma complex and treated with 10 mg/kg bw of palmidrol, revealed an increase in the granular density of cutaneous mast-cells, suggesting a decrease in mast-cell degranulation (Scarampella et al., 2001). Similar techniques were used to study mast cell activity after a local exposition with PEA analogues. Experimentally induced wounds were treated with a gel formulation containing the fatty acid amide adelmidrol (N,N0-bis-[2-

hydroxyethyl] nonandiamide]. This topical application produced a significant increase in the densitometric measurement for mast-cells in cutaneous wounds (Abramo et al 2004).

The ALIA mechanism is based on the crucial role exerted by mast cell in inflammation and even more on the protective functions activated in the body during disease. Under normal physiological conditions, mast cells are preferentially localised around nerves and blood vessels in tissues interfacing with the external environment (Maurer et al., 2003), where they perform critical protective and homeostatic functions (Boyce, 2003). Mast cell can respond to various stimuli (infectious and chemical). This facilitates its function in immunity and the response of tissue injury, realising pro-inflammatory mediators (e.g., cytokines, vasoactive amines), mediators of hyperalgesia (e.g., proteolytic enzymes, bradykinins, neuropeptides) and itch mediators (e.g., histamine and serotonin) (Boyce, 2003). PEA exerts the capability to naturally control mast cell hyperactivity, which occurs not only in inflammation, but also in inflammatory hyperalgesia (Levi- Montalcini et al., 1995) and neuropathic hyperalgesia (Theodosiou et al., 1999; Zuo et al., 2003).

Receptor hypothesis

The hypothesis of a receptor-mediated mode of action is compatible and may possibly even be synergistic with the ALIA hypothesis. The ‘‘CB2-like receptor’’ hypothesis suggests that pharmacological effects of PEA may be the result of direct or indirect stimulation of CB2 receptors (or of a yet uncharacterised CB2-like receptor). The CB-2 is a G protein-coupled receptor and it is mainly expressed in the immune cells and hematopoietic cells (Malan et al., 2002; Sokal et al., 2003)

and perhaps also by primary sensitive neurons in the dorsal horn of the spinal cord (Hohmann, 2002; Nackley et al., 2003).

This hypothesis is supported by the results obtained from several *in vivo* studies performed using different CB receptor agonist. The synthetic highly CB2 selective agonists AM 1241 and HU-308 decreased experimentally-induced oedema (Hanus et al., 1999; Malan et al., 2002; Quartilho et al., 2003). In experimental models of nociceptive pain, local or systemic administration of AM 1241, suppressed the development of thermal and mechanical hyperalgesia induced by carrageenan (Malan et al., 2002; Nackley et al., 2003; Quartilho et al., 2003) and by capsaicin (Quartilho et al., 2003), as well as the allodynia (a condition characterised by pain from stimuli which are not normally painful) produced by carrageenan (Nackley et al., 2003). In the case of neurophatic allodynia (condition in which opioids are effective only at dosages higher than those effective against thermal hyperalgesia), AM 1241 is effective at the same dose that is effective for the suppression of hyperalgesia (Malan et al., 2002). The effect is dose-dependent and CB1 receptor independent. Topical pre-treatment with HU210, a synthetic nonselective agonist of CB1 and CB2 receptors, significantly reduced pain perception and the consequent primary heat hyperalgesia after cutaneous administration of capsaicin (Rukwied et al., 2003) and also reduced the histamine-induced itching perception.

The hypothesis that PEA may act via a direct or indirect interaction with CB2 receptors is strengthened by the results obtained from different *in vivo* studies performed using a selective CB2 receptor antagonist. In fact, the administration of SR144528, a CB2 specific

receptor antagonist, eliminated the antinociceptive effects of PEA (Calignano et al., 1998, 2001; Conti et al., 2002; Farquhar-Smith and Rice, 2003). In experimental models of visceral pain, the analgesic properties of PEA are suppressed by the administration of SR144528 (Farquhar-Smith et al., 2002). Moreover, the administration of SR144528 completely blocked the inhibitory effects of PEA on carrageenan induced oedema (Conti et al., 2002). Thus the ‘‘CB2-like receptor’’ hypothesis is based on a plethora of data supporting the existence of a possible peripheral mode of action of PEA (Malan et al., 2001; Nackley et al., 2003; Quartilho et al., 2003) and involving the activation of CB2 receptors. CB2 (or CB2-like) receptor activation inhibits the release of substances involved in inflammation and stimulation of afferent nerve fibres (Sokal et al., 2003).

Entourage effect

This hypothesis was suggested in order to explain some discrepancies between data obtained *in vitro* and those obtained *in vivo* on PEA action. This hypothesis suggests that PEA is an enhancer of other endogenous compound activity, through an increase in their affinity for receptors or via inhibition of their metabolic degradation (Ben-Shabat et al., 1998; Mechoulam et al., 1998; Lambert and Di Marzo, 1999; Smart et al., 2002). Among these endogenous compounds PEA can enhance anandamide (AEA) activity and it is particularly considered because of its powerful anti-inflammatory and nociceptive effects.

Anandamide is an endogenous compound acting as a natural ligand of CB1, a G protein-coupled receptor mainly expressed in the central nervous system (brain) (Devane et al., 1992). Anandamide, locally

synthesized "on demand" by neurons in the brain (Steffens et al., 2003) increases after tissue damage in macrophages, central and peripheral neurons (Pestonjamasp and Burstein, 1998; Liu et al., 2003; Walker et al., 1999; Ahluwalia et al., 2003a). When administered peripherally, at very low dosages (0.01 ng/rat), AEA is able to inhibit carrageenan-induced oedema (Richardson et al., 1998). AEA reduces formalin-induced pain and carrageenan-induced thermal hyperalgesia via CB1 receptor activation (Calignano et al., 1998; Richardson et al., 1998) or through stimulation of CB2 receptors expressed in spinal neurons (Sokal et al., 2003). Anandamide is also effective in the same models of visceral pain in which PEA antinociceptive effects have been observed, but at a dosage (25 mg/kg) higher than that required for PEA (2.5 mg/kg). These effects appear to be mediated via CB1 receptor stimulation (Farquhar- Smith et al., 2002). Besides acting as an endocannabinoid, AEA also acts as an endovanilloid. It has been recently demonstrated that AEA also interacts, in several tissues, with a capsaicin receptor, named vanilloid receptor type 1 (VR1) or transient receptor potential vanilloid 1 channel (TRPV1) (Di Marzo et al., 2002; Ross, 2003; van der Stelt and Di Marzo, 2004). TRPV1 expression is largely associated with small diameter primary afferent fibres and it is expressed on bladder (Birder et al., 2001), kidney (Sanchez et al., 2001), spleen (Sanchez et al., 2001), heart (McIntyre et al., 2001), stomach (Nozawa et al., 2001), and mast cells (Birò et al., 1998). This receptor, unlike CB1, is involved in transducing thermal and inflammatory pain. The emerging antagonism of its actions, endocannabinoid–endovanilloid, does not facilitate the pharmacological classification of AEA. By acting at the TRPV1

receptor, AEA induces the antidromic release of neuropeptides closely involved in neurogenic inflammation, like substance P (SP) and calcitonin gene-related peptide (CGRP), and consequently exerts pro-inflammatory effects (Ahluwalia et al., 2003b; Di Marzo et al., 2002; Maccarrone et al., 2002; Ralevic, 2003). However, AEA, like other TRPV1 agonists, can also desensitise this channel, leading to paradoxical analgesic effects. Furthermore, it is well known that the activation of TRPV1 reduces itching both in humans (Lysy et al., 2003; Weisshaar et al., 2003) and in dogs (Marsella et al., 2002). Therefore, the ability of PEA to strengthen the TRPV1-mediated effects of AEA, probably by increasing the affinity of AEA and other endovanilloids for their receptor, may be considered as a possible strategy in the treatment of pruritus and pain (De Petrocellis et al., 2001).

In conclusion, the ‘‘entourage hypothesis’’ proposes that the anti-inflammatory and antinociceptive effects of PEA are in part due to the enhancement of the endocannabinoid and/or endovanilliod actions exerted by AEA or other endogenous related compounds.

Neuropathic pain

Chronic pain falls into three broad categories: pain owing to the tissue disease or damage (nociceptive pain such as osteoarthritis), pain caused by somatosensory system disease or damage (neuropathic pain), and the coexistence of nociceptive and neuropathic pain (mixed pain) (Klusakova and Dubovy, 2009). The neuropathic pain is debilitating and it develops as a result of lesion or inflammation of the nervous system. Neuropathy is afflicting millions of people worldwide

and is becoming one of the most significant health problems (Burgoyne, 2007), because it is refractory to common analgesics.

There are three categories of neuropathies: painful peripheral neuropathies, polyneuropathies and central pain syndromes. The first category includes postsurgical pain syndromes (e.g. phantom pain, stump pain, postmastectomy and postthoracotomy syndrome). Polyneuropathies includes metabolic and nutritional disorders (diabetes), drug and toxin related syndromes (neuropathy by antiretroviral agents), hereditary disease and immune disease (HIV and herpes zoster infection). The last category concerns multiple sclerosis, traumatic brain and spinal cord injury (Basic-Kes et al., 2009).

Pain is often described clinically as either spontaneous or evoked pain. An alternative terminology is that of stimulus-independent (spontaneous) or stimulus-dependent (evoked) pain (Herr, 2004). Spontaneous pain can be continuous or intermittent and it varies typically its intensity over the time.

Spontaneous continuous pain is present all of the time, while spontaneous intermittent pain is episodic (paroxysmal) and it is often of short duration. It can have different qualities, such as “shooting,” “stabbing,” “burning,” “throbbing,” or “electricshock–like” (Dworkin, 2002).

Evoked pain is typically of two types: allodynia and hyperalgesia and they are common features of peripheral nerve injury or damage (Basic-Kes et al 2009). Allodynia is a term used to describe pain in response to a non-nociceptive stimulus. There are two different kinds of allodynia: mechanical and thermal allodynia. Mechanical allodynia

may further categorized as dynamic and static; the first one occurs in response to brushing, whereas the second one is caused by a light touch or pressure (Dworkin, 2002). Thermal allodynia (hot or cold) occurs when mild temperatures are applied on damaged area (Dworkin, 2002).

Hyperalgesia is a term used to describe an increased sensitivity to a nociceptive stimulus and it can be divided in according to the nature of the stimulus: mechanical, thermal, or chemical (Herr, 2004). Hyperalgesia can be divided in two subtypes: primary hyperalgesia occurs immediately within the area of the injury, and secondary hyperalgesia occurs in the undamaged area surrounding the injury (Basic-Kes et al 2009).

Many patients can have also abnormal sensations, such as dysaesthesias and paraesthesias: the first one is an unpleasant abnormal sensation, whereas the second one is an abnormal sensation but not necessarily unpleasant. These abnormal sensations can be spontaneous or evoked. Dysaesthesias / paraesthesias include feelings of itching, crawling, numbness, tingling, and pins-and-needles sensations.

TABLE 2.
A Selection of Pain Terms Plus Definitions from the Published IASP List of Pain Terms

Pain Term	Definition
Pain	An unpleasant sensory and emotional experience associated with actual or potential tissue damage
Allodynia	Pain due to a stimulus that does not normally provoke pain
Causalgia	A syndrome of sustained burning pain, allodynia, and hyperpathia after a traumatic nerve lesion
Central pain	Pain initiated or caused by a primary lesion or dysfunction in the central nervous system
Dysesthesia	An unpleasant abnormal sensation, whether spontaneous or evoked
Hyperalgesia	An increased response to a stimulus that is normally painful
Hyperesthesia	Increased sensitivity to stimulation, excluding the special senses
Hyperpathia	A painful syndrome characterized by an abnormally painful reaction to a stimulus, especially a repetitive stimulus, as well as an increased threshold
Neuralgia	Pain in the distribution of a nerve or nerves
Neuropathic pain	Pain initiated or caused by a primary lesion or dysfunction in the nervous system
Neuropathy	A disturbance or pathologic change in a nerve; in one nerve, mononeuropathy; in several nerves, monooneuropathy multiplex; if diffuse and bilateral, polyneuropathy
Nociceptor	A receptor preferentially sensitive to a noxious stimulus or to a stimulus that would become noxious if prolonged
Noxious stimulus	A noxious stimulus is one that is damaging to normal tissues
Paresthesia	An abnormal sensation, whether spontaneous or evoked
Peripheral neuropathic pain	Pain initiated or caused by a primary lesion or dysfunction in the peripheral nervous system

Note: IASP = International Association for the Study of Pain. (Adapted from Chong, M. S., & Bajwa, Z. H. (2003). Diagnosis and treatment of neuropathic pain. *Journal of Pain and Symptom Management, 25*, S4-S11 (used with permission).

Tab.1: list of commonly used IASP pain terms and their definition (Herr, 2004)

Nerve compression: the chronic constriction injury

Chronic constriction injury (CCI) of peripheral nerve by ligatures is one of the most commonly used models with characteristic signs and symptoms found in humans. Bennett and Xie reported a rat model of painful peripheral mononeuropathy in 1988: the model loosely ties the sciatic nerve (left or right side) with four chromic gut ligatures at the mid-thigh level (Bennett and Xie, 1988). In mice this model is slightly modified: briefly chronic constriction injury in the sciatic nerve is caused by three tight chromic gut ligatures at the mid-thigh level (Costa et al 2008).

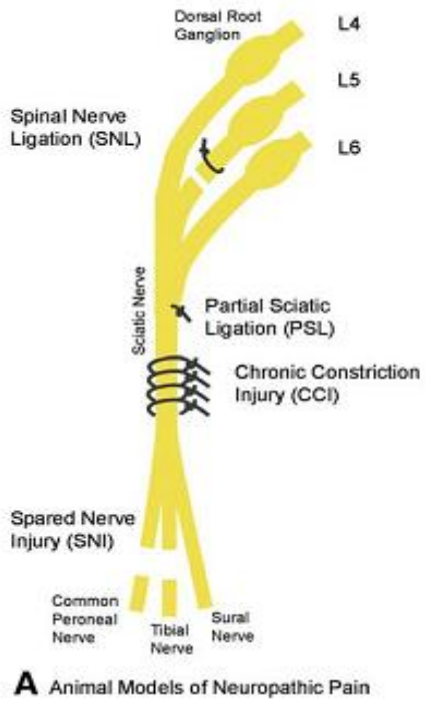


***Fig. 9**: Animal models of neuropathic pain. The chronic constriction injury in rat was induced by four loose ligatures at the mid-thigh level. The afferent fibers of sciatic nerves enter in the dorsal horn of the lumbar (L4-L5-L6) spinal cord (Campbell and Meyer, 2006, Neuron, 52, p77)*

The chronic constriction injury is a model for study neuropathic pain induced by traumatic nerve injury and related neuroinflammatory reaction in consequence of Wallerian degeneration (Bennett and Xie, 1988; Costa et al 2005). It results in the development of hyperalgesia to thermal stimulus, as indicated and measured by reduced paw withdrawal latency to heat source. Allodynia to mechanical stimuli also occurs (Comelli et al., 2008; Bettoni et al., 2008 and Costa et al., 2008). Initially, CCI of peripheral nerve, like other models of nerve compression, leads to epineurial oedema and nerve strangulation. Mechanical injury of the capillary endothelial cells and subsequent endoneurial oedema increase peripheral nerve compression

(Oaklander et al., 1987). Nerve fibres of the nerve profile's peripheral area, however, are more affected by compression than those in the central regions (Mackinnon et al., 1985). Alteration in the fibre spectrum is reported in the nerve segment distal to the CCI. Most large and heavily myelinated axons are significantly decreased by Wallerian Degeneration, while alterations in the number of small, thinly myelinated and unmyelinated axons are substantially more variable in the sciatic nerve after CCI (Basbaum et al., 1991; Coggeshall et al., 1993; Gautron et al., 1990; Munger et al., 1992 and Nuytten et al., 1992). Therefore, a large increase in the number of small, thinly myelinated axons and many unmyelinated axons are observed distal to the CCI (Guilbaud et al., 1993 and Sommer et al., 1993). Large and heavily myelinated axons undergoing Wallerian Degeneration are a robust source of molecular mediators (cytokines such as TNF-α, IL-1 α, IL-1β and IL-6) that are able to induce spontaneous activity of spared afferents and behavioural signs of neuropathic pain (Wu et al., 2001).

Different evidence indicates that the principal pathogenic mechanisms responsible for induction of neuropathic pain by CCI of peripheral nerve are associated with oedema, ischemia, macrophage activation, endoneurial ECM remodelling, cytokine and chemokine upregulation, and other manifestations of neuroinflammation (Sommer and Myers, 1995 and Uceyler and Sommer, 2006).

Diabetic neuropathic pain model

Streptozotocin is a nitrosurea derivative isolated from *Streptomyces Achromogenes* with broad-spectrum antibiotic and anti-neoplastic activity (Bono, 1976). It is a powerful alkylating agent that has been

shown to interfere with glucose transport (Wang and Gleichmann, 1998) glucokinase function (Zahner and Malaisse, 1990) and induce multiple DNA strand breaks (Bolzan and Bianchi, 2002). A single large dose of streptozotocin can produce diabetes in rodents and is used to study diabetic neuropathy (Comelli et al., 2009; Comelli et al., 2010) Alternatively, multiple small doses of streptozotocin are used (e.g. 50 mg/kg on five consecutive days) (Wodarski et al., 2009). A single high dose or multiple low doses of streptozotocin induce insulinopenic diabetes in which immune destruction plays a role, as in human Type 1 diabetes.

In Type 1 diabetes the deterioration of both glucose transport and metabolism (Wang and Gleichmann, 1998) causes a decrease of insulin production, which is associated to an increase of blood glucose level (hyperglycaemia). Hyperglycaemia is the primary pathophysiological mechanism, and it has vbeen associated with development of the secondary complications of diabetes. One of the most frequent secondary complications is diabetic polyneuropathy (DPN), which affects 50% of diabetic patients (Sima, 2003; Vincent and Feldman, 2004). Sensory, motor, and autonomic neurons can all be affected by hyperglycemia, typically starting in the lower limbs and developing from distal to proximal (Brown and Asbury, 1984; Leinninger et al, 2004). Like the human clinical condition, the induced models of diabetic neuropathy demonstrate reduced nerve blood flow (Kumar et al, 2005), nerve conduction slowing (Kumar et al 2005; Hasegawa et al 2006), accumulation of polyol pathway metabolites (Stewart et al 1967) reduced caliber of nerve fibers (Kennedy and Zochodne 2005), altered pain thresholds (Calcutt et al 2004).

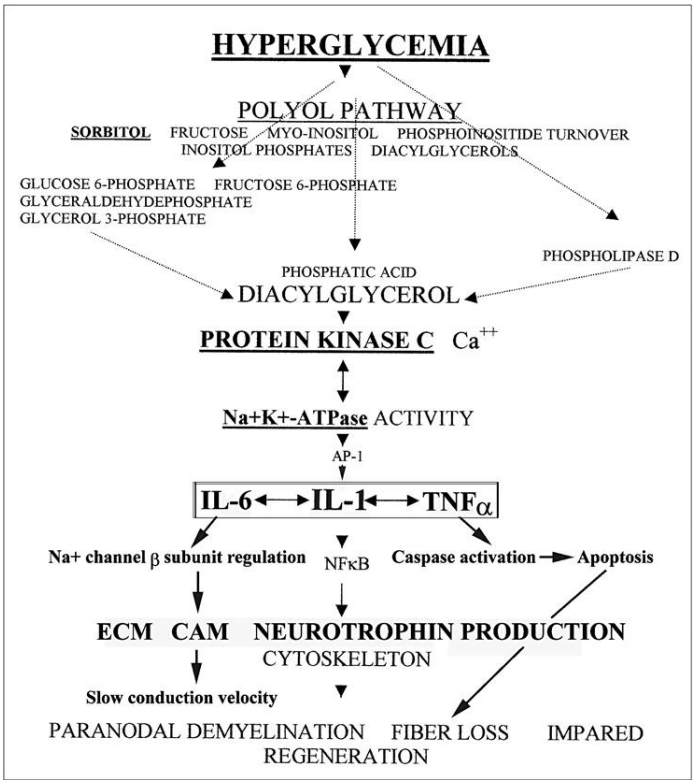


Fig. 10: Hyperglycemia leads to accelerated phosphoinositide turnover, activation of phospholipase D and changes of diacilglycerol (DAG) levels .Activated phospholipase D and DAG induce changes in the activation of protein kinase C (PKC) and expression of its isoforms in peripheral nerve. Changes in PKC activation and/or PKC isoform expression can modulate the activity of Na+, K+-ATPase. The Na+,K+-ATPase pump actively regulates intracellular concentrations of these two cations important for the function of cellular enzymes and transcription factors, such as activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB). Transcription factors AP-1 and NF-κB are involved in the initiation of the transcription of numerous genes, including proinflammatory and neuropoietic cytokines (IL-1, IL-6, TNF-α). Lack of neurotrophic support by nerve growth factor (NGF), neurotropin-3 (NT-3), brain-derived nerve factor (BDNF), insulin-like growth factor-I (IGF-I), and vascular endothelial growth factor (VEGF) is implicated in apoptosis of dorsal root ganglia sensory neurons and axonal degeneration in diabetes. In peripheral nerve, cytokines regulate functions essential for Schwann cell-axonal homeostasis and nerve regeneration, such as production of extracellular matrix (ECM) of cellular adhesion molecules (CAMs), and organization of the cytoskeleton causing degenerative changes such as fiber loss and perinodal demyelination. (Skundrik and Lisak, 2003)

Pharmacotherapy

Topical Agents

Topical Lidocaine: Several clinical trials showed efficacy and excellent tolerability of the 5% lidocain patch in patients with postherpetic neuralgia (PHN) and allodynia and in patients with allodynia caused by different types of neuropathic pain (Dworkin et al 2007; Finnerup et al 2005). The side effects of these patches are mild and their application can cause local reactions. An advantange of their use is the lack of systemic absorption, for this reason these patches can be use in older patients or in patients complex neuropathic pain. In commerce there is also the lidocaine gel (5%), which is less expensive than the patch ones. It is used showed efficacy in patients with PHN and llodynia. (Dworkin et al 2007; Finnerup et al 2005).

Topical Capsaicin: Capsaicin patch has been studied at high concentration and it was applied at patients with and painful HIV neuropathy (European Medicines Agency). Results of 2 phase 3 trials in PHN showed that a single application of the high concentration patch was more efficacious than a low concentration one. The first one reduced pain from the second week after the capsaicin application (Backonja, 2008; Qutenza http://www neurogesx.com/ngx). Similarly one clinical trial in patients with painful HIV neuropathy showed significantly decreased pain (Simpson et al., 2008); however, in a second HIV neuropathy trial, the effects of high concentration patches didn't decrease pain. (European Medicines Agency)

Application of the high-concentration capsaicin patch in patients with PHN or painful HIV neuropathy was safe and well tolerated, and adverse events were limited to transient increases in pain associated

with patch application and application site reactions (eg, erythema) (Simpson et al 2008). However, the long-term benefts of this treatment are unknown, and the safety of repeated applications of high-concentration capsaicin must be carefully evaluated because skin biopsy studies have shown transient epidermal denervation by capsaicin (Polydefkis et al., 2004) that is paralleled by a functional loss, particularly of heat pain sensation (Magerl, 2001).

Antidepressants and selective serotonin norepinephrine reuptake inhibitors (SSNRIs)

Tricyclic antidepressants (TCA), because of their efficacy, are often used during the treatment of neuropathic pain. (Finnerup et al 2005). TCAs. The side effects are dry mouth, orthostatic hypotension, constipation, and urinary retention. These effects can be reduced by starting with low dosages administered at bedtime and with slow titration to higher dosages and also by using a secondary amine TCA (nortriptyline or desipramine) (Dworkin et al 2007: Finnerup et al 2005). TCAs can give cardiac toxicity, in fact the NeuPSIG guidelines recommend prescribing TCAs with caution in patients with ischemic cardiac disease or ventricular conduction abnor-malities, limiting the dosages to less than 100 mg/d when possible, and obtaining a screening electrocardiogram for patients older than 40 years. It can take 6 to 8 weeks, including 2 weeks at the highest dosage tolerated, for an adequate trial of treatment with a TCA.

Duloxetine and venlafaxine are selective serotonin norepinephrine reuptake inhibitors (SSNRIs) that have been studied in peripheral neuropathic pain (a third SSNRI, milnacipran, has been studied only in fbromyalgia). Duloxetine has shown consistent efficacy in painful

diabetic polyneuropathy (DPN), (Dworkin et al., 2007) with effectiveness sustained for 1 year in an open-label trial. (Raskin et al., 2006). Unfortunately, duloxetine has not been studied in other types of neuropathic pain, and so its effcacy in such conditions is unknown. The most common adverse effect of duloxetine is nausea, which seems to be reduced by administering 30 mg once daily for 1 week before increasing to 60 mg once daily. Duloxetine does not seem to produce clinically important electrocardiographic or blood pressure changes (Wernicke et al., 2007)

Venlafaxine has shown efficacy in painful DPN and painful polyneuropathies of different origins but not in PHN. Typically, 2 to 4 weeks are required to titrate to an effcacious dosage (ie, 150-225 mg/d); venlafaxine is available in short- and long-acting preparations. Cardiac conduction abnormalities have been reported in a small number of patients, (Rowbotham et al., 2005) and blood pressure increases can occur; therefore, venlafaxine should be prescribed with caution in patients with cardiac disease.

Anticonvulsivants

Anticonvulsivants are often regarded as first line drugs for neuropathic pain. The most common anticonvultivants are: gabapetin, pregabalin, lamotrigine, and carbazepine. The most studied drug is gabapentin, in clinical trials this drug showed efficacy in patients with PHN, PDN, mixed neruropathic pain syndromes, phantom limb pain, Guillain- Barrè syndrome, and acute and chronic pain from spinal cord injury (Rice and Maton, 2001; Backonja et al., 1998; Serpell, 2002; Bone et al. 2002; Pandey et al., 2002; Tai et al., 2002). Gabapentin (900-3600 mg/day) significantly reduced pain compared

to placebo, and in some trials its administration caused an improvement in sleep, in mood, and in quality of life. The side effects of gabapentin include somnolence, dizziness, dry mouth and oedema: Weight gain can be found in some patients. (Gimbel et al., 2003)

Gabapentin and pregabalin each bind to voltage-gated calcium channels at the a_2-δ subunit and inhibit neurotransmitter release. Both gabapentin and pregabalin have few drug interactions, but they can produce dose-dependent dizziness and sedation. These side effects can be reduced starting the treatment with low dosages. Moreover, it's necessary a careful administration of these two drugs in patients with renal insuffciency, and dosage adjustments can be made in relation to creatinine clearance (Dworkin et al., 2010). Gabapentin pharmacokinetics are nonlinear (due to saturable absorption), and dosing requires careful titration. Gabapentin has to be administrated at low dosages at the the beginning of the treatment, and the dosages has to increase gradually until pain relief (Dworkin et al 2010).

Opioids

Opioids have been tested in several clinical trials for PDN, PHN, phantom pain and neuropathic pain of diverse aetiology (Watson et al., 2003; Gimbel et al., 2003; Huse et al., 2001). The drugs tested were: morphine, oxycodone, methadone, and levorphanol. (Basic-Kes et al.,2009) It ha been reported that opioids were efficacy in spontaneous neuropathic pain with a dose- dependent analgesic response. In a unique crossover study comparing opioids, TCAs and placebo, morphine provided statistically benefits for pain and sleep, but not for physical condition and mood (Raja et al., 2002). However, because of their long term safety, including risk of hypogonadism,

immunological changes, and opioid misuse or abuse, opioids are not recommended for routine first-line use. Costipation, nausea, and sedation are the most side effects of opioids. Nausea and sedation can be reduced starting the treatment with low dosages and titrating gradually. However, constipation remains a chronic problem. Another problem of opioids is the misuse or abuse. Particularly in patients with active or previous substance abuse (alcoholism) and a family history of abuse are more subject to misuse or abuse opioids. (Dworkin et al.,2010)

Endocannabinoids

The first endogenous ligand for cannabinoid receptors (Devane et al., 1992) was named anandamide (AEA) after the sankrit word for bliss. Several other endocannabinoids including 2-arachydonoylglycerol (2-AG) (Mechoulam et al., 1995 and Sugiura et al., 1995) noladin ether (Hanus et al., 2001), virodhamine (Porter et al., 2002) and N-arachidonoly-dopamine (Huang et al., 2002) have been described. Fatty-acid amide hydrolase (FAAH) is the principle catabolic enzyme for fatty-acid amides including AEA and *N*-palmitoylethanolamine (PEA). Anandamide also acts on endovanalloid transient receptor (TRPV1) (Ross et al., 2001), shows affinity for TRPV1 that is 5- to 20-fold lower than its affinity for CB1. AEA can also activate the nuclear peroxisome proliferator receptor-γ (PPAR- γ) (Bouaboula et al., 2005). In a study performed by Costa and colleagues (1999) anandamide (20 mg/kg, i.p.) reduced ambulatory and non-ambulatory activities, with a strong cataleptic effect, produced hypothermia and hindlimb splaying, and reduced defecation. It did not significantly increase either the tail-flick or hot-plate latencies.

A valid alternative strategy for pain relief may be the modulation of the endocannabinoid system by potentiating its actions through the inhibition of endocannabinoid (particularly anandamide) inactivation. The use of so-called 'indirect agonists' may make it possible to minimize the adverse side effects produced by the direct activation of cannabinoid receptors. AM 404, an inhibitor of anandamide reuptake prevented nociceptive behaviour in a rat model of neuropathic pain, the chronic constriction injury (Costa et al., 2006) and partial sciatic nerve ligation (Mitchell et al., 2007,). Several FAAH inhibitors (e.g., OL135, URB597) have been developed and used to investigate physiological effects of increasing accumulation of AEA and other fatty-acid amides. Systemic administration of URB597 ($0.3\,mg\,kg^{-1}$) reduced the mechanical allodynia and thermal hyperalgesia in the CFA model of inflammatory pain. It didn't reduced mechanical allodynia in the partial sciatic nerve-ligation model of neuropathic pain (Jayamanne et al., 2006)

Monoacylglycerol lipase (MGL) is a key enzyme implicated in the hydrolysis of 2-AG (Hohmann et al., 2005, Dinh et al., 2002). MGL inhibitors (e.g., URB602, JZL184) have been developed and can be used to selectively increase accumulation of this endocannabinoid. Systemic administration of URB602 elicited a dose-dependent anti-oedemigen and anti-nociceptive effect in a murine model of inflammation/inflammatory pain (Comelli et al., 2007).

Diabetes

The pancreas is an unpaired gland of the alimentary tract with mixed exocrine-endocrine function. The pancreas has en elongated shape, the

handle is formed by the head of the gland, which is closely attached to the distal two-third s of the duodenum, the barrel is formed by the body region, which is overlaid by the posterior wall of the stomach, and by the tapering tail region that ends near the spenic hilus (Fig.11).

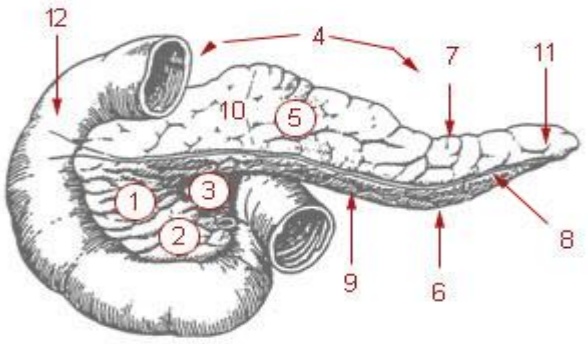


Fig.11: *Drawing of Pancreas. 1 Head of pancreas, 2 Uncinate process, 3 Pancreatic notch, 4 Body of pancreas,5 Anterior surface, 6 Inferior surface, 7 Superior margin, 8 Anterior margin, 9 Inferior margin, 10 Omental tuber, 11 Tail of pancreas and 12 Duodenum*

Macroscopically, the pancreas has a yellow- pink aspect and a soft to firm consistency depending on the level of fibrosis and fat accumulation the organ (In't Veld and Marichal, 2010).

Microscopically, the lobules are formed by a mixture of ductules and well- vascularized epithelial cell clusters that reflect the two functions of the pancreas: digestion and glucose homeostasis. Exocrine cells release a mixture of digestive enzymes and bicarbonate into the duodenu. Cluster of the endocrine cells form islets of Langerhans that are scattered throughout the exocrine parenchyma. . The islets of Langerhans are of vital importance to the body as they produce

insulin, a regulator of glucose homeostasis. Islets are pervaded by a dense network of capillaries (Goldstein and Davis, 1968) and is partly surrounded by a thin collagen capsule (Hughes et al., 2006) and glial sheet (Smith, 1975) that separates the endocrine cells from the exocrine component (Fig.12). The cell types contained in the islets are: α-cells (they release glucagon), β-cells (they release insulin), δ-cells (they release somatostatin). Endocrine cells form aggregates of various sizes with small or large form and ellipsoid or spherical structure.

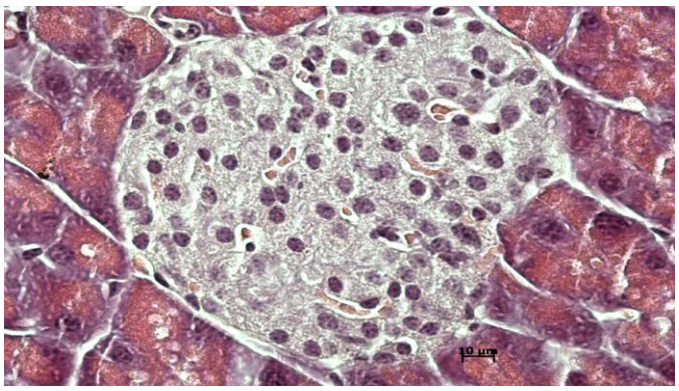


Fig.12: *Langerhans islet has an spheric or ovoid shape, they are formed by endocrine polyedric cells. Among these cells a network of capillaries can be seen. The different cellular types can be distinguished using specific immunohistocemic techniques Hematoxylin and eosin staining. Original magnification 40x.*

The term "diabetes mellitus" describes a metabolic disorder of multiple aetiology. Diabetes is characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. The effects of diabetes mellitus include long–term damage, dysfunction and failure of various organs. There are two main types of diabetes: type 1 and type 2 diabetes. The first one usually develops in childhood and adolescence and it is characterized by a failed

production of insulin. The second one usually develops in adulthood and it is characterized by an inadequate production of insulin. Type 2 diabetes is a related to obesity, lack of physical activity, and unhealthy diet. Other categories of diabetes include gestational diabetes (a state of hyperglycemia which develops during pregnancy) and "other" rarer causes (genetic syndromes, acquired processes such as pancreatitis, diseases such as cystic fibrosis, exposure to certain drugs, viruses, and unknown causes).In the short term, hyperglycemia causes symptoms of increased thirst (polydipsia), increased urination (polyuria), increased hunger (polyphagia), and weight loss. However, in the long-term, it causes damage to eyes (retinopathy), kidneys (nephropathy) and nerves (neuropathy). As well, it increases the risk of heart disease, stroke, and insufficiency in blood flow to legs. Thus, the primary goal of treatment is to bring the elevated blood sugars down to a normal range, both to improve symptoms of diabetes as well as to prevent or delay diabetic complication.

Patients with recent onset of type I diabetes (T1D) usually present a pancreas that is macroscopically normal in appearance and weight, while in patients with this chronic disease present an atrophy of the acinar cells and a decrease in overall pancreatic weight (Gepts, 1965, Loehr and Kloeppel, 1987).

As a consequence of hyperglycaemia islets of Langerhans are characterized by an inflammatory infiltrate. This infiltration was described by Gepts in 1965 when he discovered the insulitis process in pancreatic tissues from T1D patients. Insulitis is characterized by β-cell necrosis with lymphocytic and macrophagic infiltration and the pancreatic islets revealed architectural distortion.

Baik and colleagues (1999) described 4 grade of insulitis. Grade 1 insulitis occurs when lymphocytic infiltrate is at the ductal pole of the islet only. Grade 2 insulitis occurs when lymphocytic infiltrate surrounds but not invades islets. Grade 3 and 4 insulitis occur when the lymphocytic infiltrate invades the islet and when there is a strong lymphocytic infiltrate with islet destruction.

Complications of diabetes

Diabetes complications are divided into microvascular (due to damage to small blood vessels) and macrovascular (due to damage to larger blood vessels). Microvascular complications include damage to eyes (retinopathy) leading to blindness, to kidneys (nephropathy) leading to renal failure and to nerves (neuropathy) leading to impotence and diabetic foot disorders (which include severe infections leading to amputation). Macrovascular complications include cardiovascular diseases such as heart attacks, strokes and insufficiency in blood flow to legs. There is evidence from large randomized-controlled trials that good metabolic control both in type 1 and 2 diabetes can delay the onset and progression of these complications.

Neuropathy

Neuropathy is one of the many complications of both type 1 and type 2 diabetes mellitus (Little et al., 2007). The financial toll exerted by diabetes in the US alone is over $132 billion per annum, half of which is attributable to diabetic complications . The frequency of neuropathy in diabetics depends on how the neuropathy is diagnosed whether by clinical examination or incorporating one or more electrophysiological criteria. Many mechanisms lead to diabetic neuropathy. Key

aetiologies are continued oxidative stress, ischaemia and inflammation leading to axonal dysfunction and loss, associated to some extent with secondary demyelination (secondary autoimmune neuropathies, such as chronic inflammatory demyelinating polyradiculoneuropathy, are more common in the diabetic than in the non-diabetic population) (Sharma et al., 2002).

The symptoms are many, depending on which nerves are affected: for example, numbness in extremities, pain in extremities, and impotence. Decreased sensation to feet can lead to patients not recognizing cuts and developing foot infections. If not treated early, these can lead to amputation.

Classification of diabetic neuropathy:

-diabetic polyneuropathy

-painful symmetrical polyneuropathy

-autonomic neuropathy

-focal and multifocal neuropathy

Painful symmetrical polyneuropathy Neuropathic pain is a major burden in diabetic patients, and is a common complication of length dependent diabetic polyneuropathy (LDDP). Recent studies of intraepidermal nerve fibers (IENFs) showed that more-severe loss of these fibers was associated with the presence of neuropathic pain only in patients with little or no objective sign of neuropathy. Consequently, loss of IENFs cannot explain pain in all cases, suggesting that different mechanisms underpin the genesis of pain at various stages of neuropathy (Sorensen et al., 2006). From a physiological point of view, however, micro neurographic recordings from unmyelinated fibers of diabetic patients with painful and

nonpainful neuropathies revealed an abnormal ratio of mechanoresponsive to mechano-insensitive nociceptors in patients with diabetes, suggesting that mechano responsive nociceptors had lost their responsive ness to mechanical stimuli and heat (Orstavik et al., 2006). Small-fiber neuropathy in diabetes, therefore, seems to affect the receptive properties of nociceptors, leading specifically to impairment of mechano responsive nociceptors (Orstavik et al., 2006). Recent developments have shown that transmission of painful stimuli depends on activation of sodium channels that are expressed at high levels in cell membranes of nociceptive neurons of dorsal root ganglia. (Waxman, 2006) Acute painful neuropathy with allodynia is sometimes associated with cachexia and depression, especially in young adults with type 1 diabetes (Ellenberg, 1974).

Trophic changes in distal symmetrical sensory polyneuropathy

Trophic changes predominantly affecting the distal parts of the lower limbs are a major complication of LDDP. The earliest change is frequently a callus (often in the region of the metatarsal heads), which might recur despite regular foot care. In other cases, the first manifestation is a painless phlyctenular lesion. Both of these conditions are painless, and are associated with loss of pain sensation over the feet. Chronic ulcers can subsequently develop. Idiopathic bullae (*bullosis diabeticorum*), which can precede the onset of plantar ulcers, can occur in territories with sensory loss on the hands. Neuropathic osteoarthropathy is a complication of long-standing diabetic neuropathy. Painless foot deformity, sometimes of acute onset, is the main sign of this condition. Penetration of bacteria through neuropathic ulcers can lead to chronic osteomyelitis. Foot

ulceration and neuropathic osteoarthropathy are not specific to the 'diabetic foot'—similar complications of loss of pain sensate ion occur in a number of conditions, including leprosy, hereditary sensory neuropathies, alcoholic sensory poly neuropathy, (Said, 1980) and hereditary in difference to pain with normal nerve biopsy findings (Landrieu et al.,1990)

Loss of pain sensation with preservation of normal muscle strength or with subnormal strength can lead to painless trauma and development of plantar ulcers and osteoarthropathy. Vasculopathy seems to be an additional risk factor for trophic changes in patients with diabetes. (Shaw and Boulton, 1998; Akbari and LoGerfo, 1998)

Autonomic neuropathy

Autonomic dysfunction is one of the characteristic manifestations of diabetic neuropathy, and can be life-threatening (Llewelyn et al., 2005; Vinik et al., 2003). Clinical cardiovascular disturbances usually start with resting tachycardia; the heart rate might return to its normal value later on, but does not exhibit normal variations in response to changing physiological situations. Postural hypotension (a fall in systolic blood pressure of more than (Dreyfus et al., 1957) mmHg on changing from a lying to a standing position, without an increase in heart rate) can be an extremely disabling symptom of autonomic neuropathy with postural syncopes. Postural hypotension can be aggravated by tricyclic antidepressants, which are often used for treatment of chronic pain in diabetic neuropathy, and by episodes of diarrhea. Cardiac autonomic neuropathy seems to be strongly associated with increased risks of silent myocardial ischemia and

mortality. Other autonomic dysfuctions are: gastroparesis, bladder atony, impotence.

Focal and multifocal neuropathy

Focal and multifocal neuropathies are much less common than LDDP in patients with diabetes. These forms of neuropathy are usually seen after 50 years of age, and mainly in patients with type 2 diabetes. Focal neuropathies include cranial nerve involvement, limb and truncal neuropathies, and proximal diabetic neuropathy (PDN) of the lower limbs. Development of sensorimotor deficits in the territories of one or several nerve trunks, roots or plexuses, is rare in patients with diabetes and warrants exclusion of other causes of neuropathy, by nerve biopsy if necessary.

Cranial neuropathy in patients with diabetes is restricted largely to unilateral oculomotor nerve palsies. Multiple cranial nerve palsies are extremely rare.

Truncal neuropathy is usually predominantly or completely unilateral (Ellenberg, 1978; Stewart, 1989). The onset is abrupt or rapid, with pains or dysesthesias as the main features. The pain often has a radicular distribution and is almost always worse on contact and at night. Weakness of abdominal muscles also occurs. Isolated involvement of peripheral nerves of the limbs is extremely rare, with the exception of median nerve entrapment in carpal tunnel syndrome.

Patients with diabetes can present with proximal neuropathy of the lower limbs, characterized by a variable degree of pain and sensory loss, associated with unilateral or bilateral proximal muscle weakness and atrophy. The onset is acute or subacute, and the patient complains of numbness or pain of the anterior aspect of the thigh, often of the

burning type and most pronounced at night. The patient experiences difficulty in walking and climbing stairs, owing to weakness of the quadriceps and iliopsoas muscles. Wasting of the quadriceps muscle and loss of the patellar reflex occur at an early stage in the disease. The syndrome progresses over several weeks or months in most cases, then it stabilizes and spontaneous pains decrease, sometimes rapidly. In approximately one-third of patients with PDN of the lower limbs there is a definite sensory loss over the anterior aspect of the thigh, and in the others there is a painful contact dysesthesia in the distribution of the cutaneous branches of the femoral nerve, without definite sensory loss. The long-term prognosis is good, regardless of the quality of glycemic control (Coppack and Watkins, 1991). In a fifth of the patients that we investigated for this syndrome relapses occurred on the other side of the body within a few months (Said et al., 1994). The clinical features of PDN, with its frequent motor involvement, asymmetry of the deficit, and gradual yet often incomplete spontaneous recovery, differ marked from those of LDDP, in which motor signs are seen only in extreme cases, and in which the condition never improves.

In a small proportion of patients with diabetes a multifocal diabetic neuropathy (MDN) is observed, with successive involvement over several weeks or months of roots and nerves of the lower limbs, trunk and upper extremities, sometimes with a relapsing course (Said et al., 2003). The distal parts of the lower limbs are invariably involved unilaterally or bilaterally, and there are also proximal deficits in most patients. Truncal and upper limb nerves are less commonly affected.

Nephropathy

Diabetic nephropathy is the most common glomerulopathy (Molitch et al., 2004) and affects the glomeurlus (Fig. 13).

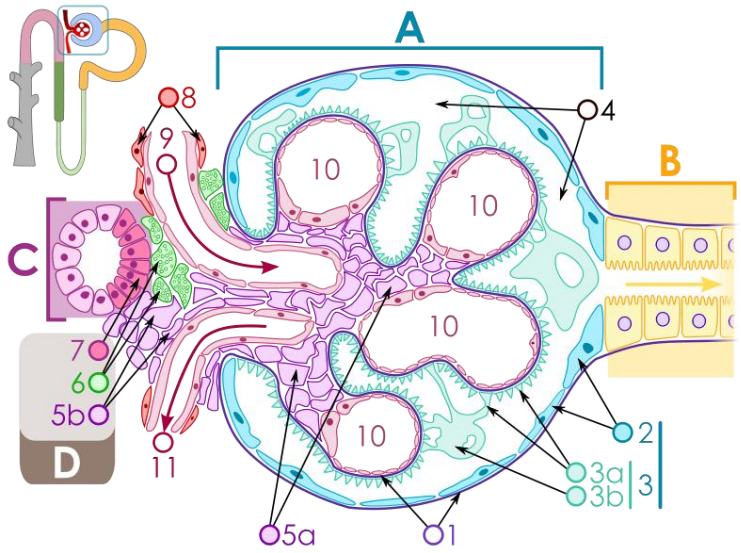


***Fig. 13**: **A** Renal corpuscle, **B** Proximal tubule, **C** Distal convoluted tubule, **D** Juxtaglomerular apparatus; **1** Basement membrane (Basal lamina), **2** Bowman's capsule - parietal layer, **3** Bowman's capsule - visceral layer, **3a** Pedicels (podocytes), **3b** Podocyte or sometimes called Bowman's cells **4** Bowman's space (urinary space), **5a** Mesangium - Intraglomerular cell, **5b** Mesangium, Extraglomerular cell, **6** Granular cells (Juxtaglomerular cells), **7**. Macula densa, **8.** Myocytes (smooth muscle), **9** Afferent arteriole, **10** Glomerulus Capillaries, **11** Efferent arteriole*

It is important to consider that hyperglycemia is a primary initiator of diabetic nephropathy. However, diabetic nephropathy holds a genetic component at two levels: first, the elevation of glycemia; and second, at establishing a genetic background where nephropathy can occur (in the presence of hyperglycemia). Only 30% of patients with type 1, and 35–40% of patients with type 2 diabetes develop diabetic nephropathy irrespective of glycemic control (Diabetes Control & Complications,

1995). The clinical history of a typical patient starts with symptoms of hyperfiltration (elevated values of glomerular filtration rate (GFR) and occasional microalbuminuria, which may last approximately 5 years. During the next ~20 years, microalbuminuria turns into progressively higher proteinuria, whereas GFR declines.

Very early, hyperglycemia increases endothelial NO synthase (eNOS) expression in afferent arteries and glomerular capillaries, which leads to vasodilation and increased GFR (Sugimoto et al., 1998). Progressively, glomerular distension causes endothelial dysfunction and hemodynamic alterations, loss of the glomerular basement membrane (GBM) electric charge and GBM thickening, decreased number of podocytes, foot process effacement and mesangial expansion have been shown to underlie the initial glomerular injury (Lehmann and Schleicher, 2000; Wolf and Ziyadeh, 2007; Munusamy and MacMillan-Crow, 2009), the initial glomerula injury eventually leads to glomerulosclerosis.

Damage to podocytes is emerging as a critical event in glomerulosclerosis (Kretzler, 2005; Reddy et al., 2008), which has lead to propose diabetic nephropathy as a disease of podocyte loss. Besides direct effects of hyperglycemia on tubular cells (Munusamy and MacMillan-Crow, 2009), glomerular damage causes tubular injury resulting in tubular cell death, epithelial to mesenchymal transition (EMT), cell infiltration, tubule degeneration and interstitial fibrosis, by different mechanisms (Kriz et al., 1998; Remuzzi et al., 2006; Wolf and Ziyadeh, 2007; Ziyadeh and Wolf, 2008): (i) the proteinuria derived from GBM alterations activates tubular cells to produce mediators (TGF-β, angiotensin-II, etc.) and proinflammatory

cytokines; (ii) growth factors derived from glomerular cells (TGF-β, insulinlike growth factor—IGF-1, angiotensin II, etc.) stimulates the uptake of proteins, which amplifies the effect of proteinuria, and activates cell death and the profibrotic program in tubule cells; (iii) microangiopathy results in reduced postglomerular blood flow to peritubular capillaries. Renal structures become gradually impaired through changes that start diffusely and spread through the glomerulus, or focally localized as focal and segmental glomerulosclerosis FSGS (Kimmelstiel–Wilson disease) (Fig. 14).

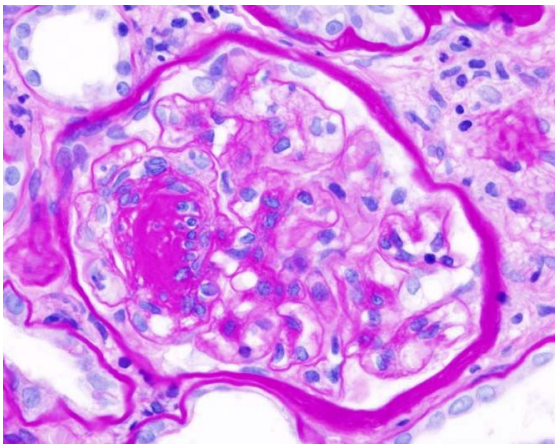


Fig.14: *Histopathological image of diabetic glomerulosclerosis with nodular (Kimmelstiel-Wilson) mesangial expansion (white arrow) PAS stain.*

Initially localized sites of focal sclerosis may extend and coalesce through the glomeruli, giving rise to a greater diffuse sclerosis. Alternatively, initial focal sclerosis may progress more rapidly towards tubule degeneration as opposed to glomerular collapse Alternatively, diffuse and focal glomerulosclerosis can be contemplated as etiologically or mechanistically different events. Typically, an early histological finding is the adhesion of a glomerular capillary to Bowman's capsule at a podocyte deprived basement

membrane point. These adhesions create gaps in the parietal epithelium that allow ectopic filtration out of Bowman's capsule into the paraglomerular, interstitial space. Two primary pathological pathways have been identified in total nephron degeneration. Through pathway I, glomerular collapse occurs before or parallels tubular degeneration, whereas through pathway II, tubular atrophy precedes glomerular degeneration (Kriz et al., 1998). Pathway I involves the propagation of glomerular adhesions at the vascular pole, and the formation of a progressively larger paraglomerular space which contains ectopic filtrate and the remains of capillary tufts, which eventually reaches the urinary pole and the tubular structure. The paraglomerular space is formed initially within the GBM, which progressively disappears as the paraglomerular space enlarges and becomes surrounded by a sheath of fibroblasts (at the interstitial side). When the paraglomerular space reaches the urinary pole, it penetrates within the tubular basement membrane and separates it from the tubular epithelium, which correlates with tubule degeneration. Focal adhesions become areas of increasing sclerosis, capillary and perfusion collapse, all of which eventually embraces the whole glomerulus. In pathway II, the paraglomerular space initiated by the original focal adhesion reaches the urinary pole before further damage occurs at the vascular pole. As such, tubular fibrosis and degeneration preceeds extensive glomerular damage. As tubule flow decreases, pressure within Bowman's capsule increases and it dilates. Finally, total tubule collapse occurs and the urinary orifice in Bowman's capsule disappears, giving rise to the physical separation of the glomerulus and the tubule, and the formation of a glomerular cyst with

substantial remnant perfusion. The paraglomerular space content is proposed to play a significant role in the initiation of damage and in the connection of glomerular and tubular disease. Paraglomerular space contains renal filtrate (or exudate), cell debris from podocytes, extracellular matrix and basement membrane material, which may prospectively trigger proinflammatory, profibrotic and cell death-inducing responses. Interestingly, these histopathological patterns have been observed both in diabetic rats and humans (Kriz et al., 1998).

Traditionally, diabetic nephropathy has been considered a glomerular disease, in which tubular damage is a consequence of primary glomerular events. However, some results challenge this concept (Lapsley et al., 1993; Hong et al., 2003; Thomas et al., 2005; Thomson et al., 2006; Singh et al., 2008). Signs of impaired renal function have been detected before the evidence of glomerular malfunction in animals and humans, including increased excretion of small proteins (Lapsley et al., 1993; Hong et al., 2003).

Hyperglycemia acts on renal endothelial and mesangial cells, podocytes and also tubular cells. The cellular consequences derived from hyperglycemia resulting from types 1 and 2 diabetes are largely similar (Kanwar et al., 2005). Also, the cellular consequences of exposure to high glucose are similar in all renal cells (Orasanu and Plutzky, 2009). Hyperglycemia initiates cell signaling pathways in renal cells (Orasanu and Plutzky, 2009), including hyperactivation of protein kinase C (Ishii et al., 1996; Koya et al., 1997), oxidative stress through an excessive production of reactive oxygen species (ROS) (Palm et al., 2003; Tesch and Nikolic-Paterson, 2006) and others. This

activation results in overexpression of (i) growth factors like transforming growth factor- β (TGF-β, Di Paolo et al., 1996; Heilig et al., 1997; Weigert et al., 2000), platelet derived growth facto (PDGF; Di Paolo et al., 1996) and connective tissue growth factor (CTGF; Connolly et al., 2003), (ii) inflammatory cytokines (Connolly et al., 2003; Lorz et al., 2009), extracellular matrix elements (fibronectin, collagens I and IV) (Connolly et al., 2003) and matrix deposition (Bolick et al., 2003), cytoskeleton reorganization (Dai et al., 2006), cell cycle arrest (Masson et al., 2006), and hypertrophy (Fan and Weiss, 2004; Masson et al., 2006). ROS attenuation *in vivo* (Brezniceanu et al., 2008) and *in vitro* (Munusamy and MacMillan-Crow, 2009) significantly reduces hyperglycaemic cell damage. Besides glucose, other mediators of hyperglycemia-associated damage include the advanced glycosylation end-products (AGEs), which result from the non-enzymatic glycation of proteins and lipids. Accumulation of advanced glycosylation end-products in the kidney is associated with the development of nephropathy (Tesch and Nikolic-Paterson, 2006), both in type 1 (Cohen et al., 2000) and type 2 diabetic mice (Lassila et al., 2004). Interaction of advanced glycosylation end-products with their membrane receptor (RAGE localised in kidney, eye, nerve, arteries and in the lung.) is necessary for their action (Flyvbjerg et al., 2004; Wendt et al., 2004). On the other hand, hyperglycemia also increases the ubiquitous glucose transporter GLUT-1 mRNA and protein, and glucose transport in mesangial cells (Schena and Gesualdo, 2005). This has been proposed as a positive feedback mechanism in the appearance of glucose-induced damage.

Hyperglycemia induces hyaluronan (an anionic, non-sulfated glycosaminoglycan) overproduction in the mesangial matrix. Hyaluronans are potential binding sites for monocytes and macrophages *in vitro* and *in vivo* (Wang and Hascall, 2004). Hyperglycemia also induces ROS-mediated apoptosis in podocytes (Susztak et al., 2006), tubule cells (Verzola et al., 2004) and endothelial cells (Tawfik et al., 2005). In the vasculature, hyperglycemia induces an oxidative stress (Giardino et al., 1996; Bellin et al., 2006) that results in endothelial dysfunction (Price et al., 2001; Yu and Lyons, 2005; Zurova-Nedelcevova et al., 2006) and impaired relaxations (Sercombe et al., 2004). High blood glucose levels are also involved in the development of dyslipidemia (Veiraiah, 2005) and atherosclerosis (Price et al., 2001). In all renal cells, high glucose and AGEs induce the production of angiotensin II and growth factors (TGF-β, CTGF, vascular endothelial growth factor— VEGF, etc.) that act in an auto or paracrine manner to activate a fibrotic and inflammatory pathway in glomeruli and tubules (Lehmann and Schleicher, 2000; Wolf and Ziyadeh, 2007). It is expected that, at least initially, all endothelial cells in the glomerular capillaries are subject to a similar glycemic stress, whereas only a few initially undergo a damage that likely translates to neighboring areas. It thus can be speculated that (i) for undetermined reasons every cell processes stimuli differently; or (ii) cell–cell and cell–matrix interactions, in conjunction with neuroendocrine influences modulate (and even null) the effect of hyperglycemia on most cells. It is only when these and certain other localized conditions (e.g. hypoxia, rupture or disorganization of basement membranes or ECM) merge in a

particular manner and localization that hyperglycemia associated damage begins. On the contrary, a homogeneous glycemic stress on all mesangial cells, inducing a similar effect in all or most cells would better correlate with the appearance of a diffuse glomerular sclerosis.

The aim of this project, founded on the previous remarks, was to characterize the anti-nociceptive effect of PEA in two murine models of neuropathy: the chronic constriction injury and diabetic neuropathy. Mast cell degranulation and Wallerian degeneration were consequently morphologically characterized in the sciatic nerve of mice with chronic constriction injury and with a painful diabetic neuropathy. In the last decade researchers had identified activated microglia as an important player in maintenance of neuropathic pain; therefore activated microglia was morphologically characterized in mice with chronic constriction injury. For this study the same animal model (CCI model and streptozotocin- induced diabetes) and the same dose (10mg/kg) and treatment of PEA employed by Costa and colleagues were used. In CCI model PEA was also administered for a short period because rapid mast cell activation after the injury was also supposed.

In addition to neuropathy, diabetes presents a large variety of compliances, such as nephropathy; therefore a morphological evaluation of kidney tissue and in particular of glomeruli was performed.

Costa and colleagues evaluated the insulin content in diabetic mice submitted to two different treatments with PEA (always administered at 10 mg/kg, i.p.). Therefore a morphological characterization of Islets

of Langerhans was performed. In particular, after a routine staining, the density of Islets of Langerhans and the area were evaluated.

Materials and Methods

Materials

Animals

All experiments were performed in accordance with Italian and European regulations governing the care and treatment with laboratory animals (Permission no. 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 (Zimmermann, 1983). All efforts were made to minimize the number of animals used and their discomfort. Male C57BL/6J mice weighing 25-30g (Harlan, Italy) were used for all experiments. Mice were housed under controlled temperature (22±1°C), humidity (60±10%) and light (12 h/day) and allowed to acclimatise at least one week before use in the experiments. Standard food and water were available *ad libitum*.

Drugs and substances

Palmitoylethanolamide was purchased by Cayman Chemical (Ann Arbor, MI, USA) and dissolved in a mixture 1:9 of ethanol and saline solution and used at the dose 10 mg/kg both for mononeuropathy and diabetic neuropathy. Streptozotocin (Sigma, Italy) was freshly prepared in citrate buffer 0.1M pH 4.5 and used at the dose 120 mg/kg.

Paraformaldehyde (Sigma, Italy) was prepared in 0.1 M phosphate buffer pH 7.4. The same buffer was used to prepare 2% paraformaldehyde and 0.2% glutaraldehyde (Electron Microscopy Sciences, USA). 1% Osmium tetrossidum (Electron Microscopy Sciences, USA) was diluted in 0.1 M phosphate buffer pH 6.0, 2% hydrogen peroxidase (Sigma, Italy) was diluted in distilled water.

The primary antibodies used are: polyclonal sheep anti- mouse mast cell protease I (anti-MMCP-I) (Moredur Scientific Ltd, UK), monoclonal rabbit anti- mouse trkA (Abcam, Cambridge, UK) and monoclonal rat anti-mouse F4/80 (AbD Serotec, Oxford, UK).

The secondary antibodies used are: biotinylated anti-sheep IgG (H+L) antibody (Vector Laboratorie, Burlingame CA, USA), anti-sheep fluorescein conjugated and anti-rabbit rhodamine conjugated antibodies (Millipore, CA, USA), and anti-rat IgG alkaline phosphatase conjugated (Sigma, Italy). Both primary and secondary antibodies were reconstituted with distilled water and stored according to manufacturer's instruction.

The avidin-biotin peroxidase kit (Vectastain Elite ABC kit, Vector, UK), the tablet BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium), the FastRed TR/ NaphtolAs-MX (4-Chloro-2-methylbenzenediazonium/3-Hydroxy-2-naphtoic acid 2, 4-dimethylanilide phosphate) (Sigma, Italy) and ImmPACT DAB peroxidase substrate (Vector Laboratories, Burlingame, CA USA) were used according manufacturer's instruction.

Acidified toluidine blue to stain mast cells was prepared in following way: 0.5% toluidine blue (Fluka, Italy) was added in a solution containing 0.1M citric acid, 0.2M dibasic sodium phosphate. Toluidine blue (Fluka, Italy) to stain myelin sheath was 0.2% in distilled water. Mayer's hematoxylin and eosin were purchased by Bio-Optica (Italy), periodic acid and Schiff reagent to perform a PAS staining were purchased by Titolchimica (Italy).

RIPA buffer (Radio Immuno Precipitation Assy buffer) was constituted by 150 mM sodium chloride (Sigma, Italy), 1.0% Triton

X-100 (Sigma, Italy), 0.5% sodium deoxycholate (Fluka, Italy), 0.1% SDS (sodium dodecyl sulphate, Sigma, Italy), and 1mM pheylmethylsulfonil fluoride PMSF (Fluka) in 50 mM Tris, pH 8.0.
All the reagents to process samples to obtain paraffin wax (melting point 58°C) and Epon-Araldite resin inclusion were purchased Electron Microscopy Sciences, USA. All the reagents used for SDS-PAGE and immunoblotting were purchased by Sigma, Italy. All phosphate buffers and all substances used in these experiments were freshly prepared.

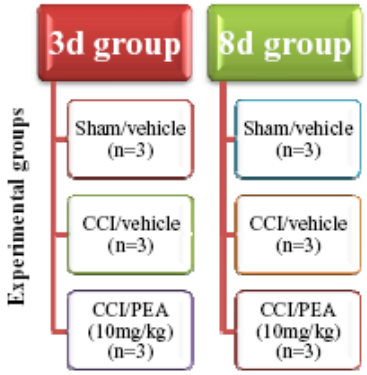
Methods: peripheral mononeuropathy

Chronic constriction injury

Neuropathic pain was induced by chronic constriction injury (CCI model) according to the modified method of Bennett and Xie (1988). Briefly, mice were anesthetized with pentobarbital (60 mg/kg, i.p.) and the right 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. The chronic constriction injury of the sciatic nerve was performed in the pharmacological lab of Dr. Costa.

Treatment

Animals were divided into two different groups: 3d and 8d group (Scheme 1).

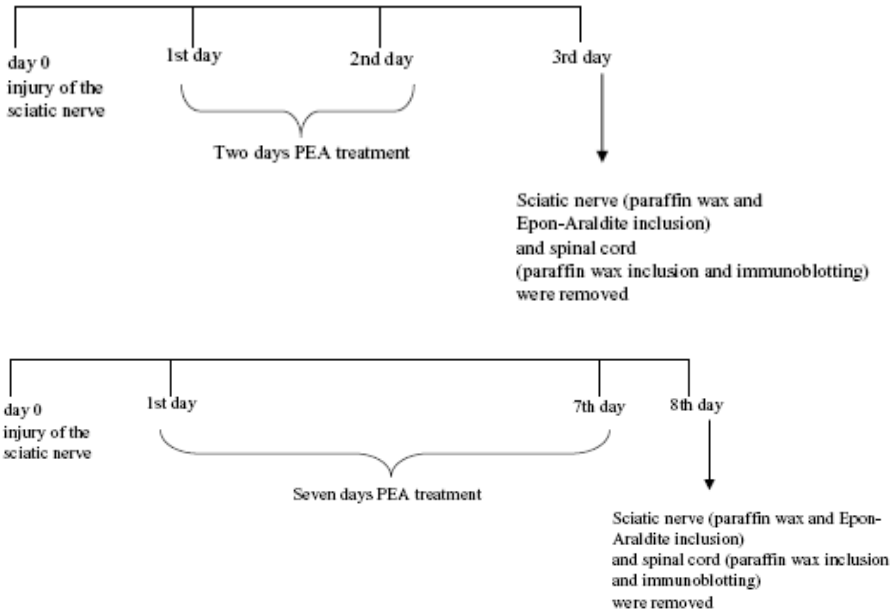


Scheme 1

In each group three experimental groups were considered: the control mice and the CCI mice treated with vehicle and the CCI mice treated with PEA (n=3 for each experimental group). In the first group (called 3d) the treatment with vehicle or PEA (10 mg/kg, i.p.) started after the injury, administered for two consecutive days and on 3rd day, 24 hours after the last administration mice were sacrificed, and the right sciatic nerve and the lumbar spinal cord (L4-L5) were removed. In the second group (called 8d) the treatment with vehicle or PEA (10 mg/kg, i.p.) started the day after the injury, administered for seven consecutive days and on 8th day, 24 hours after the last administration mice were sacrificed and the right sciatic nerve and the lumbar spinal cord (L4-L5) were removed (Scheme 2).

18 sciatic nerves were processed for Epon Araldite resin to obtain semi-thin cross-sections and the other 18 sciatic nerves were processed in paraffin to obtain longitudinal sections. The lumbar spinal cord (n=18) was processed in paraffin wax to obtain cross-section.

To protect the integrity of the sciatic nerve and of the spinal cord for the morphological analysis, animals were firstly submitted to an intracardiac perfusion with fixative.



***Scheme 2**: Mice were divided into two groups (3d and 8d), and into each group the three experimental groups were present. PEA (10mg/kg, i.p.) was administered for two or for seven days)*

Briefly, the anticoagulant heparin (100 µl, 5000 U/mL), was injected to mice and then mice were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.). The perfusion occurred with 4% paraformaldehyde fresh solution.

The spinal cord used for biochemical analysis was immediately stored at -80°C for the subsequent analysis.

The PEA treatment, the intracardiac perfusion and the collection of tissues were performed in the pharmacological lab of Dr. Costa.

Thermal hyperalgesia and mechanical allodynia

Thermal hyperalgesia and mechanical allodynia were evaluated respectively with plantar test (Hargreaves method) and with Dynamic Plantar Aesthesiometer; these evaluations were performed in all experimental groups before the chronic constriction injury (0 day), before PEA treatment started (1st day) and 24hours after the last administration with PEA (on 3rd and 8th days) by pharmacological lab of Dr Costa.

Sciatic nerve: longitudinal sections

Right sciatic nerve was removed and fixed in 4% paraformaldehyde fresh solution at room temperature for 4 hours, dehydrated in serial alcohol concentrations, clearing in a xylene solution and embedded in paraffin wax. Longitudinal serial sections (6µm) were obtained with a rotatory microtome.

Histology and immunohistochemistry

Sections were stained with toluidine blue in order to characterize mast cell morphology. Briefly, sections were deparaffinized in xylene and rehydrated in serial alcohol concentrations and soaked in a solution of 0,5% toluidine blue for 20 minutes at room temperature.

Other deparaffinized and rehydrated sections were immunostained with the antibody directed against mast cell proteases-I (MMCP-I) in order to confirm mast cell morphology. To block endogenous peroxidase, an enzyme present in the blood, sections were treated with 2% hydrogen peroxide (H_2O_2). Afterwards few washes with phosphate buffer (pH 7.4) sections were incubated with 2% bovine

serum albumin (BSA) to block non specific staining and with 0.1% triton X-100 in order to permeabilize tissue. Polyclonal sheep anti-mouse mast cell protease I (anti-MMCP I), used as primary antibody, was diluted 1:100 and sections were incubated with it at 4°C overnight.

Sections were then incubated with anti-sheep biotinylated secondary antibody (dilution 1:300) at room temperature for 2 hours and then incubated for 1 hour with avidin-biotin peroxidase. The complex was detected with diaminobenzidine (DAB) which produced a dark brown product end.

Immunofluorescence

Other deparaffinized and rehydrated sections were submitted to a double immunofluorescence (parallel approach) in order to observe the co-localization of MMCP-I and trkA receptor. Sections were incubated overnight at 4°C with 1% BSA. After that sections were washed with phosphate buffer pH 7.4 and incubated with a mixture of the following primary antibodies: anti-sheep MMCP-I (1:100, directed against mast cell proteases) and anti- mouse trkA receptor (1:200, directed against trkA receptor). The antibody mixture was employed at room temperature for 3 hours. Sections were then incubated at room temperature for 2 hours with a mixture of secondary antibodies conjugated to specific flurochromes and with a dilution of 1:200. The anti-sheep fluorescein (emit green signal) conjugated antibody was directed against MMCP-I primary antibody and the anti-rabbit rhodamine (emit red signal) conjugated antibody was directed against trkA receptor antibody.

Sciatic nerve: cross-sections

Right sciatic nerves were removed, fixed for 3 hours in a fresh mixture of 2% paraformaldehyde and 0.2% glutaraldehyde. Then sciatic nerves were post-fixed with 1% osmium tetroxidum at 4°C for 30 minutes, sciatic nerves were washed then with phosphate buffer pH 7.4 and incubated with 1% uranyl acetate at room temperature for 20 minutes. These two last passages were performed in dark condition. Sections were dehydrated in graded concentration of ethanol and soaked in propylene oxide to remove residual ethanol and then incubated in Epon- Araldite resin. Resin polymerized at 40°C for 48 hours and for 72 hours at 60°C. Sciatic nerves were then cut at LKB ultramicrotome to obtain serial semi-thin sections (1 µm).

Semi- thin sections were incubated with sodium methoxide to eliminate resin for 4 minutes and then incubated with a drop of 0,2% toluidine blue for 5 minutes. Toluidine blue was employed to characterize myelin state and to measure myelin thickness of intact axons.

Spinal cord

Histology and immunohistochemistry

The lumbar (L4-L5) spinal cord was removed because in this region the afferent fibers of the sciatic nerve arrive here. 18 lumbar spinal cord removed from each experimental group was embedded in paraffin wax (same schedule used to embed the sciatic nerve) to obtain serial cross sections (6 µm). Some deparaffinized sections were stained with Mayer's hematoxylin and eosin, while others were used for immunohistochemistry with F4/80 antibody in order to identify

activated microglia. For hematoxylin and eosin staining a standard protocol was used. For immunohistochemistry, sections were incubated with 1% BSA at room temperature for 6 hours then with primary antibody. Sections were incubated with monoclonal rat anti-mouse F4/80 at 4°C overnight (dilution 1:50). After few washes with phosphate buffer (pH 7.4) and a further incubation with 1% BSA for 45 minutes at room temperature, sections were incubated at room temperature for 2 hours with the anti-rat IgG antibody phosphatase alkaline conjugate (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 minutes, the interaction between substrate and alkaline phosphate produced a pink color. Slides were washed with distilled water in order to stop the reaction and then counterstained with Mayer's hematoxylin for 10 seconds in order to staining the nucleus of the cells.

For immunofluorescence the same schedule (without hematoxylin counterstaining) was followed but in this case the secondary antibody rodhamine conjugated was used. The antibody was employed at room temperature for 2 hours.

Western immunoblotting

18 spinal cords removed from each experimental group were incubated with RIPA buffer. 100 µl of lysis buffer were added and samples were sonicated for 5 seconds with 10 amplitude/microns potency (Soniprep 150, Sanyo). 5 µl of end product were used to dose

proteins in the samples with Bradford method and 50 µg of proteins were unloaded to perform SDS- PAGE (gel 7,5%).

F4/80 (MW=160 kD) expression was measured employing the same primary antibody used for immunohistochemistry (monoclonal anti-mouse F4/80). Nitrocellulose was incubated at 4°C overnight and the antibody was diluted 1:500 in 5% blocking solution. The anti-rat secondary antibody phosphatase alkaline conjugated was used and it was diluted 1:5000 in 5% blocking solution. The membrane nitrocelluose was incubated with it for 2 hours at room temperature. The protein was detected employing a tablet BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) dissolved in distilled water. Nitrocelluose membrane was incubated with this solution at room temperature for 20 minutes in dark condition. Then the enzyme activity was blocked with distilled water. The red level of the bands was quantified by image analysis software (Bio-Rad, gel doc 2000).

Methods: diabetic polyneuropathy

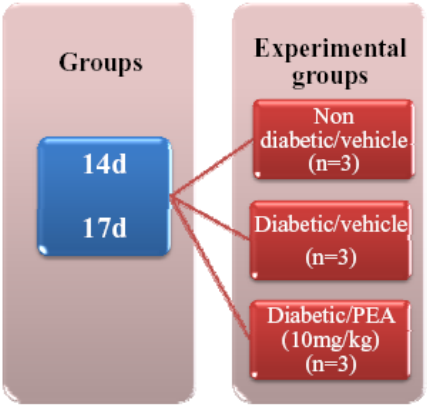
Induction of diabetes

Type-1 diabetes was induced through chemical pancreatectomy by a single intraperitoneal (i.p.) injection of streptozotocin (STZ) at 120 mg/kg, freshly prepared in citrate buffer 0.1M pH 4.5. Diabetes was verified one week later by measurement of blood glucose concentration by a glucometer (Lifescan One Touch Ultra glucose meter, Milan, Italy) on a sample of blood obtained from a tail prick. Only mice with blood glucose levels above 250 mg/dl were selected for the experiments. Control mice received an i.p. injection of citrate

buffer. Mice body weight was monitored over the whole period of the experimental study. The induction of diabetes, the monitoring of blood glucose level, the PEA treatment (explained in the next section) and the collection of the sciatic nerves, of the pancreases and of kidneys were performed in the pharmacology lab of Dr. Costa.

Treatment

Animals were divided into two different groups: 14d and 17d groups (Scheme 3).

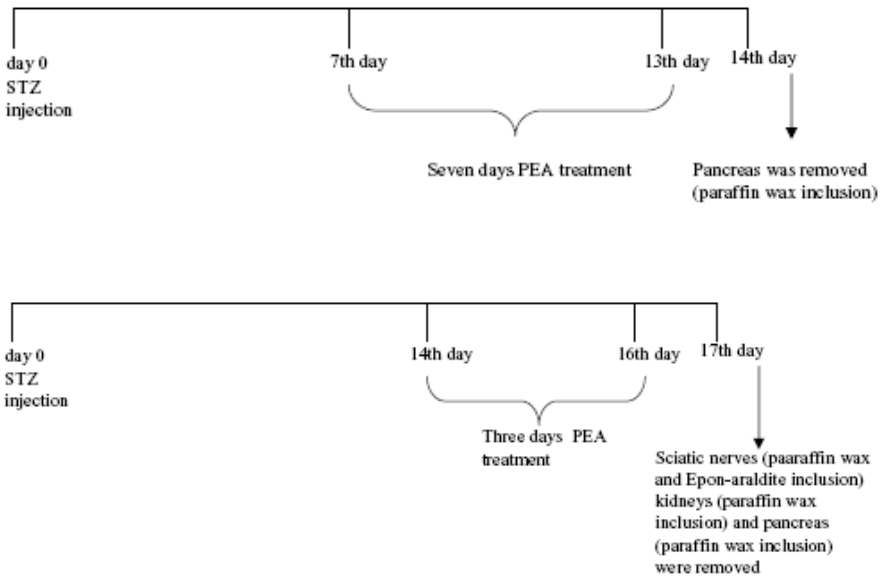


Scheme 3

In each group three experimental groups were considered: the non diabetic and diabetic mice treated with vehicle and diabetic mice treated with PEA (n=3 for each experimental group).In the first group (called 14d) PEA treatment (10 mg/kg, i.p.) started on 7th day from the STZ administration. PEA was administered for seven consecutive days. On 14th day, 24 hours after the last administration, mice were sacrificed and pancreas was removed to perform a histological analysis. Before the beginning of the treatment and 24 hours after the last administration of PEA (on 14th day) a sample of blood was taken in order to measure both glucose level and insulin content.

In the second group (called 17d) PEA treatment (10 mg/kg, i.p.) started on 14th day from STZ administration and PEA was administered for three consecutive days. On 17th day, 24 hours after the last administration, mice were sacrificed and pancreas, kidneys and sciatic nerves (both right and left) were removed to perform a histological analysis. The sciatic nerves were removed to also evaluate the content of NGF level. (Scheme 4)

Before the beginning of the treatment and 24 hours after the last administration of PEA (on 17th day) a sample of blood was taken in order to measure both glucose level and insulin content. NGF level content in the sciatic nerve and the insulin level content in the blood were evaluated by pharmacological lab of Dr Costa.



Scheme 4: *Mice were divided into two groups (3d and 8d), and into each group the three experimental groups were present. PEA (10mg/kg, i.p.) was administered for two or for seven days)*

Mechanical allodynia

Mechanical allodynia was measured with Dynamic Plantar Aesthesiometer; these evaluations were performed in all experimental groups of 17d group before the streptozotocin injection (0 day), before PEA treatment started (on 14th day) and 24hours after the last administration with PEA (on 17th day) by pharmacological lab of Dr Costa.

Histology of mouse sciatic nerve: cross and longitudinal sections

Left and right sciatic nerves (group 17d) were processed to embed them respectively in paraffin wax and in Epon Araldite resin. The same protocol and same timetable employed for peripheral mononeuropathy was used.

Longitudinal (6 µm) sections of sciatic nerves were stained with the 0,5% toluidine blue to characterize mast cell morphology. Semi-thin cross sections (1 µm) were stained with 0,2% toluidine blue to study myelin thickness and its degeneration. The protocols employed were previously described.

Histology of mouse pancreas and kidneys

Pancreas (group 14d and 17d) and kidneys (group 17d) were fixed in 4% paraformaldehyde fresh solution overnight at 4°C, dehydrated in serial alcohol concentrations, soaked in a xylene solution and embedded in paraffin wax. Pancreas and kidneys were cut with a rotary microtome to obtain respectively longitudinal and cross sections (6 µm).

Pancreas sections were stained with Mayer's haematoxylin and eosin to determine both the total number and the area of islet of Langerhans. The definition of an islet was chosen as a cluster of cells with a minimum of four visible nuclei displaying the typical characteristics of islet cells, as suggested by literature.

Kidney cross sections were stained with Periodic Acid Schiff (PAS) reagent to evaluate the general morphology of glomerular structure and with Mayer's haematoxylin and eosin to measure the glomerular area (μm^2).

Cell counting and image analysis

All sections were photographed with a light Zeiss Axioplan MC 100 microscope using AxioVision Rel 4.6 software. Images were taken with a colour digital camera (AxioCam MRc 5, Zeiss). Obtained immunofluorescence images were merged with specific software (Merge, version 2.1).The analyses were performed by an experimenter blind to pharmacological treatment.

The mast cell counting was performed on longitudinal sections of the right sciatic nerve in each experimental group. Mast cells were divided into intact mast cells, in degranulation and degranulated mast cells and the total mast cell number were divided by the total area of the nerve in which cells were counted to derive the mean number of mast cells/mm^2. The sum of mast cells in degranulation and degranulated mast cells were divided with intact mast cells to obtain the average ratio of degranulated mast cells over intact cells.

The intact axon counting was performed on transversal sections of the right sciatic nerve in each experimental group (about 30 sections for

each experimental group). 3 areas were randomly selected for the intact myelinated fibers quantification. The total number of intact axons was counted and the average was divided by the area considered (intact axons/mm^2). A total of 30 intact axons for each experimental group were considered to evaluate the myelin thickness.

About 80 sections of pancreas tissue and 20 islets of Langerhans (for each experimental group, 14d and 17d groups) were considered to determine respectively the total number of islet of Langerhans and the area of islet of Langerhans (μm^2). The total number of islets of Langerhans was divided by the pancreas area where islets of Langerhans were counted (number of islet of Langerhans/mm^2).

About 20 glomeruli of kidney cross-section of each experimental group were considered to evaluate the area of glomeruli (17d group) when the vascular pole was visible.

The thicknesses of about intact axon myelin, the area of islet of Langerhans and glomeruli were measured employing the AxioVisio Rel 4.6 software.

Statistical analysis

Data were expressed as the mean ± SEM and analyzed using one-way repeated measures analysis of variance (ANOVA) followed by Newman-Keuls test. Differences were considered significant at $P < 0.05$. All statistical analysis was performed using PRISM 4.01 (GraphPAD Software, San Diego, CA).

Results

Peripheral mononeuropathy

Effect of PEA on thermal hyperalgesia and allodynia

In order to confirm the development of thermal hyperalgesia and mechanical allodynia, two behavioural tests were performed: the plantar test (thermal hyperalgesia, Hargreaves method) and the Dynamic Plantar Aesthesiometer (mechanical allodynia) were used.

As expected all CCI mice developed hyperalgesia and allodynia one day after the injury compared to sham mice (Figs.15A,B), and CCI mice treated with a repeated administration of PEA showed a significant relief of both thermal hyperalgesia and mechanical allodynia (Figs.15A,B). In particular, PEA significantly attenuated both painful symptoms after only two administrations and restored the physiological nociceptive response to thermal stimulus after one week of treatment, while the withdrawal threshold to non-noxious mechanical stimulus wasn't superimposed on basal one (Figs.15A,B).

Effect of PEA on NGF level in the sciatic nerve

NGF content in the right sciatic nerve, evaluated with ELISA kit, was measured in the three experimental groups: control (sham/vehicle and CCI/vehicle) mice and CCI mice treated with PEA at the end of the treatment in injured sciatic nerve. As expected NGF level in the injured sciatic nerve significantly increased after 8 days from the surgery compared to control (sham/vehicle) mice, to a value of about 170% (Fig.16).

The pharmacological treatment for a whole week with PEA restored the NGF level to physiological level; in fact the NGF level was

superimposable to that obtained in control (sham/vehicle) mice (Fig.16).

Effect of PEA on mast cell activity

Fig.17 shows longitudinal sections stained with acidified (0,5%) toluidine blue in the three experimental groups in 3d and 8d gropus. The longitudinal sections were stained with toluidine blue to observe the sciatic nerve morphology and to identify the mast cells. Toluidine blue is a basic staining with metachromatic property: it stains the nucleus of the cell (Schwann cell and mast cell nucleus), and the granules of mast cells containing heparin and histamine. The Fig.17 (right side of panel A) shows a particular of the longitudinal sections of the right sciatic nerve. Mast cells with a clearly outlined cell membrane and dense granules were defined as not active, mast cells with an elongated form and with intensily blue staining are in degranulation, while mast cells with heavily stained granules scattered outside the cell membrane were defined as degranulated.

The sciatic nerve of control (sham/vehicle) mice of 3rd and 8th day were comparable, therefore we included only one image. As expected it appeared normal with no oedema or inflammation. Organized fiber and resident mast and nuclei of Schwann cells were present and uniformely distributed (Fig.17A).

Nerve injury caused oedema, which is more evident after 8 days from the injury and in the sciatic nerve of CCI mice treated with vehicle a significant recruitment of mast cells was clear: this recruitment was confined in the side region of the sciatic nerve after 3 days after the injury (Fig.17B), with an infiltration in the inner part of the nerve after 8 days after the injury (Fig.17D). At this time mast cell morphology

was changed with many degranulated cells. Sciatic nerve of PEA treated mice presented an improvement of inflammatory state and PEA appeared to protect sciatic nerve from oedema formation and also to protect mast cell against recruitment and degranulation (Figs.17C,E). This morphology was also confirmed in immunostained sections with MMCP1 antibody which is directed against mast cell proteases (Fig.18).

The density of mast cells (mast cell number/mm^2) in control (sham/vehicle) group is about ±1460 both 3 and 8 days after the surgery. The mast cell density in CCI mice significantly increased (56%) 3 days after the injury, and such increase was stable 8 days after the injury. Mice treated with two doses of PEA (3d group) showed a significantly lower density than that found in the corresponding CCI mice, whereas after a week of treatment no significant difference was found in CCI mice treated with vehicle (Fig.19A). The average ratio of degranulated mast cells over not active cells was significantly higher in the sciatic nerve of CCI mice 8 days after the injury than in control group (8 fold increase). After seven PEA administrations the average ratio of degranulated mast cells over not active cells was the same as in non pathological mice (Fig.19B).

Colocalization of mast cell proteases and trkA receptor

Fig.20 represents the double immunofluorescence performed in order to confirm the expression of trkA receptor on mast cells. The panel A represents the immunofluorescence of mast cell proteases (MMCP-1) with a green signal (Fig.20A), the panel B represents the

immunofluorescence of trkA receptor with a red signal (Fig.20B) and panel C represents the superimposition (merge) of the two previous images (Fig.20C).

Figs. 7 and 8 show representative images of longitudinal sciatic nerve immunolabeled with specific antibodies at original magnification 40x. Sciatic nerve of control (sham/vehicle) mice weren't included because of the weak signal of mast cell proteases and trkA receptor.

3 days after the surgery an elevated immunofluorescence signal of mast cells and trkA receptors expression was observed in CCI mice (Figs.21A,B), and an evident signal is present in the side region of the sciatic nerve. In the representative figure of merge (Fig.21C) an overlap of mast cells and trkA could be observed. .After a repeated treatment with PEA for two consecutive days the immunofluorescence signal was lesser than in CCI mice (Figs.21D,E) and the two signals were completely overlapped (Fig.21F).

8 days after the surgery a less difference between control mice (CCI/vehicle) and CCI treated with PEA could be observed both for mast cell proteases (Figs.22A,B) and trkA receptor (Figs.22D,E). Figs.22C,F show a complete colocalization of mast cell proteases and trkA receptor.

Effect of PEA on sciatic nerve demyelination

Wallerian degeneration of myelinated fibers occurs in the chronic constriction injury. In order to verify and confirm the Wallerian degeneration presence, transversal semi-thin sections were stained with toluidine blue and a morphological analysis was performed. Sciatic nerve of control (sham/vehicle) mice appeared essentially normal with regular scaffold, a uniform distribution of small and large

diameter myelinated fibers and with a regular proportion between myelin sheath thickness and fiber diameter (Fig.23A, only one image was included for the two times). 3 days after the injury, many signs of Wallerian degeneration occurred: in particular, endoneural oedema, 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, were detected (Fig.23B). Fiber degeneration was observed even 8 days after the injury (Fig.23D). After two PEA administrations no evident amelioration was detectable (Fig.23C); after seven PEA administrations, there was axonal regeneration evidence, although the presence of some remaining degenerating fibers and groups of small thinly myelinated fibers were observed (Fig.23E).

The average density of normal nerve fibers in the nerve of control (sham/mice) group 3 days after the surgery was 11656 fibers/mm^2, as expected for a mouse sciatic nerve, with no differences as compared to that of control mice 8 days after surgery (Fig.24A), or to that of the sciatic nerve from the paw contralateral to the injury site (data not shown). The analysis of the sciatic nerves from CCI mice highlighted, as expected, a significant reduction of the average density of normal fibers: 3 days and 8 days after the injury the nerve fiber density were respectively 2555 fibers/mm^2 and 2622 fibers/mm^2. A reduction of normal fiber density is still present in mice treated with two administrations of PEA (3d group). This reduction (4154 fibers/mm^2) was significantly lesser than in CCI mice treated with vehicle. In CCI mice treated with PEA for a week (8d group) fiber density revealed a

significant increase (5340 fibers/mm2) compared to CCI mice (Fig.24A).

The same images were used to determine myelin thickness. In CCI mice treated with vehicle the myelin thickness was significantly lower (about 40%) than in control (sham/vehicle) mice both 3 days and 8 days after the surgery. The repeated treatment with PEA for two (3d group) and seven (8d group) days protect against myelin thickness reduction, in fact the myelin thickness of CCI mice treated with PEA was comparable to that of non pathological-mice (Fig.24B)

Effect of PEA on activated microglia

Resident microglia are a type of glial cell of the brain and spinal cord and when activated, microglia plays a key role in the maintenance of neuropathic pain In order to verify the presence of activated microglia in the chronic constriction injury of the sciatic nerve, a well established model of neuropathy, transversal sections of lumbar spinal cord was immunolabeled.

Fig.25 shows representative images, stained with hematoxylin and eosin at original magnification 40x of the right side of dorsal horn of the lumbar (L4-L5) spinal cord. L4 and L5 dorsal horn region was considered because afferent fibers of the sciatic nerve arrive here.

The morphology and different cell types were appreciable in all three experimental groups both 3 and 8 days after the surgery. Tissue appeared undamaged in both 3d and 8d groups, with no oedema or inflammation. Neurons appeared bigger than glial cells (microglia, astrocytes) with a voluminous cytoplasm and nucleus.

Other transversal sections were immunolabeled and counterstained with hematoxylin, to evaluate the presence of activated microglia in

the lumbar spinal cord (L4-L5). Figs.26 and 27 show representative images of the right dorsal horn L4 and L5 3 and 8 days after the injury, respectively (only one image of control mice was included because they were comparable). Microglia after immunofluorescence appeared deep pink.

Microglia activation was more prominent in CCI mice treated with vehicle (Figs.26B,C) compared to control (sham/mice, Fig.26A) and its activation appeared in a time-dependent manner. Microglia activation could also be observed in CCI mice treated with PEA for 7 days (Figs.26D,E), but not in CCI mice treated with PEA for 3 days (Figs.12D,E). Microglia activation and microglia morphology could be observed in sections processed with immunofluorescence (inserts in the right top of Figs.26,27). Activated microglia appeared with a rounded red signal in the dorsal horn of L4 and L5 in CCI mice treated with vehicle and CCI mice treated with PEA 3 and 8 days after the surgery.

The contralateral dorsal horn of CCI mice treated with vehicle showed a less prominent activation of microglia than in control (sham/vehicle, Figs.28A,D) mice at the two different times (Figs.28B and E), while it didn't occur in CCI mice treated with PEA (Figs.28C and F).

The densitometric analysis of F4/80 was performed in order to evaluate the presence activated microglia. The F4/80 immunoblot was performed on the three experimental groups 3 and 8 days after the surgery (nitrocellulose not included). Fig.29 shows the densitometric analysis of F4/80 expression: activated microglia was significantly increased in CCI mice treated with vehicle compared to control

(sham/vehicle) mice 3 and 8 days after the injury, and this activation increase in a time-dependent manner ($P<0.001$).

The microglia activation in CCI mice treated with PEA for two consecutive days (3d group) was completely overlapped to control (sham/vehicle) mice, while the repeated treatment of 7 days (8d group) with PEA revealed a partial modulation of microglia activation (Fig.29).

Diabetic polyneuropathy

Effect of PEA on diabetes-induced mechanical allodynia

The main symptom of diabetic polyneuropathy, induced with a high dose of streptozotocin (STZ) injection (120mg/kg), is the mechanical allodynia. Mechanical allodynia was performed 14 days from the streptozotocin injection to verify its development and after three days of PEA treatment to evaluate its efficacy. As expected, diabetic mice treated with vehicle developed mechanical allodynia. In particular after two weeks from streptozotocin injection mice developed a significant decrease in paw withdrawal threshold to von Frey filament (Fig.30) compared to control (non-diabetic/vehicle) mice. This reduction remained constant even after the end of treatment both for non-diabetic and diabetic mice treated with vehicle. The repeated treatment of PEA for three consecutive days reported the mechanical threshold to physiological levels (Fig.30).

Effect of PEA on hyperglycaemia

To verify the development of hyperglycaemia and the PEA effect on it, the blood glucose content was evaluated with a glucometer 7 days

(Fig.31A) and 14 days (Fig.31B) after streptozotocin injection, and after 7 and 3 PEA administrations.

Before the PEA treatment started all mice injected with STZ developed hyperglycaemia. The hyperglycaemia was still present in diabetic mice treated with vehicle at the end of the experiment, 14 (Fig.31A) and 17 (Fig.31B) days after diabetes induction. In PEA-treated mice for 7 (Fig.31A) or 3 days (Fig.31B) the hyperglycaemia was comparable to that observed in diabetic mice.

Effect of PEA on NGF level in the sciatic nerve

In diabetic mice the NGF content decrease and this decrease is associated with the development of neuropathic pain, so the NGF content was measure in the sciatic nerve in the three experimental groups at the end of the experiment.

As expected, in diabetic mice after 14 days from the streptozotocin injection the NGF level content was significantly less than in non diabetic mice. This course was constant even after the end of the experiment, 17 days after the streptozotocin injection (Fig.32). In mice receiving PEA for three consecutive days the NGF level content in the sciatic nerve was statistically equal to control mice (non-diabetic/vehicle) and to diabetic mice treated with vehicle ($P>0.05$).

Effect of PEA on mast cell activity and on sciatic nerve demyelination

Neuropathy is a long term consequence of diabetes: the involvement of mast cell and a morphological characterization of Wallerian degeneration were performed and the results are shown in Tab 2.

The mast cell density (mast cell number/mm^2) and the normal fiber density (normal axons/mm^2) were respectively performed in longitudinal and transversal sections in the three experimental groups 17 days from the STZ injection.

The count of total mast cell stained with acidified toluidine blue didn't show any difference among the three experimental groups. The images revealed a uniform presence of non active and degranulated mast cells (images not shown).

The count of axons with an intact structure and myelin was performed on transversal semi-thin sections of the three experimental groups (17d group), stained with toluidine blue. The analysis of axons revealed that at the end of treatment diabetic mice treated with vehicle showed a significant decrease of the number of intact axons (about 70%), and that this decrease remained constant in diabetic mice treated for three consecutive days with PEA.

Effect on PEA on insulin level

Insulin content was measured in order to evaluate the insulin products in diabetic mice and after a repeated treatment with PEA. The insulin content (exspressed as µg/l), as showed in Fig 33, was evaluated following two different schemes. The first was measured in only diabetic but not allodinic mice and was measured 7 days after the streptozotocin injection, followed by an evaluation after a repeated treatment with PEA for 7 consecutive days. The second scheme consisted in an evaluation performed 14 days after the streptozotocin injection and it was measured in diabetic and neuropathic mice, followed by an evaluation after a repeated treatment with PEA for 3 days (Fig.33). As expected 7 and 14 days after the streptozotocin

injection a significantly reduction of insulin level was observed in diabetic mice compared to non diabetic mice. On 14th day a mild production of insulin could be appreciated in diabetic mice treated with PEA for 7 consecutive days. On 17th day no production of insulin occurred in diabetic mice treated with PEA for 3 consecutive days (Fig.33).

Islet of Langerhans histology

Streptozotocin is a cytotoxic substance, and exerts its toxicity destroying pancreas β-cells. Microscopically islets of Langerhans appear smaller and they decrease in number. The islet of Langerhans was characterized in the three experimental groups and after 3 (14d group) and 7 (17d group) PEA administration and the histological examination of stained pancreas are reported in Figs.34 and 35.

14 and 17 days after streptozotocin injection pancreatic tissue stained with hematoxylin and eosin appeared normal in non diabetic mice: in fact a homogeneous distribution of large and well organized islets of Langerhans appeared (Figs.34A,D and 35A,D). After diabetes induction islet of Langerhans number appeared to decrease and the islets appeared smaller than in non diabetic (Figs.34B,E and 35B,E). In mice treated with PEA for 7 consecutive days a mild improvement (Figs.34C,F and 35C,F) was observed. About 60 sections (for each experimental group) were used to count the total number of islets of Langerhans (expressed as Islet of Langerhans number/mm^2) and about 20 islets of Langerhans (for each experimental group) were used to measure the area of islets of Langerhans in each experimental group at the two different times.

The morphological analysis confirmed that the density of islets of Langerhans was significantly decreased (about 60%) in diabetic mice treated with vehicle 14 days after the streptozotocin injection .This decrease was not observed in pancreatic tissue of diabetic mice treated with PEA 14 days after the streptozotocin injection (Fig.36A). The area of islets of Langerhans of diabetic mice treated with vehicle was significantly smaller (6375±370μm^2) than in non diabetic mice treated with vehicle (8213±480μm^2). The repeated treatment for 7 consecutive days with PEA prevented the total reduction of islet of Langerhans area (7452±170μm^2) (Fig.36B).

The quantitative analysis of pancreas sections 17 days after the streptozotocin injection revealed that the density of islets of Langerhans decreased about 80% in diabetic mice treated with vehicle compared to non diabetic mice (Fig.37A). The treatment with PEA for 3 days highlighted the partial protective effect on the islets of Langerhans (Fig.37A).

As expected the area of islets of Langerhans were smaller in diabetic mice (4108±76) treated with vehicle than in non diabetic mice (8455±533) (Fig.37B). The repeated treatment with PEA for 3 consecutive days with PEA partially prevented the decrease of area of islets of Langerhans (5906±460) (Fig.37B).

Kidney histology

Nephropathy, such as neuropathy, is a consequence of chronic hyperglycaemia and we investigated the development of nephropathy in diabetic and neuropathic mice and the effect of 3 PEA administrations on the development of the first phase of nephropathy.

Representative images of glomeruli in PAS-stained sections are shown in Fig.38. PAS-stained transversal sections of diabetic mice showed an increase of mesangial matrix and subsequently an increase of the glomeruli area compared to non-diabetic mice (Figs.38A,B). The transversal sections treated with three consecutive injections of PEA appeared similar to the images observed in diabetic mice treated with vehicle (Fig.38C).

About 20 glomeruli of each experimental group were used to evaluate the area (expressed in μm^2) to confirm the increase of mesangial area. Fig.39 shows the area of the three experimental groups on 17th day after the streptozotocin injection. The area, expressed as μm^2, increased 20% in diabetic mice treated with vehicle compared to control (non-diabetic/vehicle) mice after 2 weeks from the streptozotocin injection. The same increase was measured in diabetic mice treated with PEA for three consecutive days (Fig.39).

Figures

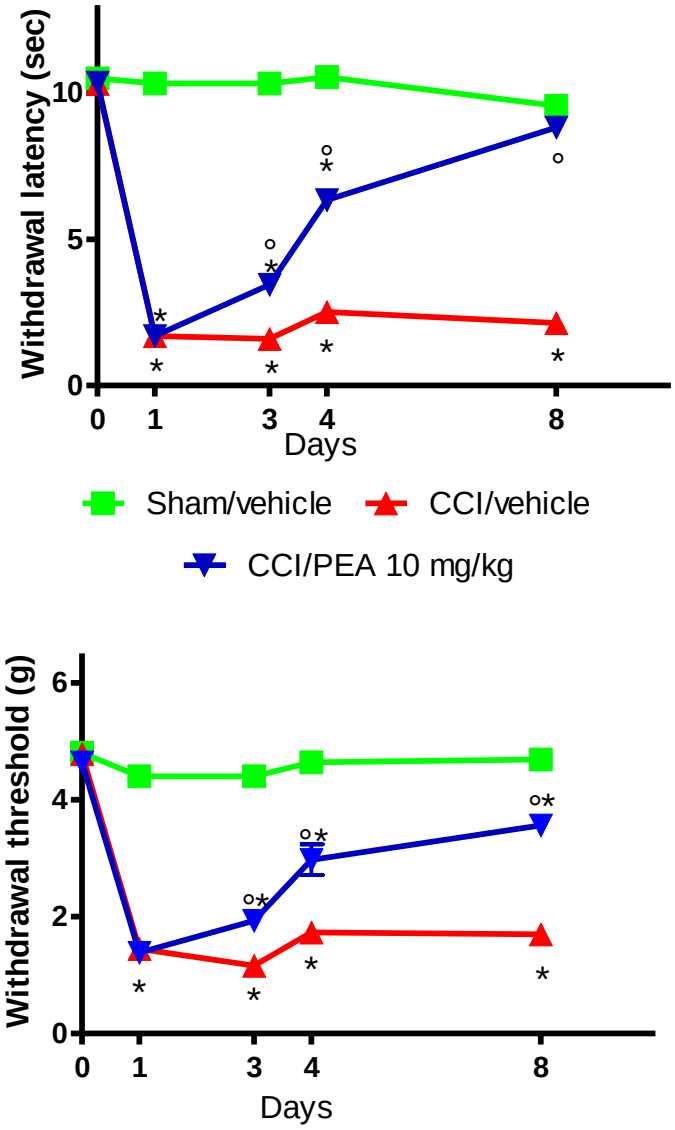


Fig.15: Effect of palmitoylethanolamide (PEA) 10 mg/kg i.p. administered to neuropathic (CCI) mice the day after the injury, on thermal hyperalgesia (A) and mechanical allodynia (B) at different time points after the treatment. Withdrawal latency to heat and mechanical allodynia of the injured paws are expressed as s and g, respectively. Data represent mean ± SEM of 9 mice. *P < 0.05, vs sham/vehicle; °P<0.01 vs CCI/vehicle. (One-way ANOVA)

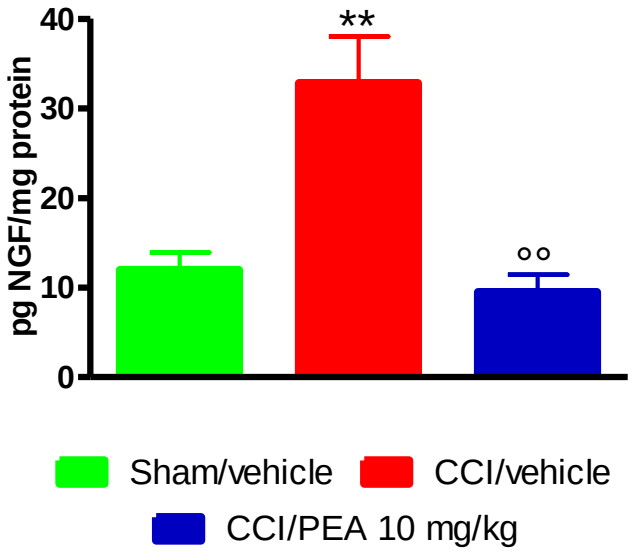


Fig.16: Effect of repeated treatment with palmitoylethanolamide (PEA) (10 mg/kg i.p., once daily for one week) to neuropathic (CCI) mice on NGF in injured sciatic nerve, 24 hours the last administration with PEA. Data represent mean ± SEM of 9 mice. **P<0.01 vs sham/vehicle; °°P < 0.01, vs CCI/vehicle (One-way ANOVA)

(Costa et al., 2008)

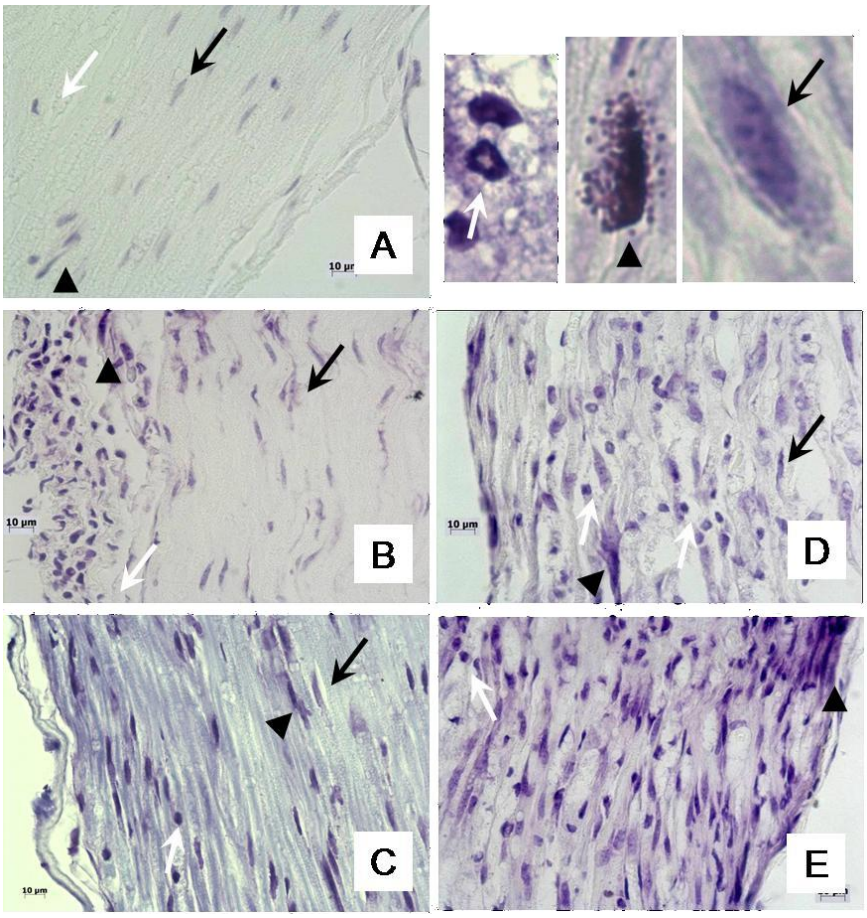


Fig.17: Light micrography of mast cell (A-E) stained with toluidine blue in longitunal sections of injured sciatic nerve in each experimental group: sham/vehicle (A, one figure of control sciatic nerve was shown for both groups), CCI/vehicle (B) CCI/PEA (10mg/kg, i.p). (C) on 3rd day; CCI/vehicle (D) CCI/PEA (10mg/kg, i.p) (E) on 8th day. Toluidine blue is basic staining with metachromatic property: it stains nucleus of Schwann cell and mast cells granules, containing histamine and heparin. Not active mast cells are identified with white arrow, degranulating mast cell are marked with black arrowhead and degranulated mast cells are marked with black arrow, as indicated in the particular at right top (original magnification 40x).

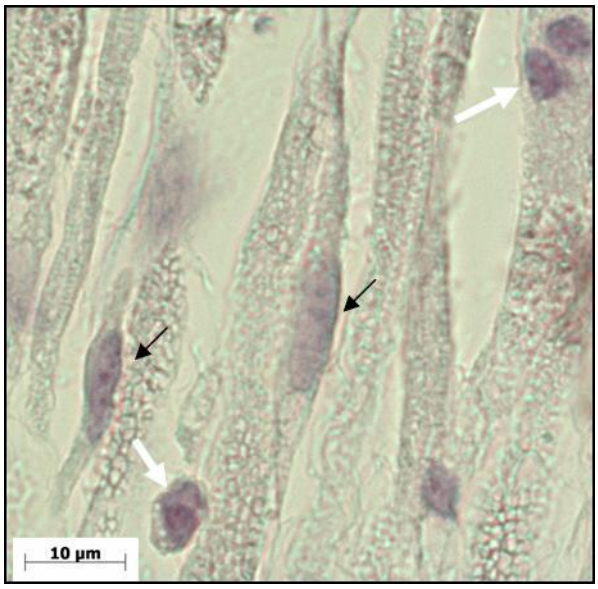


Fig.18: High magnification of mast cells and their granules stained with anti-mouse mast cell protease (anti-MMCP-I) (100x). White, black arrows and black arrowhead indicate respectively intact and degranulated mast cells.

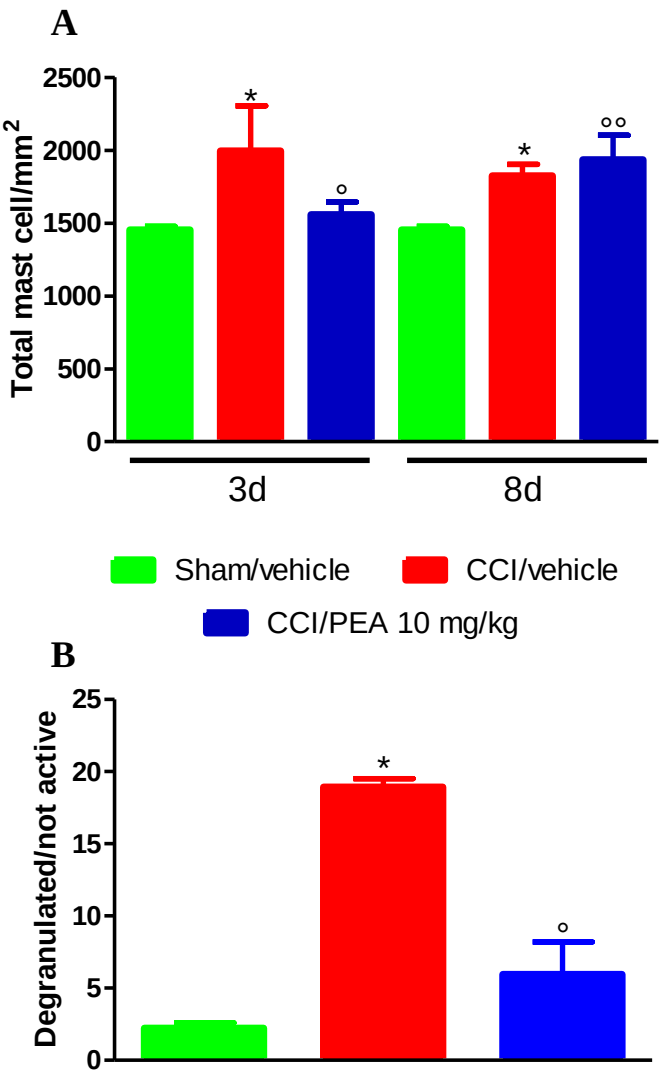


Fig.19: Density of mast cells in 3d and 8d group (A) and degranulated/non active mast cell ratio in 8d group (B) in injured sciatic nerve in the three experimental groups: sham/vehicle, CCI/vehicle and CCI/PEA (10 mg/kg, i.p.) at the two different times considered (3d and 8d). The sum of degranulated and degranulating mast cells represents the degranulated group. Data represent mean ± SEM of 5 mice. *P < 0.05, vs sham/vehicle; °P<0.05, °°P < 0.01 vs CCI/vehicle (One-way ANOVA, Newman-Keuls test)

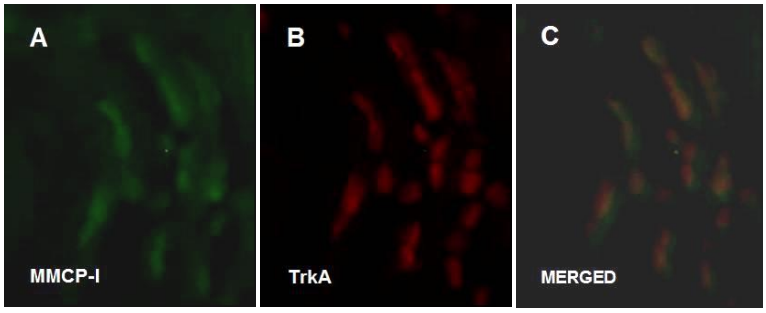


Fig.20: MMCP-I and trkA co-localization in longitudinal sections of injure sciatic nerve. Longitudinal sections were immunolabeled for mast cells proteases, MMCP-I (A) and for mast cell plasma membrane receptor, trkA (B). Panel C represent the co-localization of MMCP-I and trkA. Original magnification 40x.

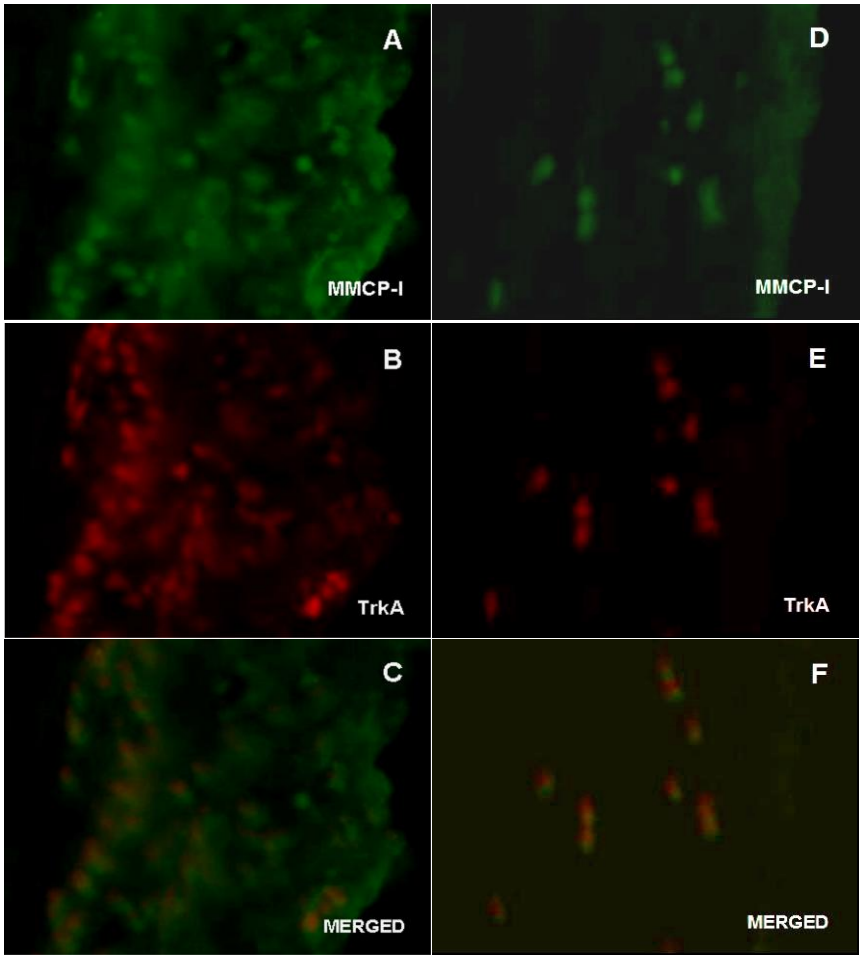


Fig.21: Immunofluorescence of longitudinal sections of injured sciatic nerve in the experimental groups 3 days after the injury: CCI/vehicle (A-B-C) and CCI/PEA (10 mg/kg, i.p.) (D-E-F). Longitudinal sections were immunolabeled with MMCP-I (A-D) and with trkA (E-F) antibodies. Panel C and F represent the co-localization of MMCP-I and trkA. Original magnification 40x.

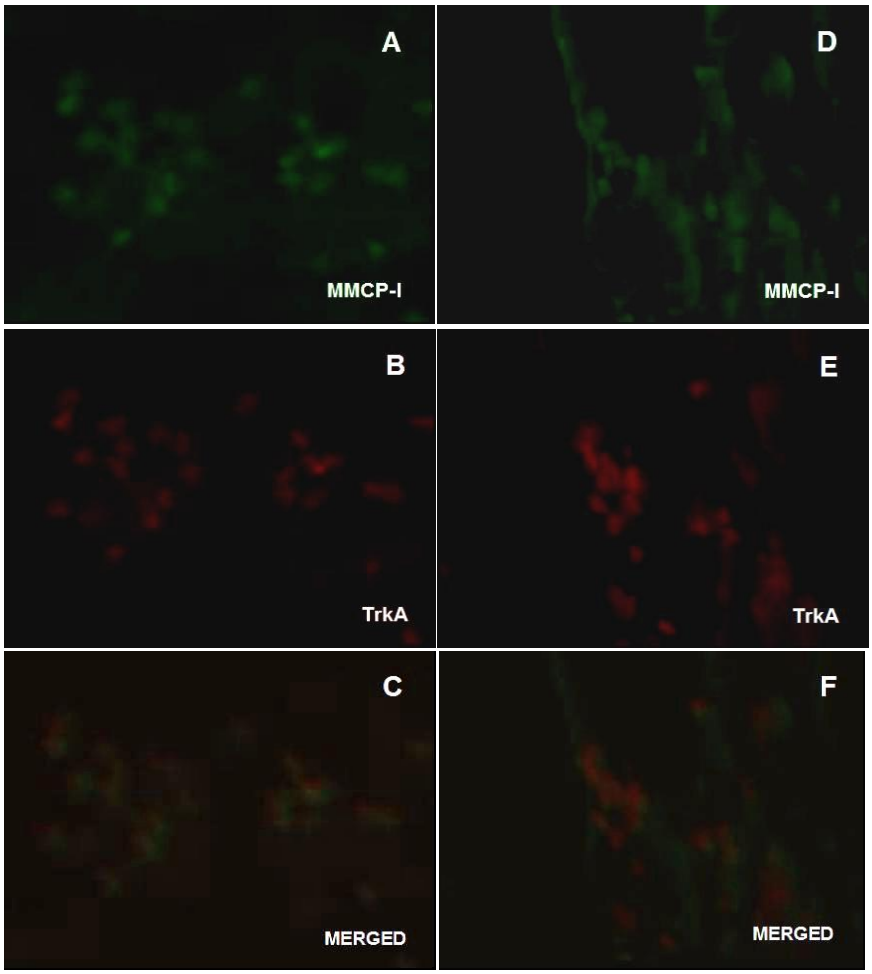


Fig.22: Immunofluorescence of longitudinal sections of injured sciatic nerves in the experimental group 8 days after the injury: CCI/vehicle (A-B-C) and CCI/PEA (10 mg/kg, i.p.) (D-E-F). Longitudinal sections were immunolabeled with MMCP-I (A-D) and with trkA (E-F). Panel C and F represent the co-localization of MMCP-I and trkA. Original magnification 40x.

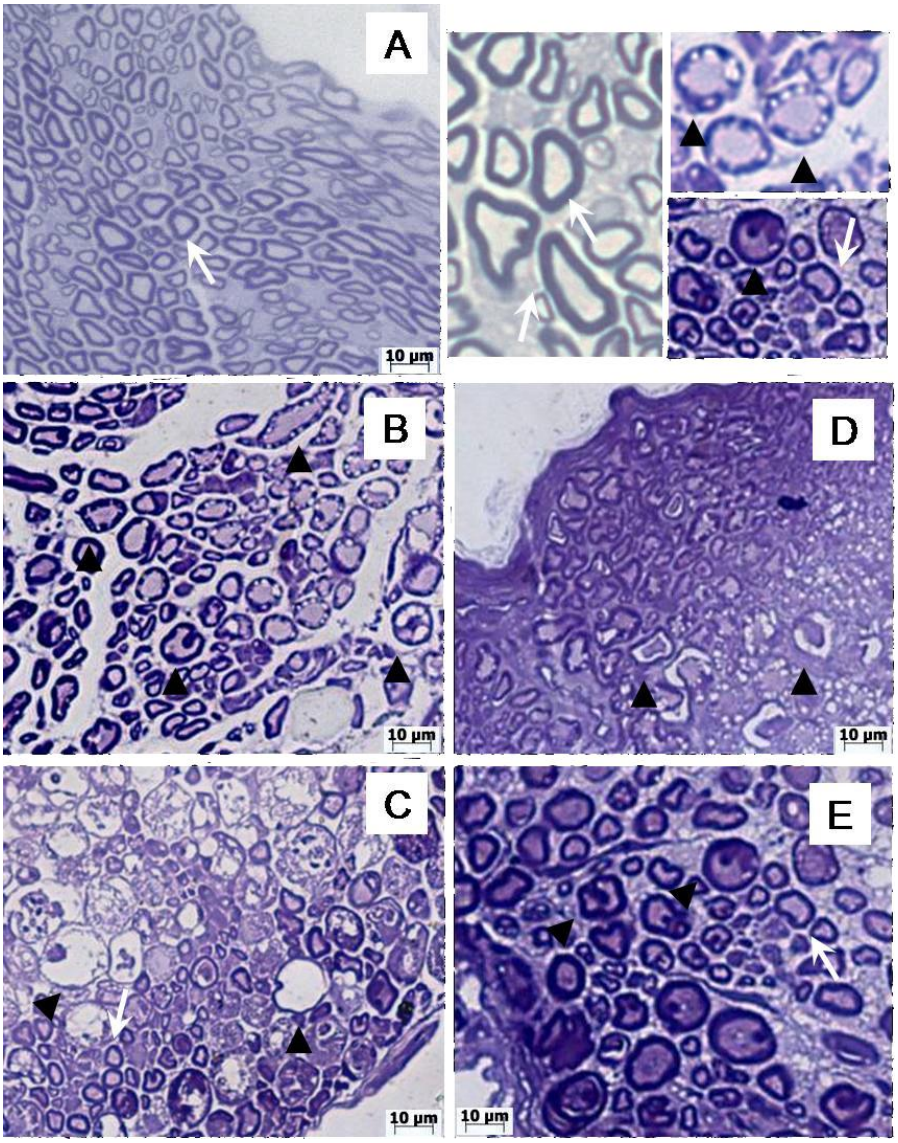


Fig.23: Light micrography of transversal semi-thin sections of injured sciatic nerve in each experimental group: sham/ vehicle (A, one figure of control sciatic nerve was shown for both groups), CCI/vehicle (B) CCI/PEA (10 mg/kg, i.p.) (C) on 3rd day; CCI/vehicle (D) CCI/PEA (10 mg/kg, i.p). (E) on 8th day. Intact myelinated fibers are indicate with white arrow, demyelinated fibers or fibers undergoing demyelination are indicated with black arrowhead as shown in the particular at right top. Original magnification 40x.

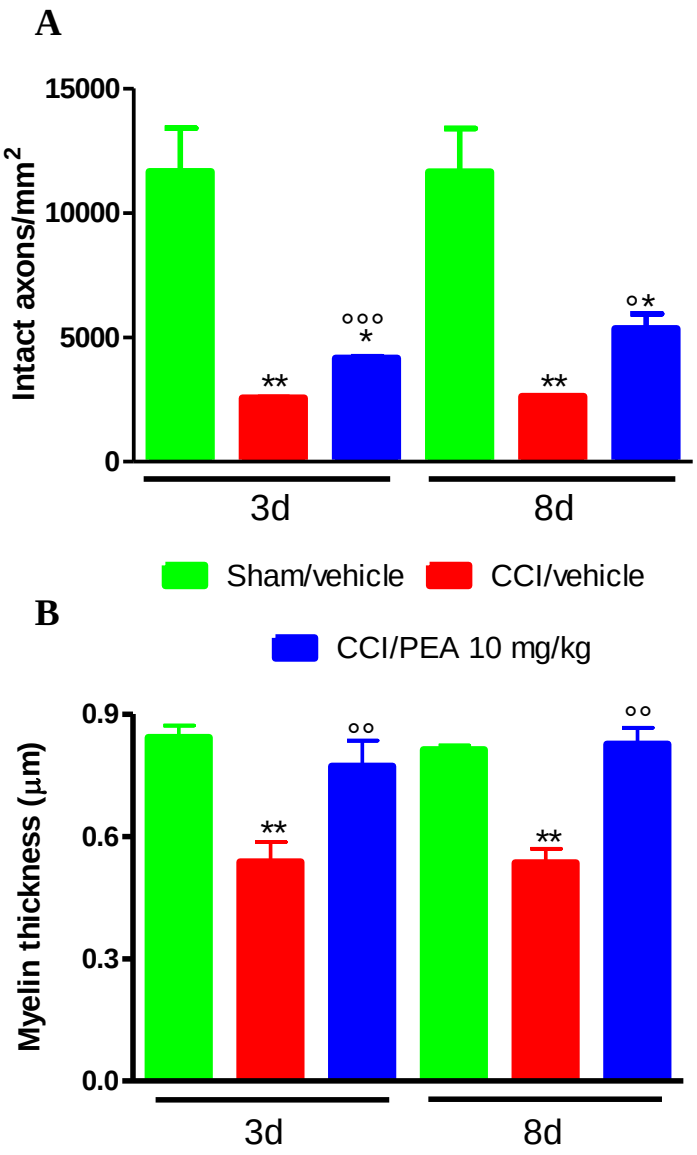


Fig.24: Density of intact axons (A) and myelin thickness of intact axons (B) in transversal semi-thin sections of injured sciatic nerve in each experimental groups: sham/vehicle, CCI/vehicle and CCI/PEA 10 mg/kg at the two different times considered (3d and 8d). Data represent mean ± SEM of 5 mice. *P < 0.05, **P<0.01 vs sham/vehicle; °P<0.05, °°P < 0.01, °°°P < 0.001 vs CCI/vehicle (One-way ANOVA, Newman-Keuls test)

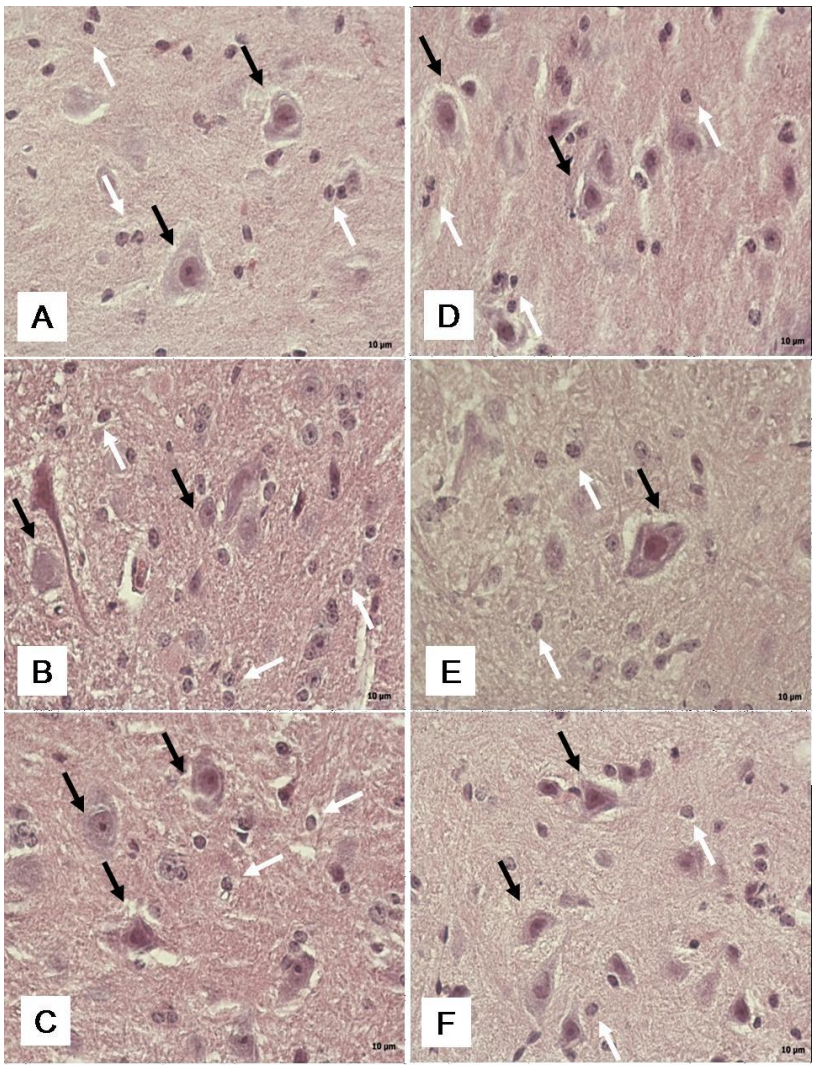


Fig.25: Representative images of transversal sections of the right dorsal horn of lumbar (L4-L5) spinal cord at the ipsilateral site. These images, stained with hematoxylin and eosin, represent each experimental group at the two different times considered: sham/vehicle (A), CCI/vehicle (B) and CCI/PEA (10mg/kg, i.p.) (C) on 3rd day; sham/vehicle (D) and CCI/vehicle (E) and CCI/PEA (10mg/kg, i.p.) (F) on 8th day. Black arrows indicate neurons, white arrows indicate glial cells. Original magnification 40x.

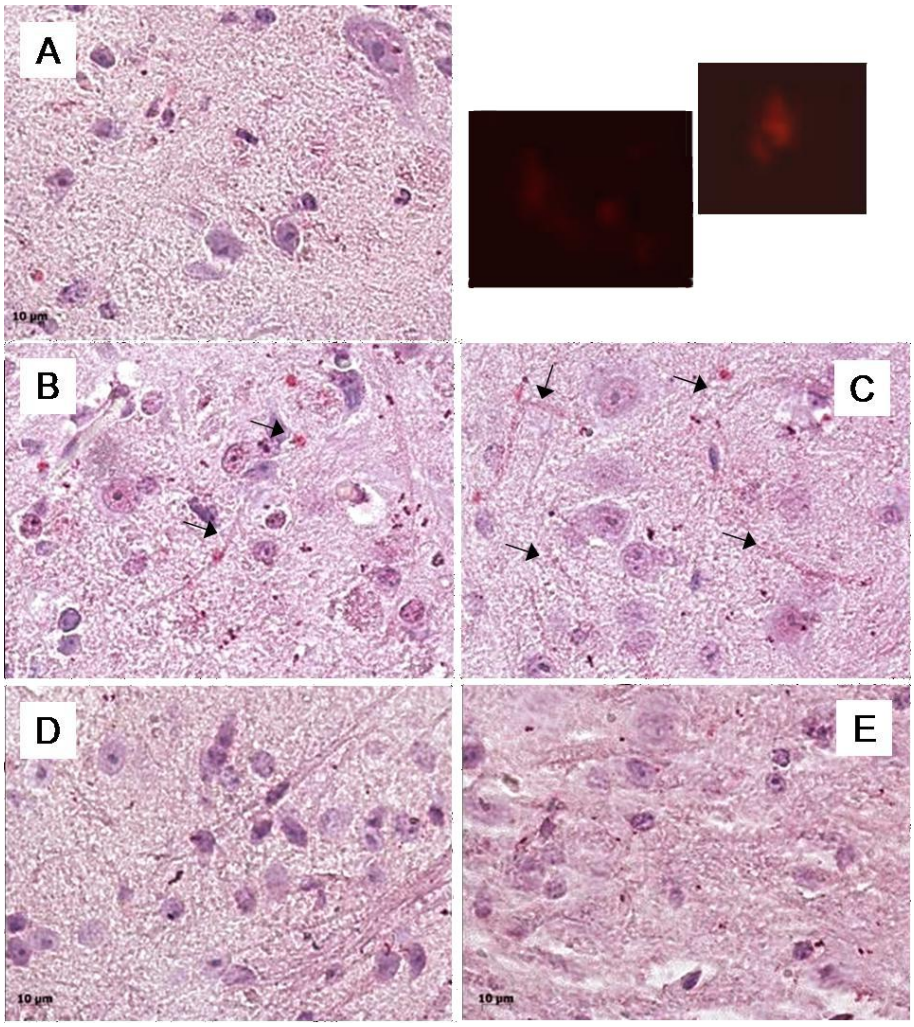


Fig.26: Representative images of transversal sections of the dorsal horn of lumbar (L4-L5) spinal cord at the ipsilateral site of the injury on 3rd day after the surgery. These images immunostained with F4/80 antibody and conterstained with hematoxylin represent each experimental group: sham/vehicle (A one figure for L4 and L5 was shown); CCI/vehicle (B L4 and C L5), CCI/PEA (10 mg/kg, i.p.) (D L4 and E L5). Black arrows indicate activated microglia, which is stained deep pink. Particular of activated microglia with a red signal because of secondary antibody conjugated to TRICT. Original magnification 40x.

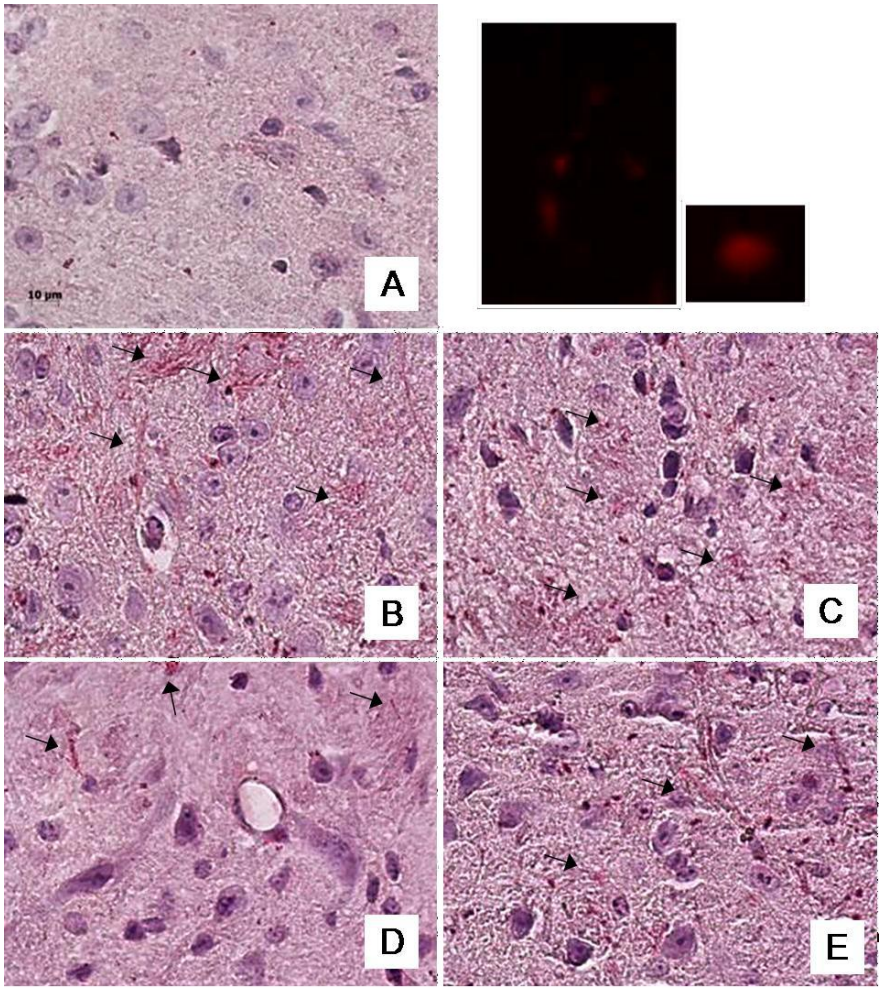


Fig.27: Representative images of transversal sections of the dorsal horn of lumbar (L4-L5) spinal cord at the ipsilateral site of the injury on 8th day from the surgery. These images immunostained with F4/80 antibody and conterstained with hematoxylin represent each experimental group: sham/vehicle (A one figure for L4 and L5 was shown); CCI/vehicle (B L4 and C L5), CCI/PEA (10 mg/kg, i.p.) (D L4 and E L5). Black arrows indicate activated microglia, which is stained deep pink. In the insert activated microglia with a red signal because of secondary antibody conjugated to TRICT. Original magnification 40x.

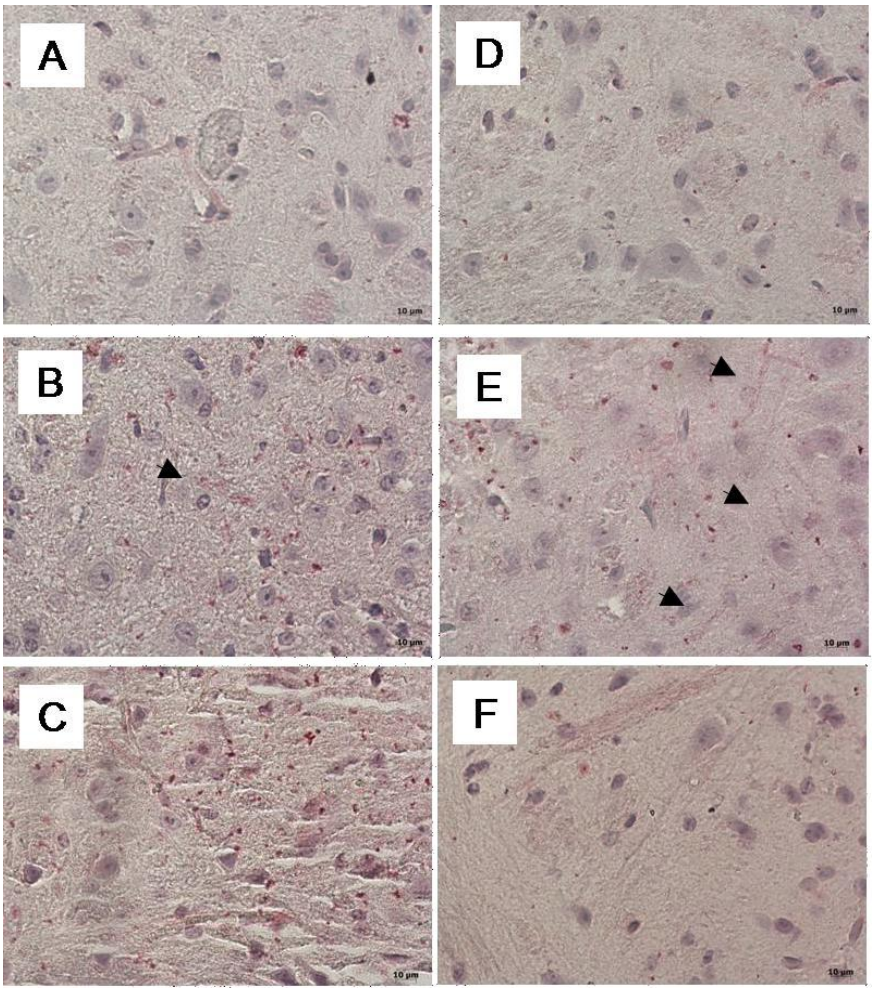


Fig.28: Representative images of transversal sections of the dorsal horn of lumbar (L4-L5) spinal cord at the contralateral site of the injury at the 3 and 8 days from the injury. These images immunostained with F4/80 antibody and conterstained with hematoxylin represent each experimental group: sham/vehicle (A l4-L5); CCI/vehicle (C L4-L5), CCI/PEA (10 mg/kg, i.p.) (E L4-L5) on 3rd day; sham/vehicle (A L4-L5); CCI/vehicle (C L4-L5), CCI/PEA (10 mg/kg, i.p.) (E L4-L5) on 8th day. Black arrows indicate activated microglia, which is stained deep pink. Original magnification 40x.

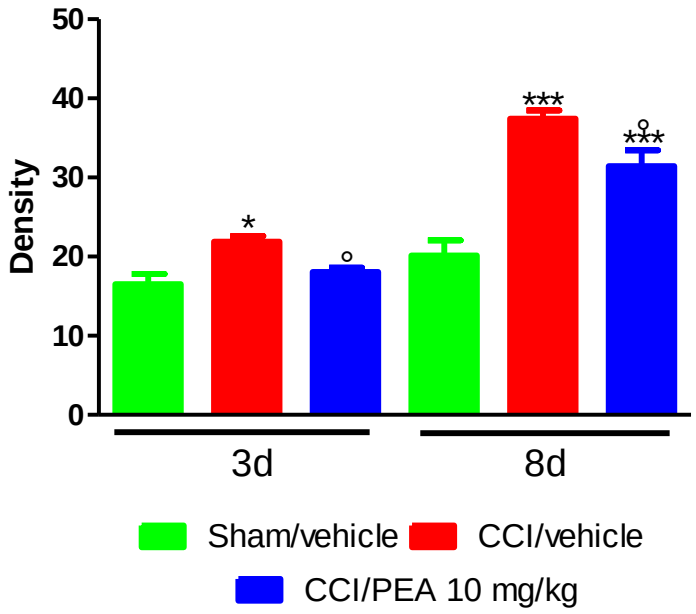


Fig.29: Densitometric analysis of F4/80 protein expression in lumbar spinal cord (L4-L5) in each experimental group at different times 3d and 8d: sham/vehicle, CCI/vehicle and CCI/PEA (10 mg/kg, i.p.). Data represent mean ± SEM of 3 mice. *P < 0.05, ***P<0.001 vs sham/vehicle; °P < 0.05, vs CCI/vehicle (One-way ANOVA, Newman Keuls test)

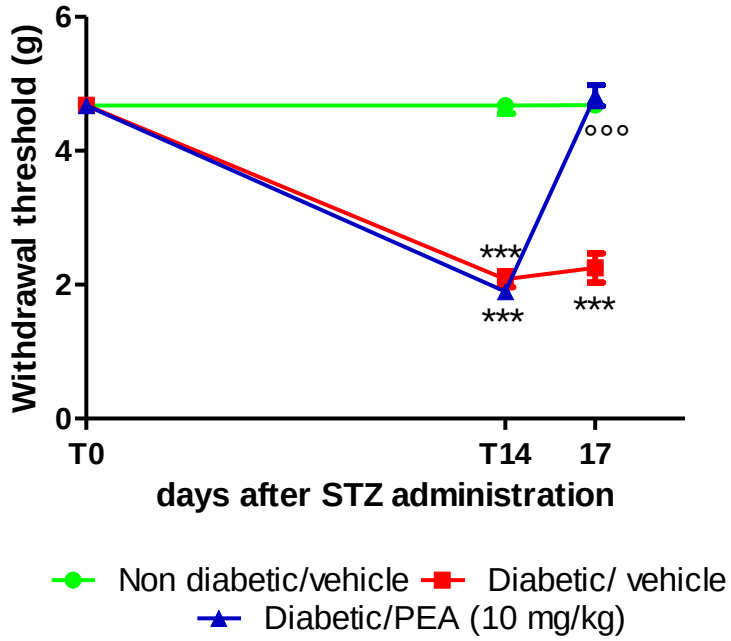
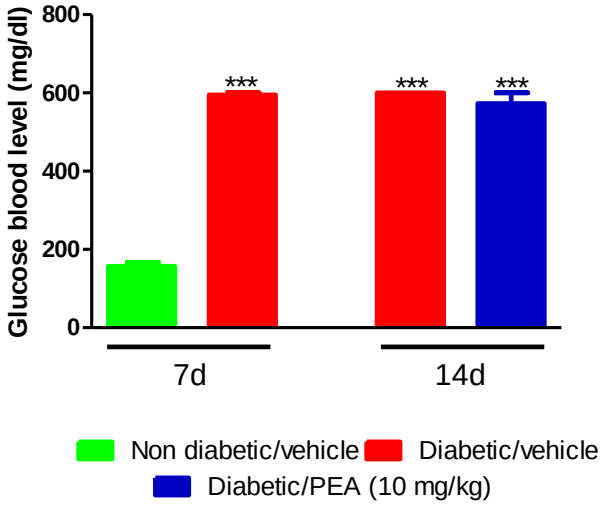


Fig.30: Effect of PEA 10 mg/kg i.p. on mechanical allodynia: PEA was administered for three consecutive days and 24h after the last administration mechanical allodynia was performed. Mechanical allodynia is expressed in g. Data represent mean ± SEM of 9 mice. ***P<0.001, vs non diabetic/vehicle; °°°P <0.001 vs diabetic/vehicle. (One-way ANOVA, Newman-Keuls test)

A



B

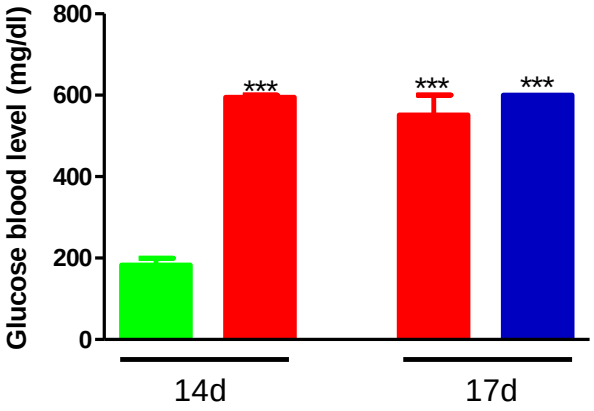


Fig.31: Effect of PEA on glucose blood concentration. PEA was administered on 7th day for seven consecutive days (A) and on 14th day for three consecutive days (B) and 24h after the last administration glucose blood concentration was performed. Glucose blood concentration is expressed as mg/dl. Data represent mean ± SEM of 9 mice. ***P < 0.001, vs non diabetic/vehicle (One-way ANOVA, Newman-Keuls test)

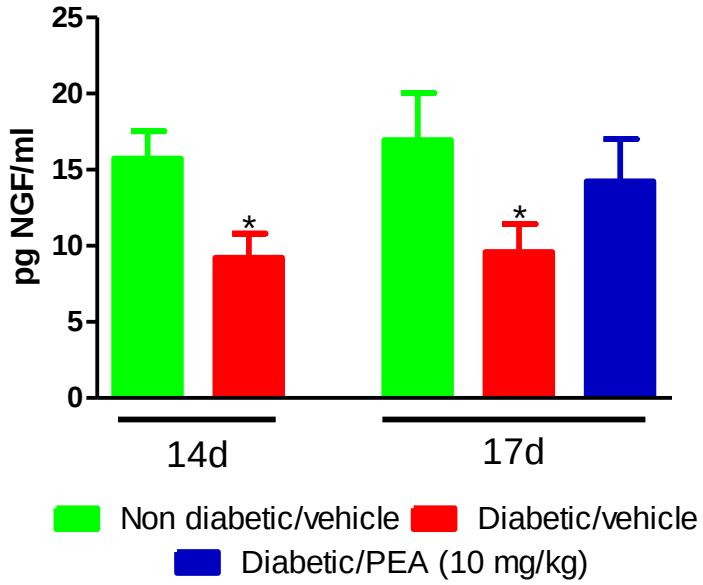


Fig.32: Effect of PEA on NGF content in the sciatic nerve. PEA was administered on 14th day for three consecutive days and 24h after the last administration NGF content was evaluated. NGF content is expressed as pg NGF/ mg of protein. Data represent mean ± SEM of 9 mice. *P < 0.05, vs non diabetic/vehicle (One-way ANOVA, Newman-Keuls test)

Experimental groups	***Mast cell number/mm²***	***Intact axons/mm²***
Non diabetic/vehicle	*582,7±72,18*	*7886±1160*
Diabetic/vehicle	*613,3±76,76*	*2501±128,7***
Diabetic/PEA	*623,0±44,79*	*2854±107,4**

Tab.2: density of mast cells and intact axons in sciatic nerve in non diabetic/vehicle, diabetic/vehicle and diabetic/PEA 10 mg/kg on 17th day, 24 hours the last administration of PEA. Data are expressed on the considered area. Data represent mean ± SEM of 9 mice. **P<0.01, *P < 0.05, vs sham/vehicle; (One-way ANOVA, Newman Keuls test)

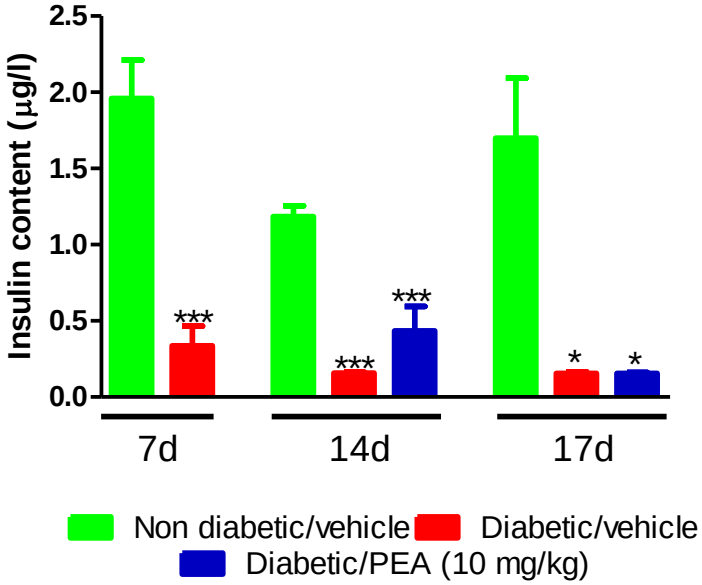


Fig.33: Effect of PEA on insulin content, 24 hors the last administration of PEA. The graphic shows the insulin content (expressed as µg/l) in the three experimental group: non diabetic and diabetic mice treated with vehicle and diabetic mice with seven (14d) or three (17d) administrations of PEA. Data represent mean ± SEM of 18 mice. ***P<0.001, *P <0.05 vs non diabetic/vehicle (One-way ANOVA, NewmanKeuls test)

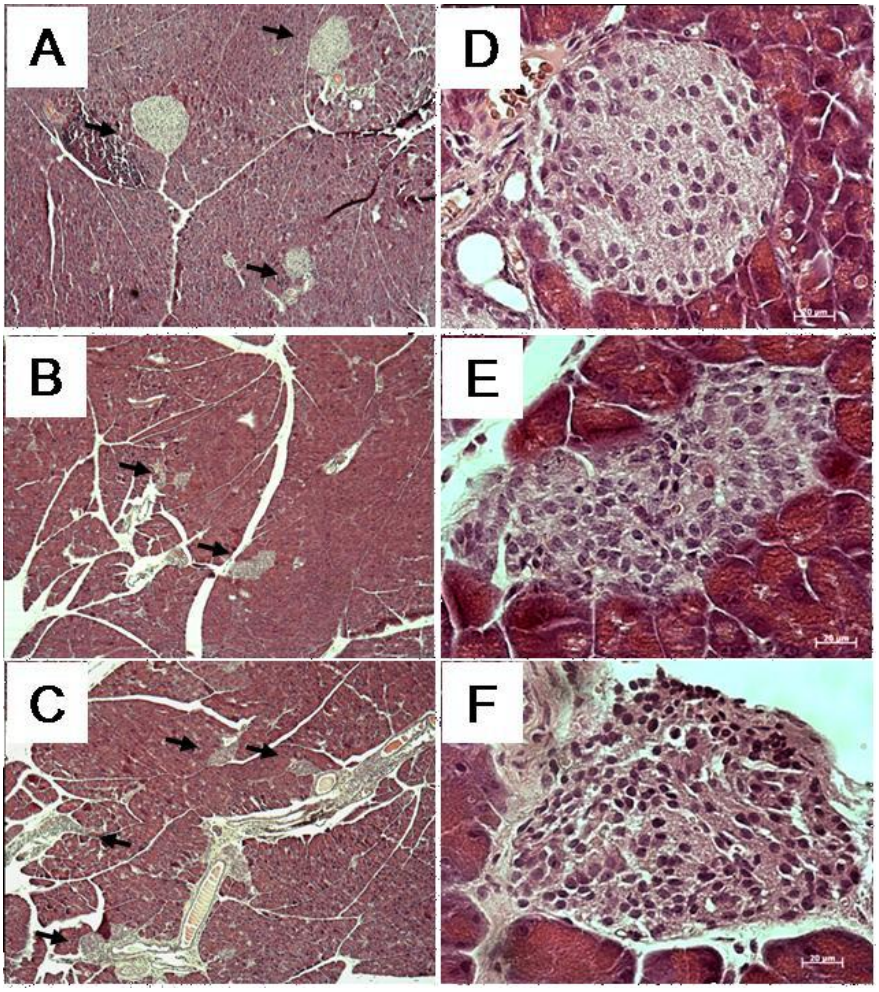


Fig.34: Hematoxylin and eosin staining on pancreas section in the following experimental groups: non diabetic/vehicle (A and D) and diabetic/vehicle (B and E) and diabetic/PEA (C and F) on 14th, 24 hours the last administration of PEA (10mg/kg). A-B-C original magnification 5x, black arrows indicate islet of Langerhans; D-E-F original magnification 40x (scale bar 20 µm)

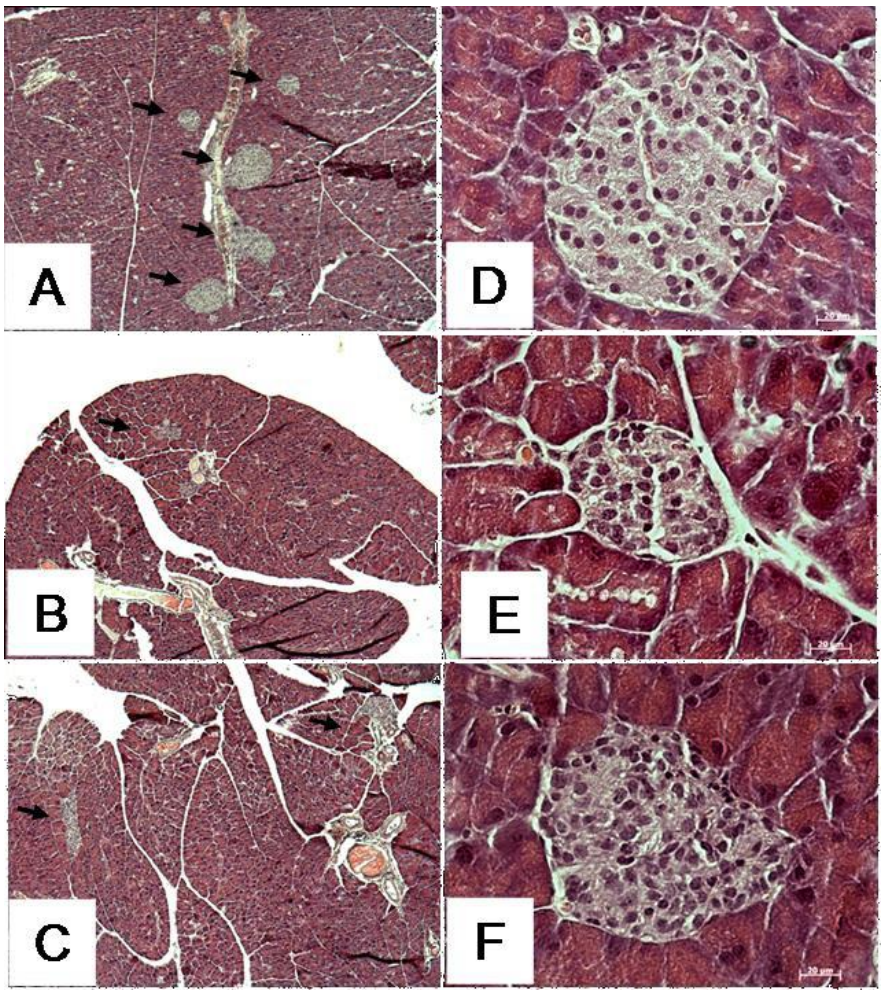


Fig.35: Representative images of pancreas tissue stained with hematoxylin and eosin in the following experimental groups: non diabetic/vehicle (A and D) diabetic/vehicle (B and E) and diabetic/PEA (10mg/kg, i.p) (C and F) on 17th, 24 hours the last administration of PEA (10mg/kg)the pancreas were collected. A-B-C original magnification 5x black arrows indicate islet of Langerhans; D-E-F original magnification 40x (scale bar 20µm)

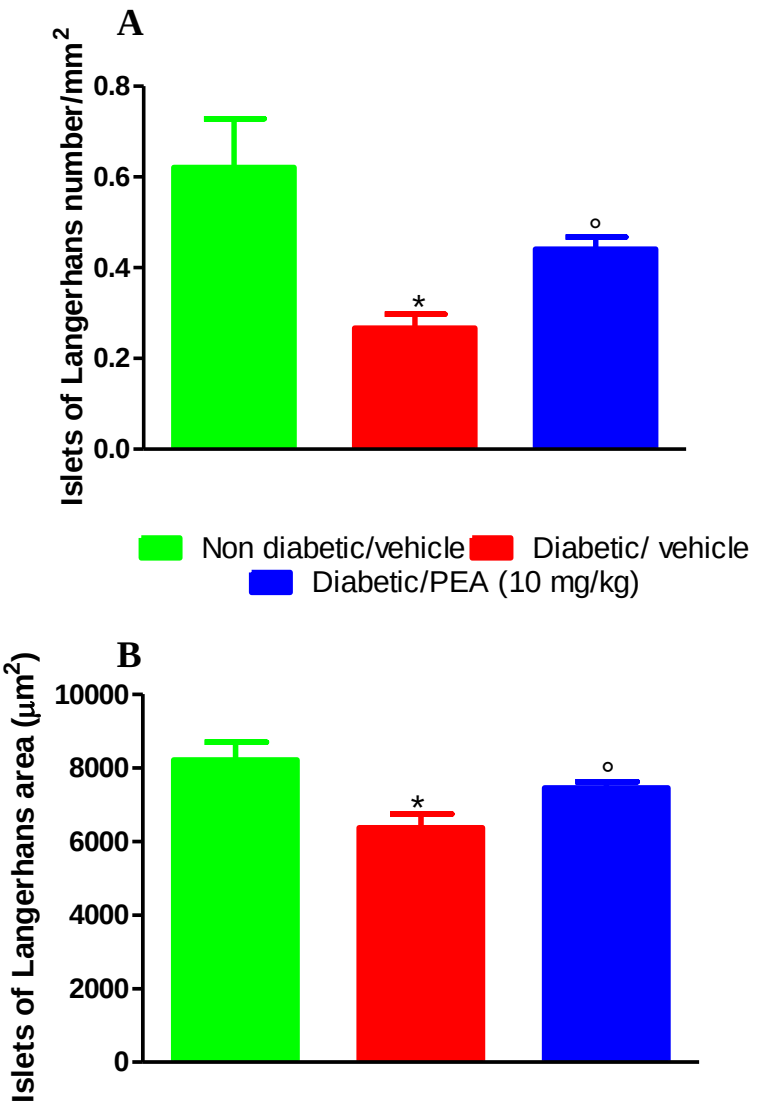


Fig.36: Effect of PEA (group 14d) on density islet of Langerhans and area of Islet of Langerhans 24 hours the last administration of PEA. The graphic shows the insulin content (expressed as µg/l) in the three experimental group: non diabetic and diabetic mice treated with vehicle and diabetic mice treated with seven administrations of PEA. Data represent mean ± SEM of 9 mice. *P <0.05 vs non diabetic/vehicle; °P<0.05 vs diabetic mice/vehicle (One-way ANOVA, Newman Keuls test)

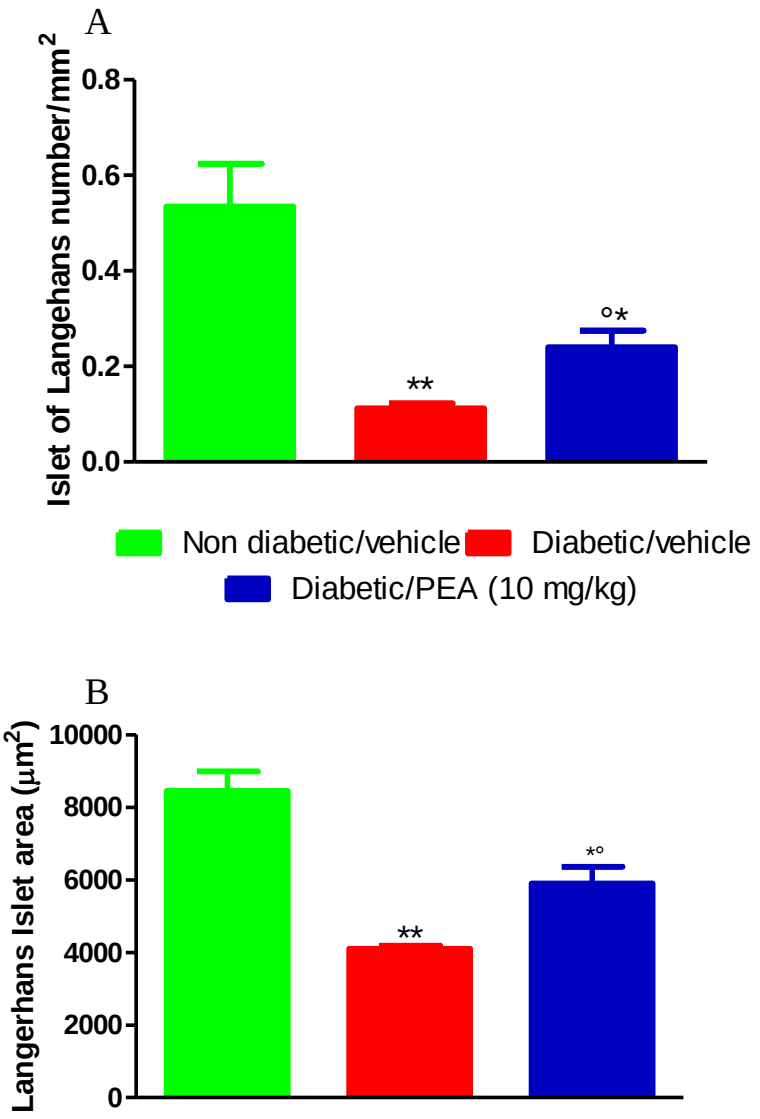


Fig.37: Effect of PEA (group 17d) on density islet of Langerhans and area of Islet of Langerhans 24 hours the last administration of PEA. The graphic shows the insulin content (expressed as µg/l) in the three experimental group: non diabetic and diabetic mice treated with vehicle and diabetic mice treated with three administrations of PEA. Data represent mean ± SEM of 9 mice. **P<0.01, *P <0.05 vs non diabetic/vehicle; °P<0.05 vs diabetic/vehicle (One-way ANOVA, Newman Keuls test)

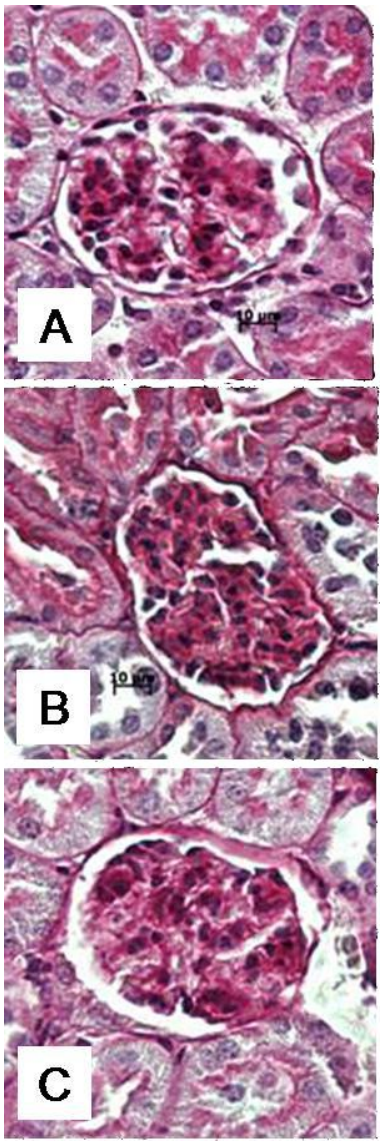


Fig.38: Representative images of glomeruli stained with PAS in the following experimental groups of group 17d: non diabetic/vehicle (A) diabetic/vehicle (B) and diabetic/PEA (10mg/kg, i.p) (C) on 17th, 24 hours the last administration of PEA (10mg/kg) kidneys were collected. Original magnification 40x (scale bar 10µm)

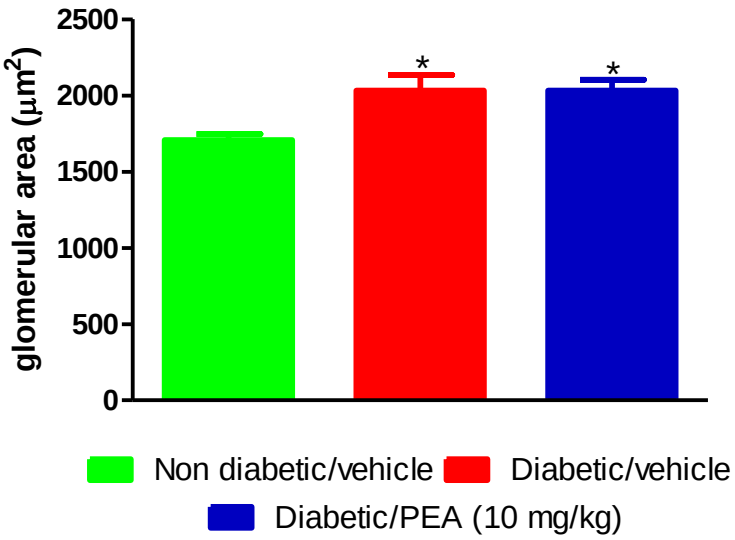


Fig.39: Effect of a repeat treatment with PEA (group 17d) on glomeruli areas 24 hours the last administration with PEA in the three experimental groups: non diabetic and diabetic mice treated with vehicle and diabetic mice treated with PEA (10mg/kg, i.p.) for three consectutive days. Data represent mean ± SEM of 9 mice. *P <0.05 vs non diabetic/vehicle (One-way ANOVA, Newman Keuls test)

Discussion

Neuropathic pain is a debilitating disease and it develops as a result of lesion or inflammation of the peripheral or central nervous system. The main symptoms of neuropathic pain are hyperalgesia (increased sensitivity to a nociceptive stimulus) and allodynia (pain in response to a non-nociceptive stimulus). Neuropathic pain is divided into three categories: painful peripheral neuropathies (e.g. trauma of the peripheral nerve) polyneuropathies (e.g. metabolic disorders such as diabetes) and central pain syndromes (such as multiple sclerosis or a trauma of the central nervous system). Neuropathy is refractory to common analgesics, because the underlying mechanisms are not fully understood, but experiments on animal models indicate that sensitization of peripheral afferents and spinal neurons are involved (Campbell, 2001; Koltzenburg and Scadding, 2001; Zimmerman, 2001). Peripheral afferents can be sensitized by inflammatory mediators (Dray, 1995; Wood and Doherty, 1997), a number of which are realesed by leukocytes which accumulate around the lesion site in injured peripheral nerve. The pro-inflammatory mediators released by leukocytes recruite mast cell, whose number increase distal to a peripheral nerve lesion (Enerbaeck et al., 1965; Sherif et al., 2001).

Mast cell are immune competent cells which possess important physiological roles that support homeostatic control, such as in tissue remodelling, wound healing, and neuroimmune response to stress (Bradding et al., 1995). Mast cells have a strategic localisation at sites directly interfacing with the external: they are located in connective tissues, in proximity to blood and lymphatic vessels (Sacchi et al., 2003), in nerves (Newson et al., 1983), in order to perform their function. The mast cells activation (degranulation) causes the release

of histamine, cytokines and proteases, which cause a pro-inflammatory microvascular environment due to increased vasodilatation, increased vascular permeability and local oedema formation, thus increasing the tissue delivery of other pro-inflammatory cells. Mast cells also produce and release the neurotrophin nerve growth factor (NGF) (Leon et al., 1994), which plays an important role in neuropathic pain. In fact NGF expression increase in different model of inflammatory pain, including complete Freud's adjuvant (Shin et al., 2009), carraggenan induced paw oedema (Aloe et al., 1992) and a rat model of cystitis (Andreev et al., 1995; McMahon et al., 1995), while NGF level decrease in diabetic neuropathy (Comelli et al. 2010).

Among the various emerging therapeutic a class of molecules able to control NGF levels is represented by ALIA compounds (from the acronym Autacoid Local Injury Antagonist), naturally-occurring lipid amides deriving from membrane fatty acides and structurally related to endocannabinoids. Palmitoylethanolamide (PEA), an endogenous lipid, is a promising compound for the cure of neuropathic pain. PEA is an endogenous fatty acid amide and it belongs to the family of the N-acyl ethanolamines (NAEs). Palmitoyletahnolamide is produced on demand within the lipid bilayer (Cadas et al, 1996). The endogenous PEA levels decreased in the spinal cord of neuropathic mice (CCI model) 3 days after the injury, as demonstrated by Petrosino and colleagues (2007). The administration of palmitoylethanolamide prevents the formation of oedema in a murine model of acute inflammation (Conti et al 2002; Costa et al 2002), also attenuates pain behaviour in a model of acute pain (Calignano et al., 1998; Conti et al

2002), inhibition of food intake (Rodriguez et al, 2001), reduction of gastrointestinal motility (Capasso et al, 2001), inhibition of cancer cell proliferation (De Petrocellis et al, 2002), and neuroprotection (Franklin et al, 2003; Lambert et al, 2001; Sheerin et al, 2004). In the mid- 1990's reaserchers demonstrated that PEA also inhibits mast cell degranulation *in vitro* (Facci et al., 1993) and *in vivo* (Mazzari et al., 1996) systems. Mazzari and colleagues demonstrated that palmitoylethanolamide (1996) administrated orally reduced the oedema formation and inflammatory hyperalgesia by down-modulating mast cell activation.

In our previous report (Costa et al., 2008), we described the anti-nociceptive effect of exogenous PEA in a well established murine model of peripheral neuropathy, the chronic constriction injury. Neuropathy was induced by the ligature of the sciatc nerve (Bennett and Xie, 1988) and exogenous PEA, administered one week, reduced hyperalgesia and allodynia. In particular this treatment reported to physiological level NGF. The same reaserch group (data not published) investigated the effect of a repeated treatment with exogenous PEA in a murine model of diabetic polyneuropathy. Diabetes, and the subsequent hyperlgyceamia, was induced through a single high dose of streptozotocin (STZ) injection in mice, destroying the β-cells in the islets of Langerhans. This model is widely used to study neuropathy, a frequent compliance of diabetes mellitus (Comelli et al., 2009; Comelli et al., 2010). After the injection of STZ, the induction of diabetes is accompanied by an alteration of painful stimuli response. Particularly, allodynia was present. Allodynia was noticed in diabetic mice 14 days after the streptozotocin injection and

remained constant even after the end of treatment. The allodinic animals also developed hyperglycaemia. When the diabetic animals developed neuropathy, the treatment with PEA started. PEA, administered for 3 consecutive days at the dose 10mg/kg, completely abolished allodynia and didn't affect hyperglycaemia. The 3 days treatment exerted a partial effect on NGF level.

Based on previous results obtained by Costa group, in this project the role of mastocytes in maintain or support the neurogenic inflammatory reaction was explored. Moreover we also investigated the therapeutic ability of PEA to prevent mastocytes degranulation in two models of neuropathy (CCI model and diabetic neuropathy), previously described.

The morphological analysis of sections of injured sciatic nerves, stained with acidified toluidine blue (Kawana et al., 2006), highlighted a massive recruitment of mast cells after three and eight days from the injury. Three days after the injury mast cells increased in the sourrounding tissue the sciatic nerve, with a prelevance of intact mast cells. On the contrary mast cells infiltrated in the inner part of the nerve after seven days from the injury, with a strong presence of degranulating and degranulated mast cells. Our results indicate that mast cells play a role in the beginning and maintenance of peripheral neuropathy. It can be hypothesized that the early steps triggering the hyperalgesia during neuropathy involved mediators produced independently from mast cells during the first period, and that the subsequent activation of mast cells, mainly due just to NGF, led to a release of many inflammatory mediators, including NGF. These results are in accordance with the behavioural tests (thermal

hyperalgesia and mechanical allodynia) performed by Costa and colleagues (2008).

The role of mast cell was demonstrated by Zuo and colleagues (2003): partial ligation of the sciatic nerve caused a change of mast cell morphology in the injured sciatic nerve in the early stages of neuropathic pain (3 hours) and the increase of mast cell number in the injured sciatic nerve after 28 days from the injury. The treatment with sodium cromoglycate, a mast cell stabiliser, resulted in a reduction of mast cell degranulation, with a reduction of neutrophils and monocytes recruitment to the injured sciatic nerve and suppression of hyperalgesia.

Intact mast cells, degranulating and degranulated mast cells were identified in accordance with previous studies (Kawana et al., 2006; Roznieckі et al., 1999). The presence and implication of mast cell was confirmed by double immunostaining images obtained from longitudinal sections showing a co-expression of mast cell proteases I (MMCP-I) and trkA receptor.

Our data showed that repeated treatment of PEA modulates mast cell recruitment and degranulation with the subsequent release of inflammatory mediators, in particular seven days PEA treatment. This outcome correlates with the anti-nociceptive effect of PEA previously described (Costa et al., 2008). The capacity of PEA to modulate mast cell degranulation seven days after the injury is ascribed to its effect on the nerve growth factor (NGF) level in CCI mice (Costa et al., 2008). Recently, De Filippis and colleagues (2011) demonstrated that palmitoylethanolamide reduced granuloma-induced hyperalgesia by modulation of mast cell activation and the release of NGF.

Afterwards a morphological analysis on transversal sections of injured sciatic nerve stained with toluidine blue was performed. The aim was to verify whether the inhibition of mast cell activity induced by PEA is accompanied by an improvement of the pattern of Wallerian degeneration, or not. Our results confirm that Wallerian degeneration occurred in injured sciatic nerve, with a decrease of density intact axons and meylin thickness. This result is in accordance with Costa and colleagues (2005), which demonstrated nerve demyelination in the same animal model. Moreover we demonstrated that the treatment with PEA, by modulation mast cell degranulation, had a protective role on intact axon density and myelin thickness.

The role of mast cells in the onset of neuropathic pain caused by metabolic disorder, such as diabetes, is poorly investigated and results in literature are controversial. From our results we can deduce that in diabetic neuropathy mast cells don't play a key role, as it occurs in chronic constriction injury.

Batbayar and colleagues (2003) demonstrated the increase of mast cells in diabetic mice with sensory neuropathy. On the contrary Nishimura and colleagues (2008) demonstrated no significant role of mast cells in a nerve compression syndrome associated with diabetes in rat model of streptozotocin-induced diabetes.

The onset of diabetic neuropathy is associated to low contents of NGF in the serum and peripheral nerve of NGF (Arrieta et al., 2005; Comelli et al., 2010), with subsequent nerve degeneration. Exogenous administration of NGF to diabetics prevents the morphological abnormalities of myelinated fibers and the progression of neuropathy (Apfel et al., 1992; Apfel et al., 1994; Tomlison et al., 1997; Schmidt

et al., 2001). Apfel and colleagues (1998) demonstrated the efficacy of the subcutaneous administration of recombinant NGF to improve the symptoms associated to diabetes in humans, but a second study, conducted by Apfel and colleague (2000), failed to confirm the early results.

In accordance with the decrease of NGF content, we observed an increase of myelinated fibers undergoing Wallerian degeneration, many fibers with an irregular myelin sheath and axon atrophy. The repeated treatment with PEA seemed to not affect Wallerian degeneration onset, despite its partial effect of NGF. Khan and colleagues (2002) investigated the role of myelinated (A-δ and A-β) and unmyelinated (C-fibers) afferent fibers in allodinic diabetic rats. The electrophysiological recordings revealed that the response of A-δ and A-β fibers afferent to mechanical stimuli was significantly augmented in diabetic rats. They demonstrated a crucial role of myelineated fibers in the development of allodynia in diabetic mice.

New emerging insights are focusing on the activation of spinal microglia in the pathogenesis of neuropathic pain. The role of activated microglia was studied in different types of neuropathy: partial nerve ligation (Coyle, 1998), chronic constriction injury (Mika et al., 2009), spared nerve injury (Luongo et al., 2010), spinal nerve ligation (Jin et al., 2003) and spinal cord ligation (Gwak and Huselbosch, 2009; Hains and Waxman., 2006). In the second part of the project the presence of activated microglia was verified in mice submitted to the chronic constricion injury of the sciatic nerve. The expression of F4/80 protein in transversal sections of the dorsal horn of the lumbar (L4-L5) spinal cord was investigated. This region was

considered because the afferent fibers from the periphery (sciatic nerve) arrive here. Morphological analysis shows that in CCI mice a time-dependent increase of activated microglia was observed in the dorsal horn of L4 and L5 spinal cord. In the contralateral horn of the spinal cord a milder activation of microglia was detected in the same region. These results were confirmed by the densitometric analysis of F4/80 expression obtained by western blotting. These results are in contrast with results reported in other articles, where in L5 spinal nerve ligation, a peak of activated microglia was already highlighted after two days from the injury (Echeverry et al., 2008; Gehrmann and Banati, 1995). In our study the signal of microglia activation, highlighted 3 days after the injury, is weak because the damage occurs on peripheral level. However the microglia activation 3 and 7 days after the injury is in accordance with the behavioural tests (hyperalgesia and allodynia) previously performed by Costa and colleagues (2008), suggesting that activated microglia play an important role in the beginning and maintenance of neuropathic pain.

The treatment with PEA for two consecutive days attenuated the limited microglia activation, restoring to physiological level as demonstrated by the expression of F4/80 protein. The treatment with PEA for seven consecutive days partially attenuated the modulation of activated microglia.

Luongo and colleagues (2010) investigated the role of a novel compound (NESS400) in a model of neuropathic pain on microglia activation. They observed that the treatment of seven days with NESS400 increased microglial cell numbers in the nerve-injured dorsal horn of the spinal cord and reduced the number of activated

microglia (pro-inflammatory). These results confirm that microglia can exist in different phenotypes: the activated microglia with pro-inflammatory effects, and resting microglia with anti-inflammatory effects.

The role of activated microglia was confirmed by Mika and colleagues (2009). They demonstrated that microglia are mainly activated in the ipsilateral dorsal horn of the lumbar spinal cord after CCI of the sciatic nerve. They also demonstrated that the preemptive and chronic intraperitoneal injections of minocycline, which selectively inhibits the activation of microglia, attenuated CCI-induced activation of microglia. Moreover, the inhibitor diminished neuropathic pain symptoms, the tactile and cold sensitivity.

Diabetes mellitus is a metabolic syndrome: the first symptom is a high blood sugar concentration that produces the classical symptoms of polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (excessive hunger). Diabetes mellitus can be divided in type-1 diabetes (body's failure to produce insulin) and type-II diabetes (cells fail to use insulin properly). If it is not cured in an adequate manner both types of diabetes have long-term complications. Diabetes doubles the risk of macrovascular disease (ischemic heart disease, stroke and peripheral vascular disease) and the risk of microvascular complications (retinopathy, neuropathy and nephropathy).

Diabetic nephropathy is a renal lesion which occurs in diabetic patients. The morphological lesions in type-I diabetes affect the glomeruli, with thickening of glomerular basement membrane (GMB) and mesangial expansion (Fioretto and Mauer, 2007). Shortly after the onset of hyperglycemia, a renal and glomerular hypertrophy develops

(Abrass et al., 1988), and a progressive thickening of the glomerular and tubular basement membranes occurs, due to the increased accumulation of matrix proteins normally present in these structures (Kim et al., 1991).

In our project, kidney sections were stained with periodic acid-Schiff (PAS) to detect the mesangial expansion due to the presence of hyaline-PAS positive material within glomeruli (Turoni et al., 2005; Yamamoto et al., 2004; Wittman et al., 2009). Our results highlight an increase of PAS positive material with an increase of glomerular area. These results are in accordance with Yamamoto and colleagues (2004): they observed an increase of glomeruli associated with an increase of PAS-positive material in diabetic mice after two week of diabetes induction. On the contrary Malatiali and colleagues (2008) observed an increase of glomerular area without an increase of PAS-positive material. This discrepancy is probably due to the different dose of STZ used; in fact Malatiali and colleagues (2008) used a low single dose of streptozotocin (55 mg/kg), while we administered streptozotocin with a single higher dose (120 mg/kg).

PEA treatment didn't affect the increase of PAS-positive material and the increase of the glomeruli area.

The chronic hyperglycaemia is associated with a decrease of insulin production. The control of blood glucose level depends on two hormones produced by pancreas: insulin and glucagone. Insulin hormone is produced by β-cell in the islets of Langerhans and it is involved in carbohydrate and fat metabolism (Zierler, 1999).

Costa and colleagues evaluated the insulin content in the following groups: the group 14d (the PEA treatment started 7 days from STZ

injection and lasted 7 days) and 17d group (the PEA treatment started 14 days from STZ injection and lasted 3 days). The insulin content was evaluated after 7, 14 and 17 days from the streptozotocin injections. As expected the insulin content is lesser in diabetic mice in respect to control mice (both groups). The treatment with PEA for three consecutive days (17d group) didn't affect insulin levels while after 7 repeated administrations (14d group) a mild production of insulin was observed.

A morphological evaluation on islet of Langerhans in the same groups was performed. In the longitudinal sections, stained with hematoxylin and eosin, of diabetic mice of 17d group a less number of islets of Langerhans were observed and the islet of Langerhas appeared smaller. The density and the area of islet of Langerhans were significantly less than in diabetic mice. The 3 PEA administrations seemed to have a partial effect: in fact the islets of Langerhans appeared more numerous and bigger. The quantitative analysis confirmed the decrease of islet of Langerhans density, and area in diabetic mice treated with vehicle and the partial effect of PEA on the density and on the area of islet of Langerhans.

In the pancreas sections of diabetic mice (14d group) stained with hematoxylin and eosin the islet of Langerhans appeared bigger than that observed in diabetic mice of 17d group. The prolonged (7 days) PEA treatment seemed to have a protective role on islets of Langerhans destruction. The quantitative analysis, performed on the density and on the area of islet of Langerhans, confirmed the time dependent presence of islets of Langerhans in diabetic mice and the protective effect of PEA on islet of Langehans. These data, obtained

from 17d and 14d groups, were in accordance with the decrease of insulin level in the blood observed on 14 and 17 days after the streptozotocin injection and after a three and seven days of PEA treatment. These results are in agreement with other studies present in literature. STZ injection (60 mg/kg) produced a significant decrease in the diameters of pancreatic islets as well as a decrease number of islets and cells per islet in diabetic rats compared to control rats in pancreatic sections (Sen et al., 2011). Yamabe and colleagues (2010) demonstrated that after a single administration of streptozotocin most cells in pancreatic islets in STZ-induced diabetic rats were destroyed, and a majority of the islets underwent shrinkage. Also, the number of islets decreased in STZ-treated rats. Streptozotocin administration causes islet atrophy through β-cell loss, which depletes the central core of islet tissue leaving an ostensibly thickened layer of peripheral islet (non-β) cells.

Probably PEA administered at early time and for a prolonged period reduces the monocytes/macrophages infiltration within the islet of Langerhans. This process, called insulitis, leads to a total infiltration of monocytes/macrophages in the islet of Langerhans with a subsequently destruction of islet. The prolonged treatment with PEA probably attenuates the infiltration of monocytes/macrophages within the islets. Our representative images of islet of Langerhans show an increase of cells near the islet of Langerhans without infiltration in the inner part in diabetic mice treated with PEA, while this infiltration reaches the inner part in diabetic mice treated with vehicle. Insulitis is a well studied process and different grade of insulitis are described: grade 0 (no infiltration), grade 1 (infiltration in the surrounding tissue

of islet), grade 3 (little infiltration within the islet) and grade 4 (strong infiltration within the islet) (Baik et al., 1999).

Mast cells and Wallerian deneration are involved in the beginning and maintenance of neuropathic pain, induced by the chronic constriction injury of the sciatic nerve and the repeated short and long treatment with PEA downmodulates mast cell recruitment and degranulation in the peripheral nervous system and this modulation has a protective effect on Wallerian degeneration. Microglia play a key role in the same neuropathic model with a peak of activated microglia 8 days after the injury and the short and long repeated treatment with PEA modulates activation of microglia in the spinal cord (central nervous system).

We demonstrated mast cells are not involved in the maintenance of neuropathic pain associated with diabetes. In this model we confirm a role of Wallerain degeneration in accordance with the decrease of NGF in the onset of neuropathic pain.

The therapeutic efficacy of PEA can be observed in the pancreas tissue. PEA administered before the onset of neuropathic pain and for a prolonged (7 days) period has a protective effect: probably due to its capacity to prevent an inflammatory state.

So given the anti-inflammatory and anti-nociceptive effect of PEA, and its capability to modulate two different targets (in the peripheral and central nervous system), this compound can be considered a strong and good alternative in the cure of peripheral mononeuropathy and diabetic polyneuropathy.

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