Prevention of opioid peptide degradation by enzyme inhibitors in a model of painful inflammation

Einfluss von Enzyminhibitoren auf den Abbau von analgetisch wirksamen Opioidpeptiden in peripherem entzündetem Gewebe

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Abbreviations

Ab	Antibody
Ala-ßNA	Alanin-ß-naphthylamine
ANOVA	Analysis of variance
APN	Aminopeptidase N
b	Bestatin
ßNA	ß-naphthylamine
CD	Cluster of differentiation
CD10	Marker of NEP
CD13	Marker of APN
CFA	Complete Freund`s Adjuvant
CNS	Central nervous system
CRF	Corticotropin-releasing factor
CXCL	Chemokine (CXC)-ligand
CXCR	Chemokine (CXC)-receptor
DRG	Dorsal root ganglion
DYN	Dynorphin A (1-17)
EC	Enzyme Commission (number) (enzyme nomenclature)
ECE	Endothelin-converting enzymes
END	ß-endorphin
FACS	Fluorescence-activated cell sorting (flow cytometry)
FITC	Fluorescein isothiocyanate
i.c.v.	Intracerebroventricular
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
i.pl.	Intraplantar
i.v.	Intravenous
kDa	Kilo-Dalton
LENK	Leucine-enkephalin
MENK	Methionine-enkephalin
mRNA	Messenger ribonucleic acid
NEP	Neutral endopeptidase
NSAID	Non-steroidal anti-inflammatory drugs
P8B	Aminophosphinic dual APN and NEP inhibitor P8B
PE	Phycoerythrin
PE-Cy5	Phycoerythrin – cytochrome 5
pNA	*p*-nitroaniline
PPT	Paw pressure threshold
RIA	Radioimmunoassay
RM	Repeated measurements
s.c.	Subcutaneous
SEM	Standard error of the mean
Suc-Ala-Ala-Phe-pNA	Succinyl-alanin-alanin-phenylalanine-*p*-nitroaniline
t	Thiorphan

1. Introduction

1.1. Peripheral opioid-mediated analgesia

Current pharmacological treatment of severe acute (e.g. postoperative) and chronic (e.g. associated with cancer) clinical pain is based on two drug classes. One of them includes non-steroidal anti-inflammatory drugs (NSAIDs) such as paracetamol and ibuprofen, which are also used in rheumatoid arthritis. Although these drugs can provide some pain relief they are not very strong analgesics and produce severe side effects such as gastrointestinal ulcers and bleeding, thromboembolic complications, kidney and liver toxicity. The second class consists of opioids such as morphine, fentanyl and methadone, which are the most potent and efficacious drugs for postoperative and cancer pain. However, their use is limited by adverse effects such as nausea, respiratory depression, analgesic tolerance, physical dependence and addiction, which result from activation of opioid receptors in the central nervous system (CNS), mostly in the brain. Opioid receptors are not restricted to the CNS but they are also present on peripheral sensory nerves. The advantage of activation of such peripheral opioid receptors is pain inhibition (analgesia or antinociception) without CNS-mediated side effects. The synthesis, signaling and analgesia upon activation of peripheral opioid receptors have been extensively examined in animal and clinical studies (reviewed in Stein et al., 2003; Machelska, 2007).

To mimic clinical inflammatory pain, many animal studies used complete Freund`s adjuvant (CFA)-induced inflammation. CFA is a suspension of heat-inactivated mycobacteria (*Mycobacterium butyricum* or *Mycobacterium tuberculosis*) in mineral oil. Injection of CFA into a rat hind paw results in a localized inflammation characterized by paw swelling, redness, increased temperature and enhanced sensitivity to noxious mechanical and heat stimuli. Within 4 - 6 days the inflammation and pain are restricted to the inoculated paw. The contralateral paw remains non-inflamed and shows no difference compared to intact paws of control animals (Stein et al., 1988). Inflammation leads to enhanced synthesis of all three opioid receptor types (μ, δ, κ) in dorsal root ganglia (DRG) and to intra-axonal transport resulting in their augmented expression in peripheral terminals of sensory neurons. After agonist binding, neuronal opioid receptors couple to inhibitory G-proteins ($G_{i/o}$) which leads to inhibition of calcium and sodium channels, and of adenylyl cyclase followed by decreased levels of cyclic adenosine monophosphate. These events are augmented in peripheral inflamed tissue and result in decreased neuronal excitability and analgesia in animals and in humans (reviewed in Stein et al., 2003; Machelska, 2007). In rodent models, injection of systemically inactive doses of μ-, δ- and κ-opioid receptor agonists into inflamed tissues

produced dose-dependent antinociception, which was reversible with opioid receptor antagonists. Similar effects were found following systemic (e.g. subcutaneous [s.c.] or intraperitoneal [i.p.]) administration of opioids with limited access to the CNS (reviewed in Sawynok, 2003; Stein et al., 2003; DeHaven-Hudkins et al., 2004; Ossipov et al., 2004; Smith, 2008). Peripherally mediated opioid analgesic effects were confirmed in numerous clinical studies. In patients with acute or chronic knee inflammation (e.g. after joint surgery or in arthritis), intraarticular injection of small, peripherally selective doses of morphine has been shown to reduce pain (reviewed in Kalso et al., 2002; Sawynok et al., 2003; Stein et al., 1991; Smith, 2008). In addition, such effects can be exerted by endogenous opioid peptides released by immune cells infiltrating inflamed tissues in animals and in patients (see 1.2, 1.3 and 1.4).

1.2. Opioid peptides in immune cells

Three families of endogenous opioid peptides, the endorphins (e.g. β-endorphin; END), enkephalins (e.g. Methionine-enkephalin [MENK] and Leucine-enkephalin [LENK]) and dynorphins (e.g. dynorphin A 1-17; DYN) were first characterized in the CNS. These peptides derive from the three respective precursor proteins proopiomelanocortin, proenkephalin and prodynorphin. All these opioid peptides share the N-terminal amino acid sequence Tyr-Gly-Gly-Phe-Met/Leu and show different affinity and selectivity for the three opioid receptors, μ (endorphins), δ (enkephalin) and κ (dynorphins). The endomorphins represent an additional group but their precursor(s) have not been identified so far (reviewed in Machelska, 2007). Within the CNS opioid peptides and their precursors are localized in structures involved in pain transmission such as thalamus, periaqueductal grey, reticular nuclei of the brainstem and dorsal horns of the spinal cord. Also peripheral sensory neurons express opioids. MENK, LENK and DYN, but not END, have been found in peripheral unmyelinated sensory axons, so called nociceptors, which specifically transmit nociceptive stimuli (Hassan et al., 1992; Carlton et al., 1997; Wu et al., 2005). Further, viral-driven delivery of MENK led to enhanced levels of the peptide in peripheral sensory neurons (Antunes bras et al., 2001) and produced antinociception in animal models of neuropathic and inflammatory pain (reviewed in Cope and Lariviere, 2006).

Opioid peptides are also abundant in cells of the immune system. Under inflammatory conditions opioid peptide-containing circulating leukocytes extravasate using adhesion molecules (selectins, intercellular adhesion molecule-1, integrins α_4 and β_2) and chemokines (CXCL1, CXCL2/3) (Fig. 1.1). Thus, the selective blockade of these adhesion molecules or

chemokines substantially decreased the number of opioid-containing immune cells accumulating in inflamed tissue (Machelska et al., 1998, 2002, 2004; Brack et al., 2004b) and in consequence abolished endogenous peripheral opioid analgesia (see paragraph 1.4). In accord with the extravasation of opioid-containing cells, mRNA of proopiomelanocortin, proenkephalin and prodynorphin as well as END, MENK, LENK and DYN are detected in leukocytes accumulating in inflamed tissues in animals (Stein et al, 1990b; Przewlocki et al., 1992; Hassan et al., 1992; Mousa et al., 2004). In early stages of CFA-induced paw inflammation (6 h) the major subpopulation of opioid-containing cells are granulocytes, whereas later (4 days) monocytes and macrophages dominate with some infiltration of T lymphocytes (Mousa et al., 2001; Rittner et al., 2001). During this course of inflammation the leukocytic content of opioids and the number of opioid-containing leukocytes increase (Rittner et al., 2001) and proopiomelanocortin mRNA and END are up-regulated in cells of lymph nodes draining the inflamed tissue (Cabot et al., 1997; Sitte et al., 2007). Furthermore, END and MENK were detected in synovial granulocytes, macrophages/monocytes, lymphocytes and plasma cells in patients with acute knee trauma or arthritis (Mousa et al., 2007). Together, these findings provide strong evidence on the presence of opioid peptides in peripheral sensory neurons and immune cells in painful inflamed tissues in animals and humans.

1.3. Secretion of opioid peptides by leukocytes

To exert their effects opioid peptides need to be secreted. In extensive *in vitro* studies it has been shown that basal release of MENK, DYN and END from lymphocytes can be stimulated with corticotropin-releasing factor (CRF), interleukin-1β (IL-1) and noradrenaline. This secretion was CRF-, IL-1- and adrenergic-receptor specific, respectively, and it was calcium dependent and mimicked by potassium, consistent with vesicular release (Schäfer et al., 1994; Cabot et al., 1997, 2001; Binder et al., 2004) (Fig. 1.1). Further, chemokines acting at CXCR1/2 receptors induced MENK and END release from human and rat granulocytes, which was dependent on inositol triphosphate receptor-triggered release of calcium from endoplasmic reticulum and (partially) on phosphinositol-3-kinase (Rittner et al., 2006). Also, immersion of non-inflamed rat hind paws in hot water resulted in increased MENK and LENK levels in the perfusate. It was suggested that opioids originated mainly from resident immune and Merkel cells, although this was not directly investigated (Yonehara et al., 1993).

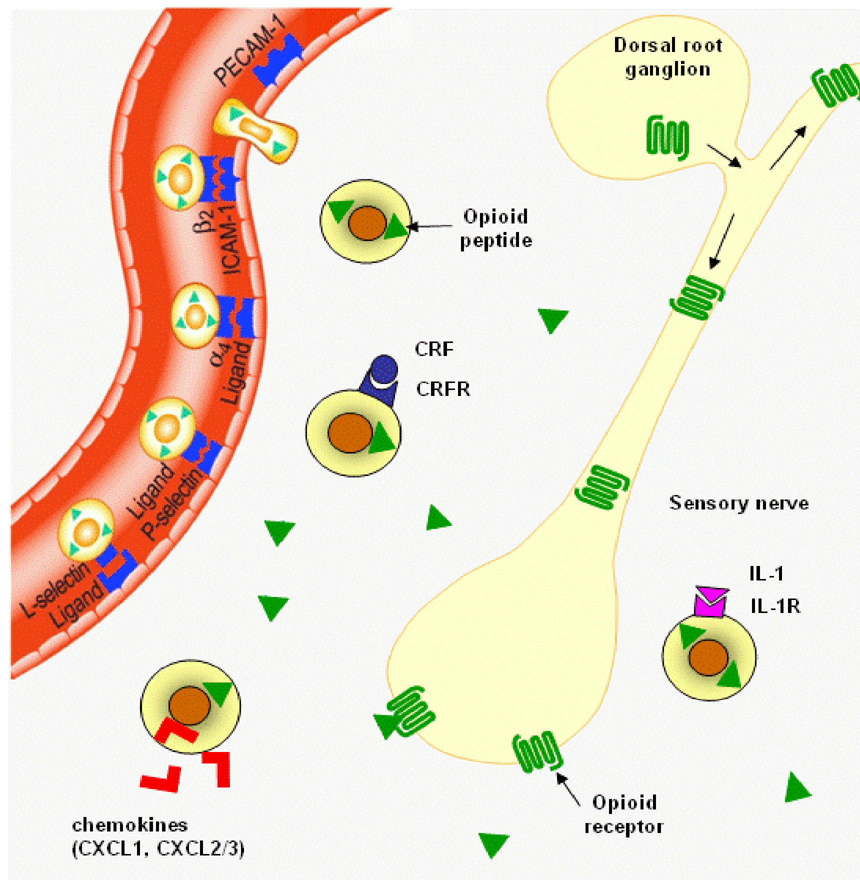

Figure 1.1. Migration of opioid-containing immune cells and opioid peptide secretion within inflamed tissue. During inflammation P-selectin, intercellular adhesion molecule-1 (ICAM-1) and platelet-endothelial cell adhesion molecule-1 (PECAM-1) are up-regulated on vascular endothelium, while L-selectin, integrins α_4 and β_2 are expressed on opioid-containing immune cells. This results in the migration of opioid-containing leukocytes through the blood vessel endothelium into the extravascular space of the inflamed tissue. In response to environmental stress or to agents such as chemokines CXCL1 and CXCL2/3, corticotropin-releasing factor (CRF) and interleukin (IL)-1 immune cells release opioid peptides. Chemokines, CRF and IL-1 act at chemokine receptors, CRF receptors (CRFR) and IL-1 receptors (IL-1R), respectively, which are expressed on opioid-containing leukocytes. Secreted opioid peptides bind to peripheral opioid receptors (produced in dorsal root ganglia and transported to peripheral endings of sensory neurons) and reduce inflammatory pain. Modified from Stein et al., 2003 and Dr. Melanie Busch-Dienstfertig.

1.4. Analgesic effects of immune cell-derived opioids

Stress is a natural stimulus triggering inhibition of pain. In rats with unilateral hind paw inflammation induced with CFA, exposure to cold (4°C) water swim stress strongly inhibited sensitivity to noxious mechanical stimuli (paw pressure) exclusively in inflamed paws. This antinociceptive effect was dose-dependently reversible by antibodies (Abs) against opioid peptides and by opioid receptor antagonists injected into the plantar surface (intraplantarly; i.pl.) of inflamed paws (Parsons et al., 1990; Stein et al., 1990a, 1990b; Przewlocki et al., 1992). Leukocytes are the source of opioid peptides because antinociception was substantially reduced by immunosuppression with cyclosporine A or whole body irradiation, by depletion of granulocytes or of monocytes/macrophages, by blocking the extravasation of immune cells following inhibition of L- and P-selectins, integrins (α_4 and β_2) or intercellular adhesion molecule-1 (Stein et al., 1990b; Przewlocki et al., 1992; Machelska et al., 1998, 2002, 2004; Brack et al., 2004a). CRF, IL-1, noradrenaline and CXCL2/3 chemokine injected into inflamed paws produced opioid-mediated antinociception, reversible by i.pl. pretreatment with selective antagonists of CRF-, IL-1-, adrenergic (α_1, α_2 and β_2)- and opioid-receptors or by Abs against END, MENK or DYN (Schäfer et al., 1994, 1996; Machelska et al., 2003; Binder et al., 2004; Ritttner et al., 2006). Furthermore, we recently showed that leukocyte-derived opioids are also essential regulators of pain resulting from injury to peripheral nerves i.e. neuropathic pain. In a mouse model of sciatic nerve injury we found that immune cells containing MENK, DYN and END accumulate at the site of nerve damage and that their activation by CRF effectively suppressed pain (Labuz et al., 2009). Also, genetic absence of T lymphocytes expressing END reduced CRF-induced antinociception, which was fully restored by transfer of wild-type mice-derived T lymphocytes (Labuz et al., 2010). Importantly, peripheral endogenous mechanisms of opioid analgesia are clinically relevant. In patients undergoing knee surgery blockade of opioid receptors by the antagonist naloxone injected into the knee joint infiltrated by opioid peptide-containing leukocytes resulted in increased postoperative pain (Stein et al., 1993; Mousa et al., 2007). This suggests that opioids are tonically released and activate peripheral opioid receptors in inflamed tissue to attenuate clinical pain. Furthermore, the intraarticular injection of CRF produced a transient but significant reduction of postoperative pain, apparently by releasing opioid peptides from synovial inflammatory cells (Likar et al., 2007).

Together, these findings clearly show that immune cell-derived opioid peptides act at peripheral opioid receptors directly in injured peripheral tissues to decrease pain associated with inflammation or nerve damage in animals and humans. However, the antinociceptive effects after swim stress or injection of IL-1 and CRF were relatively short-lasting in animal

models and in patients (10 – 30 min) (Stein et al., 1990a; Schäfer et al., 1994; Likar et al., 2007; Labuz et al., 2009), which might be related to rapid inactivation of opioid peptides by peptidases.

1.5. Peptidases degrading opioid peptides

Among peptidases localized on the surface of neurons and immune cells, the best characterized enzymes inactivating opioid peptides are aminopeptidase N (APN or CD13; EC 3.4.11.2) and neutral endopeptidase (NEP or CD10; EC 3.4.24.11), which belong to the family of zinc metallopeptidases. They are type II integral membrane proteins with a short cytoplasmic region, a transmembrane region and a large extracellular C-terminus. Their active sites are characterized by a zinc atom, which is incorporated into two histidine residues in the consensus sequence HExxH. The glutamate in this sequence transfers a hydrogen atom and activates the nucleophilic attack of the polarized zinc-bound water on the peptide bond. The specificity of the active site is essentially ensured by Van der Waals and ionic interactions between the S_2, S_1, S_1\`, and $S_2$\`subunits and the lateral chain of the corresponding P_2, P_1, P_1\` and $P_2$\` moieties of the substrate (Roques et al., 1993; Tiraboschi et al., 1999).

1.5.1. APN expression and function

APN is a 130 - 160 kDa glycoprotein widely distributed in most mammalian tissues such as kidney, intenstine, liver, lung, fibroblasts and vessels. APN is also expressed in several immune cell types such as monocytes, macrophages, granulocytes and dendritic cells (Watt et al., 1989; Shipp and Look, 1993; Jardinaud et al., 2004; Mina-Osorio, 2008). T- and B- lymphocytes in earliest stage of differentiation are APN-positive but become APN-negative upon maturation. Thus, lymphocytes in blood, spleen and tonsils were APN-negative (Riemann et al., 1999); however they expressed the enzyme in synovial fluid of rheumatoid arthritis patients (Riemann et al., 1993). In the brain APN was found in endothelial cells of microvessels, cerebral membranes and meninges (Gros et al., 1985; Solhonne et al., 1987; Noble et al., 2001). In the pig peripheral nervous system APN is associated with peripheral nerve microvessels and connective tissues including perineurium (Barnes et al., 1991).

APN belongs to exopeptidases because it removes N-terminal amino acids of peptides. It is a multifunctional protein involved in invasion and metastasis of cancer cells (Hashida et al., 2002), viral infection (Kolb et al. 1998), signal transduction (Mina-Osorio et al., 2006) and chemotaxis (Proost et al., 2007; Wulfaenger et al., 2008). The main function of APN is the degradation of peptides including the opioids MENK and LENK (Fig. 1.2) but also neurokinin A, somatostatin, vasoactive peptides (angiotensin III, lysyl-bradykinin), chemokines (e.g. CXCL8, CXCL11/12) and immunomodulating peptides (Miller et al., 1994a, b; Safavi et al., 1995; Song B et al., 2003; Jardinaud et al. 2004; Stein et al., 2003).

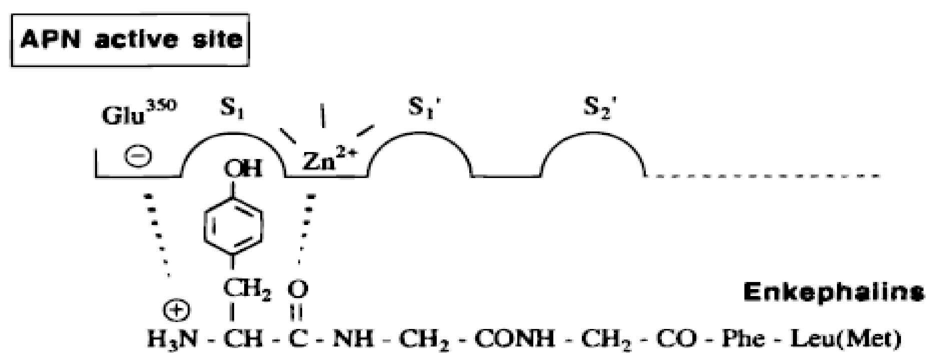

Figure 1.2. Schematic representation of interaction of LENK or MENK with the active site of APN. Adapted from Chen et al. (2000).

1.5.2. NEP expression and function

NEP belongs to a family of mammalian zinc metallopeptidases, which also include endothelin-converting enzymes (ECE), the erythrocyte surface antigen KELL, the phosphate-regulating gene on the X chromosome and recently identified damage-induced neuronal endopeptidase. NEP is a 90 - 110 kDa glycoprotein widely distributed on mammalian cells in kidney, brain, spinal cord, placenta, testes, ovaries, adrenal cortex, granulocytes, macrophages, pre-B lymphocytes, circulating and synovial T lymphocytes (Shipp and Look, 1993; Turner et al., 1997; Oefner et al., 2004; Simonini et al., 2005; Schroeter et al., 2007). In the pig and rat peripheral nervous system, NEP is located in Schwann cell membranes surrounding the DRG and in unmyelinated nerve fibers (Matsas et al., 1986; Kioussi et al., 1992).

NEP expressed on surface of neutrophils plays a role as tumor-associated antigen, it is known as common acute lymphoblastic leukemia antigen and can also act as a receptor of

the human immunodeficiency virus 1 (Roques et al., 1993). NEP belongs to endopeptidases, acting on the amino terminus of hydrophobic amino acid residues in a peptide chain. It is important in terminating the activity of peptides involved in cardiovascular functions (artrial natriuretic factor, bradykinin), inflammation (substance P, IL-1, angiotensin II), synaptic neuropeptide metabolism (neurotensin, cholecystokinin-8) and chemotaxis (fMet-Leu-Phe). Regarding opioid peptides, NEP degrades predominantly enkephalins (LENK and MENK) by cleaving their glycin-phenyl (Gly^3 – Phe^4) bond (Fig. 1.3), but a few reports also described DYN and END as NEP substrates (Graf et al., 1985; Roques et al., 1993; Song B et al., 2003).

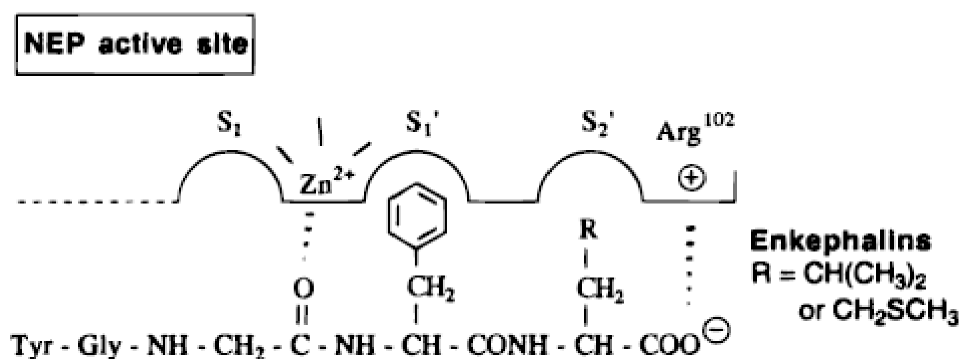

Figure 1.3. Schematic representation of interaction of LENK or MENK with the active site of NEP. Adapted from Chen et al. (2000).

1.6. APN and NEP inhibitors

1.6.1. Bestatin – APN inhibitor

Bestatin ($C_{16}H_{24}N_2O_4$) is a potent peptidase inhibitor discovered in filtrates of *Streptomyces olivoreticuli* cultures in Japan 25 years ago. It is a natural analog of the dipeptide Phe-Leu with a secondary alcohol inserted between the two amino acids (Fig. 1.4). In addition to blocking APN, bestatin can also bind to other metallopeptidases such as leucyl-, alanyl- and cystinyl-aminopeptidases. The inhibitor-enzyme complex is tight and bestatin exhibits competitive kinetics with the peptidase substrates (Scornik et al., 2001).

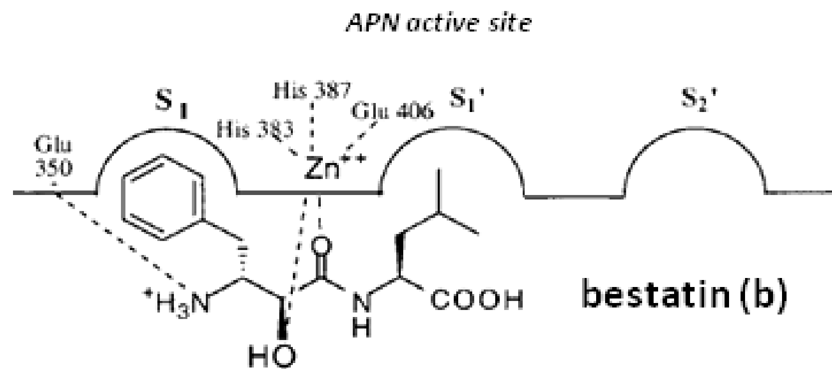

Figure 1.4. Binding of bestatin to the active site of APN. Adapted from Bauvois and Dauzonne (2006).

1.6.2. Thiorphan – NEP inhibitor

In 1980 the group of Bernard Roques developed the synthetic selective NEP inhibitor thiorphan ($C_{12}H_{15}NO_3S$). This inhibitor contains a thiol group, which interacts with the zinc atom in the active NEP site (Fig. 1.5) with high inhibitory potency in the nanomolar range (Roques et al., 1980).

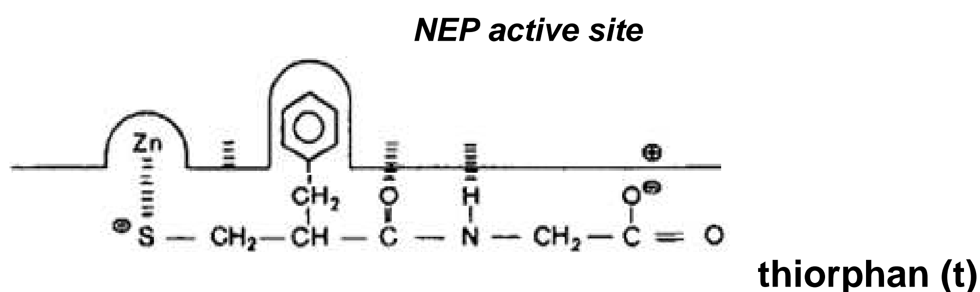

Figure 1.5. Thiorphan interacting with the active site of NEP. Adapted from Roques et al. (1980).

1.6.3. Dual inhibitors blocking APN and NEP simultaneously

It has been shown that simultaneous blockade of APN and NEP was more effective than their separate inhibition in elevating extracellular MENK levels in the CNS and in producing antinociception (see paragraph 1.7). This led to the development of dual inhibitors that block both enzymes concurrently. Dual inhibitors have the additional advantage of avoiding different pharmacokinetics and bioavailability when two "single" inhibitors are employed (Chen et al., 1998). The most extensively studied dual inhibitors are kelatorphan and RB101 (Fournie-Zaluski et al., 1984, 1992). However, the affinities of these compounds for APN are up to 100-fold lower than for NEP (Chen et al., 1998). To improve the potency of inhibiting both peptidases with similar affinities, new aminophosphinic compounds were designed. Their general formula NH_2-CH(R_1)P(O)_(OH)CH_2-CH(R_2)CONH_CH(R_3)COOH contains a phosphinic moiety as a zinc coordinating ligand. The side chains R_1 = Ph, R_2 = CH_2Ph(p-Ph), R_3 = CH_3 are responsible for optimal recognition of APN and NEP in the nanomolar range. It was found out that a small residue on R_3 optimized the recognition of NEP subsites and a free N-terminal amino group for optimal APN binding (Chen et al., 1998, 2000). One of the newest is P8B, synthesized by our collaborator Prof. B. Roques (Université Paris). P8B came out as the most promising phosphinic inhibitor blocking the activities of NEP (Ki = 2.0 nM) and of APN (Ki = 4.8 nM) in the nanomolar range *in vitro* combined with poor cross-reactivity with related zinc metallopeptidases such as ACE or ECE (Chen et al., 2000).

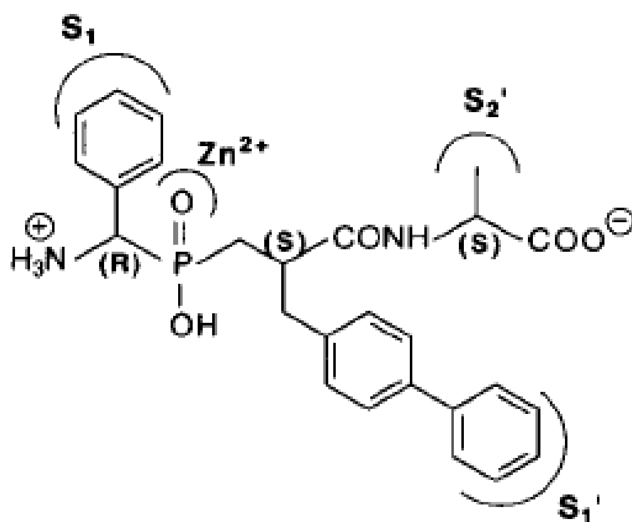

Figure 1.6. Binding of a dual inhibitor to the active site of APN/NEP. Adapted from Chen et al. (2001)

1.7. Effects of peptidase inhibitors on opioid peptide levels and pain

The actions of secreted opioid peptides can be enhanced and prolonged when their degrading enzymes are inhibited. Bestatin and thiorphan have been shown to block H^3[MENK] and H^3[LENK] degradation in rat and mouse brain slices and in rat spinal cord slices. Administration into cerebral ventricles (intracerebroventrically; i.c.v.) of bestatin or thiorphan resulted in a reduction of H^3[MENK] hydrolysis product levels in rat brain *in vivo* (Roques et al., 1980; De la Baume et al., 1983; Challet et al., 1983; Bourgoin et al., 1986; Giros et al., 1986b). These effects on enkephalin levels were associated with antinociceptive actions, which were mostly studied in healthy animals with no pathological pain. Bestatin potentiated antinociception induced by intracerebrally administered dynorphins in mice (Nakazawa et al., 1989). Intrathecal bestatin or thiorphan potentiated antinociception and increased the amount of MENK released in the spinal cord in response to i.c.v. END in rats (Suh et al., 1990). In rats bestatin or thiorphan potentiated antinociception induced by intrathecal END and increased the amount of MENK released from spinal cord (Suh et al., 1990). The i.c.v. application of thiorphan produced antinociception after thermal noxious stimulation, which was blocked by the opioid receptor antagonist naloxone (Roques et al., 1980; De la Baume et al., 1983). Further studies showed that simultaneous blockade of NEP and APN by a combination of bestatin and thiorphan was more efficient in enhancing the extracellular MENK concentrations compared with the separate inhibition of each peptidase (Bourgoin et al., 1986; De la Baume et al., 1983). Also, the simultaneous injection of bestatin and thiorphan elicited opioid receptor-mediated antinociceptive effects after thermal stimulation in healthy mice (Carenzi et al., 1983). In a model of pathological pain, the combined injection of bestatin and thiorphan into inflamed paws enhanced opioid antinociception induced by swim stress (Parsons and Herz, 1990). These findings also have clinical relevance. In arthritic patients NEP was found in plasma, synovial fluid, polymorphonuclear cells and lymphocytes (Appelboom et al., 1991; Matucci-Cerinic et al., 1993; Simonini et al., 2005), and APN was expressed on T cells in synovial tissue (Riemann et al., 1993). Intravenous injections of thiorphan or its prodrug acetorphan decreased NEP activity in plasma and cerebrospinal fluid and produced some analgesia in a small number of patients with migraine or after myelography (Floras et al., 1983; Spillantini et al., 1986). In accord with animal studies, thiorphan and bestatin produced stronger pain inhibition when applied together in cancer patients (Meynadier et al., 1988)

Following the concomitant use of selective APN and NEP inhibitors the actions of dual peptidase inhibitors were tested (see also paragraph 1.6.3). The antinociceptive action of i.c.v. MENK was potentiated by co-administration of kelatorphan. This effect was even

higher than that produced by a combination of bestatin and thiorphan (Fournie-Zaluski et al.,1984). RB101 administered systemically elevated extracellular levels of MENK-like material in the nucleus accumbens of rats (Daugé et al., 1996) and induced opioid antinociception after thermal, mechanical and chemical noxious stimuli in healthy animals (Noble et al., 1992). In CFA-induced paw inflammation RB101 injected i.v. produced opioid antinociception after mechanical noxious stimulation (Maldonado et al., 1994). A new dual aminophosphinic inhibitor RB3007 increased extracellular levels of endogenous MENK in the periaqueductal grey of rats. In mice, i.v. injection of this inhibitor decreased sensitivity to thermal and mechanical stimuli as well as formalin-induced tonic pain and produced longer-lasting antinociception compared with RB101. In CFA inflammatory pain model i.v. injection of RB3007 led to antinociception in inflamed and non-inflamed rat hind paws (Le Guen et al, 2003). Also, i.c.v. injected P8B produced opioid antinociception against thermal noxious stimulation in healthy mice (Chen et al., 1998, 2000). In none of these studies END or DYN have been examined directly, possibly because of earlier findings that ENKs were the preferred substrates of NEP and APN (Matsas et al., 1983; Hersh, 1984). However, one study reported degradation of END to gamma-END by NEP (Graf et al., 1985) and there are reports on the cleavage of DYN by APN and NEP, which could be prevented by their inhibitors (Safavi and Hersh, 1995; Song and Marvizon, 2003).

1.8. Hypothesis and objectives

Currently used pain medications have major side effects such as gastrointestinal ulcers, tolerance or addiction. Effective analgesia without adverse effects can be achieved by exogenously applied or endogenous opioids activating opioid receptors on peripheral sensory neurons. Because opioid peptides are susceptible to enzymatic inactivation their analgesic actions might be enhanced by inhibiting their degradation. Previous studies suggested that blockade of APN and NEP activity prevents the catabolism of enkephalins and leads to antinociception, but effects on END or DYN have not been examined directly. So far, these studies concerned the CNS, mostly in animal models without or only short-lasting tissue injury, and they did not examine specific cellular sources of peptidases.

In the current studies we hypothesized that blocking the opioid-metabolizing peptidases APN and NEP expressed in immune cells or in nerves in peripheral inflamed tissue will prevent opioid peptide degradation leading to the local inhibition of pain. To inactivate peptidases we used classical selective inhibitors of APN (bestatin) and of NEP (thiorphan) as well as a novel dual inhibitor P8B. As a model of pathological pain we employed CFA-induced unilateral hind paw inflammation in rats. We used cell biological (flow cytometry) and biochemical (photospectrometry, radioimmunoassay) as well as *in vivo* pain testing methods. The main goals were:

a) To characterize and quantify leukocyte subpopulations expressing APN and NEP in inflamed tissue

b) To verify the activities of leukocytic APN and NEP using their selective synthetic substrates

c) To quantify the activities of neuronal APN and NEP using their selective synthetic substrates in peripheral nerves innervating inflamed and non-inflamed tissue

d) To examine the degradation of the four opioid peptides MENK, LENK, DYN and END, and to investigate which of them can be prevented from catabolism by leukocytic and neuronal APN and NEP by using selective and dual inhibitors

e) To validate the antinociceptive effects induced by APN- and NEP-inhibitors in peripheral inflamed tissue *in vivo*

2. Animals, Materials and Methods

2.1. Animals and animal housing

Male Wistar rats (250 - 300 g) were housed in pairs in cages lined with the standard bedding in a 12 h light-dark (8 a.m. /8 p.m.) cycle with food pellets and water *ad libitum*. Room temperature was maintained at 22 ± 0.5℃ and at a relative humidity between 40% and 60%. Experiments were approved by the local animal committee (Landesamt für Gesundheit und Soziales Berlin, Germany) and performed in accordance with ethical guidelines for investigation of experimental pain in animals (Zimmermann, 1983).

2.2. Materials

Materials are listed in alphabetical order and the distributors`names are given. For more details on distributors (city, country) see Appendix.

2.2.1. Antibodies

Table 2.1: Antibodies used for the following applications: F = flow cytometry, RIA = radioimmunoassay. Other abbreviations: FITC = fluorescein isothiocyanate conjugated, PE = phycoerythrin conjugated, PE-Cy5 = PE-cytochrome 5 conjugated.

Antibody	**Application**	**Company**
Goat anti-rabbit IgG	RIA	Bachem
Mouse anti-human, crossreacting with rat CD10 (SN5c)-FITC	F	Santa Cruz
Mouse anti-human, crossreacting with rat CD13 (WM15)-FITC	F	Santa Cruz
Mouse anti-rat T lymphocytes (CD3; G4.18)-PE	F	Pharmingen
Mouse anti-rat hematopoietic cells (CD45; OX-1)-PE-Cy-Chrome 5	F	Pharmingen
Mouse anti-rat macrophages (CD163; ED2)-PE	F	Serotec
Mouse anti-rat granulocytes (RP-1)-PE	F	Pharmingen
Normal rabbit serum	RIA	Bachem
Rabbit anti-rat DYN	RIA	Bachem

Antibody	Application	Company
Rabbit anti-rat END	RIA	Phoenix Laboratories
Rabbit anti-rat LENK	RIA	Bachem
Rabbit anti-rat MENK	RIA	Bachem
Rat anti-mouse IgG 1 κ-FITC (isotype control)	F	Pharmingen
Rat anti-mouse IgG 1-PE (isotype control)	F	Pharmingen
Rat anti-mouse IgG 2a κ-PE (isotype control)	F	Pharmingen
Rat anti-mouse IgG 3 κ-PE (isotype control)	F	Pharmingen

2.2.2. Chemicals, kits and media

Table 2.2: List of chemicals, kits and media

ß-naphthylamine (ßNA)	Sigma-Aldrich
BD Trucount^{TM} Tubes	BD Biosciences
Bestatin hydrochloride	Sigma-Aldrich
Bovine serum albumin (BSA)	Sigma-Aldrich
Collagenase from *Clostridium histolyticum*	Sigma-Aldrich
Complete Freund`s adjuvant (CFA)	Calbiochem
Dimethyl sulfhoxid Hybri-MAX® (DMSO)	Sigma-Aldrich
Fast blue B (FBB) salt	Sigma-Aldrich
Fetal bovine serum (FBS)	Biochrome
HEPES	Sigma-Aldrich
H-Gly-Gly-Phe-Leu-OH	Bachem
H-Gly-Gly-Phe-Met-OH	Bachem
H-Tyr-Gly-Gly-OH	Bachem
Hyaluronidase	Sigma-Aldrich
Isofluran (1-Chloro-2,2,2-trifluoroethyl-difluoronetylether)	Abbott
L-Alanin ß-naphthylamine (Ala-βNA)	Sigma-Aldrich
Paraformaldehyde (PFA)	Sigma-Aldrich
Penicillin/streptomycin (10,000U/10,000 µg/ml)	Biochrom
Phosphate buffered saline (PBS, 0.1 M, pH 7.4)	GIBCO Invitrogen
P-nitroaniline (pNA)	Sigma-Aldrich
Rat-DYN RIA kit	Bachem

Rat-END RIA kit	Phoenix Laboratory
Rat-LENK RIA kit	Bachem
Rat-MENK RIA kit	Bachem
NH_2-CH(Ph)P(O)_(OH)CH_2-CH(CH_2Ph(p-Ph)) CONH_CH(CH_3)COOH (P8B)	Pharmaleads
RPMI-1640 medium	Life Technologies
Saponin from Quillaja bark	Sigma-Aldrich
Succinyl-alanin-alanin-phenylalanine-*p*-nitroaniline (Suc-Ala-Ala-Phe-pNA)	Bachem
Thiorphan	Sigma-Aldrich
Tri(hydroxymethyl)-aminomethan (Tris)	Sigma-Aldrich
TrueCOUNT®	Parmingen/Becton
Trypan blue stain 0,4%	GIBCO Invitrogen

2.2.3. Equipment and other materials

Table 2.3: List of equipment and other materials

Cell strainer (70 µm mesh)	BD Bioscience Discovery Labware
Fluorescence activated cell sorting (FACS Calibur)	Becton-Dickinson
Gamma-counter, Wallac Wizard 1470	Wallac
Heraeus incubator	Kendro
Microscope (Axiovert 25)	Carl Zeiss Mikroskopie
Orion PerpHecT ROSS micro combination pH electrode	Thermo Electron Corporation
Polyethylene tubes (15 and 50 ml)	Falcon
Paw pressure algesiometer	Ugo Basile
Ultrapure water systems (Direct-Q™ 5)	Millipore
Ultrasound-bath Sonorex RK 52H	Bandelin
UV-visible spectrophotometer (UV-1601)	Shimadzu Europa GmbH

2.3. Methods

2.3.1. Anaesthesia

Inhalational anaesthesia with isoflurane was used to perform all i.pl. injections and to kill the rats. This was done by placing an animal in a glass chamber on a perforated ceramic plate covering a tissue soaked with isoflurane.

2.3.2. Induction of inflammation

Rats received an i.pl. injection of 0.15 ml of CFA into the right hind paw under brief isoflurane anesthesia. This inflammatory model is routinely used in our as well as in other research groups (Stein et al., 1988; Barber and Gottschlich 1992). It is characterized by swelling, increased temperature and enhanced sensitivity to noxious pressure (hyperalgesia) in the CFA-inoculated paw. During the course of 4 days the inflammation and hyperalgesia remain confined to the CFA-injected paw and there are no significant differences in feeding behavior, body weight, body temperature and general activity compared to untreated animals (Stein et al., 1988).

2.3.3. Measurement of pH value in subcutaneous paw tissue

To measure pH in the paw and to prepare tissues for *in vitro* experiments (see below) rats were killed by isoflurane inhalation. Immediately afterwards the skin on the plantar surface of CFA-inflamed and contralateral non-inflamed paws was cut and a special pH electrode was inserted i.pl. through the incision to determine the pH.

2.3.4. Tissue preparation

2.3.4.1. Preparation of immune cell suspensions from inflamed paws

Plantar subcutaneous tissue was dissected from inflamed rat hind paws and digested in a solution containing 30 mg collagenase, 10 mg hyaluronidase, and 0.5 ml HEPES per 10 ml RPMI buffer with 2% FBS for 1 h at 37℃, as previously

(Rittner et al., 2001). Cells were dissociated from homogenates using a 70 µm mesh cell strainer and suspended in RPMI medium. Cell number and viability (90 – 95%) was examined by the trypan blue exclusion method using a Neubauer chamber. Cells were reconstituted in RPMI at a concentration of 5 x 10^6 cells in 250 µl. They were either directly transferred for flow cytometry, for enzyme activity or opioid peptide RIA.

2.3.4.2. Preparation of membranes from immune cells and kidney

Kidneys and immune cells isolated from rat inflamed paws (see paragraph 2.3.4.1) were homogenized and centrifuged at 15,000 x *g* for 15 min at 4℃ to obtain a granular pellet and supernatant containing cell membranes. The supernatant was centrifuged at 48,000 x *g* for 30 min at 4℃ to obtain a solid membrane fracti on, according to Balog (1999). The membranes from 5 x 10^6 immune cells were reconstituted in 250 µl of RPMI, and those from kidneys in 2 ml of PBS per one kidney.

2.3.4.2. Preparation of peripheral nerve suspensions

The skin and muscles of the paw were cut and sciatic nerves terminal branches of the tibial, sural and common peroneal nerves innervating the plantar subcutaneous paw tissue were dissected. The nerve tissue was cut into small pieces placed in 300 µl RPMI buffer and homogenized using 10 x power 10% pulsed ultrasound.

2.3.5. Flow cytometry

Staining of leukocytes isolated from inflamed paws was performed as previously described (Rittner et al., 2001; Machelska et al., 2002). All centrifugations of cell suspensions were run for 5 min at room temperature and 450 x *g* using a swinging bucket rotor. Antibodies were conjugated with either fluorescein isothiocyanate (FITC), R-phycoerythrin (R-PE) or phycoerythrin-cytochrome 5 (PE-Cy5), as listed below, and were used in 2-color or 3-color staining. A total of 50,000 – 90,000 events were measured per sample in a FACS Calibur apparatus. Data were analyzed using CellQuest software (Parmingen/Becton Dickinson).

Staining of cell surface markers was performed first. Cells were suspended in 1 x PBS and centrifuged. Supernatants were softly decanted to leave 50 µl of buffer per sample. Single cell suspensions were stained for 15 min at room temperature without exposure to light with anti-CD45-PE-Cy5 (12 µg/ml) to identify hematopoietic cells, with mouse anti-human (crossreacting with rat) CD13-FITC (10 µg/ml) to identify APN and with mouse anti-human (crossreacting with rat) CD10-FITC (10 µg/ml) to identify NEP. T -lymphocytes were stained with mouse anti-rat CD3-R-PE (12 µg/ml) and macrophages with mouse anti-rat CD163-R-PE (5 µl/ 1 x 10^6 cells). Unbound Abs were removed by washing twice with PBS.

For intracellular staining cell suspensions were fixed for 30 min with PBS containing 1% PFA and permeabilized in PBS containing 0.5% saponin and 0.5% BSA. After centrifugation cells were incubated for 30 min at room temperature with intracellular mouse anti-rat Ab recognizing granulocytes (RP-1-R-PE, 6 µg/ml).

Specificity of the staining was verified by incubation of cell suspensions with appropriate isotype-matched rat anti-mouse IgGs (12 µg/ml). To calculate absolute numbers of cells per paw, the stained cell suspensions were analyzed together with a known number of fluorescent beads in a TruCOUNT® tube. Numbers of cells per tube were calculated in relation to the known number of fluorescence TruCOUNT® beads and extrapolated to the whole paw. To exclude non-hematopoietic cells, only $CD45^+$ cells were analyzed.

2.3.6. APN-induced degradation of its specific substrate

APN activity was determined according to Balog et al. (1999) using Ala-βNA as a substrate. Immune cell suspensions (1.5 x 10^6 cells in 250 µl) were incubated with 200 µM of Ala-βNA in PBS (pH ranging from 5.0 – 8.0) for 4 h at 37℃ in Heraeus incubator. Because there were no significant differences between pH conditions further experiments were performed at pH 7.4, as described previously (Florentin et al., 1984). Ala-βNA was freshly prepared as a stock solution in DMSO and diluted with 1:100 PBS immediately before use. Samples were incubated in PBS without or with bestatin (5 – 20 mM) or P8B (0.1 – 10 mM) and measured in duplicates. Rat kidney membranes (100 µl) were used as a positive and PBS as a negative control. After incubation, βNA was visualized in 0.5 ml of supernatant using 0.2 ml FBB solution (1.5 mg FBB dissolved in 1 ml PBS with 10% TWEEN-20 detergent, pH 4.2).

Similar experiments were performed with membranes from immune cells (prepared from 1.5 x 10^6 cells). Samples were incubated in PBS without or with bestatin (5 – 50 µM) or P8B (1 – 10 µM) and were measured in duplicates. Cleavage of Ala-βNA in immune cell

membranes and in whole immune cells was quantified photospectrometrically by absorbance at 530 nm compared to a standard curve of βNA and was expressed in μmol / 10^6 cells / 4 h.

Suspensions of terminal nerve branches (50 μl) were incubated in PBS without or with bestatin (5 – 15 mM) or P8B (1 – 10 mM) and measured in duplicates. Rates of cleavage of Ala-βNA were measured in the supernatant (0.15 μl) and expressed in mmol / g tissue / 4 h.

2.3.7. NEP-induced degradation of its specific substrate

NEP activity was measured according to Balog et al. (2001) using Suc-Ala-Ala-Phe–pNA as a substrate. Briefly, immune cells (2.5 x 10^6 cells) were prepared (see paragraph 2.3.4.1) and suspended in PBS (pH ranging from 5.0 – 8.0). Because there were no significant differences between different pH conditions further experiments were performed at pH 7.4, similar to experiments with APN (see paragraph 2.3.6). Samples were incubated for 4 h at 37℃ in Heraeus incubator with 100 μM of Suc-Ala-Ala-Phe–pNA in PBS without or with thiorphan (8 – 10 mM) or P8B (0.1 – 10 mM) and were measured in duplicates. Rat kidney membranes (100 μl) were used as a positive and PBS as a negative control. After incubation the presence of pNA was measured photospectrometrically at 405 nm in the supernatant (0.5 ml), and concentrations were calculated from a standard curve. NEP activity was expressed as μmol / 10^6 cells / 4 h.

Similar experiments were performed with immune cell membranes (prepared from 2.5 x 10^6 cells). Samples were incubated in PBS without or with thiorphan (1 – 10 μM) or P8B (1 – 10 μM) and measured in duplicates. To assess whether peptidase inhibitors interfere with photospectrometric measurements, bestatin (0.5 – 15 mM) and thiorphan (0.1 – 10 mM) or P8B (0.1 – 10 mM) without APN and NEP substrates were also tested.

Suspensions of nerve terminal branches (50 μl) were incubated in PBS without or with thiorphan (5 – 8 mM) or P8B (1 – 10 mM) and measured in duplicates. Metabolite pNA was quantified in the supernatant (0.15 μl) and expressed in mmol / g tissue / 4 h.

2.3.8. APN- and NEP-induced degradation of opioid peptides

Freshly prepared suspensions of vital immune cells were incubated in 250 μl RPMI buffer without or with a combination of bestatin (5 mM) and thiorphan (1 mM) or with P8B (5 mM). The samples (in a total volume of 500 μl) were centrifuged at 760 x *g* for 5 min at 4℃

either immediately after addition of peptidase inhibitors (basal measurements) or after 4, 6 or 8 h of incubation. Afterwards the supernatants (400 µl) were collected and stored at –20°C until RIA measurements. Each experiment was repeated 3 - 4 times.

Freshly prepared suspensions of immune cell membranes (250 µl) or nerve terminals (50 µl) were incubated in 150 µl RPMI buffer without or with a combination of bestatin (5 µM – 10 mM) and thiorphan (1 µM – 5 mM) or with P8B (1 µM – 10 mM). After 15 min of preincubation at 37°C in Heraeus incubator 100 µl of the standard opioid peptide MENK (12.8 ng/ml), LENK, DYN or END (each at 1.28 ng/ml) was added, obtaining a total volume of 500 µl for immune cell membranes or of 300 µl for nerves. The samples were centrifuged for 5 min at 760 x *g* and 4°C either immediately after addition of opioid peptides (basal measurements) or after 4 h of incubation with the tissues and peptidase inhibitors. Subsequently, the supernatants were collected and stored at –20°C until RIA. Each experiment was repeated 3 - 8 times.

2.3.9. Measurement of opioid peptide concentrations by RIA

RIA was performed according to Cabot et al. (1997, 2001) and Binder et al. (2004). Supernatants (see paragraph 2.3.8) were thawed and analyzed for the content of MENK, LENK, DYN or END using commercially available RIA kits. Reaction tubes containing 100 µl of the respective standard opioid peptide concentrations or unknown samples were prepared in duplicates. They were incubated overnight at 4°C with 100 µl of primary Ab against the respective opioid peptide. On the second day, 100 µl of I-125-labeled opioid peptide tracer (10,000 – 12,000 cpm) was added and tubes were incubated overnight at 4°C. On the third day, 100 µl of goat anti-rabbit IgG and 100 µl of normal rabbit serum were added followed by incubation for 90 min at room temperature. Subsequently, 500 µl RIA buffer was added and tubes were centrifuged at 1700 x *g* for 20 min at 4°C. After aspiration of supernatants (except for total count tubes), radioactivity was counted in the pellets using a gamma-counter. The content of opioid peptides was calculated by comparison to the respective standard curves.

To confirm the specificity of opioid peptide Abs, each Ab was tested with RIA using: A) samples containing either MENK (12.8 ng/ml), LENK, DYN or END (1.28 ng/ml) to examine whether the Abs specifically detect their respective opioid peptides; B) samples containing a combination of bestatin (5 µM – 5 mM) and thiorphan (1 µM – 10 mM) or P8B (1 µM – 10 mM) to test whether opioid peptide Abs recognize peptidase inhibitors; C) samples containing metabolites of opioid peptides (i.e. H-Gly-Gly-Phe-Leu-OH, H-Gly-Gly-Phe-Met-OH and H-Tyr-Gly-Gly-OH; 10 ng/ml for MENK or 1 ng/ml for LENK, DYN and END) to assess whether opioid peptide Abs recognize metabolites of opioid peptides.

2.3.10. Assessment of nociceptive thresholds in vivo

These experiments were performed by my collaborator Dr. D. Labuz. Four days after CFA inoculation mechanical nociceptive thresholds were assessed using the paw pressure algesiometer (modified Randall-Selitto test), as described earlier (Stein et al., 1990a; Machelska et al., 2003; Labuz et al., 2006). Rats (n = 2 – 3 per group) were gently restrained under paper wadding, and incremental pressure was applied via a wedge-shaped, blunt piston onto the dorsal surface of the hind paw. The paw pressure threshold (PPT) (cutoff at 250 g) required to elicit paw withdrawal was determined by averaging three consecutive trials separated by 15 s intervals. The sequence of paws was alternated between animals to avoid "order" effects. A combination of bestatin (5 mg) and thiorphan (0.8 mg) or P8B (1 mg) were injected i.pl. into inflamed paws together with a control IgG (2 µg; control group) or with Abs against MENK (2 µg), LENK (0.5 µg), END (1 µg) or DYN (0.5 µg). The doses of all substances were determined in pilot experiments. The substances were dissolved in 0.9% NaCl and injected in a total volume of 100 µl under brief isoflurane anesthesia. PPTs were measured before (baseline) and 5 min after injections.

2.3.11. Statistical analysis

Sigma Stat Version 3.5 for Windows software was used for statistical analyses. Multiple comparisons for independent data were analyzed by one-way analysis of variance (ANOVA) for normally distributed data or by one-way ANOVA on ranks for not normally distributed data. Two-way repeated-measures (RM) ANOVA was used for multiple comparisons of two treatments over time. Post-hoc multiple comparisons were performed using Bonferroni *t* test or Student-Newman-Keuls test. Linear regression analysis was used to evaluate dose-dependency. Data are presented as means ± standard error of the mean (SEM). Differences were considered significant if $P < 0.05$.

3. Results

3.1. Quantitative characterization of leukocytes expressing APN and NEP in inflamed paws

Single cell suspensions were prepared from rat hind paws inoculated with CFA 4 days earlier, and processed for flow cytometry. To exclude nonhematopoietic cells and debris, cell suspensions were stained with an Ab against CD45, a hematopoietic cell marker, and gated on $CD45^+$ cells (Fig. 3.1 A). Macrophages ($CD45^+CD163^+$) dominated at this stage of inflammation constituting 31.3% of $CD45^+$ cells (Fig. 3.1 B and Fig. 3.2 B) followed by granulocytes ($CD45^+RP\text{-}1^+$) representing 16.6% (Fig. 3.1 D and Fig. 3.2 C) and by T-lymphocytes ($CD45^+CD3^+$) comprising 4.1% of $CD45^+$ cells (Fig. 3.1 F and Fig. 3.2 D). T-lymphocytes were significantly less abundant than granulocytes and macrophages ($P < 0.05$, one-way ANOVA, Bonferroni *t* test; Fig. 3.2).

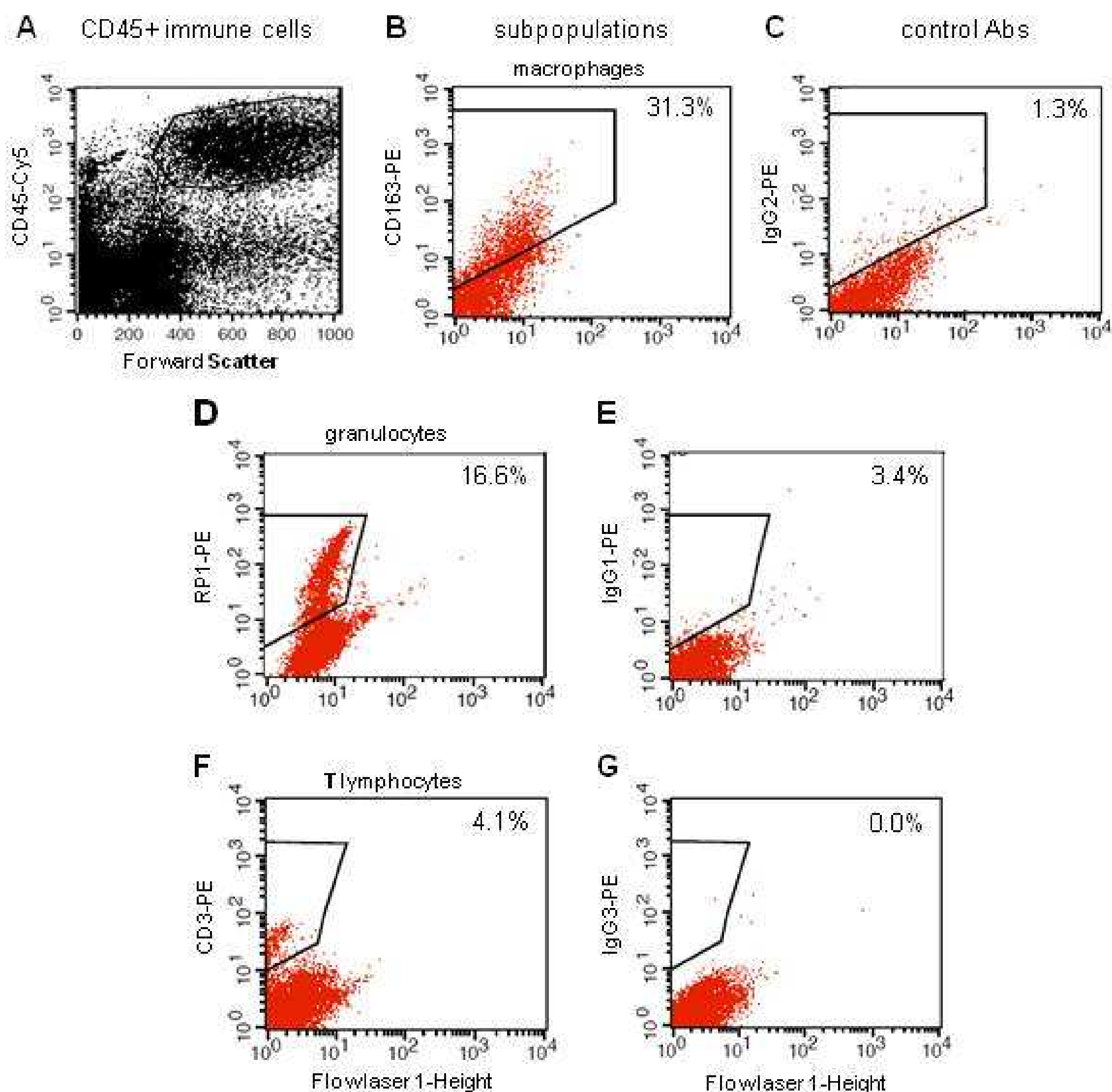

Figure 3.1. (previous page) Characterization of immune cell subpopulations in inflamed rat paws (**A**) Gating on hematopoietic cells (CD45 Ab). (**B**) Gating on macrophages (CD163 Ab). (**C**) Staining by control IgG2 Ab for anti-CD163. (**D**) Gating on granulocytes (RP-1 Ab). (**E**) Staining by control IgG1 Ab for anti-RP-1. (**F**) Gating on T cells (CD3 Ab). (**G**) Staining by control IgG3 Ab for anti-CD3. Cy5, PE and FITC represent fluorescent dyes conjugated with respective Abs.

To evaluate $CD45^+$ cell subpopulations expressing APN ($CD13^+$) or NEP ($CD10^+$) triple-color flow cytometry was employed. Of all $CD45^+$ cells 22.6% were APN-positive ($CD45^+CD13^+$) and 12% were NEP-positive ($CD45^+CD10^+$) (Fig. 3.2 A). The analysis showed that 24.2% of macrophages expressed APN ($CD45^+CD163^+CD13^+$) and 37.4% expressed NEP ($CD45^+CD163^+CD10^+$) (Fig. 3.2 B). Among granulocytes 15.2% expressed APN ($CD45^+RP\text{-}1^+CD13^+$) and 14.3% expressed NEP ($CD45^+RP\text{-}1^+CD10^+$) (Fig. 3.2 C). APN and NEP were not detected in T-lymphocytes (Fig. 3.2 D). Minimal staining (0 - 3.4%) by respective isotype-matched control Abs confirmed staining specificity of Abs against APN, NEP (not shown) and each leukocyte subpopulation (Fig. 3.1 C, E and G).

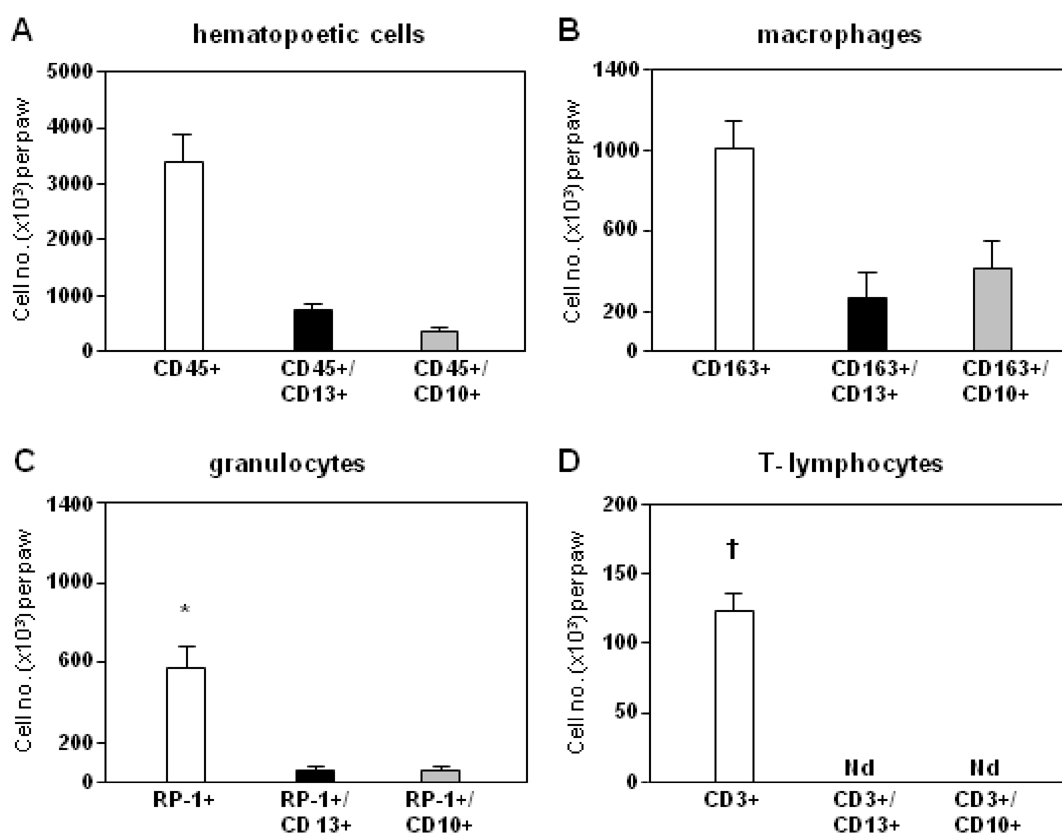

Figure 3.2. Quantification of leukocyte subpopulations expressing APN or NEP in inflamed rat paws. The cell nnumbers were calculated in relation to a fixed number of TruCOUNT® beads added to the cell suspensions. (**A**) Hematopoietic ($CD45^+$) cells and $CD45^+$ cells expressing APN ($CD45^+CD13^+$) or

NEP ($CD45^+CD10^+$). (**B**) Macrophages ($CD163^+$) and macrophages expressing APN ($CD163^+CD13^+$) or NEP ($CD163^+CD10^+$). (**C**) Granulocytes ($RP\text{-}1^+$) and granulocytes expressing APN ($RP\text{-}1^+CD13^+$) or NEP ($RP\text{-}1^+CD10^+$). (**D**) T-lymphocytes ($CD3^+$). $^*P < 0.05$, compared with $CD163^+$; $^\dagger P < 0.05$, compared with $RP\text{-}1^+$ and $CD163^+$ (one-way ANOVA, Student-Newman-Keuls test). Nd, not detected. Data are expressed as means ± SEM. N = 7 rats per group.

3.2. Role of APN and NEP in the degradation of opioid peptides released by immune cells

3.2.1. Effects of APN and NEP inhibitors on the degradation of peptidase-specific substrates

To determine the enzymatic activity of APN the degradation of its specific synthetic substrate Ala-βNA to βNA was measured photospectrometrically. No βNA was detectable at the zero time points in all experiments (Fig. 3.3). Incubation of vital immune cells with Ala-βNA for 4 h resulted in an accumulation of its metabolite βNA in inhibitor-free control groups ($P < 0.001$, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.3 A and B). When cells were treated with either bestatin (5 – 20 mM) or P8B (0.1 – 10 mM) the βNA levels in supernatants dose-dependently decreased ($P < 0.001$, Linear regression, Fig. 3.3 A and B). βNA production was abolished by 20 mM of bestatin (Fig. 3.3. A) and was diminished by about 69% by 10 mM of P8B (Fig. 3.3 B).

To determine the enzymatic activity of NEP the degradation of its specific substrate Suc-Ala-Ala-Phe-pNA to pNA was examined. Incubation of immune cells with Suc-Ala-Ala-Phe-pNA for 4 h resulted in a significant increase of pNA in inhibitor-free control groups ($P < 0.001$, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.3 C and D). pNA production was dose-dependently blocked by thiorphan (8 – 10 mM) and diminished by up to 64% by P8B (0.1 – 20 mM) ($P < 0.001$, Linear regression; Fig. 3.3 C and D).

The actions of selective inhibitors were enzyme-specific because there were no significant effects of thiorphan (10 mM) on βNA production (i.e. APN activity) or of bestatin (10 mM) on pNA production (i.e. NEP activity) ($P > 0.05$, two-way RM; data not shown). Analysis of thiorphan (1 µM – 10 mM) and bestatin (5 µM – 20 mM) or P8B (1 µM – 20 mM) without APN and NEP substrates showed no signals, suggesting that inhibitors did not interfere with photospectrometric measurements.

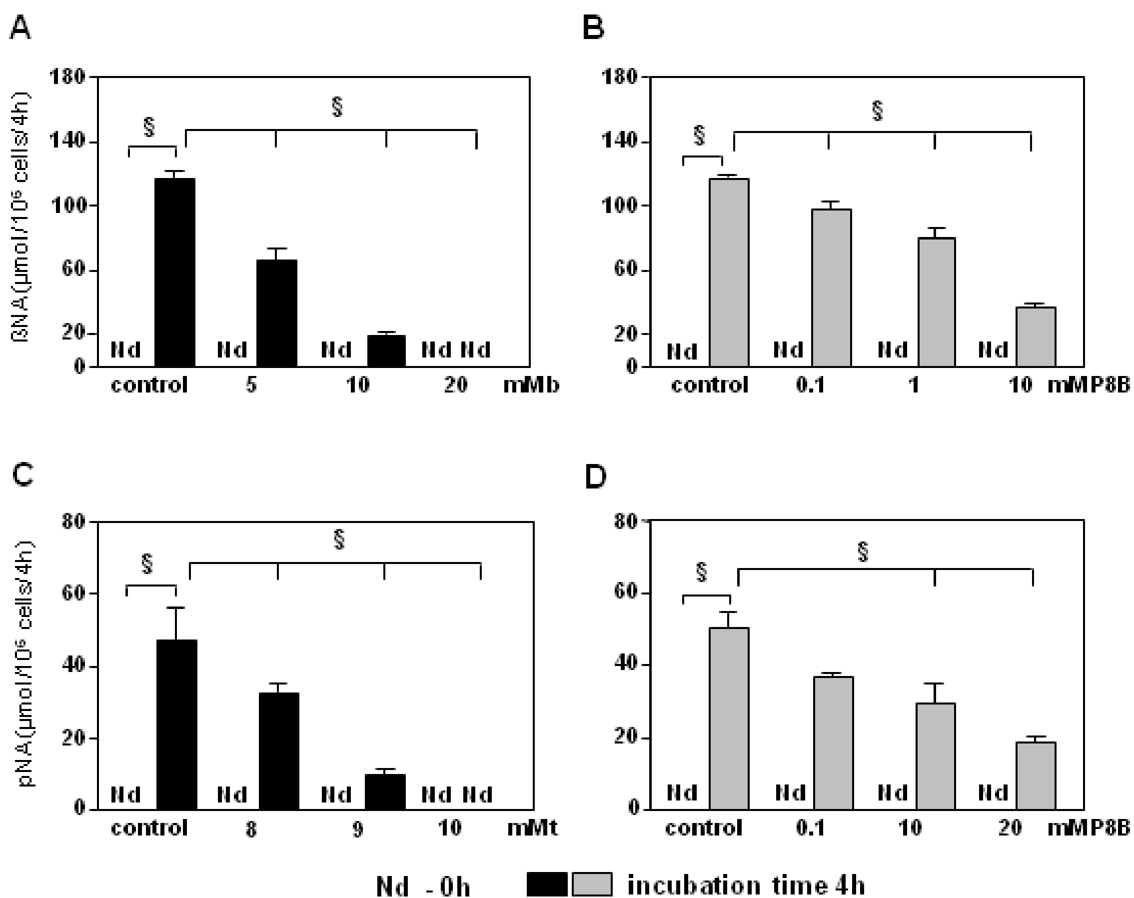

Figure 3.3. Effects of APN and NEP inhibitors on the degradation of peptidase-specific substrates in vital immune cells. (**A, B**) Incubation with Ala-βNA (200 µM) alone for 4 h resulted in accumulation of the APN metabolite βNA in the supernatant. Substrate hydrolysis was dose-dependently blocked by bestatin (b) and diminished by P8B (P < 0.001, Linear regression). (**C, D**) Incubation with Suc-Ala-Ala-Phe-pNA (100 µM) alone resulted in accumulation of the NEP metabolite pNA after 4 h. Substrate hydrolysis was dose-dependently prevented by thiorphan (t) and attenuated by P8B (P < 0.001, Linear regression). The metabolites were below the detection limit at 0h in all groups. §P < 0.001 (two-way RM ANOVA, Bonferroni *t* test). Nd, not detected. Data are expressed as means ± SEM. N = 3-8 experiments per group.

3.2.2. pH dependency of APN and NEP activity

It has been previously reported that inflammation is accompanied by acidification of the affected tissues (Punnia-Moorthy, 1988). Measurements (in co-operation with Mrs. Spahn) by i.pl. insertion of a pH electrode revealed significantly lower pH values in inflamed (7.02 ± 0.05) as compared with non-inflamed rat paws (7.43 ± 0.02) (P < 0.001, *t* test, n = 4-8).

To investigate whether the activities of APN and NEP were influenced by extracellular pH levels, immune cells isolated from inflamed paws were incubated for 4 h in RPMI-buffer under different pH conditions. As demonstrated in Fig. 3.4., changing pH values from 5.0 – 8.0 did not significantly influence the levels of βNA (indicating APN activity) and pNA (indicating NEP activity) (P > 0.05, one-way RM ANOVA).

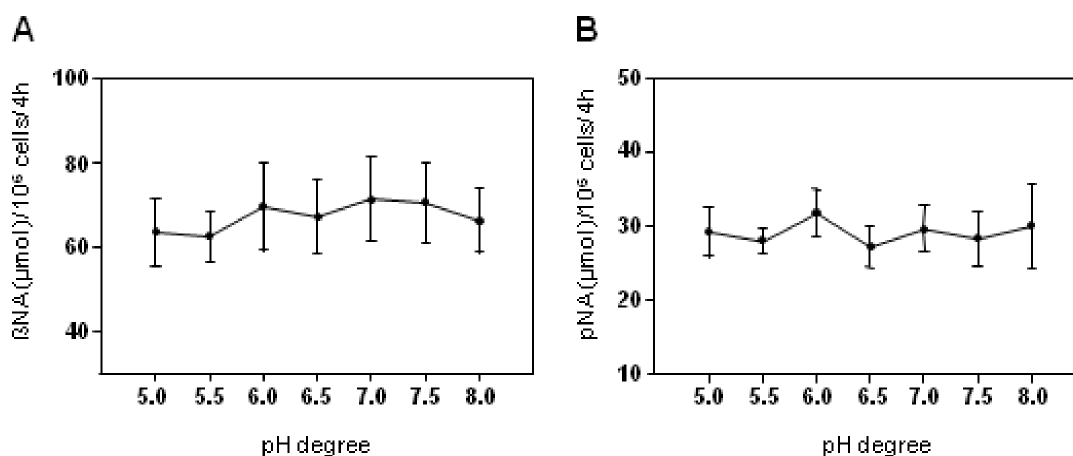

Figure 3.4. Effects of pH on the activity of APN and NEP in vital immune cells. (A) Incubation of cells with the APN substrate Ala-βNA (200 µM) at different pH values (5.0 - 8.0) did not influence accumulation of its metabolite βNA over 4 h. (B) Incubation of cells with the NEP substrate Suc-Ala-Ala-Phe-pNA (100 µM) at different pH values (5.0 - 8.0) did not influence accumulation of its metabolite pNA over 4 h (P > 0.05, one-way RM ANOVA). Data are expressed as means ± SEM. N = 3-5 experiments per group.

3.2.3. Effects of selective APN and NEP inhibitors on opioid peptides released from immune cells

The next step was to ascertain the release of endogenous opioid peptides from vital inflammatory cells. The extracellular levels of immunoreactive MENK and LENK increased 1.3 and 2.3 fold, respectively, over the first 4 h (P < 0.01) while those of DYN and END increased 2.4 and 2.1 fold, respectively, at 8 h (P < 0.05) (two-way RM ANOVA, Bonferroni *t* test; Fig. 3.5). To exclude that these increases in immunoreactivities were due to cross-reactivity of Abs with opioid peptide metabolites, the standard analogs Tyr-Gly-Gly-OH, Gly-Gly-Phe-Met-OH and Gly-Gly-Phe-Leu-OH were tested in RIA for each of the four opioid peptides. No immunoreactivities were detected (data not shown). As shown in Fig. 3.5 A and B, control MENK and LENK began to decrease at 6 h and dropped to baseline levels at 8 h (P < 0.001 and P < 0.01, respectively, two-way RM ANOVA, Bonferroni *t* test).

A combination of bestatin (5 mM) and thiorphan (1 mM) increased MENK concentrations at 4 - 8 h of incubation when compared to baseline (0 h) of the inhibitor-treated group (P < 0.01, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.5 A). When compared with control groups the inhibitors decreased MENK levels at 0 h and 4 h (P < 0.05). Also, inhibitor treatment did not increase MENK over level of control group at 6 - 8 h (P > 0.05) (two-way RM ANOVA, Bonferroni *t* test; Fig. 3.5 A). No changes of LENK levels were detected at 0 h to 6 h between groups and treatment with bestatin and thiorphan significantly elevated (by 47 %) LENK levels compared with control at 8 h (P < 0.001, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.5 B). Levels of DYN and END significantly increased over time in control and inhibitor-treated groups (P < 0.05), but were not significantly influenced by inhibitors (P > 0.05) (two-way RM ANOVA, Bonferroni *t* test; Fig. 3.5 C and D). Concentrations of inhibitors in these experiments were lower than in experiments depicted in Fig. 3.3 A and C, because a combination of bestatin and thiorphan in doses higher than 5 mM and 1 mM, respectively, led to cell death.

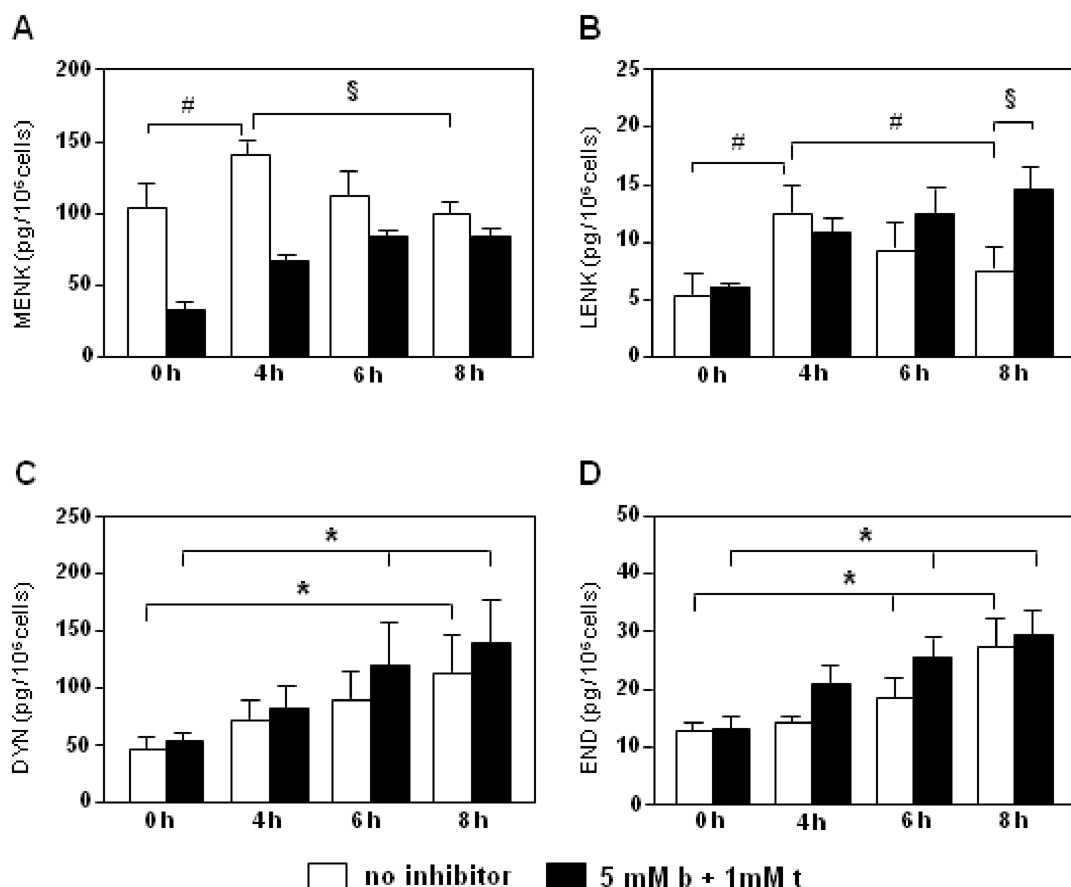

Figure 3.5. (previous page) Effects of the combination of bestatin (b) and thiorphan (t) on the levels of opioid peptides released from immune cells over 8 h of incubation. Amounts of MENK (**A**), LENK (**B**), DYN (**C**) and END (**D**) were determined in the supernatants of immune cells immediately after addition (0 h) of buffer (white bars) or of bestatin and thiorphan (black bars) and at 4 - 8 h later. $^{*}P < 0.05$; $^{\#}P < 0.01$; $^{\S}P < 0.001$ (two-way RM ANOVA, Bonferroni *t* test). Data are expressed as means ± SEM. N = 3-4 experiments per group.

3.2.4. Effects of a dual APN and NEP inhibitor on opioid peptide levels released from immune cells

We examined the effects of P8B on the levels of opioid peptides secreted from immune cells during 8 h of incubation. Similar to the above experiments (Fig. 3.5), extracellular levels of MENK and LENK in control groups increased over the first 4 h ($P < 0.001$) but began to decrease at 6 h and significantly dropped at 8 h ($P < 0.05$ for MENK and $P < 0.001$ for LENK) (two-way RM ANOVA, Bonferroni *t* test; Fig. 3.6 A and B). P8B (5 mM) increased levels of MENK at 4 - 8 h and of LENK at 8 h when compared to baseline (0 h) of the respective inhibitor-treated groups ($P < 0.05$, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.6 A and B). The inhibitor significantly decreased LENK levels compared with control groups at all time points of incubation ($P < 0.001$) and it did not increase both peptides compared with the respective control groups at 8 h ($P > 0.05$, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.6 A and B). Levels of DYN and END significantly increased over time in control groups ($P < 0.05$) and were not significantly changed by P8B ($P > 0.05$) (two-way RM ANOVA, Bonferroni *t* test; Fig. 3.6 C and D). Concentrations of inhibitors in these experiments were chosen similar to bestatin in experiments above.

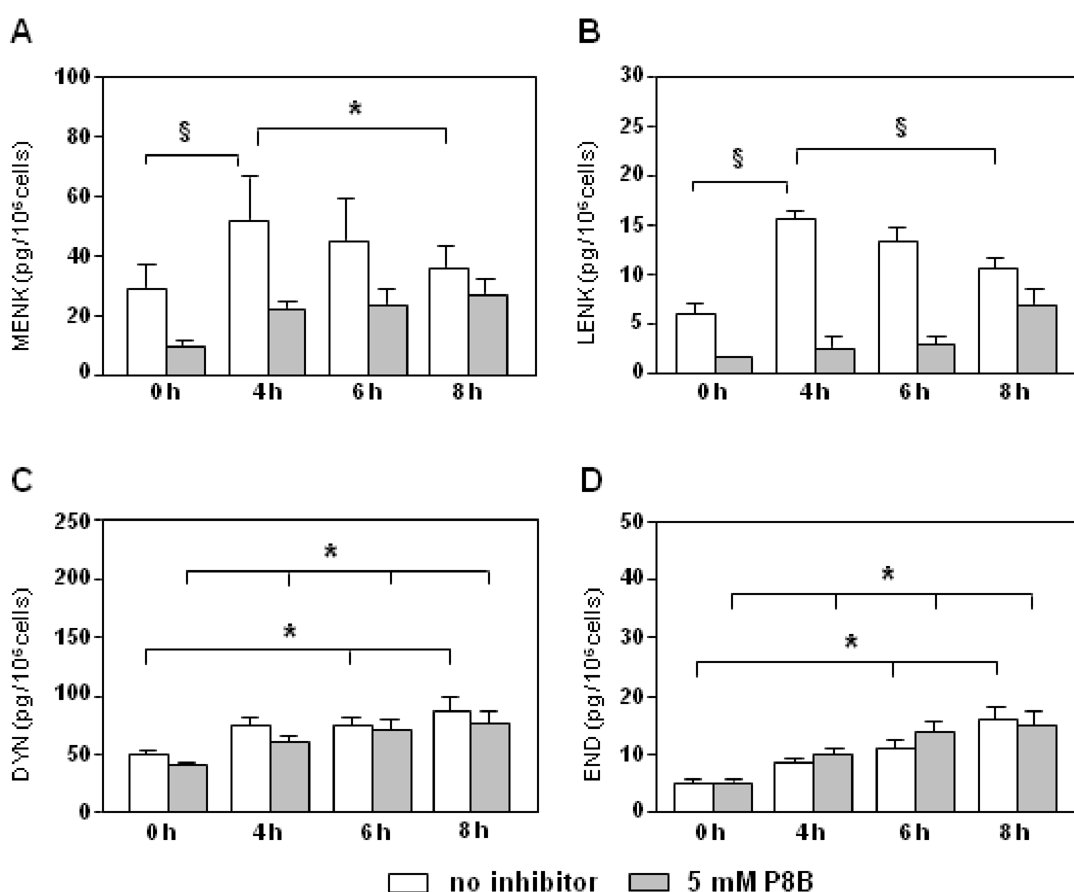

Figure 3.6. Effects of P8B on the levels of opioid peptides released from immune cells during 8 h of incubation. Amounts of MENK (**A**), LENK (**B**), DYN (**C**) and END (**D**) were determined in the supernatants of immune cells immediately after addition (0 h) of buffer (white bars) or of P8B (gray bars) and after 4, 6 and 8 h. $^{*}P < 0.05$; $^{\S}P < 0.001$ (two-way RM ANOVA, Bonferroni *t* test). Data are expressed as means ± SEM. N = 3-4 experiments per group.

3.3. APN and NEP activity on immune cell membranes

3.3.1. Effects of APN and NEP inhibitors on the degradation of peptidase-specific substrates

The next step was to study peptidase activities in immune cell membrane preparations from leukocytes infiltrating inflamed paws. APN and NEP enzymatic activities were assessed by photospectrometrically measuring the metabolites of their specific substrates (as described in paragraph 3.2.1.) and no substrate hydrolysis was detected at

0 h in any experiment (Fig. 3.7). Incubation of immune cell membrane suspensions with the APN substrate Ala-βNA for 4 h resulted in an accumulation of its metabolite βNA in inhibitor-free control experiments (P < 0.001, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.7 A and B). Co-incubation with bestatin (50 µM) or P8B (1 – 10 µM) fully abolished the generation of βNA (P < 0.001, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.7 A and B).

Incubation of immune cell membrane suspensions with the NEP substrate Suc-Ala-Ala-Phe-pNA for 4 h led to enhanced levels of pNA in inhibitor-free control groups (P < 0.001, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.7 C and D). This pNA production was fully blocked by thiorphan (1 – 10 µM) and P8B (1 – 10 µM) (P < 0.001, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.7 C and D).

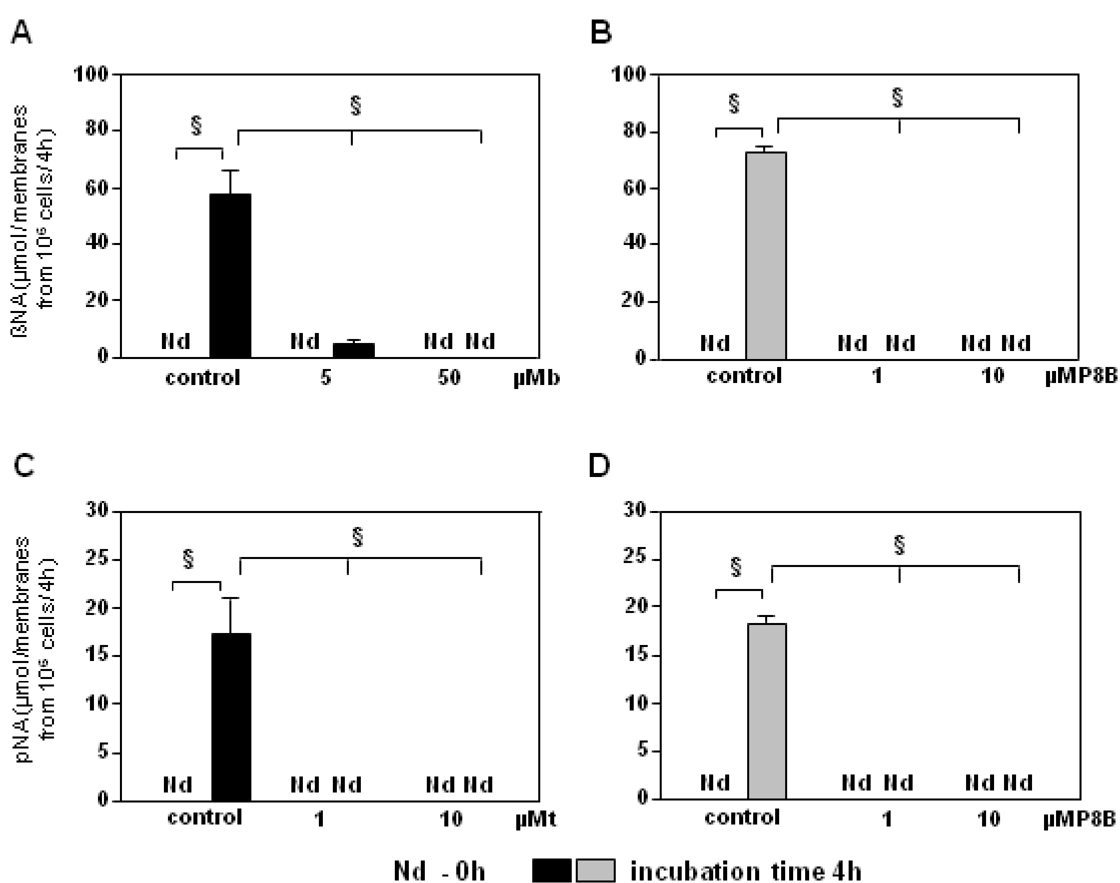

Figure 3.7. Effects of APN and NEP inhibitors on the degradation of peptidase-specific substrates in immune cell membranes. (**A, B**) APN activity was determined by the degradation of Ala-βNA (200 µM) to its metabolite βNA after 4 h of incubation in the inhibitor-free control groups. βNA production was prevented by bestatin (b) or P8B. (**C, D**) NEP activity was determined by degradation of Suc-Ala-Ala-Phe-pNA (100 µM) to its metabolite pNA after 4 h of incubation in inhibitor-free control groups. pNA production was prevented by thiorphan (t) or P8B. The metabolites were below the detection limit at time 0 h in all groups. §P < 0.001 (two-way RM ANOVA, Bonferroni *t* test). Nd, not detected. Data are expressed as means ± SEM. N = 3-4 experiments per group.

3.3.2. Effects of selective APN and NEP inhibitors on the degradation of exogenous opioid peptides

To assess the effects of selective APN and NEP inhibitors on the degradation of exogenous opioid peptides, immune cell membrane suspensions were incubated with MENK (12.8 ng/ml), LENK, DYN or END (1.28 ng/ml) in the absence or presence of combinations of bestatin (5 – 500 μM) and thiorphan (1 – 100 μM), and 4 h later opioid levels were measured with RIA. There were no statistically significant differences in basal opioid peptide levels at 0 h of incubation between inhibitor-free controls and groups containing a combination of bestatin and thiorphan ($P > 0.05$, two-way RM ANOVA; Fig. 3.8). Significantly decreased amounts of MENK (by 70%; $P < 0.001$), LENK (by 77%; $P < 0.01$), DYN (by 46%; $P < 0.05$) and END (by 39%; $P < 0.05$) (two-way RM ANOVA, Bonferroni *t* test; Fig. 3.8) were observed after 4 h of incubation without inhibitors (controls). Combinations of bestatin (5 - 500 μM) and thiorphan (1 - 100 μM) dose-dependently prevented the degradation of MENK and LENK ($P < 0.01$ and $P < 0.001$, respectively, Linear regression; Fig. 3.8 A and B). Bestatin and thiorphan did not significantly change the levels of DYN and END ($P > 0.05$, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.8 C and D).

To examine cross-reactivity of Abs, the standard MENK, LENK, DYN and END peptides were tested in the respective three complementary RIAs. MENK produced a minimal signal when tested in the LENK RIA, and therefore all subsequent LENK data were corrected by subtracting this signal. There was no cross-reactivity in any other combinations (data not shown). To determine whether opioid peptide Abs recognize peptidase inhibitors, the inhibitors were also tested in all four RIAs. Bestatin (50 μM – 5 mM) in a combination with thiorphan (10 μM – 1 mM) produced a small signal when tested with the LENK RIA, and therefore all LENK data (with inhibitors) were corrected by subtracting this signal. To assess whether peptidase inhibitors cross-react with the standard opioid peptides, MENK, serving as an example of all opioid peptides, was combined with bestatin (5 μM - 5 mM) and thiorphan (1 μM - 1 mM) in standard curves and tested with RIA. There were no differences between standard curves with and without peptidase inhibitors (data not shown).

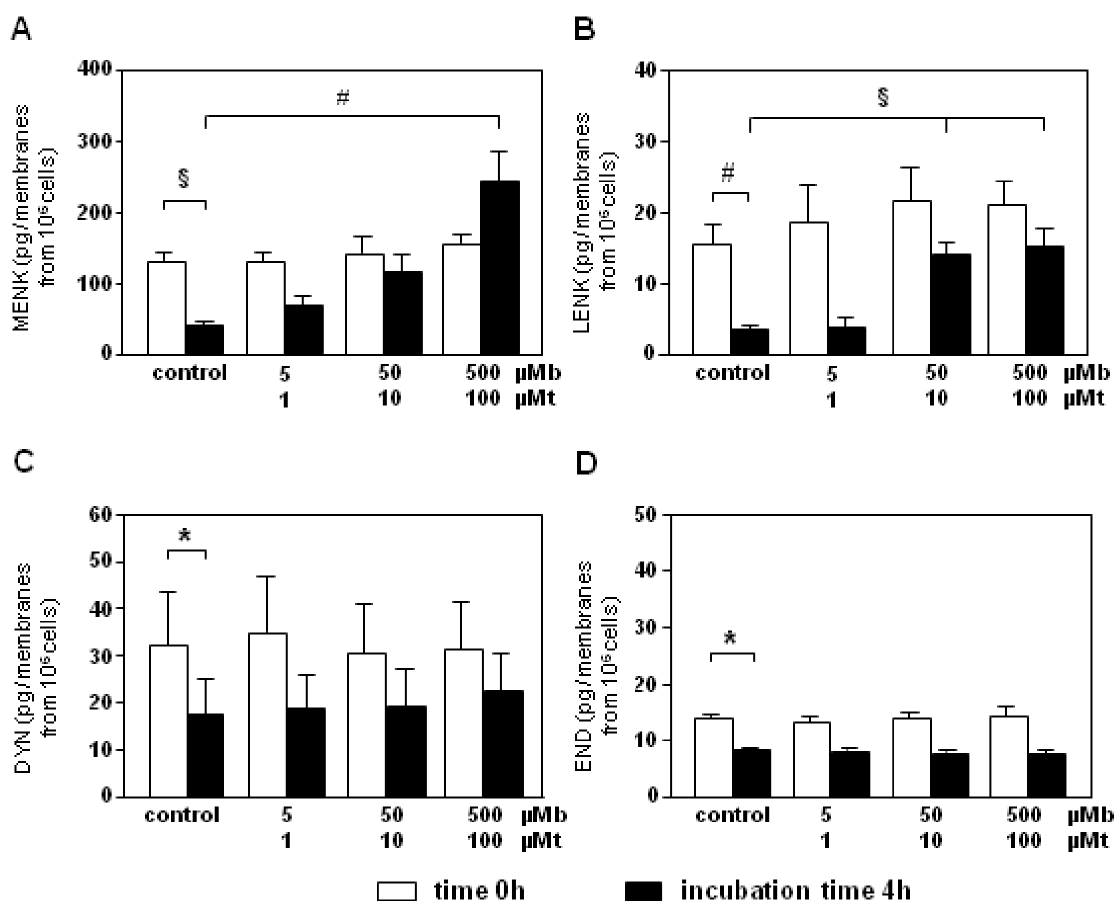

Figure 3.8. Effects of the combination of bestatin (b) and thiorphan (t) on the degradation of exogenous opioid peptides in immune cell membranes. Membrane suspensions were incubated with MENK (**A**), LENK (**B**), DYN (**C**) or END (**D**), and with control buffer or combinations of thiorphan and bestatin. Measurements were performed immediately after addition of buffer or inhibitors (white bars) and 4 h later (black bars). Inhibitors dose-dependently increased MENK and LENK concentrations ($P < 0.01$ and $P < 0.001$, respectively, Linear regression). Levels of DYN and END were not significantly changed by the inhibitors ($P > 0.05$, two-way RM ANOVA, Bonferroni *t* test). *$P < 0.05$; $^{\#}P < 0.01$; $^{\S}P < 0.001$ (two-way RM ANOVA, Bonferroni *t* test). Data are expressed as means ± SEM. N = 3-5 experiments per group.

3.3.3. Effects of a dual APN and NEP inhibitor on the degradation of exogenous opioid peptides

To investigate the effect of P8B, immune cell membrane suspensions were incubated with standard MENK (12.8 ng/ml), LENK, DYN or END (1.28 ng/ml) in the absence or

presence of P8B (1 – 100 µM), and opioid levels were measured with RIA 4 h later. There were no significant differences in basal opioid peptide levels immediately after addition of buffer or P8B (0 h) ($P > 0.05$, two-way RM ANOVA, Bonferroni *t* test; Fig 3.9). Significantly decreased amounts of MENK (by 37%), LENK (by 77%), DYN (by 47%) and END (by 37%) ($P < 0.001$, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.9) were observed after 4 h. Treatments with P8B (1 - 100 µM) dose-dependently increased MENK and LENK ($P < 0.05$, Linear regression; Fig. 3.9 A and B) but did not significantly change the levels of DYN and END ($P > 0.05$, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.9 C and D).

To examine whether P8B cross-reacts with opioid peptides, standard LENK, serving as example of all opioid peptides, was combined with P8B (5 mM) in RIA. There were no differences between the standard curves with and without P8B (data not shown).

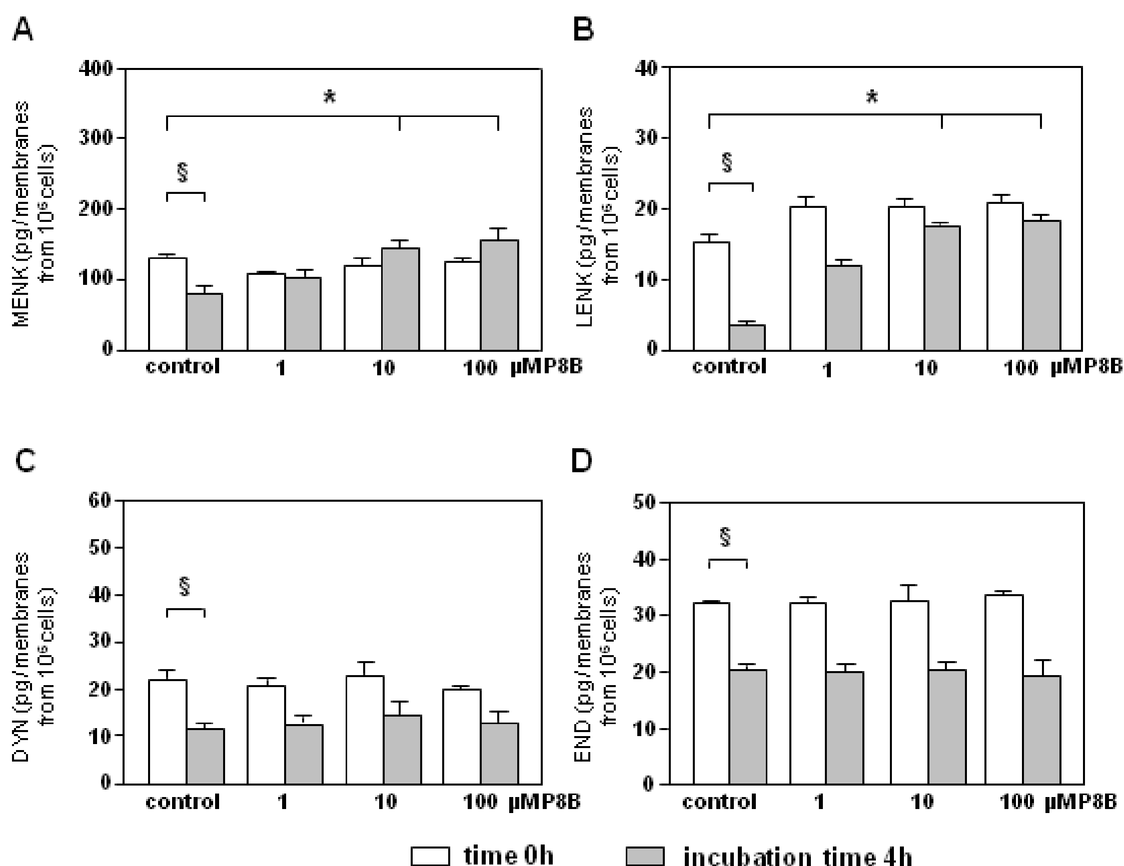

Figure 3.9. Effects of P8B on the degradation of exogenous opioid peptides in immune cell membranes. Membrane suspensions were incubated with MENK (**A**), LENK (**B**), DYN (**C**) or END (**D**) and with buffer (control) or P8B. Measurements were performed immediately after addition of buffer or inhibitor (white bars) and 4 h later (gray bars). The inhibitor dose-dependently prevented the degradation of MENK and LENK ($P < 0.05$, Linear regression) but not of END and DYN ($P > 0.05$,two-way RM ANOVA, Bonferroni *t* test). *$P < 0.05$; §$P < 0.001$ (two-way RM ANOVA, Bonferroni *t* test). Data are expressed as means ± SEM. N = 3-5 experiments per group.

3.4. Activity of APN and NEP expressed on peripheral nerves

3.4.1. Effects of APN and NEP inhibitors on the degradation of peptidase-specific substrates in nerve preparations

Similar to experiments with vital immune cells and immune cell membranes (see paragraphs 3.2.1 and 3.3.1), APN and NEP activities in suspensions of sciatic nerve terminal branches were determined photospectrometrically using peptidase specific substrates. No metabolites were detectable at the beginning (0 h) of incubations in any experiment (Fig. 3.10 and 3.11). Incubation of nerve suspensions with the APN substrate Ala-βNA for 4 h resulted in an accumulation of its metabolite βNA in inhibitor-free control groups ($P < 0.001$, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.10 A and Fig. 3.11 A and B). There was no significant difference between inflamed and non-inflamed sides ($P > 0.05$, two-way RM ANOVA; Fig. 3.10 A). Both bestatin (5 – 15 mM) and P8B (1 – 10 mM) dose-dependently diminished the generation of βNA in nerve suspensions from inflamed paws ($P < 0.001$, Linear regression; Fig. 3.11 A and B). βNA production was fully blocked by 15 mM of bestatin and diminished by about 61% by 10 mM of P8B.

Incubation of nerve suspensions with the NEP specific substrate Suc-Ala-Ala-Phe-pNA for 4 h resulted in a significant accumulation of its metabolite pNA in inhibitor-free control groups ($P < 0.001$, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.10 B and Fig. 3.11 C and D). A significantly higher (by 42%) amount of pNA accumulated over 4 h in nerve suspensions from inflamed compared to non-inflamed limbs ($P < 0.001$, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.10 B). The pNA production was dose-dependently blocked in the presence of either thiorphan (1 – 8 mM) or P8B (1 – 10 mM) in nerve suspensions from inflamed paws ($P < 0.001$, Linear regression; Fig. 3.11 C and D). NEP activity was fully blocked by 8 mM of thiorphan and was decreased by 92% by 10 mM of P8B.

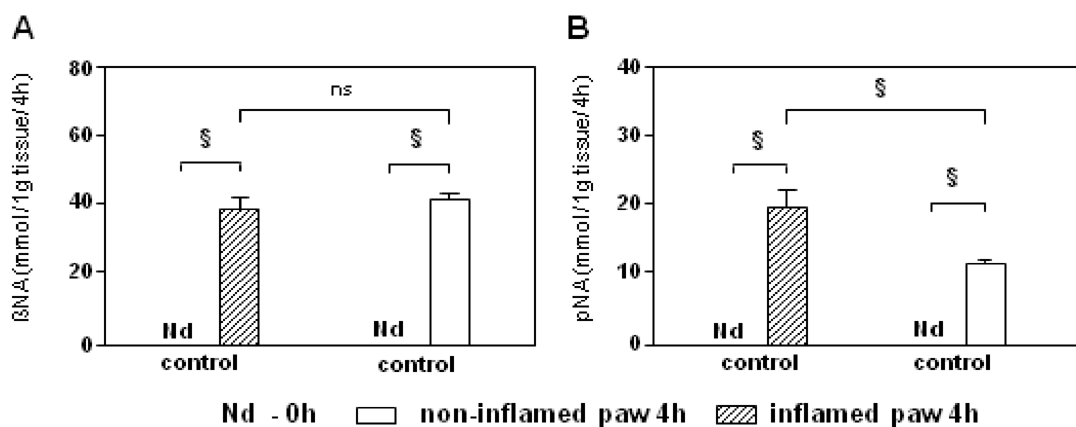

Figure 3.10. Activity of APN and NEP in suspensions of sciatic nerve terminal branches innervating inflamed and non-inflamed paws. (**A**) APN activity was determined by the degradation of Ala-ß-NA (200 µM) to its metabolite βNA after 4 h of incubation in peripheral nerves. There was no significant difference between inflamed and non-inflamed sides ($P > 0.05$, two-way RM ANOVA, Bonferroni *t* test). (**B**) NEP activity was determined by the degradation of Suc-Ala-Ala-Phe-pNA (100 µM) to its metabolite pNA after 4 h incubation in peripheral nerves. pNA production was increased in nerve suspensions from inflamed limbs. The metabolites were below detection limit at 0 time points in all groups. $^{\S}P < 0.001$; ns, not significant (two-way RM ANOVA, Bonferroni *t* test). Nd, not detected. Data are expressed as means ± SEM. N = 3-8 experiments per group.

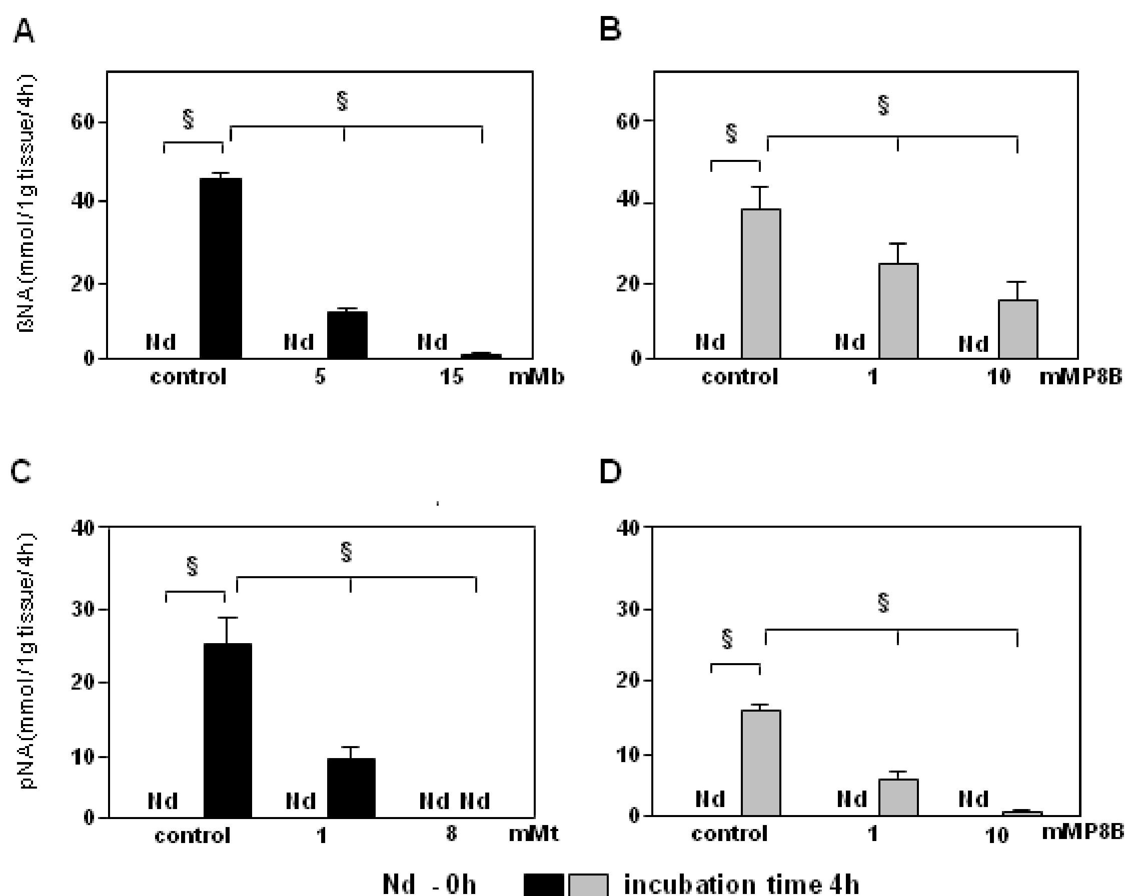

Figure 3.11. (previous page) Effects of APN and NEP inhibitors on the degradation of peptidase-specific substrates in suspensions of sciatic nerve terminal branches innervating inflamed paws. (**A, B**) Incubation with Ala-βNA (200 μM) alone for 4 h resulted in accumulation of the APN metabolite βNA in the supernatant. Prevention of substrate hydrolysis was assessed in the presence of bestatin (b) and diminished by P8B ($P < 0.001$, Linear regression). (**C, D**) Incubation with Suc-Ala-Ala-Phe-pNA (100 μM) alone resulted in accumulation of the NEP metabolite pNA after 4 h. Prevention of substrate hydrolysis was examined in the presence of thiorphan (t) or P8B ($P < 0.001$, Linear regression). The metabolites were below detection limit at 0 time points in all groups. $^{\S}P < 0.001$ (two-way RM ANOVA, Bonferroni *t* test). Nd, not detected. Data are expressed as means ± SEM. N = 3-8 experiments per group.

3.4.2. Effects of selective APN and NEP inhibitors on the degradation of exogenous opioid peptides in nerve preparations

Next we assessed the functional relevance of APN and NEP in peripheral nerves for the degradation of exogenously added opioid peptides. No significant differences in the basal opioid peptide levels were measured at 0 time points among groups ($P > 0.05$, two-way RM ANOVA, Bonferroni *t* test; Fig 3.12). Four h incubation of the suspensions of sciatic nerve terminal branches with standard MENK (12.8 ng/ml), LENK, DYN or END (1.28 ng/ml) showed 78% decrease of MENK and 79% decrease of LENK compared to 0 h ($P < 0.01$, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.12 A and B). Levels of DYN were lowered by 57% and of END by 83% ($P < 0.05$, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.12 C and D). In the samples treated with a combination of bestatin (0.5 - 10 mM) and thiorphan (0.1 - 5 mM) for 4 h, the degradation of MENK and LENK was dose-dependently prevented ($P < 0.001$, Linear regression; Fig. 3.12 A and B). The DYN degradation was also dose-dependently prevented ($P < 0.001$, Linear regression; Fig. 3.12 C) and its amounts increased significantly over the basal (0 h) levels after treatment with high concentrations of bestatin and thiorphan ($P < 0.001$, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.12 C). Bestatin and thiorphan did not significantly change the levels of END ($P > 0.05$, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.12 D).

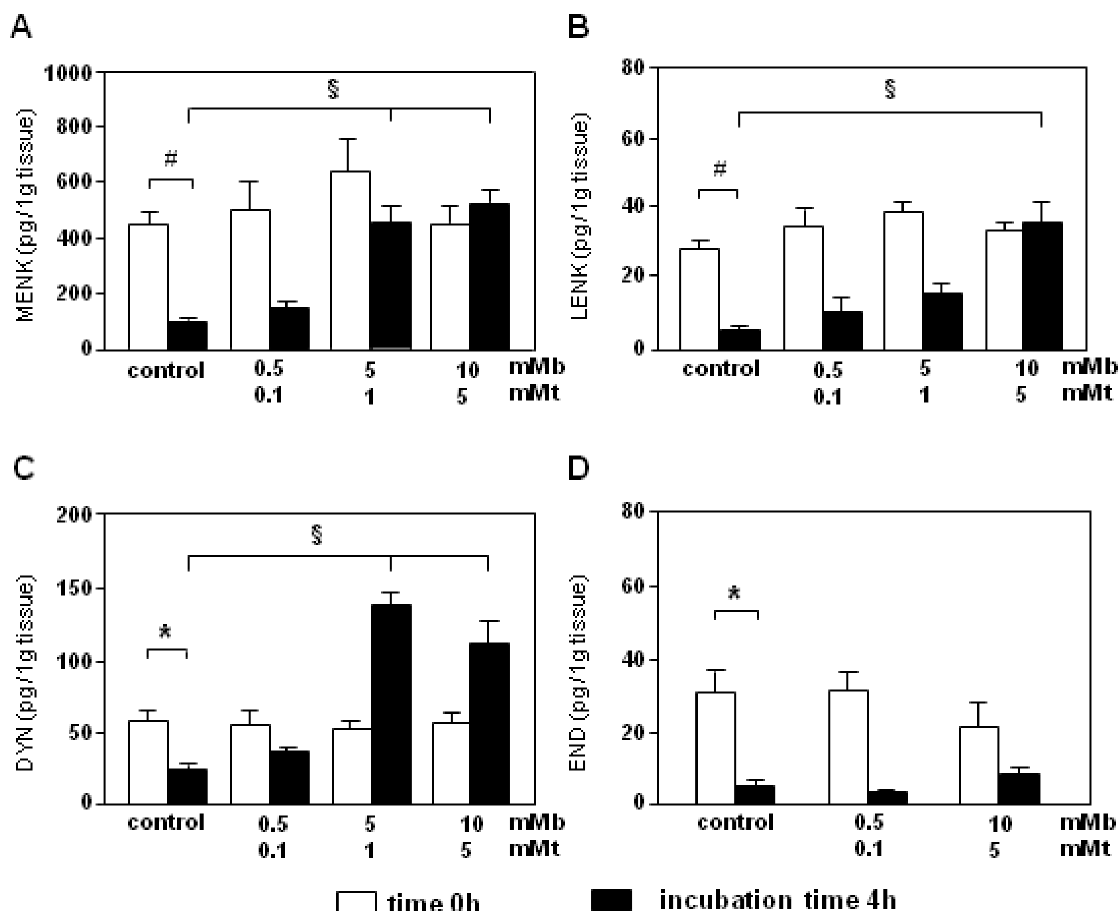

Figure 3.12. Effects of the combination of bestatin (b) and thiorphan (t) on the degradation of exogenous opioid peptides in peripheral nerve suspensions. Sciatic nerve terminal branches suspensions were incubated with exogenous MENK (**A**) LENK (**B**) DYN (**C**) or END (**D**) and with a buffer (control) or with bestatin and thiorphan. Measurements were performed immediately after addition of buffer or inhibitors (white bars) and 4 h later (black bars). Inhibitors dose-dependently prevented the degradation of MENK, LENK and DYN ($P < 0.001$, Linear regression) but not of END ($P > 0.05$, two-way RM ANOVA, Bonferroni *t* test). *$P < 0.05$; $^{\#}P < 0.01$; $^{\S}P < 0.001$ (two-way RM ANOVA, Bonferroni *t* test). Data are expressed as means ± SEM. N = 3-5 experiments per group.

3.4.3. Effects of a dual APN and NEP inhibitor on the degradation of exogenous opioid peptides in nerve preparations

Four h incubation of the suspensions of sciatic nerve terminal branches with standard MENK (12.8 ng/ml), LENK, DYN or END (1.28 ng/ml) resulted in significant degradation of MENK (by 79%; $P < 0.001$), LENK (by 88%; $P < 0.001$), DYN (by 46%; $P < 0.01$) and of END(by 74%; $P < 0.001$) compared to 0 h in inhibitor-free control groups (two-way ANOVA,

Bonferroni *t* test; Fig. 3.13). No significant differences in basal opioid peptide levels measured at 0 time points among groups (P > 0.05, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.13). Treatment with P8B (0.1 - 10 mM) dose-dependently prevented the degradation of MENK and LENK (P < 0.001, Linear regression), until enkephalin levels were not significantly different compared with the basal (0 h) control (P > 0.05, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.13 A and B). Also, DYN levels were dose-dependently elevated by P8B (P < 0.001, Linear regression; Fig. 3.13 C), and its amounts increased significantly over the basal (0 h) levels after treatment with the highest concentration of P8B (P < 0.001, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.13 C). The levels of END were not significantly altered by P8B (P > 0.05, two-way RM ANOVA, Bonferroni *t* test; Fig. 3.13 D).

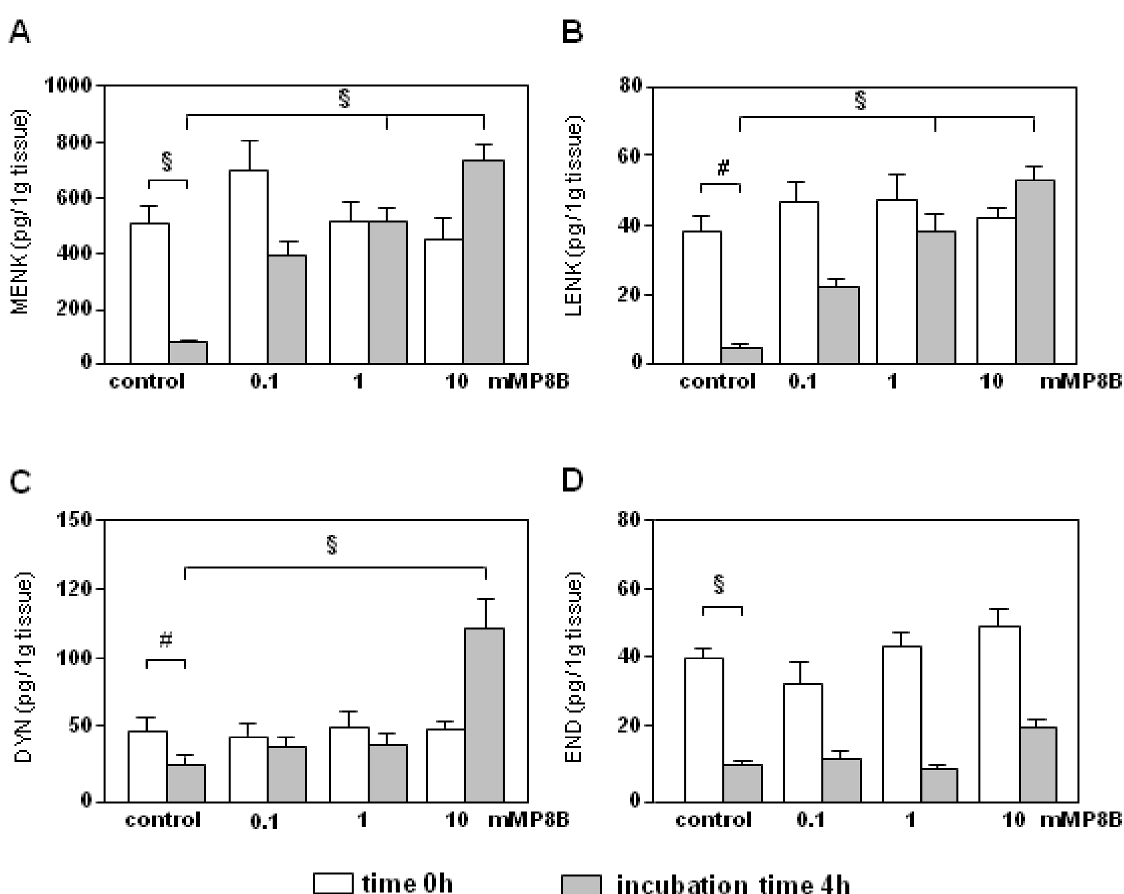

Figure 3.13. Effects of P8B on the degradation of exogenous opioid peptides in peripheral nerve suspensions incubated with exogenous MENK (**A**), LENK (**B**), DYN (**C**) or END (**D**) and with a buffer (control) or P8B. Measurements were performed immediately after addition of buffer or inhibitor (white bars) and 4 h later (gray bars). The inhibitor dose-dependently prevented the degradation of MENK and LENK (P < 0.001, Linear regression). P8B (10 mM) significantly increased levels of DYN (P < 0.001, two-way RM ANOVA, Bonferroni *t* test). Levels of END were not significantly changed by P8B (P > 0.05, two-way RM ANOVA, Bonferroni *t* test). $^{\#}$P < 0.01; §P < 0.001 (two-way RM ANOVA, Bonferroni *t* test). Data are expressed as means ± SEM. N = 3-5 experiments per group.

3.5. In vivo effects of APN and NEP inhibitors on peripheral opioid antinociception

Four days after i.pl. injection of CFA rats developed hyperalgesia characterized by significantly decreased paw pressure thresholds (PPT) in inflamed paws (37.7 ± 1.04 g) compared with contralateral non-inflamed paws (69.2 ± 1.3 g). Intraplantarly injection of a combination of bestatin (5 mg) and thiorphan (0.8 mg) or of P8B (1 mg) into inflamed paws produced antinociception represented by increased PPT in inflamed paws as compared to baseline (Fig. 3.14 A and B). Antinociceptive effects of the inhibitors were fully reversed by i.pl. co-injected Abs against MENK (2 µg), LENK (0.5 µg) or DYN (0.5 µg) but not by anti-END (1 µg) (Fig. 3.14 A and B). No changes were observed in contralateral non-inflamed paws (data not shown). These experiments were performed by Dr. Dominika Labuz from our group.

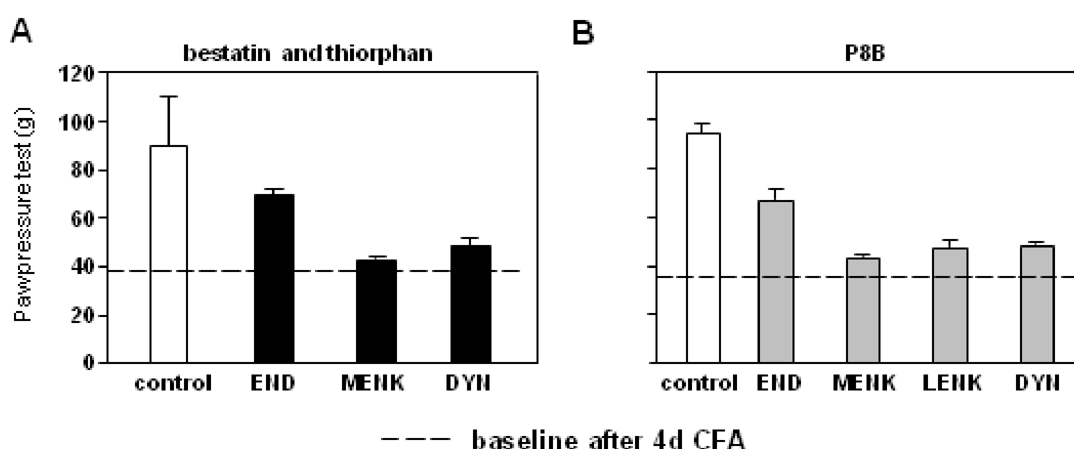

Figure 3.14. Antinociceptive effects produced by APN and NEP inhibitors injected into inflamed hind paws (white bars). (**A**) Reversibility of i.pl. bestatin (5 mg) and thiorphan (0.8 mg)-induced antinociception by co-injected anti-MENK (2 µg) or anti-DYN (1 µg). (**B**) Reversibility of P8B (0.1 mg)-induced antinociception by co-injected anti-MENK (2 µg), anti-LENK (0.5 µg) or anti-DYN (1 µg). Injection of anti-END (1 µg) only slightly decreased inhibitor-induced antinociception. Dashed lines represent baseline PPT measured 4 days after induction of inflammation but before drug injections. Data are expressed as means ± SEM. N = 2-3 rats per group.

4. Discussion

The major findings of this study are:

a) APN and NEP are expressed in granulocytes and macrophages, but not in T lymphocytes, infiltrating painful inflamed tissue.

b) Leukocytic and neuronal APN and NEP degraded their specific synthetic substrates, which could be dose-dependently prevented with selective inhibitors of APN (bestatin) and NEP (thiorphan) as well as with a novel dual inhibitor P8B. This was demonstrated in vital immune cells as well as in immune cell membranes and peripheral nerves. Furthermore, the enzymatic activity of neuronal NEP, but not of APN, was higher in nerves innervating inflamed compared to non-inflamed tissue.

c) Immune cell-released MENK and LENK, but not DYN and END, underwent degradation. Catabolism of extracellular LENK was prevented with selective APN and NEP inhibitors. This was not in the case of MENK and LENK after treatment with P8B, probably because inhibitors paradoxically diminished enkephalin levels compared to inhibitor-free groups.

d) In immune cell membranes and peripheral nerves, exogenously added MENK and LENK appeared as preferential targets for leukocytic and neuronal APN and NEP. This was demonstrated by prevention of enkephalin degradation with specific and dual peptidase inhibitors. In addition, DYN degradation was dependent on neuronal APN and NEP.

e) Both specific and dual APN and NEP inhibitors injected into inflamed rat hind paws produced antinociception which was fully reversed by a co-injection with Abs against MENK, LENK or DYN, but only partially by Ab against END.

4.1. Expression of APN and NEP in leukocytes

Our group has provided evidence that opioid peptides released from immune cells accumulating in inflamed tissues decrease inflammatory pain in animals and humans (reviewed in Busch-Dienstfertig and Stein, 2009). The focus of the present studies was to investigate the degradation of opioid peptides by APN and NEP in painful inflamed tissue following CFA application. As a source of peptidases we analyzed immune cells as they densely infiltrate such tissue at this advanced stage of inflammation (4 days following CFA). Indeed, we found numerous hematopoietic $CD45^+$ cells, among which we identified macrophages (31.3%), granulocytes (16.6%) and T lymphocytes (4.1%), confirming our

earlier studies where accumulation of macrophages together with the precursor monocytes (~55%), granulocytes (10 - 35%) and 5% lymphocytes in inflamed tissue was shown (Stein et al., 1990; Przewlocki et al., 1992; Rittner et al., 2001; Brack et al., 2004a,b). Furthermore, we extended these findings by examining the expression of APN and NEP in $CD45^{+}$ cell subpopulations with triple-color flow cytometry. This analysis revealed that APN was expressed by 15.2% granulocytes and 24.2% macrophages, while NEP was expressed by 14.3% granulocytes and 37.4% macrophages. T lymphocytes did not express any of the two peptidases. Previously, very few studies examined leukocytic expression of APN and NEP and most of them were performed in healthy animals and humans. Thus, APN was shown to be present on bone marrow monocytes, macrophages, granulocytes, dendritic and B lymphocytes, but not on thymocytes or spleen T cells in mice and only on pre-mature T lymphocytes or on thymic stromal cells in adult rats (Hansen et al., 1993; Jardinaud et al., 2004). Another study showed that T lymphocytes become APN-negative upon maturation, which resulted in its absence in the spleen or peripheral blood lymphocytes in humans (Riemann et al., 1999). NEP is expressed on early normal T- and B-cell progenitors (Shipp and Look, 1993; Mari et al., 1994). Also, NEP was shown on different T cell populations of healthy rat thymocytes (Amantini et al., 2008). Other studies that used FACS, determined NEP on granulocytes in mice spleen cells (Braun et al., 1983) and peripheral blood of patients with open heart surgery (Schroeter et al., 2007). Regarding pathological conditions, NEP was found in circulating and synovial fluid granulocytes, monocytes and in T lymphocytes of patients with juvenile chronic arthritis (Simonini et al., 2005). APN was detected with flow cytometry and Western blot in T lymphocytes in synovial fluid, but not in peripheral blood, of rheumatoid and juvenile chronic arthritis patients (Riemann et al., 1993). Other leukocyte subsets were not assessed in that study. In both studies APN and NEP expressing T lymphocytes were detected under pathological condition, but the state of inflammation is chronic, characterized by enhanced T cell infiltration. In the present study the absence of expression of both peptidases in T lymphocytes might be related to a relatively low percentage of these cells in our inflammatory model. Together, expanding those earlier observations our comprehensive analysis of both peptidases in all major leukocyte subpopulations infiltrating painful inflamed tissue demonstrated that granulocytes and macrophages are predominant populations expressing APN and NEP.

4.2. Functionality of leukocytic and neuronal APN and NEP assessed by the degradation of their specific substrates

To decrease inflammatory pain opioid peptides secreted from immune cells act at opioid receptors on peripheral sensory nerves in injured tissues (reviewed in Busch-Dienstfertig and Stein, 2009). The expression of opioid degrading enzymes APN and NEP on neuronal tissues was investigated previously. Thus, APN was shown in brain and in the spinal cord of healthy rats (Watt et al., 1989; Noble et al., 2001; Jardinaud et al., 2004). One study assessed APN expression in pig peripheral nerves using immunohistochemistry and detected it in microvessels and perineurium of femoral and splenic nerves (Barnes et al., 1991). The expression of NEP was previously intensive investigated in the CNS as well as in kidney, lungs and salivary glands (Sales et al., 1991, Dutriez et al., 1992). In peripheral nerves, NEP was demonstrated in Schwann cell membranes of the sciatic, splenic and vagus nerves from pig (Matsas et al., 1986; Barnes et al., 1991). Further, in healthy rats and under pathological conditions, NEP was detected in non-myelin-forming cells of unmyelinated fibers and very weakly with Schwann cells of large diameter fibers (Kioussi et al., 1992, 1995).

4.2.1. APN and NEP activity is not dependent on pH values

The next step was to verify the activity of APN and NEP in the sciatic nerve and leukocytes from inflamed tissue. Substrate specificity of zinc metallopeptidases essentially ensure by Van der Waals and ionic interactions between their active subunits and the corresponding moieties of the substrate. Substrate specificity is also determined by several hydrogen bonds between groups of the bound molecule and the polar residues of the peptidases (Roques et al., 1993). Thus, the possibility insists that different ph values could influence the affinity between substrates and enzymes. Most studies testing activities of APN and NEP were done in tissues from healthy animals and humans under neutral pH conditions. It was not clear whether the inflammation in our CFA model changes the function of both peptidases. Although it is generally accepted that inflammation is associated with high tissue acidity the available data are inconsistent. The pH values measured *in vivo* and *in vitro* experiments ranges from acidic 5.8 to alkaline 8.3, as summarized by Punnia-Moorthy (1988). Studies in short-lasting (6 – 8 h) inflammation induced by carrageenan, dextran and staphylococcus aureus injected into rat skin showed a small lowering of pH (by about 0.5) in inflamed tissues (Punnia-Moorthy, 1987, 1988). To mimic the *in vivo* conditions in the *in vitro*

assays we assessed pH in inflamed rat paws and found a small decrease in pH from 7.4 in non-inflamed paws to 7.0 in inflamed paws. In an *in vitro* study using natural killer leukocytes, isolated from spleen of healthy rats, a decreased APN activity in the specific substrate degradation was observed at acidic pH values (Amoscato et al., 1994). Therefore, using immune cells isolated from inflamed paws we analyzed the activities of APN and NEP, measured by the degradation of their specific substrates, within a pH range of 5.0 – 8.0. We detected no significant differences between various pH values, suggesting no major influence of the acidity on APN and NEP function in our experiments. Based on these findings, we decided to perform all *in vitro* assays at pH level of 7.4 and a temperature of 37℃, according to Florentin and colleagues (1984).

4.2.2. Prevention of APN - induced specific substrate degradation with selective and dual inhibitors

A common way to assess the enzyme activity is to evaluate the degradation of its specific substrate(s) and verify the specificity of such effect by testing selective enzyme inhibitors. We confirmed that APN was functional in tissues from painful inflamed rat paws as we recorded a significant degradation of APN specific substrate Ala-βNA to its metabolite βNA. In vital immune cells and their membranes the degradation rate was 17 and 29 µmol/h (34% - 58% of possible hydrolysis), respectively. This is a higher degradation rate of Ala-βNA than 2.1 µmol/h measured in peritoneal macrophages or 10.2 nmol/h in spleen T cells from healthy mice (Miller et al., 1994a, 1994b). However, our results are comparable to those by Balog and colleagues (1999) and Marotti and co-workers (2000), who recorded Ala-βNA hydrolysis with the rate of about 37% - 50% during 1 h in blood neutrophils of healthy humans. None of the previous studies examined APN activity in leukocytes infiltrating pathological tissues.

So far, only one study assessed APN activity in peripheral nerves from young pigs, but the influence of inflammation was not addressed (Barnes at al., 1991). We now demonstrate that Ala-βNA was hydrolyzed in sciatic nerve terminal branches innervating both non-inflamed and inflamed rat hind paws. As the Ala-βNA degradation rates were similar in nerves from both hind paws (10 mmol/h), it appears that the inflammation does not affect the activity of APN. It seems that APN is more active in neuronal tissue, but the hydrolysis rate could not compare to immune cell assays because of the method (see paragraph 2.3.6.).

Subsequently, we verified the specificity of APN action as the enzyme ability to metabolize its specific substrate was dose-dependently and fully blocked by its selective

inhibitor bestatin in vital immune cell (20 mM), their membranes (50 μM) as well as in sciatic nerves (15 mM). Such experiments using bestatin in preparations from CNS structures or peripheral nerves have not been performed previously. Nevertheless, the requirement for higher bestatin doses to block APN in intact immune cells versus their membranes was found in earlier studies. For example, bestatin (0.1 mM) decreased the generation of βNA by about 50% in intact human neutrophils, but by 88% in neutrophil membranes (Marotti et al., 2000). Also, although such comparison has not been directly addressed by Amoscato and colleagues (1994), these authors used bestatin in a dose of 3 mM to obtain 60% inhibition of APN activity using Leu-pNA as a substrate, in rat intact natural killer cells. None of those previous studies examined bestatin in a dose-dependent fashion. Interestingly, bestatin has been shown to passively enter immune cells, bind and block the cytosolic aminopeptidases (Constam et al., 1995; Scornic and Botbol, 2001) that could explain the necessity for relatively high bestatin concentrations to block APN action in intact cells.

Next we tested the effect of a new dual APN and NEP inhibitor P8B on APN action. In immune cell membranes, the APN activity was fully blocked by P8B in a concentration of 1 μM. However, in vital immune cells and the sciatic nerves the generation of βNA could be dose-dependently but partially diminished by about 69% and 61% by 10 mM of P8B, respectively. P8B was not tested to enter immune cells, but the use of higher P8B doses in intact immune cells versus their membranes is in line with our experiments using bestatin (discussed above). P8B is a new compound and was only tested by our corroborators who synthesized this inhibitor (Chen et al., 1998, 2000). These authors used *in vitro* assay in which activity of APN purified from hog kidney was tested by its ability to degrade Ala-p-nitroaniline as a specific substrate. In such conditions P8B displayed a dissociation constant K_i value of 4.8 nM, which implies that the complex of enzyme-inhibitor is stabile and therefore the affinity of the inhibitor to APN is high. The P8B selectivity toward APN was further confirmed by the finding that the activity of another peptidase aminopeptidase A was poorly inhibited by P8B displaying 1000 fold less affinity to that enzyme (Chen et al., 1998). The differences between the studies might be related to the use of a purified APN by Chen et al. (1998) versus nerves and vital immune cells as APN source, requiring higher P8B doses, in line with the experiments using bestatin, in our study. Together, we have clearly demonstrated the function of leukocytic and neuronal APN by means of its specific substrate degradation in a model of painful inflammation.

4.2.3. Prevention of NEP activity on specific substrate degradation with selective and dual inhibitors

To assess NEP activity, the degradation of several synthetic substrates, such as chromogenic (Suc-Ala-Ala-Phe-pNA) and fluorogenic (dansyl-D-Ala-Gly-Phe(pNO_2)-Gly, peptides, to their metabolites measured with photospectrometry or fluorospectrometry has been employed. The peptidase activity was tested in mouse and rat brain, spinal cord, ileum, lung as well as in human neutrophils and the corresponding membranes (Florentin et al., 1984; Marotti et al., 2000; Balog et al., 2001). We validated NEP activity in inflamed paw tissues by showing the metabolism of its specific substrate Suc-Ala-Ala-Phe-pNA to pNA. Degradation rate reached 12.5 µmol/h in vital immune cells and 4 µmol/h in their membranes. This hydrolysis rate was higher than 0.23 µmol/h in neutrophils from healthy human volunteers (Balog et al., 2001), which suggested that NEP activity is increased in inflammation. There are no other studies examining immune cells from injured tissues.

We also demonstrated that Suc-Ala-Ala-Phe-pNA was hydrolyzed at a rate of 3 mmol/h in sciatic nerve terminal branches isolated from non-inflamed and at 5 mmol/h from inflamed rat hind paw. The NEP activity in peripheral nerves was recorded previously, but the degradation rate cannot directly compare with this studies which used another specific substrate. Kioussi and colleagues (1992) could observe NEP activity as a rate 0.18 mmol/h with [^{125}I] insulin B-chain in sciatic nerves from rats without inflammation. In pig femoral nerve NEP activity was 3.7 mmol/h (Barnes et al., 1991). The two groups prove the activity with enzyme specific inhibitors phosphoramidon. Interestingly, our findings suggest that inflammation enhanced NEP activity (by 41%) in the sciatic nerve. The effects of injury on NEP activity in peripheral nerves have not been addressed so far. However, similar to our observations, NEP expression and activity was higher in wounded skin from diabetic patients compared to healthy controls (Antezana et al., 2002). An increase of peptidase activity in synovial fluid was also shown in chronic diseases such as rheumatoid arthritis in humans, which are characterized by an increase number of macrophages and mature lymphocytes (Spreedharan et al., 1990; Matucci-Cerinic et al., 1993; Simonini et al., 2005). In contrast to our observations, no differences of NEP activity were found in CNS of chronic arthritis compare to control rats (Van Veldhoven et al., 1982).

We verified the specificity of NEP activity by showing that its selective inhibitor thiorphan dose-dependently and fully prevented the formation of pNA in vital immune cells (10 mM) and their membranes (1 µM) as well as in sciatic nerves (8 mM). Previously it was shown that thiorphan (10 - 100 µM) completely abolished the hydrolysis of Suc-Ala-Ala-Phe-pNA in cultured and purified peripheral human T lymphocytes, and in human thymocytes

(Mari et al., 1992, 1994). Direct investigations of neutrophils and corresponding membranes observed 85% inhibition of NEP activity after treatment with 0.1 mM thiorphan (Marotti et al., 2000; Balog et al., 2001). Those studies used immune cells from healthy donors, which might explain lower concentrations of thiorphan than in our experiments with leukocytes from inflamed tissue. Thiorphan-dependent activity has not been so far tested in peripheral nerves under pathological conditions, but it was observed in brain and spinal cord of healthy rodents (Florentin et al., 1984; Roques et al., 1993). Thus, our results suggest that NEP expressed in the CNS and in peripheral nerves are functionally similar.

We also showed that the new dual APN and NEP inhibitor P8B fully prevented the NEP substrate degradation in immune cell membranes (1 µM) and in sciatic nerves (10 mM). Furthermore, the generation of pNA could be dose-dependently attenuated by about 42% by 10 mM of P8B in vital immune cell assay and this attenuation was improved (reaching 64%) after decreasing the cell number by half. P8B was previously tested only by our corroborators who, using NEP purified from rabbit kidney and flourophore dansyl-Gly-(pNO_2)-Phe-ßAla as NEP specific substrate, showed that P8B displayed a dissociation constant K_i value of 2.0 nM, implying that the complex of enzyme-inhibitor is stable and therefore the affinity of the inhibitor to NEP is high. In addition, the affinity of P8B to another enzyme ACE was very low (K_i value of 2500 nM) confirming NEP selectivity of the inhibitor (Chen et al., 1998, 2000). A need for relatively high P8B doses in our experiments might be related to the use of nerves and vital immune cells as NEP source versus a purified NEP by Chen et al. (1998).

In summary, we showed that NEP and APN cleave their specific substrates in immune cells and their membranes, and in the sciatic nerve terminal branches, suggesting that leukocytic and neuronal APN and NEP in inflamed painful tissue are functionally similar. This degradation was reversible by the respective peptidase specific inhibitors thiorphan and bestatin and by the new dual inhibitor P8B.

4.3. Effects of peptidase inhibitors on the levels of immune cell-derived enkephalins

4.3.1 Degradation of immune cell-derived MENK and LENK

Our group demonstrated that inflammatory pain can be decreased as a consequence of peripheral opioid receptor activation by MENK and END released from immune cells accumulating in inflamed tissue. Even though several studies examined the release of opioid peptides from immune cells, this was always done in the presence of peptidase inhibitors

(e.g. Rittner et al., 2006, 2009), and the functional relevance of leukocytic peptidases was not directly addressed so far. In the present study we found that immune cells isolated from CFA-inflamed rat paws released MENK and LENK as their extracellular levels were enhanced by 23 – 97.5 pg and 6 – 9.6 pg after 4 h of incubation *in vitro*, respectively. To further verify our results we examined whether the increased levels measured at 4 h were related to enkephalins or their metabolites. In previous studies that used HPLC, APN was shown to cleave MENK and LENK to its metabolites Tyr and Gly-Gly-Phe-Met, whereas NEP degraded both enkephalins to Tyr-Gly-Gly and Phe-Met (Venturelli et al., 1985; Giroset al., 1986b; Shibanoki et al., 1992; Miller et al., 1994a). We tested some of the synthetic analogs of these metabolites and found that Abs used in our RIA measurements did not recognize Tyr-Gly-Gly-OH, Gly-Gly-Phe-Met-OH and Gly-Gly-Phe-Leu-OH, but selectively detected MENK or LENK. Thus, the increased extracellular levels of enkephalins at 4 h were obviously related to a continuous liberation of these peptides from the inflammatory cells. Prolongation of the cell incubation time to 8 h resulted in a significant drop of accumulated extracellular levels of MENK and LENK determined in the supernatant, reaching the basal levels (at 0 h). Relatively slow leukocyte-derived enkephalin degradation was surprising because it was investigated previously that enkephalins are susceptible to a rapid catabolism by APN and NEP. This has been predominantly shown for exogenously added enkephalins (7.4 – 64.8 µM) to plasma from healthy humans or from rats and mice, and under these conditions full opioid peptide degradation (as assessed by the accumulation of their metabolites) was detected within minutes (Venturelli et al., 1985; Shibanoki et al., 1991, 1992). This quick degradation might have been related to an easier accessibility of peptidases by peptides when they were in high (micromolar) concentrations compared with the nanomolar range of MENK (2 nM) and LENK (0.23 nM) released from immune cells in our assays. Also the whole plasma as a cell mix was investigated, but there are some studies that investigated opioid peptide degradation by sorted immune cells. In murine macrophages and nylon wool-enriched splenic T cells, exogenously added MENK (even in high concentration of 0.2 mM) was degraded at a rate of 11% per hour (Miller et al., 1994a,b). Also, in healthy rat splenocytes a hydrolysis rate of 8 – 18% of the total LENK and MENK amounts added was noted per hour (Amoscato et al., 1993). The above data are comparable with the enkephalin hydrolysis rate of 7.5 - 9% per hour in our immune cell assays. We could not observed differences in peptidase-induced metabolism between endogenous enkephalins in our assays and exogenously added enkephalins in the studies above. Furthermore, these data show that addition of high amounts of MENK is not accompanied by an increase in the hydrolysis rate. One reason might be that opioid peptides bind predominately to opioid receptors that are likewise expressed on immune cells. Opioids have a higher affinity to opioid receptors (nanomolar) compared to peptidases (micromolar) (Skidgel et al., 1984).

Taken together, MENK and LENK secreted by immune cells from painful inflamed tissue are degraded by leukocytic peptidases. These findings raised the question on the contribution of APN and NEP in this opioid peptide degradation and this question was addressed using peptidase-specific inhibitors.

4.3.2 Effects of selective and dual APN and NEP inhibitors on the levels of immune cell-derived MENK and LENK

Treatments with a combination of thiorphan and bestatin or P8B paradoxically significantly decreased MENK levels at 0 h and 4 h. A tendency of MENK accumulation over time in the inhibitor treated groups was found, while opioid levels decreased between 4 and 8 h incubation time in the inhibitor-free samples. Similarly, accumulation of LENK became apparent in P8B-treated groups at 8 h. In this assays inhibitor treatment could not increased the enkephalin level compared to control group after 8 h and therefore our hypothesis could not be confirmed. In contrast, the levels of LENK were not affected at 0 - 4 h by a combination of thiorphan and bestatin. At the later stage of incubation (after 8 h), the prevention of LENK degradation was even more pronounced, resulting in an elevation of the peptide level by about 47% over the levels found in inhibitor-free groups. Moreover, LENK amount enhanced by about 13.5% compared to accumulated level at 4 h, which suggested that degradation of LENK could be displaced by immune cell released opioid peptides at 8 h. We can exclude that the inhibitors stimulate the release of LENK, because the peptide accumulation at 4 h was similar in inhibitor-treated and in the inhibitor-free control groups. These findings are in accordance with our hypothesis and verify it by showing that the combined treatment of immune cells with thiorphan and bestatin counteracts LENK degradation. At the moment, we could not solve our technical problems. Although inhibitors in these experiments were used in relatively high concentrations (1 – 5 mM), they were still lower than in experiments testing specific synthetic substrate degradation (5 – 20 mM). Adjustment of the doses was necessary to keep the immune cells viable, particularly when they were treated with bestatin and thiorphan at the same time. Although it was possible to use P8B in concentrations up to 20 mM, the concentration was matched to bestatin-treatment in our immune cell assays. Even though the same concentrations of bestatin and thiorphan were used to study the degradation of the different opioid peptides, only the levels of MENK were affected by the inhibitors, while the degradation of LENK was prevented, and none of the inhibitors at any time significantly changed the levels of immune cell-derived DYN and END (see paragraph 4.6). Thus, it appears that in certain conditions APN and NEP

inhibitors interfered only with endogenous enkephalins. Possible reasons could be that inhibitors somehow interact with enkephalins in a way that they diminish the accessibility of primary RIA Abs to the peptide or that inhibitors decrease the secretion of enkephalins from leukocytes. The second possibility was previously indicated for MENK in rat striatum slices (De La Baume et al., 1983).

Only few studies have previously examined opioid peptide hydrolysis using immune cells and all of them used leukocytes from healthy animals and exogenously applied enkephalins. In 1994 the group of Miller and colleagues demonstrated MENK catabolism in the presence of macrophages and spleen lymphocytes isolated from pathogen-free mice. Degradation of MENK was attenuated (by 50 – 60%) by treatment with bestatin (10 µM) and another APN selective inhibitor amastatin (10 – 200 µM) (Miller et al., 1994a, b). In rat splenocytes and natural killer cells the catabolism of MENK and LENK was decreased by 85 - 96% by bestatin (1 mM) (Amoscato et al., 1993). Also, hydrolysis of MENK and LENK in rat plasma was inhibited with bestatin (500 µM) and thiorphan (100 µM), although the cellular source of the peptidases was not identified (Shibanoki et al., 1991). In summary, none of the previous studies examined the impact of APN and NEP degradation of immune cell-derived opioid peptides in a pathological situation.

Our findings indicate that under painful inflammatory conditions immune cell-derived enkephalins are slowly degraded by peptidases expressed on the surface of intact leukocytes *in vitro*. This does not result from inactive peptidases because they were capable to degrade other substrates (Ala-βNA and Suc-Ala-Ala-Phe-pNA; see paragraph 4.2). Due to the unexpected effects of APN and NEP inhibitors on the levels of immune cell-derived enkephalins, in the next series of experiments we used suspensions of purified immune cell membranes.

4.4. Enkephalin degradation and its prevention in immune cell membranes

In immune cell membrane suspensions APN and NEP were functional because they cleaved their specific substrates (see paragraphs 4.2.1 and 4.2.2). Now we found that the levels of exogenously added MENK and LENK were substantially lower (by 47 - 70% and 77%, respectively) following 4 h incubation with immune cell membranes. The hydrolysis rate of enkephalins in membrane assays was 12% - 19% per hour. It seems, that degradation rate depend on tissue preparation type, which was found previously by groups of De la Baume (1983) and Chaillet (1983). They observed that metabolic pattern depends on the type of tissue preparation, for example accumulations of metabolites were higher in the brain membrane fractions compared to brain slices.

The low levels of enkephalins resulted from the action of APN and NEP because the degradation of MENK and LENK was dose-dependently and fully prevented by a combination of thiorphan and bestatin and by P8B. The interference of the inhibitors was obvious only when endogenous MENK was analyzed. Our findings indicate that MENK and LENK are targets for leukocytic APN and NEP in immune cell membrane preparations. So far there are no studies, which observed explicitly the effect of the novel inhibitor P8B on prevention of opioid peptide degradation. Also, similar experiments using membranes of leukocytes have not been performed previously. Nevertheless, our findings are in line with the studies reporting the APN- and NEP-dependent cleavage of exogenous enkephalins in intact immune cell suspensions or plasma (Venturelli et al., 1985; Giros al., 1986b; Shibanoki et al., 1992; Amoscato et al., 1993; Miller et al., 1994a, b) as discussed in detail above (see paragraph 4.3.). Concentrations of bestatin (5 µM - 0.5 mM) and thiorphan (1 µM - 0.1 mM) used in our experiments are similar to those used in the studies mentioned above using cells from healthy animals. All these studies used exogenously applied enkephalins and did not found interferences with the inhibitors, like we see in our immune cell membrane and control experiments with synthetic opioid peptides. Also, they used HPLC as a method to detecting the metabolites of enkephalin degradation, which in contrast to our RIA worked without Ab accessibility.

Taken together, in our model of CFA induced painful inflammation we could show that APN and NEP expressed on immune cells induced degradation of enkephalins. Importantly, enkephalin degradation can be fully blocked with both classical selective (thiorphan and bestatin) and novel dual (P8B) APN and NEP inhibitors. These results show that immune cells are a source to degrade opioid peptides in painful inflamed tissue, but also peripheral neurons could be a source of APN and NEP activity on opioid peptide degradation.

4.5. Enkephalin degradation and its prevention by blockade of APN and NEP in peripheral nerves

Here, the impact of peripheral neuronal APN and NEP on opioid peptide degradation in pathological situation was examined. We found that both peptidases on terminal branches of the sciatic nerve innervating such tissue are functional by means of specific synthetic substrate degradation (see paragraph 4.2.). In these experiments we demonstrated that levels of exogenously applied MENK and LENK were diminished by 78 - 85% and 79 - 88%, respectively, after 4 h incubation. Notably, neuronal APN and NEP mediated enkephalin degradation as documented by its dose-dependent and full inhibition by a combination of thiorphan (5 mM) and bestatin (10 mM) or by P8B (1 – 10 mM). Previous studies examining

a role of APN and NEP in degradation of opioid peptides were performed exclusively in the CNS. Hydrolysis of exogenous [^{3}H]LENK was inhibit by thiorphan (1 – 100 nM) in mouse striatum fraction (Roques et al., 1980). Also, hydrolysis of endogenous MENK released from rat striatum slices was prevented in the presence of 0.1 µM thiorphan (Patey et al., 1981). Further investigation in rat striatum slices examined the metabolite formation after [^{3}H]MENK degradation was inhibited by 40% in the presence of 20 µM bestatin and 29% by 0.1 µM thiorphan and the co-presence of both selective inhibitors resulted in total recovery of the enkephalin in the medium (De la Baume et al., 1983). This was supported by another study showing that bestatin (0.1 mM) or thiorphan (0.2 µM) used separately attenuated [^{3}H]MENK metabolism by 13% and 59%, respectively, while their combination decreased the hydrolysis by 76% in rat globus pallidus slices (Giros et al., 1986a). None of those previous studies examined inhibitors in a dose-dependent fashion and it seems that blockade of peptidase activity depend on the neuronal region. Also, concentrations of bestatin and thiorphan used in those studies were lower than in our assays (5 - 10 mM). This could be related to the use of CNS tissues from healthy animals in the other studies versus peripheral nerves from inflamed tissue in our experiments. Another study also showed the importance of a concomitant APN and NEP blockade. A clear protection (by 85%) of [^{3}H]MENK degradation by treatment with a combination of thiorphan (1 µM) and bestatin (20 µM) was reported in slices from rat spinal cord, while the inhibitors were inactive when applied separately. Moreover, this group investigated kelatorphan, one of the first dual inhibitors, which completely prevented the degradation of exogenously added [^{3}H]MENK as well as of endogenous MENK released from spinal cord slices (Bourgoin et al., 1986). Regarding dual APN and NEP inhibitors, in none of the previous studies P8B was used and effects of all other dual inhibitors on enkephalin degradation was tested only in the CNS. A couple of studies showed similar effects *in vivo* using microdialysis in the CNS, in awake and freely moving rats. One of them reported that systemic (i.p.) injection of another dual inhibitor RB101 increased extracellular levels of MENK in the nucleus accumbens (Dauge et al., 1996). The other found increased extracellular levels of MENK in the periaqueductal gray after i.p. administration of aminophosphinic inhibitor RB3007 (Le Guen et al, 2003).

Summing up, our current findings substantially expand earlier studies performed in the CNS of healthy animals, by demonstrating that classical selective and a novel dual inhibitor can block APN and NEP on peripheral nerves innervating painful inflamed tissue and protect MENK and LENK from the degradation.

4.6. Effect of peptidase inhibitors on the levels of DYN and END

In this study MENK and LENK came out as targets for leukocytic and neuronal APN and NEP, but in addition to enkephalins, leukocytes infiltrating inflamed tissues also contain DYN and END which contribute to the local pain inhibition (reviewed in Machelska, 2007; Busch-Dienstfertig and Stein, 2010). These opioid peptides have longer sequences of 17 amino acids (DYN) to 31 amino acids (END) and the question arose whether these opioid peptides are also targets for APN and NEP in our model of CFA induced inflammation. In the current study, immune cells from inflamed rat paws secreted DYN and END and their extracellular levels were elevated by 43 - 58% and 53 - 68%, respectively, over 8 h of incubation *in vitro*. Thus, it appears that leukocyte-derived DYN and END are not or less then liberated opioid peptides hydrolyzed by peptidases in intact immune cells. However, in immune cell membrane suspensions we observed a degradation of exogenously applied DYN (by 47%) and END (by 37 - 39%). Treatment with a combination of thiorphan and bestatin or with P8B, in doses which blocked the degradation of MENK and LENK, did not prevent the catabolism of DYN and END in immune cell membranes. In peripheral nerve preparations from inflamed rat hind paw, exogenous END were catabolized by 74 - 83% and treatment with inhibitors did not affect END level. A possible explanation could be that the degradation of DYN and END in our assays is done by other zinc metallopeptidases, which are not sensitive to the inhibitors. For example, both opioid peptides has been shown to be cleave by γ-endorphin-generating enzyme, which is mostly located in cytosol, peroxisomes and in a low level in plasma membrane of enterocytes and immune cells (Safavi et al., 1996; Song ES et al., 2003). It was examined, that END cleavage by γ-endorphin-generating enzyme has been measured only after differentiation of the cells in activated mouse macrophages and T cells (Thiele et al., 1998), which correspond with the cells taken from inflamed tissue. The hydrolysis of DYN and END was only evaluated in leukocytic membrane assays, which my explained by low expression of γ-endorphin-generating enzyme in plasma membranes and it is difficult to ascertain whether membranes, even when washed, are not contaminated by cytosolic peptidases.

Furthermore, we found that exogenous DYN was catabolized by 46 - 57% in peripheral nerve branches from inflamed rat paws. Treatment with high concentrations of inhibitors prevented the hydrolysis of DYN increasing 1.9 – 2.4-fold its levels over basal (0 h) concentrations of exogenously applied DYN. This suggests that inhibitors might have prevented the degradation of endogenous DYN previously detected in primary afferents in inflamed rat paws (Hassan et al., 1992). Thus, our present findings identify DYN as a substrate of neuronal APN and NEP in inflammation, suggesting a tissue dependent activity

of both peptidases on opioid peptide degradation. The role of APN and NEP in degradation of DYN has been not investigated in peripheral neuronal tissue so far. Previous studies were performed mostly with purified APN and NEP from kidney. In a study by Hersh (1984) kinetics of opioid peptide degradation by NEP from human and rat kidney and rat brain were investigated. It was found that DYN and END are poor substrates for NEP in contrast to MENK and LENK. Similarly, 100 times more purified kidney NEP was needed for hydrolysis of END compared with enkephalins (Graf et al., 1985). Also, Safavi and colleagues (1995) found DYN degradation by purified APN from rat kidney, but the hydrolysis rate is 70 fold lower compared to LENK. Taken together, they all demonstrated that when length of the substrate increases, the affinity of the substrate to the peptidase decreases.

In summary, in our model of peripheral painful inflammation the degradation of END could not blocked with inhibitors, which eliminate this opioid peptide as a substrate for leukocytic and neuronal APN and NEP. DYN came out as substrate for neuronal APN and NEP. This degradation was reversible by the specific inhibitors thiorphan and bestatin and by the new dual inhibitor P8B.

4.7. Analgesic effects of APN and NEP inhibitors

We followed our *in vitro* findings on leukocytic and neuronal APN and NEP activity in opioid peptide degradation and its prevention with enzyme specific inhibitors by *in vivo* investigations. Injection of a combination of thiorphan and bestatin or of P8B directly into inflamed rat paws led to amelioration of pain measured with the paw pressure test. These antinociceptive actions were fully reversed by co-injected Abs against MENK, LENK or DYN, and were slightly decreased by anti-END. This suggests that pain-attenuating effects of APN and NEP inhibitors resulted from preventing predominately enkephalins and DYN from the degradation in peripheral inflamed tissue, corroborating our *in vitro* findings in immune cells and peripheral nerves. This is supported by a study which addressed the degradation of exogenously applied MENK, LENK and DYN examining their ability to internalize μ-opioid receptors in spinal cord slices. These authors found that μ-opioid receptor internalization was induced by a mixture of inhibitors of APN and NEP and of another peptidase, dipeptidyl carboxypeptidase. END-mediated μ-opioid receptor internalization was independent of APN and NEP in that study (Song and Marvizon, 2003). In contrast to our *in vitro* findings we observed a decreased analgesic effect after anti-END Abs injection. This may explained by a blockade of APN and NEP expressed on other tissues such as fibroblasts and endothelia cells (Shipp and Look, 1993; Mina-Osario, 2008). Another study describes some remaining analgesic effects of RB101 in ENK-KO mice. Although they do not test the nature of this

remaining analgesia, this suggests that it is mediated by other opioids than enkephalins such as END and endomorphins (Noble et al., 2008). In previous behavioral investigations, only one study tested APN and NEP inhibitors in peripheral inflamed tissue. Thus, a combined i.pl. injection of thiorphan (0.2 mg) and bestatin (0.2 mg) prolonged stress-induced antinociception mediated by endogenous opioids, but did not change basal nociceptive thresholds in inflamed paws (Parson and Herz, 1990). This was probably due to insufficient dosing of inhibitors, as in our current studies to obtain substantial peripheral antinociception thiorphan and bestatin required higher doses (0.8 mg and 5 mg, respectively). Most earlier studies showed antinociceptive effects of selective ANP and NEP inhibitors following systemic or into the CNS structures injections in healthy mice and rats. A moderate potentiation of antinociception after i.c.v. injected dynorphin B or END by pretreatment with thiorphan or bestatin was found in acute pain models (Roques et al., 1980; Chaillet et al., 1983; Nakazawa et al., 1989; Suh et al., 1990). Combined i.c.v. application of both inhibitors elevated thresholds to thermal stimulation in mice and rats, which was reversible by naloxone, an opioid receptor antagonist (Carenzi et al., 1983). Furthermore, few clinical studies showed that i.v. injections of thiorphan or its prodrug acetorphan decreased NEP activity in plasma and cerebrospinal fluid and produced some analgesia in selected number of patients with migraine or after myelography (Floras et al., 1983; Spillantini et al., 1986). In cancer pain patients a combination of adjuvant with bestatin attenuated recurrence of bladder cancer (Uchibayashi et al., 1995). Also, bestatin and thiorphan applied concomitantly on the spinal cord produced significant pain inhibition in cancer patients (Meynadier et al., 1988). Our current results might have clinical implications for treatment of inflammatory pain such as arthritis.

Dual APN and NEP inhibitors have not been examined in clinical situation so far. In healthy rodents the antinociceptive effect of MENK was potentiated by i.c.v. co-administrated kelatorphan (Fournie-Zaluski et al., 1984). Intravenous injection of RB101 induced dose-dependent and naloxone-reversible antinociceptive responses in thermal acute pain tests in wild type mice (Noble et al., 1992) and these effects were much weaker in preproenkephalin-deficient mice in tests employing noxious mechanical, thermal and chemical stimulation (Noble et al., 2008). Also, aminophosphinic dual inhibitor RB3007 injected i.v. decreased in a naloxone-sensitive manner the sensitivity to acute thermal, chemical and mechanical stimuli as well as nociceptive behaviors in a formalin-induced tonic pain in mice (Le Guen et al., 2003). The only two studies assessing APN and NEP dual inhibitors in prolonged inflammatory pain reported naloxone-reversible antinociceptive effects following i.v. injection of RB101 and RB3007 in the CFA inflammatory model (Maldonado et al., 1994; Le Guen et al., 2003). P8B was only reported to produce antinociception after i.c.v. injection in acute thermal pain in mice without tissue injury (Chen et al., 1998, 2000).

Taken together our studies substantially extend previous reports which showed antinociceptive effects of APN and NEP inhibitors mostly in acute animal pain models following central and systemic injections. All previous studies showing only involvement of MENK and opioid receptors tested with a non-selective antagonist naloxone were addressed. Our data clearly show that simultaneous application of bestatin and thiorphan or P8B produced substantial antinociception against noxious pressure in a model of prolonged inflammatory pain. This effect was reversed by Abs against MENK, LENK and DYN and was slightly attenuated by anti-END. Based on our *in vitro* findings, the pain ameliorating actions were most likely mediated by enhanced levels of endogenous enkephalins and DYN as a consequence of the blockade of APN and NEP in leukocytes and peripheral nerves.

4.8. Future studies

In the present study we have characterized the expression and activity of APN and NEP in leukocytes and peripheral endings of sensory neurons in painful inflamed tissue. Using classical selective (thiorphan and bestatin) and a novel dual (P8B) inhibitors we comprehensively investigated the effects of leukocytic and neuronal APN and NEP on the degradation of four opioid peptides MENK, LENK, DYN and END. In immune cell membranes and sciatic nerves we consistently showed that degradation of MENK and LENK, but not of END, was fully prevented by the inhibitors, pointing to enkephalins as main targets of leukocytic and neuronal APN and NEP. In addition, DYN appeared the substrate of APN and NEP on peripheral nerves. However, in vital immune cells MENK levels were paradoxically diminished by selective and dual APN and NEP inhibitors, while LENK levels were decreased by the dual inhibitor. Although to examine opioid levels inhibitors were used in relatively high concentrations (1 – 5 mM), they did not affect immune cell viability. Nevertheless, they might have affected enkephalin level measurements. An alternative to synthetic peptidase inhibitors might be their endogenous counterparts, which could be tested in future investigations. A possible candidate is sialorphin, which is synthesized predominantly in submandibular gland and prostate of adult rats in response to androgen steroids and is released locally and into the bloodstream in response to environmental stress. Sialorphin was characterized as inhibitor of renal and CNS NEP and was able to prevent the degradation of MENK *in vitro* (Rougeot et al., 2003). Opiorphin is another potent endogenous APN and NEP inhibitor recently identified in human saliva. In human cell line experiments opiorphin prevented the degradation of APN and NEP specific substrates as well as of MENK (Wisner et al., 2006). Both inhibitors injected systemically (i.v. or i.p.)

elicited opioid receptor-mediated antinociception in rat models of acute and formalin-induced pain (Rougeot et al., 2003; Wisner et al., 2006).

Another attractive approach to suppress the expression and function of both peptidases is the use of siRNA. Silencing of APN expression by siRNA markedly decreased the migration and proliferation of ovarian carcinoma cells and attenuated enzymatic activity of APN in osteosarcoma cell line (Kido et al., 2003; Terauchi et al., 2007). It would be interesting to explore whether silencing of APN and NEP by siRNAs in immune cells has more favorable effects on the levels of enkephalins than synthetic inhibitors and whether it results in the inhibition of pathological pain.

In the literature it is described that both metallopeptidases cleave certain chemokines (CXCL8, CXCL11/12, fMLP), while NEP can degrade substance P and bradykinin, which are pro-nociceptive and pro-inflammatory mediators (Roques et al., 1993; Ramirez-Molina et al., 2006; Turner et al., 1997; Mina-Osorio, 2008). However, these effects were described exclusively *in vitro* and do not seem to influence pain perception *in vivo*. This is supported by numerous *in vivo* studies showing exclusively antinociceptive actions of APN and NEP inhibitors injected systemically or into the CNS structures (see paragraph 4.5) as well as by our current studies showing that thiorphan and bestatin and P8B clearly produced antinociception in inflamed paws. No involvement of interactions between APN, NEP and substance P in nociception might be attributed to sequestration and predominant degradation of substance P by its own metabolizing peptidases that are not sensitive to NEP inhibitors (Rougeot et al., 2003). Furthermore, substance P and bradykinin were described as natural inhibitors of APN, as both compounds inhibited the enzyme activity in assays using Ala-βNA or LENK as substrates. Interestingly, substance P was as potent as bestatin in blocking APN (Xu et al., 1995). Moreover, peripherally acting opioids can inhibit the release of substance P from peripheral terminals of primary afferent neurons (Yaksh, 1988) and to block the synthesis of cytokines in macrophages (Belkowski et al., 1995). On the other hand, APN was found to possess chemotactic properties. In patients with rheumatoid arthritis APN participated in lymphocytic infiltration of the synovial fluid, which was partially inhibited by bestatin (Shimizu et al., 2002). Although there is no doubt about pain-relieving actions of APN and NEP inhibitors, it would still be interesting to examine their effects on the migration of immune cells containing opioid peptides and on the levels of opioid peptides and substance P directly in the inflamed rat hind paw using microdialysis.

5. Summary

Opioids are used as pharmacological treatment of severe acute and chronic pain states. However, their actions are associated with side effects such as nausea, depression of breathing, physical dependence and addiction that result from the activation of opioid receptors in the central nervous system (CNS). In contrast opioid peptides produced naturally in the body control pain without eliciting CNS side effects. In inflammation, immune cells infiltrate the site of injury or infection and liberating opioid peptides. The released opioids bind to opioid receptors on peripheral sensory nerves resulting in the inhibition of inflammatory pain. Nevertheless, opioid peptides are susceptible to enzymatic inactivation. The degradation of opioids by peptidases such as aminopeptidase N (APN) and neutral endopeptidase (NEP) has been extensively examined in the CNS and immune cells from healthy animals and humans. Our hypothesis in the current study is that the blockade of opioid-metabolizing peptidases APN and NEP expressed on immune cells and peripheral nerves in inflamed tissue prevents opioid peptide degradation and ameliorates inflammatory pain. As a model of such condition we employed complete Freund's adjuvant-induced unilateral hind paw inflammation in rats and we investigated degradation of all four opioid peptides i.e. Met-enkephalin (MENK), Leu-enkephalin (LENK), dynorphin A (DYN) and β–endorphin (END). To assess enzymatic selectivity of opioid degradation we used bestatin and thiorphan to selectively block APN and NEP, respectively, as well as a novel inhibitor P8B that is equally selective for both peptidases. Using flow cytometry we detected APN and NEP on macrophages and granulocytes, but not on T lymphocytes from inflamed rat hind paws. The activity of leukocytic and neuronal APN and NEP was determined photospectrometrically as their ability to degrade their specific synthetic substrates Alanin-ß-naphthylamine (Ala-βNA) and Succinyl-alanin-alanin-phenylalanine-p-nitroaniline (Suc-Ala-Ala-Phe-p-NA), respectively. *In vitro* incubation of these substrates with vital immune cells, immune cell membranes or sciatic nerve suspensions led to accumulation of the metabolites βNA and pNA, indicating APN and NEP activity, respectively. The degradation of the peptidase-specific substrates was dose-dependently prevented with bestatin, thiorphan, and P8B in all three tissues preparations, suggesting that leukocytic and neuronal APN and NEP are functionally similar. Degradation of immune cell-derived MENK and LENK, but not of DYN and END, was observed between 4 h and 8 h of cell incubation, as reflected by a decrease of the extracellular peptide levels over time measured with radioimmunoassay. Unexpectedly, treatment with thiorphan and bestatin or with P8B significantly decreased the levels of MENK. In contrast inhibitor-induced prevention of LENK degradation could show at 8 h. In immune cell membranes or sciatic nerve suspensions the amounts of exogenously

applied MENK, LENK, DYN and END significantly dropped within 4 h of incubation. In both tissue preparations, the catabolism of MENK and LENK, but not of END, was dose-dependently and fully prevented with a combination of bestatin and thiorphan or with P8B alone. This shows that both enkephalins are important substrates of leukocytic and neuronal APN and NEP in peripheral inflamed tissue. Selective and dual inhibitors prevented the degradation of DYN in suspensions of the sciatic nerves, but not in immune cell membranes. This indicates that under inflammatory conditions DYN becomes a substrate of APN and NEP on peripheral nerves. Finally, concomitant injection of thiorphan and bestatin or of P8B into inflamed rat paws decreased inflammatory pain in response to noxious pressure *in vivo*. These antinociceptive effects were completely reversed by co-injected Abs against MENK, LENK and DYN, and were only slightly attenuated by anti-END.

In conclusion, blockade of ANP and NEP expressed on leukocytes and peripheral nerves from inflamed painful tissue prevented the degradation of enkephalins or DYN, and ameliorated inflammatory pain. These results provide a rationale for a promising strategy for pain control based on the enhancement of the analgesic actions of endogenous opioid peptides by inhibiting their enzymatic degradation directly in injured tissues. Thus, a combination of classical selective inhibitors bestatin (APN) and thiorphan (NEP) as well as the novel dual inhibitor P8B might represent a new class of analgesics without the untoward side effects of conventional opioid or non-opioid compounds.

Zusammenfassung

Zur pharmakologischen Behandlung bei diversen akuten und chronischen Schmerzen werden unter anderem Opioide eingesetzt, allerdings führt dieser Einsatz auch zu ungewollten Nebeneffekten wie Übelkeit, Atemstillstand und Drogenabhängigkeit, welche durch die Aktivierung von Opioidrezeptoren im Zentralen Nervensystem (ZNS) ausgelöst werden. Im Gegensatz dazu führen körpereigene Opioidpeptide zur Schmerzlinderung ohne zentral ausgelöste Nebenwirkungen zu verursachen. Bei schmerzhaften entzündlichen Erkrankungen infiltrieren Immunzellen das betroffene Gewebe und setzen endogene Opioidpeptide frei, welche an Opioidrezeptoren auf peripheren sensorischen Nerven binden und dort die Schmerzübertragung verhindern. Allerdings werden Opioidpeptide durch Peptidasen wie Aminopeptidase N (APN) und Neutrale Endopeptidase (NEP) abgebaut, was intensiv im Zentralen Nervensystem und an Immunzellen von gesunden Tieren und Menschen untersucht wurde. Die Hypothese der hier durchgeführten Studie ist, durch Inhibierung der opioid-abbauenden Peptidasen APN und NEP den Abbau von Opioidpeptiden zu verhindern, wodurch sich die endogen vermittelte Linderung von Entzündungsschmerz verbessert. Als Entzündungsmodell wurde die Freund`s Complete Adjuvants-induzierte, lokale Entzündung einer Hinterpfote bei Ratten angewandt, um die Wirkung der selektiven Inhibitoren Bestatin (APN) und Thiorphan (NEP), sowie des neuartigen Dualen-Inhibitor P8B auf die Degradation der vier Opioidpeptide Met-Enkephalin (MENK), Leu-Enkephalin (LENK), Dynorphin A (DYN) und Endorphin (END) unter entzündlichen Bedingungen zu untersuchen. Mit Hilfe der Durchflusszytometrie konnten APN und NEP in der aus lokalen Entzündung entnommenen Makrophagen und Granulozyten, aber nicht auf T-Lymphozyten, nachgewiesen werden. Die Aktivität der leukozytären und neuronalen Enzyme APN und NEP wurde spektrophotometrisch anhand der Degradation enzym-spezifischer Substrate Alanin-ß-naphthylamine (Ala-βNA) und Succinyl-alanin-alanin-phenylalanine-p-nitroaniline (Suc-Ala-Ala-Phe-p-NA) untersucht. Nach *in vitro* Inkubation dieser Substrate mit vitalen Immunzellen, Immunzellmembranen oder Nervengewebe-Suspensionen kam es zur Akkumulation der entsprechenden Metaboliten βNA und pNA nach APN respektive NEP Aktivität. Die Degradation der enzym-spezifischen Substrate konnte dosis-abhängig durch Bestatin und Thiorphan oder P8B in den drei Gewebepräparationen verringert werden, was auf eine identische Funktion der leukozytären und neuronalen Enzyme APN und NEP hinweist. Die radioimmunologische Analyse von aus Entzündungszellen freigesetzten MENK und LENK zeigte, dass die Peptidmengen im extrazellulären Medium innerhalb der ersten vier Inkubationsstunden akkumulierten; im Laufe der folgenden 4 h wurden diese Peptide degradiert während sich die Akkumulation von

DYN und END über 8 h fortsetzte. Die Behandlung mit Bestatin und Thiorphan in Kombination oder P8B verringerte signifikant die Menge von MENK, was nicht erwartet wurde. Im Gegensatz dazu konnte der Abbau von Immunzell-freigesetzten LENK durch die kombinierte Gabe von Bestatin und Thiorphan nach 8 h signifikant inhibiert werden. Die Untersuchung des Abbaus von exogen zugegebenen Opioidpeptiden (MENK, LENK, DYN und END) an isolierten Immunzellmembranen und Suspensionen des Nervus ischiadicus aus entzündetem Gewebe zeigte, dass alle Peptide über einen Zeitraum von 4 h abgebaut wurden. Der Abbau von MENK und LENK, nicht aber der von END, wurde dosis-abhängig und vollständig durch Bestatin und Thiorphan oder P8B verhindert. Das bedeutet, dass beide Enkephaline wichtige Substrate für die leukozytären und neuronalen Enzyme APN und NEP in peripherem, entzündeten Gewebe sind. Dem Abbau von DYN konnte durch den Einsatz der Inhibitoren vor allem in Suspensionen vom Nervus ischiadicus, aber nicht an isolierten Immunzellmembranen, entgegengewirkt werden. Das lässt den Schluss zu, dass DYN als Substrat von neuronalen Enzymen APN und NEP bevorzugt wird. Diese *in vitro* Befunde werden durch unsere *in vivo* Untersuchungen gestützt. Hier resultierte die Injektion von Bestatin und Thiorphan in Kombination bzw. von P8B in das entzündete Pfotengewebe in einer erheblichen Verminderung der Hyperalgesie als Antwort auf einen schmerzhaften Druckreiz. Dieser analgetische Effekt der Inhibitoren, wurde durch die Gabe von spezifischen MENK-, LENK- und DYN-Antikörpern vollständig aufgehoben, die Injektion von END-Antikörpern zeigte einen geringen Einfluss.

Zusammenfassend ist festzustellen, dass im Rahmen der durchgeführten Untersuchungen der Abbau von Enkephalinen und DYN durch die Blockade von APN und NEP in Immunzellen und peripheren Nerven aus entzündetem schmerzhaftem Gewebe verhindert werden konnte, was zur Schmerzverminderung führte. Basierend auf der Erhöhung der Aktivität endogener Opioidpeptide durch Inhibierung des enzymatischen Abbaus im entzündeten Gewebe zeigen diese Ergebnisse eine mögliche Strategie zur Schmerzkontrolle auf. Deshalb könnten die klassischen selektiven Inhibitoren Bestatin (APN) und Thiorphan (NEP) oder der neue Duale-Inhibitor P8B eine neue Klasse von Analgetika repräsentieren, deren Anwendung keine der Nebenwirkungen von konventionellen opioidhaltigen und nicht-opioidhaltigen Arzneimitteln auslösen.

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Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

For reasons of data protection,
the curriculum vitae is not included in the online version.

Publications

Labuz D, Schmidt Y, **Schreiter A**, Rittner HL, Mousa SA, Machelska H. 2009. Immune cell-derived opioids protect against neuropathic pain in mice. J Clin Invest. 119(2):278-86.

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Schreiter A, Gore C, Labuz D, Fournie-Zaluski M, Roques BP, Stein C, Machelska H. Prevention of opioid peptide degradation in peripheral inflamed tissue for pain control. (in preparation)

Posters

2006: **A. Schreiter**, D. Labuz, A. Brack, H. Rittner, H. Machelska. T cells contribute to pain control in neuritis. Berlin Brain Days, Charite Mitte, 29.10. – 01.11.06.

2007: **A. Schreiter**, D. Labuz, H.L. Rittner, A. Brack, H. Machelska. T lymphocytes contribute to opioid pain control in neuritis. 2nd International Congress on Neuropathic Pain, Berlin, Germany, 07.06. - 10.06.07.

A. Schreiter, D. Labuz, H.L. Rittner, A. Brack, Y. Schmidt, H. Machelska.. T lymphocyte-derived opioids contribute to peripheral antinociception in neuritis. INRC 2007, Berlin, Germany, 08.07. - 13.07.07.

A. Schreiter, D. Labuz, Y. Schmidt, H.L. Rittner, A. Brack, H. Machelska. Neuropathic pain control by T lymphocyte-derived opioids. Berlin Brain Days 2007, Charite, Berlin, Germany, 26.11. - 29.11.07.

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Eidesstattliche Erklärung

Ich versichere, dass ich diese Arbeit selbständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet habe. Stellen, die anderen Quellen im Wortlaut oder Sinn entnommen sind, wurden mittels Quellenangaben kenntlich gemacht. Dies gilt auch für Skizzen, Zeichnungen, Fotos und andere bildliche Darstellungen.

Berlin,

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Anja Schreiter

Appendix: Locations of companies and distributors

Table A.1: Companies and distributors

Company	City	Country
Abbott	Wiesbaden	Germany
Amersham	Buckinghamshire	UK
Bachem	Weil am Rhein	Germany
Bachem/Peninsula Laboratories	Belmont, CA	USA
BD Bioscience	Heidelberg	Germany
Biochrom	Berlin	Germany
Brand	Wertheim	Germany
Callbiochem	La Jolla, CA	USA
Carl Zeiss Mikroskopie	Göttingen	Germany
Eastman Kodak Company	Rochester, NY	USA
Eppendorf	Hamburg	Germany
Falcon	Heidelberg	Germany
GIBCO Invitrogen Corporation	Karlsruhe	Germany
Kendro	Osterode	Germany
Life Technologies	Carlsbad, CA	USA
Millipore GmbH	Eschborn	Germany
Parmingen/Becton Dickinson	Heidelberg	Germany
Pharmaleads	Paris	France
Phoenix Laboratories	Belmont, CA	USA
Santa Cruz Biotechnology	Heidelberg	Germany
Serotec	Oxford	UK
Shimadzu Europa GmbH	Duisburg	Germany
Sigma-Aldrich	Taufkirchen	Germany
Wallac Distribution GmbH	Freiburg	Germany
Ugo Basile	Comerio	Italy