1. Introduction

Traditionally, pain perception and endogeneous modulation have been attributed mostly to neuronal circuits within the central nervous system (CNS) [1]. It was shown that pain can be effectively controlled by various endogenous mechanisms, for example by interactions between opioid receptors and opioid peptides located in the dorsal horn of the spinal cord, the midbrain, brainstem, thalamus, the limbic system, and cortex [2]. Recent research revealed that, in addition to these mechanisms in the CNS, intrinsic modulation of nociception can also occur in the periphery, mediated by interactions between immune cells and peripheral sensory neurons [3, 4] (Fig. 1). This neuroimmune link has emerged during studies concerning the peripheral antinociceptive actions of locally applied exogenous opioid receptor agonists [5, 6]. A prerequisite for the manifestation of such peripheral effects seems to be inflammation, accompanied by hyperalgesia (increased sensitivity to normally painful stimuli) [7]. Under such conditions opioid receptors are upregulated on peripheral endings of sensory nerves. Their endogenous ligands, opioid peptides, are synthesized in circulating immune cells which migrate to injured sites. Under environmental stressful stimuli or in response to releasing agents (e.g. corticotropin releasing factor, CRF) immunocytes can secrete opioids. These activate peripheral opioid receptors and produce antinociception by inhibiting either the excitability of the nerves or the release of excitatory, proinflammatory neuropeptides. Because these effects occur in the periphery they are devoid of central opioid side effects such as respiratory depression, sedation, dysphoria or dependence. Targeting of immune cells containing opioids to injured tissues is a novel concept of pain control and opens potential new therapeutic approaches.

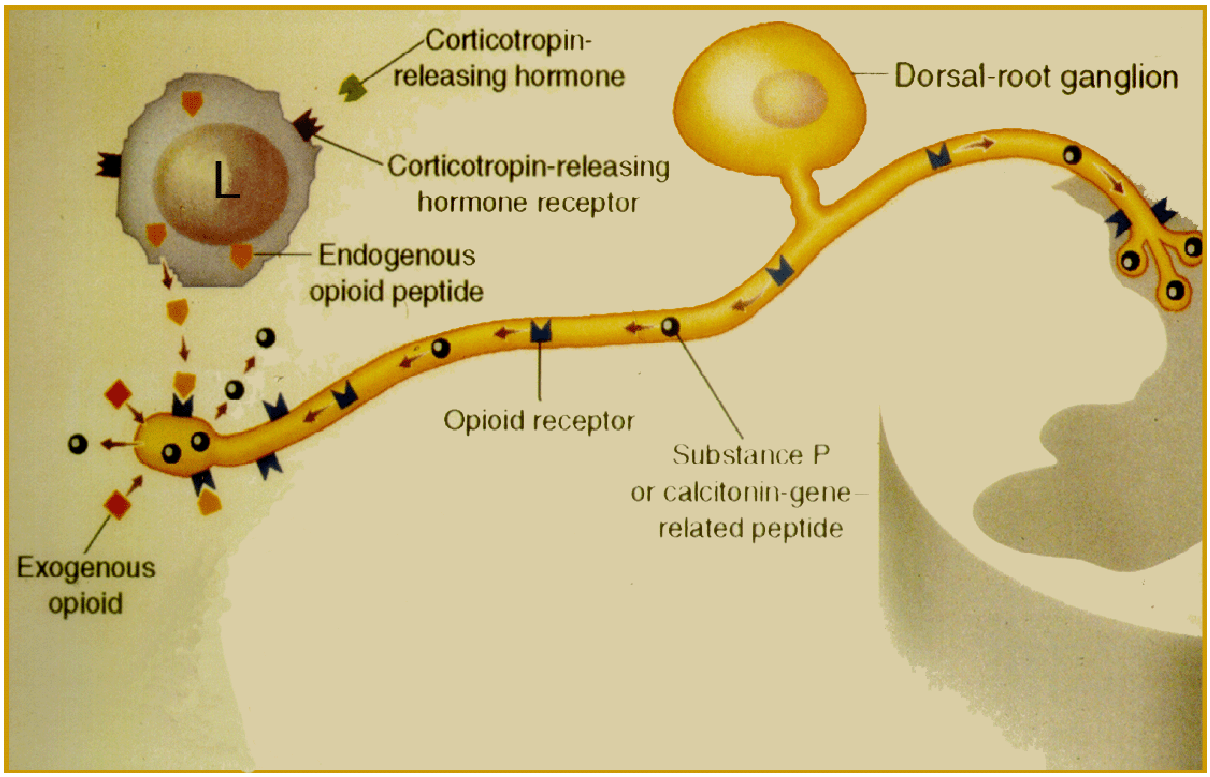

Figure 1. Interactions of opioids with peripheral opioid receptors in inflamed tissue. Opioid receptors and neuropeptides (such as substance P or calcitonin-gene-related peptide) are synthesized in the dorsal root ganglion and transported into central and peripheral processes of the primary afferent neuron. Upon activation by stress or releasing agents (e.g. corticotropin releasing hormone) immune cells secrete opioids that activate peripheral opioid receptors and produce antinociception by inhibiting either the excitability of the nerves or the release of excitatory, proinflammatory neuropeptides (e.g. substance P or calcitonin-gene-related peptide). This effect can be mimicked by exogenous opioid peptides. L, leukocyte. Adapted from Stein, C. The control of pain in peripheral tissue by opioids. N Engl J Med: 332 (32): 1685 – 90, 1995.

1.1. Peripheral opioid receptors

All three types of opioid receptors (μ-, δ-, κ-) are located on peripheral sensory neurons where their activation by opioids leads to potent antinociception in animals or analgesia (i.e. absence of pain in response to stimulation which would normally be painful) in humans [8-11]. Cell bodies of these neurons in dorsal root ganglia (DRG), express opioid receptor mRNAs and their respective receptor proteins [12]. The binding characteristics of peripheral opioid receptors are similiar to their central correlates [13]. They belong to a G-protein (G_i and/or G_0) coupled receptor family and mediate their inhibitory effects on primary afferent neurons mainly via the modulation of (high-voltage activated) calcium currents [12, 14, 15]. During peripheral tissue inflammation local opioid mediated antinociceptive effects are significantly augmented [12, 16].

Synthesis and expression of opioid receptors can be increased in the DRG [17, 18], and within several days after induction of inflammation, the peripherally directed axonal transport of opioid receptors is greatly enhanced, leading to their upregulation on peripheral terminals of sensory neurons [13, 19]. In addition, pre-existent but possibly inactive neuronal opioid receptors may undergo changes owing to the specific milieu (e.g. low pH) of inflamed tissue and, thus, be rendered active. Further, inflammation increases the number of sensory nerve terminals (sprouting) and entails a disruption of the perineural barrier thus faciliating the access of opioid agonists to their receptors [20]. Opioid receptors have also been demonstrated on immune cells [13, 21], modulating their proliferation, chemotaxis, cytokine production and mast cell degranulation [22]. However, the significance of such effects with regard to nociception has not been investigated as yet.

1.2. Peripheral endogenous opioid peptides

Opioid peptides are the natural ligands at opioid receptors. Three families have been well characterized in the CNS and neuroendocrine system, the endorphins (END), the enkephalins (ENK), and the dynorphins (DYN). Each family derives from a distinct gene, and from one of the three precursor proteins proopiomelanocortin (POMC), proenkephalin (PENK), and prodynorphin, respectively. These peptides exhibit different affinities and selectivities for the three opioid receptors: μ (β-END and Met-ENK), δ (Met-ENK and β-END), and κ (DYN) [2, 23]. Two additional highly selective μ-opioid receptor ligands, endomorphin-1 and -2, have been isolated, although their precursors are not known yet [24].

Blalock and Smith were the first to demonstrate POMC expression by leukocytes [25, 26]. Since then, POMC related opioid peptides have been found in immune cells of many vertebrates and nonvertebrates [27, 28]. PENK-derived opioid peptides have also been detected in human and rodent immune cells [29, 30]. Both the mRNA and met-enkephalin protein were detected. In conditions of painful inflammation POMC mRNA, β-END, met-ENK, and DYN are found in circulating immune cells, and lymph nodes. Within injured tissue these peptides are upregulated in lymphocytes, granulocytes, and monocytes/macrophages [22, 31-35]. These findings show that local signals can stimulate the synthesis of opioid peptides in different types of

inflammatory cells at the site of tissue injury. Upon secretion opioid peptides can activate peripheral opioid receptors to elicit local antinociception (see below).

Other peripheral loci of opioid peptide production include the adrenals and pituitary but apparently neither are involved in peripheral pain inhibition [36]. Keratinocytes can release opioids upon stimulation of endothelin-B receptors by endothelin-1 and decrease pain [37]. Also, peripheral neurons can produce opioids [3, 38], can be stimulated to overexpression by gene transfer and decrease chronic pain and inflammation in animals [39].

1.3. Migration of opioid containing cells to inflamed tissue

The recruitment of leukocytes from the circulation into areas of inflammation is a multistep process which begins with the attachment of these cells to vascular endothelium, followed by their transmigration into the inflamed tissue. This is a well-orchestrated set of events mediated by various cell adhesion and chemoattractant/activator molecules expressed by leucocytes and endothelium (Figs. 2, 3).

Initially, the circulating leukocytes are captured and roll on the endothelial cells of vessels, a process mediated primarily by selectins. Selectins are cell-surface glycoproteins located on leukocytes (L-selectin), endothelium (P-, E-selectin), and platelets (P-selectin) and interact with their ligands on endothelial or immune cells, respectively (Fig. 3). The rolling immunocytes can then be activated by chemoattractants released from inflammatory cells and endothelium which leads to an upregulation and increased avidity of leukocyte integrins, in particular $\alpha/\beta 2$ (CD11/CD18), and $\alpha 4\beta 1$ (CD49d/CD29; very late antigen-4; VLA-4) through activation of G protein-linked receptors. Integrins are a broadly expressed family of heterodimeric membrane molecules which mediate the firm adhesion of leukocytes to endothelial cells by interacting with ligands of the immunoglobulin superfamily (IgSF), e.g., intercellular adhesion molecule-1 (ICAM-1/CD54) expressed by endothelium, leukocytes and synovial lining cells [40]. Thereafter leukocytes migrate through endothelium engaging other IgSF members, predominantly platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31) on leukocytes and endothelium, and are directed to the sites of inflammation to initiate a host defence [41-43].

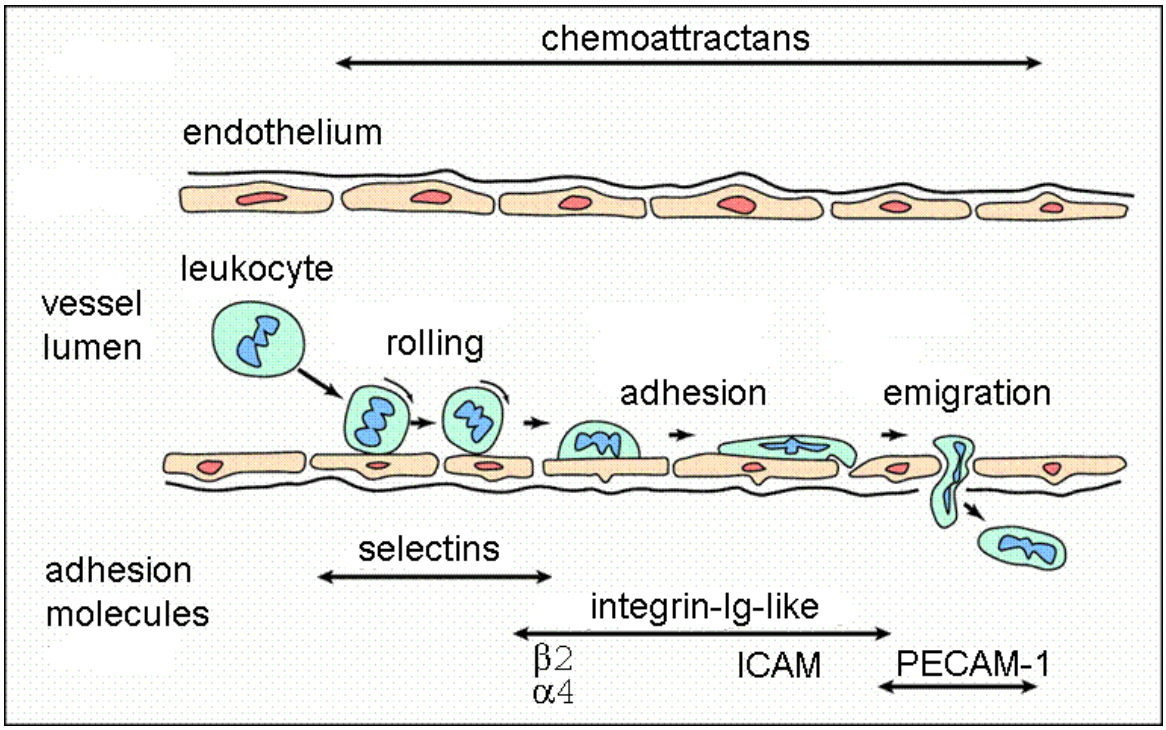

Figure 2. Leukocyte extravasation. Recruitment of leukocytes from the circulation into areas of inflammation is a multistep process mediated by various cell adhesion and chemoattractant molecules expressed by leukocytes and endothelium. Leukocytes are captured and roll on the endothelial cells of vessels, a process mediated primarily by selectins. Chemoattractants lead to an upregulation and increased avidity of lekocyte integrins, in particular β2 and α4 which mediate the firm adhesion of leukocytes to endothelial cells. Integrins interact with ligands of the immunoglobulin superfamily (e.g. β2 with ICAM-1). Thereafter, leukocytes migrate through endothelium a process mediated primarily by PECAM-1, and are directed to the sites of inflammation. ICAM, intercellular adhesion molecule-1, PECAM-1, platelet endothelial cell adhesion molecule-1.

Pharmacological or genetic interruption of these leukocyte-endothelial interactions can block immunocyte extravasation [44]. The blockade of selectins, α4 and β2 integrins was shown to decrease the migration of leukocytes to inflamed skin and/or joints [45-49], and chronic treatment with monoclonal antibodies (mAbs) against E-selectin, α4 and/or β2 integrins, ICAM-1 or PECAM-1 reduced the severity of experimental arthritis [50-53]. Conversely, no improvement or even increased severity of arthritis was observed after treatment with mAbs against β2 integrins or in P-selectin deficient mice, respectively [54, 55]. Recent research indicates that these events also govern the homing of immune cells containing opioids. Inflammation leads to an upregulation of P-selectin and PECAM-1 on vascular endothelium and, importantly, to a simultaneous migration of β-endorphin-containing leukocytes to inflamed

subcutaneous tissue. β-endorphin-containing immunocytes co-express L-selectin [56] and blocking of selectins impairs intrinsic peripheral opioid-mediated antinociception [57]. Thus, local inflammatory-induced signals not only stimulate the synthesis of opioid peptides in resident immune cells but also attract opioid-containing leukocytes from the circulation to the site of tissue injury where they release the peptides to reduce antinociception [19, 58].

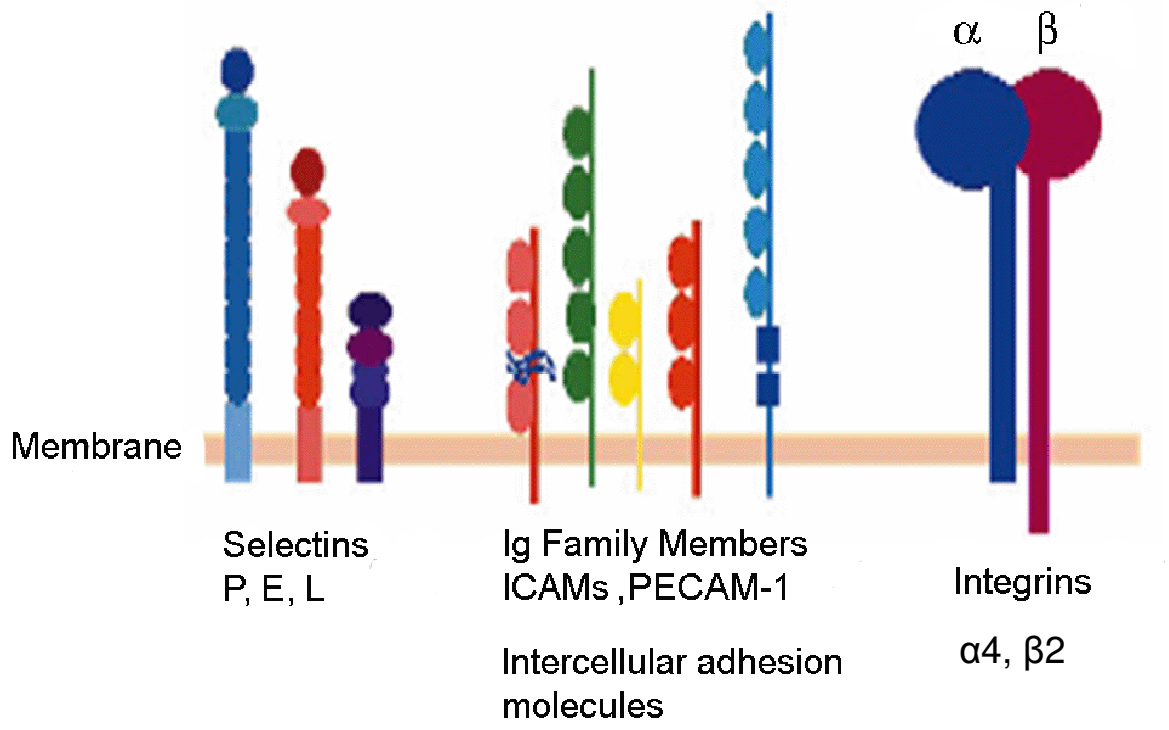

Figure 3. Structure of adhesion molecules. The selectins are transmembrane proteins that contain an N-terminal lectin domain, epidermal growth factor-type domain and two to nine complement regulatory repeats. The Ig superfamily members are structurally most diverse, but each has two to five Ig repeats in the extracellular domain and most of them are transmembrane proteins. The integrins are noncovalently linked heterodimers composed of α and β subunits, ICAMs, intercellular adhesion molecules, PECAM-1, platelet-endothelial cell adhesion molecule-1. Reprinted from Am J Med 1999, 106, Petruzelli L, Takami M, Humes D. Structure and function of cell adhesion molecules. 467-476, Copyright 1999, with permission from Experta Medica Inc..

1.4. Release of opioid peptides from immune cells

In the pituitary, β-END and other POMC-derived peptides (e.g. adrenocorticotropic hormone) can be released by CRF. This neuropeptide derives from the hypothalamus and controls the activity of the hypothalamo-pituitary-adrenal axis being the predominant regulator of the endocrine, neuroendocrine, autonomic and behavioral responses to stress. It seems to regulate the body's defence reactions to stress, preventing those actions from overshooting and from threatening homeostasis [59].

It has been shown that CRF and its receptors are not only expressed in neurons in the CNS but are also present in immunocytes, fibroblasts, and vascular endothelium [60, 61] (Fig. 1). Inflammation leads to an enhanced expression of peripheral CRF in synovial and subcutaneous tissue in animals and humans [62].. Similar to the pituitary, CRF can trigger local opioid release within peripheral inflamed tissue. CRF binding sites are upregulated in lymph nodes and inflamed paws. They are located on lymphocytes and monocytes/macrophages [63], are G protein-coupled and resemble the high affinity CRF binding sites in the pituitary [62, 63]. They are absent on primary afferent neurons [63]. *In vitro* experiments showed that CRF can stimulate the release of β-END, Met-ENK, and DYN from lymphocytes via the activation of CRF binding sites [34, 35]. This release was dose-dependent, and reversible by the selective CRF receptor antagonist α-helical CRF, indicating the receptor specifity of action. Furthermore, injection of CRF into inflamed paws was shown to produce potent antinociception *in vivo* reversible by CRF- and μ- and δ-opioid receptor antagonists as well as by monoclonal Abs against β-END and Met-ENK [34, 35, 64]. Although injection of Abs against DYN did not change CRF-induced antinociception *in vivo*, taking *in vitro* experiments into consideration, it is reasonable to assume that CRF-mediated antinociception is a combination of effects of all three opioid peptides (β-END, Met-ENK, DYN) acting mainly at μ- and δ-receptors [34, 35, 64]. Importantly, these events occur only within inflamed tissue once more underlining the importance of inflammation for manifestation of antinociception. Further, systemic administration of CRF does not change nociceptive thresholds demonstrating a peripheral site of CRF-induced antinociception [64].

1.5. Interactions of immune-derived opioids with peripheral opioid receptors

Stress is a natural stimulus to produce antinociception [4, 65]. For example, experimental foot shock, restraint and cold water swimming (CWS) can produce potent opioid antinociception in animals without tissue injury. Structual components of this endogenous system include the periaqueductal gray, the nuclei of the medulla, and the dorsal horn of the spinal cord [65, 66]. Each of these areas is densely innervated by opioidergic neurons and contains opioid receptors [67-69].

Peripheral mechanisms of intrinsic opioid antinociception are based on neuroimmune interactions and become apparent within injured tissue [4]. Local injections of a nonselective opioid receptor antagonist in small systemically inactive doses dose-dependently and stereospecifically blocked stress-induced antinociception indicating a peripheral site of action [70]. It has been shown that experimental stress (e.g. cold water swim) in animals or postsurgical stress in humans can liberate opioids within inflamed (but not noninflamed) tissue which bind to peripheral opioid receptors leading to potent and clinically relevant analgesia [71-72]. At later stages of FCA inflammation in rats (4-6 days) the most prominent opioid peptide involved in swim stress-induced antinociception is β-END, acting mainly at μ and δ opioid receptors [70]. Met-ENK and DYN or κ receptors are not involved at these later stages. At 4 days of FCA inflammation an important endogenous agent triggering swim stress-induced antinociception is CRF. This is supported by findings that swim stress-induced antinociception was abolished by α-helical CRF, Ab against CRF and CRF antisense (an oligodeoxynucleotide to inhibit CRF synthesis) injected in small systemically inactive doses directly into inflamed tissue [64, 72]. Thus, upon a stressful stimulus CRF can be secreted locally at the site of tissue injury, activate its receptors on immune cells to release opioids that subsequently occupy opioid receptors on sensory nerves resulting in reduction of antinociception [72].

That immune cells are the source of opioids is demonstrated by the abolishment of stress- and CRF-induced antinociception by immunosuppression with cyclosporine A or whole-body irradiation, and by blockade of selectin dependend opioid-containing leukocyte extravasation [9, 31, 57, 64, 73]. Thus, pain can be diminished by local paracrine interactions of the immune system with peripheral sensory neurons.

1.6. Aims of the study

There is evidence that the course of inflammation can influence the neuroimmune interactions in opioid-mediated antinociception, i.e. the degree of swim stress-induced antinociception increases with the duration of the inflammation [58]. The primary goal of this study is to define the mechanisms of intrinsic opioid antinociception at an early (6 h) compared to a later (4 days) stage of localized hindlimb FCA-induced inflammation in rats. The specific aims were: **(1)** to evaluate the development of inflammation at 6 h and 4 days after FCA injection, **(2)** to investigate the contribution of opioid peptides and opioid receptors to swim stress-induced antinociception, **(3)** to characterize the endogenous trigger for swim stress-induced antinociception at 6 h, **(4)** to establish the contribution of peripheral versus central opioid receptors to swim stress-induced antinociception, and **(5)** to evaluate the relative contribution of selectins, integrins, and the IgSF members to intrinsic peripheral opioid antinociception at 6h by: **(a)** examining the effect of fucoidin (a blocker of L-, and P-selectin), and mAbs against α4 and β2 integrins, ICAM-1 and PECAM-1 on swim stress-induced antinociception, and **(b)** by investigating the effect of mAbs against ICAM-1 on CRF-induced antinociception.