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5. ABBREVIATIONS

BAPTA/AM	1,2-*bis*(*o*-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester
CFA	complete Freund's adjuvant
CRF	corticotropin releasing factor
CINC-2	cytokine induced chemokine -2
CXCL1	keratinocyte-derived chemokine (KC) = CINC-1
CXCL2/3	macrophage inflammatory protein (MIP)-2 = CINC-3
CXCL5	lipopolysaccharide-induced chemokine (LIX)
CXCL8	interleukin (IL)-8
CXCL12	stromal derived factor-1α (SDF-1α)
CXCR/CCR	chemokine receptor CXCR (z. B. CXCR2)
DRG	DRG
fMLP	formyl-methionine-leucine-phenylalanine
ICAM-1	intercellular adhesion molecule-1
IL	interleukin (e.g. IL-1β)
IP_3	inositol 1,4,5-trisphosphate
MAPK	mitogen activated kinase
MMP-9	metalloproteinase 9
MPO	myeloperoxidase
PI3K	phosphoinositol-3-kinase

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8. DECLARATION UNDER OATH

ERKLÄRUNG

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Berlin, 3. August 2007

(Dr. H. Rittner)

1. INTRODUCTION AND AIMS

1.1. Generation and inhibition of inflammatory pain

During inflammation of peripheral tissues, numerous mediators are produced by endothelial cells, resident cells as well as leukocytes that are recruited to the site of injury. These mediators (e.g. protons, cytokines, nerve growth factor) are known to elicit pain by activation of specialized primary afferent neurons called nociceptors. Nociceptors are defined as "neurons preferentially sensitive to a noxious stimulus or to a stimulus which would become noxious if prolonged" (definition of the International Association for the Study of Pain, IASP, www.iasp-pain.org/terms-p.html) (Merskey and Bogduk, 1994). Nociceptors belong to the group of unmyelinated Aδ and C fibers originating from trigeminal and dorsal root ganglion (DRG). Aδ and C fibers transduce and propagate noxious stimuli to the dorsal horn of the spinal cord. Various neurotransmitters modulate these signals at the level of the spinal cord and later at supraspinal sites. Together with environmental and cognitive factors, the sensation of pain is elicited. Thus, "pain" is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" (definition of the IASP, www.iasp-pain.org/terms-p.html) (Merskey and Bogduk, 1994). Strictly speaking, "analgesia" is defined as the "absence of pain in response to stimulation which would normally be painful" in humans, while "antinociception" is defined as the inhibition of behavioral responses to noxious stimuli in animals. In this manuscript the terms pain and analgesia are used for reasons of readability.

Inflammatory pain is characterized by an increased response to mechanical or heat stimuli which are normally mildly painful (mechanical or thermal hyperalgesia) (Merskey and Bogduk, 1994). After tissue injury, inflammatory mediators are produced in the circulation (e.g. bradykinin) and by local resident cells (e.g. tissue macrophages and dendritic cells). The inflammatory response is amplified by migration of leukocytes into the inflamed tissue, by production of cytokines, chemokines and growth factors like nerve growth factor as well as tissue acidification. Animal models of peripheral tissue inflammation are generated by local injection of agents such as carrageenan, complete Freund's adjuvant (CFA), lipopolysaccharide, zymosan or glycogens. In our studies we used the model of CFA inflammation. CFA is injected into the right hind paw of Wistar rats inducing a local inflammatory response with edema, redness, hyperthermia and pain. Pain and analgesia can be quantified by determining mechanical or thermal nociceptive thresholds using various behavioral assays. Among these, mechanical nociceptive thresholds can be evaluated using the paw pressure test of Randall and Selitto whereby painful pressure is elicited by a blunt piston (Stein et al., 1990a). Thermal nociceptive thresholds can be measured determining the paw withdrawal latency in response to heat from a heated bulb (Hargreaves et al., 1988). The contralateral untreated paw serves as a control.

The role of leukocytes in the generation of pain is controversial. Several earlier studies suggested that granulocytes confer pain. However, these studies have used nonselective chemotactic peptides for the induction of granulocyte migration or nonselective drugs for the depletion of granulocytes (Levine et

al., 1984; Levine et al., 1985; Levine et al., 1986). In the meantime tools for selective granulocyte depletion or recruitment have been made available. Selective granulocyte migration can be achieved using certain chemokines as described in chapter 1.3. This allowed us to examine the question whether selective recruitment of granulocytes indeed causes pain (chapter 2.1).

Leukocytes secrete hyperalgesic as well as analgesic mediators. Among the analgesic mediators, endogenous opioid peptides have been most extensively studied. Opioid peptides bind to opioid receptors on peripheral sensory neurons and confer analgesia. Other analgesic mediators include anti-inflammatory cytokines, somatostatin (somatotropin release-inhibiting factor; SRIF), endocannabinoids and interleukin-1 (IL-1) (Rittner et al., 2005; Agarwal et al., 2007; Wolf et al., 2007).

1.2. Opioid receptors and their ligands in peripherally mediated analgesia

μ- (MOR), δ- (DOR) and κ-(KOR) opioid receptors are expressed in the central nervous system as well as on the peripheral sensory nerve (Table 1). Opioid receptors are synthesized in DRG and intraaxonally transported to the peripheral nerve terminals (Ji et al., 1995). Further anatomical and electrophysiological studies show expression of opioid receptors on unmyelinated C- and on myelinated Aδ-fibers (i.e. nociceptors) but not on large myelinated Aβ-fibers (Arvidsson et al., 1995; Beland and Fitzgerald, 2001).

Intraarticular or intraplantar injection of opioid agonists elicits potent analgesia in patients as well as animals, respectively (Stein et al., 1991; Schäfer et al., 1995). Studies in patients and in rats have shown that peripherally mediated opioid analgesia is more effective under inflammatory conditions (Antonijevic et al., 1995; Likar et al., 1998; Stein et al., 2003). Analgesic efficacy increases with increasing duration of inflammation (Schäfer et al., 1995; Machelska et al., 2003). Four factors contribute: 1. Inflammation increases μ-opioid receptor binding sites in DRG neurons and μ-opioid receptor axonal transport leading to an increased density of opioid receptors on the peripheral nerve endings (Mousa et al., 2001; Zöllner et al., 2003). 2. Inflammation enhances G-protein coupling to the opioid receptors and subsequent activation of the intracellular signaling cascade (Zöllner et al., 2003). 3. Inflammation destroys the perineural sheath, the barrier to diffusion of hydrophilic and high molecular weight substances, facilitating the access of agonists like morphine or opioid peptides (Antonijevic et al., 1995; Stein et al., 2003). 4. Inflammation increases the number of accessible nerve endings by sprouting of nerve terminals (Mousa et al., 2001). However, the *in vivo* regulation of opioid receptor transcription and translation in DRG is incompletely understood and seems to differ between opioid receptor types. Both electrical conduction (Puehler et al., 2004) as well as local production of nerve growth factor (Mousa et al., 2007b) was shown to contribute to μ-opioid receptor expression during different stages of the inflammatory process. Inflammation and peripheral injection of IL-1β enhance axonal transport of κ-opioid receptors (Jeanjean et al., 1994; Jeanjean et al., 1995). We therefore hypothesized that IL-1β also regulates the expression of κ-opioid receptor mRNA in the DRG which is studied in chapter 2.2.

Opioid peptide	Receptor
β-Endorphin	μ > δ
Enkephalins	δ > μ
Dynorphins	κ > μ, δ
Endomorphin-1	μ
Endormophin-2	μ

Table 1 *Naturally occurring opioid peptides and their receptors.*

Opioid peptides are the endogenous ligands of opioid receptors (Table 1). They are produced by neurons, neuroendocrine cells and leukocytes and can be released upon stimulation. Opioid peptides are known to derive from different precursors: β-endorphin from proopiomelanocortin, met-enkephalin from proenkephalin and dynorphin from prodynorphin. Endomorphins are selective μ-opioid receptor agonists but their precursors are currently unknown. Proopiomelanocortin processing occurs in the endoplasmic reticulum and the *trans*-Golgi network and involves several processing enzymes (Mousa et al., 2004). Opioid peptides and processing enzymes are detectable in circulating as well as migrating leukocytes (granulocytes, monocytes/macrophages, and lymphocytes).

The proopiomelanocortin gene consists of 3 exons: Exon 1 encodes the promoter binding sites and the mRNA cap region. Exon 2 contains the signal peptide sequence directing the nascent polypeptide to the regulated secretory pathway. Exon 3 encodes several hormones and β-endorphin. While truncated proopiomelanocortin mRNA (Exon 3 only) is readily detectable outside the CNS (Lacaze-Masmonteil et al., 1987), the presence of full length proopiomelanocortin mRNA in inflammatory cells is controversial (Lyons and Blalock, 1997). In CFA inflammation, lymphocytes migrate into the inflamed paw, release their opioid peptides and travel to local lymph nodes (Cabot et al., 1997) where proopiomelanocortin mRNA is detectable by Northern blot (Cabot et al., 1997). We, thus, quantified truncated and full length proopiomelanocortin mRNA in leukocyte subpopulations from inflamed and noninflamed lymph nodes by RACE and Light Cycler PCR (chapter 2.3).

In contrast to β-endorphin und met-enkephalin, endomorphin-1 and endomorphin-2 highly selectively bind to μ-opioid receptors (Table 1). Endomorphin-1 is more widely distributed throughout the brain, whereas endomorphin-2 is more prevalent in the spinal cord (Martin-Schild et al., 1999). In the peripheral nervous system, some nerve fibers contain endomorphin-2 in both inflamed and noninflamed tissue (Mousa et al., 2002). Outside the nervous system, endomorphin-1 and -2 are expressed in leukocytes (macrophages/monocytes, granulocytes and lymphocytes) in the circulation and at the site of inflammation. In behavioral studies, endomorphin-induced analgesia was previously studied in acute and neuropathic pain but not in inflammation (Spampinato et al., 2003; Obara et al., 2004). We, thus, investigated the effect of injected and endogenously released endomorphins in our inflammatory model (chapter 2.3).

To achieve functional relevance, opioid peptides have to be released from leukocytes. *In vivo*, such release can be induced by exposure to stress (e.g. cold water swim) (Stein et al., 1990a) or by various cytokines and hormones such as tumor necrosis factor-α, corticotropin releasing factor (CRF) and IL-1 (Czlonkowski et al., 1993; Schäfer et al., 1996). Swim stress-induced analgesia is elicited by CRF and other agents (Schäfer et al., 1996; Binder et al., 2004). The functional relevance of leukocytes is supported by studies using total-body irradiation and cyclosporine A. Both treatments induce immunosuppression and abolish stress-induced endogenous opioid analgesia. While the role of leukocytes for this type of analgesia is well established, the contribution of distinct leukocyte subpopulations has thus far not been examined (chapter 2.4).

1.3. Adhesion and chemotaxis of leukocytes

Migration of immune cells into inflamed tissue is a multi-step process governed by adhesion molecules and chemokines (Butcher and Picker, 1996; von Andrian and Mackay, 2000). First, leukocytes roll along the endothelial cell wall. This process is mediated predominantly by selectins. Second, chemokines activate leukocytes (see next paragraph) leading to the up-regulation and increased avidity of integrins. Integrins mediate the firm adhesion of leukocytes to endothelial cells (e.g. intercellular adhesion molecule-1, ICAM-1 = CD54). Finally, leukocytes transmigrate through the endothelium mediated by e.g. platelet-endothelial adhesion molecule-1. Blockade of the adhesion cascade by anti-selectin treatment (Machelska et al., 1998) or by antibodies against intercellular adhesion molecule-1 or integrins (Machelska et al., 2002; Machelska et al., 2004) inhibits the migration of opioid-containing leukocytes and markedly reduces peripherally mediated opioid analgesia. In contrast, no effect is seen after blockade of platelet-endothelial adhesion molecule-1 (Machelska et al., 2004). An intact adhesion molecule cascade is, therefore, a prerequisite for recruitment of opioid-containing leukocytes and for peripherally mediated endogenous opioid analgesia.

Chemokines are chemotactic proteins that are produced at the site of inflammation. They are presented on the inflamed endothelium and interact with chemokine receptor-expressing leukocytes. In our studies we concentrated on the early phase of inflammation characterized by an infiltrate of granulocytes. Depending on the species, granulocytes express the receptors CXCR1, 2 and/or 4, among others. In humans the best characterized CXCR1/2 ligand is CXCL8 (formerly IL-8). In rats CXCR2 is expressed on granulocytes. Its ligands include CXCL2/3 (syn. macrophage inflammatory protein-2), CXCL1 (syn. keratinocyte-derived chemokine), CXCL5 (syn. lipopolysaccharide-induced chemokine) and cytokine-induced neutrophil chemoattractant-2 (CINC-2) (Shibata et al., 1998; Piccolo et al., 1999; Moore et al., 2000; Shibata et al., 2000; Chandrasekar et al., 2001). All four are produced under inflammatory conditions. In humans and rats CXCL12 (syn. stromal derived factor-1α) is the only known ligand for CXCR4. CXCL12 plays a role in the bone marrow retention and homing of granulocytes (Martin et al., 2003). Chemokines and adhesion molecules interact closely. Chemokine receptor binding (e.g. CXCR2) enhances adhesion molecule-mediated granulocyte rolling, sticking and ex-

travasation (Zhang et al., 2001). We investigated the role of chemokines in the recruitment of opioid-containing granulocytes and in peripherally mediated opioid analgesia (chapter 2.5).

1.4. Intracellular mechanisms of granule release in granulocytes

As a first line of defense, granulocytes are recruited in the early phase of inflammation. Granulocytes are known to contain and release met-enkephalin (Vindrola et al., 1990), but its subcellular localization is unknown. Granulocytes harbor four distinct types of cytoplasmic granules: azurophil (primary), specific (secondary) and gelatinase (tertiary) granules as well as secretory vesicles (Borregaard and Cowland, 1997; Gullberg et al., 1997). Each granule or vesicle has a well characterized content: primary granules contain myeloperoxidase (MPO), elastase, and a wide array of lytic enzymes and proteins with bactericidal activities, which are released into the environment of the cell to eliminate potential pathogens. Secondary granules contain lactoferrin and several metalloproteinases including collagenase. Tertiary granules hold gelatinase. Secondary and tertiary granules fuse with the plasma membrane and harbor proteins involved in adhesion and extravasation that are translocated to the cell surface upon granulocyte activation. Secretory vesicles are storage organelles for membrane receptors, secretable proteins including albumin and alkaline phosphatase needed in the earliest phases of granulocyte-mediated inflammatory responses (Borregaard, 1988; Borregaard et al., 1993; Borregaard and Cowland, 1997). A previous study demonstrated that β-endorphin is located in secretory granules in granulocytes and other leukocyte subpopulations (Mousa et al., 2004). We now examined the subcellular localization using dual color confocal microscopy of opioid peptides and marker proteins to determine the intracellular localization in granulocytes in further detail (chapter 2.6).

Opioid peptide release from granulocytes can be induced by various releasing agents: Formyl peptide formyl-methionine-leucine-phenylalanine (fMLP) can stimulate release of met-enkephalin *in vitro* (Vindrola et al., 1990). Another potent activator of granulocytes is CXCL8. It triggers chemotaxis, oxidative burst and release of granules (Borregaard and Cowland, 1997) but its effect on opioid peptide release has not been studied thus far. Chemokine receptors couple to G_i proteins. After activation and dissociation of the heterotrimeric G-proteins, the resulting Gβγ subunits activate phospholipase C. This generates inositol 1,4,5-trisphosphate (IP_3). IP_3 binds to IP_3 receptors (IP_3R) on the endoplasmatic reticulum leading to release of Ca^{2+} from intracellular stores (Schorr et al., 1999). In a parallel pathway, Gβγ subunits activate phosphoinositol-3-kinase (PI3K)-γ independent of Ca^{2+} mobilization (Knall et al., 1997; Ito et al., 2002). Third, CXCR1/2 ligands stimulate the mitogen activated protein kinase (MAPK) family such as p38 and p42/44. This pathway is independent of Ca^{2+} mobilization and of PI3K activation (Takami et al., 2002; Chakrabarti and Patel, 2005; Fuhler et al., 2005). We examined the role of these three signaling pathways on chemokine induced opioid peptide secretion and on peripherally mediated opioid analgesia (chapter 2.6 and 2.7).