## 2. RESULTS

### 2.1. Lack of pain following selective local granulocyte recruitment

Previous literature from other groups postulated that granulocytes contribute to pain in inflammation (Levine et al., 1984; Levine et al., 1985; Levine et al., 1986). Data from our group however had provided evidence that leukocytes induce analgesia through release of opioid peptides. This is abolished by immunosuppression (Stein et al., 1990b). To selectively examine the role of granulocytes in the generation of pain we locally injected granulocyte-specific chemokines and determined nociceptive thresholds (Rittner et al., 2006b). Intraplantar injection of CXCL1 or CXCL2/3 into the noninflamed paw evoked a dose and time dependent migration of granulocytes into the tissue ((Rittner et al., 2006b), Fig. 4 A). A comparable number of infiltrating granulocytes was seen during CFA inflammation ((Rittner et al., 2006b), Fig. 4, third column). Chemokine injection did not alter mechanical or thermal nociceptive thresholds while CFA injection significantly reduced them ((Rittner et al., 2006b), Fig. 4 C, D). Granulocyte depletion by prior injection of an anti-granulocyte serum significantly reduced the number of infiltrating granulocytes in CFA inflammation, but mechanical or thermal nociceptive thresholds remained not significantly changed ((Rittner et al., 2006b), Fig. 4, forth column) ((Rittner et al., 2006a); Fig. 1 B).

Pain triggers the expression of immediate early genes like c-fos in the laminae I and II in the dorsal horn of the spinal cord (Hunt et al., 1987). c-Fos protein and c-fos mRNA expression paralleled the pattern seen for nociceptive thresholds: c-fos mRNA copies and c-Fos immunoreactive cells were high in CFA induced inflammation regardless of granulocyte depletion ((Rittner et al., 2006b), Fig. 6). In contrast c-fos mRNA copies and c-Fos immunoreactive cells were significantly lower in untreated or rats injected with CXCL2/3 0.

To exclude the possibility that chemokine-induced recruitment of granulocytes resulted in insufficient granulocyte activation we analyzed expression of CD11b, CD18 and CD62L in the paw. No difference of adhesion molecule expression was seen between CFA and CXCL2/3 treated animals. Pain can be induced by a variety of mediators and CXCL1 was shown to activate IL-1β and prostaglandin E2 as part of a pronociceptive cascade (Cunha et al., 2005). We analyzed these mediators in our inflammatory model. Content of IL-1β was highest in CFA rats, but similar levels were obtained in CFA rats depleted of granulocytes and rats injected with CXCL2/3 ((Rittner et al., 2006b), Fig. 4 E). In contrast, production of prostaglandin E2 was significantly higher in CFA as well as CFA rats depleted of granulocytes in comparison to control rats and rats injected with CXCL2/3 ((Rittner et al., 2006b), Fig. 4 F). Taken together selective migration of activated granulocytes into non-inflamed tissue does not cause pain. This might be due to low production of prostaglandin E2 following chemokine-induced granulocyte recruitment in comparison to models of inflammation.

Papers cited in this chapter:

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„Selective local PMN recruitment by CXCL1- or CXCL2/3-injection does not cause inflammatory pain"

*Journal of Leukocyte Biology 2006; 79:1022-32*

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Comment on „Neutrophils: are they hyperalgesic or anti-hyperalgesic"

*Journal of Leukocyte Biology 2006; 80:729*

## 2.2. Opioid receptor expression in DRG

Analgesic efficacy of peripherally active opioids is augmented in inflammation. Inflammation induces increased anterograde axonal transport of opioid receptors, disruption of the perineural barrier and enhanced G-protein coupling of opioid receptors on sensory neurons (Hassan et al., 1993; Antonijevic et al., 1995; Zöllner et al., 2003). In inflammation μ-opioid receptor expression in the DRG is regulated at the transcriptional level (Puehler et al., 2004). In this study we examined κ-opioid receptor regulation during inflammation (Puehler et al., 2006). κ-opioid receptor transcripts were significantly increased in ipsilateral DRG at 12 h after CFA inflammation with no changes on the contralateral side. Previous studies demonstrated that IL-1β enhances centrifugal axonal transport of opioid receptors (Jeanjean et al., 1995). We demonstrate here that CFA-induced upregulation of κ-opioid receptor mRNA can also be induced by intraplantar injection of IL-1β into the noninflamed paw. Augmented mRNA expression correlated with an increased number of κ-opioid receptor positive cells in DRG neurons compared to contralateral sides as determined by immunohistochemistry ((Puehler et al., 2006), Tab. 2).

Inflammation induced an increase in IL-1β production in paw tissue as determined by ELISA ((Puehler et al., 2006), Fig. 3). To evaluate whether IL-1β was involved in the regulation of κ-opioid receptors rats were treated systemically with IL-1 receptor antagonist. This treatment abolished the inflammation-induced rise of κ-opioid receptor transcripts in ipsilateral DRG ((Puehler et al., 2006), Fig. 4) as well as the number of κ-opioid receptor positive cells in DRG ((Puehler et al., 2006), Tab. 2). No change was seen in the composition of the inflammatory infiltrate in the paw between control animals and rats treated with IL-1 receptor antagonist ((Puehler et al., 2006), Tab. 1). In summary, IL-1β seems to specifically regulate the expression of κ-opioid receptors in DRG during CFA inflammation.

Paper cited in this chapter:

Puehler W*, **Rittner HL***, Mousa SA, Brack A, Krause H, Stein C, Schäfer M

"Interleukin-1 beta contributes to the upregulation of kappa opioid receptor mRNA in DRG in response to peripheral inflammation."

*Neuroscience 2006; 141:989-98* **contributed equally*

## 2.3. Regulation and analgesic function of the endogenous opioid peptides β-endorphin, endomorphin-1 and -2 in leukocytes

Opioid-containing lymphocytes migrate from the inflamed paw to the local lymph node. These lymphocytes contain β-endorphin, met-enkephalin and dynorphin (Cabot et al., 1997; Cabot et al., 2001). We attempted to elucidate the regulation of β-endorphin and its precursor proopiomelanocortin in immune cells from control and draining lymph nodes of rats with CFA-induced paw inflammation. Two color immunohistochemistry demonstrated that β-endorphin and proopiomelanocortin are coexpressed in lymphocytes ((Sitte et al., 2007), Fig. 1). 20% of cells were positive for β-endorphin and proopiomelanocortin and no changes were seen between control lymph nodes and inflamed lymph nodes after 24 h CFA. However β-endorphin content per cell doubled 12-48 h after CFA injection in inflamed lymph nodes compared to lymph nodes from control rats ((Sitte et al., 2007), Fig. 2). Proopiomelanocortin mRNA transcripts (both exon 1-3 and exon 2-3) encoding the signal peptide as well as the β-endorphin sequence were detectable by nested PCR albeit in low copy number ((Sitte et al., 2007), Fig. 4). Separating lymph node cells into T-cells and non-T-cells such as B cells, dendritic cells and macrophages, showed that proopiomelanocortin mRNA (exon 2-3) was expressed in both subsets ((Sitte et al., 2007), Fig. 6). Non-T cells, however, had higher levels of β-endorphin peptide compared to T cells in control and inflamed lymph nodes. In summary, lymphocytes contain β-endorphin and its precursor proopiomelanocortin and express full-length and exon 2-3 proopiomelanocortin mRNA at low copy numbers. Paw inflammation induces an up-regulation of proopiomelanocortin mRNA expression and β-endorphin content in lymphocytes from draining lymph nodes.

Endomorphins display a high degree of specificity and affinity for μ-opioid receptors. In this study we analyzed whether endomorphin-1 and -2 alleviate inflammatory pain 4-6 d after CFA injection (Labuz et al., 2006). Intraplantar injection of endomorphin-1 and -2 induced analgesia lasting for 10 min. Endomorphins displayed a comparable efficacy to β-endorphin ((Labuz et al., 2006), Fig. 1). Endomorphin-1 and -2-induced analgesia was mediated by μ-opioid receptors but not κ-opioid receptors ((Labuz et al., 2006), Fig. 2). δ-opioid receptors were involved in endomorphin-2, but not in endomorphin-1-induced analgesia. In binding experiments of DRG membranes endomorphin-1 and endomorphin-2 both bound to μ-opioid receptor while δ-opioid receptor binding could not be quantified.

We further characterized the role of endomorphins in endogenous peripherally mediated analgesia. In inflamed tissue, the number of β-endorphin positive leukocytes was significantly higher than the number of endomorphin-1 or -2 positive leukocytes ((Labuz et al., 2006), Fig. 3). Both mononuclear cells and granulocytes contained endomorphins while some nerve fibers expressed endormophin-2. Stress- and CRF-induced analgesia could be partially blocked by local injection of anti-endomorphin antibodies. However inhibition was less effective than by anti-β-endorphin antibodies ((Labuz et al., 2006), Fig. 4 and 5). Treatment with multiple injections of anti-granulocyte serum significantly reduced all leukocyte subpopulations after 4 d of CFA inflammation by 32-74% in the inflamed paw ((Labuz et

al., 2006), Tab. 2). The reduction of infiltrating leukocytes lead to impaired CRF- and swim stress induced analgesia in the inflamed paw. Taken together, endomorphins seem to have similar properties in peripherally mediated analgesia like endorphin, albeit lower potency.

Papers cited in this chapter:

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"Lymphocytes upregulate signal sequence-encoding proopiomelanocortin mRNA and β-endorphin during painful inflammation in vivo"
*Journal of Neuroimmunology 2006; 183:133-45*

Labuz D, Berger S, Mousa SA, Zöllner C, **Rittner HL,** Shaqura M, Segovia-Silvestre T, Przewlocka B, Stein C, Machelska H
"Peripheral antinociceptive effects of exogenous and immune cell-derived endomorphins in prolonged inflammatory pain."
*Journal of Neuroscience 2006; 26:4350-8*

## 2.4. Temporal and functional analysis of leukocyte subpopulations in peripherally mediated opioid analgesia

To identify the leukocyte subpopulations responsible for endogenous opioid analgesia we established a method to quantify opioid containing leukocytes by flow cytometry in inflamed tissue (Rittner et al., 2001). Subcutaneous paw tissue was enzymatically digested to obtain a single cell suspension. Cells were stained for CD45 expression to separate leukocytes from debris and noninflammatory cells (e.g. fibroblasts, muscle cells, endothelial cells). Opioid containing leukocytes were specifically labeled using a monoclonal antibody (3E7) recognizing the pan-opioid motif detectable in β-endorphin, met-enkephalin and dynorphin. Using this technique we found that 20-30% of $CD45^+$ cells also contain opioid peptides. Furthermore, the absolute number of opioid containing leukocytes significantly increased during the course of inflammation (0-96 h after CFA injection) ((Rittner et al., 2001), Fig. 3). This was paralleled by an increase in β-endorphin content in $CD45^+$ cells measured by radioimmunoassay ((Rittner et al., 2001), Fig. 4).

To analyze the functional relevance of increased recruitment of opioid-containing leukocytes and of opioid content per leukocyte we tested mechanical nociceptive thresholds before and after cold water swim stress ((Rittner et al., 2001), Fig. 6). While no change was seen in baseline hyperalgesia, we detected a significant increase in stress-induced analgesia during the course of the inflammation.

We further analyzed the composition of the inflammatory infiltrate and the leukocyte lineage of opioid peptide containing cells ((Rittner et al., 2001), Fig. 5). In early inflammation (2-6 h of CFA) 65-70% of the infiltrating cells were $HIS48^+$ granulocytes. 24-38% of the infiltrating cells were $CD68^+$ macrophages. In late inflammation (96 h post CFA) 35% of the infiltrating cells were $HIS48^+$ granulocytes and 60% were $CD68^+$ macrophages. Only 2-4% of cells were $CD3^+$ lymphocytes and no B cells were found. A similar pattern of opioid-containing leukocyte subpopulations was seen during inflammation ((Rittner et al., 2001), Fig. 5). In early inflammation 66% of $3E7^+CD45^+$ cells were $HIS48^+$ granulocytes whereas at later stages macrophages were the major source for opioid peptides (73% of $3E7^+CD45^+$ cells were $CD68^+$).

To evaluate the functional significance of these leukocyte subpopulations we selectively depleted animals and tested for peripherally mediated opioid analgesia. Granulocytes were depleted using systemic application of anti-rat granulocyte serum, which resulted in an over 90% reduction of circulating granulocytes as well as granulocytes infiltrating the inflamed paw at 2 h after induction of CFA inflammation without significant changes in the number of infiltrating macrophages (Brack et al., 2004a). This treatment almost completely abolished peripherally mediated opioid analgesia elicited by local injection of CRF ((Brack et al., 2004a), Fig. 1).

The role of macrophages in peripherally mediated opioid analgesia was examined in late inflammation (48–96 h post CFA). Monocytes/macrophages were depleted using clodronate containing liposomes (Brack et al., 2004b). These liposomes are taken up by phagocytes, liberate the toxic drug clodronate

after liposome uptake and induce apoptotic cell death. Local injection of clodronate liposomes into the inflamed paw reduced the number of macrophages and opioid containing leukocytes by 30-35% ((Brack et al., 2004b), Fig. 3). No depletion of granulocytes or lymphocytes was observed. Mechanical nociceptive thresholds before and after cold water swim stress were obtained demonstrating no influence on baseline pain but a significant reduction of stress induced analgesia ((Brack et al., 2004b), Fig. 4). In summary, granulocytes are the major opioid containing leukocyte subpopulation in early CFA inflammation while macrophages are predominant at later stages. Both subpopulations are functionally relevant *in vivo* as shown by depletion studies.

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"Opioid peptide expressing leucocytes – identification, recruitment and simultaneously increasing inhibition of inflammatory pain"

*Anesthesiology 2001; 95 (2): 500-8*

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"Tissue monocytes/macrophages in inflammation: hyperalgesia versus opioid-mediated peripheral antinociception"

*Anesthesiology 2004; 101:204-11*

## 2.5. CXCR2 ligands in the recruitment of opioid containing granulocytes and in peripherally mediated analgesia

Recruitment of granulocytes into inflamed tissue is governed by specific chemokines. In rats, CXCL2/3, CXCL1 and CINC-2 are known ligands of the CXCR2 receptor. After intraplantar CFA injection CXCL2/3 and CXCL1 production was significantly upregulated starting after 2 h reaching a peak at 12 h of inflammation ((Brack et al., 2004a), Fig. 3). CINC-2 reached significant increases at 12 h of inflammation. Leukocytes were the major source of chemokine production as seen by immunohistochemistry. Production of CXCL2/3, CXCL1 and CINC-2 returned to baseline levels at 96 h of inflammation. The corresponding receptor CXCR2 was expressed on opioid containing granulocytes but not on macrophages or T cells ((Brack et al., 2004a), Fig. 2). To test the functional relevance of these chemokines *in vivo* rats were locally pretreated with antibodies against CXCL2/3 and CXCL1. Blocking of only one chemokine did not impair recruitment of opioid containing granulocytes and had no influence on CRF-induced analgesia. However, following combined blockade of both chemokines by local antibody injection migration of granulocytes and of opioid containing leukocytes was impaired. CRF-induced analgesia was also significantly diminished ((Brack et al., 2004a), Fig. 4). In summary, CXCR2 ligands regulate the migration of opioid containing leukocytes and thereby peripherally mediated opioid analgesia. The redundancy of CXCR2 ligands makes it necessary to block at least two chemokines to obtain an effect on granulocyte migration.

Paper cited in this chapter:

Brack A, **Rittner HL,** Machelska H, Leder K, Mousa S, Schäfer M, Stein C

"Control of inflammatory pain by chemokine-mediated recruitment of opioid-containing polymorphonuclear cells"

*Pain 2004; 112 (3): 229-238*

## 2.6. Subcellular localization and MAPK dependent release of opioid peptides from primary granules of granulocytes

We have previously shown that opioid peptides are stored in vesicular structures in leukocytes (Mousa et al., 2004) but the exact location in granulocytes was unknown. Granulocytes harbor 4 different types of granules: azurophil (primary), specific (secondary) and gelatinase (tertiary) granules as well as secretory vesicles (Borregaard and Cowland, 1997; Gullberg et al., 1997). For each of these we evaluated marker proteins: myeloperoxidase and CD63 for primary granules, lactoferrin for secondary granules, gelatinase for tertiary granules and albumin for secretory vesicles (Rittner et al., 2007). Using confocal microscopy we demonstrated that β-endorphin and met-enkephalin colocalized with myeloperoxidase ((Rittner et al., 2007), Fig. 4 A-C) and CD63 ((Rittner et al., 2007), Fig. 4 D-F) but not with lactoferrin, gelatinase or albumin indicating that opioid peptides are stored in primary granules of granulocytes.

Chemokines like human CXCL8, a ligand of CXCR1/2, induce the release of granules from granulocytes. Granulocyte activation by chemokines translocates granules to the plasma membrane to facilitate the release. This centrifugal translocation was also observed for opioid containing primary granules in response to CXCL8 stimulation ((Rittner et al., 2007), Fig.4 A-F). Preincubation of granulocytes with the p38 MAPK inhibitor completely reversed this translocation of granules indicating a role of p38 MAPK in this process ((Rittner et al., 2007), Fig. 4 G-I).

To study the above findings *in vivo* we injected CXCL2/3 locally into the inflamed paw and measured mechanical nociceptive thresholds. CXCL2/3 induced a dose dependent elevation of mechanical nociceptive thresholds. This analgesic effect was dependent on β-endorphin and met-enkephalin, because the effect was partially blocked by coinjection of specific antibodies ((Rittner et al., 2007), Fig. 6 B). To analyze the role of MAPK in the release of opioid peptides *in vivo,* we locally injected a p38 MAPK inhibitor in rats with CFA induced inflammation. CXCL2/3-induced analgesia was significantly reduced ((Rittner et al., 2007), Fig. 7 A). A small but significant increase in nociceptive thresholds was seen after injection of the p38 MAPK inhibitor alone.

Paper cited in this chapter:

**Rittner HL**, Labuz D, Richter JF, Brack A, Schäfer M, Stein C, Mousa SA

"Inhibition of inflammatory pain by CXCR1/2 ligands requires p38 MAPK activation and release of opioid peptide-containing primary granules from polymorphonuclear cells"

*Brain Behavior Immunity 2007 in press*

## 2.7. Role of intracellular calcium and PI3K activation in CXCR1/2 ligand-induced release of opioid peptides from granulocytes *in vitro* and *in vivo*

CXCR1/2 ligands like human CXCL8 are not only chemotactic but they induce oxidative burst and release of granules from granulocytes. We, thus, examined the role of CXCR1/2 ligands in the release of opioid peptides from granulocytes (Rittner et al., 2006c). Human CXCL8 but not CXCL12, the ligand of CXCR4, dose dependently stimulated the release of the opioid peptides β-endorphin and met-enkephalin ((Rittner et al., 2006c), Fig. 2 A).

In rat granulocytes, the CXCR2 ligand CXCL2/3 elicited dose dependent release of met-enkephalin ((Rittner et al., 2006c), Fig. 5 A, left panel) and of β-endorphin ((Rittner et al., 2006c), Fig. 5 A, right panel) while CXCL12 had no effect. Interestingly, both receptors, CXCR2 and CXCR4, were expressed on rat granulocytes ((Rittner et al., 2006c), Fig. 4 A+B). Both chemokines had chemotactic effects ((Rittner et al., 2006c), Fig. 4 C). However, only CXCL2/3 stimulation resulted in intracellular calcium mobilization ((Rittner et al., 2006c), Fig. 4 D).

To study the signaling requirements in opioid peptide release after CXCR1/2 stimulation, the role of calcium mobilization was examined. Opioid peptide release could be stimulated by addition of calcium ionophore which augments intracellular calcium through influx from outside of the cell. Incubating the cells with 1,2-*bis*(*o*-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester (BAPTA/AM) or thapsigargin, which chelates intracellular calcium or blocks the release of calcium from intracellular stores, respectively, abolished CXCL8 induced opioid peptide release ((Rittner et al., 2006c), Fig. 3 C+D and 5 C). In contrast, CXCL2/3-induced opioid peptide release was unchanged if extracellular calcium was chelated using EDTA ((Rittner et al., 2006c), Fig. 3 B and 5 B). Blockade of $IP_3$-receptors using 2-aminoethoxyphenylborate also significantly inhibited the release of opioid peptides ((Rittner et al., 2006c), Fig. 3 E). Taken together, mobilization of calcium from intracellular stores through the cascade Gβγ subunits – phospholipase C – $IP_3$ binding to its receptor on the endoplasmatic reticulum seems to be necessary for opioid peptide release after CXCR1/2 stimulation. Release can also involve calcium independent pathways such as activation of PI3K. Inhibitors of the PI3K like wortmannin and LY294002 partially reduced opioid peptide release from rat and human granulocytes ((Rittner et al., 2006c), Fig. 3 F and 5 D+E). Another calcium independent signaling pathway involves MAPK activation. Preincubation with an inhibitor of p38 MAPK but not with an inhibitor of p42/44 MAPK also significantly decreased opioid peptide release ((Rittner et al., 2007), Fig. 3 B).

To determine the *in vivo* relevance of these findings the effect of locally injected CXCL2/3 on nociceptive thresholds was evaluated. CXCL2/3 dose dependently increased mechanical nociceptive thresholds ((Rittner et al., 2006c), Fig. 1 A). This analgesic effect was dependent on μ- and δ-opioid receptors since it was fully reversed by μ- and δ-opioid receptor specific antagonists ((Rittner et al., 2006c), Fig. 1 C-E). Furthermore, CXCL2/3 induced analgesia was dependent on the presence of

granulocytes because analgesia was abolished by prior granulocyte depletion ((Rittner et al., 2006c), Fig. 6 B). Local adoptive transfer of allogenic granulocytes dose dependently reconstituted CXCL2/3 induced analgesia in granulocyte depleted rats ((Rittner et al., 2006c), Fig. 6 C).

This model was further used to study the intracellular calcium requirement for opioid peptide release *in vivo*. Before adoptive transfer the granulocytes were treated *ex vivo* with BAPTA/AM to chelate intracellular calcium. Granulocytes pretreated with the chelator could not reconstitute CXCL2/3 induced analgesia in granulocyte depleted animals ((Rittner et al., 2006c), Fig. 6 D). In summary, CXCL2/3 induced analgesia is mediated through release of opioid peptides from granulocytes. This release is regulated by mobilization of intracellular calcium as well as activation PI3K and p38 MAPK.

Paper cited in this chapter:

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"Pain control by CXCR2 ligands through Ca2+-regulated release of opioid peptides from polymorphonuclear cells"

*FASEB Journal 2006; 20:2627-9*