

## Pain and the immune system

H. L. Rittner\*, A. Brack and C. Stein

*Klinik für Anästhesiologie und operative Intensivmedizin, Charité – Universitätsmedizin Berlin, Campus Benjamin Franklin, Hindenburgdamm 30 D-12200, Berlin, Germany*

*\*Corresponding author. E-mail: heike.rittner@charite.de*

In inflammation, leucocytes containing opioid peptides migrate into the tissue. Opioid peptides can be released and bind to opioid receptors on peripheral nerve terminals, which counteracts inflammatory pain. Migration of opioid peptide-containing leucocytes is controlled by chemokines and adhesion molecules. Neurokinins, such as, substance P also contribute to the recruitment of these cells. Opioid peptide release from granulocytes can be stimulated by chemokines, such as, CXCR2 ligands. The release is dependent on intracellular calcium and activation of phosphoinositol-3 kinase and p38 mitogen activated kinase. Endogenous opioid peptides produced by leucocytes not only confer analgesia but recent evidence supports the concept that they also prevent the development of tolerance at peripheral opioid receptors. This review presents the discoveries that led to the concept of analgesia produced by immune-derived opioids.

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During inflammation of peripheral tissues, numerous mediators are produced by endothelial cells, resident cells, and leucocytes that are recruited to the site of injury. Many of these mediators (e.g. protons, cytokines, and 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](http://www.iasp-pain.org/terms-p.html)).<sup>22</sup> Nociceptors belong to the group of unmyelinated A $\delta$  and C fibres originating from the trigeminal and dorsal root ganglion. A $\delta$  and C fibres transduce noxious stimuli into action potentials and propagate these 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 eventually elicited.

Inflammatory pain is characterized by an increased response to mechanical or heat stimuli which are normally only mildly painful (mechanical or thermal hyperalgesia).<sup>22</sup> 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 leucocytes into the inflamed tissue, by production of cytokines, chemokines, growth factors (e.g. nerve growth

factor), and tissue acidification. Intraplantar injection of complete Freund’s adjuvant (CFA) can be used to study inflammatory pain in rodents.

Leucocytes are the source not only of hyperalgesic but also of analgesic mediators. Among the best-characterized and clinically relevant systems are the endogenous opioid peptides and receptors.

### Opioid peptides in leucocytes

In peripheral inflamed tissue, opioid peptides such as  $\beta$ -endorphin, met-enkephalin, dynorphin, and endomorphins are produced by leucocytes and released upon certain types of stimulation.<sup>17,39</sup> Opioid peptides can bind to opioid receptors on sensory neurons. These receptors are synthesized in dorsal root ganglia and are transported intra-axonally to peripheral nerve endings. Three types of opioid receptors such as  $\mu$ -(MOP),  $\delta$ -(DOP), and  $\kappa$ -(KOP) are expressed in sensory neurons.<sup>24,26,27</sup> Agonist binding elicits potent exogenous or endogenous analgesia in inflamed tissue.<sup>39</sup>

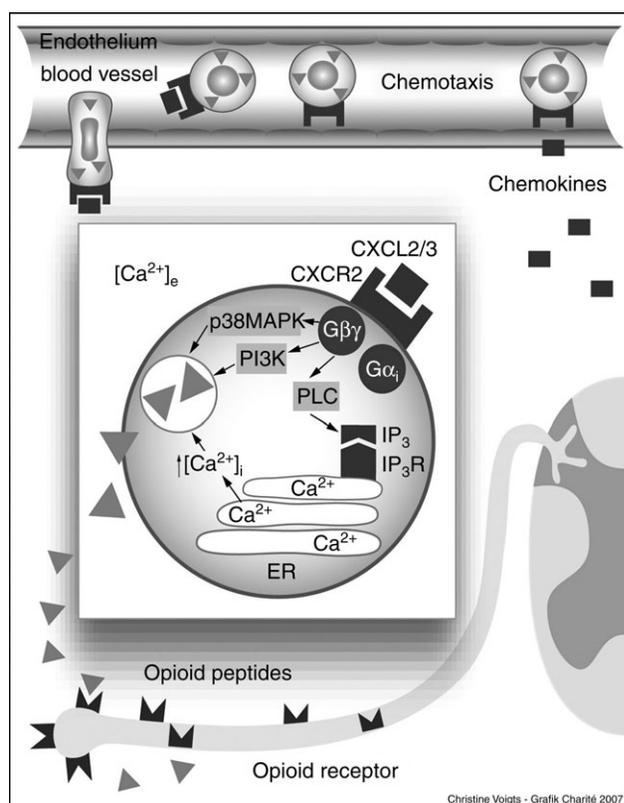
### Role of leucocytes containing opioid peptides in inflammation

Opioid peptides are found in many leucocyte subpopulations including lymphocytes, monocytes, and granulocytes in the peripheral blood, in inflamed and non-inflamed lymph nodes, and also at the site of experimentally induced or

clinical inflammation.<sup>6 23 36</sup> In early inflammation, granulocytes are the major source of opioid peptide production.<sup>30</sup> Later in the inflammatory course, monocytes and macrophages are the predominant supply of opioid peptides. Selective depletion of granulocytes and monocytes/macrophages significantly impairs opioid-mediated antinociception implicating the functional relevance of these leucocyte subpopulations.<sup>3 4</sup> Reconstitution of granulocytes after depletion using local injection of allogenic cells re-establishes peripherally mediated opioid antinociception.<sup>31</sup>

### *Chemokines regulating migration of opioid peptide-containing leucocytes*

Chemokines are chemotactic mediators controlling cell trafficking under physiological and pathological conditions. Chemokines are not only important under various inflammatory conditions but also play a role in pain and analgesia. Although many studies examined the hyperalgesic action of chemokines, recent evidence also points towards their antinociceptive effects.



**Fig 1** Intracellular mechanisms of opioid peptide release from granulocytes. Opioid peptide-containing granulocytes migrate into inflamed tissue. Opioid peptides are released and bind to opioid receptors on peripheral sensory neurons leading to analgesia. CXCL2/3 produces antinociception by triggering opioid peptide release. CXCL2/3-induced opioid release requires elevation of intracellular Ca<sup>2+</sup> via flux from the endoplasmic reticulum (ER). In addition, activation of phosphoinositol-3-kinase (PI3K) and p38 mitogen-activated kinase (MAPK) are involved. PLC, phospholipase C; IP<sub>3</sub>, inositol 1,4,5-triphosphate.

Chemokines released from leucocytes and endothelial cells upregulate and increase the avidity of adhesion molecules and thereby migration into inflamed tissue.

In early inflammation, CXCL1 and CXCL2/3, binding to their receptor CXCR2 on granulocytes, are expressed in CFA inflammation.<sup>4 5</sup>

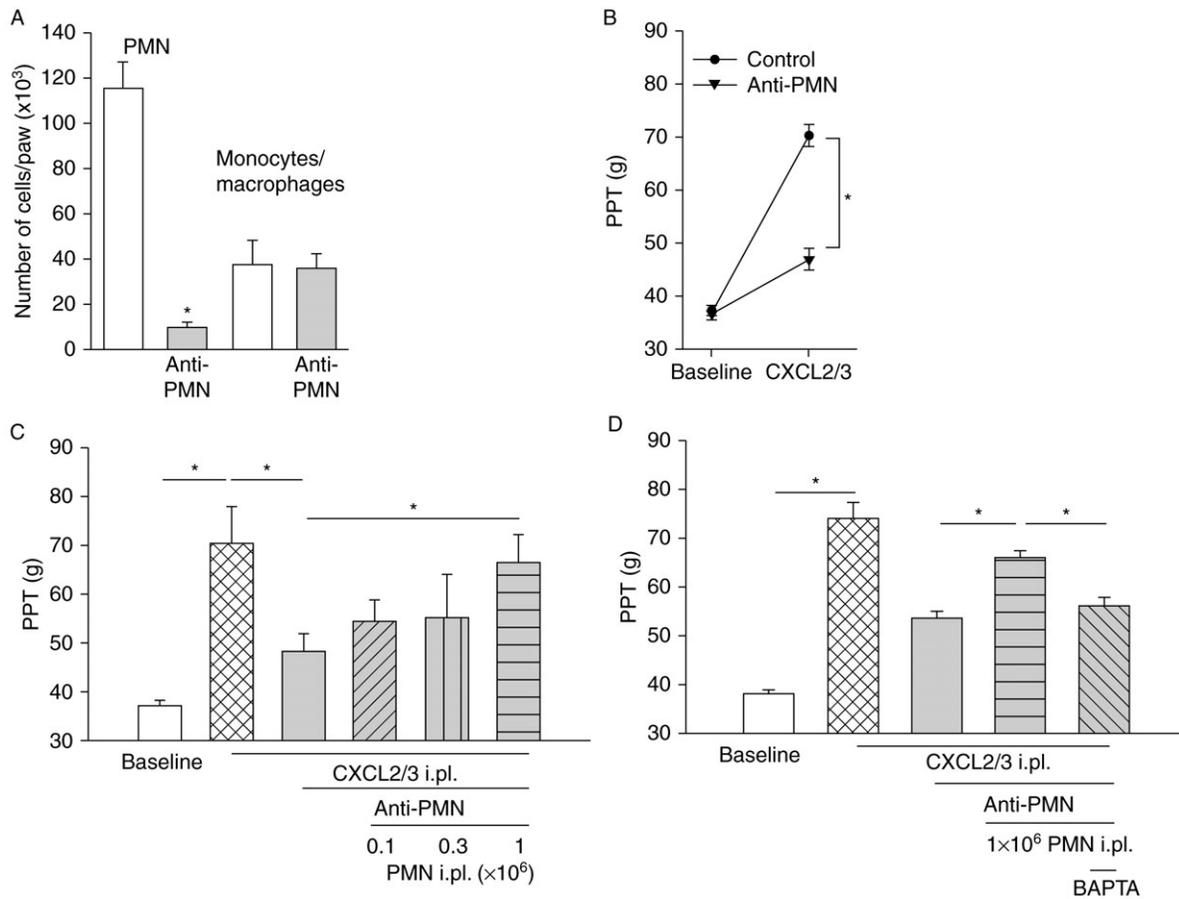
CXCR2<sup>+</sup> granulocytes also contain opioid peptides. Pre-treatment of rats with antibodies against CXCL1 or CXCL2/3 substantially decreases the number of opioid-containing granulocytes but not of monocytes/macrophages accumulating in the inflamed tissue<sup>4</sup> and in consequence abolishes antinociception mediated by endogenous opioid peptides. An intact chemokine cascade is, therefore, a prerequisite for peripherally mediated endogenous opioid antinociception and inhibition of only one of the steps is sufficient to impair this mechanism.

### *Neuropeptides regulating the migration of opioid-containing leucocytes*

Leucocyte recruitment is not only mediated by chemokines but also by other mediators such as complement or neuropeptides which can act as chemoattractants. Substance P, binding to NK<sub>1</sub> receptors,<sup>7</sup> is one of these neuropeptides. It was originally described as a mediator in pain transmission in the central nervous system and in neurogenic inflammation in peripheral tissue.<sup>14 25</sup> NK<sub>1</sub> receptor antagonists were initially designed for treatment of pain but were unsuccessful in the clinic.<sup>13</sup> Enhancement of leucocyte migration by substance P occurs by three distinct mechanisms: (i) direct chemotactic effects on monocytes and granulocytes,<sup>8 35</sup> (ii) increased expression of adhesion molecules,<sup>28</sup> and (iii) augmentation of local chemokine production.<sup>29</sup> In CFA inflammation, we demonstrated that NK<sub>1</sub> receptor antagonists reduced the migration of opioid-containing leucocytes without changing the expression of the adhesion molecule ICAM-1 on endothelial cells or the production of local chemokines/cytokines.<sup>33</sup> This treatment impaired stress-induced peripheral opioid-mediated antinociception. Therefore, NK<sub>1</sub> receptor antagonists seem to act peripherally by directly inhibiting the recruitment of opioid-containing leucocytes to the site of inflammation.

### *Secretion of opioid peptides from leucocyte subpopulations in vitro*

Granulocytes harbour four different types of granules: azurophil (primary), specific (secondary) granules, gelatinase (tertiary) granules, and secretory vesicles.<sup>2</sup> Marker proteins for each of these include myeloperoxidase and CD63 for primary granules, lactoferrin for secondary granules, gelatinase for tertiary granules, and albumin for secretory vesicles.<sup>32</sup> Using confocal microscopy, we demonstrated that β-endorphin and met-enkephalin co-localized with myeloperoxidase and CD63 but not



**Fig 2** Effects of granulocyte depletion and reconstitution with adoptively transferred granulocytes on CXCL2/3-induced antinociception. Rats were pretreated with i.v. anti-granulocyte (polymorphonuclear cells) serum (grey bars); control animals received non-immune rabbit serum (white bars). Two hours after complete Freund's adjuvant, the number of leucocytes in the paw (A) was quantified by flow cytometry. Paw pressure threshold (PPT) (B) was measured before (baseline) and after intraplantar injection of CXCL2/3. (C) Different numbers of allogenic glycogen-recruited peritoneal granulocytes from normal animals were injected into the inflamed paws of granulocyte-depleted rats. PPT was obtained 15 min later and again after intraplantar CXCL2/3 (white bar: before CXCL2/3 without granulocyte depletion; cross-hatched bar: effect of intraplantar CXCL2/3 without granulocyte depletion; grey bars: granulocyte depletion; striped bars: granulocyte reconstitution). (D) Effect of *ex vivo* BAPTA/AM (an intracellular calcium chelator) or solvent pre-treatment before allogenic granulocyte transfer. Rats were granulocyte-depleted and reconstituted as described in (C) using  $1 \times 10^6$  granulocytes and CXCL2/3-induced PPT elevation was measured thereafter. \* $P < 0.05$ . Data are means (SEM). Reproduced with permission from Rittner and colleagues.<sup>32</sup>

with lactoferrin, gelatinase, or albumin, indicating that opioid peptides are stored in primary granules and are released together with bactericidal enzymes such as myeloperoxidase.

Opioid peptide release from granulocytes can be stimulated by various agents including chemokines binding to CXCR1/2 receptors.<sup>31</sup> Chemokine receptors couple to G<sub>i</sub> proteins. After activation and dissociation of the heterotrimeric G-proteins, the resulting Gβγ subunits activate several signalling pathways including phosphoinositol-3-kinase-γ, p38 mitogen-activated kinase and mobilization of calcium from the endoplasmic reticulum (ER).<sup>15 34</sup>

One of the first steps in the secretion of opioid peptides is the translocation of granules to the plasma membrane. Stimulation with CXCR1/2 ligands leads to such a translocation.<sup>32</sup> Chemokine-induced opioid peptide release is

dependent on the liberation of calcium from intracellular stores (ER) but is independent of extracellular calcium. Phosphoinositol-3-kinase and p38 mitogen-activated kinase are also involved in this release<sup>31 32</sup> because block of p38 mitogen-activated kinase inhibits the translocation of primary granules to the plasma membrane and inhibition of phosphoinositol-3-kinase attenuates opioid secretion. In summary, intracellular calcium, phosphoinositol-3-kinase, and p38 mitogen-activated kinase control the CXCR2-mediated opioid peptide release from granulocytes (Fig. 1).

#### *Analgesic effects in vivo*

Release of opioid peptides from granulocytes and subsequent antinociceptive effects *in vivo* can be achieved using local injection of CXCR2 ligands such as

CXCL2/3.<sup>31</sup> Depletion of granulocytes abolishes this peripherally mediated opioid antinociception (Fig. 2A and B). The latter effect can be reconstituted by adoptive transfer of untreated but not by BAPTA (an intracellular calcium chelator)-pretreated granulocytes from donor rats (Fig. 2C). Using this model, we demonstrated *in vivo* that CXCL2/3-induced antinociception is mediated by opioid peptide secretion that is dependent on calcium release from the ER (Fig. 2D).

### *Opioid tolerance in vivo*

Normal animals rapidly develop tolerance to opioid analgesic effects after chronic morphine application. In animals with CFA-induced paw inflammation, however, no tolerance to the acute intraplantar injection of fentanyl is seen after chronic morphine application.<sup>41</sup> In normal rats, opioid receptors are localized predominantly on the membrane of sensory neurons during chronic morphine treatment. However, in animals with ongoing CFA inflammation and continuous morphine treatment, receptors are more rapidly internalized and recycled to the neuronal membrane. In addition, receptor signalling is enhanced, indicating that the increase in receptor recycling preserves the function of opioid receptors and counteracts the development of tolerance.<sup>41</sup> One of the reasons for this difference might be the availability of endogenous immune cell-derived opioid peptides.<sup>12</sup> To study this hypothesis, rats were either treated with local antibodies against opioid peptides or the availability of endogenous opioid peptides was reduced by prior immune ablation using cyclophosphamide. Both approaches restored opioid tolerance in rats with CFA inflammation.<sup>41</sup> Taken together, the continuous availability of endogenous opioid peptides from leucocytes in inflammation apparently preserves signalling of  $\mu$ -opioid receptors and consequently counteracts the development of opioid tolerance in inflammatory pain.

## Clinical implications and perspectives

Peripherally mediated endogenous opioid analgesia has been amply demonstrated in clinical settings: opioid receptors are expressed on peripheral terminals of sensory nerves in human synovia.<sup>24 38</sup> Upon activation by agonists (e.g. morphine) they mediate analgesia in patients with chronic rheumatoid and osteoarthritis, bone pain, and after dental, laparoscopic, urinary bladder, and knee surgery.<sup>18–20 39</sup> Opioid peptides are found in human synovial lining cells, mast cells, lymphocytes, and macrophages. The prevailing peptides are  $\beta$ -endorphin and met-enkephalin, while only minor amounts of dynorphin are detectable.<sup>24</sup> Stimulation of opioid peptide release using intraarticular injection of corticotropin releasing factor induced a transient naloxone-sensitive analgesic effect.<sup>21</sup> Blocking intraarticular opioid receptors by the local administration of the antagonist naloxone resulted in

significantly increased postoperative pain in patients undergoing knee surgery.<sup>37</sup> These findings suggest that in a stressful (e.g. postoperative) situation, opioids are tonically released within inflamed tissue and activate peripheral opioid receptors to attenuate clinical pain.

Treatment of patients with opioids sometimes leads to the development of opioid tolerance in clinical settings,<sup>10</sup> however there are few rigorous clinical studies. Inflammatory diseases such as chronic arthritis, inflammatory neuropathy, or cancer are accompanied by an infiltration of opioid-containing leucocytes. Taking our experimental findings into account, endogenous secretion of opioid peptides might counteract the development of opioid tolerance. Therefore, peripherally acting opioids could be used for prolonged treatment of inflammatory pain that might not necessarily be associated with the development of opioid tolerance. Peripherally acting opioids lack the central side-effects of opioids including sedation, dizziness, and nausea and vomiting.

Chemokine receptor antagonists are currently under investigation: for example, CCR1 is responsible for leucocyte recruitment into inflamed tissue and is expressed in the synovia of patients with rheumatoid arthritis and on monocytes in active multiple sclerosis.<sup>1 9 40</sup> The important role of chemokines in the trafficking of opioid-containing cells to injured tissues and in the release of opioid peptides in inflamed tissue indicates that anti-chemokine strategies for the treatment of inflammatory diseases may in fact carry a significant risk to exacerbate pain.

In addition, mitogen-activated kinase inhibitors are explored for the treatment of rheumatoid arthritis, allergy, or cancer.<sup>11</sup> Findings in neuropathic and inflammatory pain in animals have led to the concept that these inhibitors might also be useful for the treatment of pain in patients.<sup>16</sup> In our model of inflammatory pain, we observed an impaired release of opioid peptides with p38 mitogen-activated kinase inhibition. Therefore, interference with this system in patients should be carefully evaluated regarding the effects on pain.

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