4. Discussion

The major findings of these studies are that the central versus peripheral components of endogenous antinociception substantially change with the development of inflammation, and that different adhesion molecules contribute to the peripheral component of this intrinsic pain inhibition. In particular these data demonstrate that **(1)** the FCA-induced unilateral hindpaw inflammation develops progressively within 6 h to 4 days. **(2)** the magnitude of intrinsic opioid antinociception does not substantially change within 6 h to 4 days. **(3)** At 6 h all three opioid receptors (μ, δ, and κ) and all three opioid peptides (β-END, Met-ENK, and DYN) play a role while at 4 days β-END acting at μ- and δ-receptors seems to be the main peptide responsible for peripheral intrinsic antinociception [70]. **(4)** CRF appears to be a major trigger of intrinsic opioid antinociception both at 4 days [64, 72] and at 6 h. **(5)** Whereas at 6 h both peripheral and central opioid receptors are involved, at 4 days endogenous antinociception is mediated exclusively by peripheral opioid receptors. **(6)** Blockade of L- and P-selectin, β2 integrins and ICAM-1, but not of α4 integrins and PECAM-1, decreases opioid CWS-induced antinociception. **(7)** ICAM-1 blockade substantially diminishes CRF-induced antinociception. Thus, peripheral opioid mechanisms of pain control become more prevalent with the chronicity of inflammation. L- and P-selectin, β2 intergrins and ICAM-1, but not α4 integrins and PECAM-1, are important molecules governing intrinsic opioid mediated pain control in injured tissue.

4.1. Development of inflammation

Six h and 4 days after injection of FCA rats developed inflammation confined to the inoculated paws, and characterized by hyperalgesia (decreased basal nociceptive thresholds), swelling (increased paw volume), and hyperthermia (increased paw temperature), in line with previous studies [57, 75]. Within the course of inflammation no significant changes in hyperalgesia or hyperthermia could be detected whereas paw volume appeared to be slightly but significantly higher at 4 days. These findings are consistent with studies showing a progression of inflammation within the first 12 h after inoculation with FCA and a constant degree of hyperalgesia beginning at 6 h [16,

[75]. Previous studies have also examined the development of FCA-inflammation at the cellular level showing a progressive increase in the immigration of leukocytes to inflamed tissue [58]. Moreover, these cells have been shown to produce opioid peptides. Thus, Cabot et al. have previously shown POMC mRNA, β-END, Met-ENK, and DYN in circulating leukocytes [34, 35], as well as mRNAs and the corresponding opioid peptides in inflamed tissue at 4-6 days after FCA [31, 32]. At early stages of inflammation (2-6 h) opioid peptides are mainly produced by granulocytes whereas monocytes and macrophages become the predominant opioid-containing cell type at later stages (4 days) [58]. This is in line with the kinetics of other types of inflammation such as infections by pathogens or wound healing [58, 78]. At later stages of inflammation paw tissue is also infiltrated by lymphocytes that are mostly activated/memory rather than naive-type T-cells [19, 34]. This is consistent with the widely held view that acivated/memory cells migrate to peripheral inflamed tissue, while naive cells typically do not enter peripheral but migrate to lymphoid tissue [19, 79]. Further, with the duration of inflammaton there is not only an increased number of opioid-containing leukocytes but also the content of the peptide in these cells is enhanced [58]. In parallel, FCA-induced inflammation leads to an enhanced transport and accumulation of opioid receptors at the peripheral terminals of sensory neurons [13, 19]. Thus, the model of FCA-induced inflammation is a progressively developing process in which peripheral opioid receptors are up-regulated and opioid peptides are processed and present both in circulating and resident inflammatory cells at the site of injury.

4.2. Opioid mechanisms of intrinsic antinociception

To examine whether opioid-producing leukocytes are of functional relevance I used the CWS test. To evaluate intrinsic opioid antinociception in relation to the development of inflammation I compared CWS-induced antinociception between 6 h and 4 days after FCA. I found that antinociception appeared only in inflamed paws and its degree was not substantially changed within this course of inflammation, in line with previous studies [9, 16, 58, 70] These results are consistent with immunohistochemical and flow cytometry findings showing that opioid-containing leukocytes infiltrate only inflamed tissue [19, 33, 56, 58] and that in this tissue peripheral

opioid receptors are markedly up-regulated [13, 19, 58]. Apparently, endogenous opioid-peptides can activate their peripheral receptors to exert a local inhibition of nociception. That immune-derived opioids are of crucial importance is demonstrated by the lack of intrinsic antinociception in noninflamed tissue, obviously innervated by sensory neurons containing opioid receptors and peptides [13, 17, 19, 32, 33, 39]. The antinociceptive effect of CWS was slightly (although in the majority of experiments not significantly) less at 6 h as compared with 4 days, similar to an earlier study [58]. However, in that study Rittner et al. showed that within a larger time frame, i.e. 2 h to 4 days, the degree of intrinsic antinociception increases significantly and proportionally to the number of opioid producing leukocytes [58]. Again, this points to a crucial role of the progression of inflammation in the generation of peripheral opioid-mediated antinociception. While it was not discriminated between opioid peptides in this earlier study [58] I now demonstrate that at 6 h β-END, Met-ENK, and DYN activating μ, δ, and κ receptors all play a role while at 4 days β-END acting at μ and δ receptors is the predominant peptide. These findings are in line with our immunohistochemical experiments demonstrating that at 6 h there are comparable quantities of β-END-, Met-ENK-, and DYN-expressing cells, whereas at 4 days β-END-containing cells appear prevalent [80]. On the other hand, considering the complexity and multiplicity of endogenous opioid systems [2], at later stages Met-ENK and DYN may be activated by other environmental stimuli, or releasing pathways [81, 82]. The involvement of endomorphins remains to be evaluated. Interestingly, at 6 h all three opioid receptor (μ-, δ-, and κ-) antagonists attenuated antinociception to a similar degree. This may result from coincident blockade of the same signaling pathways by μ, δ, and κ receptors colocalized on the same neurons [17], or from interactions between the receptors subsequent to their potential oligomerization [83]. Studies have shown that such heterodimers have distinct physical and functional properties compared with single receptors [84]. The heterodimerization of μ and δ opioid receptors to form μ - δ heterodimers as well as of δ and κ opioid receptors to form δ – κ heterodimers was demonstrated. They synergistically bind highly selective agonists and potentiate signal transduction [85, 86]. Taken together, it appears that at earlier stages of inflammation, when there are fewer opioid producing cells Met-ENK and DYN need to be engaged in addition to β-END to effectively control pain.

Based on previous studies at later stages of inflammation [72] I now tested the hypothesis that locally produced CRF is a trigger of peripheral stress-induced antinociception also at 6 h after FCA. I found that the local administration of the CRF receptor antagonist dose-dependently attenuated CWS-induced antinociception to the same degree as antibodies against opioid peptides and opioid receptor antagonists. Furthermore, mimicking the action of endogenous CRF, the intraplantar administration of CRF produced dose-dependent antinociception which was abolished by coadministration of NLX. These effects were restricted to the peripheral inflamed tissue because no changes were observed in contralateral noninflamed paws, and equivalent systemic injections were without effects. These findings are in line with earlier studies at 4 days which have shown that CRF activates its receptors expressed on inflammatory cells leading to secretion of opioids [34, 35, 63, 64]. Thus, an important endogenous agent triggering peripheral CWS-induced opioid antinociception appears to be CRF, independent of the stage of inflammation.

A striking observation of the present study is that stress-induced antinociception is only partially reversed (by about 50 %) at 6 h after FCA while at 4 days it is fully abolished by peripherally selective doses of NLX. The exclusive involvement of peripheral opioid mechanisms in intrinsic antinociception at 4 days is consistent with earlier studies using NLX in peripherally selective doses, or quarternary naltrexone and naloxone methiodide, peripherally restricted opioid receptor antagonists [36, 70, 72, 80]. That peripheral opioid-mediated mechanisms are only partially involved at 6 h is also demonstrated by my findings that neither of the selective opioid receptor antagonists or opioid peptide antibodies completely abolished endogenous antinociception. In fact, higher doses of these compounds were less effective with a tendency to produce U-shaped responses. Such effects are described for many drugs acting at G-protein coupled receptors, and have been attributed to compensatory responses, or conformational changes of the receptor when a certain dose of a drug is exceeded [87]. Also, concomitant application of all three selective antagonists did not produce stronger inhibition of this antinociception. To examine the remaining antinociception at 6 h I tested NLX in doses also acting centrally [77], and found that it dose-dependently and fullly blocked this effect. In line with previous studies, this strongly indicates that opioids in the central nervous system play a

significant role at this acute stage of tissue injuy [65, 66, 69]. It was shown that peripheral and central components of opioid systems can be activated simultaneously to control pain [77]. They can interact and potentiate their effects leading to potent and measurable antinociception [88]. Together it seems that, in contrast to later stages, in early inflammation, when there are relatively few opioid-producing immune cells [58] (and present study), less peripheral opioid receptors [13], and a not fully developed inflammatory milieu (e.g. low pH) [3, 78], central opioid sources have to be engaged to guarantee sufficient pain control. At later stages products of activated immune cells such as proinflammatory cytokines might feed back to the brain to alter neuronal activity and pain processing. These notions are supported by one recent study demonstrating the close interplay of central and peripheral mechanisms in intrinsic pain inhibition and pointing towards a neuroimmune interaction in the sense of a feedback mechanism [89]. Stimulation of nociceptive afferents, strong enough to reach the central nervous system, apparently provokes opioid producing immune cells to migrate to the injured tissue, where they release opioids to produce antinociception. Thus, Schmitt et al. where able to show that the homing of opioid containing leukocytes to peripheral damaged tissue is reduced if the nociceptive stimulus is not “realized” in the central nervous system because of preemptive spinal blockade (by morphine) of neurotransmission leading to a decrease in intrinsic peripheral antinociception. Together, these observations point to a close interplay between peripheral and central mechanisms indicating that peripheral opioid mechanisms of pain inhibition gain functional relevance with the chronicity of inflammation.

What are the peripheral sources of opioids contributing to this endogenous antinociceptive effect? DYN was found in peripheral sensory neurons [32] and viral-driven ENK overproduction in these neurons resulted in decreased chronic pain in rats with polyarthritis [39]. However, in my model peripheral sensory neurons do not seem to be major contributors because neither Met-ENK nor DYN are involved in CWS-induced antinociception at 4 days [70, 80], and others could not detect any opioid peptides in peripheral sensory neurons at 6 h after FCA [80]. It is unlikely (but remains to be investigated) that opioids derived from the pituitary and adrenal glands are involved at 6 h. At 4 days these sources have definitely been excluded as functionally relevant [36]. However, at 4 days CWS- and CRF-induced antinociception is completely

abolished by immunosuppression with cyclosporine or whole body irradiation [31, 64, 70]. Overall, these observations strongly suggest that immune cells are the major peripheral source of opioid peptides at later stages of inflammation.

4.3. Contribution of adhesion molecules to intrinsic opioid antinociception

To investigate the contribution of leukocytes as a source of opioids to provide intrinsic pain control at early inflammatory stages I examined adhesion molecules. Selectins, integrins, ICAM-1 and PECAM-1 are sequentially engaged in directing leukocytes from the circulation to inflammatory sites. Selectins are the most important initiators of adhesion and lead to tethering and rolling of leukocytes along the vessel endothelium. To stop rolling and firmly adhere to the endothelium, the cells use integrins and their ligands, particularly α4/β1, and β2 interacting with ICAM-1. Finally, PECAM-1 is thought to be critical for leukocyte transendothelial migration [42, 90]. To assess functional relevance of these adhesion molecules in the generation of intrinsic antinociception we examined the effects of blockade of L-, and P-selectin (by fucoidin) and of α4 and β2 integrins, ICAM-1 and PECAM-1 (by mAbs) on CWS-induced antinociception. The main finding of these experiments is that blockade of L- and P-selectin and of ICAM-1 was very efficient in the attenuation of the peripheral component of CWS-induced antinociception. In addition, anti-ICAM-1 strongly diminished CRF-induced antinociception. In contrast, blocking of β2 integrins only slightly decreased while blockade of α4 integrins or of PECAM-1 was ineffective in reducing CWS-induced antinociception.

Selectins

The abolishment of the peripheral component of CWS-induced antinociception by simultaneous blockade of L- and P-selectins (producing a similar effect to that of NLX in a peripherally selective dose) is consistent with the need of concomitant blockade of several adhesion molecules to achieve significant effects on leukocyte migration and arthritic inflammation [45, 46, 48, 49, 51]. It is also in line with a previous study where Machelska et al. showed a dose-dependent blockade of CWS-induced antinociception by fucoidin apparently resulting from the decreased content of β-END and of β-END-containing leukocyte number in inflamed tissue [57]. I was unable to determine the relative importance of L- versus P-selectin because both are targeted

by fucoidin [91]. Also, the functional contribution of E-selectin to intrinsic opioid antinociceptioin awaits further evaluation because selective blockers are not commercially available at present.

ICAM-1

Effects of anti-ICAM-1 were potent and similar to the effects of NLX on CWS- and CRF-induced antinociception (only slightly less compared to the effect of NLX). My finding that a high dose of anti-ICAM-1 tended to lose its blocking effect is in line with other studies reporting stimulatory effects of monoclonal antibodies [92, 93], and with their therapeutic effects that strongly vary with their dosage [94, 95]. Notwithstanding, anti-ICAM-1 is very effective in attenuating intrinsic opioid analgesia in my model. Thus, simultaneous blockade of several adhesion molecules is not absolutely neccessary to interfere with the generation of antinociception. In contrast, to achieve an optimal effect on inflammation, ICAM-1 had to be blocked simultaneously with other adhesion molecules [45, 96]. Thus, my findings point to a specific role of ICAM-1 in intrinsic pain control. This is confirmed by our recent study showing simultaneous upregulation of endothelial ICAM-1 and of opioid-containing cells as well as by anti-ICAM-1 impaired extravasation of these cells to inflamed tissue by anti-ICAM-1 [97].

Integrins

Here I found that anti-α4 was ineffective and anti-β2 only slightly decreased CWS-antinociception. Because β2 integrins use ICAM-1 to orchestrate leukocyte extravasation [98] the small effect of anti-β2 on CWS-induced antinociception is intriguing in light of a clear involvement of ICAM-1 (see above). It has been shown that integrins interact with each other, which can result in mutual up- or downregulation [99]. Thus, it is conceivable that blocking β2 alters α4-mediated functions (and vice versa) in a sense that α4-mediated cell migration is increased. This would explain why I observed no significant changes in the CWS-induced antinociception following the blockade of either α4 or β2 alone. This is in line with the highest effeciveness of concomitant integrin blockade on leukocyte migration in inflamed joint [48].

PECAM-1

Although a single blockade of PECAM-1 has been reported to decrease experimental inflammation [53] in my studies a wide dose range did not influence CWS-induced

antinociception indicating no involvement of PECAM-1 in this effect. This is in spite of an upregulation of endothelial PECAM-1 in inflamed tissue in my model [100]. It is conceivable that concomitant blockade of PECAM-1 with other adhesion molecules might be more effective. However, in view of our present findings on ICAM-1 it seems that a single adhesion molecule blockade might be sufficient to interfere with the generation of intrinsic antinociception.

Together, blockade of selectins, integrins and ICAM-1 attenuates only the peripheral component of CWS-induced antinocicetption. Apparently, this results from the decreased migration of opioid-containing leukocytes to inflamed tissue [57, 97, 100]. Further, the remaining (not blocked by anti-adhesion molecule treatments) centrally mediated intrinsic opioid analgesia is independent of leukocyte extravasation. These observations strongly indicate immune cells as a major source of opioids to provide intrinsic peripheral pain inhibition at early as well as later stages of inflammation (Fig. 21).

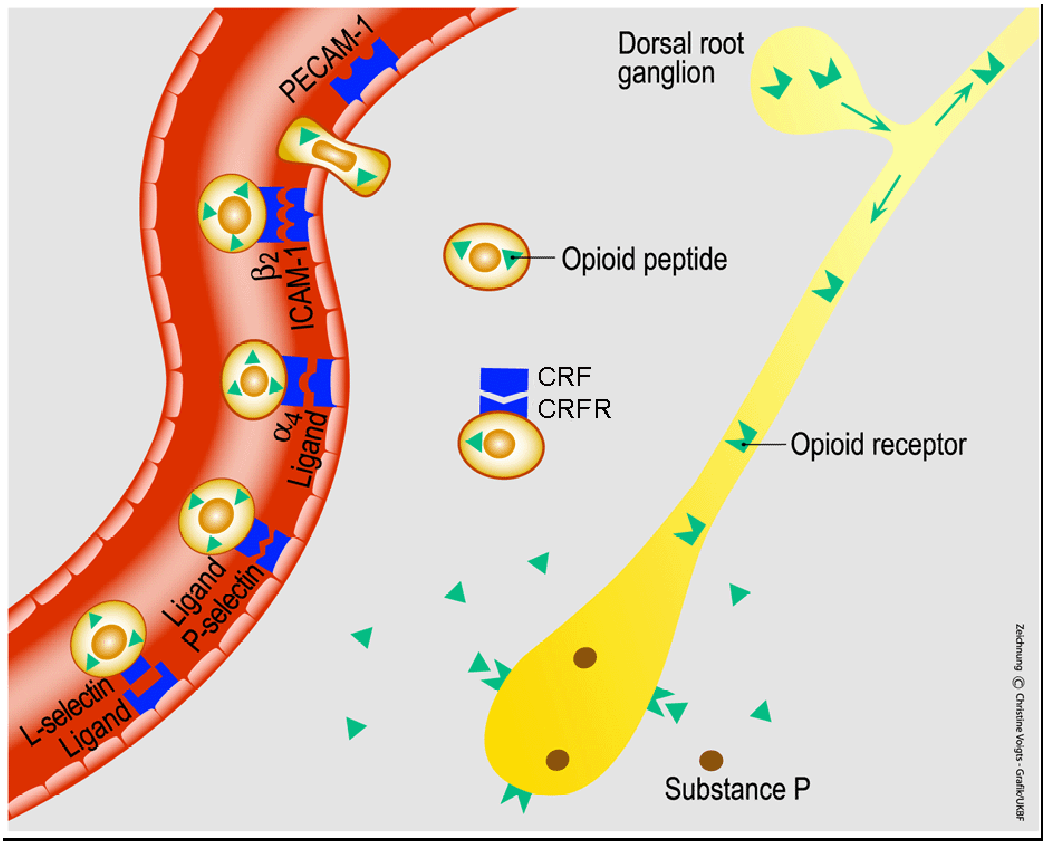

Figure 21. Immune mechanisms in peripheral opioid antinociception in inflammation. P-selectin, ICAM-1 and PECAM-1 are upregulated on vascuar endothelium. L-selectin, α4 and β2 integrins are co-expressed by immune cells containing opioid peptides. L- and P-selectin mediate rolling of opioid-containing cells along the vessel wall. Alpha4 and β2 integrins mediate the firm adhesion of these leukocytes to endothelial cells by interacting with ligands of the immunoglobulin superfamily, e.g. ICAM-1 expressed by endothelium. In consequence opioid-containing leukocytes transmigrate through the vessel wall. In response to stress or corticotropin releasing factor (CRF), the cells secrete opioid peptides. CRF elicits opioid peptide release by activating CRF receptors on leukocytes. Opioid peptides bind to opioid receptors on primary afferent neurons, leading to antinociception. CRFR, CRF receptor. Adapted from Stein C, Schäfer M, Machelska H. Attacking pain at its source: new perspectives on opioids. Nature Medicine, 2003, Vol. 9, 8: 1003 – 1008.

4.4. Adhesion molecules and inflammation

Because the blockade of selectins, integrins and IgSF members was shown to modulate various inflammatory responses [50-52, 55] I evaluated their effects on macroscopic parameters of inflammation such as hyperalgesia, paw swelling and elevated paw temperature in my model. Blockade of L- and P-selectin and of ICAM-1 resulted in a statistically significant decrease in paw edema, while paw temperature

was slightly attenuated by anti-ICAM-1. Similar, others found reductions in inflammation in animals and humans in response to such treatments [52, 102-104]. This could result from impaired migration of immunocytes to inflamed tissues [45, 46, 51, 97, 104-106] or attenuated mast cell- or cytokine-dependent enhances in vascular permeability [107, 108]. A lack of antiinflammatory effects in response to either anti-α4 or anti-β2 can be explained by necessity of concomitant blockade of these integrins as showed by others and discussed above [45, 51]. In contrast, it is important to note several studies reporting that blockade of adhesion molecules or deletion of their genes not only did not improve but even exaggerated inflammation [55, 96, 109, 110]. Notwithstanding, in my studies effects of anti-adhesion treatments on macroscopic inflammation are apparently independent of their effects on CWS-induced antinociception. Blockade of selectins and ICAM-1 abolished or strongly attenuated the peripheral component of CWS-induced antinociception, respectively, but had only a moderate effect on paw volume. Blockade of selectins did not influence paw temperature that was slightly decreased by ICAM-1 but only in the dose that had no effect on CWS-induced antinociception. Also, this inflammatory parameter was decreased by anti-β2 which had only a minimal effect on CWS-induced antinociception and no effect on paw edema. Furthermore, none of the treatments changed hyperalgesia. This could be explained by a lack of influence on the migration of cells containing hyperalgesic cytokines or transmitters (e.g., 5-hydroxytryptamine, histamine, nerve growth factor, substance P or calcitonine gene-related peptide) [111-113]. In addition, these adhesion molecule treatments are unlikely to block the release of nerve-derived substance P and/or calcitonin gene-related peptide. Although the blockade of selectins, integrins, and ICAM-1 decreased the immigration of opioid-containing leukocytes to inflamed paws [57, 97] the hyperalgesia is not decreased [100]. This may be due to a lack of sensitivity of the paw pressure assay. It is also possible that the endogenous opioid system is not essential for tonic pain control (i.e. without a stressor) in my model. This would be consistent with several studies using opioid receptor antagonists or mice lacking either opioid receptors or opioid peptides, which have shown no changes in basal nociceptive transmission in acute and inflammatory pain [114, 115]. Consistently, it was shown that immune cells

need to be stimulated by stress or CRF to release opioids and to produce antinociception [34, 35, 64, 72, 79].

In summary, it seems that in my model selectins and ICAM-1 play a crucial role in the recruitment of opioid-containing cells and come into play when this intrinsic system is activated in order to cope with inflammatory pain. Further, my findings point to a specific role of these adhesion molecules in intrinsic pain control. Blockade of adhesion molecules has been proposed as a novel anti-inflammatory strategy [45, 50, 51, 53, 102]. My studies indicate that selectin and ICAM-1 blockade can result in severely impaired pain control. Thus, endogenous pain inhibition may be harmed following anti-selectin and anti-ICAM-1 treatments.