

# Modulation of Peripheral Endogenous Opioid Analgesia by Central Afferent Blockade

Thomas K. Schmitt, M.D.,\* Shaaban A. Mousa, Ph.D.,† Alexander Brack, M.D.,\* Diego K. Schmidt, M.D.,\* Heike L. Rittner, M.D.,\* Martin Welte, M.D.,‡ Michael Schäfer, M.D.,‡ Christoph Stein, M.D.§

**Background:** Peripheral tissue injury causes a migration of opioid peptide-containing immune cells to the inflamed site. The subsequent release and action of these peptides on opioid receptors localized on peripheral sensory nerve terminals causes endogenous analgesia. The spinal application of opioid drugs blocks the transmission of nociceptive information from peripheral injury. This study investigates the influence of exogenous spinal opioid analgesia on peripheral endogenous opioid analgesia.

**Methods:** Six and forty-eight hours after initiation of continuous intrathecal morphine infusion and administration of Freund's complete adjuvant into the hind paw of rats, antinociceptive and antiinflammatory effects were measured by paw pressure threshold, paw volume, and paw temperature, respectively. Inflammation and quantity of opioid-containing cells were evaluated by immunocytochemistry and flow cytometry. Cold water swim stress-induced endogenous analgesia was examined 24 h after discontinuation of intrathecal morphine administration.

**Results:** Intrathecal morphine (10  $\mu$ g/h) resulted in a significant and stable increase of paw pressure threshold ( $P < 0.05$ ) without changing inflammation, as evaluated by paw volume, paw temperature, and flow cytometry ( $P > 0.05$ ). At 48 but not at 6 h after Freund's complete adjuvant, the number of  $\beta$ -endorphin-containing cells and cold water swim-induced antinociception were significantly reduced in intrathecal morphine-treated rats compared with those treated with intrathecal vehicle ( $P < 0.05$ ).

**Conclusions:** These findings suggest an interplay of central and peripheral mechanisms of pain control. An effective central inhibition of pain apparently signals a reduced need for recruitment of opioid-containing immune cells to injured sites.

ENDOGENOUS pain control mechanisms are not limited to the central nervous system.<sup>1</sup> Peripheral analgesic effects of exogenous and endogenous opioids have been demonstrated both in animals and humans, especially under inflammatory conditions.<sup>2–5</sup> Following tissue injury, opioid-containing immunocytes preferentially home to the

inflamed site to release  $\beta$ -endorphin (END) and met-enkephalin (ENK).<sup>6,7</sup> These peptides can activate peripheral opioid receptors on sensory nerve terminals to produce analgesia.<sup>8</sup> Selectins and intercellular adhesion molecule-1 play an important role in the site-directed recruitment of opioid-containing cells to injured tissue.<sup>9–12</sup>

Extensive interactions between the nervous and immune systems have been described.<sup>13,14</sup> Thus, the question arises whether peripheral mechanisms of endogenous analgesia are influenced by the central nervous system. To detect such potential interactions, we used continuous intrathecal application of morphine in a rat model of Freund's complete adjuvant (FCA)-induced inflammation of the hind paw. By activation of presynaptic  $\mu$ -opioid receptors, intrathecal morphine inhibits the release of excitatory neurotransmitters and blocks nociception at the spinal level.<sup>15,16</sup> To examine endogenous peripheral opioid analgesia, we used cold water swim (CWS) stress, an extensively characterized environmental stimulus, to activate intrinsic peripheral opioid systems in this model. After CWS, the animals show a robust increase of nociceptive thresholds selectively in the inflamed paw, which is mediated by immune cell-derived END interacting with opioid receptors on sensory nerve terminals.<sup>3,8,17</sup> The aims of the current study were (1) to create a sufficient central blockade of nociceptive input without influencing the peripheral inflammatory process and (2) to examine the influence of central antinociception on the immigration of opioid-containing cells and CWS-induced peripheral analgesia at both early and late stages of the inflammation. We hypothesized that effective central nociceptive blockade would decrease the number of opioid cells in the inflamed tissue and preempt endogenous peripheral analgesia.

## Materials and Methods

### Induction of Hind-paw Inflammation

Male Wistar rats (weight, 250–300 g) were housed individually and kept in a temperature-controlled room ( $22 \pm 1^\circ\text{C}$ ) with a 12-h alternating light–dark cycle. To induce inflammation, rats were sedated by brief halothane anaesthesia and received an intraplantar injection of 150  $\mu$ l FCA (Calbiochem, San Diego, CA) into the right hind paw. This treatment consistently produces a unilaterally localized inflammation and hyperalgesia of the inoculated paw within 6 h after the injection, lasting for at least 6–8 days.<sup>8,17</sup> Experiments and animal care were in accordance with standard ethical guidelines<sup>18</sup> and were approved by the animal care committee of the

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\* Resident, † Research Associate, ‡ Professor, § Professor and Chairman. Dr. Schmitt's current address: Institut für Anästhesiologie und Notfallmedizin, Westfälisch-Klinikum Kaiserslautern, Germany.

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Address reprint requests to Dr. Stein: Klinik für Anaesthesiologie und operative Intensivmedizin, Freie Universität Berlin, Hindenburgdamm 30, D-12200 Berlin, Germany. Address electronic mail to: christoph.stein@medizin.fu-berlin.de. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

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#### *Nociceptive Thresholds*

Before and after induction of inflammation, mechanical nociceptive thresholds were assessed using a paw pressure algometer (Ugo Basile, Comerio, Italy). The paw pressure threshold (PPT) required to elicit hind-paw withdrawal was determined by averaging three consecutive trials separated by 10 s, with an arbitrary cutoff at 250 g. The sequence of left and right paws was alternated between animals to preclude "order" bias. In separate groups ( $n = 7$  each), baseline PPT measurements were taken, and 10 min later the animals were subjected to CWS at  $1-2^{\circ}\text{C}$  for 1 min.<sup>3,17</sup> At 1, 5, 15, 30, and 45 min after CWS, PPTs were reevaluated. The experimenter was blinded to the experimental conditions used, and rats were randomly allocated to the various treatments. Data are represented as percent of maximum possible effect ( $\% \text{ MPE} = [\text{PPT}_{\text{treated}} - \text{PPT}_{\text{pretreated}}] / [250 - \text{PPT}_{\text{pretreated}}] \times 100$ ).

#### *Motor Performance Test*

Motor function was tested using the combined behavioral score originally described for evaluation of spinal cord dysfunction.<sup>19</sup> The combined behavioral score combines six categories assigned to different weights on a nominal scale (movement and weight-bearing of the limbs [0-5-15-25-40]; toe spread [0-2, 5-5], righting [0-5-10-15], extension withdrawal [0-2, 5-59], placing [0-2, 5-5], and movement on an inclined plane [0-5-10-15]) into a total score ranging from 0 for a normal rat to 90 for a completely paralyzed rat.

#### *Evaluation of Inflammation*

Potential antiinflammatory effects of the treatments were assessed by monitoring paw volume (PV) and paw temperature (PT) with a plethysmometer (Ugo Basile) and a contact thermometer (Cooper Instrument Corporation, Middlefield, CT), respectively. The PV data are presented as percent change of baseline ( $[\text{PV treated} / \text{PV pretreated}] \times 100$ ), and PT data are presented as temperature difference to baseline ( $\text{PT treated} - \text{PT pretreated}$ ). To evaluate the immune cell infiltrate, subcutaneous paw tissue was analyzed by flow cytometry for the number of hematopoietic ( $\text{CD45}^{+}$ ) cells and the percentage of granulocytes ( $\text{RP-1}^{+}$ ), monocytes ( $\text{ED1}^{+}$ ), and lymphocytes ( $\text{CD3}^{+}$ ) in this fraction. Data are presented as number of cells per 100 mg wet weight of tissue.

#### *Drugs*

Morphine hydrochloride (Merck, Darmstadt, Germany) was diluted with isotonic saline to a final concentration of  $1 \mu\text{g}/\mu\text{l}$ . Single injections used  $10 \mu\text{l}$  of this solution; osmotic pumps (Alzet/Durect Corp., Cupertino, CA) were filled with 2 ml to deliver a constant rate of  $10 \mu\text{l}/\text{h}$ ,

which was determined following pilot experiments and according to the literature.<sup>20</sup> The pumps were filled 8 h before the experiment and incubated in sterile saline containers at  $37^{\circ}\text{C}$  overnight, according to the manufacturer's guidelines. Lidocaine, 2% (Braun, Melsungen, Germany), was used to test catheter function.

#### *Antibodies*

For flow cytometry, we used mouse monoclonal anti-rat opioid 3E7 (subtype immunoglobulin  $\text{G}_{2a}$  [ $\text{IgG}_{2a}$ ]; Gramsch Laboratories, Schwabhausen, Germany), mouse  $\text{IgG}_{2a}$ , rat antimouse  $\text{IgG}_{2a+b}$  PE (BD Biosciences, Heidelberg, Germany), mouse antirat CD3-FITC/PE (T cells), mouse antirat RP-1-PE (granulocytes), mouse antirat CD45-CyChrome (all hematopoietic cells), and mouse antirat ED1-FITC (monocytes-macrophages) (all BD Biosciences and Serotec, Oxford, United Kingdom). For immunohistochemistry, we used polyclonal rabbit antirat END, polyclonal rabbit antirat ENK (Peninsula Laboratories, Belmont, CA), and biotinylated secondary goat antirabbit (Vector Laboratories, Burlingame, CA).

#### *Implantation of Intrathecal Catheters and Osmotic Pumps*

Rats were handled and trained in the test situation for 3 days before intrathecal catheterization. Anesthesia was induced and maintained with 2% halothane *via* a loose-fitting plastic mask. The intrathecal catheters were prepared according to a method described elsewhere.<sup>21</sup> Briefly, a polyethylene tube (PE 10; Portex, Hythe, United Kingdom) was cut in 200-mm lengths and inserted intrathecally for 15 mm in the cervical direction through an incision at the L3-L4 level. The animals were allowed 4 days to recover. Animals showing signs of neurologic damage (combined behavioral score  $> 10$ ) were excluded from the study. Drugs were injected intrathecally in a volume of  $10 \mu\text{l}$  followed by  $5 \mu\text{l}$  of vehicle to flush the catheter, immediately before the continuous administration by osmotic pumps and the concomitant induction of inflammation. Using a 25-mm polyethylene tube (PE 60; Portex) attached to the flow moderator, the pump was connected to the proximal catheter tip by heated glue. The connection was also coated with a two-component acrylic glue. After 6 or 48 h, respectively, the pumps were disconnected and removed, and the amount of the remaining fluid was determined following aspiration. Animals showing signs of insufficient pump function (disconnection, leakage, or difference of the remaining volume from the expected value) were excluded from the study. The correct placement of the catheter was tested by an injection of  $10 \mu\text{l}$  lidocaine 2% (Braun) 24 h before the experiment. Only animals with an immediate yet reversible paralysis of their hind limbs were included in the study. After the experiments (each animal was used only once), rats were killed and the correct position of the catheter

tip was confirmed at autopsy by an experienced investigator who was blinded to the experimental results. Rats were randomly investigated histologically for correct catheter position in relation to the spinal cord.

#### Flow Cytometry

**Tissue and Cell Preparation.** Tissue preparation and flow cytometry were performed as previously described.<sup>3</sup> At 6 and 48 h after FCA, animals ( $n = 6$  per group) were killed, and subcutaneous paw tissue was harvested from the plantar surface, leaving the deep flexor tendon *in situ*. To obtain a single cell suspension, the tissue was cut into 1–2-mm pieces. Fragments were digested for 1 h at 37°C with 10 ml/g tissue of RPMI 1640 medium (Invitrogen GmbH, Karlsruhe, Germany) containing 30 mg collagenase, 10 mg hyaluronidase, and 0.5 ml 1 M HEPES (Sigma, Deisenhofen, Germany). The digested pieces were pressed through a 70- $\mu$ m nylon filter (BD Biosciences) to remove particles.

**FACS Staining.** Single cell suspensions obtained from one paw were fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature and washed with PBS. Cells were permeabilized with saponin buffer (0.5% saponin, 0.5% bovine serum albumin, 0.05%  $\text{NaN}_3$ ; all Sigma) and subsequently stained with 3E7 in saponin buffer (20  $\mu$ g/ml) for 30 min at room temperature. This monoclonal antibody was originally raised against END and recognizes the pan-opioid sequence Tyr-Gly-Gly-Phe at the N-terminus of opioid peptides.<sup>22</sup> Afterwards, cells were incubated with a secondary rat-antimouse IgG<sub>2a+b</sub> PE-conjugated monoclonal antibody (1.5  $\mu$ g/ml) for 15 min at room temperature. Negative controls included the replacement of the primary antibody with an isotype-matched irrelevant antibody (mouse IgG<sub>2a</sub>) at the same concentration, or the omission of the secondary antibody. Separate aliquots of the cell suspensions from the same paw were labeled with fluorescein isocyanate (FITC), PE, or Cy-Chrome-conjugated surface markers to differentiate subpopulations of monocytes-macrophages (ED1), granulocytes (RP-1), and T cells (CD3) and then fixed in 1% paraformaldehyde in PBS. To calculate absolute numbers of cells per paw, the stained cell suspension was transferred to a TruCOUNT<sup>®</sup> tube (BD Biosciences) containing a known number of fluorescent beads. FACS events from the fluorescent TruCOUNT beads and stained cells were collected simultaneously using the FACScan. Numbers of CD45<sup>+</sup> cells per tube were calculated in relation to the known number of fluorescent TruCOUNT beads and extrapolated for the whole paw. At least 30,000 FACS events were collected per paw. Data were analyzed using CellQuest software (BD Biosciences).

**Immunohistochemistry.** Six or 48 h after FCA inoculation, respectively, rats ( $n = 8$  per group) were deeply anesthetized with halothane and perfused transcardially with 100 ml of 0.1 M PBS (pH 7.4) and with 300 ml of

cold PBS containing 4% paraformaldehyde and 14% saturated picric acid (pH 7.4; fixative solution). The skin with adjacent subcutaneous tissue was removed, post-fixed for 3 h at 4°C in the fixative solution, and cryoprotected overnight at 4°C in PBS containing 10% sucrose. The tissue was then embedded in tissue-Tek compound (O.C.T.; Miles Inc., Elkhart, IN) and frozen. Sections (7- $\mu$ m thick) prepared on cryostat were mounted onto gelatin-coated slides. Each experiment was repeated three times. Immunocytochemical staining of the sections was performed with a Vectastain avidin-biotin peroxidase complex kit (Vector Laboratories) as described previously.<sup>9</sup> Unless otherwise stated, all incubations were performed at room temperature, and PBS was used for washing (three times for 10 min) after each step. The sections were incubated with PBS, 0.3%  $\text{H}_2\text{O}_2$ , and 10% methanol for 45 min to block endogenous peroxidase. To prevent nonspecific binding, the sections were incubated for 60 min in PBS containing 0.3% Triton X-100, 1% bovine serum albumin, 4% goat serum, and 4% horse serum (block solution). The sections were then incubated overnight at 4°C with polyclonal rabbit antirat END or anti-ENK antibody (1:1,000 dilution), respectively. Thereafter, the sections were incubated for 1 h with the appropriate biotinylated secondary goat anti-rabbit antibody. Sections were then incubated with avidin-biotin-conjugated peroxidase for 45 min. Finally, the sections were washed and stained with 3',3'-diaminobenzidine tetrahydrochloride (Sigma) containing 0.01%  $\text{H}_2\text{O}_2$  in 0.05 M Tris-buffered saline (pH 7.6) for 3–5 min. After the enzyme reaction, the sections were washed in tap water, dehydrated in alcohol, cleared in xylene, and mounted in DPX (Merck). To demonstrate specificity of staining, the following controls were included: (1) preabsorption of diluted anti-END or anti-ENK with 5  $\mu$ g/ml of purified END or ENK, respectively (Peninsula Laboratories, Belmont, CA) for 24 h at 4°C; (2) omission of either the primary antisera, the secondary antibodies, or the avidin-biotin complex. Immune cells containing opioids were counted by an observer blinded to the experimental protocol, using a Zeiss microscope (objective  $\times 20$ ; eyepiece  $\times 10$ ). The mean number of stained cells in four sections per sample and 10 squares (384  $\mu\text{m}^2$  each) per section were calculated.

#### Experimental Protocols

Baseline PPTs were obtained and then reevaluated at 6, 12, 24, 48, and 72 h after initiation of intrathecal morphine (10  $\mu$ g/h) and concomitant FCA administration ( $n = 7$ ). Control rats received intrathecal isotonic saline (10  $\mu$ l/h) or subcutaneous morphine (10  $\mu$ g/h) ( $n = 7$  each). Motor function, PT, and PV were evaluated immediately after PPT to avoid stress-induced influences on the algometry. To evaluate endogenous peripheral opioid analgesia, intrathecal treatments were discontinued after 6 or 48 h, respectively, and 24 h later (to assure



a complete recovery from spinal analgesia) animals were subjected to CWS. At 1, 5, 15, 30, and 45 min thereafter, PPTs were reevaluated ( $n = 7$  per group).

### Statistical Analysis

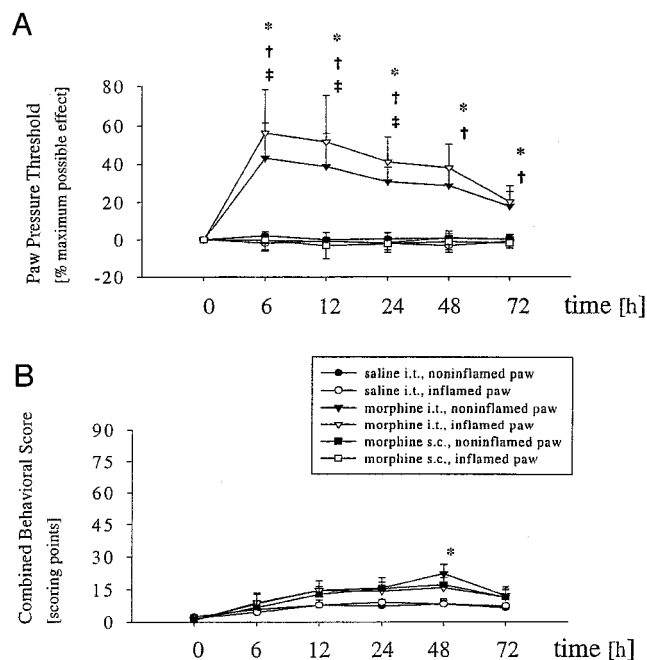
All data were initially tested for normality of distribution (Skewness and Kurtosis tests). Data are represented as means  $\pm$  SD. Differences between morphine- and saline-treated rats were assessed by analysis of variance (ANOVA) followed by a *post hoc* Dunnett test if the normality test and the equal variance test were passed. Otherwise, the Kruskal-Wallis Test (ANOVA on ranks) was used. *Post hoc* multiple pairwise comparisons were performed by the Student-Newman-Keuls method. If group sizes were unequal, *post hoc* comparisons were performed by Dunn test. Differences between inflamed and noninflamed paws within animals were evaluated by the paired *t* test. The Bonferroni correction was used for repeated measurements. Differences were considered significant at  $P < 0.05$ . For CWS testing, the area under the curve was calculated (Pharm/PCS Version 4.2; MCS, Philadelphia, PA).

## Results

A total of 166 animals were enrolled; 40 animals had to be excluded because of neurologic damage, catheter dislocation, pump dysfunction, or infection. Consequently, the results were calculated from a total of 126 animals.

### Antinociception and Inflammation during Intrathecal Morphine Treatment

Continuously administered intrathecal morphine caused a significant increase in PPT of both inflamed and noninflamed paws compared with either baseline, intrathecal saline-treated rats, or subcutaneous morphine-treated rats over the entire observation period of 72 h ( $P < 0.05$ , ANOVA; fig. 1A). The analgesic effect decreased slightly (but not significantly) over time and was significantly higher (except at 72 h) on the inflamed than on the noninflamed paw ( $P < 0.05$ , paired-*t* test; fig. 1A). Neither intrathecal nor subcutaneous morphine administration caused significant motor disturbance (fig. 1B). The PV and PT increased significantly over time in inflamed paws regardless of the intrathecal or subcutaneous treatment ( $P < 0.001$ , ANOVA; fig. 2). The number of CD45<sup>+</sup> cells in inflamed paws was not different between intrathecal morphine- and intrathecal saline-treated rats, but was significantly higher at 48 than at 6 h ( $P < 0.05$ , ANOVA; fig. 3A). The subpopulations of immune cells in inflamed paws were not different between intrathecal morphine- and saline-treated rats (fig. 3B). Similar to our previous studies,<sup>3</sup> the pattern of the infiltrating cells changed in that granulocytes (RP-1<sup>+</sup>)



**Fig. 1.** Time course of analgesia and motor function. (A) In intrathecal morphine-treated rats, paw pressure threshold (PPT) was significantly increased in both paws. The inflamed paw of intrathecal morphine-treated rats is compared with control groups (†), baseline (\*), and the contralateral noninflamed paw (§; mean  $\pm$  SD;  $n = 7$  per group; \**P*, †*P*, and §*P* < 0.05, analysis of variance). (B) Combined behavioral scores increased slightly but not significantly in all groups.

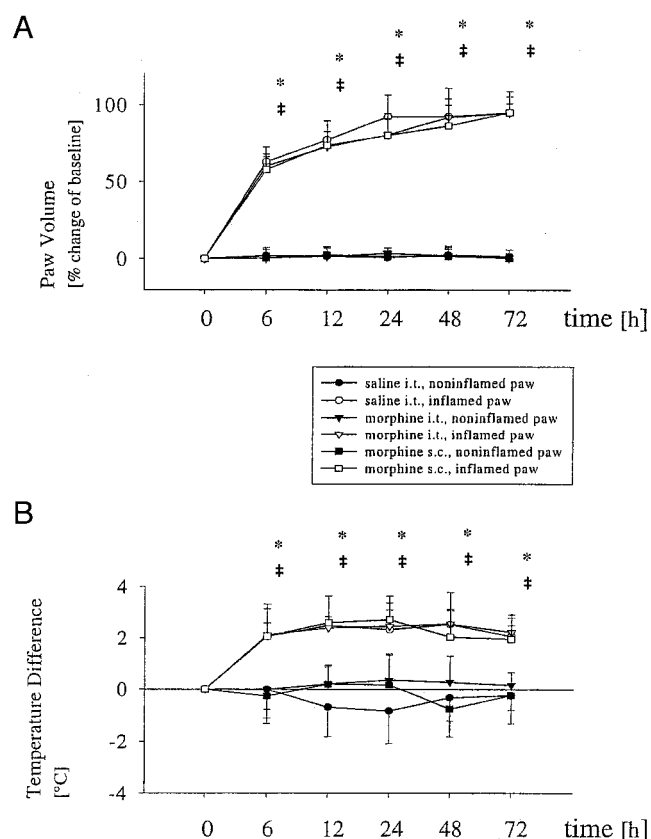
were the predominant population at 6 h, whereas monocytes-macrophages (ED1<sup>+</sup>) were predominant at 48 h ( $P < 0.05$ , ANOVA; fig. 3B).

### Opioid-containing Immune Cells within Inflamed Tissue

The number of 3E7<sup>+</sup>CD45<sup>+</sup>, END<sup>+</sup>, and ENK<sup>+</sup> cells increased from 6 to 48 h of inflammation ( $P < 0.05$ , Kruskal-Wallis; figs. 4 and 5). There was no significant difference in the number of 3E7<sup>+</sup>CD45<sup>+</sup> cells between intrathecal morphine- and intrathecal saline-treated rats (fig. 4A), as measured by flow cytometry. However, immunocytochemistry revealed that the number of END<sup>+</sup> cells was significantly lower in intrathecal morphine- than in intrathecal saline-treated rats after 48 h ( $P < 0.05$ , Kruskal-Wallis; figs. 4B, 5A, and 5B). ENK<sup>+</sup> cells did not show a significant difference between intrathecal treatments (figs. 4C, 5C, and 5D). Control experiments did not show opioid staining.

### Peripheral Endogenous Analgesia

The PPT increased significantly after CWS ( $P < 0.05$ , ANOVA) in inflamed but not in noninflamed paws (fig. 6). After 6 h there was no significant difference between intrathecal morphine and intrathecal saline treatments (figs. 6A and 6B). However, after 48 h the CWS-induced PPT increase was significantly less in the intrathecal

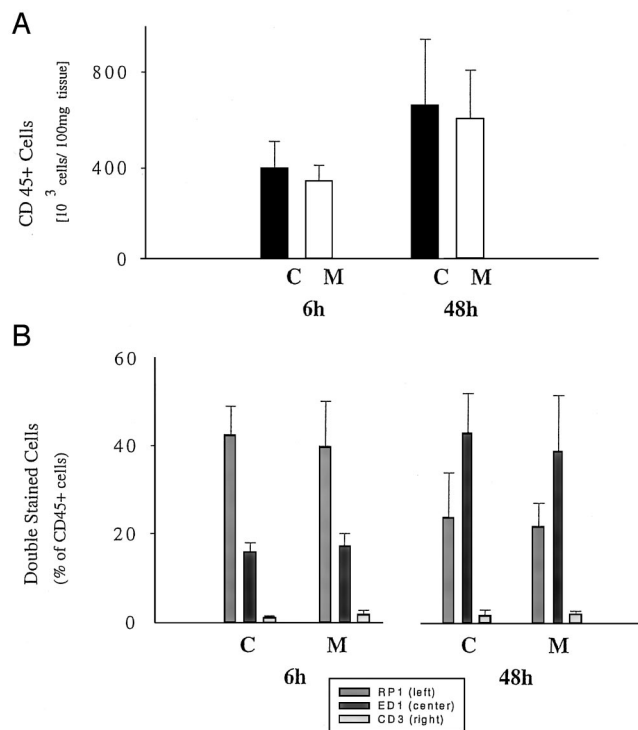


**Fig. 2.** Time course of paw volume and paw temperature. Volume (A) and temperature (B) of inflamed paws increased significantly in all groups. The inflamed paw of intrathecal morphine-treated rats is compared with baseline (\*) and the contralateral noninflamed paw (‡; mean  $\pm$  SD;  $n = 7$  per group; \* $P$  and ‡ $P < 0.05$ , analysis of variance). There were no significant differences in inflamed paws between groups at any time point. Noninflamed paws did not change in volume (A) or temperature (B).

morphine than in the intrathecal saline group, as demonstrated by a significantly reduced PPT elevation at 5 min (fig. 6C;  $P < 0.05$ , Dunnett test) and by a significantly smaller area under the curve (fig. 6D;  $P < 0.05$ , ANOVA).

## Discussion

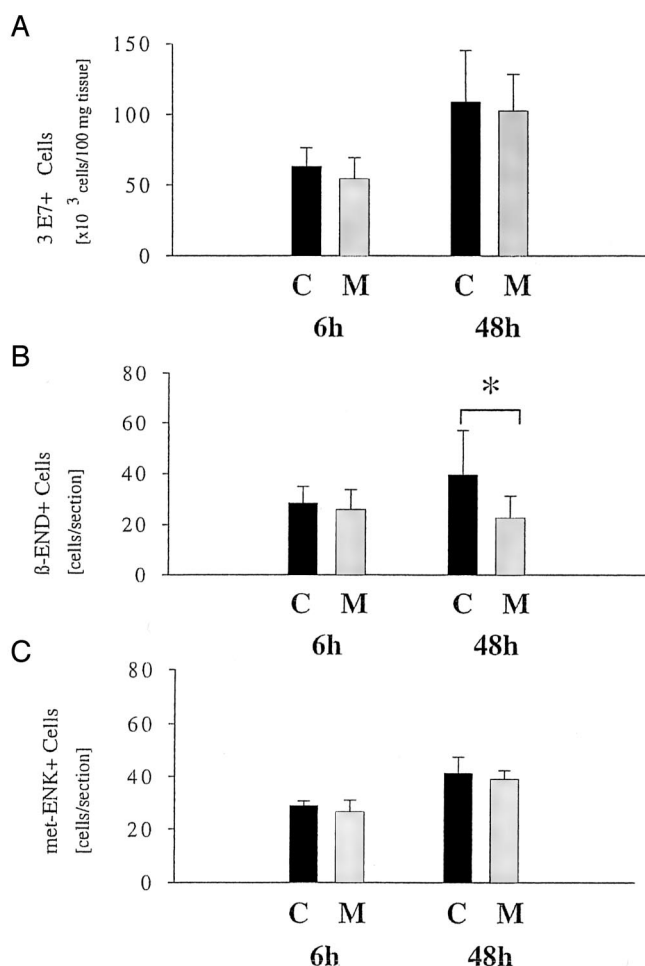
Opioid analgesia can be produced in the central nervous system and in the periphery *via* activation of opioid receptors by exogenous or endogenous opioids. In the periphery, endogenous analgesia is elicited by immunocytes entering inflamed tissue and releasing opioid peptides that activate up-regulated opioid receptors on sensory nerve terminals.<sup>1</sup> The trafficking of opioid cells apparently occurs in a site-directed manner since they express adhesion molecules that govern their recruitment to damaged tissue<sup>9,10,12</sup> and since END-containing lymphocytes are of the memory type.<sup>6,11</sup> Here we examined whether central nociceptive blockade diminishes



**Fig. 3.** Characterization of the inflammatory infiltrate by flow cytometry at 6 and 48 h after Freund's complete adjuvant inoculation. (A) Total numbers of CD45<sup>+</sup> cells increased significantly, without differences between intrathecal saline (C) and intrathecal morphine (M) groups (means  $\pm$  SD;  $n = 6$  per group; \* $P < 0.05$ , analysis of variance; not shown in the graph). (B) At 6 h, granulocytes (RP-1, left) were the most abundant compared with monocytes–macrophages (ED-1, center) and lymphocytes (CD3, right). At 48 h, monocytes–macrophages were most abundant compared with granulocytes and lymphocytes. There were no differences between intrathecal treatments (mean  $\pm$  SD,  $n = 6$  per group).

the recruitment of opioid-containing immune cells to the peripheral site of injury.

By activation of presynaptic  $\mu$ -opioid receptors, intrathecal morphine inhibits the intraspinal release of excitatory neurotransmitters and produces antinociception.<sup>15,16</sup> Consistently, in our hands, 10  $\mu$ g/h intrathecal morphine caused a significant elevation of PPTs over 72 h without motor impairment. The systemic (subcutaneous) application of the highest dose of morphine did not affect PPT, confirming the spinal mediation of these effects. The intrathecal morphine-induced PPT elevations were always higher in the inflamed than in the noninflamed paws. This has been observed in other models of inflammation and has been ascribed to possible central changes of opioid or adrenergic systems.<sup>23–26</sup> In those studies, additive–synergistic interactions of exogenous opioids with spinal descending adrenergic pathways,<sup>23</sup> with spinal microglia,<sup>25</sup> or with supraspinal endogenous opioid peptides<sup>26</sup> were postulated. Spinal opioid effects on peripheral inflammation have also been suggested.<sup>27</sup> However, such effects were not apparent in our study since we could not detect any gross changes in



**Fig. 4.** Opioid-containing immune cells in inflamed paw tissue. (A) The number of 3E7<sup>+</sup>CD45<sup>+</sup> cells, measured by flow cytometry, increased significantly over time (means  $\pm$  SD;  $P < 0.05$ , Kruskal-Wallis;  $n = 6$  per group; not shown in the graph), without significant differences between intrathecal saline (C) and intrathecal morphine (M) treatments. (B) At 48 h, the number of  $\beta$ -endorphin ( $\beta$ -END+) cells, analyzed by immunohistochemistry, was significantly lower in intrathecal morphine-treated rats than in intrathecal saline-treated rats (means  $\pm$  SD;  $*P < 0.05$ , Kruskal-Wallis). (C) The number of met-enkephalin (met-ENK+) cells, analyzed by immunohistochemistry, increased significantly over time (mean  $\pm$  SD;  $P < 0.05$ , Kruskal-Wallis; not shown in the graph), without significant differences between intrathecal treatments ( $n = 8$  per group).

PV, PT, or cell infiltrates in the paw. The slight decrease of morphine-induced PPT elevations over time may indicate tolerance development. Importantly, however, at both 6 and 48 h of intrathecal morphine administration (the time points of major interest), PTTs were clearly and significantly elevated, confirming continuous spinal nociceptive blockade.

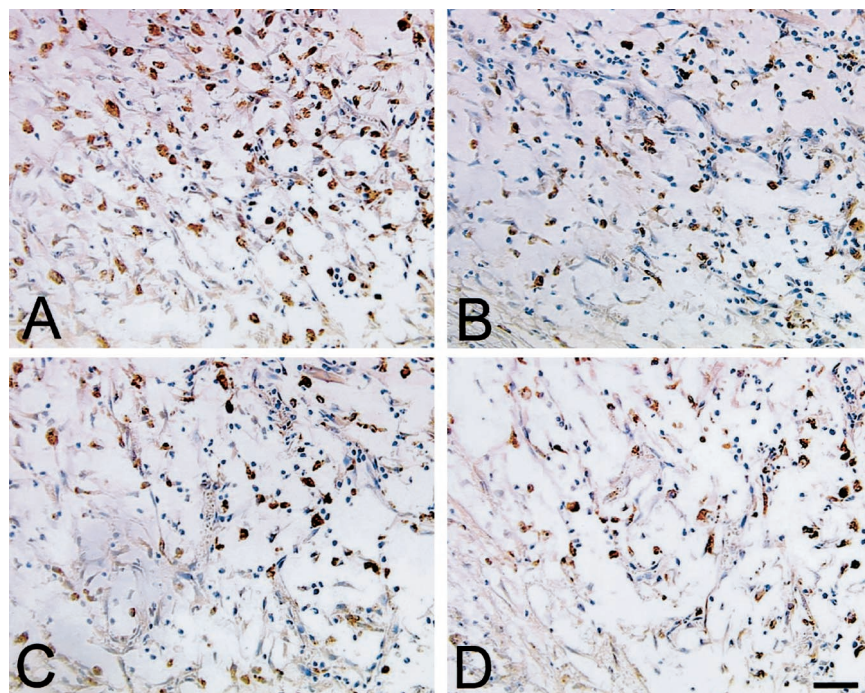
In the current study we found that granulocytes and monocytic cells are the major producers of opioid peptides during early and later stages of inflammation, respectively. These findings are consistent with our previously published data and have been discussed in detail.<sup>3</sup> As in this earlier study, we used a double-staining pro-

cedure for the identification of hematopoietic (CD45<sup>+</sup>) opioid (3E7<sup>+</sup>) cells by FACS to analyze opioid-containing immune cells in further detail. The monoclonal antibody (3E7) used for intracellular staining recognizes the pan-opioid sequence Tyr-Gly-Gly-Phe at the N-terminus of all opioid peptides.<sup>22</sup> This analysis did not yield significant differences in the composition of cell subpopulations or the number of opioid-containing cells between intrathecal morphine- and intrathecal saline-treated groups at either of the time points examined. However, the various opioid peptides are not equally relevant for the generation of endogenous analgesia. Our previous studies have shown (1) that inflamed tissue contains mostly END and ENK but nearly undetectable amounts of dynorphin<sup>2,8</sup>; (2) that immune cells contain and release END and ENK but very little dynorphin<sup>6,7</sup>; and (3) that END is the predominant peptide responsible for the generation of intrinsic (CWS-induced) analgesia during late stages of inflammation.<sup>8,17</sup> Therefore, it was necessary to further differentiate between END and ENK, the peptides of major interest. This was performed using immunocytochemistry since the available selective polyclonal antibodies are not applicable for intracellular staining by FACS.<sup>3</sup> We found no difference in ENK but a significant reduction of END cells in the intrathecal morphine-treated group after 48 h. This suggests that central morphine analgesia (*i.e.*, lack of central nociceptive transmission) leads to a selective inhibition of the migration of END cells to the inflamed site or to a selective down-regulation of END production in immune cells. Effects on cell migration seem less likely since the composition of cell subpopulations was not different between intrathecal treatments at either early or late stages of the inflammation. A change in END production, however, might explain both our immunohistochemical and flow cytometric findings: END and ENK can be simultaneously present in granulocytes, monocytes, and lymphocytes.<sup>28</sup> If END content but not ENK content decreases in a particular immune cell, these differences would be detected by immunohistochemistry because END- and ENK-specific antibodies were used. In contrast, staining with the 3E7 antibody recognizes both END and ENK, and therefore an isolated decrease of END but not ENK would still yield a positive fluorescence signal. This could explain the apparent difference between the immunohistochemical and flow cytometric results. While regulation of transcription and translation of the respective opioid precursors proopioidmelanocortin and proenkephalin have not been fully characterized in immune cells, there is evidence for differential gene regulation.<sup>28,29</sup> Thus, central opioid analgesia (or lack of central nociceptive processing) might lead to a selective down-regulation of END production in immune cells, but the mechanisms involved remain to be elucidated.

Simultaneously with the reduction of END cells, CWS-induced analgesia was significantly attenuated at 48 h.



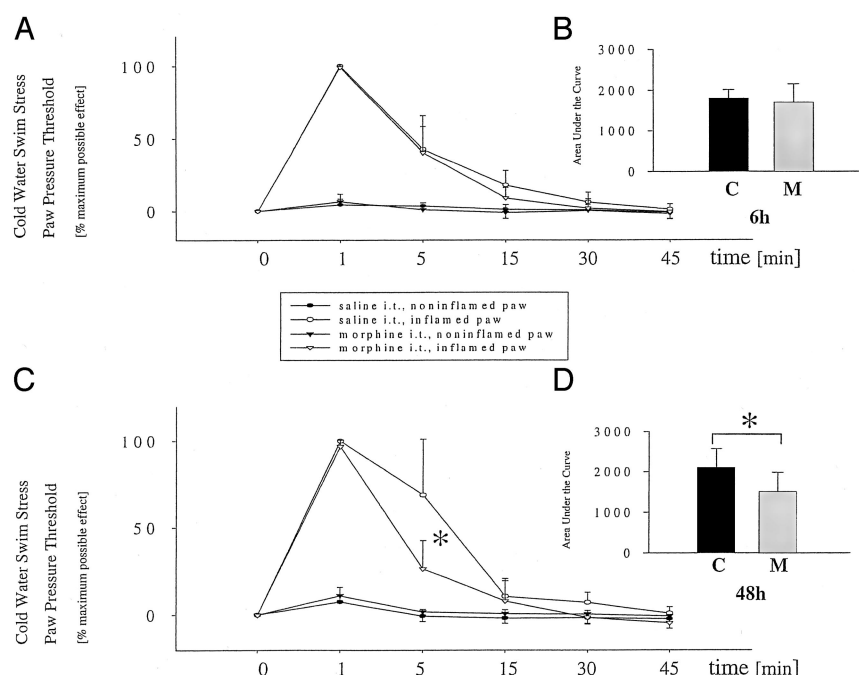
Fig. 5. Immunohistochemistry of  $\beta$ -endorphin (END<sup>+</sup>) and met-enkephalin (ENK<sup>+</sup>) cells in inflamed paws after 48 h of intrathecal treatment. The number of END<sup>+</sup> cells was significantly reduced ( $P < 0.05$ , Kruskal-Wallis test) in intrathecal morphine-treated (B) versus intrathecal saline-treated (A) rats. No significant difference was detectable in ENK<sup>+</sup> cells between intrathecal morphine (C) versus intrathecal saline (D) treatments ( $n = 8$  per group). Bar = 20  $\mu$ m.



This was mainly caused by a shorter duration rather than by a reduced maximum of the CWS-induced antinociceptive response. This is most probably a result of the fact that CWS produced the maximum possible effect at 1 min in all groups (*i.e.*, all animals reached the cutoff of 250 g) so that a further increase in the intrathecal saline-treated groups was technically undetectable. Alternatively, this finding is certainly consistent with a larger releasable pool of END in the intrathecal saline-treated animals but a limited number of available opioid receptors. Future studies will have to elucidate the underlying

mechanisms in further detail. Notwithstanding, the overall CWS-induced antinociceptive effect, as determined by the area under the curve, was significantly reduced in the intrathecal morphine-treated animals at 48 h. Since there were no significant differences between intrathecal treatments in CWS-induced analgesia or the number of opioid-containing cells at 6 h, it appears that the processes leading to a reduced presence of opioids in the inflamed tissue require time to become functionally effective. Together, these data show that the content of END cells and the generation of intrinsic local analgesia

Fig. 6. Antinociceptive effects following cold water swim (CWS) stress after intrathecal morphine treatment. After 48 h (C) but not after 6 h (A), PPT elevations were significantly lower in intrathecal morphine-treated compared with intrathecal saline-treated rats at 5 min after CWS (means  $\pm$  SD;  $n = 7$ ;  $^*P < 0.05$ , analysis of variance). The area under the curve was significantly decreased in intrathecal morphine-treated (M) rats compared with intrathecal saline-treated (C) rats at 48 h (D) but not at 6 h (B) (means  $\pm$  SD;  $n = 7$ ;  $^*P < 0.05$ , analysis of variance).



in injured tissue is significantly reduced in animals treated with central nociceptive blockade.

In summary, our findings suggest a neuroimmune interaction in the sense of a feedback mechanism. Stimulation of nociceptive afferents, strong enough to reach the central nervous system, apparently provokes opioid-containing immune cells to migrate—directed by adhesion molecules—to the injured tissue, where they release END to produce analgesia.<sup>9,11,12</sup> On the other hand, the END production or the homing of END cells to peripheral damaged tissue is reduced if the nociceptive stimulus is not “realized” in the central nervous system because of preemptive spinal blockade of neural transmission. Conceivably, such processes need time to become effective, which may explain why we did not observe differences at early stages (6 h) of the inflammatory reaction. Further studies will have to elucidate the molecular links between nociception and the site-directed traffic of opioid cells to injured tissue. Clinical studies will have to determine whether spinal anesthesia-analgesia influences the potency of endogenous peripheral mechanisms of pain control, e.g., in the postoperative setting.<sup>2</sup>

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