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Neurokinin-1 Receptor Antagonists Inhibit the Recruitment of Opioid-containing Leukocytes and Impair Peripheral Antinociception

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Background: **Neurokinins (*e.g.*, substance P) contribute to pain transmission in the central nervous system, peripheral neurogenic inflammation, and leukocyte recruitment in inflammation. Leukocyte recruitment involves (1) up-regulation of adhesion molecule expression through neurokinin-1 (NK_1) receptors on endothelial cells, (2) augmented chemokine production, or (3) chemotaxis through NK_1 receptors on leukocytes. In inflammation, leukocytes can trigger endogenous antinociception through release of opioid peptides and activation of opioid receptors on peripheral sensory neurons. The authors hypothesized that NK_1 receptor antagonists impair recruitment of opioid-containing leukocytes and stress-induced antinociception.**

Methods: **Rats were treated intraperitoneally and intrathecally with peripherally restricted (SR140333) or blood-brain barrier–penetrating (L-733,060) NK_1 receptor antagonists and were evaluated for paw pressure thresholds, numbers of infiltrating opioid-containing leukocytes and leukocyte subpopulations, expression of adhesion molecules, NK_1 receptors, and chemokines 24–48 h after complete Freund adjuvant–induced hind paw inflammation.**

Results: **Systemic and peripherally selective, but not intrathecal, NK_1 receptor blockade reduced stress-induced antinociception (control: 177 ± 9 g, L-733,060: 117 ± 8 g, and control: 166 ± 30 g, SR140333: 89 ± 3 g; both $P < 0.05$, *t* test) without affecting baseline hyperalgesia. In parallel, local recruitment of opioid-containing leukocytes was decreased (L-733,060 and SR140333: 56.0 ± 4.3 and 59.1 ± 7.9% of control; both $P < 0.05$, *t* test). NK_1 receptors were expressed on peripheral neurons, infiltrating leukocytes and endothelial cells. Peripheral NK_1 receptor blockade did not alter endothelial expression of intercellular adhesion molecule-1 or local chemokine and cytokine production, but decreased polymorphonuclear cell and macrophage recruitment.**

Conclusions: **Endogenous inhibition of inflammatory pain is dependent on NK_1 receptor–mediated recruitment of opioid-containing leukocytes.**

MANY cellular inflammatory mediators contribute to the generation of pain.[1,2] However, leukocyte recruitment also contributes to peripheral antinociception.[3] In complete Freund adjuvant (CFA)–induced inflammation, leukocytes containing opioid peptides (*e.g.*, β-endorphin and metenkephalin) migrate to inflamed tissue. After exposure to a cold water swim stress (CWS) or local injection of various releasing agents, opioid peptides can be liberated from leukocytes and bind to nearby opioid receptors on peripheral sensory neurons resulting in unilateral antinociception.[3–5] Both polymorphonuclear leukocytes (PMNs) and monocytes/macrophages cause opioid-mediated antinociception in different stages of inflammation.[6–8] Blockade of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) or selectins inhibits the recruitment of opioid-containing leukocytes into the paw and CWS-induced antinociception.[9–11] In early stages (*i.e.*, 0–12 h after CFA injection), chemokines (*i.e.*, CXCR2 ligands) are produced at the site of inflammation, mediate the recruitment of opioid-containing PMNs, directly trigger the release of opioid peptides from PMNs, and induce peripheral opioid-mediated antinociception.[5,6] In contrast, at later stages (*i.e.*, 24–96 h after CFA), molecular mechanisms for the recruitment of opioid-containing leukocytes have not yet been identified. PMNs are predominantly recruited in early inflammation (0–12 h after CFA), whereas macrophages are the principal leukocyte subpopulation at later stages (beyond 24 h after CFA).[8]

Leukocyte recruitment is classically mediated by chemokines, but other mediators, such as complement or neuropeptides, can also act as chemoattractants. Substance P is one of the neuropeptides and was originally described as a mediator in pain transmission in the central nervous system and in neurogenic inflammation in peripheral tissue.[12,13] Three neurokinin (NK_{1-3}) receptors have been identified. Substance P preferentially binds to NK_1 receptors.[14,15] NK_1 receptors are expressed on neurons in the peripheral and central nervous system as well as on leukocytes, endothelial cells, and keratinocytes.[16–18] Neurokinin receptor agonists enhance leukocyte migration by three distinct mechanisms: (1) direct chemotactic effects on monocytes and PMNs,[19–22] (2) binding to NK receptors on endothelial cells and increasing the expression of ICAM-1 and several selectins,[17,23,24] and (3) augmentation of local chemokine production (*e.g.*, CC chemokine ligand 2 [CCL2]; synonym: monocyte chemoattractant protein 1).[25]

In this study, we examined whether the blockade of NK_1 receptors (1) impairs opioid-mediated peripheral antinociception; (2) reduces recruitment of opioid-containing leukocytes; and (3) influences endothelial adhe-

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sion molecule expression, local chemokine production, and/or migration of PMNs or monocytes/macrophages at 24 or 48 h of CFA inflammation. To further characterize the site of action of NK_1 receptor agonists, we used systemic administration of blood-brain barrier–penetrating (L-733,060) and nonpenetrating NK_1 receptor antagonists (SR140333) as well as intrathecal injection.[26–28]

Materials and Methods

Animals and Model of Inflammation

Animal protocols were approved by local authorities and are in accordance with the guidelines of the International Association for the Study of Pain.[29] Male Wistar rats weighing 180–240 g were injected with 150 μl CFA (Calbiochem, La Jolla, CA) into the right hind paw (intraplantar) during brief isoflurane anesthesia and developed an inflammation confined to the inoculated paw. Experiments were conducted at 0–48 h after inoculation of CFA. All further injections were also performed during brief isoflurane anesthesia.

Measurement of Paw Pressure Threshold

Mechanical nociceptive thresholds were assessed using the paw pressure algesiometer (modified Randall-Selitto test; Ugo Basile, Comerio, Italy).[30] Rats were handled for 4 days before testing. On the day of testing, rats were held under paper wadding, and incremental pressure was applied *via* a wedge-shaped, blunt piston onto the dorsal surface of the hind paw by an automated gauge. The pressure required to elicit paw withdrawal, the paw pressure threshold, was recorded (cutoff at 250 g). The average of three trials, separated by 10-s intervals, was calculated. The same measurement was performed on the contralateral paw in alternated sequence to preclude order effects. To examine endogenous mechanisms of antinociception, paw pressure thresholds were determined at baseline conditions and 1 min after swimming in 2°–4°C cold water for 1 min.[8] An increase in paw pressure threshold was interpreted as mechanical antinociception. All behavioral experiments were performed by an examiner blinded to the treatment protocol.

Implantation of Spinal Catheters

Implantation was performed under continuous isoflurane anesthesia as described.[31] In brief, a 150-mm polyethylene tube (PE 10; Portex, Hythe, United Kingdom) was inserted intrathecally for 25 mm in cervical direction through an incision at the L3–L4 level. During the recovery period of 2 days, animals showing neurologic damage (*e.g.*, paralysis of the hind limbs) were excluded from the study. To ensure intrathecal localization of the catheter, 10 μl lidocaine, 2% (Braun, Melsungen, Germany), followed by a 10-μl solvent flush was applied, and the animals were monitored for development and reversal of paralysis of both hind limbs.

Fluorescence-activated Cell Sorting

Antibodies. All hematopoietic cells were stained by mouse anti-rat CD45 Cy5 phycoerythrin monoclonal antibody (clone OX-1, identifies the leukocyte common antigen, 4 μg/ml; BD Biosciences, Heidelberg, Germany).[32] PMNs were identified by mouse anti-rat RP-1 phycoerythrin monoclonal antibody (12 μg/ml). Macrophages were stained by mouse anti-rat CD68 fluorescein isothiocyanate monoclonal antibody (formerly called ED1; 2 μg/ml; Serotec, Oxford, United Kingdom). Opioid-containing cells were labeled by mouse 3E7 monoclonal antibody (recognizing the pan opioid sequence, 20 μg/ml; Gramsch Laboratories, Schwabhausen, Germany) followed by rabbit anti-mouse immunoglobulin (Ig)G_{2a+b} phycoerythrin antibody (15 μg/ml; BD Biosciences).[5,7] NK_1 receptor was stained by rabbit anti-rat NK_1 receptor serum (1:100; Chemicon, Hampshire, United Kingdom) at 4°C for 30 min and subsequently by goat anti-rabbit IgG- fluorescein isothiocyanate antitbody (15 μg/ml; Vector Laboratories, Burlingame, CA). Specificity of staining was controlled by isotype-matched control antibodies (mouse IgG_{2a}, 20 μg/ml, BD Biosciences; and rabbit IgG 4 μg/ml, Santa Cruz, Santa Cruz, CA).

Leukocyte Staining. Cell suspensions from paw tissue were prepared and stained as described.[5,7,32] Briefly, subcutaneous paw tissue was enzymatically digested and was pressed through a 70-μm nylon filter (BD Biosciences). Staining with anti-rat CD45 Cy5 phycoerythrin was done without permeabilization. For intracellular stains using 3E7, anti-rat–CD68 fluorescein isothiocyanate, and anti-rat RP-1 phycoerythrin, cells were fixed with 1% paraformaldehyde and permeabilized with saponin buffer. Permeabilized cells were incubated with the aforementioned primary and secondary antibodies. Replacement of the primary antibodies with isotype-matched irrelevant antibodies was used for negative controls.[11]

To calculate absolute numbers of cells per paw, fluorescence-activated cell sorting events from fluorescent TruCOUNT beads and $CD45^+$ stained cells were collected simultaneously, and the number of $CD45^+$ cells per tube was calculated accordingly. For quantification, 70,000 fluorescence-activated cell sorting events were acquired. Data were analyzed using CellQuest software (all BD Biosciences).

Enzyme-linked Immunosorbent Assay

All experiments were performed 0–48 h after intraplantar injection of CFA. Paw tissue was retrieved, the skin was removed, and subcutaneous tissue was cut into small pieces and processed as described.[6] Substance P, CXC chemokine ligand 1 (CXCL1; synonym: keratinocyte-derived chemokine), CCL2, and interleukin-1β (IL-1β) concentrations were measured by commercially available sub-

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stance P, mouse CXCL1, rat CCL2, and rat IL-1β enzyme-linked immunosorbent assay kits according to manufacturers' instructions (Bachem/Peninsula, London, United Kingdom; Biosource International, Nivelles, Belgium; and R&D, Minneapolis, MN, respectively). To measure CXCL1, a rat CXCL1 peptide standard was used as described.[6] Optical density was measured by Spectra Max (Molecular Devices, Ismaning, Germany). Data were analyzed by the Softmax program (Molecular Devices).

Immunofluorescence

The expression of ICAM-1 and NK_1 receptor in inflamed paw tissue was analyzed in rats (n = 5/group) treated with NK_1 receptor antagonists immediately before induction of inflammation and in control animals. Twenty-four hours later, rats were deeply anesthetized with halothane and perfused transcardially (0.1 M phosphate-buffered saline [PBS], followed by fixative solution: PBS containing 4% paraformaldehyde, pH 7.4). The skin with adjacent subcutaneous tissue was removed from both hind paws, postfixed for 30 min at 4°C in the fixative solution, and cryoprotected overnight at 4°C in PBS containing 10% sucrose. The tissue was embedded in Tissue Tek compound (OCT; Miles, Elkhart, IN), frozen, cut into 7-μm sections, mounted onto gelatin-coated slides, and processed for immunofluorescence.[33] The sections were incubated with mouse anti–ICAM-1 (1:200) alone or in combination with rabbit polyclonal anti–NK_1 receptor antibody (1:500) and then with secondary antibodies. After incubation with primary antibodies, the tissue sections were washed with PBS and then incubated with the appropriate secondary antibodies: a Texas red conjugated goat anti-rabbit antibody in combination with fluorescein isothiocyanate conjugated donkey anti-mouse. The sections were then washed with PBS, mounted in Vectashield or Vectashield mounting media containing 4′,6 diamidino-2-phenylindole (DAPI) (both Vector Laboratories, Burlingame, CA), and viewed under Zeiss LSM510META confocal laser scanning system (Carl Zeiss Imaging, Thornwood, NY). To demonstrate specificity of staining, the following controls were included as mentioned in details elsewhere[10] by omission of either the primary antisera/antibodies or the secondary antibodies. Cell types were identified based on morphologic criteria. The number of ICAM and NK_1 receptor expression in endothelial cells was quantified by an observer blinded to the experimental protocol using a Zeiss microscope (objective 20×).[10] The mean number of ICAM- or NK_1 receptor–positive vessels per section was calculated from counting 3 sections per animal and 5 squares (384 mm^2).

Experimental Protocols

Systemic Application of Nk_1 Receptor Antagonists. Rats were injected intraperitoneally every 12 h with 20 mg/kg L-733,060 (dissolved in sterile water and further diluted in up to 500 μl normal saline; Tocris, Bristol, United Kingdom) or 10 mg/kg SR140333 (dissolved in dimethyl sulfoxide and further diluted in up to 500 μl normal saline; Sanofi, Paris, France). Doses were chosen based on previous publications[34,35] and our pilot data. Control animals received solvent only (sterile water or dimethyl sulfoxide in normal saline, respectively). CFA was injected intraplantarly after the first injection, and experiments were performed 24–48 h later.

Intrathecal Application of Nk_1 Receptor Antagonists. Separate groups of rats were injected intrathecally with 0.5 mg/kg L-733,060 (20 μl every 12 h) followed by 10 μl NaCl (0.9%) flushes.[36] Doses were chosen in pilot experiments and did not produce any toxic effects. Control animals received solvent only. Immediately after the first injection, paw inflammation was induced by CFA, and tissue was harvested 24 h later.

Statistical Analysis

Data are presented as raw values or percentage control of baseline (mean ± SEM). Missing values are due to injection-related hematoma formation distorting fluorescence-activated cell sorting quantification of leukocyte subpopulations or due to accidental death by isoflurane overdose. Normally distributed data were analyzed by *t* test. Otherwise, the Mann–Whitney rank sum test was used. Multiple measurements were analyzed by one-way analysis of variance for normally distributed data and by two-way repeated-measures analysis of variance when dependent and independent factors were present. *Post hoc* comparisons were performed by the Student-Newman-Keuls and Holm-Sidak method, respectively. Differences were considered significant if $P < 0.05$.

Results

Stress-induced Antinociception Is Reduced by NK_1 Receptor Antagonists

Neurokinin-1 receptor blockade did not significantly change baseline paw pressure threshold in inflamed (fig. 1) or noninflamed paws (data not shown). At 24 h of inflammation, CWS-induced antinociception was significantly reduced by both intraperitoneal L-733,060 and intraperitoneal SR140333 treatments (figs. 1A and B), but not by intrathecal L-733,060 (fig. 1C). Furthermore, systemic treatment with L-733,060 did not alter baseline hyperalgesia at 48 h of inflammation, whereas it significantly reduced CWS-induced antinociception (table 1).

Recruitment of Opioid-containing Leukocytes Is Reduced by Blockade of NK_1 Receptors

Cold water swim stress–induced antinociception is mediated by opioid-containing leukocytes at the site of inflammation.[8,30] In the inflamed paw, 213 ± 11 × 10^3 $3E7^+CD45^+$ opioid-containing leukocytes per paw were

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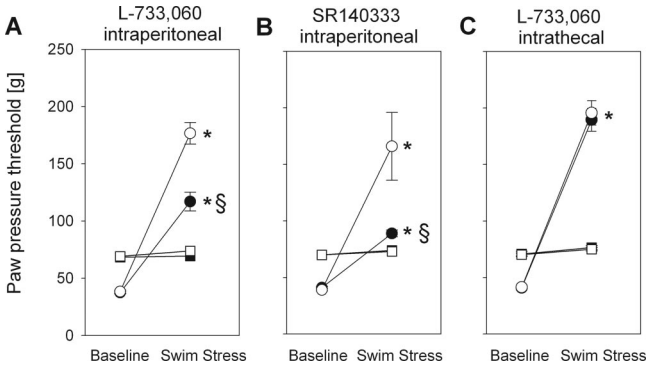

Fig. 1. Peripheral neurokinin-1 receptor blockade impaired stress-induced opioid-mediated antinociception. Wistar rats were treated with neurokinin-1 receptor antagonists (*A*: 20 mg/kg intraperitoneal L-733,060 [n = 6/group]; *B*: 10 mg/kg intraperitoneal SR140333 [n = 6/group]; *C*: 0.5 mg/kg intrathecal L-733,060 [control n = 7, drug n = 6]) immediately before and at 12 h after intraplantar injection of complete Freund adjuvant for induction of inflammation. Control animals received complete Freund adjuvant and solvent injections. Paw pressure thresholds were determined 24 h after induction of inflammation before (baseline) and after cold water swim stress (CWS) (* $P < 0.05$, baseline-CWS [F = 319 for intraperitoneal L-733,060, F = 196 for intraperitoneal SR140333, F = 458 for intrathecal L-733,060], § $P < 0.05$, drug × baseline-CWS [F = 23.2 for intraperitoneal L-733,060, F = 39.9 for intraperitoneal SR140333], $P > 0.05$, drug × baseline-CWS for intrathecal L-733,060, two-way repeated-measures analysis of variance). Inflamed (●, ○) and noninflamed contralateral paw (■, □), neurokinin-1 receptor antagonist (●, ■), and solvent control (○, □). Data are presented as mean ± SEM.

present at 24 h after CFA, as quantified by flow cytometry. At 24 h of inflammation, intraperitoneal L-733,060 or intraperitoneal SR140333 administration significantly reduced the number of opioid-containing leukocytes in the paw by 41% and 44%, respectively (figs. 2A and B), whereas no effect was observed after intrathecal L-733,060 treatment (fig. 2C). Systemic treatment (L-733,060 intraperitoneally) also significantly decreased the number of opioid-containing leukocytes in the paw at 48 h after CFA (control: 100 ± 7.4% *vs.* intraperitoneal L-733,060: 55 ± 7.2%; $P < 0.05$, *t* test).

Expression of NK_1 Receptors and Substance P at the Site of Inflammation

Levels of substance P were low in noninflamed paws (fig. 3A). At 24 and 48 h of inflammation, substance P

Table 1. Neurokinin-1 Receptor Blockade Reduces Stress-induced Antinociception but Not Baseline Hyperalgesia at 48 h of Inflammation

	Control	Intraperitoneal L-733,060
Baseline paw pressure threshold, g	36.9 ± 1.2	36.4 ± 1.3
CWS paw pressure threshold, g	170.3 ± 15.7*	125.0 ± 9.1*†

Data are presented as mean ± SEM.

* $P < 0.05$, baseline–cold water swim stress (CWS) (F = 172.2), † $P < 0.05$, drug × baseline-CWS (F = 6.9), two-way repeated-measures analysis of variance (n = 6/group).

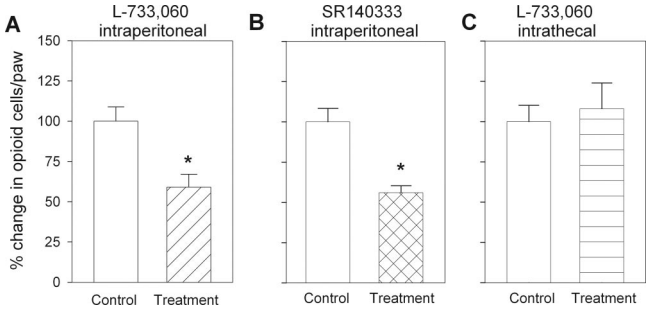

Fig. 2. Peripheral neurokinin-1 receptor blockade decreased the number of opioid-containing leukocytes at the site of inflammation. Rats were injected with solvent (control) or neurokinin-1 receptor antagonists (treatment) as well as complete Freund adjuvant as described in figure 1 (*A*: intraperitoneal L-733,060 [control n = 8, drug n = 7]; *B*: intraperitoneal SR140333 [n = 8/group]; and *C*: intrathecal L-733,060 [control n = 8, drug n = 6]). Opioid-containing $3E7^+CD45^+$ cells were quantified by flow cytometry 24 h after induction of inflammation (* $P < 0.05$, *t* test *vs.* solvent control). Data are presented as mean of percentage control ± SEM.

content in the paw was significantly increased, with a 17.5-fold increase at 24 h. NK_1 receptor expression was observed on endothelial cells as shown by colocalization with ICAM-1, peripheral neurons, and on $CD45^+$ leukocytes in the inflamed paw (figs. 3B–D).

Endothelial Adhesion Molecule Expression and Local Chemokine/Cytokine Content after NK_1 Receptor Blockade

Intercellular adhesion molecule 1 and NK_1 receptor immunoreactivities on endothelial cells were quantified. Both were significantly up-regulated at 24 h of inflammation (fig. 4, representative examples; and table 2). Blockade of NK_1 receptors using intraperitoneal SR140333 treatment did not alter ICAM-1 or NK_1 receptor expression at 24 h (table 2). At 24 h inflammation, intraperitoneal SR140333 treatment did not alter the content of CXCL1 (PMN specific), CCL2 (monocyte specific), or IL-1β (table 3).

NK_1 Receptor Blockade Reduces Migration of PMNs and Macrophages

At 24 h of CFA, similar numbers of monocytes/macrophages and PMNs per paw were detected by flow cytometry (245 ± 18 × 10^3 $CD68^+$ macrophages per paw and 219 ± 16 × 10^3 $RP1^+$ PMNs per paw; fig. 5). Treatment with intraperitoneal L-733,060 or intraperitoneal SR140333 significantly reduced infiltrating PMNs and macrophages (CFA 24 h: figs. 5A, B, D, and E; CFA 48 h: table 4). $CD3^+$ lymphocytes did not change (data not shown). Intrathecal administration of L-733,060 did not influence overall leukocyte migration or recruitment of leukocyte subpopulation at 24 h after CFA (figs. 5C and F).

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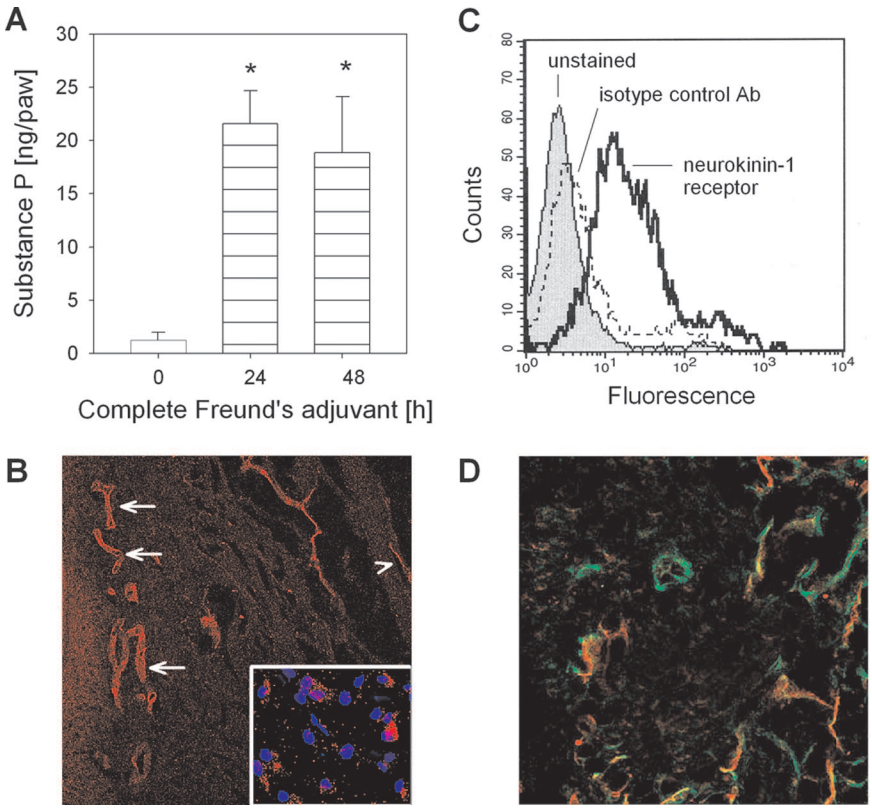

Fig. 3. Expression of substance P and neurokinin-1 (NK_1) receptors in the inflamed hind paw. (*A*) Substance P content was measured in subcutaneous paw tissue 0–48 h after complete Freund adjuvant injection (CFA) by enzyme-linked immunosorbent assay (* $P < 0.05$, F = 7.6, analysis of variance, Student-Newman-Keuls method *vs.* 0 h CFA; n = 6/group). Data are presented as mean ± SEM. (*B*) NK_1 receptor expression (*red*) in the inflamed paw was demonstrated by fluorescent immunohistochemistry 24 h after CFA injection. *Arrows* point to NK_1 receptor immunoreactive endothelial cells, and *arrowhead* points to neurons (magnification 20×). In the *inset*, NK_1 receptor expression is shown on infiltrating cells (nuclei stained in *blue*, magnification 40×). Representative sections are shown. (*C*) NK_1 receptor expression on $CD45^+$ cells in the paw was measured by flow cytometry 24 h after CFA injection (*gray histogram* = unstained control; *dotted line* = isotype control antibody; *thick black line* = anti-NK_1 receptor antibody). To exclude nonhematopoietic cells and cell debris, cells were pregated on $CD45^+$ leukocytes (data not shown). A representative histogram is shown. (*D*) To verify expression of NK_1 receptor on endothelial cells, inflamed paw tissue was analyzed by fluorescent immunohistochemistry for NK_1 receptor (*red*) and intercellular adhesion molecule 1 (*green*) 24 h after CFA injection. Coexpression of both markers is shown in *yellow* (magnification 20×).

Discussion

In this study, we demonstrated that NK_1 receptor antagonists (1) impaired stress-induced peripheral opioid-mediated antinociception but did not alter baseline inflammatory hyperalgesia and (2) reduced migration of opioid-containing leukocytes without changing expression of ICAM-1 on endothelial cells or local chemokine/cytokine production. Systemic administration of blood-brain barrier–penetrating (SR140333) and nonpenetrating (L-733,060) NK_1 receptor antagonists was equally effective, but intrathecal injection (L-733,060) was ineffective. Taken together, NK_1 receptor antagonists seem to act peripherally by directly inhibiting the recruitment of opioid-containing leukocytes to the site of inflammation.

Baseline inflammatory hyperalgesia was not reduced by administration of NK_1 receptor antagonists (figs. 1A and B and table 1). These findings are in line with previous animal studies[15,37] as well as studies in human pain patients.[12] Intrathecal application of NK_1 receptor antagonists was also ineffective in our study (fig. 1C), but it has been reported to reduce hyperalgesia in other models.[37–39] In those models, antihyperalgesic effects were short lasting (maximum of 6 h after injection), whereas we measured nociceptive thresholds at 24–48 h of inflammation and 12 h after the last injection of the NK_1 receptor antagonist. Long-lasting antihyperalgesic effects were only achieved by intrathecal injection of saporin-conjugated substance P that destroys NK_1 receptor–expressing spinal neurons.[40] Inflammatory hyperalgesia is induced by pronociceptive mediators,[2,41] including cytokines (*e.g.*, IL-1β[42]) and chemokines (*e.g.*, CXCL1[43] and CCL2[44,45]). In our studies, NK_1 receptor blockade did not change the local expression of representative hyperalgesic cytokines or chemokines (table 3), in line with the unaltered baseline nociceptive thresholds (fig. 1 and table 1).

In contrast to this lack of change in baseline hyperalgesia, stress (CWS)–induced antinociception was reduced by NK_1 receptor antagonists (figs. 1A and B and table 1). Our previous studies demonstrated that this form of antinociception is mediated by opioid peptide release from leukocytes at the site of inflammation and is fully blocked by opioid receptor antagonists in late stages of inflammation.[8,30,46] In the current study, we observed a comparable decrease in opioid-containing leukocytes in the inflamed paw using both blood-brain barrier–penetrating (L-733,060) and nonpenetrating (SR140333) NK_1 receptor antagonists, whereas intrathecal injection (L-733,060) was ineffective. The intrathecally injected dose of L-733,060 was previously used in rats and was shown to effectively inhibit NR2B tyrosine phosphorylation in the spinal cord in the CFA model.[36] In addition to intrathecal injection of L-733,060, we also examined intrathecal injection of SR140333 (up to 6

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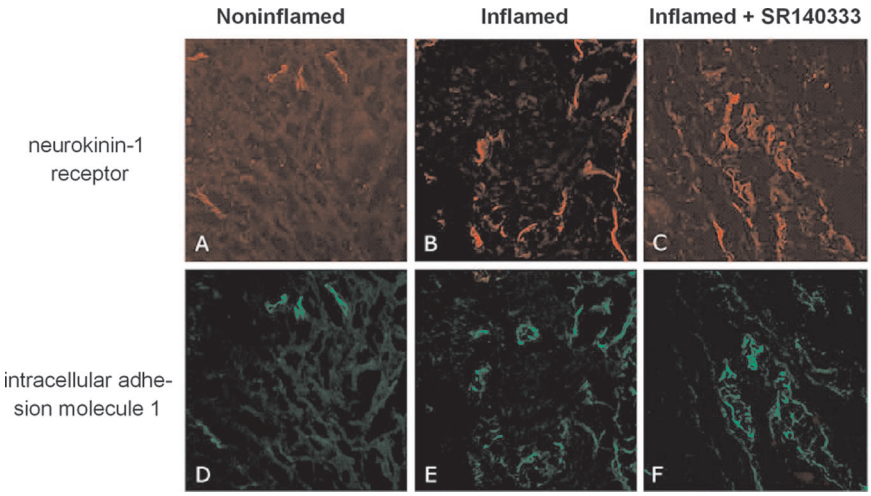

Fig. 4. Peripheral neurokinin-1 receptor blockade did not alter the expression of intercellular adhesion molecule 1 and neurokinin-1 receptors on endothelial cells at the site of inflammation. Rats (n = 5/group) were treated with complete Freund adjuvant for 24 h and with either neurokinin-1 receptor antagonist SR140333 (10 mg/kg intraperitoneal every 12 h; *C* and *F*) or solvent only (*B* and *E*). Noninflamed tissue was obtained from control animals without complete Freund adjuvant injection (*A* and *D*). Tissue was analyzed by fluorescent immunohistochemistry for neurokinin-1 receptor (*red*; *A–C*) and intercellular adhesion molecule 1 (*green*; *D–F*) (magnification 20×). Representative sections are shown.

μg/kg). This treatment did not reduce migration of opioid-containing leukocytes or stress-induced antinociception either, but we did not present the data because we observed neurotoxicity (*e.g.*, paralysis) at the highest doses. CWS-induced antinociception is mediated exclusively by locally secreted opioid peptides in subcutaneous inflammation.[30,47] In another paradigm, swim stress has been shown to involve IL-1 receptor activation,[48] but major differences exist between the two models: (1) presence *versus* absence of inflammation, (2) species (rats *vs.* mice), (3) type of swim stress (cold *vs.* warm water), and (4) tests of nociception (Randall-Selitto *vs.* hot plate). In particular, in our model CWS-induced antinociception cannot be blocked by an antibody against IL-1 or by an IL-1 receptor antagonist, indicating that this form of antinociception is not IL-1 dependent.[49] Taken together, our data show that NK_1 receptor antagonist treatment correlates with impaired recruitment of opioid-containing leukocytes as well as a decrease in opioid-mediated antinociception. This seems to involve a peripheral site of action (*i.e.*, outside the central nervous system). Conceivably, peripheral NK_1 receptors could be blocked on leukocytes, endothelial cells. or peripheral sensory neurons.

Table 2. Intercellular Adhesion Molecule-1 and Neurokinin-1 Receptor Expression Are Up-regulated at 24 h of Inflammation but Unaltered by Concomitant Neurokinin-1 Receptor Blockade

	Intercellular Adhesion Molecule 1	Neurokinin-1 Receptor
Noninflamed, vessels/section	1.90 ± 0.19	1.72 ± 0.13
CFA, vessels/section	5.54 ± 0.32*	4.69 ± 0.33*
CFA + SR140333, vessels/section	4.46 ± 0.30*	4.16 ± 0.34*

Data are presented as mean ± SEM.

* $P < 0.05$, noninflamed *vs.* complete Freund adjuvant (CFA) and noninflamed *vs.* CFA + SR140333, $P > 0.05$ CFA *vs.* CFA + SR140333, analysis of variance, *post hoc* comparison, Student-Newman-Keuls method (intercellular adhesion molecule 1: F = 5.9, neurokinin-1 receptor: F = 4.3; n = 5/group and 3 sections/animal).

To further elucidate the mechanisms of reduced migration of opioid-containing leukocytes, we measured substance P content and NK_1 receptor expression in the inflamed paw. Local substance P content increased during CFA-induced inflammation (fig. 3A). Potential sources of substance P are the peripheral sensory neurons and infiltrating leukocytes.[13,50,51] In addition to increased substance P amounts, we detected NK_1 receptor expression on all infiltrating leukocytes, on neurons, and on endothelial cells (figs. 3B–D), confirming previous studies.[16,17,20,52] While neurogenic inflammation contributes to leukocyte infiltration, it is unclear whether blockade of NK_1 receptors on peripheral sensory neurons influences leukocyte recruitment.[53–55] However, NK_1 receptors have previously been shown to enhance leukocyte recruitment by increasing adhesion molecule expression on endothelial cells,[17,23,24] by augmenting chemokine production[25] or by directly inducing chemotaxis of leukocytes.[19–22]

Adhesion molecules (*i.e.*, ICAM-1, selectins, and integrins) have previously been shown to mediate the recruitment of opioid-containing leukocytes, and their blockade can impair peripheral opioid-mediated antinociception during CFA-induced inflammation.[9,11] In the current study, expression of ICAM-1 on endothelial cells was increased at later stages of inflammation (24 h after CFA; table 2). Neither the CFA-induced up-regulation of

Table 3. Neurokinin-1 Receptor Blockade Does Not Influence Local Chemokine or Cytokine Expression at 24 h of Inflammation

	CXCL1	CCL2	IL-1β
CFA, ng/paw	2.79 ± 0.30	0.39 ± 0.05	1,768 ± 368
CFA + SR140333, ng/paw	3.10 ± 0.58	0.44 ± 0.04	1,838 ± 383

Data are presented as mean ± SEM.

$P < 0.05$, differences between chemokines/cytokine, $P > 0.05$ for drug and drug × chemokines/cytokine, two-way repeated-measures analysis of variance (F = 36.7, n = 6).

CCL2 = CC chemokine ligand 2; CFA = complete Freund adjuvant; CXCL1 = CXC chemokine ligand 1; IL-1β = interleukin-1β.

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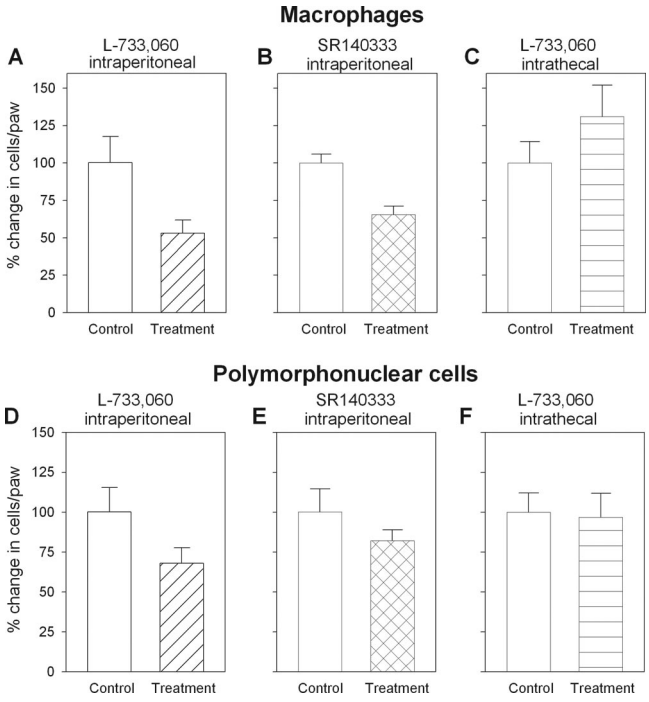

Fig. 5. Peripheral neurokinin-1 receptor blockade decreased the number of infiltrating leukocytes in the inflamed paw. Rats were treated (intraperitoneal L-733,060 [control n = 8, drug n = 7], *A* and *D*; intraperitoneal SR140333 [n = 8/group], *B* and *E*; and intrathecal L-733,060 [n = 7/group], *C* and *F*) as described in figure 1. $CD68^+CD45^+$ macrophages (*A–C*) and $RP1^+CD45^+$ polymorphonuclear cells (*D–F*) were quantified by flow cytometry 24 h after complete Freund adjuvant injection ($P < 0.05$ for drug, not shown in the figure [F = 8.2 for intraperitoneal L773,060 and F = 8.6 for SR140333], $P > 0.05$ for drug intrathecal L-733,060, $P > 0.05$ for drug × leukocyte subpopulation for all drugs, two-way analysis of variance). Data are presented as mean of percentage control ± SEM.

ICAM-1 nor that of NK_1 receptors on endothelial cells was altered by peripheral NK_1 receptor blockade (fig. 4 and table 2). Previous *in vitro* studies have demonstrated that substance P increased the expression of ICAM-1[16,17] as well as NK_1 receptors.[56,57] In *in vivo* models of inflammation, the effects of NK_1 receptor antagonists vary between experimental models: They decreased NK_1 receptor but not ICAM-1 expression in a mouse model of pancreatitis.[58] In contrast, they reduced ICAM-1 expression in experimental autoimmune encephalomyelitis.[59] In our model, NK_1 receptor antagonists reduced the recruitment of opioid-containing leukocytes, but they did not alter the expression of ICAM-1 or NK_1 receptors on the inflamed endothelium. We cannot fully exclude the possibility that NK1 receptor antagonists alter the expression of other adhesion molecules such as selectins or integrins and, thereby, impair peripheral antinociception.

Table 4. Neurokinin-1 Receptor Blockade Reduces Migration of Infiltrating Leukocytes at 48 h of Inflammation

	Control	L-733,060
Macrophages, %	100 ± 7.1	42.7 ± 9.8*
PMNs, %	100 ± 13.7	67.3 ± 10.4*

Data are presented as percentage control of baseline ± SEM.

* $P < 0.05$ for drug (F = 8.2), $P > 0.05$ for leukocyte subpopulation and drug × leukocyte subpopulation, two-way repeated-measures analysis of variance (n = 8/group).

PMNs = polymorphonuclear cells.

As a second hypothesis, NK_1 receptor antagonists might decrease local chemokine production and thereby impair selective leukocyte recruitment. Substance P has been shown to up-regulate chemokine production in leukocytes and epithelial cells *in vitro*.[60,61] In experimental pancreatitis, local chemokine expression was reduced by an NK_1 receptor antagonist.[62] We examined a PMN- and a monocyte/macrophage-specific chemokine in our model (*i.e.*, CXCL1[63] and CCL2,[64] respectively), and their expression was not altered by NK_1 receptor antagonists (table 3). Multiple chemokines are responsible for PMN and monocyte/macrophage recruitment,[65] and alterations in other chemokines might occur after treatment with NK_1 receptor antagonists. Although this possibility cannot be excluded, our previous study demonstrated that PMN-specific chemokines (*i.e.*, CXCR2 ligands) were regulated as a group and did not show relevant differences between individual chemokines.[6] Taken together, in our model NK_1 receptor antagonists reduce the migration of opioid-containing leukocytes, but this effect does not seem to be mediated through alterations in adhesion molecule expression on endothelial cells or in local chemokine production.

Third, substance P binding to NK_1 receptors on leukocytes might directly induce migration of opioid-containing leukocytes. This migration might be reduced by NK_1 receptor blockade. Several studies demonstrated that NK_1 receptor agonists induce chemotaxis of PMNs and monocytes/macrophages *in vitro*.[19,66,67] Previous studies have shown that PMNs and monocyte/macrophage migration to the site of inflammation is impaired after treatment with NK_1 receptor antagonists or in NK_1 receptor–deficient mice.[20,24,68,69] In line with these studies, the number of infiltrating macrophages and PMNs as well as of opioid-containing leukocytes was reduced in our model (figs. 2 and 5 and table 4).

In conclusion, we have shown that NK_1 receptor antagonists reduce the recruitment of opioid-containing leukocytes into the inflamed paw and thereby impair opioid-mediated antinociception in CFA inflammation. NK_1 receptor agonists such as substance P seem to directly induce preferential recruitment of opioid-containing monocytes/macrophages while no indirect effects (*i.e.*, alterations in adhesion molecule expression or chemokine production) are observed. Most likely, NK_1 receptor antagonists act directly on NK_1 receptor–expressing, opioid-containing leukocytes, but the effects might also be mediated indirectly through NK_1 receptors on peripheral neurons. Taken together, NK_1 receptor agonists are important in the recruitment of opioid-con-

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taining leukocytes and the generation of peripheral opioid-mediated antinociception. The impairment of endogenous opioid-mediated peripheral analgesia might be an additional explanation for the lack of efficacy of NK_1 receptor antagonist in human studies.[12]

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