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Local upregulation of corticotropin-releasing hormone and interleukin-1 receptors in rats with painful hindlimb inflammation [1]

Shaaban A. Mousa [a,c], Michael Schäfer [a,c,*], W. Mark Mitchell [b], Ahmed H.S. Hassan [a], Christoph Stein [c]

[a] *Preclinical Pharmacology Laboratory, Division of Intramural Research, National Institute on Drug Abuse, NIH, 4940 Eastern Ave., Bld. C, Baltimore, MD 21224, USA*

[b] *Neuroscience Branch, Division of Intramural Research, National Institute on Drug Abuse, Baltimore, MD 21224, USA*

[c] *Department of Anesthesiology and Critical Care Medicine, The Johns Hopkins University, School of Medicine, Baltimore, MD 21287-8711, USA*

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Abstract

Opioid peptides derived from immune cells produce analgesia by activating opioid receptors on peripheral sensory nerves in inflammation. Corticotropin-releasing hormone (CRH) and interleukin-1β (IL-1β) can release these opioids. Here we show that both corticotropin-releasing hormone and interleukin-1β elicit receptor-specific antinociception in inflamed paws of rats by an opioid-mediated mechanism. Autoradiographic studies demonstrate ^{125}I-CRH and ^{125}I-IL-1β binding sites on immune cells in lymph nodes and inflamed paws. This binding is of high affinity and displaceable by the respective unlabeled agonist and antagonist ligands but not by opioid or adrenergic compounds. ^{125}I-CRH and ^{125}I-IL-1β binding sites are absent on nerves and in non-inflamed subcutaneous tissue but their number is greatly enhanced in inflamed paws and lymph nodes. This upregulation of binding sites for the opioid-releasing agents corticotropin-releasing hormone and interleukin-1β likely represents part of the body's local response to combat inflammatory pain.

Keywords: Neuro-immune interaction; Analgesia; Nociception; Opioid; Neuropeptide; Cytokine

1. Introduction

Under inflammatory and other pathological conditions, various types of immunocytes have been shown to produce and contain opioid peptides in culture (Sibinga and Goldstein, 1988; Sharp and Linner, 1993) and in situ (Stein et al., 1990; Przewlocki et al., 1992). Opioid-containing immune cells have also been detected in human arthritis, suggesting clinical implications of these findings (Wiedermann et al., 1992; Stein et al., 1993). Indeed, immune-derived opioids (predominantly β-endorphin) are released during environmental stressful stimuli and inhibit pain in animals (Stein et al., 1990; Przewlocki et al., 1992) and in humans (Stein et al., 1993) by interacting with opioid receptors located on peripheral sensory nerve terminals (Stein et al., 1990; Hassan et al., 1993). These findings strongly suggest that, beyond their well known functions to fight and protect against pathogens and antigens, immune cells can also serve to decrease pain (Stein, 1995).

Opioid peptides can be actively released by stimulating immunocytes with corticotropin-releasing hormone (CRH) or interleukin-1β (IL-1β) in vitro (Smith et al., 1986; Heijnen et al., 1991; Schäfer et al., 1994). Together with previous reports on binding of corticotropin-releasing hormone (Singh and Fudenberg, 1988; Webster and De Souza, 1988; Webster et al., 1990) and interleukin-1 (Uhl et al., 1989; Dinarello, 1994) to splenic and circulating immunocytes, these findings strongly suggest the presence of corticotropin-releasing hormone and interleukin-1β receptors on immune cells in inflamed tissue. The present study investigated in vivo antinociceptive effects and binding of corticotropin-releasing hormone and interleukin-1β in a model of chronic inflammatory pain. Using autoradiography, we examined subcutaneous tissue, lymph nodes and spleen to determine quantitative differences in inflamed versus non-inflamed tissue and the anatomical localization of the binding sites. The specificity and selectivity of this binding were assessed by displacement and cross-competi-

* Corresponding author. Tel.: 410-550-3399; fax: 410-550-1648.

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tion experiments. Finally, a possible binding of corticotropin-releasing hormone to CRH binding protein, rather than CRH receptors, was examined by comparing the binding of ovine corticotropin-releasing hormone (which binds to the receptor but not to the binding protein) to that of rat/human corticotropin-releasing hormone (which binds to both) (Lowry, 1995).

2. Methods

2.1. Animals

The guidelines on ethical standards by the International Association for the Study of Pain were followed. Animal facilities were accredited by the American Association of Laboratory Animal Care and experiments were approved by the Institutional Animal Care and Use Committee of DIR/NIDA/NIH in accordance with the Institute of Laboratory Animal Resources, National Research Council, Department of Health, Education and Welfare, Publication (NIH) 85-23, revised 1985. Adult male Wistar rats weighing 180–250 g were obtained from Charles River Breeding Laboratories and housed individually in cages lined with ground corn cob bedding. Room temperature was maintained at 22 ± 0.5°C and a relative humidity between 40% and 60%. A 12 : 12 h (7 a.m./7 p.m.) light/dark cycle was used, with food and water available ad libitum. Rats received an intraplantar injection of 0.15 ml Freund's complete adjuvant into the right and 0.9% saline into the left hindpaw under brief halothane anesthesia. Control animals were anesthetized but not injected. Animals were handled at least three times before any testing was performed.

2.2. Parameters of inflammation

Paw volume and paw temperature were assessed before and 4 days after Freund's complete adjuvant injection. The volume was assessed by submerging the hindpaws to the tibiotarsal joints into a water filled Perspex cell of a plethysmometer (Ugo Basile, Comerio, Italy). The volume of displacement, which is equal to the paw volume, was then read on a digital display. The surface temperature of the plantar skin was measured with an Infrared radiation thermometer (Ultrakust, Ruhmannsfelden, Germany). These parameters were taken twice and the average calculated.

2.3. Algesiometric testing

Nociceptive thresholds were evaluated by use of a paw pressure test with the observer blind to the experimental condition employed. Animals ($n = 6–12$ per group) were gently restrained and incremental pressure (maximum 250 g) was applied onto the dorsal surface of the hindpaw. The pressure required to elicit paw withdrawal, the paw pressure threshold was determined. Baseline paw pressure thresholds were tested before and 4 days after inoculation with Freund's complete adjuvant. In separate experiments (4 days Freund's complete adjuvant), rats received an intraplantar injection of either vehicle or different doses of corticotropin-releasing hormone (0.1–1.5 ng) or interleukin-1β (0.2–2 ng). Paw pressure thresholds were determined at the time of the maximal effect (5 min after injection). Finally, the attenuation of these effects by α-helical CRH (0.1–2 ng) or interleukin IL_1 receptor antagonist (1–50 ng), respectively, and by naloxone (0.01–5 μg) was examined.

2.4. Reagents

Rat/human ^{125}I-CRH, ^{125}I-Tyr°-ovine CRH and human recombinant ^{125}I-IL-1β (specific activities 2200–4200 Ci/mmol) were obtained from DuPont-New England Nuclear, Boston, MA. Unlabeled CRH, CRH antagonist (α-helical-CRH), (−)-naloxone hydrochloride and (−)-propranolol were purchased from Sigma Co. (St. Louis, MO). Recombinant human interleukin-1β and recombinant human interleukin IL_1 receptor antagonist were obtained from R and D Systems, Minneapolis, MN and Freund's complete adjuvant was purchased from Calbiochem (La Jolla, CA).

2.5. Tissue preparation

Four days after intraplantar injection of Freund's complete adjuvant (or after anesthesia in controls), rats were killed by decapitation. Popliteal lymph nodes, subcutaneous tissue from hindpaws and spleen were rapidly removed. The tissues were immersed in cold isopentane, stored at −80°C, and sectioned (20 μm) using a cryostat (Hacker instruments, Fairfield, NJ) at −16°C. Paw tissue was cut along the longitudinal axis and lymph nodes were cut in the sagittal plane. Tissues were thaw mounted onto precleaned chrome alum/gelatin subbed microscope slides, dried in a desiccator at 4°C, and then stored at −80°C.

2.6. Characterization of CRH binding sites

Slide-mounted tissue sections were brought to room temperature and preincubated for two 15-min periods in 50 mM Tris-HCl (pH 7.4) containing 5 mM $MgCl_2$, 2 mM EGTA, 0.1% bovine serum albumin (Fraction V, Sigma), aprotinin (100 kallikrein units/ml), and 0.1 mM bacitracin (Sigma) at room temperature in order to dissociate endogenous ligands, followed by 2 h incubation in the same buffer containing 0.1 nM ^{125}I-Tyr°-ovine CRH or 0.1 nM ^{125}I-rat/human CRH, respectively. Controls were incubated in the same medium with the addition of 1 μM corticotropin-releasing hormone, 5 μM α-helical-CRH, 200 nM interleukin-1β, 5 μM naloxone or 1 μM propranolol,

respectively. These concentrations were previously found to significantly inhibit binding of the respective specific ligands (Hassan et al., 1993; Chai et al., 1990; Takao et al., 1991). In further competitive displacement experiments, consecutive adjacent sections were incubated with increasing concentrations of unlabeled corticotropin-releasing hormone (10 pM–1 mM) in the presence of 0.1 nM ^{125}I-Tyr^{0}-ovine CRH. After incubation, tissue sections were washed for three 5 min periods in Dulbecco's phosphate-buffered saline containing 1% bovine serum albumin and 0.01% Triton X-100 at pH 7.4 and 4°C, dipped in deionized water, and dried rapidly under a stream of cold air.

2.7. Characterization of IL-1 binding sites

Slide mounted tissue sections were brought to room temperature and preincubated for two 15-min periods in RPMI-1640 (Sigma) with 20 mM HEPES (Sigma) and 0.1% bovine serum albumin at room temperature in order to dissociate endogenous ligands, followed by 3 h incubation with 0.2 nM ^{125}I-IL-1β in RPMI-1640 containing 50 μg/ml gentamicin, 20 mM HEPES, 1 mg/ml sodium azide (Sigma), aprotonin (100 kallikrein units/ml), 10 μM bacitracin and 0.1% bovine serum albumin at 37°C. Controls were incubated in the same medium with the addition of 200 nM interleukin-1β, 200 nM interleukin IL_1 receptor antagonist, 1 μM corticotropin-releasing hormone, 5 μM naloxone or 1 μM propranolol, respectively. These concentrations were previously found to significantly inhibit binding of the respective specific ligands (Hassan et al., 1993; Takao et al., 1991; Mackenzie et al., 1989). In further competitive displacement experiments, consecutive adjacent sections were incubated with increasing concentrations of unlabeled interleukin-1β (0.1 nM–400 nM) in the presence of 0.1 nM ^{125}I-IL-1β. After incubation, tissue sections were washed for three 5-min periods in Dulbecco's phosphate-buffered saline containing 1% bovine serum albumin and 0.01% Triton X-100 at pH 7.4 and 4°C, dipped in deionized water, and dried rapidly under a stream of cold air.

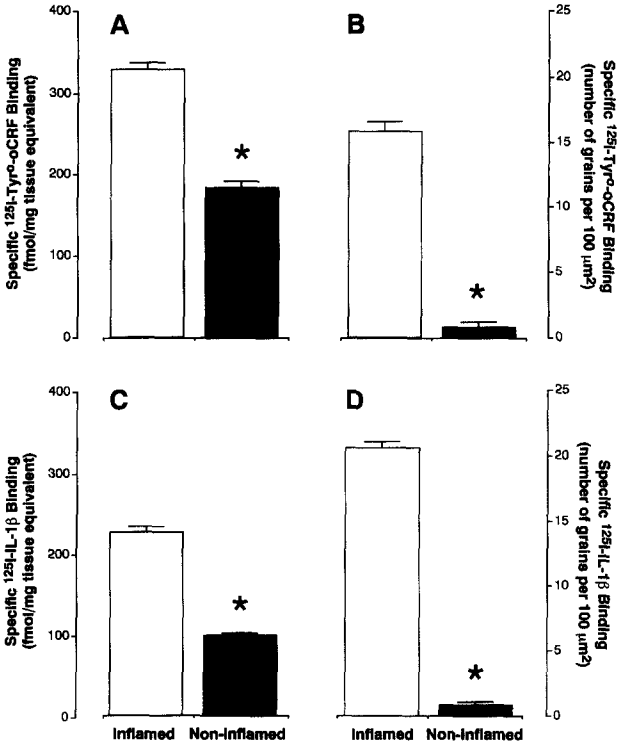

Fig. 2. Specific ^{125}I-Tyr^{0}-ovine CRH (^{125}I-oCRH) (A, B) and ^{125}I-IL-1β (C, D) binding, as assessed in autoradiographic films (A, C) and by counting of autoradiographic silver grains in photoemulsion-coated sections (B, D), in inflamed and non-inflamed subcutaneous paw tissue. Data represent means and standard errors of five experiments ($n = 5$ per experiment). Specific ^{125}I-oCRH and ^{125}I-IL-1β binding was calculated by subtracting non-specific binding (determined in the presence of 1 μM CRH or 200 nM IL-1β, respectively) from total binding. Asterisks denote significant differences between inflamed and non-inflamed paws (* $P < 0.05$; Wilcoxon test). Experiments using ^{125}I-rat/human CRH yielded similar results (data not shown).

2.8. Autoradiographic processing and development

The labeled slide-mounted sections and ^{125}I autoradiographic standards (Amersham, Arlington Heights, IL) were apposed to Tritium-Hyperfilm (Amersham). Seven to 14

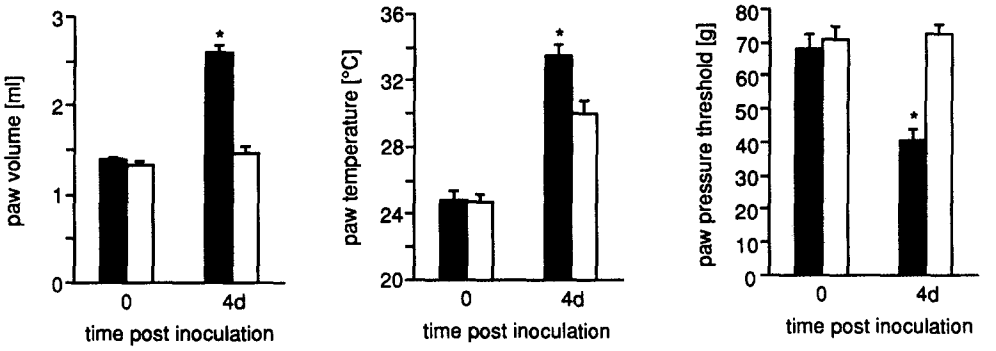

Fig. 1. Volume (left), temperature (center) and thresholds to noxious pressure (PPT) (right) of inflamed (hatched bars) and non-inflamed (open bars) paws before and after unilateral inoculation with FCA. Data represent means and standard errors ($n = 12$). Asterisks denote significant differences between inflamed and non-inflamed paws (* $P < 0.05$; Wilcoxon test).

days later they were tank developed for 5 min in GBX developer (Eastman Kodak Co., Rochester, NY), washed for 1 min in stop bath, fixed in GBX fixer (Kodak) for 5 min, washed in running water at 20°C for 20 min, and then air dried. The tissue was finally stained with thionin. Selected sections were processed for light microscopic autoradiography. They were exposed to paraformaldehyde vapors at 80°C for 2 h to fix and maintain the ligand receptor complexes before being individually dipped into Kodak NTB-3 emulsion diluted with distilled water (1 : 1). After 7-14 days exposure at 4°C, emulsion-coated slides were developed in GBX developer at 20°C for 4 min, fixed in GBX fixer for 8 min, and counterstained with thionin.

2.9. Data analysis

In autoradiographs prepared with Tritium-Hyperfilm, optical density readings and construction of standard curves were carried out using a Loats PC-based computerized image analysis system (Loats Associates, Westminster, MD). Measurements were made within 1 mm^2 in regions of interest (in paw and lymph nodes) or over entire sections (in spleen) in three sections per animal and tissue. Five animals were used for each experiment. The optical density obtained was related to fmols bound/mg tissue equivalent by reference to a standard curve. In autoradiograms prepared with NTB-3 emulsion, grains (black) and cells (blue staining) were counted in the same anatomical regions. In each section, 20 areas of 100 μm^2 were counted using a X100 oil immersion objective on a Zeiss microscope equipped with a grid containing eyepiece. Five animals (3 sections per animal) were used for each experiment. Specific binding was defined as the difference between total binding of the radioactive ligand and residual binding in the presence of both labeled and unlabeled ligand. In the displacement experiments, data were transformed into Scatchard and Hill plots by using the EBDA/LIGAND computer program and dissociation constants (K_d), maximal number of binding sites (B_{max}) and Hill coefficients were calculated. These experiments were performed in duplicate and for each concentration of unlabeled ligand, 3 rats, 2 sections per rat and 20–30 sections of 100 μm^2 were counted. Multiple comparisons between groups were made using the Friedman analysis of variance.

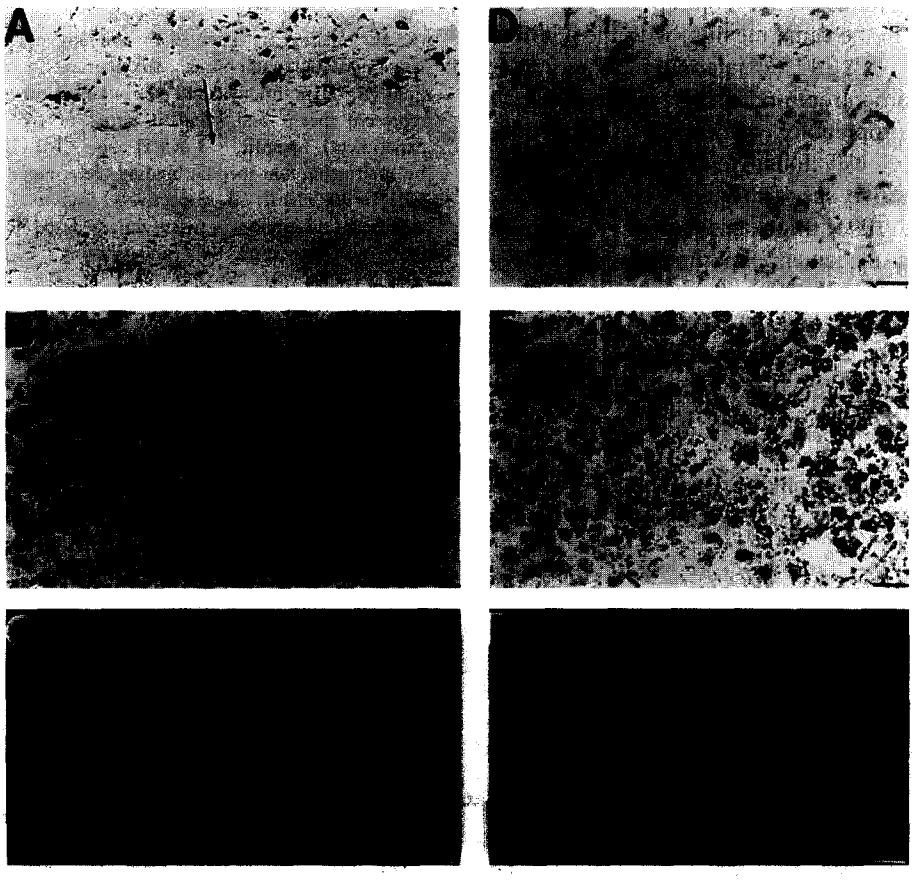

Fig. 3. Photoemulsion autoradiography of ^{125}I-Tyr°-ovine CRH (A, B) and ^{125}I-IL-1β (D, E) binding sites in non-inflamed (A, D) and inflamed (B, E) subcutaneous paw tissue. (F) shows displacement of ^{125}I-IL-1β binding by unlabeled IL-1β in inflamed tissue. Displacement of ^{125}I-Tyr°-ovine CRH by unlabeled CRH yielded similar results (not shown). Note the absence of binding on the nerve (arrow) in (A) and binding on lymphocytes (large arrow) and macrophages (small arrow) in (B, E). Darkfield photomicrographs of tritium-sensitive ultrofilm show the distribution of ^{125}I-Tyr°-ovine CRH binding in the inflammatory foci (arrow) of subcutaneous paw tissue (C) and of non-specific binding in the presence of 1 μM unlabeled CRH (F). The distribution of ^{125}I-IL-1β binding was similar (not shown). Bars represent: 10 μm (A), 5 μm (B, D, E) and 280 μm (C, F).

Post hoc tests were performed using the Wilcoxon test for dependent variables and the Mann-Whitney *U*-test for independent variables. Differences were considered significant when $P < 0.05$ (two-tailed).

3. Results

3.1. Parameters of inflammation

Both volume and temperature of the inoculated paws increased significantly from baseline levels ($P < 0.01$; Wilcoxon test) (Fig. 1). The volume of non-inflamed paws did not change during the observation period ($P > 0.05$; Wilcoxon test) (Fig. 1). The temperature of non-inflamed paws increased, probably due to a general hyperthermia caused by the inflammation, but remained significantly below that of inoculated paws ($P < 0.05$; Wilcoxon-test) (Fig. 1).

3.2. Algesiometry

Baseline paw pressure thresholds were similar in both paws ($P > 0.05$; Wilcoxon test) (Fig. 1). Four days after inoculation, paw pressure thresholds did not change in non-inflamed paws ($P > 0.05$; Wilcoxon test) but decreased significantly in inflamed paws ($P < 0.05$; Wilcoxon test) (Fig. 1). Similar to our previous studies (Schäfer et al., 1994), intraplantar injection of corticotropin-releasing hormone and interleukin-1β significantly elevated paw pressure thresholds above baseline in inflamed but not in non-inflamed paws (data not shown). Corticotropin-releasing hormone- and interleukin-1β-induced paw pressure threshold elevations were dose-dependent and significantly antagonized by α-helical-CRH and interleukin IL_1 receptor antagonist, respectively, and by naloxone (data not shown).

3.3. Paw tissue

Consistent with our previous studies, intraplantar injection of Freund's complete adjuvant produced a marked infiltration of inflammatory foci within the plantar subcutaneous tissue with lymphocytes and monocytes/macrophages (Stein et al., 1990; Przewlocki et al., 1992). These signs remained confined to the inoculated paw. Autoradiographic films showed that more than 80% of the total binding was displaceable by the respective unlabeled ligands (Fig. 3). The specific binding of ^{125}I-Tyrz°-ovine CRH and ^{125}I-IL-1β was significantly higher in inflamed than in non-inflamed paw tissue ($P < 0.05$, Wilcoxon test) (Fig. 2A and C). Examination by light microscopy of photoemulsion-coated sections revealed that CRH and IL-1β binding sites were mainly associated with cells of subcutaneous inflammatory foci (Fig. 3B and E), while binding was practically absent in non-inflamed subcutaneous tissue (Fig. 3A and D) and on nerves in non-inflamed (Fig. 3A) and inflamed paws (not shown). Some binding was observed on muscle fibers, which likely accounts for the radioactivity detected on autoradiographic films of non-inflamed tissue (Fig. 2A and C). The morphological appearances of the majority of labeled cells are consistent with lymphocytes and macrophages (Fig. 3B and E).

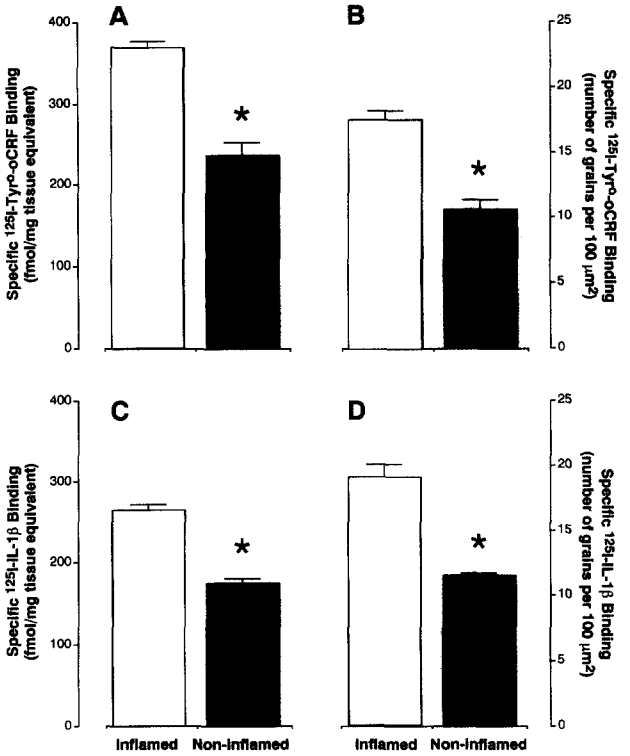

Fig. 4. Specific ^{125}I-Tyr°-ovine CRH (^{125}I-oCRH) (A, B) and ^{125}I-IL-1β (C, D) binding, as assessed in autoradiographic films (A, C) and by counting of autoradiographic silver grains in photoemulsion-coated sections (B, D), in inflamed and non-inflamed popliteal lymph nodes. Data represent means and standard errors of five experiments ($n = 5$ per experiment). Specific ^{125}I-oCRH and ^{125}I-IL-1β binding was calculated by subtracting non-specific binding (determined in the presence of 1 μM CRH or 200 nM IL-1β, respectively) from total binding. Asterisks denote significant differences between inflamed and non-inflamed lymph nodes (* $P < 0.05$; Wilcoxon test). Experiments using ^{125}I-rat/human CRH yielded similar results (data not shown).

3.4. Lymph nodes

Intraplantar injection of Freund's complete adjuvant produced enlargement of ipsilateral (inflamed) but not contralateral (non-inflamed) lymph nodes at 4 days after inoculation. Histologically, the cortex was enlarged, lymph follicles were expanded and their population of cells was increased compared to non-inflamed lymph nodes (data not shown). Autoradiographic films showed that more than 80% of the total binding was displaceable by the respective unlabeled ligands (Fig. 5) and that specific binding of ^{125}I-Tyr°-ovine CRH and ^{125}I-IL-1β was significantly

higher in inflamed than in non-inflamed lymph nodes for both ligands ($P < 0.05$, Wilcoxon test) (Fig. 4A and C). Examination by light microscopy of photo-emulsion coated lymph node sections revealed a high density of ^{125}I-Tyr°-ovine CRH and ^{125}I-IL-1β binding sites in the outer cortex and a notable absence of binding in the central medulla (Fig. 5A and B). These binding sites appeared as clusters of autoradiographic grains over cells of the lymph follicles (Fig. 5B). Non-specific binding was low and uniformly distributed (Fig. 5C and F). The number of specific binding sites per 100 μm^2 was significantly higher in inflamed than in non-inflamed lymph nodes ($P < 0.05$, Wilcoxon test) (Table 1) but there was no significant difference in the number of labeled cells per 100 μm^2 ($P > 0.05$, Wilcoxon test) (Table 1). Therefore, the number of specific binding sites per cell was higher in inflamed than in non-inflamed lymph nodes for both ligands ($P < 0.05$, Wilcoxon test) (Table 1). Autoradiograms of non-inflamed lymph nodes of Freund's complete adjuvant-pretreated rats were not significantly different from those of normal animals ($P > 0.05$; Mann-Whitney U-test) (data not shown).

Table 1
Lightmicroscopic quantification of CRH- and IL-1β-binding sites in popliteal lymph nodes

	Inflamed LN		Non-inflamed LN	
	^{125}I-oCRH	^{125}I-IL-1β	^{125}I-oCRH	^{125}I-IL-1β
Grains/100 μm^2	17.6±0.6	19.4±0.8	10.6±0.7 *	11.5±0.1 *
Cells/100 μm^2	3.3±0.1	3.2±0.1	3.1±0.1	2.9±0.1
Grains/cells	5.4±0.2	6.1±0.3	3.5±0.3 *	4.0±0.1 *

Number of specific binding sites and of labeled cells per 100 μm^2, and calculated number of specific binding sites per cell. Values represent means and standard errors of five experiments. Twenty areas of 100 μm^2 per section and three sections per animal ($n = 5$ per experiment) were counted. The number of specific ^{125}I-oCRH and ^{125}I-IL-1β binding sites was determined by subtracting non-specific binding sites, determined in the presence of 1 μM unlabeled CRH or 200 nM IL-1β, respectively, from total binding sites. Asterisks denote significant differences between inflamed and non-inflamed LN (* $P < 0.05$; Wilcoxon test).

3.5. Spleen

The densitometric evaluation of entire spleen sections revealed no significant differences ($P > 0.05$, Mann-Whit-

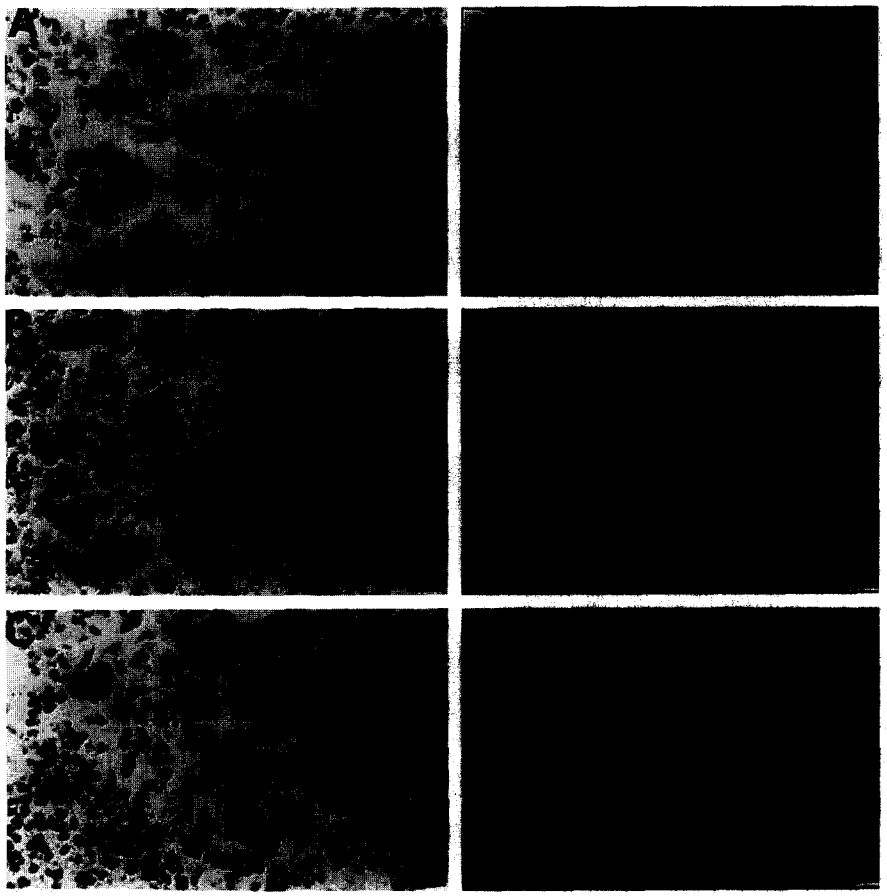

Fig. 5. Photoemulsion autoradiography of ^{125}I-Tyr°-ovine CRH (A, B) binding sites in non-inflamed (A) and inflamed (B) popliteal lymph nodes. (C) shows displacement of ^{125}I-Tyr°-ovine CRH binding by unlabeled CRH in inflamed tissue. Experiments using ^{125}I-rat/human CRH and ^{125}I-IL-1β yielded similar results (data not shown). Darkfield photomicrographs of tritium-sensitive ultrofilm show the distribution of ^{125}I-IL-1β binding sites in non-inflamed (D) and inflamed (E) lymph nodes. Note ^{125}I-IL-1β-associated silver grain deposits in the outer cortex (oc) and a lack of binding in the inner medulla (m). (F) shows non-specific binding in the presence of 200 nM IL-1β. The distribution of ^{125}I-Tyr°-ovine CRH binding was similar (data not shown). Bars represent 5 μm (A, B, C) and 280 μm (D, E, F).

ney *U*-test) in specific ^{125}I-Tyr°-ovine CRH or ^{125}I-IL-1β binding between Freund's complete adjuvant-pretreated and normal animals on autoradiographic films, as well as on darkfield photomicrographs (data not shown).

3.6. Competition studies

Both autoradiographic films and photoemulsion experiments revealed that in all tissues ^{125}I-Tyr°-ovine CRH binding was displaced by unlabeled corticotropin-releasing hormone and by α-helical CRH ($P < 0.05$, Wilcoxon test, compared to sections incubated with ^{125}I-Tyr°-ovine CRH only), but not by interleukin-1β, naloxone or propranolol ($P > 0.05$, Wilcoxon test) (Fig. 6A, C and E). Both unlabeled interleukin-1β (Fig. 3F) and interleukin IL_1 receptor antagonist displaced ^{125}I-IL-1β binding in all tissues ($P < 0.05$, Wilcoxon test, compared to sections incubated with ^{125}I-IL-1β only), while corticotropin-releasing hormone, naloxone or propranolol had no effect ($P > 0.05$, Wilcoxon test) (Fig. 6B, D and F). IL-1β and CRH binding was not

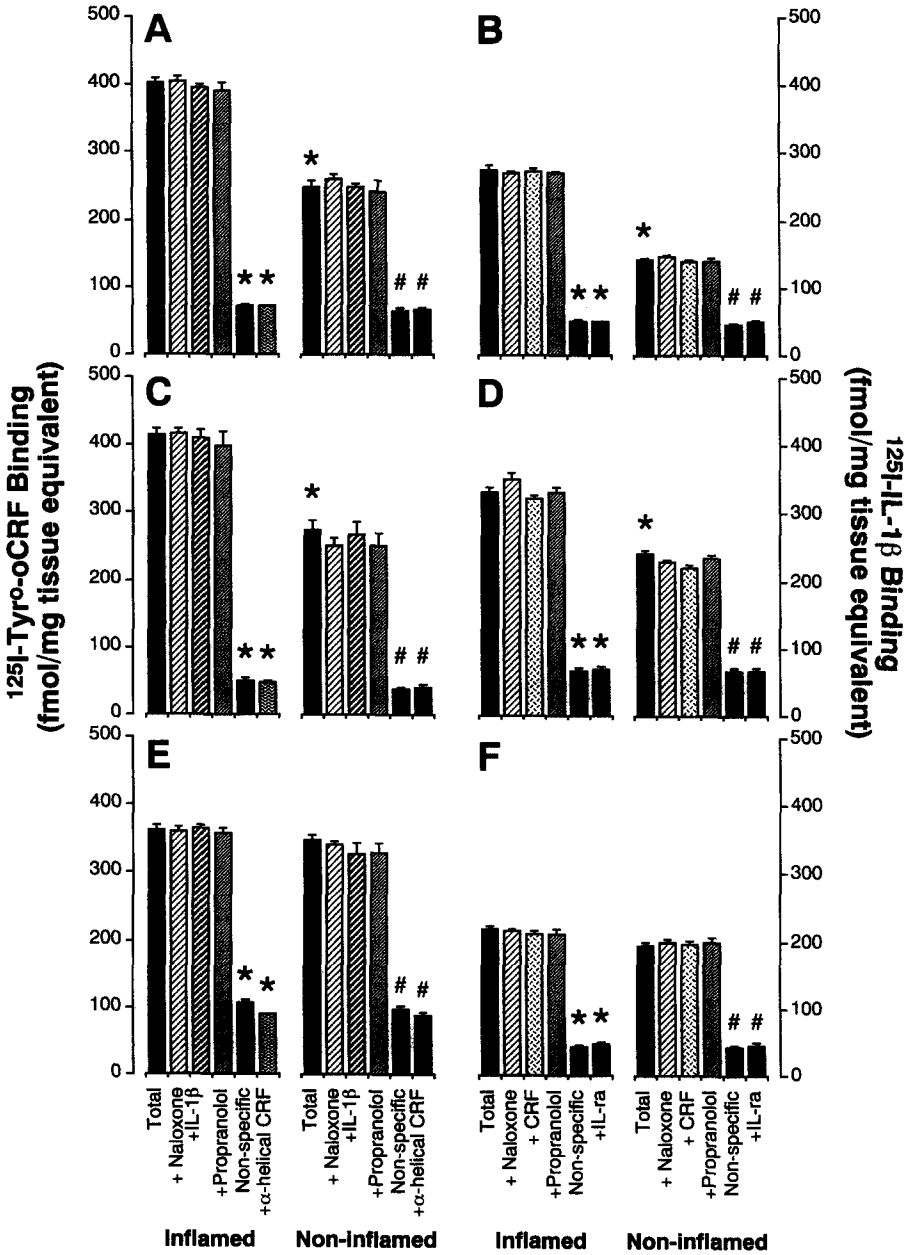

Fig. 6. ^{125}I-Tyr°-ovine CRH (^{125}I-oCRH) (A, C, E) and ^{125}I-IL-1β (B, D, F) binding in autoradiographic films in the presence of unlabeled homologous agonist and antagonist ligands and in the presence of opioid (naloxone) and adrenoceptor (propranolol) ligands in inflamed and non-inflamed paws (A, B), lymph nodes (C, D) and spleen (E, F). Binding in the presence of homologous unlabeled agonists (CRH and IL-1β) represents non-specific binding. Data represent means and standard errors of five experiments (n = 5 per experiment). Significant differences at $P < 0.05$ are denoted by * (comparison to total binding in inflamed tissue) and by # (comparison to total binding in non-inflamed tissue). Counting of autoradiographic silver grains in photoemulsion-coated sections yielded similar results (data not shown).

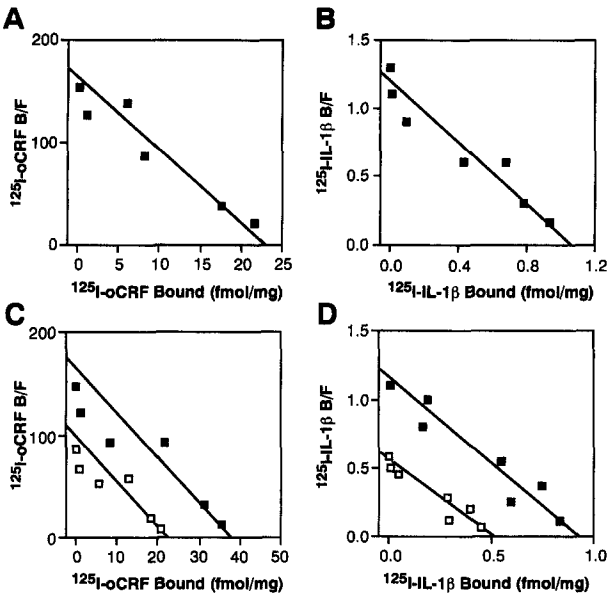

Fig. 7. Characterization of ^{125}I-Tyr°-ovine CRH (^{125}I-oCRH) (A, C) and ^{125}I-IL-1β (B, D) binding sites by Scatchard analysis in inflamed subcutaneous tissue (A, B) and in inflamed (filled symbols) and non-inflamed (open symbols) lymph nodes (C, D). Tissue sections were incubated with 0.1 nM ^{125}I-oCRH or with 0.1 nM ^{125}I-IL-1β in the presence of increasing concentrations of unlabeled CRH or IL-1β, respectively. Specific ^{125}I-oCRH and ^{125}I-IL-1β binding was determined by subtracting non-specific binding (determined in the presence of 1 μM CRH or 400 nM IL-1β, respectively) from total binding. Data are from one representative experiment. Scatchard curves were calculated as best fit by the computer program EBDA/LIGAND. B/F denotes the ratio between bound and free ligand. K_d and B_{max} are given in Table 2.

different between non-inflamed paws of Freund's complete adjuvant-treated rats and those of untreated controls ($P > 0.05$, Wilcoxon test) (data not shown). Similar results were obtained in films, photoemulsion and competition studies when ^{125}I-rat/human CRH was used instead of ^{125}I-Tyr°-ovine CRH (data not shown). The displacement of both radiolabeled ligands by increasing concentrations of their respective unlabeled ligands demonstrated that the specific binding was saturable and of high affinity in all tissues studied (inflamed subcutaneous tissue, inflamed and non-inflamed lymph nodes). Transformation of the data revealed linear Scatchard plots and Hill coefficients of greater than 0.94, indicating that each radioligand bound to a single class of non-cooperative sites (Table 2) (Fig. 7). The binding affinities (K_d) of both ligands were similar in the different tissues. The B_{max} of both binding sites was about 2-fold higher in inflamed compared to non-inflamed lymph nodes (Table 2).

4. Discussion

The first set of experiments demonstrates that, upon intraplantar inoculation with Freund's complete adjuvant, signs of inflammation and pain are prominent by 4 days after inoculation but they remain confined to the inoculated paw. CRH and IL-1β binding sites are detectable on inflammatory cells within the subcutaneous tissue. This binding is saturable, of high affinity and specific since it is displaceable by the homologous unlabeled agonist and antagonist ligands. CRH and IL-1β binding is virtually absent on nerves and in non-inflamed subcutaneous tissue and neither compound binds to opioid or adrenergic receptors. Both ovine and rat/human corticotropin-releasing hormone display the same binding patterns, indicating that the binding sites labeled here represent corticotropin-releasing hormone receptors rather than CRH binding protein (Lowry, 1995). The present findings extend previous studies on CRH binding in tissue homogenates, in that they provide evidence for the anatomical localization of these binding sites and show that they are detectable not only in polyarthritic Lewis rats with abnormally deficient hypothalamic corticotropin-releasing hormone responses (Crofford et al., 1992), but also in normal animals with localized hyperalgesic inflammation. Non-inflamed subcutaneous tissue contains only muscle fibers and isolated scattered cells that bind corticotropin-releasing hormone and interleukin-1β, the latter being in agreement with the presence of interleukin-1 receptor mRNA in normal skin (Deyerle et al., 1992). Taken together, our findings indicate the presence of abundant and specific corticotropin-releasing hormone and interleukin-1 receptors on inflammatory cells but not on nerves within peripheral inflamed tissue.

Where do the endogenous ligands of these receptors originate and what are their functions? Corticotropin-releasing hormone (Crofford et al., 1992; Karalis et al., 1991; Schäfer et al., 1996) as well as interleukin-1 (Dinarello, 1994) are present in immune cells and/or neurons within peripheral inflamed tissue. Both pro- and antiinflammatory actions have been reported for corticotropin-releasing hormone (Karalis et al., 1991; Wei and Thomas, 1993), while interleukin-1 is commonly referred to as a proinflammatory agent (Dinarello, 1994). In inflamed tissue, corticotropin-releasing hormone has been shown to produce analgesia (Hargreaves et al., 1989), whereas in non-inflamed tissue interleukin-1β elicits hyperalgesia (Ferreira et al., 1988; Davis and Perkins, 1994; Fukuoka et

Table 2
Properties of CRH- and IL-1β-binding sites as determined by Scatchard and Hill analysis

	^{125}I-oCRH			^{125}I-IL-1β		
	Inflamed paw	Inflamed LN	Non-inflamed LN	Inflamed paw	Inflamed LN	Non-inflamed LN
K_d (pM)	156 ± 29	258 ± 46	254 ± 49	92 ± 12	88 ± 17	100 ± 13
B_{max} (fmol/mg)	23.7 ± 4.2	38.1 ± 4.7	22.1 ± 1.7	1.1 ± 0.04	0.94 ± 0.04	0.52 ± 0.01
Hill coefficient	0.96	0.99	0.99	0.94	0.96	0.99

al., 1994). Some authors have invoked neuronal corticotropin-releasing hormone (Hargreaves et al., 1989) or interleukin-1 receptors (Fukuoka et al., 1994) to explain these actions. However, our results provide no support for these hypotheses since neuronal binding sites were absent in both inflamed and non-inflamed peripheral tissue. Therefore, an indirect mechanism, possibly through modulation of mediators (Ferreira et al., 1988; Davis and Perkins, 1994; Schäfer et al., 1994), seems more likely.

Our observations extend previous reports that have found high affinity binding of corticotropin-releasing hormone (Singh and Fudenberg, 1988; Webster and De Souza, 1988; Webster et al., 1990) and interleukin-1β (Uhl et al., 1989; Dinarello, 1994) to splenic and circulating immunocytes in that we have now shown a topical regulation of these receptors by localized painful inflammation. Furthermore, both corticotropin-releasing hormone and interleukin-1β, when injected into inflamed tissue, induce analgesic effects which are reversible by the respective antagonists and by immunosuppression (Schäfer et al., 1994). The fact that naloxone antagonizes these effects but does not displace CRH and IL-1β binding in the tissue, is consistent with the concept that the in vivo effects of corticotropin-releasing hormone and interleukin-1β are not due to a direct action at opioid receptors, but that they result from secondary opioid peptide release from immune cells (Schäfer et al., 1994). Similarly, adrenoceptor antagonists can attenuate corticotropin-releasing hormone-induced opioid peptide release in vivo (Heijnen et al., 1991) but do not displace CRH binding in the tissue. Thus, corticotropin-releasing hormone may act indirectly through sympathetic activation in vivo (Heijnen et al., 1991), in addition to its direct opioid releasing action at CRH receptors on immune cells. The opioid peptides subsequently penetrate the damaged perineurial sheath (Antonijevic et al., 1995) and activate opioid receptors on peripheral sensory nerves to produce potent local analgesia under stressful conditions (Stein et al., 1990; Przewlocki et al., 1992; Stein et al., 1993; Stein, 1995). Under baseline conditions, however, this mechanism is apparently not potent enough to completely overcome the nociceptive inflammatory stimuli, since there is a certain degree of residual hyperalgesia (see Fig. 1C and D; vehicle). Notwithstanding, our present and previous findings are consistent with the notion that both corticotropin-releasing hormone and interleukin-1β can activate their receptors on immunocytes to release opioids and inhibit pain within inflamed tissue.

This concept is further supported by in vitro studies demonstrating that both agents can release opioids from immunocytes in long-term cultures (Smith et al., 1986; Heijnen et al., 1991) and in ex-vivo cell suspensions prepared from lymph nodes (Schäfer et al., 1994). Similar to the in vivo studies, the corticotropin-releasing hormone and interleukin-1β-induced opioid release from lymph nodes is specific to CRH- and IL-1-receptors, respectively (Schäfer et al., 1994). Consistently, our present results demonstrate CRH and IL-1β binding in lymph nodes which is specific and localized mainly on cells of lymph follicles in the cortices. This anatomical distribution is in line with in situ hybridization studies of interleukin-1 receptor mRNA (Deyerle et al., 1992) and, together with morphological characteristics, suggests that these cells are mostly lymphocytes (Hoshi et al., 1989).

While the anatomical localization of corticotropin-releasing hormone and interleukin-1β receptors is similar, both CRH and IL-1β binding is enhanced in inflamed lymph nodes. Our analysis of binding relative to tissue weight and to the number of cells per unit area indicates that the binding per mg tissue and per cell is elevated. Consistently, the B_{max} of both binding sites is about 2-fold higher in inflamed than in non-inflamed lymph nodes. The binding affinity, however, is not different. Thus, inflammation of the paw apparently causes not only an increase in the total number of lymphocytes in the draining lymph nodes (Stünkel et al., 1988) but also an enhanced expression of CRH and IL-1β binding sites. Together with the enhanced production of endogenous corticotropin-releasing hormone and interleukin-1β in inflamed tissue (Dinarello, 1994; Karalis et al., 1991; Schäfer et al., 1996), this indicates an upregulation of both agonists and receptors and is in line with previous observations for interleukin-1 in vitro (Kawaguchi et al., 1992). Whether the increased number of binding sites seen in the lymph nodes represents an increased number of functional receptors remains to be determined. This is a particularly important consideration in the case of interleukin-1 because the ligands used in the present study bind to both type I and type II interleukin-1 receptors (Dinarello, 1994). Since it has been suggested that most of the biological actions of interleukin-1 are mediated by the type I receptor and that the type II receptor is not a signaling receptor (Colotta et al., 1993; Sims et al., 1993), it is conceivable that part of the binding seen here represents non-functional receptors.

Our results in the spleen are consistent with other studies demonstrating corticotropin-releasing hormone receptors (Webster and De Souza, 1988; Webster et al., 1990), but they are at variance to one report which was unable to detect IL-1 binding (Takao et al., 1993). This discrepancy may be due to technical differences, since the above study was performed in homogenates while we used tissue sections. Nevertheless, our data show that the abundance of CRH and IL-1β binding sites in spleen or non-inflamed lymph nodes is not different between treated and untreated rats, which is in agreement with previous reports examining interleukin-2 receptors (Stünkel et al., 1988) and with the notion that Freund's complete adjuvant-induced inflammation remains confined to the injected paw without evidence for generalized disease within the observation period employed here (Stein, 1995).

In summary, we have shown that both corticotropin-releasing hormone and interleukin-1β elicit receptor-specific antinociception in inflamed tissue by a mechanism involv-

ing opioids. Abundant, specific CRH and IL-1β binding sites of high affinity, are primarily localized on immune cells within inflamed subcutaneous tissue and lymph nodes. Both CRH and IL-1β binding is greatly enhanced in inflamed tissue. This increase is apparently due to a greater number of cells bearing such binding sites and of binding sites per cell, and it remains regionally confined in this model of localized inflammation. In context with previous studies (Schäfer et al., 1994; Schäfer et al., 1996) we interpret this upregulation as part of the body's response to combat inflammatory pain (Stein, 1995). This localized response consists of an upregulation of opioid receptors on nociceptive nerve terminals (Stein et al., 1990; Hassan et al., 1993), an accumulation of opioid containing immune cells (Stein et al., 1990; Przewlocki et al., 1992; Stein et al., 1993) and, as shown here, an upregulation of binding sites for opioid releasing agents such as corticotropin-releasing hormone and interleukin-1β.

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