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Remodelling of spinal nociceptive mechanisms in an animal model of monoarthritis

Reza Sharif Naeini,[1] Catherine M. Cahill,[1,6,†] Alfredo Ribeiro-da-Silva,[3,4] Henri A. Ménard[5] and James L. Henry[1,2,*]
[1]Department of Physiology, McGill University, Montreal, QC, H3A 1A1, Canada
[2]Department of Psychiatry, McGill University, Montreal, QC, H3A 1A1, Canada
[3]Department of Pharmacology & Therapeutics, and [4]Department of Anatomy and Cell Biology, McGill University, Montreal, QC, H3A 1A1, Canada
[5]Division of Rheumatology, Department of Medicine, McGill University, Montreal, QC, H3A 1A1, Canada
[6]Current address: Department of Pharmacology & Toxicology, Queen's University, Kingston, Ontario, Canada

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Abstract

Intra-articularly injected complete Freund's adjuvant creates in rats a chronic monoarthritis suitable for studying neuronal plasticity and chronic pain. Using such a model, we report electrophysiological and morphological evidence of alterations in somatosensory synaptic function. In arthritic rats, the baseline activity of dorsal spinal cord wide dynamic range or nociceptive-specific neurons was greater than in control animals. Moreover, neuronal responses elicited by an innocuous stimulation with von Frey filaments applied to the arthritic joint were greater in amplitude and produced the afterdischarge that normally characterizes a nociceptive response. In contrast to the response in control animals, passive movement of the arthritic joint produced an increase in the amplitude of the response of these neurons to iontophoretic application of glutamate receptor agonists over a time frame of 10–30 min. This potentiation was blocked by pretreatment with a neurokinin-1 (NK-1) receptor antagonist, suggesting the involvement of substance P. Ultrastructural analysis of the dorsal horn revealed that movement of the arthritic joint also induced NK-1 receptor internalization, indicative of nociception. Morphological examination revealed significantly increased expression of substance P and its receptor within the superficial dorsal horn of monoarthritic animals. These unique functional and chemical changes reflect alterations in both presynaptic and postsynaptic mechanisms in nociceptive transmission at the spinal level. Thus, although treatment of arthritis should obviously target its peripheral aetiology, targeting its central components is a logical therapeutic complementary objective.

Introduction

Pain is an important protective mechanism to minimize tissue damage; however, chronic pain lacks this teleological advantage and poses a burden to a significant proportion of the population. Inflammatory pain mechanisms are a feature of a variety of chronic pain states including arthritis, back pain and temporomandibular joint disorder. Under conditions of chronic inflammatory pain, normally innocuous sensory stimuli are often perceived as painful (allodynia), and mild noxious sensory stimuli perceived as deeply painful (hyperalgesia). Both hyperalgesia and allodynia are thought to arise from sensitization of peripheral nociceptors (peripheral sensitization) and spinal dorsal horn neurons (central sensitization; Treede *et al*., 1992).

Peripheral sensitization corresponds to a sustained activity of primary afferent fibres and features an increase in the efficacy of synaptic transmission between primary afferent fibres and dorsal horn neurons (Woolf & Salter, 2000). Central sensitization is less well understood. Several studies have implicated cooperation between substance P (SP) and NMDA-mediated events in the development and maintenance of inflammation-induced central sensitization (Cumberbatch *et al*., 1995). Other suggested mechanisms include (i) changes in cytoarchitecture and interneuronal connections in the spinal cord (Woolf *et al*., 1992; Nakatsuka *et al*., 1999; Woolf & Costigan, 1999); (ii) changes in the quantity and release of neurotransmitters from sensory fibres projecting from the periphery (Oku *et al*., 1987; Hanesch *et al*., 1995; Allen *et al*., 1997; Sasaki *et al*., 1998); (iii) changes in receptor expression within the spinal cord (Abbadie *et al*., 1997; Honoré *et al*., 1999, 2000); and (iv) changes in ion channel distribution and properties (Guo & Huang, 2001; Saegusa *et al*., 2001; Voilley *et al*., 2001). While many mechanisms probably contribute to the development of sensitization and synaptic hyperexcitability of sensory processes, one of our greatest challenges now is to determine what factors contribute to its maintenance, and how these phenomena contribute to the genesis of chronic pain states.

In recent years, significant progress has been made in our understanding of the generation of pain caused by acute inflammatory processes (reviewed in Schaible *et al*., 2002). Extracellular recordings of dorsal horn neurons *in vivo,* following stimulation of an acutely inflamed joint, demonstrated that they were hyperexcitable (Menétrey & Besson, 1982; Grubb *et al*., 1993; Neugebauer *et al*., 1993, 1994) and that not only did neurokinin-1 (NK-1) receptors play an important

Correspondence: Dr Catherine M. Cahill, at †present address below.
E-mail: cathy.cahill@queensu.ca

**Current address*: Michael G. DeGroote Institute for Pain Research and Care Health Sciences Centre, McMaster University, Hamilton, Ontario, Canada.

†*Present address*: Department of Pharmacology & Toxicology, Queen's University, Kingston, Ontario, Canada.

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role in this hyperexcitability (Neugebauer *et al*., 1995; Traub, 1996; De Felipe *et al*., 1998; Ma *et al*., 1998) but their expression was up-regulated during chronic polyarthritis (Honoré *et al*., 1999).

In contrast, less is known about the mechanisms underlying the maintenance of chronic arthritic conditions during stages of severe bone erosion and cartilage destruction. Hence, although the phenomena of hyperexcitability are similar in acute and chronic inflammatory arthritis, this does not necessarily imply that the same mechanisms are at work.

In this paper, we have used a multidisciplinary approach we examined: (i) differences in postsynaptic excitability of dorsal horn spinal cord neurons in arthritic and control animals; (ii) responses of dorsal horn neurons to both innocuous and noxious sensory stimuli; and (iii) neurochemical and morphological correlates that might account for the changes in the observed functional synaptic transmission. Together, these data suggest that chronic arthritic pain is characterized by a sustained hyperexcitability of spinal dorsal horn neurons, similar to that occurring during the acute phase of arthritis. We provide further evidence for modulation of sensory transmission to both noxious and non-noxious stimuli and emphasize that these phenomena are partially mediated via activation of NK-1 receptors.

Materials and methods

Animals

Adult male Sprague-Dawley rats weighing 275–300 g were obtained from Charles River (St-Constant, QC, Canada) and kept on a 12 : 12-h light : dark cycle. Guidelines from the Canadian Council on Animal Care were strictly followed and all procedures with animals were approved by the McGill University Animal Care Committee.

The monoarthritis model

Complete Freund's adjuvant (CFA, *Mycobacterium butyricum*) was injected in a volume of 25 μL (135 μg) into the left tibiotarsal joint of rats briefly anaesthetized with halothane. Control animals received an equal volume injection of the vehicle (saline, mineral oil and Tween 80). To validate the model, various physiological parameters including ankle circumference and mechanical withdrawal threshold were assessed at various intervals prior to and for 21 days following CFA injection. Withdrawal thresholds were measured with von Frey filaments applied to the ankle joint in ascending strength beginning at 0.25 g and continuing with filaments of increasing strength. It should be noted that the observed changes were confined to the injected joint and present throughout the 21-day time course. In electrophysiological experiments, the ankle was gently palpated to identify the joint space. A mark was subsequently placed on the joint with a felt tip marker and mechanical stimuli via von Frey filaments were applied to that area. As with experiments in awake animals, von Frey filaments were applied in ascending order of increasing strength. Radiograms were obtained using a Faxitron X-ray machine (Faxitron X-ray Corporation, model 43855 A; 45 kV, 7-s exposure). All data are from experiments performed before or at the 21st day postinjection. The occasional animals with clinical evidence of polyarthritis were excluded from this study.

Electrophysiological experiments

Rats were anaesthetized with sodium pentobarbital (initial bolus dose 60 mg/kg, i.p., supplemented with 10 mg/kg/h, i.v. by continuous delivery; Abbott Laboratories Ltd, Montreal, Canada). The left jugular and the right femoral veins were catheterized for infusion of anaesthetic and drugs, respectively. Body temperature was maintained at 37.5 °C using an infrared heating lamp as necessary. Supraspinal descending influences were eliminated by transection of the spinal cord at T9 at least 2 h before recordings. Spinal shock was minimized by injecting xylocaine 1%, 0.05 mL (Astra, Mississauga, Ontario, Canada) into the spinal cord before the transection. Electrophysiological recordings were performed at vertebral levels T13–L2.

Single-unit spikes were recorded extracellularly using seven-barrelled glass microelectrodes (tip diameter 5–7 μm) at depths of 150–900 μm from the dorsal surface of the spinal cord. A solution of 2.7 M NaCl was placed in the central recording barrel (impedance 2.5–5 MΩ). Neuronal signals were displayed on a digital oscilloscope and fed into a window discriminator whose output was processed by an interface connected to a personal computer for subsequent analyses with Spike 2.0 software (CED, England). Signals were also digitized and recorded on video tape for off-line analysis. Throughout the experiment spike size and configuration were continuously monitored on the oscilloscope to confirm that activity of the same neuron was being recorded and to ensure that the position of the recording electrode was kept constant near the observed neuron.

Identification and classification of neurons

Neurons were classified functionally according to their responses to natural stimulation of the cutaneous receptive field (Pitcher & Henry, 2000). Briefly, once a stable, single-unit recording was obtained, the following stimuli were used to functionally characterize each neuron: (i) light touch and moderate pressure using a calibrated clip (0.2 N for 3 s); and (ii) noxious mechanical stimulation using a calibrated clip. Identified units were classified into three categories: (i) non-nociceptive neurons that responded only to non-noxious stimuli such as touch and/or pressure stimulation; (ii) wide dynamic range (WDR) neurons that responded to both noxious and innocuous stimulation; and (iii) nociceptive-specific neurons that responded only to noxious stimuli. All the units that responded to a noxious stimulus displayed a characteristic slow-decaying afterdischarge, as previously described (De Koninck & Henry, 1991).

The neurons tested in this study included WDR and non-nociceptive neurons receiving inputs from the ankle joint. In arthritic rats, some nociceptive-specific neurons may have been included as their activation threshold is reduced following inflammation (Neugebauer *et al*., 1995), making them indistinguishable from WDR neurons. Although not analysed in this study, receptive field sizes were measured in each rat; a greater receptive field size was consistently observed in the arthritic animals, often encompassing the whole leg. Baseline activity of all neurons was recorded at the beginning of the recording session, before any stimulus was given to the receptive field. Baseline activity was recorded from 16 to 20 neurons per group (three neurons per animal).

Iontophoresis

Each of the peripheral barrels of the microelectrode was filled with either *N*-methyl-D-aspartate (NMDA; 50 mM in 100 mM NaCl) or α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA; 1 mM in 100 mM NaCl). The pH of each solution was adjusted to 7.3–7.4. Iontophoretic application of drug was with negative current, ranging from −20 to −70 nA, and lasted for 8–10 s. A retaining current of +5 to +10 nA was applied to solutions between ejections to counteract diffusion of the drugs. Recordings were obtained from

5–13 neurons per neuronal classification group (one neuron per animal). For analyses of the responses to mechanical stimulation or to iontophoretic application of glutamate receptor agonists, the total number of spikes in the response of dorsal horn neurons was determined in each case. Ongoing baseline activity was determined as the number of spikes over an 8- to10-s period immediately preceding the evoked response. This baseline activity was subtracted from the number of spikes occurring during the evoked response.

Statistical analysis of electrophysiological data

Descriptive statistics used mean ± SEM. As indicated in the text, comparative parametric statistics use the unpaired *t*-test. When appropriate, two-way ANOVA followed by Tukey's *post hoc* analyses were also performed. Significance was set at $P < 0.05$.

Immunofluorescence detection of SP and the NK-1 receptor in the superficial dorsal horn of the spinal cord

Rats ($n = 4$ per group) were anaesthetized with sodium pentobarbital (70 mg/kg, intraperitoneal) and perfused through the left cardiac ventricle with 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) at 4 °C. Lumbar spinal cords were removed, postfixed in the same fixative solution by immersion for 1 h at 4 °Cand then cryoprotected in 30% sucrose in PB at 4 °C for a minimum of 16 h. Transverse serial sections (50 μm) were cut on a freezing sledge microtome and collected in 0.1 M phosphate-buffered saline with 0.2% Triton X-100 (PBS-T).

Sections were treated with 1% sodium borohydride in PBS for 30 min followed by incubation with 10% normal horse serum and 10% normal goat serum in PBS-T for 1 h at room temperature. Sections were then incubated for 48 h at 4 °C with an affinity-purified C-terminal-directed polyclonal anti-NK-1 receptor antibody (1 : 100, generous gift from Dr James Krause, Neurogen Corp., Branford, CT, USA; Ardelt *et al*., 1996) and a rat anti-SP monoclonal antibody coded NC1/34 (Medicorp, Montreal, Canada). These antibodies have both been extensively characterized and used (Cuello *et al*., 1979; Ardelt *et al*., 1996). Sections were subsequently incubated with goat antirabbit biotinylated IgG (Vector Laboratories, Burlington, ON, Canada) for 2 h at room temperature followed by a 2-h incubation with a rhodamine red-conjugated antirat IgG antiserum (Jackson Laboratories, Bar Harbor, MA, USA) and streptavidin conjugated to Alexa 488 (Molecular Probes, Eugene, OR, USA) in 5% normal horse serum in PBS-T. Sections were then mounted onto gelatin-coated slides using Vectashield mounting medium and stored at −20 °C until examined with a Zeiss LSM 510 laser scanning confocal microscope.

Images were obtained using a 63× plan-apochromatic oil-immersion objective and represent optical sections of 1 μm thickness. For quantification, files were converted to TIFF format and SP was quantified using a MCID Elite image analysis system (Imaging Research Inc., St. Catharines, ON, Canada). The density of SP-immunopositive varicosities was quantified using a modified version of functions originally designed for autoradiographic quantification. Analyses were performed in at least three sections per experiment for three conditions: control with movement and CFA ipsilateral with and without movement. The data are expressed as the number of varicosities per 6000 μm^2 (60 × 100 μm rectangle within the centre of the superficial dorsal horn). A correction factor was employed by the software to correct for overlapping or clustered varicosities. Statistical analyses were performed using a one-way ANOVA followed by Friedman's *post hoc* analysis. Statistical significance was set at $P < 0.05$.

NK-1 receptor immunolabelling for electron microscopy

Rats ($n = 3$ per group) were anaesthetized and perfused as described with a mixture of 4% paraformaldehyde, 15% picric acid (volume/volume) and 0.1% glutaraldehyde in PB, pH 7.4. Lumbar spinal cords were removed and postfixed in the same fixative for 1 h at 4 °C followed by cryoprotection in 30% sucrose in PB at 4 °C for 12 h. Spinal cord segments L4–L5 were snap-frozen by immersion in liquid nitrogen and immediately thawed in PB at room temperature. Transverse sections (50 μm) were cut using a Vibratome series 3000 (Technical Products International Inc., St. Louis, MO, USA) and collected in PB. Spinal cord sections were treated for 30 min with 1% sodium borohydride in PB followed by incubation in a solution consisting of 0.5% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Sections were then incubated in rabbit anti-NK-1 receptor antiserum (1 : 100) in 0.1% BSA in PBS for 48 h at 4 °C. After washing, sections were incubated for 10 min with 8% BSA and 2% gelatin in PBS followed by incubation overnight at 4 °C with colloidal gold (1 nm)-conjugated goat antirabbit IgG (1 : 50, Biocell) diluted in the same buffer. Sections were then fixed for 10 min with 2% glutaraldehyde in PBS and rinsed with 0.2 M citrate buffer, pH 7.4. Immunogold particles were intensified with silver for 10 min using an IntenSE M kit (Amersham Pharmacia Biotech, Inc.). Sections were postfixed for 1 h at room temperature with 1% OsO_4 in PB and dehydrated with increasing concentrations of ethanol. Sections were then flat-embedded in Epon between acetate sheets. After polymerization of the Epon, the sections were examined by light microscopy and the selected fields were re-embedded and trimmed for electron microscopic examination. The ultrathin sections were collected on formvar-coated one-slot grids, counterstained with uranyl acetate and lead citrate, and observed with an electron microscope (Philips 410; Philips Electron Optics Canada, Scarborough, ON, Canada).

Ultrathin sections from three grids from three different Epon blocks were quantified for each animal. Random fields in laminae I–II were analysed. A dendritic profile was considered NK-1 receptor-immunoreactive if it contained at least three gold particles. For each field, the following parameters were quantified: (i) the total number of NK-1 receptor-immunogold particles on dendritic profiles; (ii) the perimeter and surface area of NK-1 receptor immunoreactive dendritic profile; and (iii) the total number of immunogold particles either intracellular or associated with the plasma membrane of immunoreactive dendritic profile. A gold particle was considered to be associated with the plasma membrane when it either contacted or overlaid it. Particles not in contact with the plasma membrane, even if in close proximity, were classified as intracellular. A minimum of 50 dendrites per section was counted for naïve, CFA-contralateral and CFA-ipsilateral sections with and without ankle joint extension. To obtain these parameters, images were obtained with a Megaview II video camera attached to a computer equipped with imaging software (Analysis 3.0; Soft Imaging System, CA, USA).

Comparison between the total mean number of immunogold particles per unit area and the percentage of immunogold particles that were intracellular was performed by using the unpaired *t*-test and the χ^2 test, respectively. Statistical significance was set at $P < 0.05$.

Results

Characterization of the model

There was a significant increase in the circumference of the ipsilateral ankle immediately after CFA injection (Fig. 1A) and it persisted throughout the 21-day period of experimentation. In vehicle-injected rats, an initial increase in ankle circumference had dissipated within

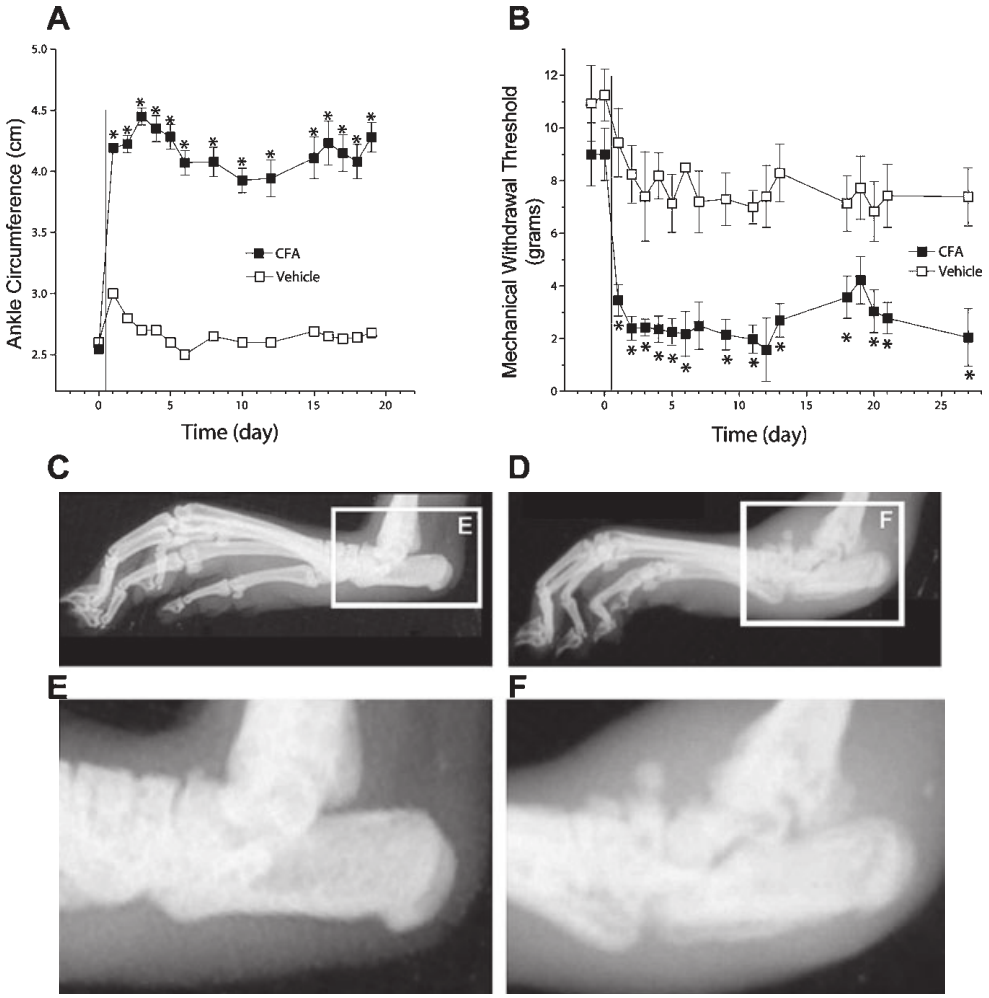


FIG. 1. Characterization of CFA-induced monoarthritis. Parameters monitored included (A) ipsilateral ankle diameter and (B) mechanical withdrawal threshold measured as the pressure needed to elicit a paw withdrawal response using von Frey filaments. Radiograms of the ipsilateral ankle joint of (C) vehicle-injected rats and (D) CFA-injected rats; (E and F) higher magnifications of (C) and (D), respectively, are presented to show the details of the injected joints. Data are expressed as mean ± SEM for $n = 5$–8 for each group. *$P < 0.01$ vs. vehicle-injected group.

5 days. A significant decrease in the mechanical withdrawal thresholds was observed in CFA-injected rats compared to vehicle-injected rats (Fig. 1B). This change was evident immediately following the CFA injection and persisted for the entire length of the study. Monoarthritic animals also demonstrated a significant loss of ankle flexibility as well as a significant increase in vascular permeability throughout the time-course of experimentation (data not shown). Radiographic imaging of the ipsilateral joint 21 days following the injection of CFA demonstrated peri-articular soft-tissue swelling and a destructive erosive arthritis (Fig. 1C–F).

Increased excitability of sensory mechanisms

To determine whether the arthritis was associated with changes in neuronal activity in the sensory spinal cord, electrophysiological experiments were performed to determine the baseline activity of dorsal horn neurons. Mean spontaneous discharge rate of single spinal dorsal horn WDR neurons was greater in CFA-injected than in vehicle-injected rats. Discharge rate was measured as the number of extracellular field potential spikes recorded when no stimulus was applied to the receptive field of the neuron. A greater baseline neuronal activity was observed when tested on day 3 (17.4 ± 3.7 spikes/s in 16 CFA-injected rats vs. 7.2 ± 2.4 spikes/s in 18 vehicle-injected rats. The corresponding values at day 21 were 19.3 ± 3.0 spikes/s in 18 CFA-injected rats vs. 10.2 ± 2.0 spikes/s in 20 vehicle-injected rats (Fig. 2).

Nociceptive responses to mechanical stimulation

In view of the observation that the level of baseline activity of spinal WDR neurons was greater in the monoarthritic rats, it was important to establish whether this might also be the case for synaptic inputs to spinal neurons. Thus, we examined first the synaptically elicited excitation of dorsal horn neurons induced by innocuous mechanical stimulation of the receptive field (arthritic ankle). Ratemeter histograms illustrate recordings from single WDR neurons in control (Fig. 3A) and monoarthritic rats (Fig. 3B). Mechanical stimulation with von Frey filaments up to 90 g, applied to the joint, produced a consistently greater response in spinal neurons of monoarthritic rats than in control rats. Importantly, an innocuous stimulus as low as 12 g elicited an afterdischarge in dorsal horn neurons in monoarthritic rats that persisted for ≥1 min following termination of the 5-s period of stimulation. This afterdischarge was blocked by administration of a selective NK-1 receptor antagonist, CP-96,345 (Pfizer Central

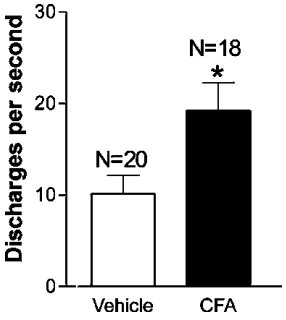


FIG. 2. Mean spontaneous discharge activity of single spinal dorsal horn WDR neurons in day 21 CFA-injected and vehicle-injected rats. Discharges were measured as the number of extracellular field potential spikes emitted by the cell when no stimulus was applied to the receptive field. Data are expressed as mean ± SEM for $n = 16$–18 per group; $^*P < 0.05$ vehicle vs. CFA-injected group.

Research, Groton, Connecticut, USA; data not shown). In naïve animals, this type of afterdischarge is only produced in nociceptive neurons (WDR and nociceptive-specific) in response to noxious stimulation of the peripheral receptive field (De Koninck & Henry, 1991; Radhakrishnan & Henry, 1995).

This change in neuronal character was confirmed using a detailed parametric study of responses of spinal neurons to a broad range of intensities of mechanical stimulation to the arthritic joint. Hence, responses to mechanical stimulation ranging from 1.15 to 90 g were evaluated. Figure 3C, depicting the magnitude of the response, and Fig. 3D, depicting the duration of the response, illustrate the cumulative data from these parametric studies in control and monoarthritic rats. In terms of the magnitude of the response, the difference between the two groups appeared at very low intensities of 1.15 g (20.0 ± 4.0 spikes in CFA rats compared to 1.5 ± 4.0 spikes in vehicle rats) and became significant at 12 g (206.7 ± 12.7 spikes in CFA rats compared to 28.9 ± 3.3 spikes in vehicle rats). This difference between the two groups remained as the intensity was further increased to 90 g (475.0 ± 28.0 spikes in CFA rats compared to 303.0 ± 24.0 spikes in vehicle rats; Fig. 3C).

A parametric study was expanded to include examining specific changes in the afterdischarge of the spinal neurons (Fig. 3D). The data show that there was a concomitant increase in the duration of the afterdischarge in the monoarthritic animals to normally innocuous stimuli. The greatest difference between the two groups was once again at the lowest intensities. The data in Fig. 3D also indicate that the duration of the response to a 12-g stimulus in the monoarthritis group is similar to that induced by much stronger stimulation in the control group. Thus, even a low intensity stimulus induces a persisting response in spinal sensory neurons in the monoarthritis model. There was also a difference between the monoarthritic and control rats in the responsiveness of WDR neurons to noxious mechanical stimulation produced by a 2100-g pinch (Fig. 3D). The pinch produced a significantly greater duration of afterdischarge in the monoarthritis animals compared to controls.

Postsynaptic mechanisms increase synaptic responses

The previous observations prompted a series of micropharmacology experiments focusing specifically on the period following direct synaptic input. To this aim, the excitability of postsynaptic neurons was measured *in situ* by their responses to direct iontophoretic application of glutamate receptor agonists *in vivo*. The selective glutamate receptor agonists AMPA and NMDA were iontophoretically administered onto single neurons by automatically controlled 6-s applications of current at 30-s intervals (Fig. 4). This caused a brief increase in the firing rate of dorsal horn neurons that lasted throughout the period of application in both vehicle-injected and CFA-injected rats and that ended within 1–2 s of the end of current application (Fig. 4A–C). The responses of dorsal horn neurons to the agonists were then determined before and after joint extension. Responses to AMPA and NMDA were normalized to the period just preceding joint extension, as the responses remained consistent in that period. Synaptic input, evoked by extending the arthritic joint for a period of 3 s using a force of 100 g, was used to determine the effects of joint movement on neuronal excitability. As illustrated in Fig. 4, ankle extension in control rats had no effect on the response of a WDR neuron to AMPA. For comparison, this procedure was carried out also studying non-nociceptive neurons of arthritic animals. Ankle extension was without effect on the AMPA-induced response in non-nociceptive neurons in monoarthritic rats (Fig. 4C). However, the same extension induced a long-lasting potentiation of the response to AMPA in a WDR neuron of a monoarthritic rat (Fig. 4B). This potentiation occurred immediately following joint extension: 138 ± 5% of baseline in WDR neurons of 13 monoarthritic rats vs. 105 ± 3% in WDR neurons of seven vehicle-injected rats and 92 ± 4% in non-nociceptive neurons of five monoarthritic rats. All animals were tested 10 s following ankle extension. Cumulative data from the WDR neurons tested illustrate that the duration of the postsynaptic potentiation typically exceeded 10 min (Fig. 4D). The NMDA-induced effects showed a similar potentiation of the response (Fig. 4E) but only evident in WDR and not in non-nociceptive neurons of monoarthritic animals and not in WDR neurons of control animals. Overall, those data indicate an increase in postsynaptic excitability of dorsal horn neurons more selectively in WDR neurons (or nociceptive specific neurons) as opposed to non-nociceptive neurons.

NK-1 receptor involvement in synaptic changes

One step in identifying the mechanisms underlying these changes in postsynaptic excitability of spinal nociceptive neurons was based on a previous observation that these neurons, unlike non-nociceptive neurons, receive input from SP-containing primary sensory nerve terminals (De Koninck *et al*., 1992). It was important therefore to determine whether SP (NK-1) receptors played any role in this increase in postsynaptic hyperexcitability. When the selective NK-1 receptor antagonist CP-96,345 (5 mg/kg, i.v., $n = 5$) was administered 15 min prior to ankle extension, the potentiation of the evoked responses to application of AMPA was significantly than that following similar administration of the inactive isomer CP-96,344 (5 mg/kg, i.v., $n = 6$; Fig. 4F). A similar reduction of the potentiation of responses to NMDA was observed following administration of the NK-1 receptor antagonist, but not of the inactive isomer (Fig. 4G; $n = 5$ with CP-96,345 and $n = 6$ with CP-96,344). In addition, it was noted that there was a decrease in the ongoing discharge of the neurons following the administration of CP-96,345 and CP-96,344, but this decrease was short-lasting (< 1 min) and did not reach significance. Thus, joint movement via ankle extension induced a potentiation of postsynaptic responses in the arthritic animals and showed a substantial participation of the NK-1 receptor and presumably also the release of SP.

NK-1 receptor distribution and trafficking

The nociceptive-like response of spinal dorsal horn neurons to innocuous stimuli (i.e. low intensity von Frey hairs and passive

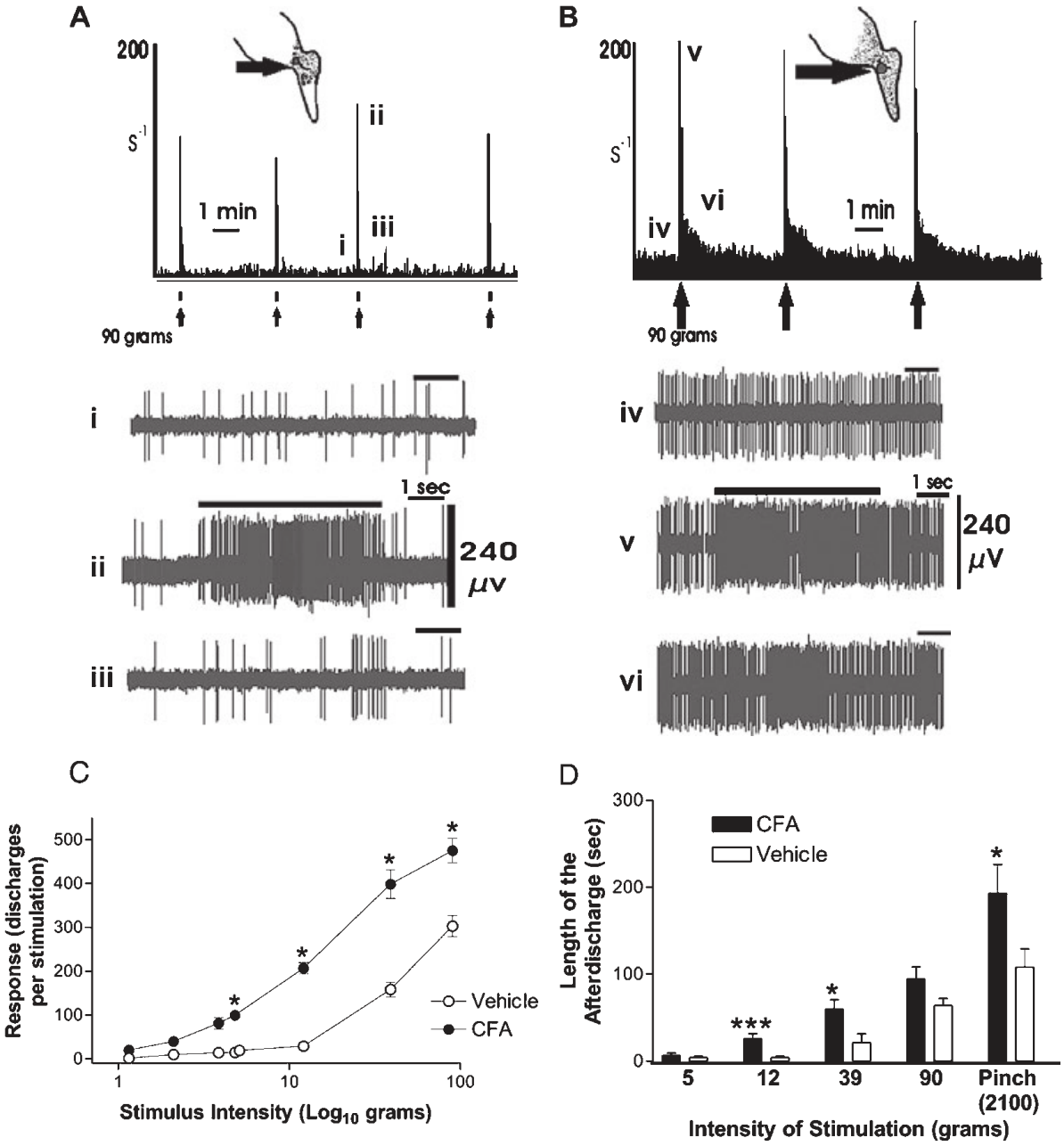
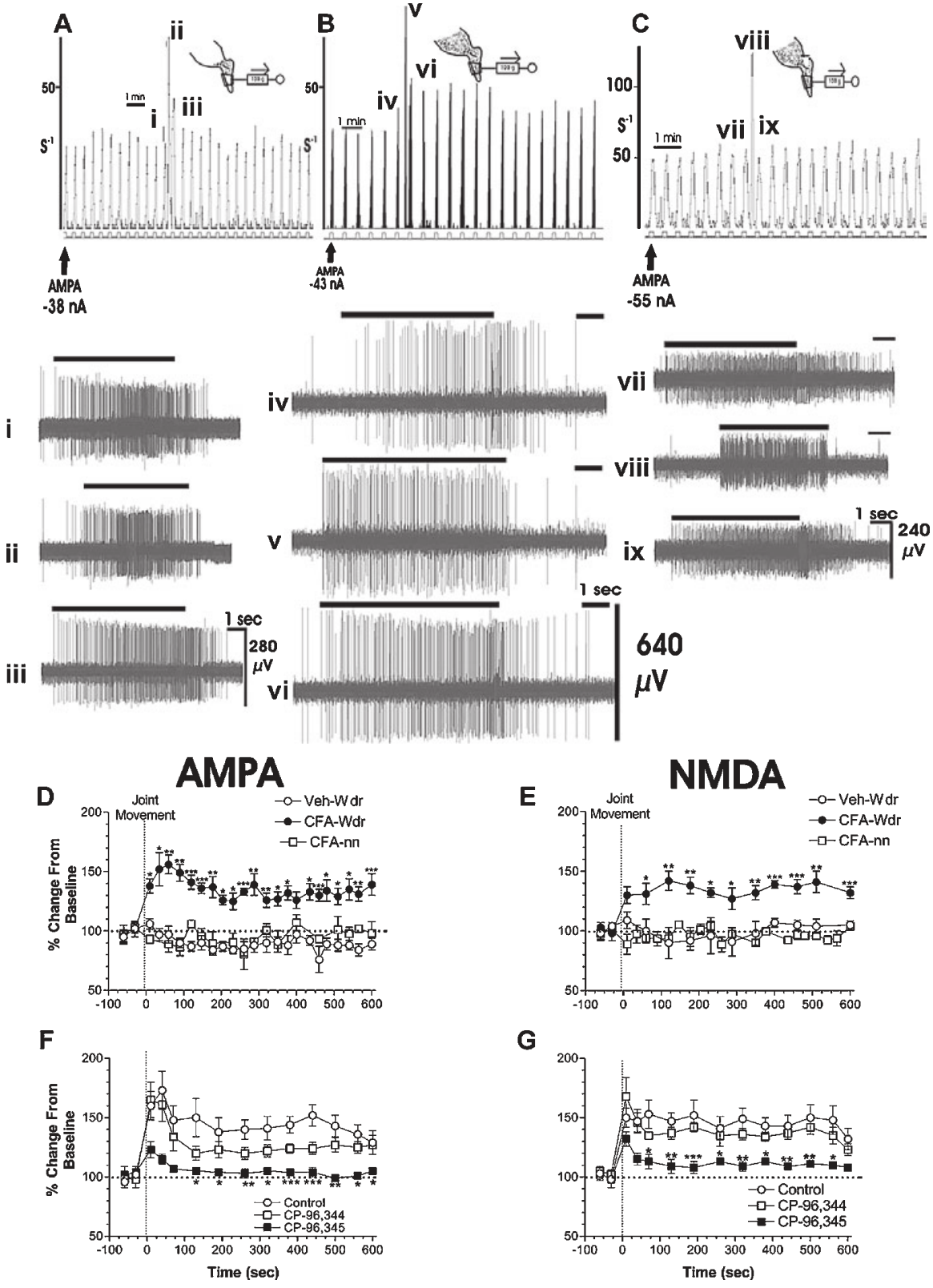


FIG. 3. Electrophysiological data of arthritic animals. Ratemeter histograms in (A) and (B) illustrate recordings from single WDR neurons in control and monoarthritic rats, respectively. Upward arrows indicate times of 5-s applications of 90-g stimuli to the respective cutaneous receptive fields. Larger horizontal arrows in the insets indicate the respective sites of application of the stimuli and the hatched areas indicate the cutaneous receptive fields. (i–vi) Oscilloscope records illustrate the extracellular spike discharges recorded from the neurons illustrated in A and B, taken at the times indicated in the ratemeter histograms; the long horizontal bars above (ii) and (v) indicate the periods of the 5-s stimulation. Data in (C) and (D) illustrate cumulative data from parametric studies in control ($n = 9$) and monoarthritic rats ($n = 6$) following either von Frey hair application or pinch (2100 g). Data are expressed as mean ± SEM with $*P < 0.05$ and $***P < 0.001$.

FIG. 4. Responses to iontophoretic application of glutamate receptor agonists following joint movement. (A–C) In representative ratemeter histograms, AMPA was delivered with direct current through the respective electrode barrel at 30-s intervals. The insets show the respective receptive fields (shaded areas of the paw) as well as the direction of the extension. Oscilloscope records illustrate the extracellular spike discharges recorded from the neurons illustrated in A–C, taken at the times indicated in the upper panel (i–ix) in the ratemeter histograms. The long horizontal bars above the oscilloscope records indicate the periods of iontophoretic application of AMPA. For iontophoretic application, direct current was applied for 6 s at 30-s intervals. Passive extension of the joint was performed using a 100-g spring for 3 s. Amplitudes of neuronal responses to AMPA were normalized to the period preceding joint extension, as the responses remained consistent in that period. Recordings are presented for (A) a WDR neuron from a control animal, (B) a WDR neuron of a monoarthritic animal, and a (C) non-nociceptive neuron of a monoarthritic animal. (D) Cumulative data; Veh, vehicle; Wdr, WDR neurons; nn, non-nociceptive neurons. Results are expressed as a percentage of the responses to AMPA before joint extension. (E) Responses to passive extension of the arthritic ankle were also evaluated in response to iontophoretic application of NMDA. (F and G) The responses to NMDA and AMPA were also evaluated following the NK-1 receptor antagonist CP-96,345, 5 mg/kg i.v. Data are expressed as mean ± SEM; significance is illustrated by differences from the Veh–Wdr group: $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

extension of the knee with a 100-g force) prompted the next series of experiments to determine whether there was an anatomical correlate of this functional shift. This correlate was examined by performing confocal and electron microscopic analyses on the cellular and subcellular distribution of NK-1 receptors in control and monoarthritic rats. The question addressed was whether the passive extension of the joint induced internalization of the NK-1 receptor in the monoarthritic model, in parallel to the observed functional changes in postsynaptic excitability. In all animals, 30 min were allowed between ankle extension and perfusion for fixation; this interval was chosen because this time period demonstrated maximum receptor internalization following NK-1 receptor activation *in vivo*. The confocal microscopy



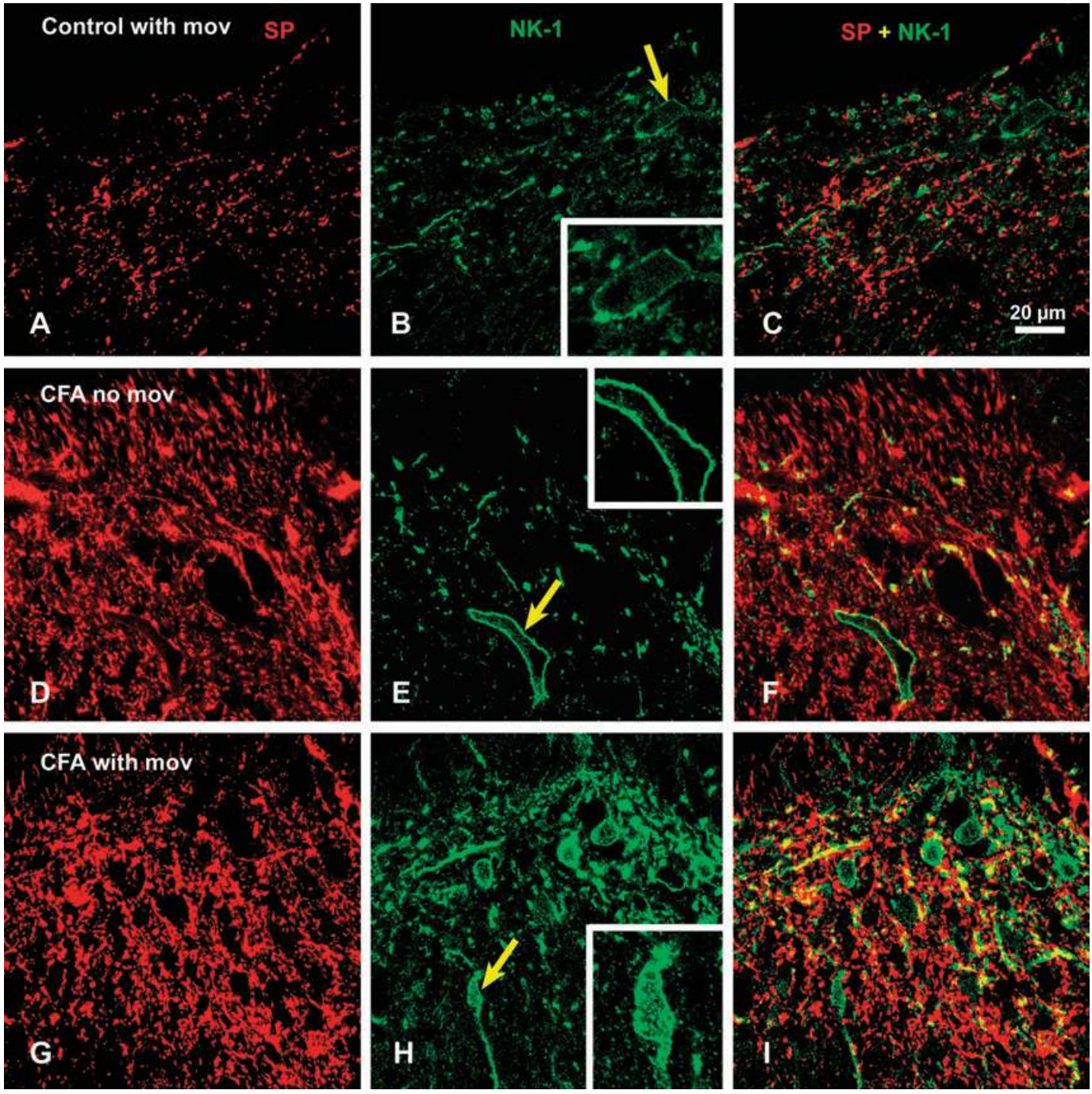


FIG. 5. Confocal microscopic images of (A, D and G) SP and (B, E and H) NK-1 receptor immunoreactivity in the rat spinal dorsal horn of (A–C) control and (D–I) monoarthritic animals; panels D–F are from the spinal cords ipsilateral to the monoarthritis without ankle movement extension and panels G–I are ipsilateral with movement. Panels C,F and I illustrate double immunolabelling of NK-1 receptor and SP immunoreactivity. Insets in panels b, e and h represent high magnifications of NK-1 receptor staining of a representative cell in each of the conditions. Scale bar, 20 µm.

data illustrating NK-1 receptor and SP distribution are shown in Fig. 5. NK-1 receptor immunoreactivity was observed in all superficial laminae with highest densities in lamina I and outer lamina II (Fig. 5B, E and H) whereas SP immunoreactivity (Fig. 5A, D and G) was observed in all superficial laminae with the highest density in lamina I and outer lamina II, with lower amounts in inner lamina II. Superimposing NK-1 receptor and SP labelling demonstrates the extensive overlap between peptide and receptor expression (Fig. 5C, F and I).

To investigate possible presynaptic changes contributing to this remodelling of sensory transmission, we analysed SP levels in the dorsal horn. Upon close examination, SP immunoreactivity was observed to be associated with axonal fibres and varicosities. Analyses of SP-immunopositive varicosities showed a significant increase in SP levels in the superficial dorsal horn of monoarthritic animals ipsilateral to the CFA-injected ankle compared to either control animals or to the contralateral spinal cord of CFA-injected rats (Fig. 6).

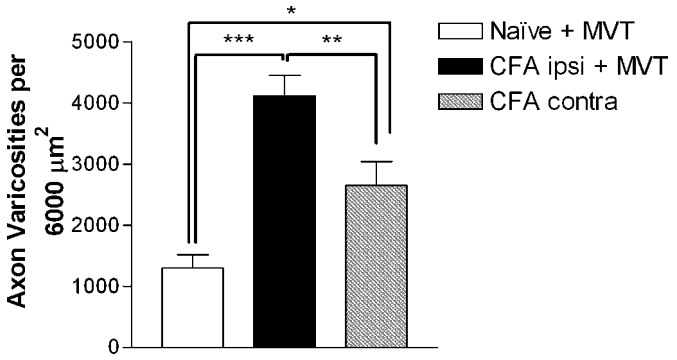


FIG. 6. Quantification of SP immuno-fluorescent labeling in the superficial dorsal horn of naïve and CFA-injected rats. Note the increase in SP labeling in the ipsilateral spinal cord of CFA-injected rats compared to the contralateral side of CFA-injected rats and to control animals: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

As previously demonstrated for control and CFA-induced polyarthritic animals (Honoré *et al*., 1999), NK-1 receptor expression was confined to the plasma membrane of non-noxious stimulation of control animals. Figure 5B inset demonstrates that joint movement of control animals did not alter NK-1 receptor internalization. However, ankle extension of monoarthritic joints caused extensive NK-1 receptor internalization (Fig. 5H inset) but not without manipulation of the ankle (Fig. 5E inset). Ultrastructural analyses confirmed that NK-1 receptor immunoreactivity was primarily localized to plasma membranes of neuronal processes in control (Fig. 7A) and monoarthritic rats (Fig. 7B), although the receptor expression was significantly augmented in the ipsilateral spinal cord of monoarthritic rats compared to controls (Fig. 7D). Interestingly, following extension of the ankle in the arthritic rat, NK-1-immunoreactive processes in the dorsal horn demonstrated internalized receptors (Fig. 7C and E), a feature associated with NK-1 activation by its ligand (Schmidlin *et al*., 2001).

Discussion

In rats with a chronic monoarthritis, our data show a modification of the response of dorsal spinal cord neurons to peripheral stimuli that would usually be considered innocuous. Our experimental protocol is concerned exclusively with the electrophysiological properties of neurons with receptive fields from the arthritic joint. It is to be contrasted with previous studies devoted to either normal healthy animals, to models of acute inflammatory pain (Menétrey & Besson, 1982; Schaible *et al*., 1991; Neugebauer *et al*., 1993, 1994) or to models of systemic chronic polyarthritis (Honoré *et al*., 1999). In these studies, the authors have already suggested that there are distinctive differences between acute and chronic arthritic inflammatory pain states, including the shift from the sensitization of primary afferent neurons to a sensitization of spinal cord neurons. What is unique about the present approach is the quantitative documentation of the electrophysiological and neurochemical changes associated with chronic pain at a stage when the chronic arthritis is at maximal severity as illustrated by the ongoing joint destruction.

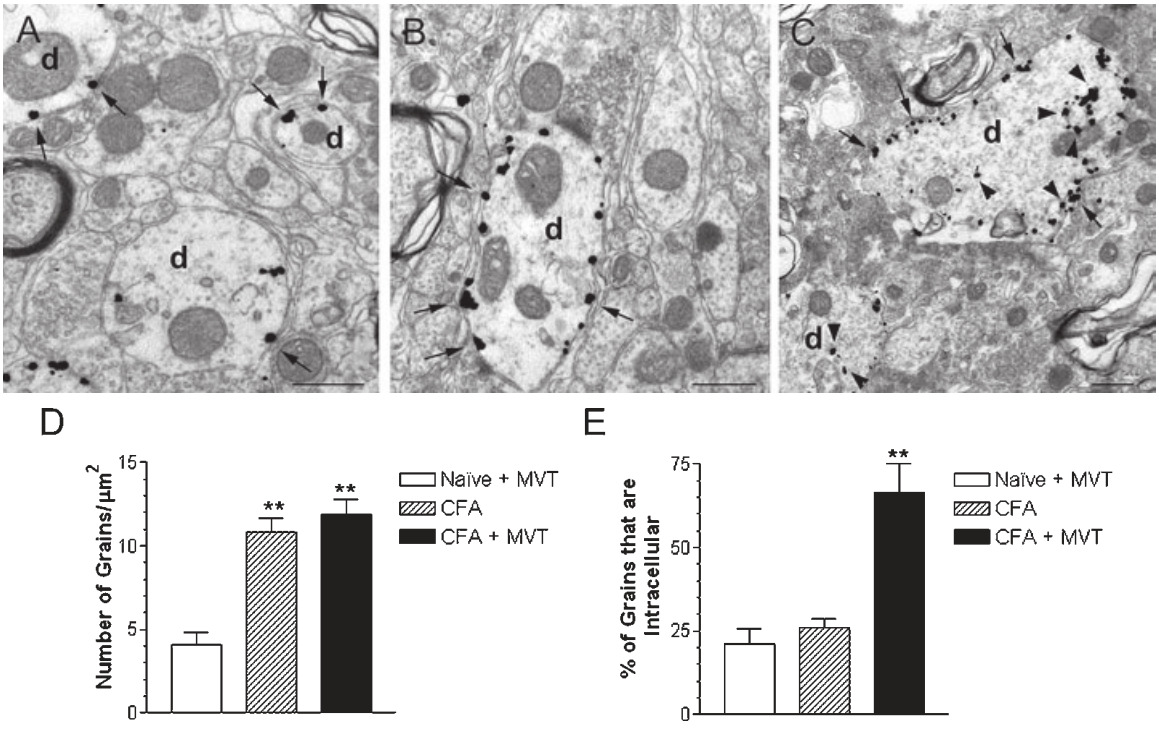


FIG. 7. Ultrastructural analysis of NK-1 receptor in arthritic and control animals following ankle extension. The electron micrographs show NK-1 receptor, as immunogold particles, in dendrites (d) in laminae I–II of the rat spinal dorsal horn. Photomicrographs demonstrate NK-1 receptors in lamina I of (A) a naïve rat with ankle extension, (B) CFA-treated with no ankle extension and (C) ipsilateral spinal cord of a CFA-treated rat with ankle extension. In all conditions, tissue samples were taken for histological processing 30 min after a passive extension of the ipsilateral ankle in naïve rats or after extension of the inflamed ankle in CFA-injected rats. CFA-treated rats with no ankle extension were perfused 30 min after anaesthesia. Arrows indicate plasma membrane-associated receptors whereas arrowheads indicate intracellular receptors. (D) Ultrastructural analysis revealed a significant increase in the number of gold particles per unit area of labelled dendritic profiles between CFA and naïve rats ($**P < 0.01$). (E) However, the percentage of gold particles associated with intracellular compartments was significantly higher in CFA rats following joint movement ($**P < 0.01$). Scale bars, 2 µm.

We have observed a change in spontaneous discharge, in postsynaptic excitability and in the synaptically elicited responses to innocuous and noxious peripheral stimuli. The morphological data showed changes in SP in nerve terminals and NK-1 receptors in the superficial laminae of the sensory spinal cord. Hence, the functional changes observed could be linked to both structural and chemical changes within the dorsal spinal cord. Our interpretation of those data is that there is remodelling of the neural substrate of nociception at the spinal level.

Previous electrophysiological studies have shown that the response of spinal cord neurons to peripheral stimuli increases in an acute inflammatory pain state (Hylden *et al*., 1989; Haley *et al*., 1990; Simone *et al*., 1991; Dougherty *et al*., 1992; Stanfa *et al*., 1992; Neugebauer *et al*., 1994). Additional studies convincingly demonstrated the rapid occurrence of central sensitization of superficial dorsal horn neurons in an acute model of monoarthritis of the knee (Neugebauer & Schaible, 1990; Schaible *et al*., 1991). We have extended those observations in showing that spinal cord neurons can remain in a state of hyperexcitability over weeks with the progression of the arthritic state. Enhanced excitability was demonstrated by the augmentation in mean spontaneous discharge of single WDR neurons and via ratemeter histograms of the response to innocuous mechanical stimuli to the receptive field. Indeed, evoked responses to mechanical stimuli were all significantly greater in monoarthritic animals than in controls. In addition, there was a significant increase in the length and amplitude of afterdischarge following cessation of the peripheral stimuli in monoarthritic animals. They were always dramatically greater over the range of both innocuous and noxious mechanical stimuli. This included responses to von Frey hair stimulation well below the withdrawal threshold of control animals. In fact, despite the reasonable assumption that the greatest difference would occur at the highest stimulus intensities (hyperalgesia), the highest percentage difference in the magnitude of the response between monoarthritic and control rats actually occurred at the lowest intensities, which potentially correlates with the phenomenon of allodynia in the awake animal.

The transient period of increased excitability following the iontophoretic application and passive joint movement lasted >20 min and no such movement-induced excitation was evident in control animals. Such a modulatory change corresponds to central sensitization and has been attributed to sustained input from sensory C-fibres resulting in an increase in the synaptic strength between primary afferents and dorsal horn neurons (Woolf & Salter, 2000). In other rodent models of arthritis, peptide-containing C-fibres are sensitized (Mannion *et al*., 1999). In avian models, these fibres can be activated by joint movement (Gentle, 1997). Finally, it is also known that repetitive firing of C-fibres leads to enhanced synaptic transmission (Mendell, 1966). Hence, it is reasonable to suggest that movement of an arthritic joint may generate an increase in neuronal excitability.

Since central sensitization was initially postulated (Mendell, 1966; Woolf, 1983; Dickenson, 1990), several studies have suggested that activation of serine and/or threonine and tyrosine kinases lead to phosphorylation of the NMDA receptor (Chen & Huang, 1992; Yu *et al*., 1997; Woolf & Costigan, 1999). Increased responsiveness would then be largely mediated by a facilitated transmission through the phosphorylated NMDA receptor which, with coactivation of NK-1 receptors, would lead to increased neuronal excitability. Our results are compatible with this concept as they show a selective modulation of postsynaptic mechanisms in nociceptive neurons occurring via an NK-1 receptor-dependent mechanism. An additional contribution made by the present study was the observation that a normally innocuous passive extension of the ankle is enough to trigger NK-1 receptor internalization, indicating that joint movement elicited the release of SP from afferent terminals in animals with chronic arthritic pain.

Given that relationship and our findings, extension of the arthritic joint was definitively a noxious stimulus in our model. This may have implications for the occasional bad response to physiotherapy regimen in humans where 'innocuous' passive movements of arthritic joints worsen the condition, maybe by enhancing the excitability of spinal nociceptive pathways. Of course, there is no information on what would happen if the passive movement was continuous or repeated at regular intervals. Could desensitization and analgesia be the result? That might be something to explore in view of the world-wide success of the continuous passive motion (CPM) machine used in the postoperative period of knee surgery (Salter, 2004).

In conclusion, a chronic monoarthritic pain state produces a significant 'remodelling' of sensory mechanisms at the spinal level, including an increase in SP in presynaptic terminals and of NK-1 receptors in postsynaptic dorsal horn neurons. We demonstrate that these changes enable synaptic modulation at low intensities of stimulation, a phenomenon that should normally occur only in response to noxious stimulation (Meyer & Campbell, 1981). Because sensory fibres have been shown to be sensitized during inflammation (Andrew & Greenspan, 1999), it is also possible that they remain excited for several minutes following the innocuous stimulation and continue releasing SP in the spinal cord. This remodelling may be of long duration, perhaps permanent, and might, with the changes in synaptic modulation, contribute to the chronicity of arthritic pain in humans. Taken together, the data provide evidence that SP and its receptor in the dorsal horn are related not only to nociceptive sensory transmission or acute inflammatory pain states, as is widely accepted, but may also be involved in the persisting remodelling of spinal nociceptive mechanisms that underlie the chronicity of the pain of arthritis.

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Abbreviations

AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; CFA, complete Freund's adjuvant; NK-1, neurokinin-1; NMDA, *N*-methyl-D-aspartate; PB, phosphate buffer; PBS-T, phosphate-buffered saline with 0.2% Triton X-100; SP, substance P; WDR, wide dynamic range.

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