British Journal of Pharmacology (2006) 149, 56–64
 0007–1188/06 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Vitamin A active metabolite, all-trans retinoic acid, induces spinal cord sensitization. I. Effects after oral administration

EA Romero-Sandoval[1], C Molina, M Alique, V Moreno-Manzano[2], FJ Lucio and JF Herrero

Departamento de Fisiología, Facultad de Medicina, Universidad de Alcalá, Madrid, Spain

Background and purpose: Retinoic acid is an active metabolite of vitamin A involved in the modulation of the inflammatory and nociceptive responses. The aim of the present study was to analyze the properties of spinal cord neuronal responses of male Wistar rats treated with all-trans retinoic acid (ATRA) p.o. in the normal situation and under carrageenan-induced inflammation. We also studied the expression and distribution of cyclooxygenases (COX) in the spinal cord.
Experimental approach: Properties of spinal cord neurons were studied by means of the single motor unit technique. The expression of COX enzymes in the spinal cord was assessed by Western blot analysis and immunohistochemistry.
Key results: Intensity thresholds for mechanical and electrical stimulation (C-fibers) were significantly lower in animals treated with ATRA than vehicle, either in normal rats or in rats with inflammation. The size of cutaneous receptive fields was also larger in animals treated with ATRA in the normal and inflammatory conditions. The expression of COX-2 enzyme, but not COX-1, was significantly higher in animals treated with ATRA. COX-2 labeling was observed in dorsal horn cells and in ventral horn motoneurons.
Conclusions and implications: In conclusion, the oral treatment with ATRA in rats induces a sensitization-like effect on spinal cord neuronal responses similar to that observed in animals with inflammation and might explain the enhancement of allodynia and hyperalgesia observed in previously published behavioral experiments. The mechanism of action involves an over-expression of COX-2, but not COX-1, in dorsal and ventral horn areas of the lumbar spinal cord.
British Journal of Pharmacology (2006) **149**, 56–64. doi:10.1038/sj.bjp.0706829; published online 17 July 2006

Keywords: vitamin A; retinoic acid; pain; hyperalgesia; COX; inflammation; Western blot; spinal cord; electrophysiology; immunohistochemistry

Abbreviations: ATRA, all-trans retinoic acid; COX, cyclooxygenase; SMU, single motor unit

Introduction

Peripheral tissue inflammation leads to sensitization of nociceptors, on exposure to inflammatory mediators, and to central sensitization due to a facilitation of synaptic inputs into the dorsal horn of the spinal cord. As a consequence there is an exaggerated sensation of pain called hyperalgesia (Treede *et al*., 1992). The initiation and maintenance of central sensitization involve numerous mechanisms and neuromediators whose release triggers and maintains a state of high neuronal activity. The expression of cyclooxygenase-2 (COX-2; Samad *et al*., 2001), for example, is enhanced rapidly in the spinal cord during sensitization, along with the production of prostaglandins like prostaglandin E2 (PGE2). Interleukin-1β (IL-1β) is also upregulated following lipopolysaccharide administration (Watkins *et al*., 1994) or inflammation (Safieh-Garabedian *et al*., 1995) and induces, in turn, a fast upregulation of COX-2 either in peripheral tissues (Maier *et al*., 1990) or in the spinal cord (Samad *et al*., 2001). The mechanisms underlying the upregulation of IL-1 or COX-2 are not known, and some endogenous systems involved in the initiation of sensitization are still unidentified. Retinoids might be one of these unidentified systems.

Active metabolites of vitamin A, such as all-trans retinoic acid (ATRA; Duester *et al*., 2003), play an essential activity in the embryological development of several tissues and organs (Gudas *et al*., 1994), including the brain and the spinal cord (Solomin *et al*., 1998; Duester *et al*., 2003). Retinoids are also

Correspondence: Dr JF Herrero, Departamento de Fisiología, Campus Universitario, Universidad de Alcalá, Alcalá de Henares, 28871 Madrid, Spain. E-mail: juanf.herrero@uah.es
[1]Current address: Baptist Medical Center, Wake Forest University, Department of Anesthesiology, Pain Mechanisms Laboratories, Medical Center Blvd. Winston Salem, NC 27157, USA.
[2]Current address: Neural Regeneration. Regenerative Medicine Department, Centro de Investigacion Principe Felipe, Av., Autovia del Sales, 16 Camino de las Moreras, 46013 Valencia, Spain.
Received 9 March 2006; revised 18 May 2006; accepted 12 June 2006; published online 17 July 2006

present in the brain and spinal cord of adult rats and mice (Zetterstrom *et al*., 1994; Werner and Deluca, 2002) and are involved in functions such as spatial learning and memory (Misner *et al*., 2001; Cocco *et al*., 2002). In addition, retinoids are involved in the mechanisms underlying the inflammatory reaction, and have been related to the generation and expression of nitric oxide (NO; Hirokawa *et al*., 1994; Grosjean *et al*., 2001; Seguin-Devaux *et al*., 2002), prostaglandins (Hill *et al*., 1996; Devaux *et al*., 2001) and cyclooxygenases (COX; Nusing *et al*., 1995; Kanekura *et al*., 2000; Li *et al*., 2002).

In a previous work carried out in our lab (Romero-Sandoval *et al*., 2004) we considered that retinoids might be involved in the processing of nociceptive information, especially in situations of hyperalgesia due to inflammation. This hypothesis was based in on three observations: (i) Retinoids are present in the spinal cord and brain; (ii) Retinoids have a relationship with mediators of inflammation, which are also involved in the generation or maintenance of sensitization and pain due to inflammation (see e.g. Kanekura *et al*., 2000; Grosjean *et al*., 2001; Devaux *et al*., 2001); (iii) The topical use of retinoids is associated with rash, pruritus and pain (Lowe and Plosker, 2000). In this study, we observed in behavioral experiments that animals with inflammation treated with ATRA p.o. showed a more intense development of allodynia and hyperalgesia than control animals. Also, the recovery to baseline data was slower in animals treated with ATRA. We also observed an over-expression of COX-2 in neuroblastoma cells and in the whole spinal cord of animals treated with ATRA.

These results suggest that ATRA is actively implicated in the generation and/or maintenance of inflammation-induced sensitization. The aim of the present study was to analyze whether the oral administration of ATRA, induces changes in spinal cord neuronal responses compatible with those observed in inflammation-induced sensitization. Central sensitization manifests as changes in the properties of spinal cord neurons. These changes include decreased thresholds for natural and electrical stimulation and enlarged cutaneous receptive fields (Woolf, 1983; McMahon and Wall, 1984; Schaible and Schmidt, 1985; Laird and Cervero, 1989; Woolf and King, 1990; Dubner and Ruda, 1992; Treede *et al*., 1992). Therefore, we first studied the influence of ATRA on spinal cord neuronal nociceptive responses to mechanical and electrical stimulation of normal rats and of rats with inflammation. In the second part of the study, we analyzed whether the changes observed were associated with a modification of the expression of COX enzymes either in the hemicord ipsilateral or contralateral to the inflammation, like in inflammation-induced sensitization, by means of Western blot and immunohistochemistry techniques. Preliminary data have been published in abstract form (Molina *et al*., 2005).

We conclude that the oral treatment with ATRA in rats induces a sensitization-like effect on spinal cord neuronal responses similar to that observed in animals with inflammation and might explain the enhancement of allodynia and hyperalgesia observed in previously published behavioral experiments. The mechanism of action involves an over-expression of COX-2, but not COX-1, in dorsal and ventral horn areas of the lumbar spinal cord.

Methods

Electrophysiological experiments

Electrophysiological experiments were performed on adult male Wistar rats (240–340 g) divided in four experimental groups: normal animals treated with ATRA ($n=17$), normal animals treated with vehicle ($n=11$), animals with carrageenan-induced inflammation treated with ATRA ($n=20$) and animals with inflammation treated with vehicle ($n=9$). Inflammation was induced 15 h before the experiment under brief halothane anesthesia (5% in oxygen for induction, 2% for maintenance) by the intraplantar administration of 100 μl of carrageenan λ (10 mg ml^{-1} in distilled water; Sigma, St Louis, MO, USA) in the right hind paw. Another 100 μl of saline were injected in the left hind paw as a control for inflammation. The effectiveness of carrageenan in the induction of inflammation was assessed by measuring the volume of the paw by plethysmometry (Letica plethysmometer, Panlab SL; Barcelona, Spain) before the administration of carrageenan and after the experiment.

ATRA (Tretinoin, Sigma) was suspended in carboxymethylcellulose (Sigma) 0.5% and administered orally in a final volume of 0.5 ml. A similar amount of carboxymethylcellulose was administered to the animals in the control group of experiments. The treatment with either ATRA or vehicle p.o. lasted for 4 days and consisted of a dose of 15 mg kg^{-1} the first 2 days and a dose of 10 mg kg^{-1} the following 2 days. The doses and protocol of administration were chosen according to the results observed in behavioral experiments performed previously in our lab (Romero-Sandoval *et al*., 2004).

The preparatory surgery has been described previously in detail (see e.g. Mazario *et al*. (2001); Romero-Sandoval *et al*. (2002)), and was performed under halothane anesthesia (5% in oxygen for induction and 2–3% for maintenance). The surgery consisted in the cannulation of the trachea, one carotid artery to register the blood pressure, and two superficial branches of the jugular veins, for the administration of α-chloralose and fluid therapy. The animal was moved to an appropriate frame and anesthesia was continued with α-chloralose (Sigma) 50 mg kg^{-1} initial dose supplemented with 20 mg kg^{-1} h. Core temperature was maintained close to 37°C by means of a feedback-controlled blanket system. Blood pressure was continuously monitored and systolic value did not fall below 100 mm Hg throughout the experiment. The preparation was allowed to rest for at least an hour before recordings started. The recording of single motor units (SMU) has, as a main advantage, the possibility of recording direct spinal cord neuron responses with a minimum preparatory surgery. The activity of spinal cord units is, therefore, recorded under better physiological conditions and the results are very reproducible. The technique has been described in detail several times (Herrero and Headley, 1991; Solano and Herrero, 1997, 1999), briefly, SMUs activated by noxious mechanical and electrical stimulation were recorded by means of a bipolar tungsten electrode inserted percutaneously into muscles of the right hind limb. Isolation of motor units was performed by moving the electrode with a micromanipulator while a mild pressure was applied to the paw. A window discriminator and

a standard system for extracellular recordings were used to count the number of spikes. The SMUs were not specifically selected and the first isolated unit was the unit used for the study. Once the unit had been isolated, the preparation was allowed to rest for 10 min before its properties were identified using the following protocol: (1) Determination of the mechanical threshold by means of von Frey hairs applied to the most sensitive area of the cutaneous receptive field (usually first toe). The series of hairs used were: 5, 7, 10, 15, 20, 25, 30, 35, 40, 50, 60, 80, 100 and 200 mN. (2) Determination of the threshold to activate C-fiber volley by electrical stimulation (2 ms width square pulses) by means of two intradermal fine needles (0.2 mm) inserted in the most sensitive area of the receptive field (Solano and Herrero, 1997). Data from electrical stimulation were analyzed by counting the responses evoked between 150 and 650 ms after each pulse (C-volley inputs; Herrero and Cervero, 1996). An original recording of responses observed after an electrical stimulus is represented in Figure 1a. Each test was repeated three times at 3 min intervals and the averaged value was considered for analysis. (3) Mapping of the field using a 500 mN hair, a force capable of inducing nociceptive withdrawal reflexes in conscious animals and considered as a noxious stimulus (Romero-Sandoval *et al*., 2004). An illustration of the technique and an original recording is shown in Figures 1b and c. The area of the receptive fields was marked on the skin and copied onto tracing paper. Dorsal and plantar areas were only considered for analysis and measured using a digitizing tablet. On-line collection of data and off-line numerical analysis were performed by computer using commercial software (CED, UK; Graph Pad, Instat, Prism, USA). Data are presented as mean ± s.e.m. Statistical significance was calculated using the one-way analysis of variance (ANOVA) with *post hoc* Dunnett's test.

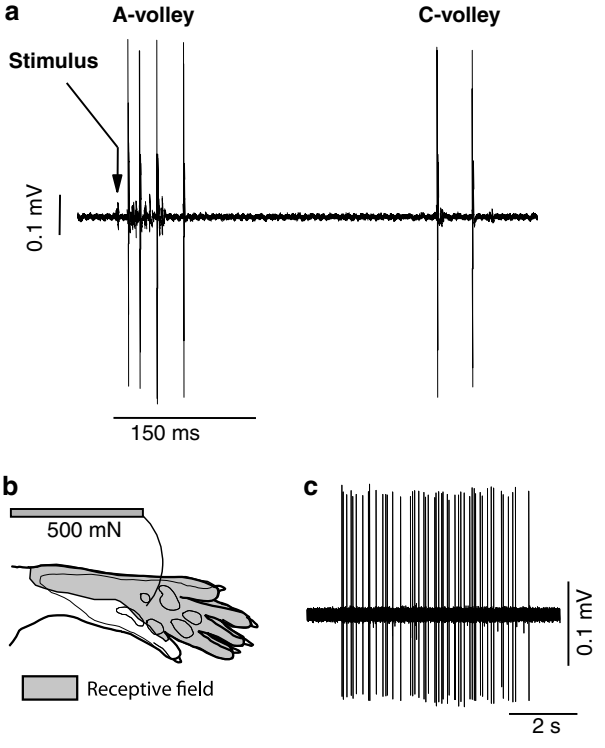

Figure 1 Illustration of the methods followed in electrophysiological experiments. (**a**) Original recording of A- and C-volley responses recorded after an electrical stimulus. The threshold for activation of C-fiber volley was determined by the application of 2 ms width square electrical pulses. Data from electrical stimulation were analyzed by counting the responses evoked between 150 and 650 ms after each pulse. (**b**) Cutaneous receptive fields were mapped on dorsal and plantar sides of the paw using a 500 mN filament. (**c**) Illustration of an original recording of an unit activated by the application of a 500 mN filament on the most sensitive area of the receptive field during 5 s.

Western blot analysis of COX expression

Fourteen male Wistar rats were treated with ATRA ($n=7$) or vehicle ($n=7$) following the same protocol as the electrophysiological experiments. Soft-tissue inflammation was also induced following the above-described protocol, injecting 100 ml of carrageenan λ (10 mg ml^{-1} in distilled water; Sigma) in the right hind paw. Another 100 μl of saline were injected in the left hind paw as a control for inflammation. The segment of the spinal cord corresponding to the lumbar enlargement was dissected under halothane anesthesia (same concentration as before), removed, hemisected along the midline and shock-frozen immediately in liquid nitrogen for Western blot analysis. Tissue samples were homogenized in a solution containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1% deoxycholic acid, 0.1% SDS, 1% Triton-X-100 and protease inhibitors 1 mM phenylmethylsufonyl fluoride, 10 μg ml^{-1} aprotinin, 2 μg ml^{-1} leupeptin and the phosphatase inhibitor 0.2 mM $NaVO_4$. Samples were then sonicated on ice and centrifugated at 14 000 r.p.m. for 30 min at 4°C, quantified using Bradford Assay (Bradford, 1976) and the supernatant collected and stored at −20°C. On the day of the assay 30 μg of every sample were thawed, mixed with equal amount of 2X Loading Buffer (0.0625 M Trizma, pH 6.8; 2% w/v SDS; 5% w/v 2-mercaptoethanol, 10% w/v glycerol; 0.002% w/v bromophenol blue), heated to 95°C for 5 min and fast cooled. The samples were separated in a SDS–PAGE gel (8% polyacrylamide). The proteins were then transferred onto a nitrocellulose membrane (Optiman Ba-S 85, Cheicher and Chevell, Germany). After the transfer, the membranes were blocked in TBST (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.1% Tween-20) containing 5% dried non-fat milk and then incubated for 24 h at 4°C with the primary antibody (rabbit anti-COX-2 and anti-COX-1, Cayman Chemical, Ann Arbor, MI, USA) at a concentration of 1–2 μg ml^{-1} in TBST containing 5% dried non-fat milk. The membranes were then washed three times with TBST and then incubated for 1 h at room temperature in TBST containing horseradish peroxidase-linked antiimmunoglobulin (Chemicon, Temecula, CA, USA; 1:5000 dilution). After three washes in TBST, immunoreactive products were detected by chemiluminiscence using the Super Signal West Pico Chemiluminescent Substrate Kit (Pierce, Rockford, IL, USA) and Kodak X-ray films. Blots were subsequently probed with monoclonal anti-β-tubulin (Sigma) as a loading control. Results are shown as the mean ± s.e.m. of the ratio of the densitometric values of COX to β-tubulin, Statistical significance was calculated using the one-way analysis of variance (ANOVA) with *post hoc* Dunnett's test.

COX-2 immunohistochemistry

Following the same protocols for the administration of ATRA and for carrageenan-induced inflammation described in the electrophysiological experiments, 12 male Wistar rats (six animals treated with vehicle and six animals treated with ATRA) were deeply anesthetized (halothane, same concentration as above) and perfused transcardially with fixative containing 0.01 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in ice-cold 0.1 M PBS (pH 7.4). The lower lumbar spinal cord was removed and post-fixed in the same fixative for an additional 10 min. Spinal cord segments were cryoprotected in 30% sucrose in 0.1 M PBS at 4° C for 24 h, sectioned at 4 μm intervals in a cryostat, mounted on pre-subbed slides and air dried. Tissue sections were fixed with a freshly prepared 2% paraformaldehyde solution in PBS for 10 min at room temperature and washed in PBS (pH 7.4). Preincubation was made in blocking solution (PBS containing 10% sheep serum and 0.5% Triton-X-100) for 30 min at room temperature. Sections were then washed in PBS and incubated overnight in anti-COX-2 (1 : 100 in PBS containing 10% sheep serum; Cayman Chemical, Ann Arbor, MI, USA). Following a further wash in PBS containing 0.02% of Tween-20 (PBST), HRP- or FITC- conjugated anti-rabbit IgG (1 : 500 in PBS) was pipetted onto the sections that were incubated at room temperature for 2 h. The slides were then washed in PBST and, in the case of secondary HRP-conjugated antibody, we first follow the instructions of VECTOR Universal Kit (Vector, Burlingame, CA, USA) to finally develop with DAB/urea mix (*Sigma FAST*; Sigma). The excess fluid was blotted from around the tissue before it was mounted in Mowiol 4.88 (Sigma). All incubations took place in a humidity chamber. In the control experiments the primary antibody was omitted from the incubation medium to determine the amount of non-specific binding. COX-2 positive neurons were quantified in at least four sections per animal for comparison of regional variations and differences between hemicords and treatments. However, the number of positive small cells observed in all areas of the dorsal horn of animals treated with ATRA was too high to be quantified. As the number of these positive cells was much larger, and evident by visual inspection, than that observed in animals treated with vehicle comparisons between hemicords and treatments were made with the number of motoneurons counted in the ventral horn.

In all series of experiments, the animals were used for one procedure only and were humanely killed on completion of testing by an overdose of sodium pentobarbital (Euta-Lender, Normon). All experimental procedures were performed in accordance with European Union legislation and were approved and supervised by the University Animal Care facility. All efforts were made to minimize animal suffering and to reduce the number of animal used.

Results

Electrophysiological experiments

Thresholds. The study of thresholds for mechanical stimulation showed significant differences in animals treated with ATRA when compared to animals treated with vehicle (Figure 2). In normal animals treated with vehicle, the mean threshold intensity was 64 ± 1.6 mN whereas in animals treated with ATRA the threshold was significantly lower: 54 ± 1.5 mN ($P<0.001$). Likewise, in animals with inflammation the threshold intensity was significantly lower in rats pre-treated with ATRA: 30 ± 1.2 vs 24 ± 1.5 mN ($P<0.01$). The administration of carrageenan induced a similar increase in the paw volume of animals treated with vehicle and ATRA: 1.4 ± 0.1 (pre-volume: 1.63 ± 0.1; post-volume: 3.02 ± 0.1 ml) and 1.5 ± 0.1 ml (pre-volume: 1.72 ± 0.1; post-volume: 3.22 ± 0.1 ml). In both cases, the volume of the paw was significantly larger when compared to the pre-inflammation volume ($P<0.001$).

The activation of C-fiber mediated responses by electrical stimulation also showed a significant lower threshold intensity for the units studied in animals treated with ATRA when compared to control animals (Figure 3). In normal animals the threshold intensities were 0.6 ± 0.1 mA in the group of animals treated with vehicle and 0.28 ± 0.1 mA ($P<0.05$) for animals treated with ATRA. In animals with inflammation the thresholds were 0.2 ± 0.05 in control rats and 0.08 ± 0.03 ($P<0.05$) in rats treated with ATRA (Figure 3).

Receptive fields

Figures 4a and b show the distribution of cutaneous receptive fields of the units studied with a 500 mN von Frey hair, as well as the quantified areas in the four groups of animals. In normal animals treated with vehicle, the receptive fields obtained with 500 mN hair were located on the lateral region of the paw, including the fourth and fifth

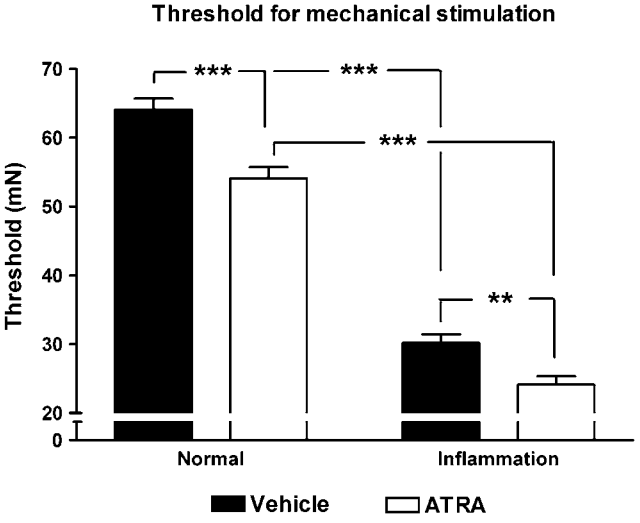

Figure 2 Threshold intensities for mechanical stimulation in animals treated with vehicle or ATRA in the normal and inflammatory conditions. As previously observed, the threshold intensity for the activation of the units was lower in animals with soft tissue inflammation. In normal animals treated with oral ATRA, the threshold was significantly lower than in vehicle-treated rats. Likewise, the threshold was lower in animals with inflammation treated with ATRA when compared to control rats. (*$P<0.05$; **$P<0.01$; ***$P<0.001$, statistical significance was calculated using the one-way analysis of variance, ANOVA with *post hoc* Dunnett's test).

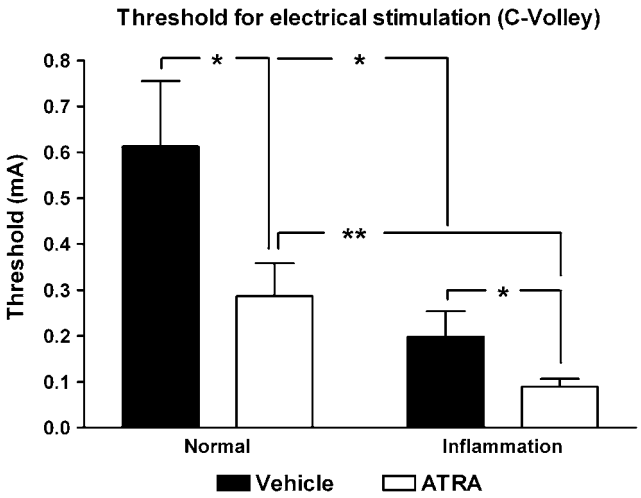

Figure 3 Threshold intensities for electrical stimulation in animals treated with vehicle or ATRA in the normal and inflammatory conditions. The threshold intensity for the activation of C-fibre volley was lower in animals treated with ATRA in comparison to vehicle treated animal. As for mechanical stimulation, the difference was observed both in the normal and inflammatory situations. In addition, the threshold observed in normal animals treated with ATRA was very similar to that seen in the control group of rats with inflammation (statistical significance and layout as for Figure 2).

toes, both on the dorsal and plantar surfaces, and the quantified area was of $1.3 \pm 0.3\,cm^2$. In normal animals treated with ATRA, the cutaneous receptive fields were larger, with an area of $2.9 \pm 0.3\,cm^2$ ($P<0.01$), and included zones of the last three toes, and medial and back areas of the paw. These fields were very similar in shape and size to those of units from animals with inflammation treated with vehicle, which quantified area was of $2.99 \pm 0.3\,cm^2$. The area of the cutaneous receptive fields of animals with inflammation treated with ATRA were again larger than those recorded in animals treated with vehicle, with a mean area of $4.5 \pm 0.3\,cm^2$ ($P<0.01$), and included the last four toes and most of the dorsal and plantar sides of the paw (Figure 4b).

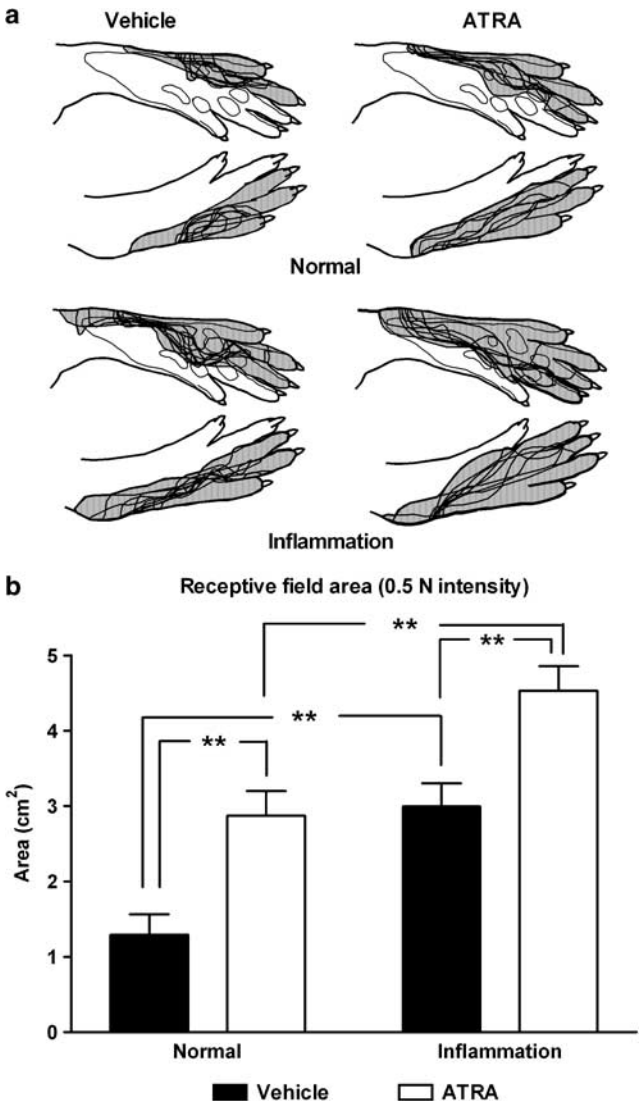

Figure 4 Cutaneous receptive fields of rats treated with vehicle and ATRA in the normal and inflammatory conditions. (**a**) Extension of cutaneous receptive fields represented for each unit on the dorsal and plantar faces of the paw of animals treated with vehicle or ATRA in the normal and inflammatory conditions. Note the similarities in the shape and size of fields in normal animals treated with ATRA and animals with inflammation treated with vehicle. (**b**) Quantification of the areas of the fields in the four experimental conditions. The size of fields from units studied in animals treated with ATRA were larger than those in animals treated with vehicle either in normal rats or in rats with inflammation (statistical comparison and layout as for Figure 2).

Modulation of the expression of COX enzymes by ATRA

Low basal levels of COX-1 and COX-2 protein expression were detected by Western blot in the normal non-sensitized hemicords (Figure 5). A slight and non-significant increment of the expression of both isoenzymes was observed in the hemicords under sensitization of animals treated with vehicle. In animals treated with ATRA, however, a significant enhancement of the expression of COX-2 ($P<0.05$), but not of COX-1, was observed in both hemicords, control and sensitized, as compared to the respective hemicords of animals treated with vehicle (Figure 5).

As Western blot analysis showed an enhancement of COX-2, but not COX-1 expression either in the control or sensitized hemicord, we only studied the distribution of neuronal COX-2-like immunoreactivity in animals treated with vehicle or with ATRA. Immunohistochemistry experiments performed on animals treated with vehicle showed a variable but always very low COX-2-like immunoreactivity either in the normal or in the sensitized hemicord. The sections in Figure 6a illustrate typical results observed in an animal with carrageenan-induced sensitization. However, in animals treated with ATRA there was a dense labeling spread throughout the whole cord (Figure 6a), confirming the upregulation of the COX-2 enzyme expression observed in Western blot experiments. Small size positive cells were observed throughout the superficial and deep dorsal horns (Figure 6b). Likewise, large size, labeled motoneurons were observed in all areas of the ventral horn (Figure 6b). In animals treated with ATRA, quantitative analysis of cells did not show any significant difference of the regional distribu-

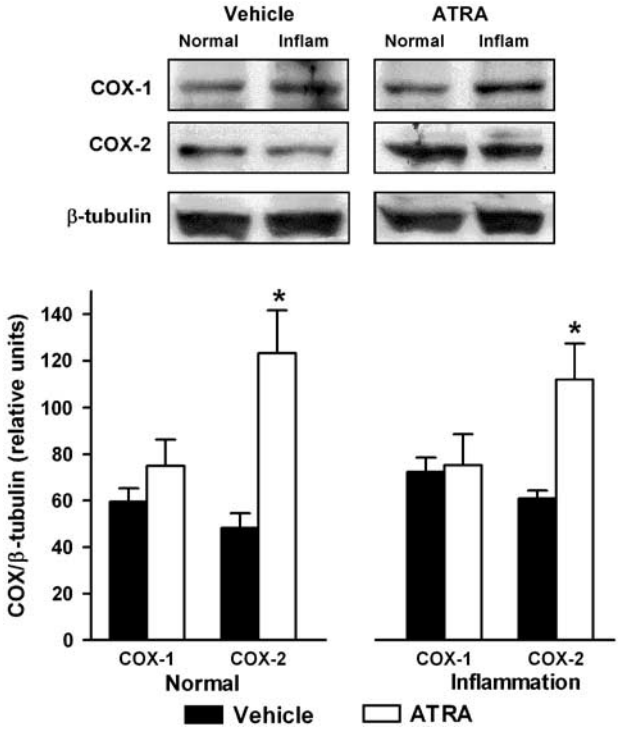

Figure 5 Expression of COX enzymes in the lumbar segment of the spinal cord of rats treated with ATRA or vehicle. The hemicords ipsilateral (Inflam) and contralateral (Normal) to the inflamed paw were studied separately. The results show a significant enhancement of COX-2, but not COX-1, expression both in the ipsilateral and contralateral hemicords of animals treated with ATRA when compared to that of animals treated with vehicle. Data are expressed as relative values of COX protein (Western blot analysis) normalized for β-tubulin protein levels (*$P<0.05$, comparison vs vehicle, using the one-way analysis of variance, ANOVA with *post hoc* Dunnett's test). A typical Western blot is shown in the upper panel of the figure.

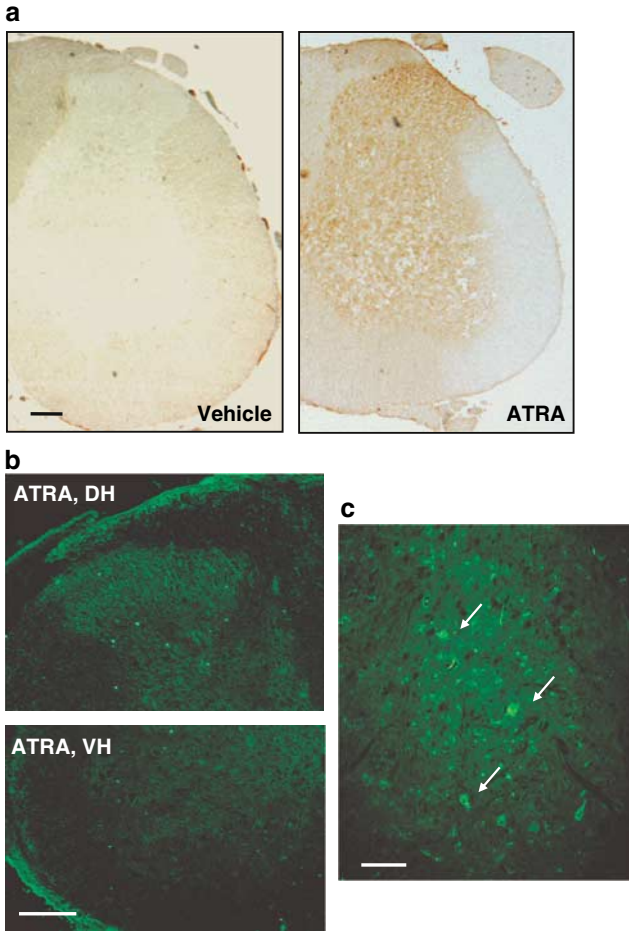

Figure 6 Effect of ATRA on spinal cord COX-2 expression of animals with carrageenan-induced inflammation. Immunohistochemistry for COX-2 in 4 μm sections of the rat lumbar segment of the spinal cord showed a rather homogeneous enhancement of immunoreactivity on the spinal cord of animals treated with ATRA when compared to vehicle treatment (**a**). A high number of positive cells were observed both on dorsal and ventral horn cells (**b**). Detailed image (**c**) shows labeling on ventral horn motoneurons (arrows). (**a**, **b**) scale bar: 250 μm; (**c**) scale bar: 50 μm.

tion of positive cells (dorsal vs ventral horn) nor of the number of positive cells between the normal hemicord (mean number of positive motoneurons counted per section: 17 ± 2 cells) and the hemicord under sensitization (24 ± 3 cells). The number of counted positive cells was, however, significantly higher in animals treated with ATRA when compared to animals treated with vehicle, either in the normal hemicord (mean number of positive motoneurons counted per section: 17 ± 2 cells in ATRA vs 10 ± 2 cells in vehicle; $P<0.05$) or in the cord with sensitization (mean number of positive neurons counted per section: 24 ± 3 cells in ATRA vs 9 ± 3 cells in vehicle; $P<0.01$).

Discussion

The recording of SMUs activated by peripheral noxious stimulation is a fine technique to study the spinal cord nociceptive neuronal responses. These experiments allow physiological and pharmacological *in vivo* studies of nociception in a less invasive manner than the direct recording of spinal cord neurones (Herrero and Cervero, 1996; Herrero and Headley, 1996; Solano and Herrero, 1999), lowering the alteration of the processing of nociceptive information observed in other experiments due to the spine surgery (Herrero and Headley, 1996). The technique has also been used to study the changes that occur in the spinal cord neuronal processing of nociceptive information in situations of inflammation-induced sensitization (Solano and Herrero, 1999). In this situation, peripheral tissue inflammation leads to a peripheral sensitization of nociceptors, on exposure to inflammatory mediators, and to a central sensitization, due to a facilitation of synaptic input into the dorsal horn of the spinal cord. As a consequence there is hyperalgesia and increased pain sensitivity (Treede *et al.*, 1992). Central sensitization manifests itself as changes in the properties of spinal cord neurons. These changes include decreased thresholds for natural and electrical stimulation and an enlargement of cutaneous receptive fields (Woolf, 1983; McMahon and Wall, 1984; Schaible and Schmidt, 1985; Laird and Cervero, 1989; Woolf and King, 1990; Dubner and Ruda, 1992; Treede *et al.*, 1992). We have previously observed in behavioral experiments that animals with carrageenan-

induced inflammation treated with ATRA show a more intense and long lasting development of allodynia and hyperalgesia than control animals (Romero-Sandoval *et al*., 2004). The present experiments show that ATRA induces changes on spinal cord neuronal responses of normal non-inflamed animals similar to those observed after inflammation-induced sensitization, that is lowered thresholds and enlargement of cutaneous receptive fields. In other words, the results suggest that the administration of ATRA induces sensitization-like changes in the properties of spinal cord neuronal activity. In fact, the size of receptive fields was virtually identical in animals with inflammation and in normal animals treated with ATRA; likewise, the threshold for C-volley activity was very similar in these two experimental groups. Furthermore, in animals with inflammation treated with ATRA both thresholds studied were lower and the size of fields larger than in animals treated with vehicle, indicating that ATRA is also effective in inflammation, or that it enhances the changes induced by inflammation. The sensitization induced by ATRA in these experiments might be the explanation for the enhancement of allodynia and hyperalgesia observed in behavioral experiments performed in our lab (Romero-Sandoval *et al*., 2004). It may also explain the sensation of pain reported with the topical use of retinoids (Lowe and Plosker, 2000).

The changes observed on the nociceptive system during inflammation involve the major classes of chemical mediators that participate in nociceptive processing. It is, therefore, difficult to study the mechanism or mechanisms underlying the effect of ATRA. In addition, ATRA is involved in the regulation of many neuromodulators participating in the nociceptive and inflammatory responses, such as opioid receptor and c-fos mRNAs (Jenab and Inturrisi, 2002). However, the modulation of COX and prostaglandin activity by ATRA and other retinoids is well reported in the literature (Nusing *et al*., 1995; Hill *et al*., 1996; Kanekura *et al*., 2000; Devaux *et al*., 2001; Li *et al*., 2002). Also, the implication of COX enzymes and prostaglandins in the changes of the nociceptive system leading to sensitization is well reported (see Schaible and Grubb, 1993; Millan, 1999; Vanegas and Schaible, 2001 for review). In addition, we observed in our previous study (Romero-Sandoval *et al*., 2004) that ATRA induces an upregulation of the expression of COX enzymes in two different lines of neuroblastoma cells and that of COX-2 in the whole spinal cord of rats. Based on these observations, we decided to study whether the treatment with ATRA followed in the present experiments was associated with a change in the expression of COX-1 and COX-2 in the hemicord under sensitization and in the control hemicord of the same animal. The results from these experiments show a clear enhancement of the expression of COX-2, but not COX-1, in both hemicords. This might be one of the mechanisms underlying the effect of sensitization observed after the administration of ATRA in electrophysiological experiments.

A small increment of the COX-2 enzyme was observed in inflamed animals not treated with ATRA when compared to normal non-inflamed animals. The increment was not significant and much lower than that seen after ATRA treatment. Nonetheless, an enhancement of COX-2 expression in cultured cells after the administration of inflammatory mediators is well documented in the literature (Hla and Neilson, 1992; O'Sullivan *et al*., 1992; Seibert *et al*., 1994; Ichitani *et al*., 1997). The small carrageenan-induced enhancement of COX-2 expression observed in our experiments might suggest that our system detected the large increments induced by ATRA but it was not sensitive enough to detect smaller increments. Supporting this interpretation is the non-significant increment of COX-2 expression in the spinal cord observed in previous studies using a similar technique (Goppelt-Struebe and Beiche, 1997).

In order to confirm our findings, and to study the distribution of COX-2 expression throughout the spinal cord, we carried out some spinal cord immunohistochemistry, following the same protocol of ATRA treatment as in previous series of experiments in normal animals. In these experiments, we observed an important increment of COX-2-like immunoreactivity in the spinal cord of animals treated with ATRA when compared to that of animals with vehicle. The results seem to confirm that the protocol of treatment with ATRA followed in the present experiments induced an upregulation of COX-2 expression in the spinal cord. Labeling was not restricted to dorsal horn neurons, but it was observed in all areas of the spinal cord, including motoneurons as observed in previous studies of COX-2 expression in inflammation-induced sensitization (Willingale *et al*., 1997).

Finally, the upregulation of COX-2 in the spinal cord observed in the present experiments was associated with the oral treatment of ATRA and with changes in the properties of spinal cord neurons similar to those seen in inflammation. However, although an over-expression of COX-2 might explain the phenomenon of sensitization, this was similar in normal animals and in animals with inflammation, and so it seems unlikely that COX-2 over-expression was the only system involved in the ATRA-mediated generation of the sensitization. It seems more sensible to think that other systems might also be implicated. This is supported by the high number of inflammatory mediators involved in the inflammatory response and also related to the activity of retinoids (see above and Introduction). All the results taken together show that Vitamin A and its metabolites might be one of the unidentified systems responsible for the initiation of sensitization and hyperalgesia and, therefore, may provide a promising strategy for the treatment of pathological pain states.

In conclusion, the oral treatment with ATRA in rats induces an effect of sensitization on spinal cord neuronal responses similar to that observed in animals with inflammation and might explain the enhancement of allodynia and hyperalgesia observed in previously published behavioral experiments. The mechanism of action involved in this effect is associated with an over-expression of COX-2, but not COX-1, in all areas of the lumbar spinal cord.

Acknowledgements

This work was supported by grants from the Spanish Ministerio de Educacion y Ciencia (Grants SAF2005-06242-

C03-01 and 03). We also thank Mr Lawrence Baron for English and scientific revisions of the manuscript. M Alique is a fellow of the Spanish Ministry of Science and Technology.

Conflict of interest

The authors state no conflict of interest.

References

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254.

Cocco S, Diaz G, Stancampiano R, Diana A, Carta M, Curreli R *et al*. (2002). Vitamin a deficiency produces spatial learning and memory impairment in rats. *Neuroscience* **115:** 475–482.

Devaux Y, Seguin C, Grosjean S, De Talance N, Schwartz M, Burlet A *et al*. (2001). Retinoic acid and lipopolysaccharide act synergistically to increase prostanoid concentrations in rats *in vivo*. *J Nutr* **131:** 2628–2635.

Duester G, Mic FA, Molotkov A (2003). Cytosolic retinoid dehydrogenases govern ubiquitous metabolism of retinol to retinaldehyde followed by tissue specific metabolism to retinoic acid. *Chemico-Biol Interact* **143:** 201–210.

Dubner R, Ruda MA (1992). Activity-dependent neuronal plasticity following tissue injury and inflammation. *Trends Neurosci* **15:** 96–103.

Goppelt-Struebe M, Beiche F (1997). Cyclooxygenase-2 in the spinal cord: localization and regulation after a peripheral inflammatory stimulus. *Adv Exp Med Biol* **433:** 213–216.

Grosjean S, Devaux Y, Seguin C, Meistelamn C, Zannad F, Mertes PM *et al*. (2001). Retinoic acid attenuates inducible nitric oxide synthase (NOS2) activation in cultured rat cardiac myocytes and microvascular endothelial cells. *J Mol Cell Cardiol* **33:** 933–945.

Gudas LJ, Sport MB, Roberts AB (1994). Cellular biology and biochemistry of the retinoids. In: Sporn MB, Roberts AB, Goodman DS (eds). *The Retinoids* 2nd edn. Raven Press: New York, pp 443–520.

Herrero JF, Cervero F (1996). Changes in nociceptive reflex facilitation during carrageenan-induced arthritis. *Brain Res* **717:** 62–68.

Herrero JF, Headley PM (1991). The effects of sham and full spinalization on the systemic potency of μ- and κ-opioids on spinal nociceptive reflexes in rats. *Br J Pharmacol* **104:** 166–170.

Herrero JF, Headley PM (1996). Reversal by naloxone of the spinal antinociceptive actions of a systemically-administered NSAID. *Br J Pharmacol* **118:** 968–972.

Hill EM, Bader T, Nettesheim P, Eling TE (1996). Retinoid-induced differentiation regulates prostaglandin H synthase and cPLA2 expression in tracheal epithelium. *Am J Physiol* **270:** L854–L862.

Hirokawa K, Oshaughnessy KM, Ramrankha P, Wilkins MR (1994). Inhibition of nitric oxide synthesis in vascular smooth muscle by retinoids. *Br J Pharmacol* **113:** 1448–1454.

Hla T, Neilson K (1992). Human cyclooxygenase-2 cDNA. *Proc Natl Acad Sci USA* **89:** 7384–7388.

Ichitani Y, Shi T, Haeggstrom JZ, Samuelsson B, Hokfelt T (1997). Increased levels of cyclooxygenase-2 mRNA in the rat spinal cord after peripheral inflammation: an *in situ* hybridization study. *Neuroreport* **8:** 2949–2952.

Jenab S, Inturrisi CE (2002). Retinoic acid regulation of mu opioid receptor and c-fos mRNAs and AP-1 DNA binding in SH-SY5Y neuroblastoma cells. *Mol Brain Res* **99:** 34–39.

Kanekura T, Higashi Y, Kanzaki T (2000). Inhibitory effects of 9-cis-retinoic acid and pyrrolidinedithiocarbamate on cyclooxygenase (COX)-2 expression and cell growth in human skin squamous carcinoma cells. *Cancer Lett* **161:** 177–183.

Laird JM, Cervero F (1989). A comparative study of the changes in receptive-field properties of multireceptive and nocireceptive rat dorsal horn neurons following noxious mechanical stimulation. *J Neurophysiol* **62:** 854–863.

Li M, Song S, lippman SM, Zhang XK, Liu X, Lotan R *et al*. (2002). Induction of retinoic acid receptor-beta suppresses cyclooxygenase-2 expression in esophageal cancer cells. *Oncogene* **21:** 411–418.

Lowe MN, Plosker GL (2000). Bexarotene. *Am J Clin Dermatol* **1:** 245–250.

Maier JA, Hla T, Maciag T (1990). Cyclooxygenase is an immediate-early gene induced by interleukin-1 in human endothelial cells. *J Biol Chem* **265:** 10805–10808.

Mazario J, Gaitan G, Herrero JF (2001). Cyclooxygenase-1 vs cyclooxygenase-2 inhibitors in the induction of antinociception in rodent withdrawal reflexes. *Neuropharmacology* **40:** 937–945.

Mcmahon SB, Wall PD (1984). Receptive fields of rat lamina 1 projection cells move to incorporate a nearby region of injury. *Pain* **19:** 235–247.

Millan MJ (1999). The induction of pain: an integrative review. *Prog Neurobiol* **57:** 1–164.

Misner DL, Jacobs S, Shimizu Y, De Urquiza AM, Solomin L, Perlmann T *et al*. (2001). Vitamin A deprivation results in reversible loss of hippocampal long-term synaptic plasticity. *Proc Natl Acad Sci USA* **98:** 11714–11719.

Molina C, Alique M, Romero-Sandoval A, Lucio J, Herrero JF (2005). Retinoic acid enhances nociceptive responses after oral and intrathecal administration. Society for Neuroscience. 35th Annual Meeting in Washington, DC, November. *SFN Abstracts* **12:** 16.

Nusing RM, Mohr S, Ullrich V (1995). Activin A and retinoic acid synergize in cyclooxygenase-1 and thromboxane synthase induction during differentiation of J774.1 macrophages. *Eur J Biochem* **227:** 130–136.

O'sullivan MG, Huggins EM, Meade EA, Dewitt DL, Mccall CE (1992). Lipopolysaccharide induces prostaglandin H synthase-2 in alveolar macrophages. *Biochem Biophys Res Commun* **187:** 1123–1127.

Romero-Sandoval EA, Mazario J, Howat D, Herrero JF (2002). NCX-701 (nitroparacetamol) is an effective antinociceptive agent in rat withdrawal reflexes and wind-up. *Br J Pharmacol* **135:** 1556–1562.

Romero-Sandoval EA, Alique M, Moreno-Manzano V, Molina C, Lucio FJ, Herrero JF (2004). The oral administration of retinoic acid enhances nociceptive withdrawal reflexes in rats with soft-tissue inflammation. *Inflamm Res* **53:** 297–303.

Safieh-Garabedian B, Poole S, Allchorne A, Winter J, Woolf CJ (1995). Contribution of interleukin-1 beta to the inflammation-induced increase in nerve growth factor levels and inflammatory hyperalgesia. *Br J Pharmacol* **115:** 1265–1275.

Samad TA, Moore KA, Sapirstein A, Billet S, Allchorne A, Poole S *et al*. (2001). Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature* **410:** 471–475.

Schaible HG, Grubb BD (1993). Afferent and spinal mechanisms of joint pain. *Pain* **55:** 5–54.

Schaible HG, Schmidt RF (1985). Effects of an experimental arthritis on the sensory properties of fine articular afferent units. *J Neurophysiol*. **54:** 1109–1122.

Seguin-Devaux C, Devaux Y, Latger-Cannard V, Grosjean S, Rochette-EGLY C, Zannad F *et al*. (2002). Enhancement of the inducible NO synthase activation by retinoic acid is mimicked by RARalpha agonist *in vivo*. *Am J Physiol Endocrinol Metab* **283:** E525–E535.

Seibert K, Zhang Y, Leahy K, Hauser S, Masferrer J, Perkins W *et al*. (1994). Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. *Proc Natl Acad Sci USA* **91:** 12013–12017.

Solano RE, Herrero JF (1997). Cutaneous responsiveness of rat single motor units activated by natural stimulation. *J Neurosci Methods* **73:** 135–140.

Solano RE, Herrero JF (1999). Response properties of hind limb single motor units in normal rats and after carrageenan-induced inflammation. *Neuroscience* **90:** 1393–1402.

Solomin L, Johansson CB, Zetterstrom RH, Bissonnette RP, Heyman RA, Olson L *et al*. (1998). Retinoid-X receptor signalling in the developing spinal cord. *Nature* **395:** 398–402.

Treede RD, Meyer RA, Raja SN, Campbell JN (1992). Peripheral and central mechanisms of cutaneous hyperalgesia. *Prog Neurobiol* **38:** 397–421.

Vanegas H, Schaible HG (2001). Prostaglandins and cyclooxygenases in the spinal cord. *Prog Neurobiol* **64:** 327–363.

Watkins LR, Wiertelak EP, Goehler LE, Smith KP, Martin D, Maier SF (1994). Characterization of cytokine-induced hyperalgesia. *Brain Res* **15:** 15–26.

Werner EA, Deluca HF (2002). Retinoic acid is detected at relatively high levels in the CNS of adult rats. *Am J Physiol Endocrinol Metab* **282:** E672–E678.

Willingale HL, Gardiner NJ, Mclymont N, Giblett S, Grubb BD (1997). Prostanoids synthesized by cyclo-oxygenase isoforms in rat spinal cord and their contribution to the development of neuronal hyperexcitability. *Br J Pharmacol* **122:** 1593–1604.

Woolf CJ (1983). Evidence for a central component of post-injury pain hypersensitivity. *Nature* **306:** 686–688.

Woolf CJ, King AE (1990). Dynamic alterations in the cutaneous mechanoreceptive fields of dorsal horn neurons in the rat spinal cord. *J Neurosci* **10:** 2717–2726.

Zetterstrom RH, Simon A, Giacobini MM, Eriksson U, Olson L (1994). Localization of cellular retinoid-binding proteins suggests specific roles for retinoids in the adult central nervous system. *Neuroscience* **62:** 899–918.