Assessment of c-Fos and pERK as Markers of Spinal Neuronal Activity in a Pain Model of Rheumatoid Arthritis

Nicole Nova, Duygu Belkis Baş and Camilla Svensson

Section for Molecular Pain Research
Department of Physiology and Pharmacology, Karolinska Institute

Pain in chronic inflammatory diseases, such as rheumatoid arthritis (RA), is regarded as a major clinical problem. Based on preclinical data, one can hypothesize that RA autoimmune factors induce constant noxious stimuli of the peripheral nerves and the spinal neurons, developing hypersensitivity. If so, RA patients could potentially be more susceptible to neuropathic pain, which would persist subsequent to anti-inflammatory treatment. The aim of the study was to use c-Fos as a marker to investigate spinal neuronal excitability during and after the inflammatory phase of RA, in order to explain the perseverance of post-inflammatory chronic pain. The study was performed using a CAIA (collagen antibody induced arthritis) mouse model. Spinal cords were harvested from CAIA and age-matched control mice 9, 13 and 29 days after induction of arthritis and processed for immunohistochemistry. The lumbar part of the spinal cords was cut and incubated with an anti-rabbit antibody against c-Fos, and anti-mouse antibody against pERK, and visualized using fluorescent labeled secondary antibodies. Immunoreactive neurons were quantified using fluorescence microscopy. The distribution of c-Fos and pERK positive cells in the Rexed Laminae were determined using a mouse histology atlas. The results showed that the increase in number of c-Fos immunoreactive neurons was most pronounced in the deep dorsal horn (Rexed laminae IV-V) on day 9, coinciding with the inflammatory phase, and less on day 13 and 29 after CAIA induction. Although the number of c-Fos positive neurons decreases when signs of RA disappear, the amount is still increased as compared to control mice on day 29. The results for pERK marking were not significant, due to poor antibody labeling, as pERK might have dissociated in long-term storage tissues. In conclusion, c-Fos expression in the spinal dorsal horn is elevated subsequent to induction of CAIA, indicating that RA induces long-term activation of dorsal horn neurons that persists even after the inflammation subsides.

INTRODUCTION

Rheumatoid arthritis

Chronic inflammation and pain related diseases are considered to be a major health problem, and therefore it is important to find new treatment strategies for pain relief. Approximately 20% of the population is diagnosed with a chronic inflammatory and pain related disease [1]. Rheumatoid arthritis (RA), an autoimmune chronic disease with inflammation of the joints leading to joint destruction, is affecting approximately 1% of the population [2].

RA primarily affects the diarthrodial joints of the hands and feet. It is mediated by infiltration of phagocytes, cytokines, and chemokines into the synovial fluid. The result is loss of cartilage and bone tissue [2]. The etiology of RA is not yet well understood, but certain factors are known to contribute to the predisposition. There is a

correlation between the major histocompatibility complex II (MHC II) and RA. MHC II proteins are primarily on the surface of white blood cells called antigen presenting cells (APC). These cells are responsible for the elimination of pathogens via phagocytosis, and to present the antigens to the T-cells. In RA however, the altered MHC II genes causes the APC cells to degenerate tissues of the joint. The distinction between the pathogens and the host cells is lost [3]. Moreover, the antibody-producing B-cells form synovial germinal centers (GCs) in lymph nodules, presenting CD4+ T-cells with antigens. Together with recruited follicular dendritic cells (FDCs) the cells are closely packed in the GC for efficient cell communication. The GCs in RA patients are the main sites for tissue destruction. RA is considered to be an autoimmune disease, primarily due to the rheumatic factor (RF), which is an autoantibody that binds to Fc on IgG. RA diagnosis is primarily based on findings of RF and auto-antibodies attacking proteins containing citrulline, called anti-citrullinated protein antibodies (ACPAs) [4].

Pain in RA is regarded as nociceptive and induced locally due to the peripheral inflammation of joints [1]. However, the peripheral pathology does not correspond to the amount of pain experienced by the patient [1]. Thus, pain transduction and processing at the level of the spinal cord must be considered in order to gain a better understanding of chronic pain.

Pain sensitization

Pain sensitization in the spinal cord, or spinal sensitization, is the hyperactivity of the nociceptive neurons exposed to noxious stimuli and plays a central role in the induction of chronic pain [5]. Factors that contribute to constant noxious stimuli at the site of inflammation are free radicals, tumor necrosis factor-α (TNF-α), interleukins 1β (IL-1β) and 6 (IL-6), and nerve growth factors (NGFs) [1, 6]. The neurons exposed to such stimuli are nociceptors, categorized in Aβ-, Aδ-, or C-fibers. Aβ-fibers are covered with myelin sheaths (fastest action potential signal), Aδ-fibers are only partially myelinated and C-fibers are not myelinated (slowest action potential signal). The C fibers are commonly regarded as the fibers related to chronic pain because of their slow conductivity generating a diffuse pain sensation for a longer period of time [5]. The constant transduction of neurotransmitters, i.e. excitatory amino acid glutamate on AMPA and NMDA receptors, or the neuropeptide substance P (SP) on NK-1 receptor, or GABA on GABAβ receptor, causes a windup effect facilitating hyperalgesia (weak painful stimuli perceived as very painful) or allodynia (non-painful stimuli perceived as painful), and thus the patient becomes more sensitive to pain stimulation [5].

Animal model CAIA

Chronic inflammatory pain studies require a well-representing animal model. In this study the animal model involved collagen antibody induced arthritis (CAIA). Recent studies by Svensson and Nandakumar suggest that the CAIA model [7] is well suiting for nociceptive research. The mice develop a pathological picture resembling the hypersensibility and pathology observed in RA patients. In the CAIA model, RA is induced by intravenous injection with arthritogenic monoclonal antibodies (mAbs) targeted for six different triple helical epitopes (J1, C1, U1, D3, F4 and E8) on collagen II, which is the main component of

cartilage [3]. As a result, the inflammatory response is initiated by recruitment of macrophages with Fc receptors, and neutronphils with the anti-collagen II antibodies. In turn, these cells release TNF-α and IL-1β, which are the mediators of RA [1].

c-Fos and pERK

There are a number of markers used in pain research. Two commonly used markers of nociceptive neuron stimuli in the dorsal horns of the spinal cord, where incoming primary nociceptive afferent neurons terminate, are the protein c-Fos of the proto-oncogene *c-fos*, and phosphorylated extracellular signal-regulated kinase (pERK). The former has been used for more than twenty years (1987), and the latter for approximately ten years (1999) [8]. It has been reported that elevated levels of c-Fos and pERK are shown in central sensitization, in particular, spinal sensitization located in the dorsal horns of laminae I, II, IV and V [9]. The mechanism underlying c-Fos is that it binds to another oncogene protein called Jun to form a complex. This complex then binds to a site called the AP-1 DNA site and triggers gene transcription for cellular adaptation [9]. Thus, c-Fos is only expressed in the nuclei of neurons and can be easily detected using immunohistochemistry.

ERK is a member of the mitogen-activated protein kinase (MAPK) family, and activated via phosphorylation by the enzyme *ERK kinase* [8]. In turn, pERK enters the nucleus and activates the transcription factor cAMP-response element binding protein (CREB) via phosphorylation. Moreover, a noxious stimulus in the unilateral dorsal horn also deduces an activation of phosphorylation of CREB in the contralateral dorsal horn. However, it is not due to pERK since it is only active unilaterally [10]. Another possibility might be the PKA pathway that induces pCREB contralaterally. CREB activation leads to the transcription of various genes, i.e. *c-fos, Cox-2,* and *neurokinin-1,* as well as genes regulating synaptic plasticity after tissue injury and hypersensitivity [11].

These markers are specific to noxious stimuli transmitted via C- and Aδ-nerve fibers due to extreme thermal, chemical, mechanical, and electrical conditions [5]. However, they are not activated under normal conditions and rarely expressed under stimuli such as light touch, warm water, and Aβ nerve fiber activation, and are thus considered to be potential markers for chronic inflammation and persistent pain perception. When exposed to slight electrical stimuli, the Aβ-fibers are activated but no pERK is expressed [8]. Increasing the electrical stimulus activates the Aδ-fibers and a pERK expression is detectable in the dorsal horn region of the spinal cord. When the electrical stimulus is increased further to activate the C-fibers the pERK expression becomes even more abundant [12]. Furthermore, not only are c-Fos and pERK expressions drastically elevated due to high intensity noxious stimuli under normal conditions, but they are also expressed in low intensity noxious stimuli under pathological conditions [13]. These factors are therefore both relevant in acute pain under normal conditions as well as chronic pain under chronic inflammatory conditions, i.e. rheumatoid arthritis.

The purpose of this investigation was to examine if there is a change in the number of c-Fos and pERK immunoreactive neurons, in the dorsal horn of the spinal cord, during and after the inflammatory phase of RA in order to gain an insight into the perseverance of post-inflammatory chronic pain. Previous work has shown that

increased reactivity and prolonged activation of spinal sensory neurons (spinal sensitization) is an important component of chronic pain. Analgesic treatment of pain associated with chronic inflammatory diseases, including RA, is frequently insufficient or associated with side effects [4]. Therefore, it is highly important to increase our knowledge about how chronic inflammatory pain is generated and maintained in order to identify new targets for pharmacological treatments of chronic pain.

MATERIALS AND METHODS

Animal study

The tissues used in this study were taken from mice at different times subsequent to induction of CAIA, and also used in another study in the Svensson laboratory (performed by Johanna Petterson and Duygu Belkis Baş). The cocktail used for establishment of CAIA was an anti-CII arthritogenic monoclonal antibody (Ab) cocktail: M2139 (J1 epitope), CIIC1 (C1 epitope), CIIC2 (D3 epitope) and UL-1 (U1 epitope) (a gift from Dr. Rikard Holmdahl). Male QB (25-30g) was used in this study. Three CAIA mice and three controls were used in the study for each time point. The intravenous injection of 4 mg of anti-CII mAb cocktail in PBS, or only PBS in control mice (300 µl) was made in the tail vein. Spinal cords were harvested from aged matched controls and arthritis mice 9, 13 and 29 days subsequent to CAIA induction. The mice were deeply anesthetized with isoflurane and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde (PFA). Spinal cords were removed, postfixed for 24 hours in 4 % PFA and cryoprotected in 20 % sucrose in PBS for 48 hours and then stored in 30 % sucrose in PBS with 0.5 % PFA. The lumbar spinal cords were cut at the caudal and rostral ends of the lumbar enlargement (lumbar enlargement measured 0,7 cm from the caudal end). The tissue specimens were embedded in OCT and snap frozen in -50^{o}C isopentan.

Immunohistochemistry

Spinal cord sections (30 µm thick) were cut in a -20^{o}C cryostat and transferred to a multi well plate containing PBS (20 consecutive sections were transferred to each well). The sections were then transferred to anti-freeze solution for long-term storage.

Antibody probing

On the day of the experiment, sections of the middle lumbar region of the spinal cord (L3-4) were taken from three different mice on day 9, and three mice on day 13 and three mice on day 29 after CAIA induction, along with three corresponding age-matched control mice for each day. Ten sections were used for immunohistochemistry per animal, and six sections were taken into account for analysis. The sections were transferred from an anti-freeze medium to a mesh holder and washed with PBS 3 x 5 min, and then permeabilized with PBS-TX (0.2% Triton-X100) for 10 min. The non specific sites were blocked with 5% goat serum (serum from species that secondary antibody been raised in) in PBS-TX for (1:200) 1 hour at room temperature (RT). Then primary antibodies (polyclonal c-Fos rabbit and monoclonal pERK mouse) were added in 5% goat serum in PBS-TX (1:1000) and the samples were incubated for 48 hours (4°C) on a shaker. The samples were then washed 3 x 5 min with PBS-TX. Meanwhile, the secondary antibodies (Alexa 488 and 594 conjugated antibodies at 1:250, Invitrogen) were prepared in 5% goat serum and PBS-

TX (1:200), and centrifuged 13500 rpm for 10 min. The supernatant was added to the samples and incubated for 1 hour in darkness at RT, and then washed with PBS for 3 x 5 min. The sections were put on slides with a PBS drop and let to dry for 1 hour. Then the slides were carefully cleaned in dH_2O and left to dry for ~30 min. One drop of cover slip mounting medium (Prolong Gold with DAPI #P36935, Invitrogen) was added and cover glass placed on top. The slides were then left to dry overnight in darkness (at RT).

Tyramid Signal Amplification (TSA)

The tissues that showed most results from the antibody probing were selected for tyramid signal amplification for a clearer image (day 9 CAIA and control). The tissues were rinsed with PBS for 3 x 5min. The tissues were then permeabilized with 0.2 % PBS-Triton X-100 for 10 min at RT, and then rinsed with PBS again for 5 min. Then the samples were incubated with peroxidase quenching buffer (2 % H_2O_2 in PBS) for 1 hour at SC. Another incubation period was performed with 1 % blocking buffer (10 mg/ml, component D) for 1 hour at RT. A third incubation of the samples with primary antibody c-Fos (anti-rabbit Santa Cruz) diluted in 1 % blocking buffer 1:10000 for 48 hours at 4 °C. The tissues were rinsed with PBS for 3 x 5min. An incubation period with HRP conjugate (1:100 in 1 % blocking buffer, component C) for 1 hour at RT was performed. The tissues were rinsed with PBS for 3 x 5min, followed by an incubation period with tyramide solution (1:100 in amplification buffer/0.0015 % H_2O_2) for 10 minutes at RT. The tissues were again rinsed with PBS for 3 x 5min. Then they were mounted on glass slides with the mounting medium Prolong Gold with DAPI #P36935.

RESULTS

The number of c-Fos immunoreactive neurons in the lumbar region of the spinal cord of mice (fig. 1, 3) is significantly higher following induction of CAIA, as compared to controls. The increase was most pronounced in the deep dorsal horn (laminae IV-V) on day 9, coinciding with the inflammatory phase of the model (fig. 2, 3). Allodynia persists beyond the inflammatory phase (fig. 2) and though the number of c-Fos positive neurons decreases when the signs of arthritis disappear, the number is still significantly increased as compared to control mice on day 29.

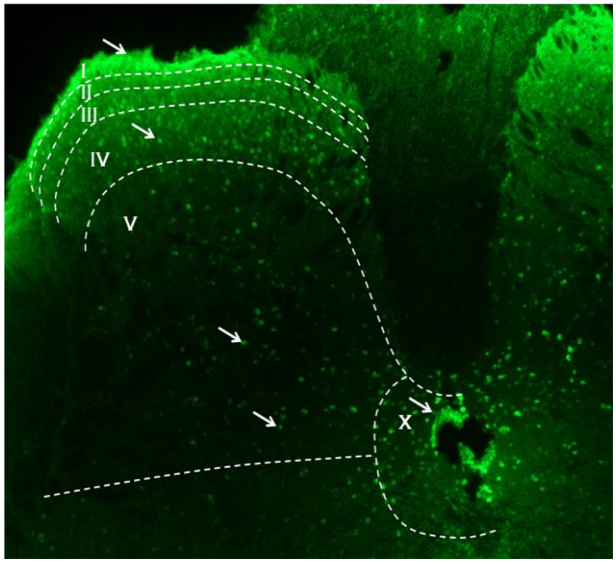

Figure 1. Immunohistochemistry of c-Fos expression shows neuronal activity in the lumbar spinal cord of mice. The arrows show active neurons in the dorsal horn (Rexed laminae I-V) and around the central canal (X).

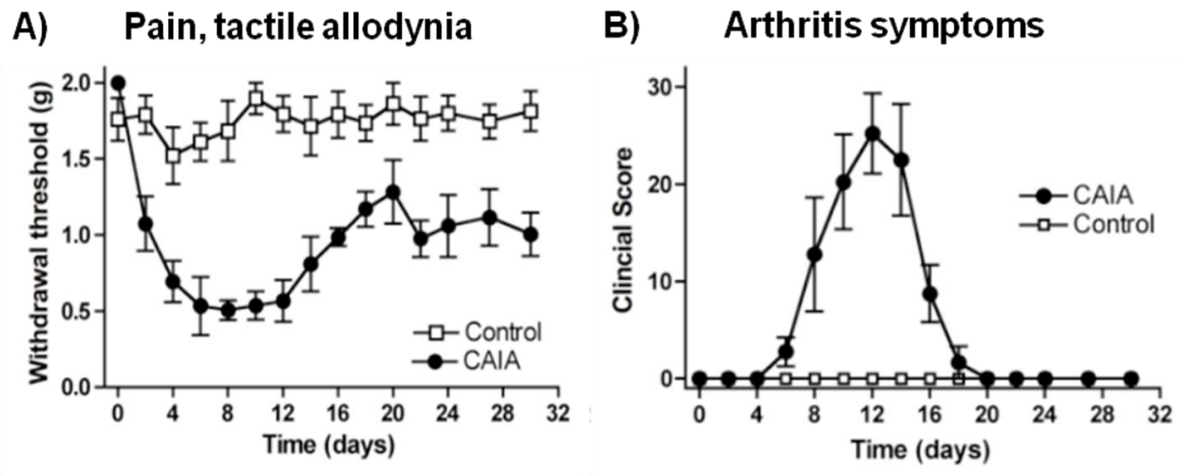

Figure 2. Collagen antibody induced arthritis leads to persistent pain in mice **(A)**, even after cessation of inflammation on day 20 **(B)**.

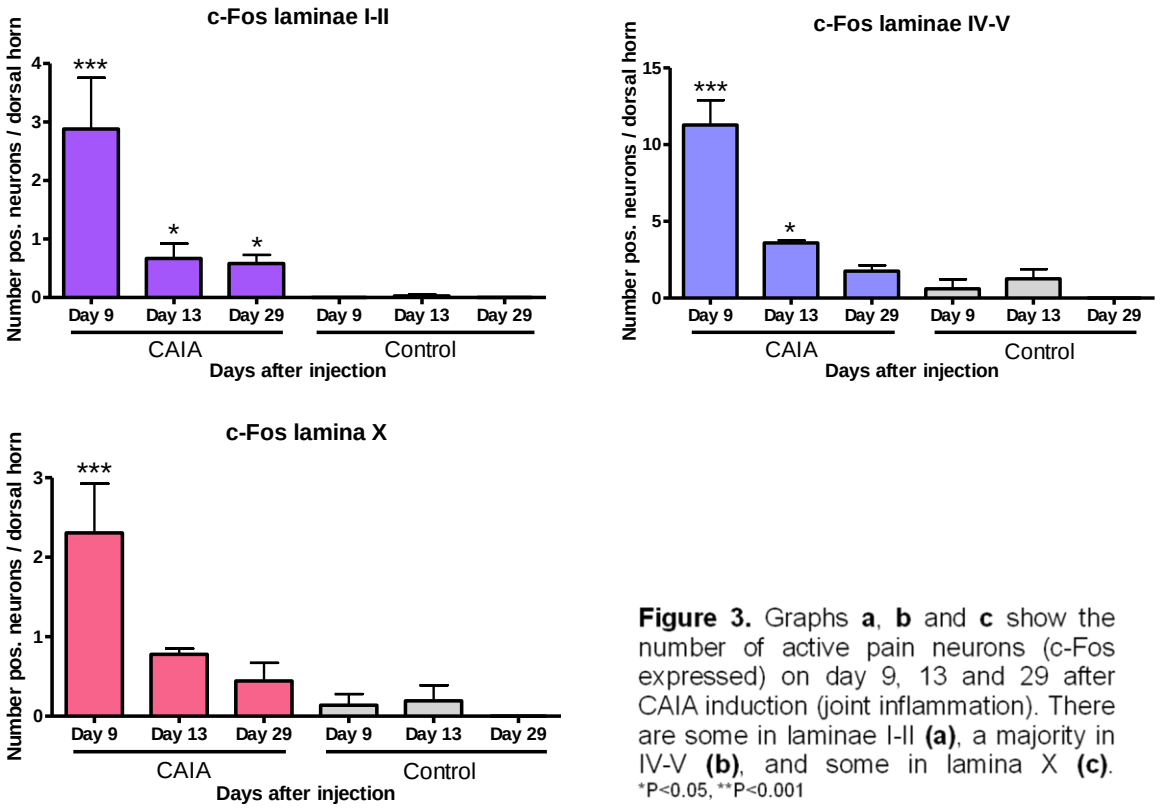

Figure 3. Graphs **a**, **b** and **c** show the number of active pain neurons (c-Fos expressed) on day 9, 13 and 29 after CAIA induction (joint inflammation). There are some in laminae I-II **(a)**, a majority in IV-V **(b)**, and some in lamina X **(c)**.
*P<0.05, **P<0.001

DISCUSSION

The results for c-Fos show a clear difference in terms of number of excitatory neurons in the lumbar segment of the spinal cord in day 9 after induction of CAIA compared to day 13 and 29 (fig. 3). This suggests that there is a peak of c-Fos activation in neurons relatively early than expected. It might be possible that the actual peak is before day 9. In future studies, one would investigate the amount of c-Fos produced in days prior to day 9. Some mouse samples from day 3 are going to be cut and processed for immunohistochemistry.

Unfortunately, the results for pERK as a marker for neuronal excitability did not show any significant results. The antibody is recognizing the phosphorylated state of the kinase, and there is a suspicion that the lack of positive signal is due to spontaneous dephysphorolation in the specimens, which have been in storage in antifreeze solution for 6-7 months. Also, the primary antibody used was a monoclonal one and therefore there were fewer secondary fluorescent antibodies attached to the sites, transmitting weaker signals. One can conclude that pERK as a marker of neuronal excitation is a little more unstable when it comes to long storage specimen. However, c-Fos could still be used as a marker for spinal sensitization even with samples that are 6 months old, stored in antifreeze media at -20ºC.

Worth noting, is that c-Fos is more easily induced under acute noxious stimulation than pERK. A nociceptive stimuli with a duration of <10 seconds is enough to see c-Fos expression, but not pERK. Moreover, non-noxious stimuli such as repetitive colorectal swelling at a low pressure can cause c-Fos activation. Therefore, c-Fos is not as specific to chronic nociceptive as pERK [8]. This makes pERK immunoreactivity rarer than c-Fos, which can also explain why c-Fos is more frequently produced and easier to observe in tissues when performing immunohistochemistry. Additionally, there is a difference in terms of the time of expression of c-Fos and pERK. The expression of c-Fos is first detected 30 min after noxious stimuli induction, and has its peak at 1-2 hours after induction. However, the expression of pERK is first detected 1 min after noxious stimuli and has its peak after 3 min [8]. This could also explain the lack of pERK expression when observing tissues older than 9 days.

When comparing the different laminae, there majority of c-Fos expression is located in IV and V. This is mainly due to the larger size of the laminae, holding more neurons. However, in laminae I-II the density of the neurons is higher, as shown when applying TSA on the sections (fig. 1). The excitatory nociceptive afferent fibers are located in laminae I-II and IV-V. Although, lamina X is associated with visceral pain, because nerve fibers from the gastro intestinal (GI) system terminate in lamina X, it was still an interesting finding that this region was correlated with neuropathic pain as well.

In conclusion, c-Fos expression in the spinal dorsal horn is elevated subsequent to induction of rheumatoid arthritis. The increase was most pronounced on day 9 after induction of CAIA, compared to day 13 and 29. This suggests that neuroexcitability is highly elevated during the phase of inflammation, and though the number of c-Fos positive neurons decreases when the signs of arthritis disappear, the quantity is still higher as compared to control mice on day 29. This study indicates that arthritis induces long-term activation of dorsal horn neurons that persists even after the inflammation subsides.

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REFERENCES

1. Gaffo A, Saag KG, Curtis JR. Treatment of rheumatoid arthritis. Am J Health Syst Pharm 2006;63:2451–2465.

2. Schaible, H-G., Richter, F., Ebersberger, A., Boettger, M. K., Vanegas, H., Natura, G., Vazquez, E., von Banchet, G-S. (2009). Joint pain. Exp brain Res. 196:153-162.

3. Firestein, G.S. (2003). Evolving concepts of rheumatoid arthritis. Nature. 423:356-361.

4. Cantaert, T., De Rycke, L., Bongartz, T., Matteson E.L., Tak P.P., Nicholas, A., Baeten, D. (2006). Citrullinated Proteins in Rheumatoid Arthritis Crucial . . . but Not Sufficient! Arthritis & Rheumatism. Vol. 54, No. 11, November 2006, pp 3381–3389.

5. Hansson, P., (1998). Nociceptiv och neurogen smärta. Pharmacia & Upjon AB, Stockholm. 52-63.

6. Nandakumar, K.S. and Holmdahl, R. (2007). Antibody-induced arthritis: disease mechanisms and genes involved at the effector phase of arthritis. Arthritis Res Ther. 8:223.

7. Nandakumar, K. S. and Holmdahl R. (2005). Efficient promotion of collagen antibody induced arthritis (CAIA) using four monoclonal antibodies specific for the major epitopes recognized in both collagen induced arthritis and rheumatoid arthritis. J Immunol Meth. 304:126-136.

8. Ru-Rong, J., Yong-Jing, G. (2009). C-fos and pERK, which is a better marker for neuronal activation and central sensitization after noxious stimulation and tissue injury? *Open Pain J*. 1; 2: 11–17.

9. Hunt SP, Pini A, Evan G. Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. Nature 1987;328:632–634.

10. Ji RR, Rupp F. Phosphorylation of transcription factor CREB in rat spinal cord after formalin-induced hyperalgesia: relationship to c-fos induction. J Neurosci 1997;17:1776–1785.

11. Lonze BE, Ginty DD. Function and regulation of CREB family transcription factors in the nervous system. Neuron 2002;35:605–623.

12. Rauscher FJ 3rd, Sambucetti LC, Curran T, Distel RJ, Spiegelman BM. Common DNA binding site for Fos protein complexes and transcription factor AP-1. Cell 1988;52:471–480.

13. Ji RR, Baba H, Brenner GJ, Woolf CJ. Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. Nat Neurosci 1999;2:1114–11.