



**Karolinska  
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Chronic inflammation and pain:  
Assessment of c-Fos and ATF-3 as markers of  
spinal neuronal activity in a pain model of rheumatoid arthritis

**Nicole Nova**

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**Supervisors**

**Camilla Svensson, PhD**

Assistant Professor

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

**Per Alstergren, DDS, PhD**

Specialist in Oral Physiology

Institution of Odontology, Dental School, Karolinska Institutet, Huddinge



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## Abstract

Chronic inflammatory pain in rheumatoid arthritis (RA) is a major clinical problem. RA factors induce constant noxious stimuli of peripheral nerves and spinal neurons, developing hypersensitivity and nociceptive pain, which persists subsequent to anti-inflammatory treatment. The aim was to use the proto-oncogene protein c-Fos, the kinase pERK, and the transcription factor ATF-3 as markers to investigate spinal neuronal excitability during and after the inflammatory phase of RA, in order to explain the persistence of post-inflammatory chronic pain. Spinal cords of collagen antibody induced arthritis (CAIA) mice and age-matched control mice were cut and processed for immunohistochemistry. Immunoreactive neurons were located and quantified using fluorescence microscopy. The results showed that the increase in number of c-Fos immunoreactive neurons was most pronounced in the deep dorsal horn (DH) during the inflammatory phase, and less so after the peak of inflammation. Although the number of c-Fos positive neurons decreases when signs of arthritis disappear, the number is still elevated compared to the control. As for pERK, there were no active cells detected in CAIA or the control, and thus it was excluded from the study due to suspected experimental errors. Regarding ATF-3, there was no significant difference between CAIA and the control during and after the inflammatory phase. Further studies need to be undertaken to confirm these negative findings. In conclusion, the c-Fos expression in the spinal DH is elevated subsequent to induction of CAIA, indicating that arthritis induces long-term activation of DH neurons that persists even after the inflammation subsides. However, ATF-3 does not appear to be a good marker for neuronal excitability in the spinal cord for the CAIA mouse model.



## Abbreviations

**ACPA** = anti-citrullinated protein antibody, **AMPA** =  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, **ANOVA** = analysis of variance, **anti-CII** = anti-collagen type II, **AP-1** = activator protein 1, **APC** = antigen presenting cell, **ATF-3** = cyclic AMP-dependent transcription factor, **BSA** = bovine serum albumin, **CAIA** = collagen antibody induced arthritis, **cAMP** = cyclic adenosine monophosphate, **CREB** = cAMP-response element binding protein, **DAPI** = 4', 6-diamidino-2-phenylindole, **DH** = dorsal horn, **DRG** = dorsal root ganglion, **FDC** = follicular dendritic cells, **GABA** = gamma-aminobutyric acid, **GC** = germinal center, **GI** = gastrointestinal system, **HRP** = horseradish peroxidase, **IL** = interleukin, **mAb** = monoclonal antibody, **MAPK** = mitogen-activated protein kinase, **MHC** = major histocompatibility complex, **Neu-N** = neuronal nuclei, **NGF** = nerve growth factor, **NK-1** = neurokinin 1, **NMDA** = N-Methyl-D-aspartic acid, **OCT** = optimum cutting temperature, **pAb** = polyclonal antibody, **PBS** = phosphate-buffered saline, **PBS-TX** = PBS Triton-x, **pERK** = phosphorylation of extracellular signal-regulated kinase, **PFA** = paraformaldehyde, **PKA** = protein kinase A, **RA** = rheumatoid arthritis, **RF** = rheumatic factor, **RT** = room temperature, **TNF- $\alpha$**  = tumor necrosis factor- $\alpha$ , **TSA** = tyramide signal amplification



## Introduction

### Rheumatoid arthritis

Chronic inflammation and pain-related diseases are considered to be a major health problem, mainly due to the fact that the analgesic treatment currently available on the market is frequently insufficient [1]. About 20% of the population is diagnosed with a chronic inflammatory and pain-related disease [2]. Therefore, it is very important to find new treatment for chronic pain relief.

Rheumatoid arthritis (RA), an autoimmune chronic disease with inflammation of the joints leading to joint destruction, is affecting approximately 1% of the population [3]. 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 [3]. 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 cause the APC cells to degenerate tissues of the joint. The distinction between the pathogens and the host cells is lost [4]. Moreover, the antibody-producing B-cells form synovial germinal centers (GCs) in lymph nodules, presenting CD4<sup>+</sup> 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 autoantibodies attacking proteins containing citrulline, called anti-citrullinated protein antibodies (ACPAs) [5].

Pain caused by RA is nociceptive and induced locally due to the peripheral inflammation of joints [2]. However, the peripheral pathology does not correspond to the amount and persistence of pain experienced by the patient [2]. Therefore, the transduction and processing of pain occurring in the central nervous system (CNS), specifically in the dorsal horn of the spinal cord (where the afferent nociceptive nerve fibers enter) 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. Factors that contribute to constant noxious stimuli at the site of inflammation are cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukins 1 $\beta$  (IL-1 $\beta$ ) and 6 (IL-6), and nerve growth factors (NGFs) [2, 6]. The neurons exposed to such stimuli are nociceptors, categorized in A $\beta$ -, A $\delta$ -, or C-fibers. A $\beta$ -fibers are covered with myelin sheaths (fastest action potential signal), A $\delta$ -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 [7]. The constant transduction of neurotransmitters, i.e. the excitatory amino acid glutamate on AMPA and NMDA receptors, or the neuropeptide substance P (SP) on the NK-1 receptor, or GABA on the GABA $\beta$  receptor, cause a wind-up effect facilitating hyperalgesia (weak painful stimuli perceived as extremely painful) or allodynia (non-painful stimuli perceived as painful). The pain threshold is lowered and one becomes more sensitive to pain stimuli [7].



## **Animal model CAIA**

Chronic inflammatory pain studies require a representative animal model. In this study the animal model applied was collagen antibody induced arthritis (CAIA). Former studies suggest that the CAIA model is well-suited for nociceptive research [8]. The mice develop a pathological picture resembling the hypersensitivity and pathology observed in human RA patients. In the CAIA model, arthritis 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 [4]. As a result, the inflammatory response is initiated by recruitment of macrophages with Fc receptors, and neutrophils with the anti-collagen II antibodies. In turn, these cells release the cytokines TNF- $\alpha$  and IL-1 $\beta$ , which are the mediators of joint inflammation in RA [2].

## **c-Fos and pERK**

There are a number of target proteins used as markers in pain research. Two commonly used markers of nociceptive neuron stimuli in the dorsal horn (DH) of the spinal cord, where incoming primary nociceptive afferent neurons enter, are the proteins c-Fos and pERK. The former is a proto-oncogene protein belonging to the AP-1 group of transcription factors, and it is upregulated in response to cell proliferation, differentiation, and damage [9]. The latter is a phosphorylated extracellular signal-regulated kinase (pERK) and it has been used as a marker of pain for 13 years, whereas c-Fos has been used for 25 years [10]. It has been reported that elevated levels of c-Fos and pERK are shown in central sensitization, in particular, spinal sensitization located in the DH of Rexed laminae I, II, IV and V [11]. 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 [11]. 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 it is activated via phosphorylation by the enzyme ERK kinase [10]. 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 DH also deduces an activation of phosphorylation of CREB in the contralateral DH. However, it is not due to pERK, since it is only active unilaterally. Another possibility might be the PKA pathway that induces pCREB contralaterally [12]. Nevertheless, CREB activation leads to the transcription of various genes, i.e. c-fos, Cox-2, and NK-1, as well as genes regulating synaptic plasticity after tissue injury and hypersensitivity [13].

These markers are specific to noxious stimuli transmitted via C- and A $\delta$ -nerve fibers due to extreme thermal, chemical, mechanical, and electrical conditions [7]. However, they are not activated under normal conditions and rarely expressed under stimuli such as light touch, warm water, and A $\beta$ -nerve fiber activation, and are thus considered to be potential markers for chronic inflammation and persistent pain perception. When exposed to weak electrical stimuli, the A $\beta$ -fibers are activated but no pERK is expressed [10]. Increasing the electrical stimulus activates the A $\delta$ -fibers and a pERK expression is detectable in the DH region of the spinal cord. When the electrical stimulus is increased further to activate the C-fibers, the pERK expression becomes even more abundant [14]. 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 [15]. These factors are highly relevant in acute pain under normal conditions, as well as

chronic pain under chronic inflammatory conditions, and it is therefore interesting to know whether they could be used as markers in this study concerning RA specifically.

## **ATF-3**

A previously used marker of nerve injury in the dorsal root ganglion (DRG) is the cyclic AMP-dependent transcription factor (ATF-3) [16]. As the case with pERK, it also belongs to the cyclic AMP responsive element binding (CREB) protein family [17]. ATF-3 initiates a transcription, after the CREB complex has been activated in the nuclei from a stress response, i.e. nerve injury, by binding to a Jun protein, forming a complex, which in turn binds to a DNA site [17]. It has been reported that elevated levels of the other proteins mentioned earlier (c-Fos and pERK) are expressed in central sensitization, especially in the DH of the spinal cord in laminae I, II, IV and V [11]. However, no prior studies have looked into using ATF-3 as a marker in the DH of the spinal cord instead of the DRG. It would be interesting to know whether the ATF-3 expression in a different anatomical site is valid, and if it can explain the persistence of chronic pain in RA.

## **Aim**

The purpose of the investigation was to examine the number of c-Fos-, pERK-, and ATF-3-active neurons, in the DH of the spinal cord, during and after the inflammatory phase of arthritis, in order to gain an insight into the persistence of post-inflammatory chronic pain. Increased activity and prolonged activation of spinal sensory neurons is an important component of chronic pain [7]. Analgesic treatment of pain associated with chronic inflammatory diseases, including RA, is frequently insufficient [1]. Therefore, it is highly important to increase our knowledge about the mechanism and maintenance of chronic inflammatory pain, in order to identify new targets in the CNS for pharmacological treatments of chronic pain.

## Materials and Methods

### Animal study

The already cut tissues used in this study were prepared in a former research project in the Svensson laboratory performed by Johanna Pettersson and Duygu Belkis Baş. In their animal study, the experiments were performed according to the regulations by the ethical committee for animal experiments: Stockholms Norra djurförsöksetiska nämnd. The animals were kept in the Medical Inflammation Research (MIR) division in the department of Medical Biochemistry and Biophysics (MBB). The housing was climate controlled with a 12h non-reversed cycle of daylight, and provision of food and water ad libidum. The tissues came from 18 male [B10.Q X DBA/1] F1 mice (QB mice), weighing 25-30g and 6-12 weeks old. The cocktail used in the former study for the establishment of CAIA was an anti-CII arthritogenic monoclonal antibody (mAb) cocktail: M2139 (J1 epitope), CIIC1 (C1 epitope), CIIC2 (D3 epitope), and UL-1 (U1 epitope) [7]. The intravenous injection (i.v.) of anti-CII mAb cocktail in PBS (4mg) for the CAIA mice, and the i.v. injection of only PBS (300ul) for the controls, was made in the tail vein. After 5 days, the intraperitoneal injection (i.p.) of LPS in PBS (25ug) was made on CAIA mice, and the controls received only PBS instead.

Spinal cords were later harvested from the CAIA mice, and their aged-matched controls, on day 9, 13 and 29 subsequent to the intravenous injections. 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 24h in 4% PFA, and cryoprotected in 20 % sucrose in PBS for 48h, 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 0,7cm from the caudal end). The tissue specimens were embedded in OCT and snap frozen in -50°C isopentane. The spinal cords were cut into sections (thickness: 30 µm) in a -20°C cryostat microtome, and transferred into antifreeze solution and kept at -20°C for long-term storage.

## Immunohistochemistry

### ANTIBODY PROBING

Several antibodies were used for the different targets when performing immunohistochemistry (table 1).

Target	Primary antibody	Secondary antibody	Blocking reagent
c-Fos	Rabbit pAb (Santa Cruz Biotechnologies #sc-52) [1: 1000]	Alexa 488 goat anti-rabbit (Invitrogen) [1:250]	5 % goat serum
pERK	Mouse mAb (Cell Signaling #9106) [1:1000]	Alexa 594 goat anti-mouse (Invitrogen) [1:250]	5 % goat serum
ATF-3	Rabbit pAb (Santa Cruz Biotechnologies #sc-188) [1:1000]	Alexa 488 goat anti-rabbit (Invitrogen) [1:250]	5% goat serum and 1% BSA
Neu-N	Mouse mAb (Chemicon Mab337) [1:1000]	Alexa 594 goat anti-mouse (Invitrogen) [1:250]	5% goat serum and 1% BSA

**Table 1.** The complete list of the antibodies used for the different target proteins and factors when performing immunohistochemistry. The blocking reagent solutions contained serum from the species where the secondary antibodies had been raised in. BSA was used to lower background noise (yielded clearer results in pilot studies), because it has the property of binding to non-specific proteins, allowing the antibodies to bind entirely to the desired targets.

**c-Fos and pERK**

On the day of the experiment for this study, 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.

**ATF-3**

Sections of the middle lumbar region of the spinal cord (L3-4) were taken from three different mice on day 13 and three mice on day 29 after CAIA induction, along with three corresponding age-matched control mice for day 13 and 29 respectively. Ten sections were used for immunohistochemistry per animal, and six sections were taken into account for analysis. The primary antibody for Neu-N was also used on the same sections when performing antibody probing for ATF-3, in order to easily detect neuron nuclei from background noise.

**Standard protocol for conventional immunohistochemistry**

The sections were taken from the antifreeze medium and washed in PBS (3x5min), and then permeabilized with PBS-TX (0.2% Triton-X100) for 10min. The non-specific sites were blocked with the blocking reagent (table 1) in PBS-TX [1:200] for 1h at room temperature (RT). Primary antibodies (table 1) were added in the blocking reagent (table 1) in PBS-TX [1:1000] and the samples were incubated for 48h (4°C) on a gentle shaker. The samples were then washed in PBS-TX (3x5min). Meanwhile, the secondary antibodies (table 1) were prepared in the blocking reagent (table 1) and PBS-TX [1:200], and centrifuged 13500 rpm for 10min. The supernatant was added to the samples and incubated for 1h in darkness (RT), and then washed with PBS (3x5min). The sections were put on slides with a PBS drop on top and left to dry for 1h. The slides were carefully cleaned in dH<sub>2</sub>O and left to dry for ~30 min. One drop of cover slip

mounting medium (Prolong Gold with DAPI #P36935, Invitrogen) was added and a cover glass placed on top. The slides were then left to dry overnight in darkness (RT).

#### TYRAMID SIGNAL AMPLIFICATION (TSA) – c-Fos

The sections that had most immunoreactive neurons after image analysis were selected for TSA to yield a clear image for an esthetic representation. The tissues were rinsed with PBS (3x5min), and then permeabilized with 0.2% PBS-Triton X-100 for 10min, and then rinsed with PBS again for 5min. The samples were incubated with peroxidase quenching buffer (2% H<sub>2</sub>O<sub>2</sub> in PBS) for 1h (RT). Another incubation period was performed with 1% blocking buffer (10mg/ml, component D) for 1h (RT). A third incubation of the samples was performed with primary antibody against c-Fos (anti-rabbit Santa Cruz Biotechnologies #sc-52) diluted in 1% blocking buffer [1:10000] for 48h at 4°C. The tissues were washed in PBS (3x5min), and incubated with HRP conjugate [1:100] in 1% blocking buffer (component C) for 1h (RT). The sections were again rinsed with PBS (3x5min), followed by an incubation period with tyramide solution [1:100] in amplification buffer (0.0015% H<sub>2</sub>O<sub>2</sub>) for 10min (RT). The tissues were then rinsed with PBS (3x5min). The tissues were mounted on glass slides with mounting medium Prolong Gold with DAPI #P36935.

#### Image analysis

All slides were blinded and observed using a fluorescence microscope in a dark room. Six sections, with the DH intact, from each animal were chosen for analysis. For the c-Fos and pERK sub-study, the immunoreactive nerve cells were counted and plotted on a diagram of the lumbar part of the spinal cord of mice using a mouse histology atlas. The mean was calculated for each group of six sections per slide, each slide representing one animal.

For the ATF-3 sub-study, cell counting was performed using the software *ImageJ*. Four to five confocal images were captured using a confocal microscope system (LSM 710, Zeiss) of each spinal cord section at a magnification of 10x with exposure time to 0.6 – 1.0 s. The images were put together using the mosaic function. Two pictures were taken for each section: 1) for ATF-3, and 2) for Neu-N, at their corresponding light wavelength depending on the type of the fluorescent molecule attached to the secondary antibodies. The Neu-N picture was used as a control when counting the cells, as Neu-N is present in every nerve nucleus. Then using the cell analysis function in *Image J* the cells could be counted in each lamina. In order to correctly distinguish immunoreactive neurons from artefacts, some images of random sections were selected and merged in *Adobe Photoshop* with a corresponding captured image in DAPI staining (which stains all nerve cells in the spinal cord section) to see the overlaps.

## **Statistical analysis**

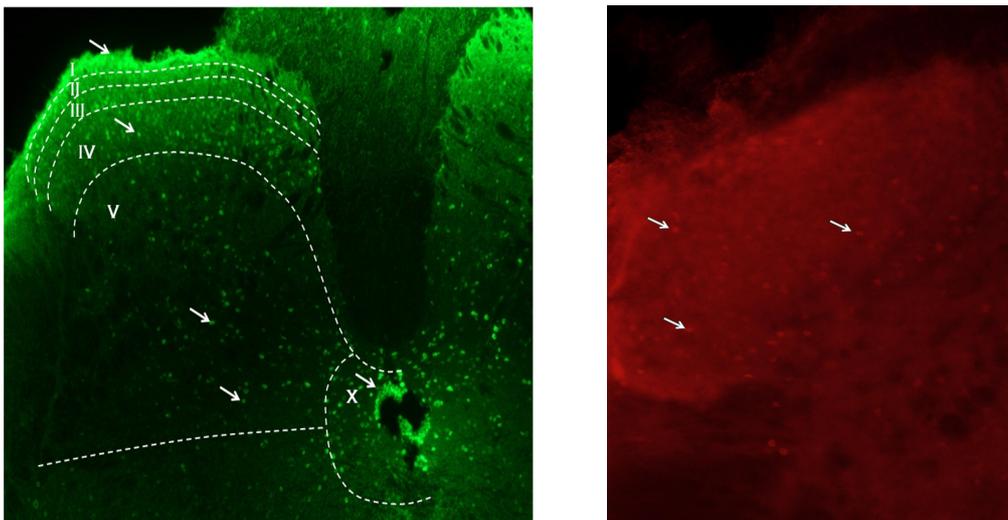
The mean was calculated for the six sections per animal. The total number of animals used in this study was 18: 1) three CAIA and three controls for day 9, 2) three CAIA and three controls for day 13, and 3) three CAIA and three controls for day 29. However, for the ATF-3 sub-study only three CAIA and three controls for day 13 and three CAIA and three controls for day 29, as there were too few sections of day 9 left in storage. The data was plotted in graphs using the software *Prism*.

Statistical significance was analyzed using a two-way ANOVA (as there were two factors: CAIA against control, and time of CAIA induction) in the software *Prism*, using the Bonferroni post test (comparing selected pairs of columns) to compare the CAIA and control pairs of group means.

## Results

### Localization of c-Fos and pERK in the spinal cord

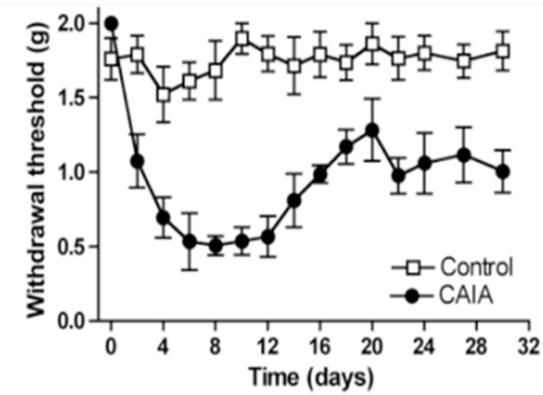
The number of c-Fos immunoreactive neurons in the lumbar region of the spinal cord of mice (fig. 1, 3) is significantly higher following induction of CAIA, as compared to the controls. The increase was most pronounced in the deep DH (laminae IV-V) on day 9, coinciding with the inflammatory phase (fig. 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.



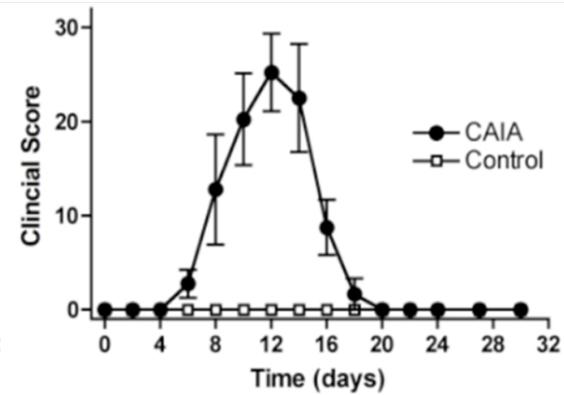
**Figure 1.** Immunohistochemistry: TSA (to the left), and conventional immunohistochemistry (to the right) of c-Fos expression shows neuronal activity in the lumbar spinal cord of mice. Left: image captured at 10x of the left dorsal horn and the central canal. The arrows show active neurons in the dorsal horn (Rexed laminae I-V) and around the central canal (X). Right: an image captured of the left dorsal horn (20x). Arrows indicate immunoreactive neurons.



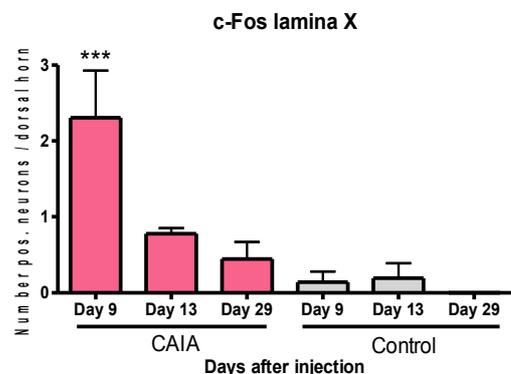
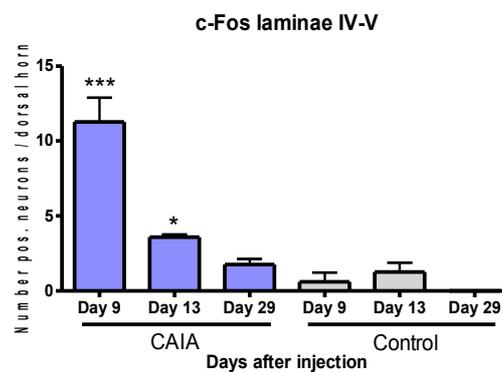
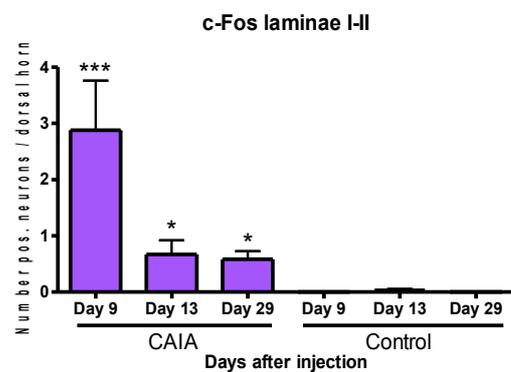
**A) Pain, tactile allodynia**



**B) Arthritis symptoms**

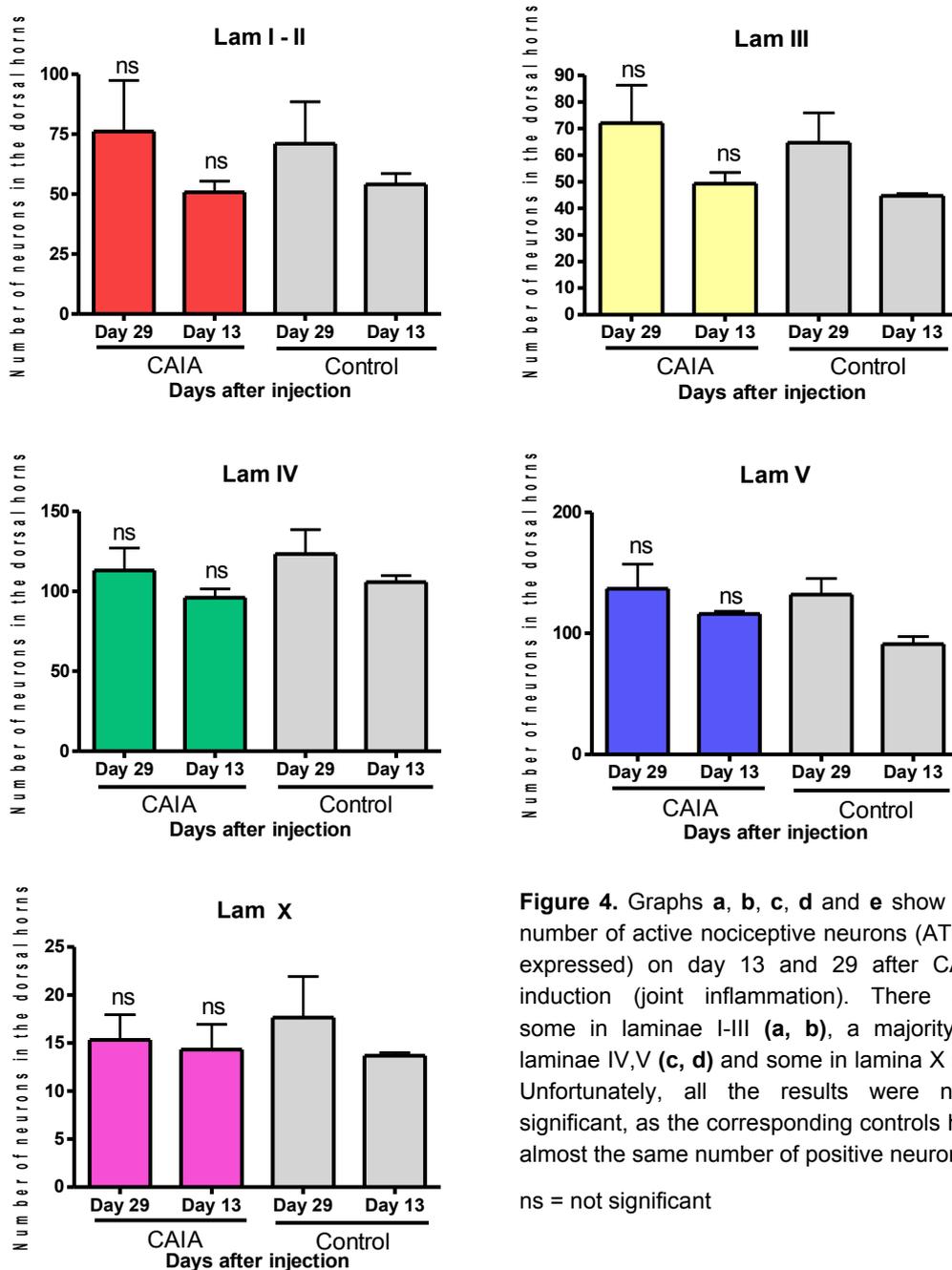


**Figure 2.** This data has been given by Baş DB. **(A)** The withdrawal threshold of the paw from the pain stimulus (poked with an optic fiber) on each day of observation after CAIA induction. The threshold decreases remarkably for CAIA mice (experience more pain) especially in the first 12 days. **(B)** Arthritis symptoms for each time point after CAIA induction. The extreme values in both graphs correlate. Induction of CAIA leads to persistent pain in mice, even after cessation of inflammation on day 20.



**Figure 3.** Graphs a, b and c show the number of active 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). This data is retrieved from using conventional immunohistochemistry, and not TSA, as shown in fig. 1.

\*P<0.05, \*\*P<0.001



**Figure 4.** Graphs **a**, **b**, **c**, **d** and **e** show the number of active nociceptive neurons (ATF-3 expressed) on day 13 and 29 after CAIA induction (joint inflammation). There are some in laminae I-III (**a**, **b**), a majority in laminae IV,V (**c**, **d**) and some in lamina X (**e**). Unfortunately, all the results were non-significant, as the corresponding controls had almost the same number of positive neurons.

ns = not significant

## **Localization of ATF-3 in the spinal cord**

Allodynia persists beyond the inflammatory phase (fig. 2). However, the number of ATF-3 immunoreactive neurons in the lumbar region of the spinal cord of mice (fig. 4) is not significantly higher following induction of CAIA, as compared to controls, in all laminae I-V, X. The results were non-significant, as the corresponding control had almost as many positive neurons as the CAIA mice. Nevertheless, there is still a pattern; namely, the numbers in both test groups seem to increase after the peak of inflammation (day 13-29). However, this contradicts the hypothesis that a marker of nerve injury, and possibly also a marker of chronic pain, should coincide with the inflammatory phase (day 13) and not necessarily after the peak of inflammation, or the case with c-Fos, before the peak of inflammation.

## Discussion

### **c-Fos and pERK**

The results of c-Fos show a clear difference in terms of number of excitatory neurons in the lumbar segment of the spinal cord on day 9 after CAIA induction compared to the days 13 and 29 (fig. 3). This suggests that there is a peak of c-Fos activation in neurons earlier than expected. There is also the possibility that the actual peak is before day 9. In future studies, one should also investigate the amount of c-Fos produced in days prior to day 9. This contradicts the hypothesis that the neuronal expression of a marker of chronic nociceptive pain, should coincide with the inflammatory symptoms. Nevertheless, an upregulation of c-Fos in the CNS, before the arthritis symptoms appear, indicate that there is a relation between peripheral nerve injury in the joints and activation of nerves in the CNS. The arthritis symptoms appear some days later possibly due to the latency of recruitment of the humoral immune system to the site of injury.

Unfortunately, immunohistochemistry with pERK did not mediate any results. The antibody used only recognizes the phosphorylated state of the kinase, and we suspect that the lack of a positive signal is due to spontaneous dephosphorylation in the specimens, after storage for 6-7 months. In conclusion, pERK as a marker of neuronal excitation is unreliable when it comes to using long-term storage of specimen. Additionally, c-Fos is more easily induced under acute noxious stimulation than pERK. A nociceptive stimulus with a duration of <10s is enough to see c-Fos expression, but not with pERK. Moreover, non-noxious stimuli such as repetitive colorectal swelling at low pressure can trigger c-Fos activation. Therefore, c-Fos is not as specific to chronic nociceptive pain as pERK [9]. This makes pERK immunoreactivity rarer, although more specific than c-Fos, which is an advantage to specifically detect activation of a noxious nature. Additionally, there is a difference in expression latency, where c-Fos is first detected 30min after noxious stimuli and has a peak at 1-2h after the induction, and



the expression of pERK is first detected 1min after noxious stimuli and has a peak only after 3min [9]. This could also explain the lack of pERK expression when observing tissues that were extracted 9 days later and beyond. Antibody probing would work in a fresh tissue (frozen section), but the morphology would be bad if not fixed with PFA.

When comparing the different laminae, the majority of c-Fos expression is located in IV and V. This is mainly due to the larger anatomical size of the laminae. 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. Lamina X is associated with visceral pain, a type of nociceptive pain, because that is where the nerve fibers from the gastrointestinal (GI) system enter. Interestingly, this region had active neurons subsequent to CAIA induction as well.

### **ATF-3**

The ATF-3 expression in the spinal DH is not significantly higher in CAIA mice compared to the control. Further studies need to be undertaken, although ATF-3 has been known to be a good marker of nerve injury when analyzing DRGs [14]. The aim of this study was to analyze DH of the spinal cord instead. Unfortunately, the results for ATF-3 are not significant.

Pilot studies on ATF-3 immunohistochemistry were made on test tissue to ensure optimal clarity of the confocal images. However, the background noise was still quite high (higher than with c-Fos), making it harder for the beholder to distinguish the individual cells. Nevertheless, since this study was blinded, and there were corresponding Neu-N and DAPI images for comparison, this minor error should be neglected, although still worth mentioning. To fully ensure it had not influenced the results it would be advisable to do antibody probing using the TSA method instead of the conventional one, to highlight the active neurons from the background noise.



Interestingly, there seems to be an increase of ATF-3 expression from day 13 to day 29 in both CAIA mice and the control. This could possibly mean that there is an accumulation of ATF-3 over a period of time after nerve injury, whereas c-Fos is expressed before the peak of inflammation. However, the nerve injury is not necessarily caused by CAIA itself, but rather the act of the injection made, since the controls also show an increase of ATF-3.

## **Conclusion**

To summarize, c-Fos expression in the spinal DH 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 after CAIA induction. This suggests that neuro-excitability 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 DH neurons that persists even after the inflammation subsides. However, pERK results were completely negative for both CAIA and control, possibly due to the experimental error of using too old tissues for immunohistochemistry, and were therefore excluded from the study. Furthermore, ATF-3 expression in the spinal DH is not significantly higher in CAIA mice compared to control. Further studies need to be undertaken to confirm this.

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