# c-fos expression in the rat spinal cord in response to facet joint stimulation with formalin.

Ray Hayek. BSc(Hons) MChiropract. PhD., Ali S. BSc MSc PhD., Holland R J. BSc., Sidaway G

E. BSc. & Bassett JR. PhD.

c/o Dr Ray Hayek, Director Centre for Chiropractic, Macquarie University 2109, Sydney Australia.

# Introduction

In recent years, there have been significant advances in our understanding of the physiological and biochemical processes involved in pain processing at the spinal level. The elucidation of these complex processes has meant a shift away from the concept of pain as a simple "hard-wired" system with a pure "stimulus-response" relationship. More recent theories of pain incorporate the processes that occur within the nervous system after prolonged, noxious stimulus. These include both descending higher cortical and local spinal inhibitory mechanisms. Trauma to peripheral nerve tissues can lead to functional changes within more central regions of the nervous system, these influence subsequent responses to sensory input. There is increasing recognition that long-term functional changes occur in pain processing pathways after prolonged noxious input. This "plasticity", characteristic of the nervous system, alters the body's response to further peripheral stimuli both noxious and benign.

Improvements to therapeutic interventions offered to human patients depend on a clear understanding of the tissues of the spine and their innervation. The loading and mobility which is provided by facet joints during motion, in conjunction with their role in strengthening the articular pillar of the vertebral column, suggest that facet joints are likely to be a significant source of pain associated with spinal disorders.

The sensory information from Tl to T4 facet joints in the rat, project to laminae I-IIV, VII, VIII, IX & X (Hayek R. 2000). These WGA-HRP descriptive studies demonstrate the direct anatomical connections to these same laminae from the facet joints. The question arises then: What role do facet joints have in the generation and propagation of spinal pain? This pain is most probably mediated via activating intemeurons, which result in an enhanced response to the painful stimuli from spinal structures.

1. Fos has been used extensively as a marker to detect activated neurones. Once expressed, the c-fos protein enters the nucleus of the neuron and participates in protein complexes, which in turn interact with DNA to produce a cascade of proteins. Neuron cell bodies expressing c-fos can be readily detected using standard microscopy methods and then counted. Experiments were performed to investigate the level and extent of c-fos expression after exogenous stimulation of facet joints at Tl to T4, with formalin.

## Aims

* + To determine the role of facet joints in the generation and propagation of spinal pain
  + To examine the distribution of c-fos expression after individual pain stimulation to the Tl/2, T2/3 and the T3/4 facet joints.

## Methods:

Experiments were performed on adult rats (400-S00g,), anaesthetised with ketamine (i.p. 60mg/kg) and placed in a stereotaxic frame. Dissections of the posterior and posterior lateral thoracic spinal region were performed to expose the facet joints (Tl to T4 one side joint injection, 8 animals per

joint, N=24). The joint space proper was exposed to an injection of SµL of 5% formalin in saline for 2 hours. The animal's respiratory rate, hindlimb withdrawal reflex, mucous membrane and rectal temperature were monitored at 15-minute intervals for the subsequent 2 hours while under anaesthesia. The joint was then irrigated with saline, the animal overdosed with anaesthetic (i.p. 60mg/kg of Nembutal), then cardially perfused with saline followed by a fixative (4% paraformaldehyde) and the spinal cord removed. Sections were frozen cut at 40µm transversely for the immunohistochemical reaction. The expression of the immediate early gene c-fos in the spinal cord was detected with biotin/streptavidin immunocytochemistry.

**Fos Immunohistochemistry:**

Spinal cord sections were cut at 40µm transversally and the c-fos immunohistochemical procedure applied. This procedure involved several steps, which include cord sections washed for 30 minutes in a solution of 50% ethanol in distilled water. The sections were then washed for 30 minutes in 5% normal horse serum (NHS) in O.lM PB solution. Tissues were then incubated in a-fos diluted to

1:2000 in PB and stored for 3 days at 4°C. Following this incubation, the sections were washed with O.lM PB for 30 minutes then rinsed for 20 minutes in 20ml of nickel intensifying agent (0.1ml 2% Nickel Ammonium Sulfate, made up to 20ml with PB (pH 7.4). This was followed by a rinse with the same fresh mixture and with 20ml of glucose oxidase for 20 minutes. The reaction was terminated once the nickel intensified Fos-labelled nuclei could be seen at 1OX with bright field microscopy, by rinsing sections with O.lM PB (pH 7.4). Sections were then mounted on gelatine coated slides, air dried for 24 hours, dehydrated and DPX mounted.

Sections from thoracic spinal cord, thoracic ganglia were examined using brightfield illumination microscopy for cytoarchitectural boundaries and the location of c-fos reaction product. All central observations were traced onto a spinal cord outline using the drawing tube or digitally mapped.

**Control Experiments:**

Control experiments (N=l2) were performed at Tl to T4 facet joints using an infusion of saline over two hours. In order to determine whether to c-fos expression was directly related to noxious stimulation and not to surgical intervention, this group of experiments was necessary. All animals utilised for this group received all of the steps outlined above including anaesthetic and surgery however, no formalin injections were delivered into the facet joint (n=6: 2 rats per joint) or the dorsal primary ramus (n=6: 2 rats per dorsal primary ramus). Fos-positive cells were then identified with light microscopy because of their characteristic, nickel intensified, darkly stained nuclei. All sections were mapped on spinal cord outlines.

**Cell Counting Methods:**

The ventral border of the superficial dorsal horn (laminae I and II) and the boundary of the reticular part oflamina V were the most easily defined. The ventral border of the nucleus proprius (laminae III and IV) was defined by a line drawn tangentially across the dorsal part oflamina V to the medial border of the dorsal horn. The ventral border of the neck of the dorsal horn (lamina V) was defined by a parallel line drawn from the ventral border of the dorsal columns to the lateral border of the dorsal horn. The remainder of the hemisection was defined as the ventral gray (laminae VII­ X).

**Results:**

The location ofFos like immunoreactive neurones (Fli) in the spinal cord of the upper thoracic spinal cord following facet joint, was qualitatively and quantitatively similar in all groups (Tl to T4). The laminae and dorsomedial position ofFli neurones at a given rostrocaudal segment showed little variation in between groups and across spinal levels. Almost no cytoplasmic staining and scant filamentous staining of the cytoplasm in the cells lining the dorsal horn entry zone was noted. Consistent with the known nuclear location of the fos protein, Fli neurones were easily

recognised by their diffusely stained nuclei and unlabelled nucleoli. No cytoplasmic staining of cells in the substantia gelatinosa and nucleus proprius sites, occasionally associated with immunostained dendrites and terminals, was seen. In all experiments, the cross-reactive staining was easily distinguished from the diffuse Fli neurones. Immunoreactive cellular nuclei appeared as dark, round to oval structures. However, gradations in labelling density were noted. Some immunoreactive cells stood out starkly from the surrounding background, whereas other labelled nuclei in the same region showed less intense immunostaining.

The base level ofFli neurones in control experiments (no formalin infusion) was extremely low, with less than 2 very lightly stained nuclei present in every second or third 40µm sections analysed. Those cells that were observed in spinal cord cross-sections of control experiments were primarily located within laminae I and II of the dorsal horn. In contrast, infusion of formalin into the joint space proper evoked dramatic Fli neurones within the gray matter. The greatest number of evoked Fli neurones was consistently observed at the spinal segmental level, which directly innervates the facet joint stimulated. Fli neurones were largely confined to the side of formalin stimulation with rostrocaudal distribution up to 2 spinal segments rostrally and I segments caudally. Fli neurones were very seldom observed contralateral to the side of stimulation. Occasionally, however, I to 2 cells were noted to be stained for c-fos expression and were limited to the cap of the dorsal horn.

It was consistently observed after two hours of formalin stimulation at Tl/2 facet joint, that dense fos labelling was present predominantly in the superficial dorsal horn (laminae I to III). The staining was greatest in terms of numbers ofFli positive cells and the intensity of staining was consistently observed along the dorsomedial margin oflaminae I to Ill, extending into the neck of the dorsal horn. The rostrocaudal distribution of formalin-evoked Fli was confined to the upper thoracic and lower cervical spinal segments, however, the rostrocaudal number of positive cells diminished in the dorsal horn away from the site of joint stimulation. This pattern ofFli neurones observed in 8 animals experiments clearly demonstrates consistency of the quality and quantity of c-fos expression (table I), secondary to joint stimulation. A three-dimensional schematic reconstruction within the ipsilateral dorsal horn at Tl demonstrates the distribution ofFli neurones.

Cell counts in each nuclear region for each animal was performed.Variations exist in the number of the Fli neurones at each segmental level. However, the average segmental demonstration ofFli neurones is presented, with the percentage contribution of neurones, at different spinal segmental levels in response to Tl/2 facet joint stimulation. It appears that a large proportion of sensory neurones responsive to noxious stimulation occurs at the same level ofjoint stimulation (Tl, 55.1%). However, neighbouring spinal segmental levels also appear to be responsive to noxious joint stimulation, particularly the rostral segments, which included CS (27 %) and C7 (7.3%), while caudal segments responded at T2 with 8.8% and T3 with 1.6%. Average data across these 8 experiments demonstrates a pattern ofrostrocaudal distribution ofFli neurones where rostral contributions are far greater than caudal. Variability in animal responses to facet joint stimulation at a Tl/2 is revealed by the range in total cell counts observed per animal. Response variability and rostrocaudal distribution of Fli neurones among the eight rats stimulated was clearly observed across the 8 experiments. The pattern of distribution suggests that the majority of a facet joint's noxious sensory input is distributed to two spinal segments with minimal noxious sensory contribution in the next 2 segments rostral and 1 segment caudal to those that receive the majority of sensory input.

In formalin-administered facet joints, rostrocaudal distribution oflabelling extended 4 to 5 spinal segments around the site of stimulation, with the greatest rostrocaudal spread in neurones of the superficial dorsal horn. Labelling was also consistently observed within the superficial layers of the dorsal horn (laminae I - III and to a lesser extent IV). Labelled neurones were not detected in

control rats administered with saline instead of formalin over 2 hours. In these noxious situations, the projection of the sensory input to the spinal cord was multisegmental.

**Discussion**

Considerable ipsilateral, symmetrical labelling of cells was seen in all experimental series. Rostrocaudal spread of the labelling, within the thoracic spine was consistently similar across joint stimulation experiments. The rostrocaudal distribution extended 4 to 5 spinal segments, around the site of stimulation. The greatest rostrocaudal spread was found in neurones of the superficial dorsal horn. While a greater concentration was noted consistently within the dorsomedial aspects of the superficial laminae of the dorsal horn. Fli neurones, observed subsequent to noxious stimulation in all experiments are consistent with previous reports which state formalin stimulation of somatic structures is a reliable method for studying sense innervation (Menetrey, et al., 1989; Tokunaga et al., 1995).

Nociceptive-specificity appears to provide preferential c-fos expression, to noxious stimuli from joint studies. Consistently Fli neurones were observed within the superficial layers of the dorsal horn (laminae I-Ill and to a lesser extent IV). These laminae contain projection neurones that are directly responsive to and mediate painful stimuli through the spinothalamic tract. Other associated neurones that may comprise excitatory and inhibitory characteristics, within these laminae, are activated and may have the potential to augment painful stimuli. Therefore, activation of these interneurones may result in an enhanced responsiveness to sensory input from the joint or nerve stimulation. Spino-spinal projections to the PAG at the brainstem level receives direct input from superficial layers of the dorsal horn. The c-fos observations made in these experiments on deep spinal structures of Tl to T4, suggest that the next relay centre of noxious input from deep somatic structures is likely to be the ventrolateral PAG.

Data from this group of stimulation experiments reveal that in noxious situations the projection of the sensory input to the spinal cord is multisegmental. A sufficiently painful stimulus arising from any of the joints studied is likely to produce an 'overwhelming' phenomena associated with bombardment of the local spinal circuitry rostrocaudally. It has been suggested that secondary to formalin stimulation, an early phase response in expression ofFli neurones is due to the high levels of activity in a primary afferents. A second late phase response follows the continuous low level of C-fibre activity reflected in the induced inflammation of the tissues stimulated. The release of excitatory amino acids and prostaglandins may, therefore, be differentially involved in these two phases (Malmberg et al., 1995). Non-steroidal anti-inflammatory agents along with steroids produce analgesia in the late phase (Shibata et al., 1989), but have little or no effect on the early phase response produced by formalin stimulation, supports the argument that inflammation and prostaglandins are involved in the late phase response (Tokunaga et al., 1995). The central mechanisms for hyperalgesia may also contribute to the late phase response. The blockade of peripheral sensory inputs by local anaesthetic during the early phase of formalin response in rats, prevents the early response in neurons, but was demonstrated to have no influence on the generation of second phase. This suggests that the activity in the early phase of the formalin response is not a pre-requisite for the formalin response observed in the second phase.

Expression of c-fos may play a key role in the spinal processing ofnociception. Fos proteins may regulate the transcription of genes such as preprodynorphin and preproenkephalin (Tokunaga et al., 1995), thereby contributing to the long-term alterations in spinal pain processing. The immediate up regulation in c-fos mRNA, may in tum regulate the expression of the late onset preprodynorphin gene and the subsequent production of the dynorphin family of peptides, and, therefore, exert a modulatory action on nociception.

**Conclnsions**

Fli neurons observed secondary to noxious stimulation in these groups of experiments are consistent with other observations secondary to painful stimulation and may represent, for the first time reported, the initial stages of pain processing from facet joints and their nerves, in the rat. The diverse spread ofFli neurons observed in these experiments provides an explanation for the common empirical observations of both patients and clinicians, that the source of spinal pain is difficult to localise.

Rostrocaudal spread of c-fos expression was consistently similar across joint stimulation experiments. The greatest rostrocaudal spread was found in neurones of the superficial dorsal horn. Nociceptive-specificity appears to provide preferential c-fos expression, to noxious stimuli from facet joint studies. The c-fos observations made in these experiments on deep spinal structures suggest that the next relay centre for noxious input is likely to be the ventrolateral PAG in the brain stem. Further, these experiments reveal that in noxious situations the projection of the sensory input to the spinal cord is multisegmental. A sufficiently painful stimulus arising from any of the joints studied is likely to produce an 'overwhelming' phenomena associated with bombardment of the local spinal circuitry rostrocaudally.

For the first time the pattern ofc-fos expression from stimulation studies at the central projection of sensory afferent sites, from facet joints of the upper thoracic spine has been studied. This work provides a foundation for further studies on both these structures and their corresponding sympathetic ganglia. The thoracic spine and sympathetic chain present an ongoing challenge for chiropractic researchers to explore and propose mechanisms for the empirical observations of visceral therapeutic effects, following spinal manipulative therapy

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