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Research Report

A semi-quantitative analysis of Fos expression by mustard oil

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Abstract

In the present study, a semi-quantitative analysis of Fos expression by mustard oil was performed. For this purpose, mustard oil was applied to the skin of the right hind foot of Wistar rats at various concentrations: 5%, 10%, 30%, 50%, 80% and 100% in liquid paraffin. The distribution and number of activated Fos-positive cells in the stimulated side (ipsilateral) and contralateral side of the spinal cord were investigated following the application. The ED50 of the response was also determined. The number of Fos-labelled cells gradually increased in a dose dependent manner in both sides of superficial layers (laminae I–II) of the spinal cord with increasing concentration of mustard oil. The increase between the doses was found significant in the ipsilateral superficial layers. The increase was significant in the contralateral superficial layers at concentrations above 50%. Very few Fos-labelled cells were observed around the central canal region in all concentrations. Higher doses of the mustard oil did not increase the number of activated cells in the deeper layers. However, the expression in the deeper layers (laminae III–X) does not show a consistent trend. Also, none of the concentrations used produced labelling in neurons of the deep ventral horn neurons or in motor neurons. Forty percent (40%) of mustard oil gave an approximately 1/2 maximum response i.e. an approximate ED 50. This may be important for studies using intrathecal application of antagonist following the mustard oil activation of skin nerve fibres.

Theme: Sensory system
Topic: Pain modulation: pharmacology

Keywords: c-Fos; Quantification; Mustard oil; Spinal cord; Rat

1. Introduction

The c-*fos* proto-oncogene encodes a nuclear phosphoprotein, Fos that can be expressed in spinal neurons by noxious peripheral stimulation [11]. This study also showed that the distribution of Fos immunoreactive neurons in the spinal cord was in close proximity to the central terminals of the activated C-fibre primary afferents. The other important result of this study was that the expression of Fos has not been found in dorsal root ganglia, the gracile nuclei or within motor neurons following physiological stimulation of primary sensory neurons. The following studies addressed issues such as the stimulus parameters and modalities that would cause Fos expression and the distribution of neurons in which expression could be induced. These studies demonstrated that the expression of Fos is directly related to intensity of the stimulus, its modality and/or the degree to which the stimulus is provoked [1,3,36].

The time course of Fos induction appears to be fairly constant irrespective of the stimulus modality investigated. Typically, synthesis of Fos protein can be detected within 30 min of the start of stimulation and reaches its maximal level within 2 h [10,36]. *Fos* mRNA expression reaches a maximal level within 15 min and then declines to basal levels within 4 h following electrical-induced seizure [21]. However, some differences do appear if the disappearance of Fos protein following different stimulus types is studied. For example, injection of formalin into one hind paw, caused activation of Fos which disappears between 8 and 16 h later whilst noxious heat-activated Fos persists for 24 h [37]. After short periods (3 s–20 min) of electrical stimulation of a cutaneous nerve, at an intensity which

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would recruit both myelinated and unmyelinated fibres, Fos disappears within a period of hours. Whereas, continuous stimulation for a longer period (4.5 h) at the same intensity gives rise to Fos synthesis, which qualitatively is judged to have reduced by less than a half of its stimulated level 24 h later [3]. These differences suggest that Fos expression may show different time courses dependent on the duration and intensity of stimulation.

Alterations in the duration of a stimulus of any one modality have a profound effect upon the number and intensity of labelling [1,3]. In addition to alterations in the numbers of neurons expressing Fos, changes in the laminar distribution are also found to be dependent on the stimulus duration and the time point sampled after stimulus application [36].

Harris [9] has reviewed evidence for the increased Fos expression in spinal neurons on the side contralateral to the noxious stimulus in awake and in prolonged stimulus received animals.

The mechanism of the induction of Fos in spinal sensory neurons is not completely clear. However, c-*fos* is expressed within neurons following calcium influx [18,19]. Morgan and co-workers [20] have shown that neuronal excitation results in a rapid and transient induction of c-*fos*. Experiments employing receptor selective drugs have demonstrated that activation of glutamate receptors [32,33], cholinergic receptors [7] and adrenergic receptors [8] induce c-*fos* or its protein product-Fos. Many of the stimuli that result in expression of c-*fos* in neurons are known to involve nerve growth factor [4,6,31], peptides [22–24,27,28,33], second messenger systems such as, cAMP and Protein kinase C [13] and nitric oxide synthase [14].

Mustard oil is a chemical irritant which activates C-fibres polymodal and high threshold mechanoreceptive afferents with minimal activation of Aδ-myelinated nociceptive afferents [38]. It has been demonstrated that cutaneous C-fibres of hind foot run in tibial, superficial peroneal, saphenous and sural nerves of sciatic nerve, which project to a separate and distinct region of the superficial dorsal horn extending from lumbar spinal segments L2 to caudal portion of the L5 [34,35].

In the present study, the effects of a range of mustard oil concentrations on the activation of Fos in the spinal cord of rats were investigated. ED 50 was also determined for the activation of Fos by this stimulus to establish more accurately the change induced by the intrathecal antagonists in mustard oil experiments.

2. Material and methods

2.1. Animals

21 adult Wistar rats of either sex were used in this study. All rats were obtained from the breeding stock in the Veterinary Pathology Department, University of Liverpool. They were housed in standard cages (North Kent Plastics Ltd., Home Gardens, Dartford) and in animal rooms at 20 °C with lighting for 12 h each day and 55% humidity. Animals were maintained with food and water ad libitum. Due to the possibility that stress and sensory stimulation could possibly lead to Fos induction, stress and animal handling were minimised. Anaesthesia was induced with halothane (Fluothane, Zenica) 4% in air and then long-term stable anaesthesia established with ethyl carbamate (Urethane, Sigma) at a dose of 1000 mg/kg IP. Mustard oil at concentrations of 5%, 10%, 30%, 50%, 80% and 100% diluted as appropriate with liquid paraffin was tested. For this purpose, the right hind foot was coated with mustard oil once after receiving deep anaesthesia. Three rats were used at each concentration and for vehicle-treated controls Two hours after the sensory stimulation, animals were perfused with an artificial cerebral spinal fluid (ACSF).

2.2. Fixation and tissue sectioning

The perfusion was followed by fixation. In all experiments, cold 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) was used for fixation in a volume of 300–500 ml. Following dissection, the tissue was post-fixed in the same fixative for 2–3 h and cryoprotected with 20% sucrose in PBS at 4 °C overnight. The required part of the tissue was taken from the spinal cord. This usually consists of the lumbar two to lumbar five segment regions (L2–L5). Tissue blocks were placed on the cooling plate of a freeze knife microtome and embedded with Cryo-M-Bed medium (Bright Instruments). The tissue was cut transversally into 40-μm thick sections and these were collected into the 10 test tubes containing 10 ml of PBS with the help of a paintbrush. They were collected serially such that each tube contained sample section spaced at 400-μm intervals throughout the length of the block. They were then washed for 2–3 h in PBS.

2.3. Immunohistochemistry for Fos

To reveal the expression of Fos, an antibody raised against the gene product, which was a generous gift from Dr. Evan [11], was used in the staining protocol. Suppression of endogenous peroxidase was done by treatment of the sections with 0.3% H_2O_2 in PBS for 30 min. Sections were washed for 30 min in PBS (three changes of the buffer) and then incubated with Fos antibody (1:1000 dilutions). This and subsequent antisera were prepared in working dilutions using PBS containing 0.5% Triton X-100 and 2.5% bovine serum albumin. Incubation in the primary antisera was carried out at 4 °C in the fridge overnight. The sections were washed for 1 h (four changes of the buffer) in PBS following each incubation. Sections were then incubated in biotinylated antispecies antisera (1–2 h in room temperature) then incubated in streptavidin HRP (1–2 h in room temperature) followed by chromogen staining. Stained sections were washed in PBS and distilled water and