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Correlation of noxious evoked c-*fos* expression in areas of the somatosensory system during chronic pain: Involvement of spino-medullary and intra-medullary connections

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Abstract

Chronic pain induces functional alterations of the endogenous pain control system namely in the modulation of nociceptive transmission at the spinal cord. We used the c-*fos* expression as a tool to study correlated neuronal activation, induced by bending the inflamed paw of monoarthritic animals, between the spinal dorsal horn and medullary centers belonging to the endogenous pain control system, namely the lateralmost reticular formation of the ventrolateral medulla (VLMlat), the lateral reticular nucleus (LRt), the dorsal reticular nucleus (DRt), the *nucleus tractus solitarius* (Sol) and the rostroventromedial medulla (RVM). Awake monoarthritic rats were subjected to 4 min of paw bending followed by anaesthesia and perfusion either immediately or 2 h later. The numbers of Fos immunoreactive neurons in the spinal dorsal horn and in the medulla oblongata were significantly correlated mainly immediately after stimulation: lamina I correlated with the VLMlat, LRt, Sol and RVM; lamina II correlated with the VLMlat, LRt and Sol; and laminae IV-V correlated with the VLMlat and LRt. Between medullary pain control centers significant correlations occurred immediately and 2 h after bending at the VLMlat-Sol and LRt-Sol, at the VLMlat-LRt and VLMlat-RVM in animals perfused immediately, and at the VLMlat-DRt and LRt-RVM in animals perfused 2 h later. These data demonstrate that the mobilization of a chronically inflamed paw triggers intense correlated neuronal activity in several areas of the somatosensory system, indicating functional relevant links in pain control.

Keywords: Fos; Spinal cord; Brainstem; Chronic pain

Chronic pain is characterized by several features including central sensitization, with receptive field expansion of spinal cord neurons and changes in neuronal excitability [32,35]. This hypersensitivity of nociceptive neurons is not restricted to the spinal cord, also affecting supraspinal structures involved in descending pain modulation [40]. Following peripheral inflammation, neurons of the rostroventromedial medulla (RVM) increase the responsiveness to innocuous and noxious stimuli [23,39]. A triggering effect from the periqueductal grey, but also from spinal nociceptive input [27], is likely to activate descending modulation from the RVM through fibres targeting laminae I, II, IV-V and X [10,22,27]. Other medullary nuclei involved in pain modulation are also likely to be affected by chronic pain. The lateralmost reticular formation of the caudal ventrolateral medulla (VLM), designated VLMlat, is especially devoted to pain modulation. It projects exclusively to spinal laminae involved in nociceptive transmission, namely laminae I, IV-V and X, and receives projections from lamina I, forming closed reciprocal loops [38]. Furthermore, the thresholds for pain inhibition from the VLMlat are lower and the analgesia is more intense and long-lasting than from other VLM areas, such as the lateral reticular nucleus (LRt) [14]. Another common role of the VLMlat and the LRt is cardiovascular control [34,37]. Besides nociceptive modulation, the LRt is also involved in providing adequate motor responses to noxious stimulation [37] mediated by anatomical connections both with the dorsal and the ventral horns [17,20]. The *nucleus tractus solitarius* (Sol), with a well-known involvement in cardiovascular control, also participates in pain modulation [11,28] through direct projections to the superficial dorsal horn. Nociceptive input arising from laminae I, IV-V, VI-X is likely to trigger descending modulation from

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the Sol [9,24]. The dorsal reticular nucleus (DRt) is an unique pain facilitating centre [19]. It projects mainly to laminae I and IV-V, and receives projections from laminae I and IV-VII [20], establishing a closed loop relevant in pain modulation.

Several connections between the aforementioned brainstem pain control centres provide the anatomical substrate for integrated responses to noxious stimulation. For example, the VLMlat establishes reciprocal connections with the LRt [41], Sol [6] and RVM [34], and the DRt is reciprocally connected with the Sol and RVM [18]. Several anatomical and physiological data support the fact that pain sensation is the result of a complex integrative network combining nociceptive and autonomic inputs, the latter of which are difficult to address when the studies are performed under anaesthesia. Taking advantage of the c-*fos* protooncogene technique to monitor the activation of large neuronal populations, we correlated Fos expression, between spinal dorsal horn and the medullary pain control centres referred above (VLMlat, LRt, Sol, DRt and RVM), and also between these centres, in response to brief bending of a monoarthritic paw in awaken animals [5,29]. This stimulus was chosen because it induces noxious-evoked activation of several areas of the somatosensory system [25,31]. In order to evaluate the pattern of the immediate and delayed response to bending the inflamed paw, the animals were perfused after the stimulus or 2 h later.

Male Wistar rats (300–330 g; Charles River, Barcelona, Spain) were used according to the procedures presented in Fig. 1. Experiments were carried out in accordance with the European Community Council Directive (86/609/EEC) and the ethical guidelines for pain investigation in animals [43]. To minimize stress, animals were gently handled by the experimenter for 10 min during 5 days prior to injections into the left tibiotarsal joint of 50 μl of saline or Complete Freund's Adjuvant solution (CFA) [4], under 5% isoflurane anaesthesia. After the intra-articular injections, the animals were daily handled during the next 14 days, and at this time point, subjected to no further manipulation ($n = 3$ for saline-, $n = 6$ for CFA-injected animals) or to 4 min of continuous flexion/extension of the injected paw ($n = 3$ for saline-, $n = 12$ for CFA-injected animals). Following this stimulation, animals were perfused either immediately ($n = 6$ for CFA-injected animals) or 2 h later ($n = 3$ for saline-, $n = 6$ for CFA-injected animals). Animals were perfused as previously described [28] after neat isoflurane anaesthesia, which took about 3–4 min. The medulla oblongata and spinal segments L_4-L_5 were removed, post-fixed, cryoprotected and coronal 40 μm frozen sections were serially collected in PBS [28]. One in every three sections was immunostained for Fos by incubation with a rabbit anti-Fos polyclonal antibody (Ab5; Oncogene, Germany) at 1:10.000, overnight, preceded by 2 h of immersion in a PBS solution, containing 0.3% Triton X-100 (PBST), 0.1 M glycine and 10% normal swine serum. Following washes in PBST, sections were incubated in biotinylated swine anti-rabbit antibody (Dako, Denmark) followed by an ABC solution (Vectorstain Elite, Vector, USA), both at 1:200, during 1 h, and ultimately reacted for DAB. Additional consecutive spinal and medullary sections immunoreacted for Fos were counterstained with formol-thionin to delimit spinal layers or medullary nuclei, as described elsewhere [28]. Fos-immunoreactive (Fos-IR) nuclei occurring in 10 sections taken at random from L_4-L_5 segments and in all the medullary sections were plotted in camera lucida drawings, by a blind procedure. Ten randomly taken spinal sections were used for each animal and the delimitation of laminae I, II, III, and IV-V (Fig. 2A) was performed in the sections that were Fos-immunoreacted and thionin-counterstained. All the medullary sections were used and the nuclei were delimited according to Paxinos and Watson [26]. The following medullary areas were considered: the VLMlat, the LRt comprising the magnocellular and the parvocellular components, the DRt, the Sol (Fig. 2B) and the RVM, comprising the nucleus raphe magnus and the adjacent gigantocellular reticular nucleus (Fig. 2C). The numbers of Fos-IR neurons in each animal were counted and Pearsons product moment correlation was used to determine the correlation coefficients (r) between dorsal horn laminae and medullary nuclei or between these medullary areas (SigmaStat vr 2.0).

Fos-IR neurons were recognized by the dark brown DAB precipitate and occurred in the spinal dorsal horn (Fig. 2D) and

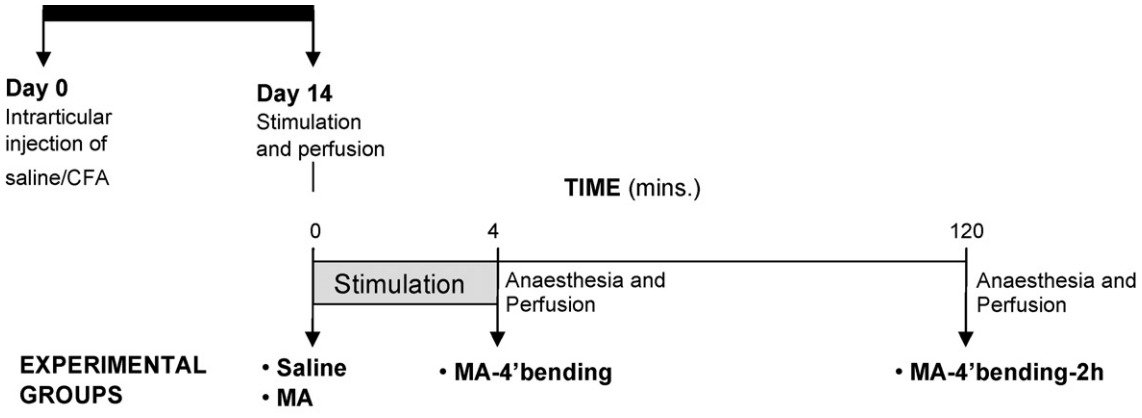

Fig. 1. Schematic overview of the experimental procedures. Stimulation consisted on bending during 4 min the injected paw, 14 days after intra-articular injection. Experimental groups: Saline – animals injected with saline; MA – animals injected with CFA and non-stimulated; MA-4′ bending – animals injected with CFA, stimulated, anaesthetised (3–4 min) and perfused; MA-4′ bending-2h – animals injected with CFA, stimulated, anaesthetised and perfused 2 h latter. The "Saline group" includes non-stimulated and stimulated animals perfused after bending or 2 h latter since similar numbers of Fos-IR neurons were obtained.