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Molecular biology of pain

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Noxious stimulation is followed by a rapid change in gene expression within the post-synaptic neurones of the dorsal horn of the spinal cord. These post-synaptic events are detectable for several hours after stimulation and are thought initially to reflect the direct activation of post-synaptic neurones by incoming sensory afferents. In contrast, non-noxious stimulation has only a very limited effect on gene transcription suggesting that it is the incoming unmyelinated, polymodal nociceptor C-fibres and the small group of small diameter myelinated Aδ nociceptors that mediate the central effects on gene transcription. As the C-fibres, in particular, are highly topographically organized it follows that new gene expression in cord neurones is tightly linked to the peripheral site of stimulation and indeed the type and location of neurones within the dorsal horn are predictably related to the nature of the tissue which is stimulated—for example, skin, muscle or viscera.

This review focuses on the expression of the immediate early gene (IEG) *c-fos* [16], which appears rapidly within the spinal cord after noxious stimulation. This gene codes for a protein (Fos) which forms part of the AP1 transcription factor complex [14, 24, 64] which may, in turn, control the expression of other genes, the products of which could form the substrate for long-term changes in neuronal excitability. We will attempt to relate Fos protein expression to possible changes in the processing of incoming sensory information.

C-fos and *c-jun* were originally described as a class of genes rapidly and transiently expressed in cells after various forms of stimulation and hence the name "immediate early genes" [15]. In the central nervous system the IEG *c-fos* and *c-jun* [7, 62] are expressed after only specific types of stimulation, some of which are outlined below [28, 44, 79, 81]. The events from cell surface stimulation leading to IEG expression in the nucleus are complex and involve multiple second messenger pathways. The details of these pathways have been described elsewhere [8, 22, 36, 37, 48, 58] and will not be specifically addressed here, but in general those neurotransmitters associated with the processing of nociceptive information, such as glutamate and substance P, increase the concentration of Ca^{2+} in the post-synaptic neurones leading to *c-fos* activation [19, 66].

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Key words

Pain, experimental. Pain, mechanism. Theories of anaesthetic action, cellular mechanisms.

FOS EXPRESSION IN THE SPINAL CORD

In the following section we will consider two hypotheses: (a) that Fos expression is a useful marker of the effect of peripheral noxious stimulation on post-synaptic spinal cord neurones [11, 63]; (b) that Fos expression may be important to the development of a pain state, as part of the *adaptive* response of the spinal cord to continuous or subsequent nociceptive input, or both.

Fos as a marker of noxious stimulation

Minutes after peripheral noxious stimulation, there is rapid expression of *c-fos* mRNA in post-synaptic dorsal horn neurones of the spinal cord. Within 1–2 h of transcription the protein product of the gene, Fos, can also be found in these same neurones [28]. Protein synthesis inhibitors have no effect on the initial production of *c-fos* mRNA indicating that the pathways leading to transcription are already in a state of readiness to respond and do not require other proteins to be synthesized first [65]. Fos-positive neurones are restricted to laminae 1 and 2 of the dorsal horn with some labelling in lamina 5. Laminae 1, 2 and 5 are known to receive input from the unmyelinated C- and Aδ-fibres which are known to respond to noxious stimulation. To achieve Fos expression in these superficial laminae of the spinal cord it is essential to use *noxious* stimulation. Non-noxious stimulation is largely ineffective in inducing Fos protein expression. No Fos expression is seen in neurones of the dorsal column nuclei, ventral horn or, importantly, in the stimulated dorsal root ganglion cells themselves. Thus only a subset of post-synaptic neurones express Fos after sensory stimulation.

EFFECT OF ANALGESIC AGENTS ON FOS EXPRESSION IN ACUTE PAIN

Williams and colleagues extended these original observations on the induction of Fos protein in neurones of the spinal cord in rats [75–79]. In one set of experiments, under barbiturate anaesthesia, Fos protein appeared in laminae 1–2 of the ipsilateral lumbar spinal cord within 30 min of noxious thermal stimulation to one paw, peaking at 2 h. However another peak of Fos expression was seen within the

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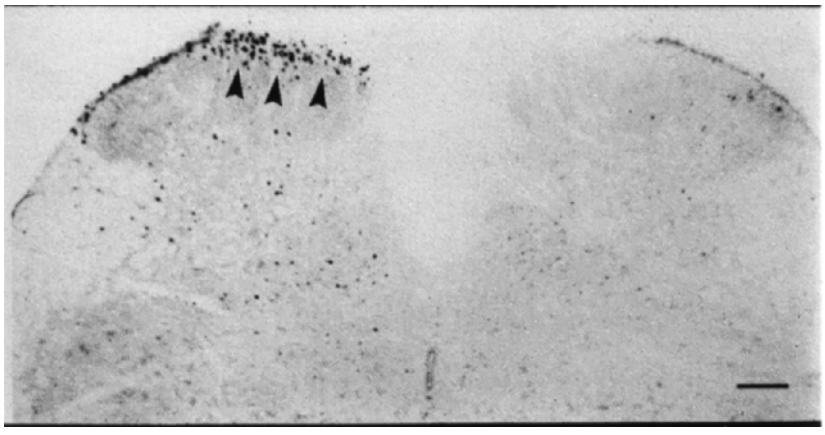

Figure 1 A 40 μm thick section of spinal cord at L4 immunostained for Fos protein 2 h after stimulation of the left hind paw of an anaesthetized rat by immersion for 20 s in gently stirring water at 52 °C. Fos expression is predominant in laminae 1 and 2 as shown by the arrows. See also fig. 4. Bar = 100 μm. From Williams and colleagues [78] with permission.

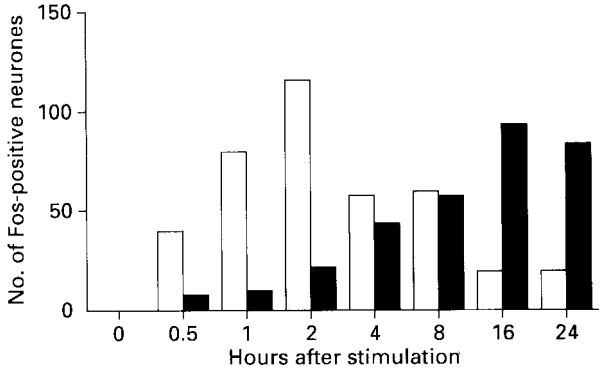

Figure 2 Fos cell counts after thermal stimulation in superficial (□, laminae 1–2) and deep laminae (■, laminae 3–10). This figure shows a superficial "wave" of Fos at 2 h and a second "wave" of Fos peaking at 16 h more deeply. For clarity only the response of the ipsilateral horn is shown and the standard errors of the mean have been omitted. After 4 h the Fos expression becomes bilateral deeply but there is little or no spread superficially. Adapted from Williams and colleagues [78].

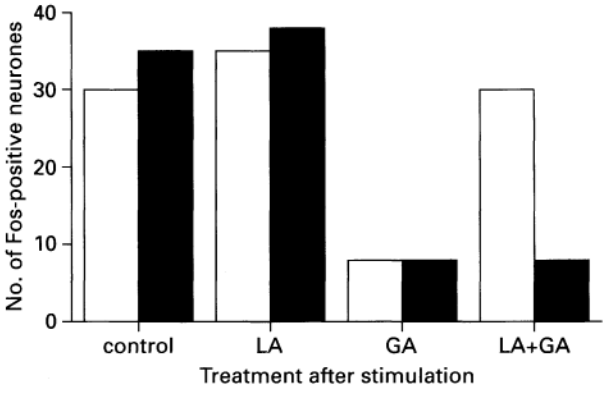

Figure 3 Fos-positive neurones per section of rat lumbar cord 8 h after heat stimulation of the left hind paw, with and without local anaesthetic (LA) block of the sciatic nerve, continuous general anaesthesia (GA), or both (LA + GA). Local anaesthesia block of the peripheral nerve does not suppress the Fos response at 8 h, indicating that input from the periphery is not required for continued expression. General anaesthesia does reduce the later Fos expression, presumably by activating inhibitory mechanisms in the spinal cord. For clarity the standard errors of the mean have been omitted. Adapted from Williams and colleagues [78].

deep laminae (5–10), commencing at 8 h, and peaking at 16 h, this "second wave" of labelling started ipsilaterally in the spinal cord and spread to become bilateral (see fig. 2).

The activation of neurones in the deeper laminae of the spinal cord is unlikely to be the result of a monosynaptic event. The initial thermal stimulation at 52 °C stimulates small diameter sensory fibres within the sciatic nerve which terminate in laminae 1, 2 and 5. Fos-positive neurones were found in other laminae both ipsilaterally and contralaterally suggesting a polysynaptic mechanism. The first "wave" of Fos expression is known to be dependent on input from the sciatic nerve, because *pre-emptive* local anaesthetic block will abolish all Fos staining in the area of the dorsal horn corresponding to the innervation of the sciatic nerve [75]. When a local anaesthetic block of the ipsilateral sciatic nerve was performed 1 h after thermal stimulation, there was still a second-wave response; in fact there was some evidence that the number of Fos-positive cells may have actually slightly *increased* (see fig. 3). If instead of blocking the sciatic nerve, the animals were kept under continuous anaesthesia after the noxious thermal stimulus there were fewer Fos-positive neurones in the spinal cord than in those animals allowed to recover from anaesthesia after thermal injury. The combination of the two treatments (*post*-injury local anaesthetic block and continuous general anaesthesia) resulted in a similar number and pattern of Fos-positive cells in the spinal cord to those animals which had received only the general anaesthesia.

Comment

These experiments suggest that if Fos is taken to be a reliable index of the efficacy of noxious stimulation then analgesia may only be effective when given just before, during or immediately after stimulation. It also suggests that Fos expression could be used to monitor the effectiveness of pre-emptive analgesia in animal models.

EFFECTS OF OTHER ANALGESIC AGENTS ON FOS EXPRESSION IN ACUTE PAIN

Opiates given pre-emptively (i.e. before the injury) reduce the extent of Fos expression in a dose-dependent manner [40, 59]; though, interestingly, even with very high doses of systemic morphine, there is still substantial Fos expression indicating that substantial nociceptive traffic has occurred. In contrast, it has been shown that morphine given *after* the injury, as with post-injury local anaesthetic block, is ineffective in preventing Fos expression [74].

Since the neurotransmitter glutamate acting at the NMDA (N-methyl-D-aspartate) receptor, plays a central role in afferent transmission and in the pathogenesis of pain states [18], one might expect a reduction in Fos expression in the spinal cord with NMDA antagonism. Interestingly, this only seems to be true in pain models where there is an intense afferent nociceptive barrage (presumably involving large amounts of glutamate release), such as after formalin injection into the paw [32]. In some other pain states, such as after thermal injury, NMDA receptor antagonists seem to be less effective in suppressing Fos expression [73, 81]. Alpha$_2$ agonists have also been shown to be potent analgesics [55, 56], and it has been shown that the alpha$_2$ agonist medetomidine strongly suppressed spinal cord Fos expression after noxious stimulation when given 12 min before the stimulation. Interestingly, however, medetomidine had no effect if it was given only 5 min beforehand [57].

USEFULNESS OF FOS EXPRESSION AS A LONG-TERM MARKER OF NOCICEPTION IN CHRONIC PAIN MODELS

Inflammatory pain models

Fos expression in the spinal cord has been described in a rat model of arthritis [1–6]. In this model, arthritis is induced by the injection of Freund's adjuvant into the base of the tail. After about 10 days, polyarthritis affects the hindlimb joints and behavioural changes such as decreased locomotion and hyperalgesia to paw pressure appear. The symptoms peak at 3 weeks and continue for up to 11 weeks after inoculation. The numbers of Fos-positive neurones were greatest in the lumbar segments L3–4 (corresponding to the innervation of the arthritic hindlimbs), and at 3 weeks (corresponding to the severity of the disease state). The pattern of Fos-positive neurones was also different from that seen in acute pain models with most of the Fos-positive cells found in laminae 5 and 6 and less than 5 % of the total in laminae 1–2. Lamina 5 contains the wide dynamic range (WDR) neurones which seem to play a role in integrating information from the periphery and also show marked sensitivity in pain states [60]. The axons of these neurones form the spinothalamic tracts [80]. Electrophysiological evidence indicates that afferent input from the inflamed joints and tissues continues in the arthritic model [40] and Fos protein might be expected to be seen in laminae 1–2 but this was not the case. Nevertheless, arthritic rats given a mechanical stimulus over the arthritic ankle joints under anaesthesia did show a normal pattern of Fos response with large increases in laminae 1–2 as well as lamina 5. This suggests that Fos expression in superficial laminae may be related to a phasic nociceptive input while deeper neurones respond to a tonic nociceptive input.

When arthritic rats are treated with morphine before further mechanical stimulation, the greatest suppression of Fos is in superficial laminae indicating that it is the phasic component that is sensitive to morphine [3]. In contrast, if unstimulated arthritic rats are given repeated doses of naloxone, there is a trend for increases in Fos count in the deeper laminae. This suggests that there may be tonic activity of endogenous antinociceptive systems in situations of chronic arthritis [3]. The arthritis in this model can be reduced by inducing immunological tolerance in the rats using an injection of dilute Freund's adjuvant given 1–3 weeks before the main injection [21]. In these animals the Fos count in lamina 5 is also reduced and continues to show a high degree of correlation with the disease state [4].

Treatment of the established arthritic rats at 3 weeks with non-steroidal anti-inflammatory drugs (NSAID) (aspirin or paracetamol) improves symptoms but surprisingly does not decrease Fos numbers in the spinal cord [5]. It is unclear why Fos expression and symptoms do not correlate after NSAID treatment, but it is pointed out that though the animals were less hyperalgesic, the arthritic joints showed very little decrease in size. In contrast with treatment of the arthritic rats at 3 weeks, NSAID *do* decrease Fos numbers and symptoms if started early in the disease at 1 week. Recently it has been shown that prostaglandin production contributes to transmission of noxious information in the spinal cord [39]. In accordance with this observation, intrathecally administered NSAID have been shown to decrease Fos expression in laminae 1–2 in response to mechanical stimulation in the arthritic model.

Neuropathic pain models

The pattern of Fos expression in chronic nerve injury (neuropathic) models of pain is very different from that seen in arthritis and other models of chronic inflammatory pain. An interesting feature is that Fos expression is now seen in laminae 3 and 4 of the spinal cord as well as the other layers more generally associated with nociceptive input (see fig. 4). As has been described earlier, laminae 3–4 are the sites of termination of large diameter non-nociceptive myelinated afferents (Aβ) and the neurones here do not usually express Fos [28]. However, after sciatic nerve injury, it has been shown that low intensity Aβ stimulation will now elicit Fos expression when previously it would not [26, 43]. It is increasingly being recognized that this normally innocuous Aβ input may play a large part in the generation of neuropathic states and helps to explain the allodynia seen clinically and in models of pain [49, 83] (see figs 4 and 5).

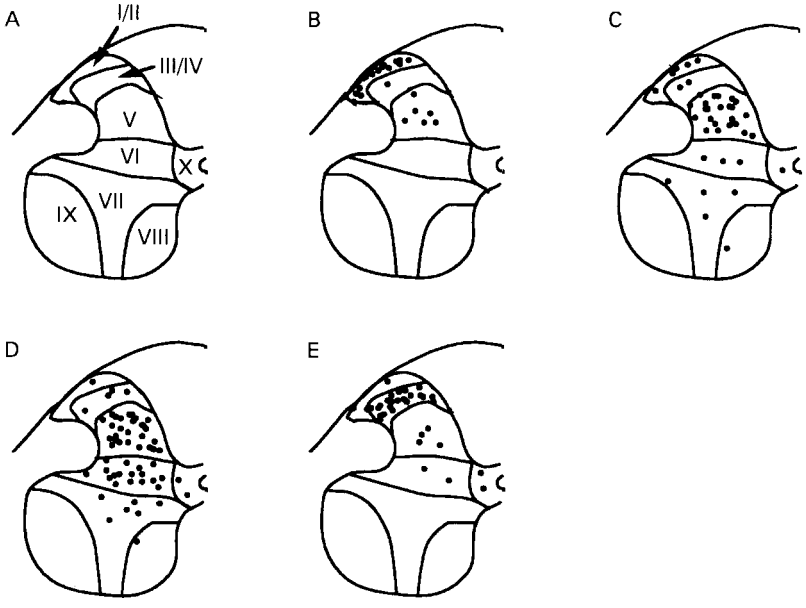

Figure 4 A schematic diagram illustrating the different patterns of Fos expression in the L4 spinal cord seen in various pain models. Each dot represents three Fos-positive cells. (A) The division of the spinal cord into Rexed's laminae [80]. Noxious information is carried by the Aδ and C-fibre and they synapse in laminae 1, 2 and 5. In contrast, non-noxious stimulation is carried by Aβ fibres which terminate in laminae 3 and 4. (B) The pattern of Fos expression 2 h after acute thermal injury to one paw. There is Fos expression mainly in laminae 1, 2 and 5 ipsilateral to the injury (see also fig. 1). (C) What happens after 16 h after the same stimulus as in (B). Fos expression is now mainly in the deeper layers and also becomes bilateral (not shown). (D) The typical pattern of Fos expression 3 weeks after induction to arthritis where the Fos expression is widespread but mainly in the deeper layers especially in lamina 5. There is comparatively very little expression of Fos in laminae 1–2 despite the excessive C-fibre-mediated noxious input known to be present in this model. (E) Fos expression in a model of neuropathic pain 14 days after loose ligation of the sciatic nerve where it is known many of the symptoms are mediated by spontaneously discharging Aδ and Aβ fibres. The Aδ fibres terminate in layers 1 and 5 while the Aβ fibres terminate in laminae 3 and 4, the latter being the site of the greatest Fos expression in this model. See also fig. 5. Drawn from data in Abbadie and Besson [1], Williams and colleagues [78] and unpublished observations.

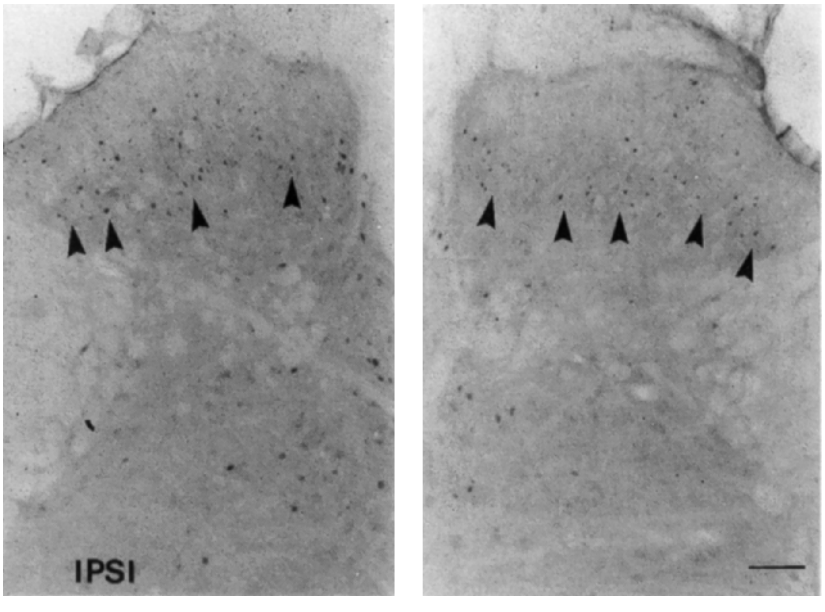

Figure 5 Photomicrograph of Fos expression in the spinal cord 14 days after loose ligation of one sciatic nerve. There is persistent bilateral Fos expression throughout the spinal cord, but mainly in laminae 3 and 4 as shown by the arrows. These layers are usually only associated with non-noxious input and do not usually express Fos. The presence of Fos expression in these layers after nerve injury correlates with the presence of Aβ mediated allodynia in this model [9, 10] [Munglani and Hunt, unpublished observations]. IPSI = side of spinal cord ipsilateral to the injury. Bar = 100 μm.

The spinal cord in neuropathic pain states may be subject to a similar afferent barrage from the periphery as in inflammatory pain states. However, in the former case the nerve impulses are generated from neuromas at the sites of nerve injury and also from the dorsal root ganglion. There is some

evidence that this peripheral afferent input helps to maintain the pain state and the persistent Fos expression seen in the spinal cord [12, 13, 17, 29, 31].

Earlier nerve injury or previous nociceptive barrages will lead to enhanced Fos expression in the spinal cord in response to subsequent noxious and non-noxious stimulation [35, 68, 69, 77]. For example, there are enhanced numbers of Fos-positive neurones in the dorsal horn when saphenous nerve stimulation follows section of the adjacent sciatic nerve. This parallels the electrophysiological and behavioural data showing saphenous nerve allodynia and hyperalgesia in the presence of sciatic nerve damage [33, 61, 71, 77]. Other electrophysiological phenomena are also paralleled by changes in Fos expression in the spinal cord. For example, the ability of "diffuse noxious inhibitory control mechanisms" (DNIC) to reduce pain perception by a concurrent painful stimulus has also been demonstrated to be followed closely by changes in Fos expression [46].

Comment

Patterns of Fos expression in both acute and chronic pain models seem to complement known behavioural and neurophysiological data.

Consequences of Fos activation in the spinal cord

Does Fos activation lead to important functional changes? Furthermore, does the activation of Fos contribute to the pain state or mediate the adaptive responses of the spinal cord to the peripheral insult? It has been shown recently that some 20–40 % of Fos-positive cells expressed after noxious stimulation are also GABA or glycine positive [72]. The latter two compounds have inhibitory roles within the CNS. The proteins Fos and Jun dimerize to form AP1-protein complex which then subsequently binds to the AP1 binding site of DNA to effect the transcription of other genes [16]. The AP1 binding site is found in the genes of the opioid family such as preprodynorphin, preproenkephalin as well as nerve growth factor and a number of neuropeptides including cholecystokinin (which is known to antagonize the action of morphine and endogenous opioids) and neuropeptide Y (which is thought to have an analgesic role at the level of the spinal cord) [25, 45, 47, 65, 84].

Fos increases in the dorsal horn in both arthritic and the nerve injury model are accompanied by increases in dynorphin (an endogenous opioid acting at the kappa receptor) [20, 27, 30, 41, 42, 53]. The increased expression of dynorphin occurs in both local circuit and projection neurones which receive nociceptive afferent input [50, 52, 70]. Dynorphin causes hyperalgesia when directly applied to the spinal cord in large doses and it had been suggested that hyperalgesia seen in these pain states was due to the expression of dynorphin [20, 34]. More recently, however, dynorphin has been shown to have a tonic *analgesic* action under both normal and inflammatory conditions [23, 67]. Since the preprodynorphin gene has several AP1-like binding sites and potentially may bind Fos (as a component of the AP1-transcription factor complex) it was tempting to speculate that Fos might lead directly to dynorphin expression. Certainly Fos is expressed in the same dorsal horn cells as those expressing dynorphin following noxious stimulation [54] and in a recent *in vitro* study activation of prodynorphin was completely blocked by an injection of a *c-fos* antisense DNA (hence blocking DNA transcription) [38, 51]. Furthermore, in a formalin pain model it has been shown that pre-emptive antisense *c-fos* administration *reduces* Fos and dynorphin staining and *increases* pain behaviour [82]. These observations suggest that Fos activation, as well as being a marker of nerve cell activity, may result directly in the activation of analgesic opioids in the spinal cord.

Summary

We have attempted to define some of the patterns of expression of the IEG Fos in pain-related states. On one level, Fos may be used simply as marker of afferent stimulation and disease state, and in this respect Fos activation may be a useful tool after nociceptive stimulation to examine the effectiveness of different analgesic regimens. For example, certain analgesics such as opioids, alpha$_2$ agonists and local anaesthetics are more effective when given pre-emptively or early in the injury rather than later on. Furthermore, the persistent expression of Fos in the presence of high dose pre-emptive opioids is disturbing and yet it may explain variable success of studies attempting to show pre-emptive analgesia with opioid-based analgesic regimens. We suggest that Fos expression, as well as defining the magnitude and the duration of insult to the spinal cord seems also to signal the adaptive responses of the nervous system to nociceptive insult. Though we have focused on only one IEG, *c-fos*, and attempted to relate appearance to known functional changes within the spinal cord, there are in fact many more genes known to be upregulated with the same or slower kinetics (e.g. Fos B, FRA-1, FRA-2, Jun B, Jun D, NGFI-A). Increased understanding of the role of these genes is likely to lead to many novel targets in the search for normalization or restoration of spinal cord function in pain states and after nerve injury.

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