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Functional changes in the rat spinal cord following sciatic nerve injury

Author
Chi, Shu-ing Hsu

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Functional Changes in the Rat Spinal Cord Following Sciatic Nerve Injury: Mapping by c-fos Expression

by

Shu-Ing Hsu Chi

DISSERTATION

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Dedicated to the memory of my father, Chi, ko-Gee
and my mother, Hsue, Ai-Chung

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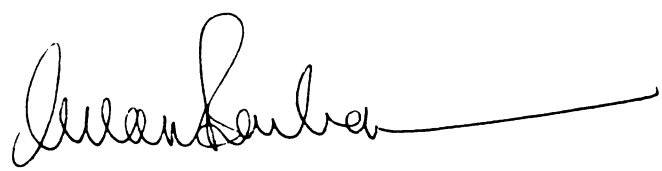
Functional Changes in the Rat Spinal Cord Following Sciatic Nerve Injury: Mapping by Fos Expression

Chi, Shu-Ing Hsu

ABSTRACT

Major nerve trunk injury is often associated with chronic pain in humans. This could result from a long term increase in ongoing activity and sensitivity developed in the injured nerve and in the spinal cord after nerve injury so that normal inputs now evoke exaggerated responses. However, the overall distribution and temporal pattern of the activated neurons in the spinal cord after nerve injury are not known. Here I use c-fos protein immunocytochemistry as a marker for neuronal activity to study the activated spinal neurons following sciatic nerve transection injury.

Compared to tissue injury, nerve injury induces higher Fos expression predominantly in superficial laminae 1,2 and deep laminae 5,6,7 of the spinal cord throughout the one month study period. The pattern of Fos expression is time and lamina dependent. Fos expression, particularly in the deep laminae, is mainly maintained by abnormal inputs from the injured nerve. The initial brief injury discharge also contributes to chronic (48hrs) Fos expression, but only in the superficial dorsal horn. Fos expression in two weeks after nerve injury is suppressed by the GABA-B agonist baclofen, but not by the opioid agonist, morphine. The spinal neurons in the regions associated with pain transmission are chronically activated and their c-fos expression is not sensitive to the opioid treatment. I propose that activity in these neurons contributes to the nerve injury associated chronic pain in human.



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INTRODUCTION

Estimates of the incidence of severe chronic pain following peripheral nerve injury in man range from 2.5 to 60% (Bonica,etal 1979, Jensen, et al,1985). Neuroma formation after nerve injury is often associated with neuropathic pain in human, as seen in casualgia and phantom limb pain. Although the mechanism of chronic pain is still not clear, both hyperactivity and hypersensitivty in the injured nerve or in the central nervous system is thought to contribute to the spontaneous and evoked paresthesia pain (Richards, 1967, Wall and Gutnick, 1974, Wall, 1981, Devor, 1989).

Neuroma Formation

Structure: Following transection of a nerve trunk, very rapid and persistent changes in structure and function take place in both the peripheral and central nervous system (Review see Wall,1989; Ludborg, 1988). Within hours, the first wave of sprouts from cut end have been observed, then disappear but are followed by second wave of sprouting within 2 days (Ludberg, 1988.). When regeneration of these axon sprouts towards their target organs is prevented, they form together with the proliferated Schwann cells a neuroma on the proximal stump (Cajal,1928; Young, 1942; Aguayo et al, 1973; Morris et al; 1972; Spencer, 1971). During an early stage, lasting about 1 week, there is retrograde degeneration for a few millimeters from the cut and a massive generation of many sprouts from each cut axon, many of which curve back along the parent fibers (Cajal, 1928; Fried and Devor, 1988). Most fibers produce multiple sprouts during the first week after nerve section ; they are initially unmylinated, some then becoming myelinated after 5 days. During the period 1-4 weeks after the injury, the majority of emitted sprouts disappears, while the remaider enlarge. After this time, the morphological features stabilize.

Physiology When an axon is cut acrss there is an immediate violaent and repitive high frequency discharge in all types of axons, but this injury discharge disappear whithin seconds and axons become silent and relatively insensitive to all sorts of stimulifor some hours (Waxman and Wall,1973; Wall et al; 1974). Within hours of an axon having been cut, sprouts begin to grow out. The sprouts from all types of sensory fibers become spontaneous active (increase resting discharge) and are abnormally sensitive to a range of stimuli (i.e. gentle mechanical pressure, sympathetic effent activty or circulating catecolamines, thermal and blood flow) in animals and man (Wall and Gutnick, 1974; Scadding, 1982; Nystrom and Hagbarth, 1981; Fields,1987; review in Devor,1989).

Normal axons are relatively insensitive to mechanical distortion and the sympathetic system normally has little affect in determing peripheral sensitivity. However, there is evidence that sypathetic fiber supply large myelinated mechanoreceptors (Akoev, 1980). Moreover, sympathetic stimulation increased a resting discharge of slowly adating I receptors (Tahmoush, 1981). In particular, following damage to the nerve membrane, the membrane of the axon beyond the damage or the sproutss from damaged nerve become extremely sensitive to norepinephrine liberated locally (Wall and Gutnick; 1974).

The abnormal activity developed in the neuroma is time dependent and varies greatly among diffent nerves, fiber types and different location along the length of a single fiber. About 20% of all myelinated affarents fire spontaneously in rat neuroma, peak ingabout 2 weeks post injury then decreasing to about 5% after one month (Govrin-Lippmann and Devor,1978). Besides ongoing activity, Scadding (1981) has recorded evoked activity from single nerve fibers from myelinated and C fibers in mouse neuroma. He reportes an early rise of all these activities within 24 hours after nerve section and then a peak at 3 days, dropping between days 6-11 and then rising to a much higher peak at 12-21 days with a return to a lower level by 25-30 days.

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In addition to developing hyperactivity in the neuroma sprouts, the fiber sprouts lie in close relation to each other and to parent fibers (Aguayo and Bray, 1975), though gap juctions and synaptic junction between axons have not been seen (Aguayo et al, 1973; Morris et al; 1972; Spencer, 1971). Some neuroma fibers often become ephatically (electically) coupled in acute (Granit et al, 1944) and chronic injured nerve (Seltzer and Devor, 1979; Blumberg and Janig, 1982; Devor and Bernstein, 1982; Lisney and Pover, 1983; Lisney and Devor,1987).

Sympathetic Nervous System

In addition to possible hypersensitivity of a damaged peripheral nerve to sympathetic activity, nerve trauma is ofen associated with increased activity in the sympathetic nerveous system. Sympathetic postganglionic neurons are known to exhibit long-term changes in sensitivity following injury (Briggs, et al, 1985). Causalgia is a classic example. It is characterized by burning pain which is exacerbated by cold, loud noises, and slight mechanical stimulaition. It is dramatically and virtully completely relieved by sympathetic block (Richards, 1967). The cause of the increase in sympathetic activity is not known. However, it is known that electrical stimulaiton of affarent fibers in peripheral nerve influences the activity of sympathetic postganglionic neurons. Stimulation of large myelinated primary afferents inhibits and stimulation of small diameter nociceptive afferents excites sympathetic effents (Horeyseck and Janig, 1974). This arrangement has the potential for a positive feedback loop: primary afferent norciceptor activation excites sympathetic efferents, which feedback to and excite the primary afferent norciceptors. This loop operates to cause a gradual build-up of pain intensity, exacerbated by sympathetic outflow and stopped by blocking the sympathetic nervous system (Fields, 1987).

Dorsal Root Ganglia

Another common location of abnormal neuronal activity is in dorsal root ganglia (DRG) (Wall & Devor,1983, Burchiel, 1984). In rat , two days after sciatic nerve section, the number of active fibers is increased but only in A fibers but not in C fibers. The increased activity rises to maximum after about 3 weeks and declines slowly over subsequent months but never ceases as long as the neuroma exists.

Since NGF is synthesized and released in abnormally large amounts from the proximal and distal stumps in the hours and days after nerve injury (Windebank and Puduslo, 1986; Heumann, et al; 1987), it has been proposed that nerve growth factor (NGF) may play a special role in the emergence of ectopic neural discharge, either by acting locally at the emerging pacemaker site or after retrograde transport to the DRG (Devor, 1989).

Central Nervous System

Following peripheral injury, the processing of sensory signals in the spinal cord and brain may be altered for hours, days or even permanently (Review see Wall, 86; Devor,1989). Four key changes have been reported: 1) increased ongoing discharge: It has been proposed that hyperactivity results from the loss of inhibition, which is due to the decrease of dorsal root potential and primary afferent depolarization following peripheral nerve cut (Wall and Devor, 1981). Furthermore, Woolf and Wall (1982) have demonstrated the diminished primary afferent A-fiber mediated inhibition. 2) increased response to previously effective peripheral stimuli, 3) the appearance of response to previously ineffective types of stimuli (e.g. response of previously "nociceptive selective" neurons to light touch) and 4) the appearence of responses to stimulation outside of the zone that previously evoked responses (ie. RF expansion of some dorsal horn neurons) (Devor and Wall,1978,1981; Lisney, 83; McMahon and Wall, 1983; Markus, et al, 1984).

Other central changes include: a long term temporal change in chemical components (e.g.SP, CGRP, CCK, FRAP, VIP,PHI) (Barbut, et al,1981; Jessel, et al; 1979), a decrease of opiate receptors in primary afferent terminals, particularly in lamina 2 (Field, et al; 1980, Csillik,et al;1982), degeneration of central presynaptic terminals (Grant and Arvidsson, 1975; Knyihar and Csillik, 1976) and active synaptic reorganization (LaMontte andKapadia,1987) in the spinal cord . All these changes would influence the remaining inputs on the neurons and may contribute to the central sensitization process.

Fos Expression

The c-fos gene is the cellular homolog of the oncogene (v-fos) carried by the FBJ and FBR murine osteogenic sarcoma viruses. It encodes a nuclear protein (Fos) that is associated with chromatin and exhibits a DNA-binding activity in vitro (Curran, et al, 1984; Sambucetti and Curran, 1986). In most cell types, the basal level of c-fos expression is relatively low, however, it can be induced rapidly (mRNA: within 5 minutes, protein about15 min) and transiently (half life 1-2 hours) by a diverse range of extracellular stimuli (Curran, et al; 1984; Muller, et al; 1984; review see Curran,1988, Sharp and Sagar, 1990).

Many different types of stimuli have been shown to elicit c-fos expression in the nervous system (Morgan, et al,1987; Sagar, et al, 1988). For instance, Fos expresson has been shown to be elevated including seizure (Morgan, et al, 1987; Sagar, et al, 1988), cerebral ischemia (Jorgensen,etal, 1989), trauma (Dragunow and Robertson, 1988) and nociceptive stimulation (Hunt,etal, 1987, Presley, 1990, Menetrey, 1989).

The precise role of Fos in neuronal physiology is unknown, the rapid induction of the fos gene in a wide variety of cell types in respose to different stimuli would suggest a generalized role in signal transduction and can be viewed as "third messenger" molecules in a stimulus-transcription coupling cascade (Curran,et al, 1985; Franza, etal, 1987; review in Morgan and Curran, 1988). The genes such as preproenkephalin (Morgan and

Curran,1989) and preprodynorphin (Draisci and Iadarola, 1989) are the targets for the stimulus-transcription coupling cascade.

In summary: It is generally accepted that tthe hyperactivity and hypersensitivity developed in the injured axons and in the central nervous system (i.e.spinal dorsal horn cells) after peripheral nerve injury is associated with chronic pain in man. Peripheral nerve block usually relieves neurophathic pain in man immediately. Subsequent peripheral abnormal inputs in the injured nerve may play a crucial role in maintaining the state of central hyperactivity. Although single neuron studies have characterized some of the responses of spinal cord dorsal horn cells after peripheral injury, the overall distribution and temporal pattern of activated spinal neurons after peripheral injury is not known. Through the technique of immunocytochemically labelling neurons which express the protein product of the c-fos proto-oncogene, it is, however, possible to monitor the activity of large populations of neurons activated by various stimulation

In my thesis, Fos expression is used as a marker for activated neurons. Temporal patterns of neuronal activity in the lumbar spinal cord of the awake rat will be mapped by immunocytochemical localization of c-fos protein following sciatic nerve transection and ligation (neuroma model).

In chapter I: Identify those populations of neurons in the spinal cord that are activated following nerve injury, and how this activity changes with time during a month study period. Second: Identify possible sources for the Fos induction.by determing whether hyperactivity in injured axon maintains central hyperactivity (Fos expression) in neuroma model.

In chapter 2: Evaluate the contributions of injury discharges to chronic Fos expression.

In chapter 3: How analgesic drugs (morphine and baclofen) affect Fos expression associated with chronic nerve injury.

In chapter 4: How baclofen affect formalin-evoked Fos expression and pain behavior.

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CHAPER I

Fos-like Immunoreactivity (FLI) in Rat Spinal Cord Following Sciatic Nerve Injury

Abstract

Peripheral nerve injury is associated with hyperactivity in both injured axons and in spinal dorsal horn cells. In this study we used immunocytochemical localization of the Fos protein product of the c-fos proto-oncogene to map the temporal pattern of the nerve injury evoked activity in the spinal cord. Sciatic nerve section induced a persistent (up to month) elevation in the number of Fos-like immunoreactive neurons, predominantly in the superficial laminae 1,2 and deeper laminae 5,6,7. The numbers of labeled neurons in different laminae varied over time The expression in the superficial dorsal horn (laminae I and II) was oscillatory, with peaks at 2 hours, 2 days and 2 weeks after nerve transection. Furthermore, within 3 days after nerve injury, the distribution of labelled neurons shifted from the medial half to lateral half of the superficial dorsal horn, with the most densely labelled cells now concentrated on the central portion of the superficial dorsal horn at caudal L5. In contrast, the increased numbers and spatial pattern of labelled neurons in laminae 5,6,7 were less changed over the 4 week period studied. Sciatic nerve block with local anesthetic significantly decreased Fos-expression when administered at 2 days or 2 weeks post nerve injury. Systemic action of local anesthetics, at 2 days reduced Fos expression only in laminae 1,2 at 2 weeks, reduced Fos-expression throughout the gray matter of the spinal cord.

These results demonstrate that compared to tissue injury, peripheral nerve injury induces a prolonged hyperactivity in regions of the spinal cord that are associated with the transmission of nociceptive messages. The increased activity in the spinal cord is mianly maintained by the abnormal activity present in the injured peripheral nerve. A nerve injury induced central sensitization may, however, also contribute to the hyperactivity observed in the superficial dorsal horn particularly in the early stage of nerve injury. Such persistent central activation may be important in maintaining the chronic pain seen after nerve injury.

Introduction

Peripheral nerve injury results in long term changes in the injured afferent fibers (Review see Janig, 1988). For example, Wall and colleagues (1974, 1978) and Scadding(1981) reported that sprouting fibers in the neuroma that forms at the end of a severed nerve become spontaneously active, are very sensitive to mechanical stimulation and can fire after local application of adrenergic drugs . Since peripheral nerve injury in humans may be accompanied by spontaneous pain which can sometimes be relieved by sympatholytic therapy (Jensen, et al, 1983, Kallio, 1950, Ochoa, et al,1985; Betcher, 1953; Richards, 1967; Loh and Natham, 1978; Bonica, 1979,1990; Nathan,1947,1983), it has been proposed that sympathetically- maintained neuropathic pain may be based, in part, on the abnormalities similar to those described in the neuroma model. In fact, local anesthetic blockade of an injured peripheral nerve, which blocks the hyperactivity and hypersensitivity in the sprouting fibers of the neuroma, can eliminate the spontaneous pain associated with peripheral nerve injury in humans (Noordenbos, 1959; Sunderland, 1968; White and Sweet, 1969; Blumberg and Janig, 1981).

Based on these observations, it has generally been considered that the etiology of pain secondary to peripheral nerve injury lies primarily in the periphery. There are, however, also many central changes in the spinal cord occur following injured peripheral

axons (review see Wall,1984, Devor, 1988). The changes include increased onging discharge which may result from a decline of postsynaptic inhibition and primary afferent depolarization associated with presynaptic inhibition (Wall and Devor, 1981; Woolf and Wall, 1982); receptive field expansion of some dorsal horn neurons (Devor and Wall,1981; Lisney, 83; McMahon and Wall, 1983; Markus, et al, 1984), altered central conections by unmasking of latent synapse (Devor and Wall,1981,Seltzer and Devor, 1984), change in chemical componet, i.e. neuropetides, (Barbet, at al; 1980; Jessel, etal; 1979); a decrease in opiate receptors (Field, et al, 1980); C-terminal atrophy (Knyihar and Csillik, 1976), transganglionic degeneration (Grant and Arvidsson,1975), sprouting and synaptic reorganization (Molander, et al, 1988; LaMontte and Kapadia,1989), increased sensitivity i.e.increased response to previously effective stimuli and the appearce of response to previously ineffective types of stimuli (Woolf, 1983, Woolf and McMahon, 1985). Such central changes might, of course, also contribute to the production of persistent neuropathic pain.

Although single neuron studies have characterized some of the responses of spinal cord dorsal horn cells after peripheral injury, the overall distribution of spinal cord neurons whose activity changes with time is not known. Through the technique of immunocytochemically labelling neurons which express the protein product of the c-fos proto-oncogene it is, however, now possible to monitor the activity of large populations of neurons activated by various stimulation (Morgan, 1987; Hunt, 1987; Sagar, 1988; Menetray, 1989; Sharp; 1989). Thus, acute noxious stimulation (hindpaw formalin injection) evokes a short term expression of Fos protein in regions of the spinal cord that have been implicated in the processing of nociceptive information (Preseley, 1990, Gogas, 1991; Chi, 1991). Since infraorbital nerve section was reported to result in a more prolonged expression of Fos (2 weeks) (Sharp, 1989), it was of interest to determine whether this approach could be used to monitor the prolonged changes that develop in the presence of a neuroma. In this study, therefore, we have examined the expression of Fos-

like immunoreactivity (FLI) in the spinal cord for up to four weeks following peripheral nerve injury. We also evaluated the contribution of ongoing activity in the injured afferents to the persistence of the Fos expression.

Materials and Methods

Neuroma surgery

Male Sprague-Dawley rats (260-320 g, 62-82 days old, Bantin and Kingman, Fremont CA) were anesthetized with Nembutal (50 mg/kg) and the left sciatic nerve exposed in the mid-thigh. The sciatic nerve was then cut and after 5-6 min after nerve cut, the proximal stump tightly ligated with a 6-0 silk suture. After the nerve was ligated, the overlying muscle and skin were sutured and the animals allowed to recover. In a separate, sham-operated group of rats, the sciatic nerve was exposed but neither cut nor ligated.

Immunocytochemistry

At various times after surgery the rats were reanesthetized and perfused for immunocytochemistry with the following solutions: 100 ml of 0.1M phosphate buffer followed by 260-320 ml of phosphate-buffered 4% paraformaldehyde solution. The lumbar spinal cord was removed, post-fixed for 6-8 hrs in the same fixative and then cryoprotected overnight in 30% phosphate-buffered sucrose. Fifty micron frozen sections through the lumbar spinal cord were collected and processed by the avidin-biotin (ABC) method for immunocytochemical localization of the Fos protein (Hsu, et al, 1981). We used a rabbit polyclonal antiserum directed against an in vitro translated c-fos gene (kindly provided by Dr. Dennis Slamon, UCLA). Details of the antiserum have been described previously (Menetrey et al, 1989).

Cell counts

Since the sciatic nerve projects heavily to the L_5 segment we concentrated our analysis at this level of the cord. To quantitate the pattern of cells that expressed FLI, an outline of the L_5 segment was first drawn with a camera lucida attachment under dark field illumination. Sections of L_5 spinal cord were then examined with bright field illumination and the distribution of FLI-positive cells plotted onto the drawing, again by a camera lucida.

To quantify changes in different laminae of the spinal cord, the drawings were divided into three regions based on functional differences: 1) the superficial dorsal horn, laminae I and II, which predominantly contains nociresponsive cells (Fitzgerald, 1981), the nucleus proprius, laminae III and IV, which predominantly contains cells selectively responsive to non-noxious stimuli (Wall, 1967; Pomeranz et al, 1968; Price and Browe, 1973; Menetrey et al, 1977); and 3) and the deeper laminae, V, VI and VII which also contain nociresponsive cells (Price and Browe, 1973; Menetrey et al, 1977; Molinari, 1982; Meyers and Snow, 1982b) , many of which have been implicated in less localizable pain conditions.

Only those cells with nuclear staining which was easily identified from background at 10X were included in the drawing. Labeled cells from three histological sections in each rat were counted and averaged. The counts were made by an investigator who was blind to the particular treatment that the animals received. Statistical comparisons between groups were done by one-way analysis of variance (ANOVA) and post hoc comparisons were done by Scheffe F-test and Fisher PLSD test.

Nerve blocks

At two days and two weeks after sciatic nerve section the rats were randomly assigned into three groups: untreated controls, controls for systemic action of local anesthetics and sciatic nerve block experimental group. Under halothane anesthesia, the systemic control rats received bupivacaine (0.5% without vasoconstrictor, 2.0-3 0 mg/kg) whcih injected subcutaneously to the back of the neck. The experimental groups received

an equivalent volume of bupivicaine which was applied to the sciatic nerve at the level of the neck of the femur, by percutaneous injection. Immediately after recovery from halothane anesthesia (2.5 %, 4 min) motor block (presence of foot drop and loss of placing reflex) could be detected. Sensory block (i.e., failure to respond to needle stick applied to the footpad) appeared 10-20 min after the onset of the motor block. The injection produced a motor block lasting 102-120 min (range, n=5) and a sensory block lasting 88±2 min (mean±SEM, n=3) in unoperated normal rats which was consistent with previous finding, 90 min in rat (Akerman, et al, 1988). Neither sensory nor motor block was detectable when the same dose of local anesthetic was injected subcutaneously in the neck.

Inspection of the sciatic nerve 2 days after section revealed that about 1 cm of nerve proximal to the cut end was swollen. This portion of nerve could not be blocked by direct application of even large amounts of local anesthetics (0.2 - 0.5 ml 0.5% bupivicaine or 2% lidocaine); that is, animals still responded to probing of the neuroma, under pentobarbital anesthesia. In fact, only 3 of 17 rats with 2 day neuromas were successfully blocked by applying the local anesthetics more proximally. Although results from all rats are described below, only the 3 rats with complete blocks were included in the statistical analysis. Complete block was more easily achieved in the 2 week neuroma. The duration of the block of the 2 week neuroma was, however, shorter than in the 2 day neuroma (60 min, n=4, compared to 90 min at 2 day, n=3). Since the half-life of the FLI is about 2 hours (Muller, et al, 1984; Chi, et al., 1991), in order to detect a local anesthetic-induced decrease in Fos-expression it was necessary to block the nerve for at least 4 hours. The bupivicaine injections were, therefore, repeated three times, over a period of four hours in the 2 day neuroma. To prolong the block in the 2 week neuromas we used 4 injections spaced over 6 hours. Although caution was taken not to touch the neuroma area during application of local anesthetic, it was possible that the manipulation during the injection procedure might evoke expression of FLI. Rats that received saline injection by an identical technique,

however, showed no significant increase in FLI compared to those which did not receive injections (data not shown).

At the end of the 4 or 6 h period of local anesthetic block, the rats were anesthetized with Nembutal (60 mg/kg) and perfused for immunocytochemistry. To test the effectiveness of the blocks, the sciatic nerve was rapidly exposed, within 5 min just prior to perfusion. Complete block was indicated in all cases by failure of the rat to respond to pinching the neuroma and the adjacent proximal segment of the nerve (i.e., withdrawal of the leg,abdominal contraction or increase in respiration). The same stimulus applied to the contralateral nerve always produced these responses. Also, all rats in the local anesthesia control group (n=7) responded to pinching the neuroma.

Results

Sham-operated -induced FLI

As reported previously (Presely, et al, 1990), in normal rats FLI is only observed in a few cells in laminae III and IV of the lumbard cord bilaterally. Sham-operated rats had a transient increase in FLI in the neurons of lateral half of the dorsal horn mostly in rostral lumbar cord (L1-3), which corresponds to the termination site of the afferent projections from the incision area in the thigh (Figure). A few cells were at L_5 segment.

Nerve Lesion-induced FLI

The pattern of FLI after acute nerve section was consistent with the known topographic projection of the sciatic nerve. Thus, within 2 hours after nerve section, the greatest density of cells was in the medial two-thirds of the superficial laminae of the dorsal horn in L4-L6 segments (Figure). Considerable numbers of labelled cells were also recorded in deeper laminae (V,VI and VII) (see below). In some rats, Fos expressed in lamina IX and motor neurons.

In both group, labelled cells were found at all lumbar levels and the expression pattern was varied with different lumbar level. There was also considerable labelling in contrlateral side at all lumbar level, but with an ipslateral predominance. Although the cells were most likely neuronal, it is possible that some were glial.

Temporal Changes in FLI

Superficial laminae (I & II)

As mentioned above, in the sham-operated control group, few FLI cells were found at L_5 and little increase was seen over the one month study period (Figure). In contrast in the neuroma group, we found a persistently elevated FLI in superficial laminae and the increased FLI in laminae I and II fluctuated over the one month study period. Specifically, there were peaks of expression recorded at 2 h, 12 h, 2 d and 2 week following nerve section (Figure). The spatial distribution of labelled cells in the superficial laminae also changed over time. By 3 days, the dense staining in the medial 1/3 of superficial laminae had disappeared. Instead, there was usually a dense cluster of cells in the central portion of laminae I and II, particularly at caudal L_5 (Figure). This pattern persisted at 1 month.

Laminae III & IV

The temporal pattern of Fos-expression in laminae III and IV was similar in the sham-operated and neuroma groups. Thus the expression was relatively low within 12 hours of cutting the nerve. The number of cells, however, increased at 2 days and reached high expression at 3 days. This level persisted throughout the one month study period (Figure).

Laminae V, VI & VII

In contrast to the variation in expression observed in superficial dorsal horn, we found there was a stable and persistent increased number of FLI neurons in the deep laminae after nerve section (details in Figure). The number of Fos-positive cells in these laminae in the sham-operated group of rats remained low throughout the one month period.

Effects of Local Anesthetic on Nerve Lesion-induced FLI

In the three rats in which complete nerve block of the 2 day neuroma was possible, FLI was reduced in both superficial and deep laminae, by >80%, without a significant reduction in the expression in laminae III and IV. In rats with incomplete block (i.e., rats that demonstrated a weak response to pinch of the sciatic nerve (n=11), there was still a significant inhibition of the expression, approximately 70%, but only in the superficial laminae ($p<0.001$, n=11). In the 2 week neuroma, nerve block significantly decreased expression in all laminae. In the deep laminae, the reduction of FLI was up to 90% (Figure).

In the 2 day neuroma (Figure), injection of bupivicaine subcutaneously in the neck, to control for a systemic action of the local anesthetic also significantly reduced Fos expression, but only in the superficial laminae (50%). Note that this injection did not alter the reflex responses to pinching the neuroma. The systemic action of bupivicaine also reduced Fos-expression in the 2 week neuroma (Figure). The reduction of FLI was comparable (50%) but was found in all laminae. The systemic action of bupivicaine was not different for the 4 h (n=3) and 6 h (n=4) blocks at 2 weeks[data not shown].

Discussion

Using Fos-expression as a marker for neuronal activity, we have mapped the pattern of central neuronal activity in specific groups of spinal neurons for a four week period following peripheral nerve injury. Compare to tissue injury (sham-operated controls), nerve injury induced a prolonged increased Fos expression in lamina 1,2 5,6 and 7 for at least one month. The degree of increased Fos expression in these regions has been shown to be positively related to the pain behavior induced by formalin stimulation (Gogas, et al, 1991, Chi, et al, 1991). On the contrary to the low level of Fos expression in the

sham-operated animals, the nerve lesion-evoked Fos expression is above the level which would show the pain behavior in the formalin study suggesting that the Fos expressed neurons following nerve injury may be related to pain. Since the half-life of the FLI in the spinal cord is approximately 2 hours (Chi et al., 1991), a persistence of Fos-expression following nerve injury suggests that there is either a continuing peripheral input or that a central change contributes to the Fos expression.

Govrin-Lippman and Devor (1978) reported that the ongoing activity in A-beta and A-delta fibers in a neuroma in the rat peaks at about 2 weeks post-injury and then decreases. Only about 5% of fibres are active at one month. This fluctuation in peripheral activity may account for some of the increase in Fos-expression which we observed over the first two weeks. By contrast, Scadding (1981) demonstrated evoked activity (mechanical and adrenaline sensitivity) from all types of fibers in mouse neuroma within 24 hours of nerve section. He also recorded spontaneous activity that fluctuated over a period of 4 weeks. The oscillatory ongoing and evoked activity observed in that study is similar to the temporal pattern of Fos-expression which we observed following nerve injury. Of note, however, we only recorded this oscillatory pattern of FLI in the superficial laminae; the expression in the deep laminae was less changed. Since A-beta fibers terminate densely in deeper laminae (V,VI,VII), but not in laminae I or II, A-beta fibers in the neuroma may be an important source of the inputs that drive and maintain the non-oscillatory Fos-expression in the deeper laminae.

The local anesthetic studies indicate that central changes that are produced by peripheral nerve injury are unlikely to be the major contributor to the persistence of Fos expression. In particular, local sciatic nerve blockade profoundly decreased FLI, both in the superficial and deep laminae of the spinal cord, suggesting that the abnormal activity generated in the injured nerve end is the main stimulus for the induction of FLI in the spinal cord. This increased abnormal activity could be from sensory sprouts as well as from sympathetic efferent sprouts which has shown to activate primary afferent fibers

particularly in damaged nerve membrane (Devor and Janig, 1981, Roberts and Elardo,1985). That peripheral nerve block reversed the observed hyperactivity (i.e., Fos expression) to basal levels is consistent with the clinical observation that local anesthetic block rapidly eliminates neuropathic pain (Noordenbos, 1959; Sunderland, 1968; White and Sweet, 1969; Campbell, 1988;).

Systemic action of local anesthetics, however, also produced a decrease, to about half, in the numbers of FLI neurons. At 2 days after nerve injury the decrease was only found in the superficial laminae. At 14 days, however, the expression in all laminae was decreased by the systemic action of local anesthetics. One possible explanation for this difference is that the neural mechanisms underlying the spinal cord changes produced 2 weeks nerve injury are particularly sensitive to local anesthetic. This hypothesis is, in fact, consistent with the studies of Woolf and McMahon (1985) which showed that a systemic action of local anesthetics is sufficient to suppress C-fiber evoked activity in the spinal cord after chronic injury. However, it is also possiblr that the low concentration of systemic local anesthetic may not have effect on the neuroma sprouts which require much higher concentration (Chabal et al, 1989) it may have effect on the central portion of nerve (Bridenbaugh and Greene, 1988). Therefore, the systemic effect of local anesthetics to reduce the Fos-expression may be mediated by reducing activity in sensitized spinal neurons as well as in the central terminals of the injured nerve. The remaining of low expression of Fos after nerve block may have been maintained by the increased activity in the dorsal root ganglion (Wall and Devor, 1983, Burchiel, 1984).

It is interesting to note that a group of dense labeled neurons were found in the bondery between sciatic and femeral nerve territory that is in central portion of superficial laminae. Local sciatic nerve block only partially reduced Fos expression in this region suggesting that inputs from peripherl as well as central sources contribute to the fos induciton. Following complete peripheral nerve section, mechanical hyperalgesia and allodynia develops between denervated area and the border of surrounding normal

sensation in man (Sunderland and Kelly; 1948; Seddon, 1972; Inbal, et al; 1987) and in animal (Trotter and Devies,1909; Denny-Brown, 1965; Kingery and Vallin, 1989). The hyperactivity in the central border may relate to hyperalgesia developed in its peripheral receptive field of medial paw. Since it was found not correlate well with the degree of peripheral collateral sprouting of femoral nerve to the adjacent denervated sciatic zone, a mechanism of central disinhibition was suggested (Kinger and Vallin, 1989). However, it is also possible that the peripheral activity from the sproutings of femoral nerve may participate to the central reorganization.

In conclusion, we have demonstrated that a high level of c-fos expression persists in the spinal neurons in the neuroma model. These results are consistent with studies demonstrating that nerve injury results in prolonged hyperactivity in both the injured axons and in spinal neurons. The increase in spinal neural activity after injury may be mainly due to the increased activity in sprouting afferents trapped in the neuroma and/or to a widespread increase in sensitivity of spinal neurons,which lowers their firing threshold, so that they now respond to previously ineffective inputs . With this dynamic map of activated neurons in the spinal cord following nerve injury, we may study more presisely about what spinal neurons activated at speific time and what factors cause the activation. In the accompanying paper we specifically evaluate the contribution of the massive injury discharge generated at the time of the nerve section itself, to the temporal pattern of c-fos expression in this model.

Figure legend

Fig. 1 The time dependent of laminar distribution of Fos positive cells at L5 level after sciatic nerve sectilion.(A).2-hour (B).1-day, (C).2-day, (D) 2-week. Fos expression was throughout laminae I to VII. Note that after 2 days of nerve section, the distribution of labelled cells shiefted from the medial half to lateral half of the superficial dorsal horn and with a group of dense staining cells locate in the central portion of superficial laminae 1 and 2 (arrows).sg, substantia gelatinosa. Scale bar, 200um.

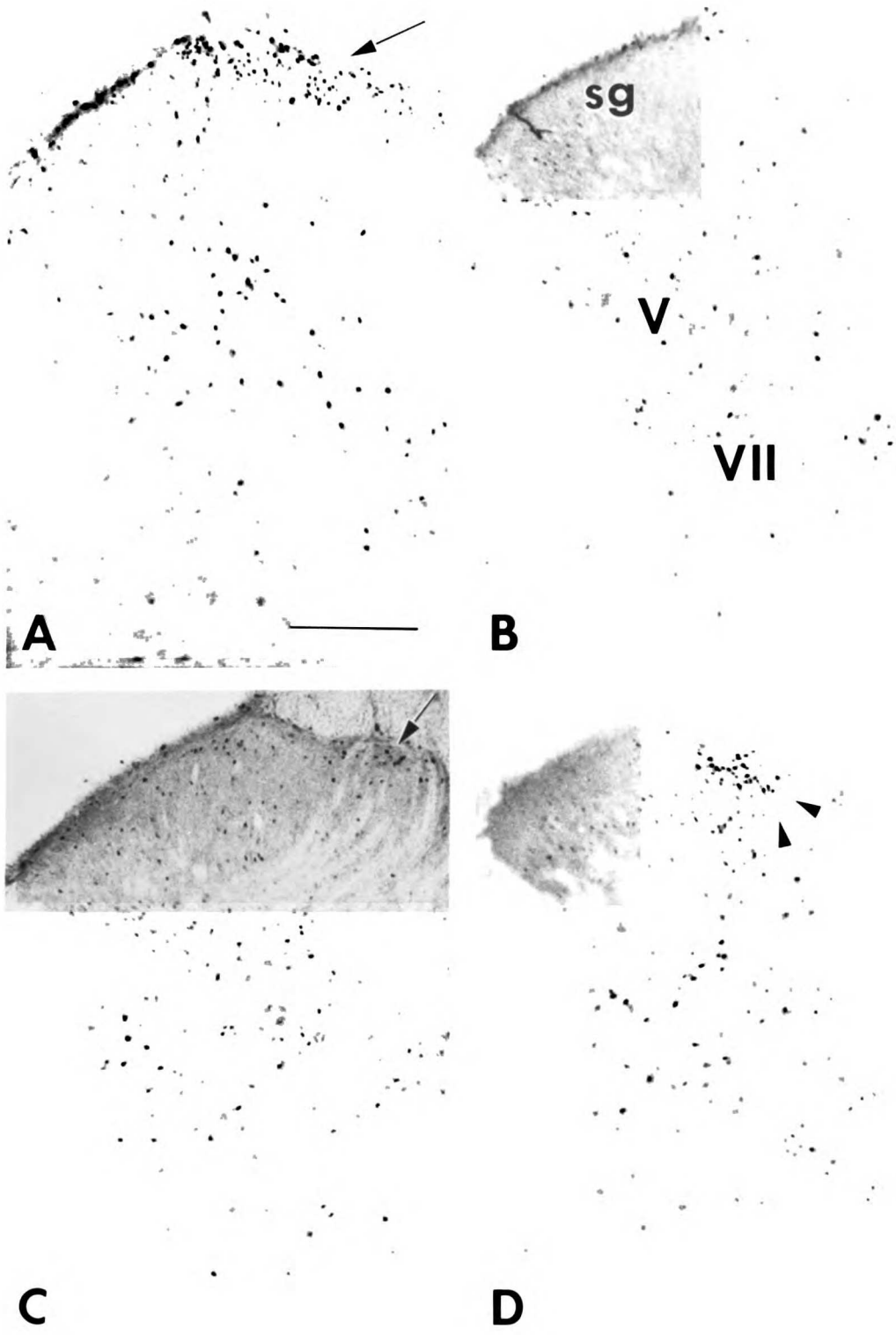

sg
V
VII
A
B
C
D

Fig. 2. The higher magnification of Fos expression in the superficial dorsal horn from Fig. 1 (A). 2 hours. (B) 2 weeks. DC, dorsal column; sg, substantia gelatinosa. Scale bar, 100um.

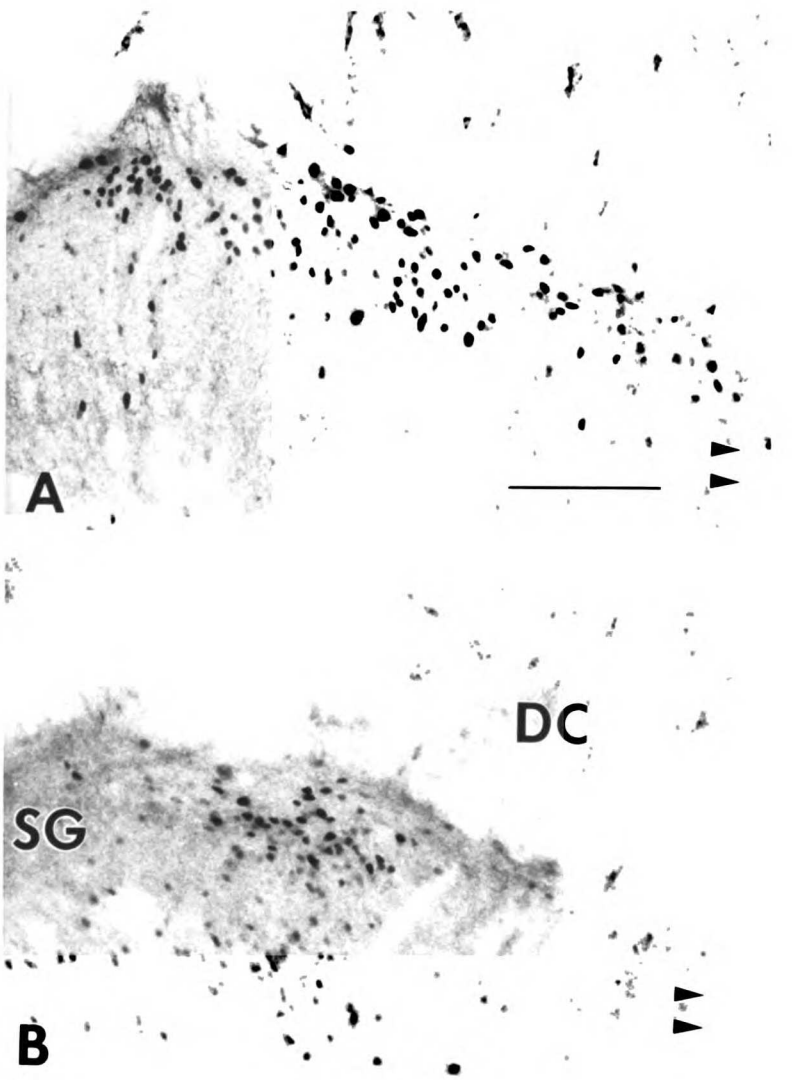

A
DC
SG
B

Fig. 3. The 2-hour pattern of Fos expression at lumbar spinal cord level. (A) Control: Acute incision injury in the mid thigh (Sham-operated) induces a transient increased expression mostly in L1-L3 and a few Fos positive cells at L4-5. (B) Neuroma: Acute nerve section and ligation induces a high expression of Fos mostly in L4-6. (*=lesion side)

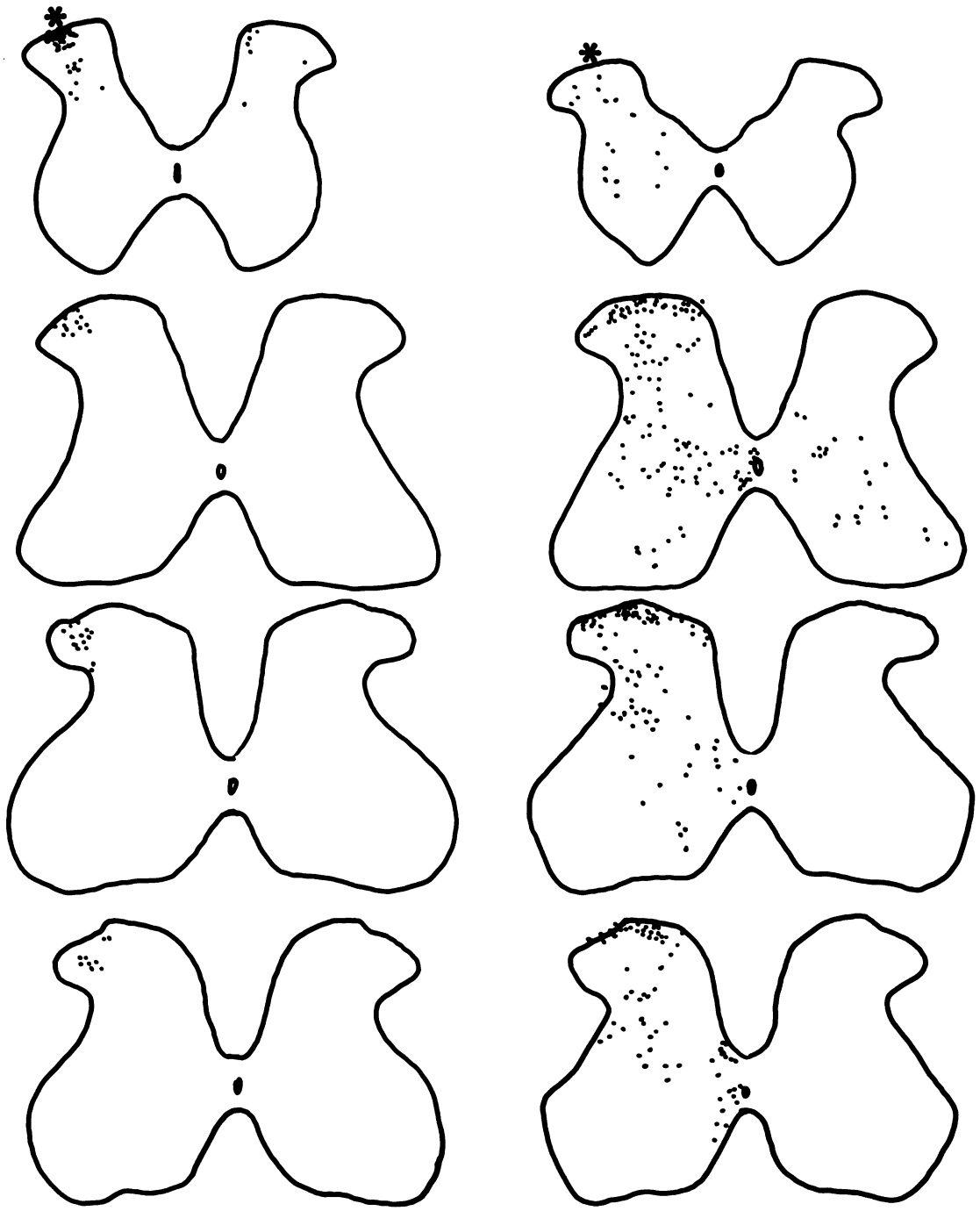

Control
2 hr
Neuroma

Fig. 4. The 2-week pattern of chronic Fos expression. (A) In sham-operated control group, the expression is mostly in the laminar 3. (B) In neuroma group, Fos expression was throughout laminae I to VII.

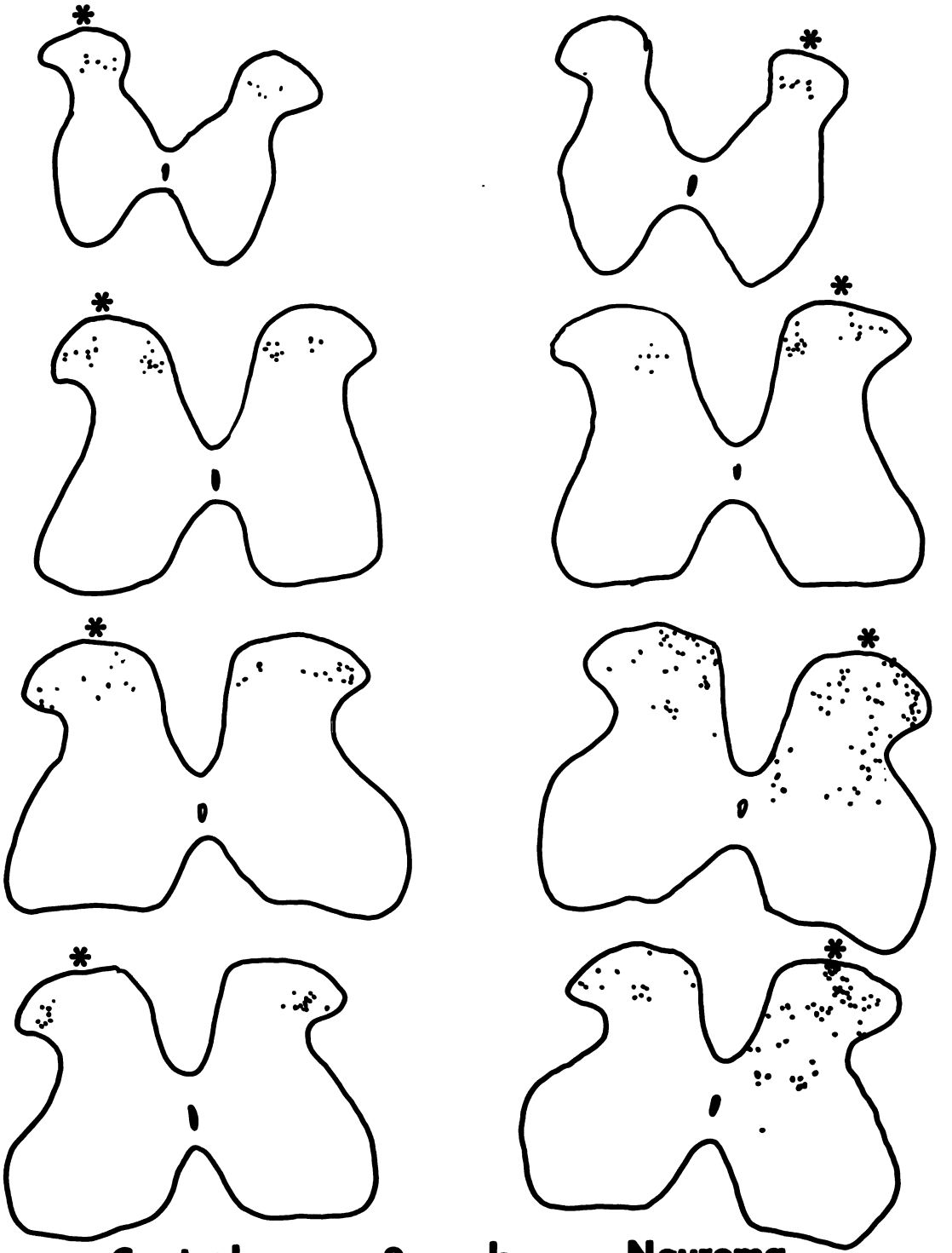
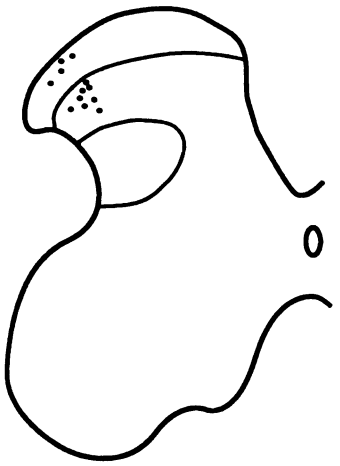
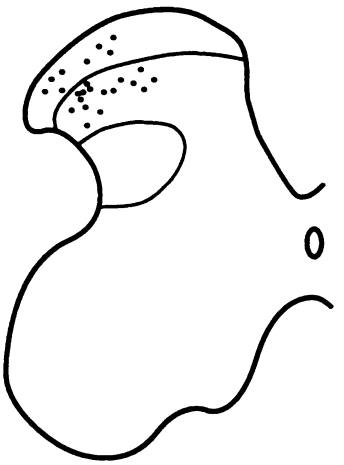

Control
2 week
Neuroma

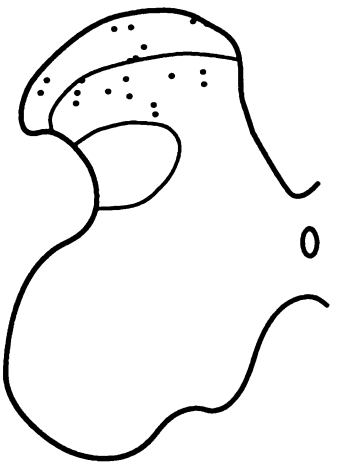
Fig. 5. Temporal pattern of Fos expression in sham-operated rats at L5 level by camera lucida drawings. Most of Fos expression is in laminae 3 and 4. After 2 weeks, most Fos expression in laminae 1 and 2 locates medially.



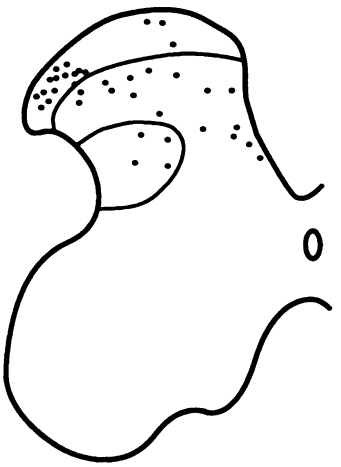
2 HOUR



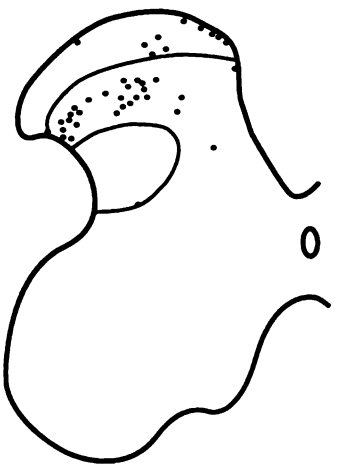
1 DAY



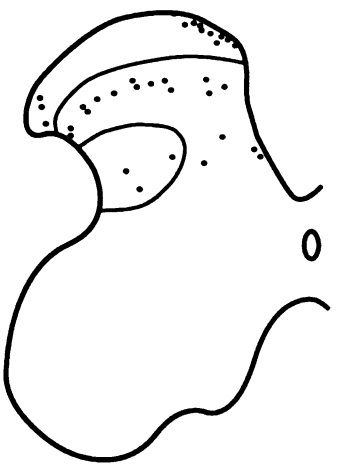
2 DAY



1 WEEK

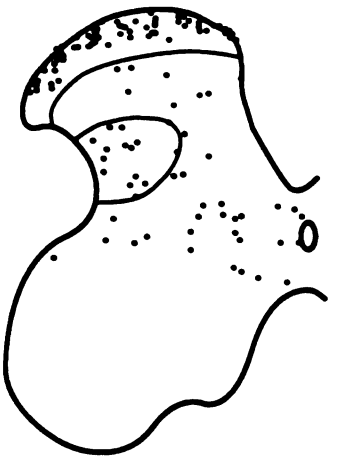


2 WEEK

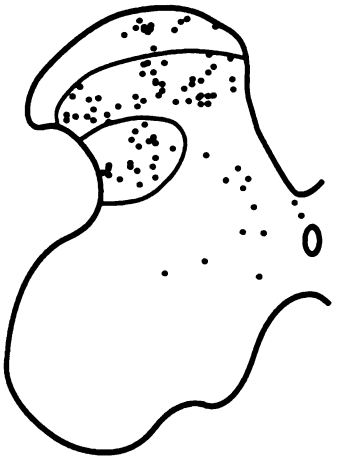


4 WEEK

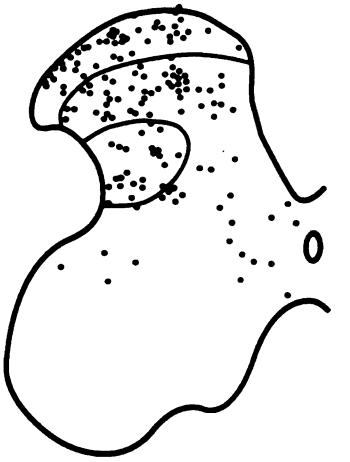
Fig. 6. Temporal pattern of Fos expression at L5 level after nerve injury by camera lucida drawings. The increased Fos expression was throughout lamina 1 to 7.



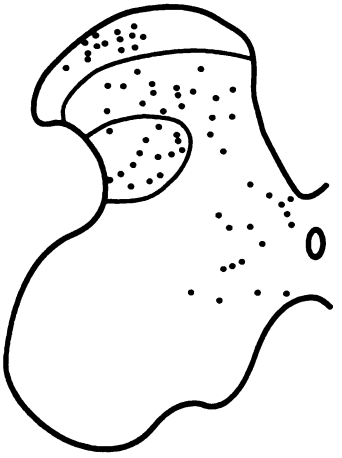
2 HOUR



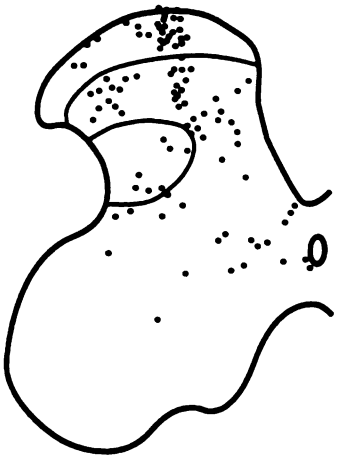
1 DAY



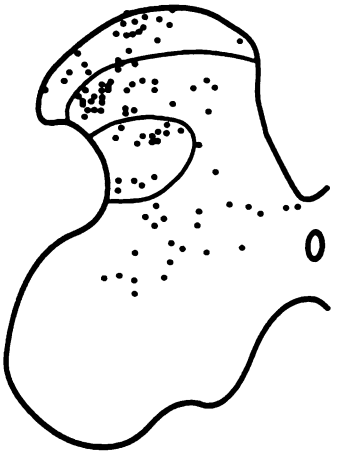
2 DAY



1 WEEK



2 WEEK



4 WEEK

Fig.7. Quantification of temporal and laminar dependent Fos expression at L5 segment level (mean ± S.E.M.). Compare to sham-operated group, neuroma group has significantly high expression in laminae 1,2,5,6 and 7 (ANOVA, $P<0.001$). In neurona group, Fos expression in superficial laminae 1 and 2. was oscillatory, with peaks at 2hr (67± 21 cells), 2 days (47±8 cells) and 2 weeks (30±4 cells).

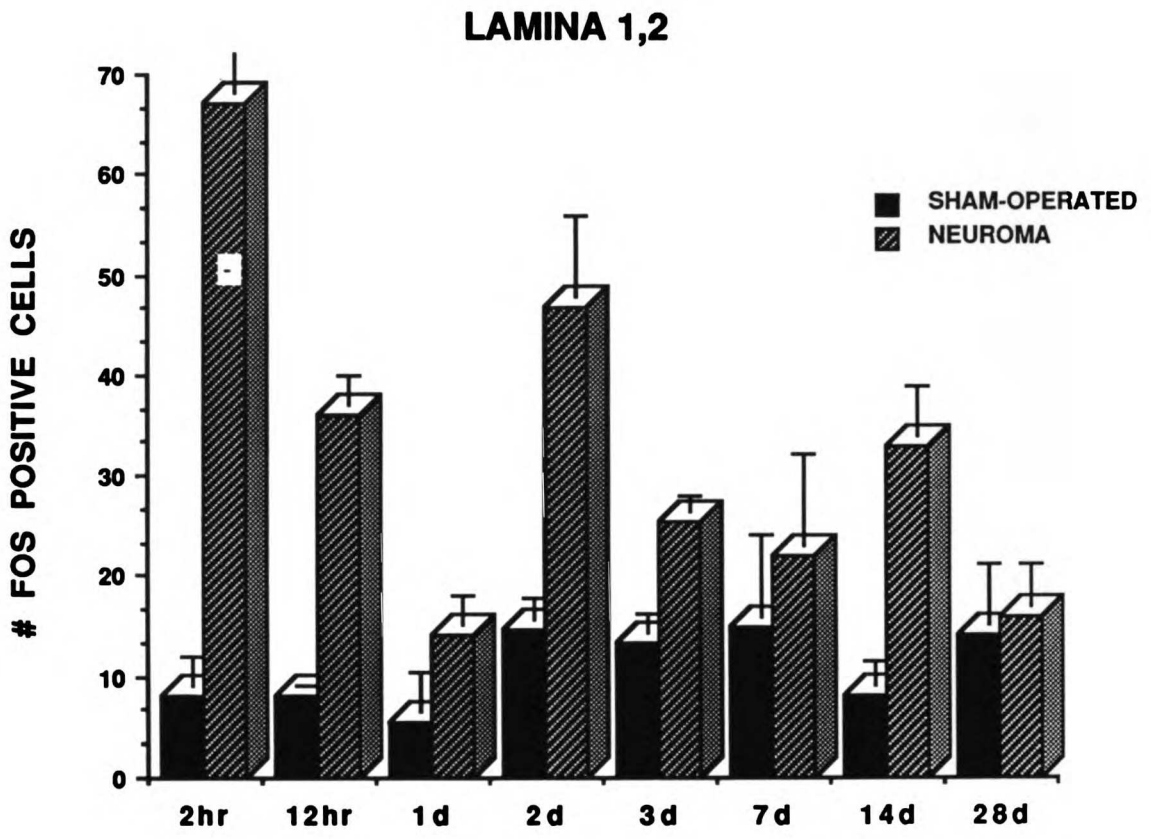

LAMINA 1,2
FOS POSITIVE CELLS
70
60
50
40
30
20
10
0
SHAM-OPERATED
NEUROMA
2hr
12hr
1d
2d
3d
7d
14d
28d

Fig.8 The temporal pattern of Fos expression in laminae 3 and 4 was not significant different between sham-operated and neuroma group (ANOVA, $p>0.1$). The expression started to increase after 2 hours and reached high expression at 3 days. This high level persisted with slowly declined in next 3 weeks.

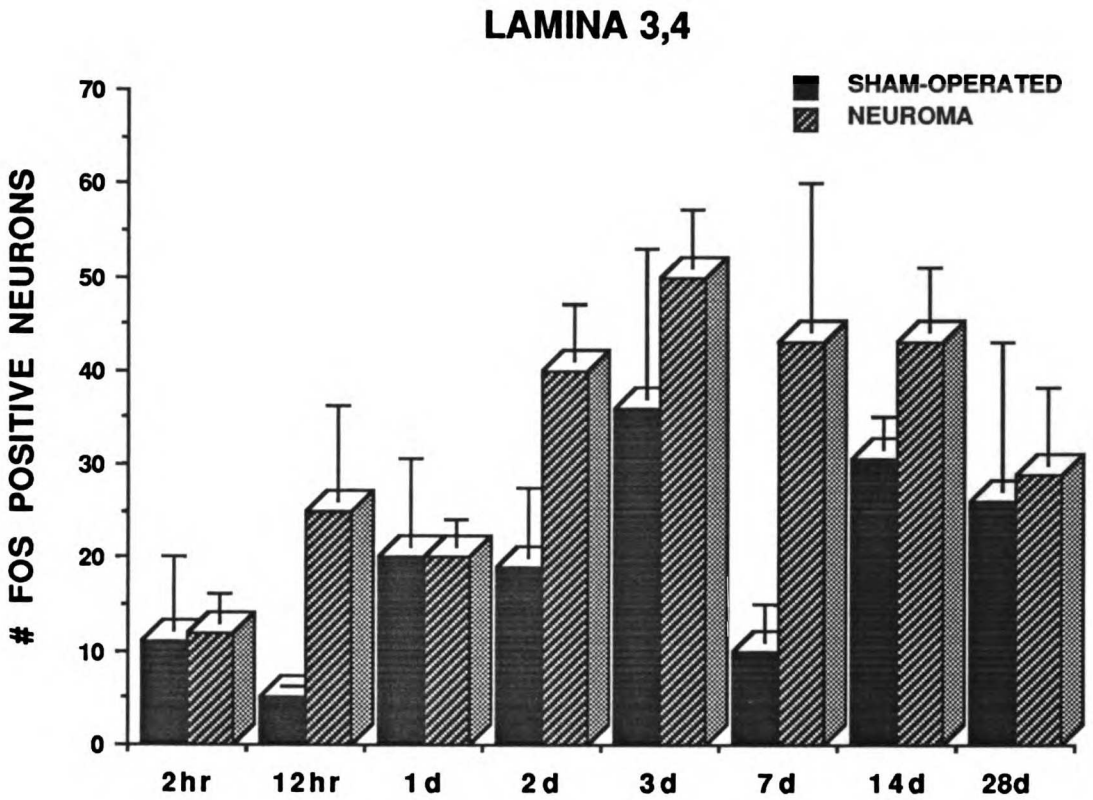

LAMINA 3,4
SHAM-OPERATED
NEUROMA
FOS POSITIVE NEURONS
70
60
50
40
30
20
10
0
2hr
12hr
1d
2d
3d
7d
14d
28d

Fig.9. In the neuroma group, a high expression of Fos in deep laminae 5,6, and 7 is maintained throughout one month period with small increase at 2 days (43±2 cells) and 2 weeks (46±8 cells). (neuroma; n=3, control: n=3, except 12hr, n=2).

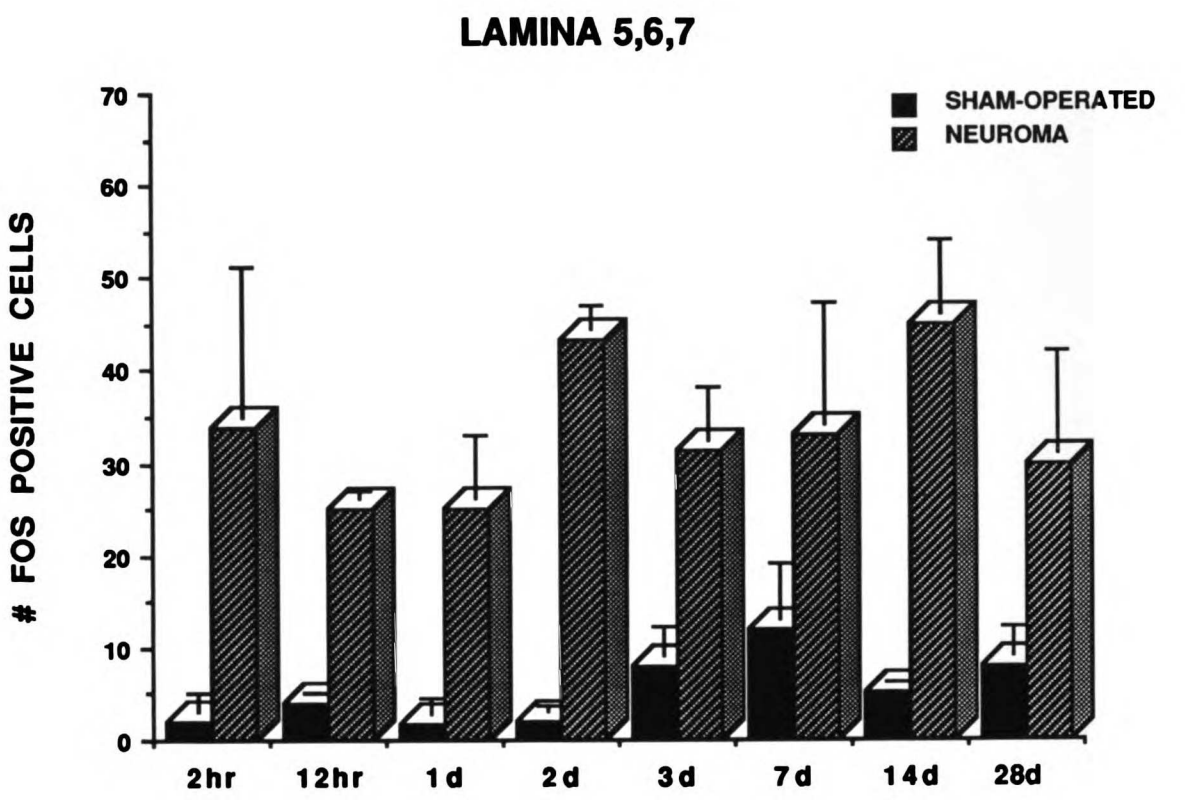

LAMINA 5,6,7
SHAM-OPERATED
NEUROMA
FOS POSITIVE CELLS
70
60
50
40
30
20
10
0
2hr
12hr
1d
2d
3d
7d
14d
28d

Fig. 12. The effect of local anesthetics on the Fos expression at L5 level in 2 weeks neuroma. (A) untreated, (B) systemic action of bupivicaine, (C) and (D) sciatic nerve block. Arrows indicate laminar 2, sg, substantia gelatinosa. Scale bar, 200 um.

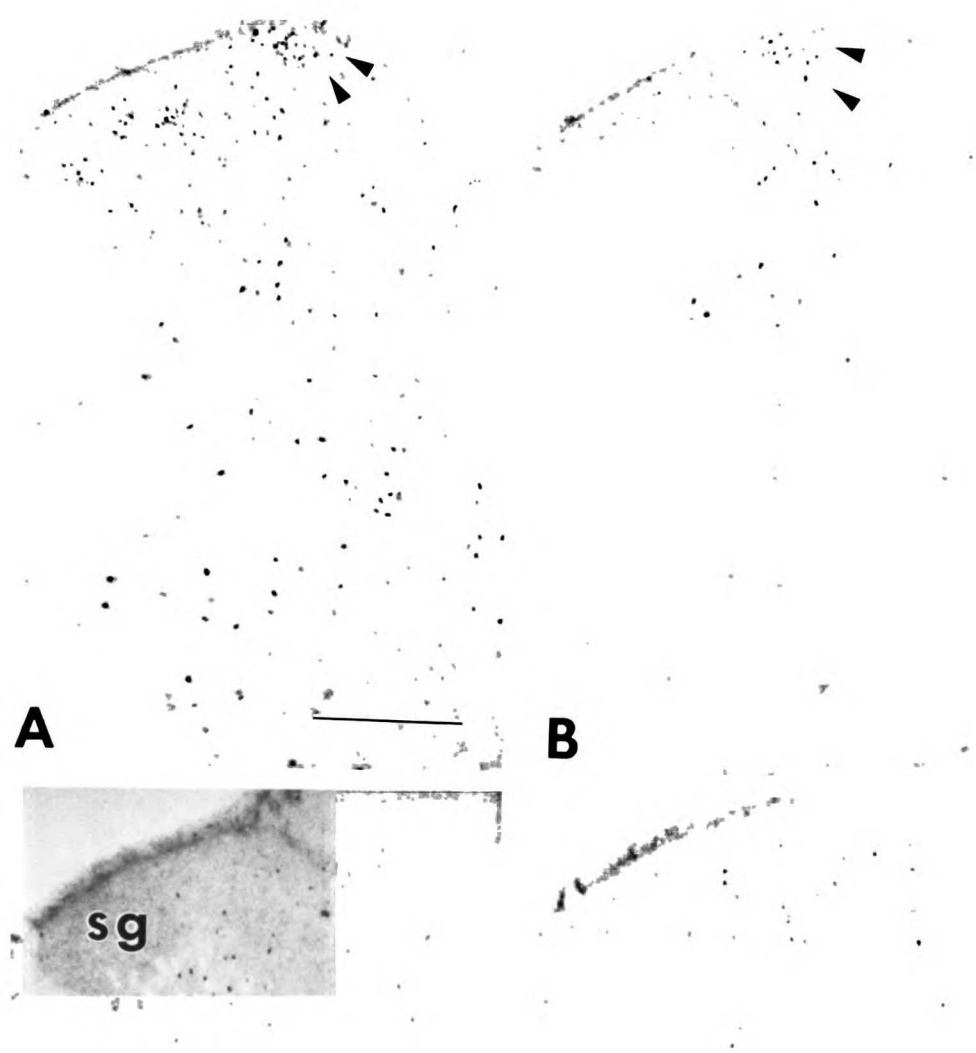

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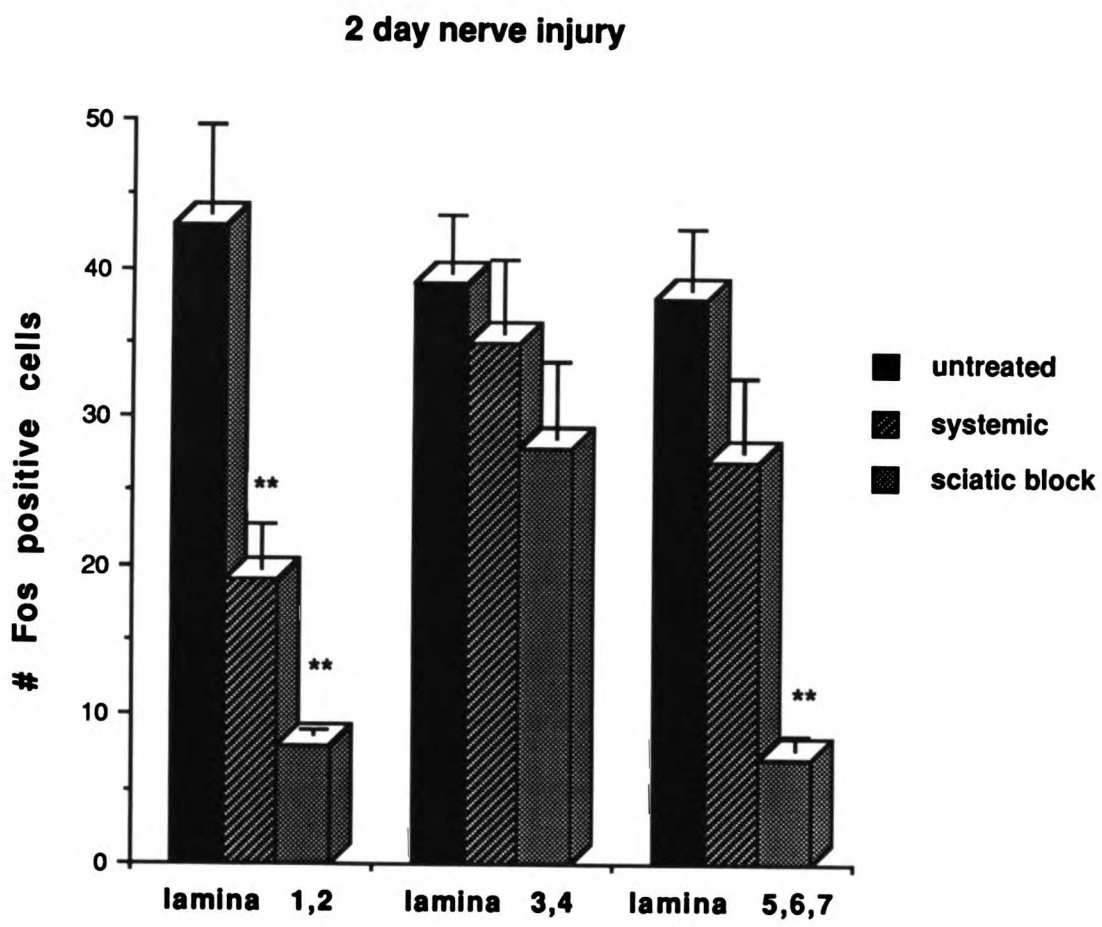
Fig.11. The quantification of effect of sciatic nerve block on Fos expression in 2 days neuroma (mean±S.E.M.). Compare to untreated control (n=6), complete nerve block (n=3) reduced Fos expression in superficial laminae and deeper laminae by about 82% ($p<0.05$) without significantly reducing Fos expression in lamina 3 and 4. The effect of systemic action of local anesthetics (n=7) was only on the superficial laminae. The Fos expression in this region was reduced to 46% ($p<0.05$).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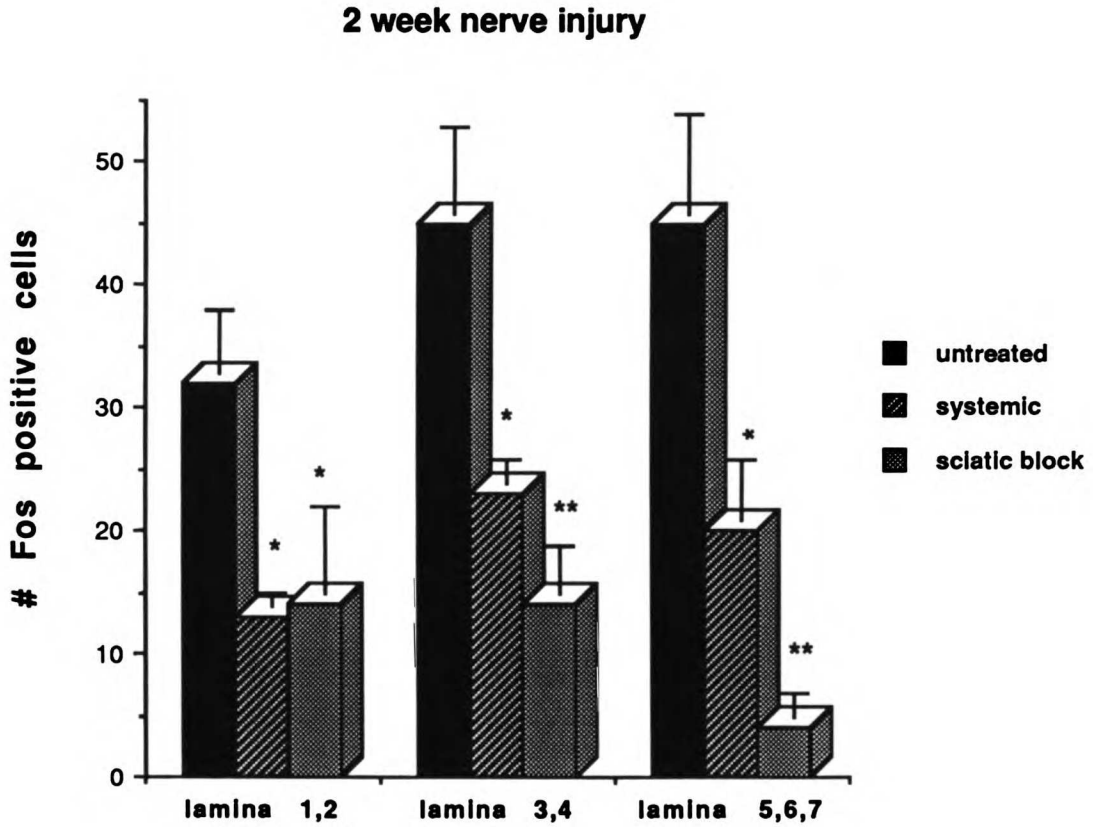
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CHAPTER II

Local Anesthetic Pre-Block Attenuates the Nerve Injury-Induced C-fos Expression in the Spinal Cord of the Rat

Abstract

In the accompanying report, we demonstrated that peripheral nerve injury (sciatic nerve section) induces a prolonged increased Fos expression, a protein product of the c-fos proto-oncogene, in the lumbar spinal cord of the rat. The temporal pattern of Fos-expression varied in different lamina of the cord. Importantly, the number of labelled cells could be reduced by local anesthetic blockade of the developing neuroma. In this paper we demonstrate that lidocaine anesthetic administrated, just prior to nerve section, which blocks the nerve injury discharge, significantly reduces the number of Fos-like immunoreactive neurons recorded at two days after nerve section. The effect of this anesthetic pre-block was most pronounced on neurons in the superficial dorsal horn. These results indicate that the initial brief injury discharge contributes to a prolonged increase in the activity of spinal cord neurons and provides support for the hypothesis that central sensitization, together with abnormal hyperactivity in the injured peripheral nerve, contributes to nerve injury-evoked pain.

Introduction

Cutting a peripheral nerve produces a very brief, vigorous barrage of impulses in the sectioned nerve (Waxman and Wall, 1973, Wall, et al, 1974). It has been suggested that this type of injury-induced activity in peripheral nerve triggers long-lasting changes in central nervous

system excitability (Hardy et al., 1950). In support of this hypothesis, experimental studies have shown that thermal injury (Woolf et al., 1983, 1984; Woolf & McMahon, 1985), inflammation (Wall et al., 1988) and nerve stimulation at intensities that activate C-fibers (Wall & Woolf, 1984, Woolf &wall, 1986, Cook, et al, 1987) facilitate nociceptive flexion reflexes. Since this prolonged facilitation of the flexion reflex that results after a C-afferent volley is not accounted for by increases in the excitability of primary afferent terminals, Cook and colleagues (1986) concluded that central changes are involved, at the level of spinal interneurons.

The importance of the nerve injury discharge has also been observed in clinical studies. For example, McQuay and colleagues (1988) demonstrated that preoperative local anaesthetic block decreases the requirement for postoperative pain medication. Other evidence suggests that phantom limb pain might be due, at least in part, to longterm consequences of the massive C-fiber discharge that occur in the peripheral nerve at the time of the amputation. In fact, Bach and colleagues (1988) demonstrated that preoperative lumbar epidural blockade significantly reduced the incidence of phantom limb pain in the first year after amputation.

We recently demonstrated that peripheral nerve lesion in the rat induces a chronic elevation in the expression of fos protein-like immunoreactivity (FLI) in neurons in those laminae of the spinal cord associated with transmission of nociceptive information (Chi et al., 1989, 1991). In the present study we evaluated the contribution of the nerve injury discharge to the development of nerve-lesion induced FLI, by pre-transection application of local anaesthetic, proximal to the site of section.

Materials and Methods

Male Sprague-Dawley rats (280-320 g, 67-75 days) were anesthetized with Nembutal (50 mg/kg, i.p.) and the left sciatic nerve exposed at the mid-thigh. To anesthetize the nerve prior to transection, we applied 0.05 - 0.1 ml of a 0.2% lidocaine solution directly onto the nerve; another 0.1 ml was injected percutaneously so that a proximal segment of the sciatic

nerve at the level of the iliac crest would also be blocked. Five minutes after the local anesthetic injection, the sciatic nerve was cut about 5mm distal to the block. Another 0.05 ml dose of lidocaine was then applied to the proximal end of the cut nerve and five minutes later the nerve was ligated with a 6-0 silk suture. Animals that received local anesthetic were included in the study only if the nerve section and ligation did not produce nocifensor responses, i.e. no withdrawal reflex in the extremity, no abdominal contractions and no increase in respiration.The control group received the same volum of saline and under the same procedure as experimental group. To test whether the nerve block also has systemic effect, via systemic absorption, we injected another group of rats with the same doses of lidocaine, subcutaneously, in the dorsum of the neck. This route of administration of local anesthetic did not block nocifensorresponses.

After the severed nerve was ligated, the overlying muscle and skin were sutured and the animals returned to their cages. Our previous study demonstrated that FLI was most pronounced two days after nerve section, in the L_5 spinal cord segment (Chi, 1989). Therefore, immunocytochemistry of the L_5 segment was performed at 2 days. Rats were anesthetized and perfused with 100 ml of 0.1 M phosphate buffer followed by 300 ml of phosphate buffered 4% paraformaldehyde fixative. The lumbar spinal cord was removed, post-fixed for 6-8 hours in the same solution and then cryoprotected in 30% phosphate-buffered sucrose for 18-24 hours. Fifty micron thick frozen sections through the lumbar spinal cord were collected and processed by the avidin-biotin method (Hsu, et al, 1981) for immunocytochemical localization of FLI, using a rabbit polyclonal antiserum directed against an in vitro translated c-fos gene (kindly provided by Dr. Dennis Slamon of UCLA). Details of the antiserum have been described elsewhere (Menetrey, 1989).

Cell counts

Since the sciatic nerve projects heavily to the L_5 segment we concentrated our analysis at this level of the cord. To quantitate the pattern of cells that expressed FLI, an outline of the L_5 segment was first drawn with a camera lucida attachment under dark field

illumination. Sections of L_5 spinal cord were then examined with bright field illumination and the distribution of FLI-positive cells plotted onto the drawing, again by a camera lucida.

To quantify changes in different laminae of the spinal cord, the drawings were divided into three regions based on functional differences: 1) the superficial dorsal horn, laminae I and II, which predominantly contains nociresponsive cells (Fitzgerald, 1981), the nucleus proprius, laminae III and IV, which predominantly contains cells selectively responsive to non-noxious stimuli (Wall, 1967; Pomeranz et al, 1968; Price and Browe, 1973; Menetrey et al, 1977); and 3) and the deeper laminae, V, VI and VII which also contain nociresponsive cells (Price and Browe, 1973; Menetrey et al, 1977; Molinari, 1982; Meyers and Snow, 1982b) , many of which have been implicated in less localizable pain conditions

Only those cells with nuclear staining which was easily identified from background at 10X were included in the drawing. Labeled cells from three histological sections in each rat were counted and averaged. The counts were made by an investigator who was blind to the particular treatment that the animals received. Statistical comparisons between groups were done by one-way analysis of variance (ANOVA) and post hoc comparisons were done by Scheffe F-test.

Results

The number of cells observed in the three regions examined was comparable, approximately 50 to 70 cells per 50 micron section . Local anesthetic, applied to the nerve 5 min prior to nerve section, significantly decreased (by >70%) the expression of Fos in the superficial laminae (I & II) at two days post nerve injury ($p<0.05$, $n=4$). In contrast, local anesthetic injection of the nerve did not significantly affect FLI in the nucleus proprius (III & IV) or in the deeper laminae (V, VI and VII) (both $p>0.3$ by ANOVA)(details in figure legend).

An inhibitory effect of lidocaine on Fos expression in the superficial laminae of the dorsal horn was also observed when the anesthetic was given "systemically". That is, subcutaneous injection of lidocaine at the back of the neck also significantly reduced Fos cells by 53% in the superficial laminae (I & II) ($p<0.03$, $n=4$) but not in the nucleus proprius (laminae III and IV) or in the deeper laminae (V-VII) of the spinal cord. Although the reduction was somewhat less than that in animals in which the lidocaine was administered to the nerve, the difference between local and remote lidocaine treatment was not significantly different.

Disscusion

Compare to tissue injury, peripheral nerve injury induced a long term increased Fos-like immunoreactivity (FLI) in spinal cord (Sharp, et al, 1989, Chi, et al, 1989). Two days after sciatic nerve section, Fos staining neurons associated with nerve injury were mainly found in superficial laminae1,2 and deep laminae 5,6,7. Since Fos expression is transient with half life 1-2 hours (Muller, et al, 1984), the persistent Fos expression indicates that there are continuous inputs to the spinal cord for the Fos induction. The possible sources may be from abnormal ongoing activity and evoked activity which were found in the spinal cord (Wall and Devor, 1981, Woolf and Wall, 1982, Woolf, 1983) and in the injured nerve following peripheral injury (Wall and Gutnick, 1974; Govrin-Lippmann and Devor,1978; Scadding, 1982).

In accompany paper, we have shown that local nerve block profoundly suppressed the nerve lesion induced Fos expression. Furthermore, the systemic action of the local anesthetics affects only in the superficial laminae but not in the other laminae in the early stage of nerve injury. The data suggest that in addition to the abnormal inputs from injured nerve, central factors may participate in maintaining the chronic Fos expression particularly in the superficial laminae. Here the results showing that the initial massive injury discharge

contributes to the chronic Fos expression in the superficial dorsal horn, but no effect on the other laminae which is consistent with our previous findings.

A major distinction between cells located superficially and those located deeper is that only the latter receive a significant monosynaptic input from large-diameter fibers (Brown, et al, 1977b, 1980a,1981; Scheibel and Scheibel, 1968). Thus, large-diameter fiber input from the neuroma may maintain FLI in the deeper laminae. In contrast, superficial laminae, the regions rich in various peptides (Hokfelt, etal, 1975, DeBiasi and Rustioni, 1988), receive a large amount of monosynaptic inputs from the peptide-rich small myelinated and unmyelinated afferents (Rethelyi, 77; Ralston and Ralston, 79; Light and Perl,79). The massive discharge produced by nerve section and ligation would initiate that release (White and Helme, 1985) which may mediate the effect of brief injury discharge on the long term Fos expression in these region.

The low concentration of systemic action of local anesthetics which was inject subcutaneously at a site remote to the nerve transection also blocked the Fos expression in the superficial dorsal horn. Although it could be argued that the dose of anesthetic necessary to block the afferent barrage is small enough that the low systemic concentration of local anesthetics could be effective, it did not markly effect the spinal nociceptive reflexes elicited by nerve transection. We favor the hypothesis that the effective blockade is in fact in the spinal cord. This hypothesis is consistent with the observation that uptake of lidocaine from a subcutaneous injection is sufficient to significantly suppress C-fiber activity in spinal cord neurons with chronic nerve injury (Woolf and McMahon, 1985). Our results implicate that there is rapid change in the spinal cord after peripheral nerve injury which makes the spinal cord as acceptible to the low concentration of local anesthetics as with chronic nerve injury. This immediate change of spinal cord is also found in the destructive transneuronal effect which occur within minute after nerve transection (Sugimoto, et al ; 1987).

Preblock the massive injury discharge inputs to the cord results in a decrease of activated neurons in the superficial dorsal horn, an area of spinal cord thought to process nociceptive information. The decrease of activity in pain-related region may account for that the less postoperative pain is found in man with preblock or premedication before surgery. The exact mechanism for why brief injury discharge triggers the long term change in activity in the superficial dorsal horn is still not clear. Two possible mechanisms may be involved that is sensitization and degeneration .

Excitatory amino acids such as glutamate is also found abundant in C-fibers and in superficial dorsal horn (DeBiasi and Rustioni, 1988). The potentiation (wind-up) of dorsal horn neurons can in fact be blocked with NMDA receptor antagonist APV and Kynurenate (Davis and Watkins, 1983; Dickenson and Sullivan, 1987). Thus, following nerve injury, neurons in the superficial dorsal horn receive a pronounced peptidergic and gulatamatergic that, in combination, may modify the long term responsiveness of those neurons to subsequent inputs. These results bear some similarity to the phenomenon of long term potentiation which has been demonstrated in the hippocampus.

However, the neuron in the superficial laminae which monosynaptically receive C-fibers inputs may expose to excessive of glutamate due to the massive release during injury discharge. The neurotoxicity of glutamate which triggers a delayed type (2 days) neuronal death has been well documented and fos protein may be involved in the cascade of degeneration process was suggested (review see Choi, at al, 1990). In fact, excessive excitation of neurons caused by chemical convulsants or electrical stimulation is known to induced degeneration of postsynaptic neurons along seizure pathways (Schwob, et al, 1980; Collins and Olney, 1982). Furthermore, many rapid transneuronal destruction was found in the superficiald dorsal horn following peripheral nerve section and which may be mediated by the excessive release of excitatory aminao acid (Sugimoto, et al; 1987). Therefore, some neurons in the superficial laminae which expressed Fos may be related to the degeneration change.

Therefore, to prevent the massive injury discharge transmits to the cord at least has two effects: preventing massive excitation and preventing massive sensitization. Unfortunately, most injuries in the clinic occur accidently, how to rescue and to reverse this potential harmful cascade triggered by the massive excitation and sensitization would be important. Excitatory inhibitors such as glutamate antagonist Mk-801 has shown to have protective effects if given earlier enough (Foster, et al, 1988). Further study of what are these activated spinal neurons following nerve injury may provide important information in understanding the mechanism of neuropathic pain.

Figure legend

Fig. 1. The laminar dependent effect of preblock of massive injury discharge on spinal Fos expression at 2 days after nerve section. (A) Two days after nerve cut (presaline treated group), there were considerable Fos positive cells in laminar 1-7. The degree of Fos expression in the three different regions at L5 level was similar: 62±9 labeled neurons in superficial laminae (arrow); 55±8 in laminae 3 and 4; 50±13 in deep laminae 5,6 and 7 (arrows) (n=4).(B) lidocaine (s.c neck): The systemic action of lidocaine reduced expression only in the superficial laminae 1 and 2. (C) and (D) Sciatic nerve preblock (15 min) profoundly suppressed Fos expression also only in the superficial laminae and no effect on the deeper laminae (arrows). sg. substantia gelatinosa. Scale bar, 200um.

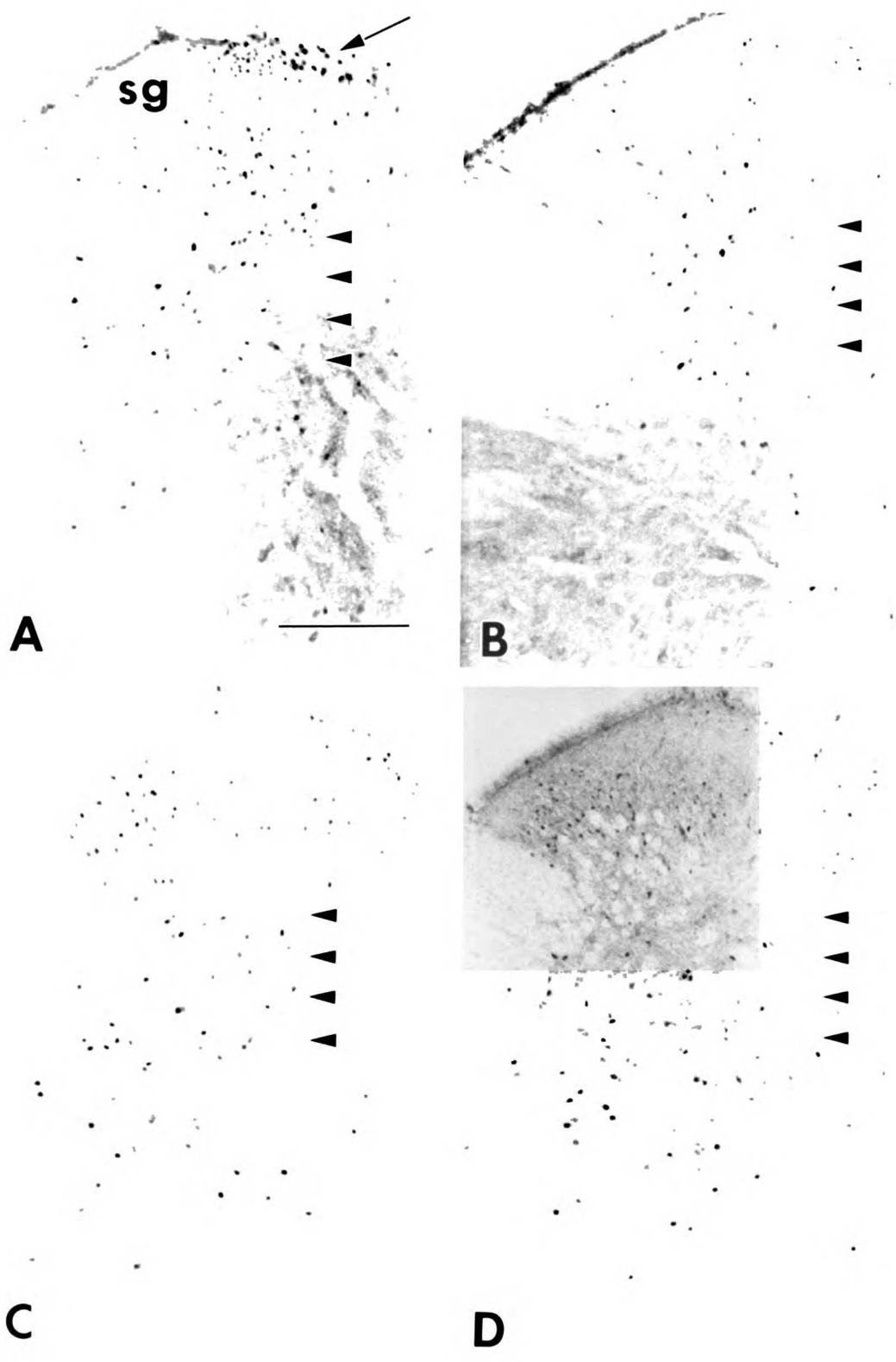
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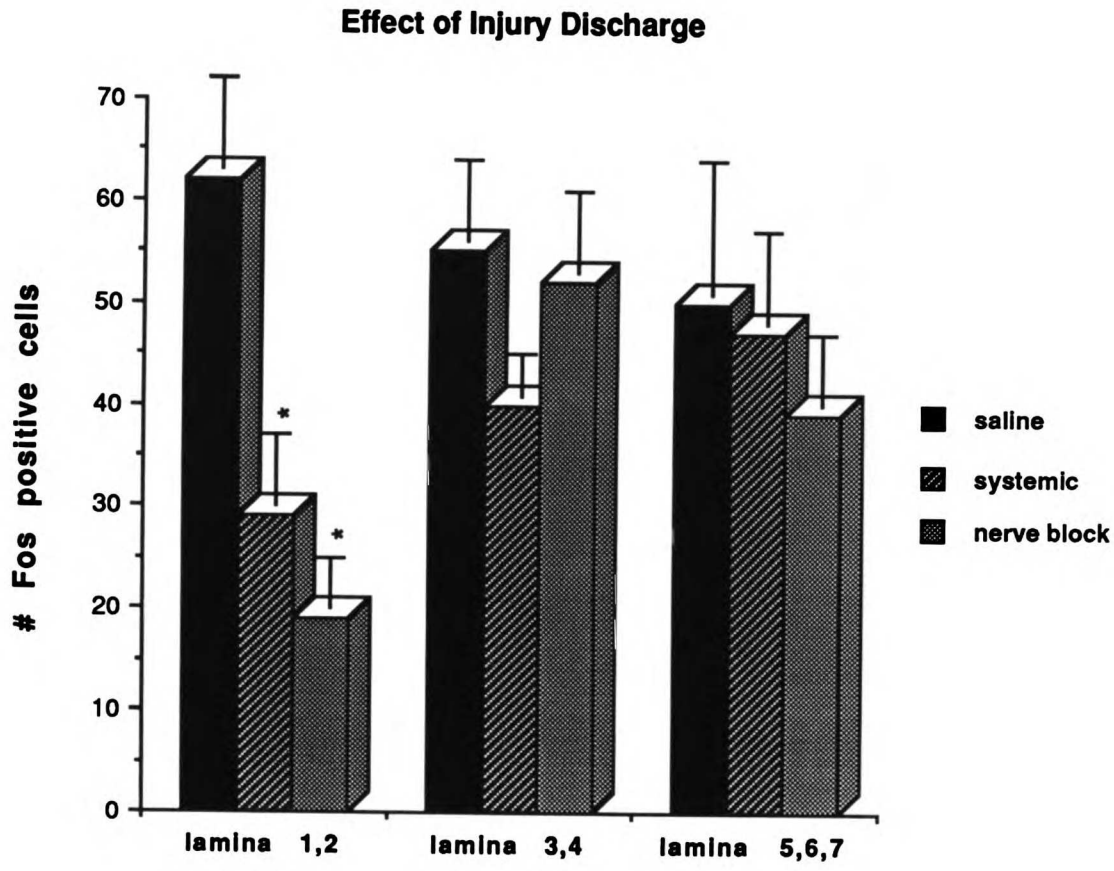
Fig. 2 The quantitative effect of preblock of injury discharge on spinal Fos expression at 2 days after nerve section (mean±S.E.M.). Compare to saline control (n=4), the preblock of massive discharge from nerve cut and ligation by local anesthetics significantly decreased 77% of the expression in the superficial laminae ($P<0.05$, n=4), but no effect on the other laminae 3,4, and 5,6 7 (ANOVA, $p>0.3$, n=4). The systemic action of lidocaine has similar effect as sciatic nerve block. The Fos expression was reduced by 53% and only the superficial laminae was affected ($p< 0.05$, n=4) (**= Scheffe-F-test).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saline
systemic
nerve block

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CHAPTER III

Effects of baclofen and morphine on the persistent expression of Fos in rat spinal cord following peripheral nerve injury

Abstract

We have previously demonstrated that sciatic nerve section and ligation evokes a long term increased expression of the fos protein product of the c-fos proto-oncogene in spinal cord cells of the rat. In this study we examined the ability of the GABA-B agonist, baclofen, and of the mu-opioid agonist, morphine, to modify expression of this Fos-like immunoreactivity (FLI) present two weeks after the peripheral nerve injury. Systemic administration of L-baclofen significantly suppressed the expression of FLI in the superficial dorsal horn and in deeper laminae (V through VII). Morphine had no effect. These results suggest that changes in the nociceptive areas of the spinal cord after nerve injury can be modulated by GABAergic mechanisms, but are unresponsive to mu-opioid agonists. The results are discussed with regard to the reported effects of baclofen and morphine for the treatment of neuropathic pain.

Introduction

The rat neuroma model, produced by sciatic nerve section and ligation, has been employed to study peripheral and central neuroanatomical and neurophysiological changes that result from peripheral nerve injury (reviw in Wall, 1984, Devor, 1988, Bonica, 1979, Dykes and Lamour,1988, Hylden, et al, 1987). In particular, it has been demonstrated that the sprouting fibers, trapped in the proximal stump of the cut nerve, become spontaneously active, mechanosensitive and responsive to local or circulating alpha-adrenoceptor agonists (Wall and

Gutnick, 1974; Govrin -Lippman and Devor, 1978, Scadding,1981). This increased spontaneous and evoked peripheral neural activity has been proposed to contribute to the neuropathic pain that is often present after peripheral nerve injury (Siegfried and Zimmermann, 1981, Wall, 1981, Loe and Nathan, 1978, Sunderland, 1976, Wall and Gutnick, 1974). The ability of peripheral local anesthetic blockade to reduce pain following peripheral nerve injury (Chabal, etal, 1989, Noordenbos, 1959; Sunderland, 1968; White and Sweet, 1969; Wall and Devor, 1978; Blumberg and Janig, 1981) supports this interpretation.

By immunocytochemically staining cells for the fos protein product of the c-fos proto-oncogene, it is possible to monitor the activity of populations of cells in the CNS in response to various peripheral stimuli. Recently, we used this technique to determine the distribution of spinal cord cells whose activity is influenced by spontaneous activity in a neuroma (Chi, et al, 1989; 1991). We found that after sciatic nerve section and ligation, there is increased expression of Fos-like immunoreactivity (FLI) in regions of the spinal gray matter mediating nociception (laminae I, II, V , VI and VII). The increased FLI persisted for several weeks. In a related study, we demonstrated that application of local anesthetic to the neuroma significantly reduced Fos expression (Chi, et al, 1989; 1991), suggesting that the peripheral abnormal hyperactivity arising in the neuroma wasresponsible, at least in part, for the spinal cord neuronal FLI.

We have also recently demonstrated that two pharmacologically distinct agents, morphine and baclofen (a GABA-B agonist), can reduce the expression of Fos, evoked by formalin (s.c) induced tissue injury (Presley, 1990, Gogas, 1991, Chi, 1991). In this model, morphine and baclofen dose-dependently decreased both pain behavior and FLI in superficial and deep laminae of the spinal cord gray matter. Since the pharmacological control of neuropathic pain in humans has proven difficult (Tasker, et al, 1983, Arner and Meyerson,1988), it is of particular interest to evaluate the effect of these different drugs on the expression of Fos-like immunoreactivity in the neuroma preparation, a model of neuropathic pain. In the present study we therefore evaluated the effect of baclofen and

morphine on the long-term expression of FLI in the nerve injury model. We report that baclofen significantly reduces long-term (two-week) expression of Fos-like immunoreactivity after sciatic nerve section but that morphine is without effect.

Methods

Male Sprague-Dawley rats (280-300 gm, 82-87 days, Bantin and Kingman, Fremont, CA) were anesthetized with Nembutal (50 mg/kg). The left sciatic nerve was exposed in the mid-thigh, cut and ligated tightly with 6-0 silk suture and then the overlying muscle and skin was sutured with 3-0 silk. Sham-operated rats had the sciatic nerve exposed, after which the overlying muscle and skin were sutured. Two weeks after nerve injury, the effect of the different drugs on the expression of Fos-like immunoreactivity was tested. The doses of baclofen and morphine used were those which had been observed to significantly reduce, by more than 50%, the numbers of Fos-like immunoreactive cells evoked by subcutaneous formalin injection (Presley, 1990, Chi, 1991). To assure adequate drug levels beyond the half-life of the Fos protein, approximately 1-2 hours (Muller, 1984, Chi, at al, 1991), the drugs were administered twice during the four-hour period prior to perfusion for immunocytochemistry. The rats were randomly assigned to one of the following groups: L-baclofen: (Ciba-Geigy city). 7.5 mg/kg ip followed by 5.0 mg/kg two hours later; D-baclofen: the stereoisomer, 7.5 mg/kg ip followed by 5.0 mg/kg ip two hours later; morphine sulfate(Antra) : 5.0 mg/kg; s.c in dorsum of neck; with repeat dose at 2 hours. Two control groups were included. One group received two injections of a similar volume of saline (ip) separated by two hours; the second group received no treatment prior to perfusion .

At the end of the four-hour period, the rats were anesthetized with Nembutal (60mg/kg) and perfused intracardially with 100ml of 0.1 M phosphate buffer, followed by 300ml of a phosphate-buffered 4% paraformaldehyde fixative. The lumbar spinal cord was removed, postfixed for 6 hours, cryo-protected overnight and then 50 micron transverse

frozen sections were cut. The sections were immunostained for the Fos protein using the avidin-biotin method (Hsu et al, 1981) and a rabbit polyclonal antiserum directed against the product of an *in vitro* translated c-fos gene (kindly provided by Dr. Dennis Slamon at UCLA). Details of the antiserum have been described previously (Menetrey, etal, 1989).

Since the largest number of labelled cells observed 2 weeks after sciatic nerve section are in the L_5 segment of the spinal cord (Chi, et al, 1989), we concentrated our studies in this region. To analyze the distribution of labelled cells in different regions, we first sketched an outline of the L_5 segment using a camera lucida attachment under dark-field illumination. The sections were then examined with bright-field illumination and the distribution of Fos-like immunoreactive (FLI) cells plotted. Only those cells with nuclear staining which was easily discernable from background at 10X magnification were included in the drawing. Labelled cells from three sections in each rat were counted and averaged.

To quantify changes in different laminae of the spinal cord, the drawings were divided into three regions based on functional differences: 1) the superficial dorsal horn, laminae I and II, which predominantly contains nociresponsive cells (Fitzgerald, 1981) the nucleus proprius, laminae III and IV, which predominantly contains cells selectively responsive to non-noxious stimuli (Wall, 1967; Pomeranz et al, 1968; Price and Browe, 1973; Menetrey et al, 1977); and 3) the deeper laminae, V, VI and VII which also contain nociresponsive cells, many of which have been implicated in less localizable pain conditions (Price and Browe, 1973; Price and Mayer, 1974, Menetrey et al, 1977; Molinari, 1982; Meyers and Snow, 1982b). Sections were coded. The investigator who plotted the distribution of the Fos-immunoreactive cells was blind to the treatment received. Statistical comparisons between Fos-like immunoreactivity (FLI) in different treatment groups were made by one way ANOVA and Scheffe F-test for post-hoc analysis.

Results

The pattern of expression of Fos-like immunoreactivity in the spinal cord at two weeks after sciatic nerve section was similar to that previously described (Chi et al. 1989). Labelled cells were found in laminae I through VII of the ipsilateral gray matter and to a lesser extent in the contralateral gray matter. The distribution and numbers of FLI cells in the different regions of the ipsilateral spinal cord were as follows. In laminae I and II: 33 ± 8 (mean± SEM, n=3) FLI cells, concentrated in the lateral two-thirds of the superficial dorsal horn. As described previously, the distribution is somewhat lateral to the central termination of sciatic afferents; which is concentrated in the medial two-thirds of laminae I and II (Chi, etal, 1989). There were 55 ± 11 FLI cells in laminae III and IV; and 57 ± 12 cells in the deeper laminae V, VI and VII.

Systemic L-baclofen significantly reduced the number of FLI-positive cells in all laminae (I-VII) of the spinal cord. The decrease in staining was 54% and 56% for the superficial and deep laminae, respectively (both, $p<0.01$; n= 4). There was, however, no significant decrease in the number of FLI-positive cells in laminae III and IV ($p>0.1$; n= 4). Systemic D-baclofen also reduced Fos-like immunoreactivity ipsilateral to the injured nerve and the pattern and degree of reduction was very similar to that observed for L-baclofen (laminae I, II, and, laminae V-VII, both $p<0.01$; n=4 and laminae III, IV $p>0.1$, n= 4). Finally, morphine had no effect on the numbers of FLI cells. ($p>0.1$; n=4)

Discussion

This study demonstrates that there is a significant difference in the ability of the GABA-B agonist baclofen and of morphine to affect the expression of Fos-like immunoreactivity (FLI) after nerve injury. Baclofen significantly inhibited expression of Fos-like immunoreactivity after nerve injury, while morphine had no effect. The pattern of inhibition by baclofen was very similar to that observed previously on the expression of FLI in response to formalin injection. Specifically, there was a more pronounced inhibition of Fos expression in laminae I, II and V-VII. Although there was inhibition of Fos in the

nucleus proprius, the reduction was not statistically significant. These results indicate that baclofen is most effective on nociresponsive cells, i.e., cells which directly or indirectly receive small diameter primary afferent input.

The fact that L-baclofen was effective in the neuroma model as well as after formalin stimulation is consistent with other studies demonstrating that the circuitry through which spinal cord GABAergic interneurons inhibit the transmission of nociceptive information is not disrupted by peripheral nerve injury (Jessel et al, 1979). On the other hand, it was surprising that D-baclofen, although ineffective in the formalin model, was able to reduce expression of Fos-like immunoreactivity in the sciatic neuroma model. This might be explained by an enhancement of the sensitivity of spinal cord cells to GABAergic regulation after nerve injury. Interestingly, and perhaps consistent with this hypothesis is the fact that in the neuroma model (Woolf and McMahon, 1985) there appears to be a significantly enhanced sensitivity to the low dose of systemic anesthetics

Since the neuroma is very sensitive to mechanical stimulation, reduced mobility after drug administration could conceivably have reduced evoked activity arising in the neuroma, and consequently reduced Fos expression. L-baclofen, in fact, did cause some flaccidity in the nerve injury rats, this possibility must be considered. D-baclofen, however, significantly reduced Fos expression but did *not* produce signs of flaccidity. We conclude that inhibition of Fos by L-baclofen is not secondary to reduced mechanical stimulation of the neuroma.

In contrast to baclofen, morphine had no effect on the expression of FLI in the neuroma model. This observation is consistent with the observation that opiates often produce only minimal relief of neuropathic pains in humans (Arner and Meyerson, 1988). The findings of the lack of effect of morphine in the neuroma model could be explained by decrease in the number of (effective) opioid receptors presynaptically and postsynaptically after nerve injury (Fields, et al; 1980). However, it is also possible that the activated neurons are not regulated by the opioids. Other factors such as effects on coupling

mechanisms may, therefore, underlie the failure of morphine to reduce Fos expression in the peripheral nerve injury model. It would be important to know what other pain modulating systems (i.e. 5-HT; catcholamine) might change after nerve injury. These pharmacological studies may also help to understand what spinal neurons are activated following nerve injury and what are the regulations of these neurons.

In conclusion, we have demonstrated that the persistent expression of FLI observed two weeks after sciatic nerve injury can be blocked by the GABA-B ligand, baclofen, but is unresponsive to the opioid agonist, morphine. These results are consistent with the common clinical experience that opioids are often ineffective for the treatment of neuropathic pains in humans. Of interest, a recent clinical report that intrthecal baclofen reduces chronic pain in patients with spinal lesion (Herman and D'Luzansky, 1990). While baclofen can be effective in other lancinating pains, the use of baclofen is unfortunately severely limited by the marked flaccid paralysis that occurs. Our results indicate, however, that development of new GABA-B ligands or lower the dose of baclofen by combining with other pain modulating drugs may yet prove beneficial to the treatment of neuropathic pain in humans ..

Figure legend

Fig. 1. The differential effect of analgesic drugs on the chronic Fos expression in 2 weeks neuroma. (A) Saline, i.p. (B) Morphine, s.c (C) L-baclofen, i.p (D) D-baclofen, i.p. laminar 2 (arrows). s,g: substantia gelatinosa. Scale bar, 200um.

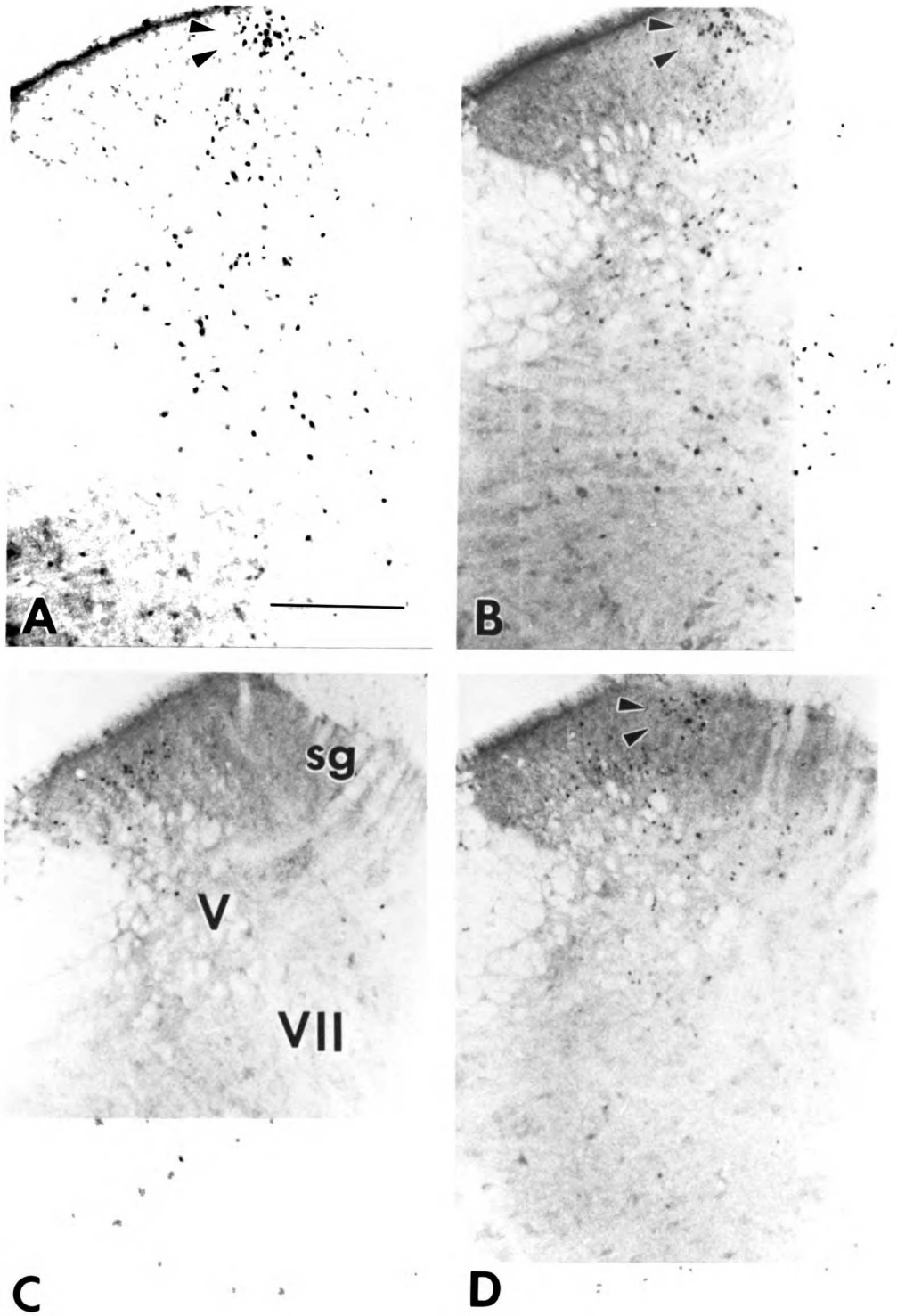

A
B
sg
V
VII
C
D

Fig. 2. The quantitative effect of baclofen on spinal Fos expression at 2 weeks after nerve section (mean±S.E.M.). Compare to saline control (50±4, n=3), L and D baclofen (7.5mg/kg, 1x; 5 mg/kg, 1x; i.p) significantly decreased about 50% of Fos positive cells in both superficial and deep laminae ($P<0.05$, n=4). The reduction of Fos expression was not significant in laminae 3 and 4 (ANOVA, $p>0.1$, n=4).

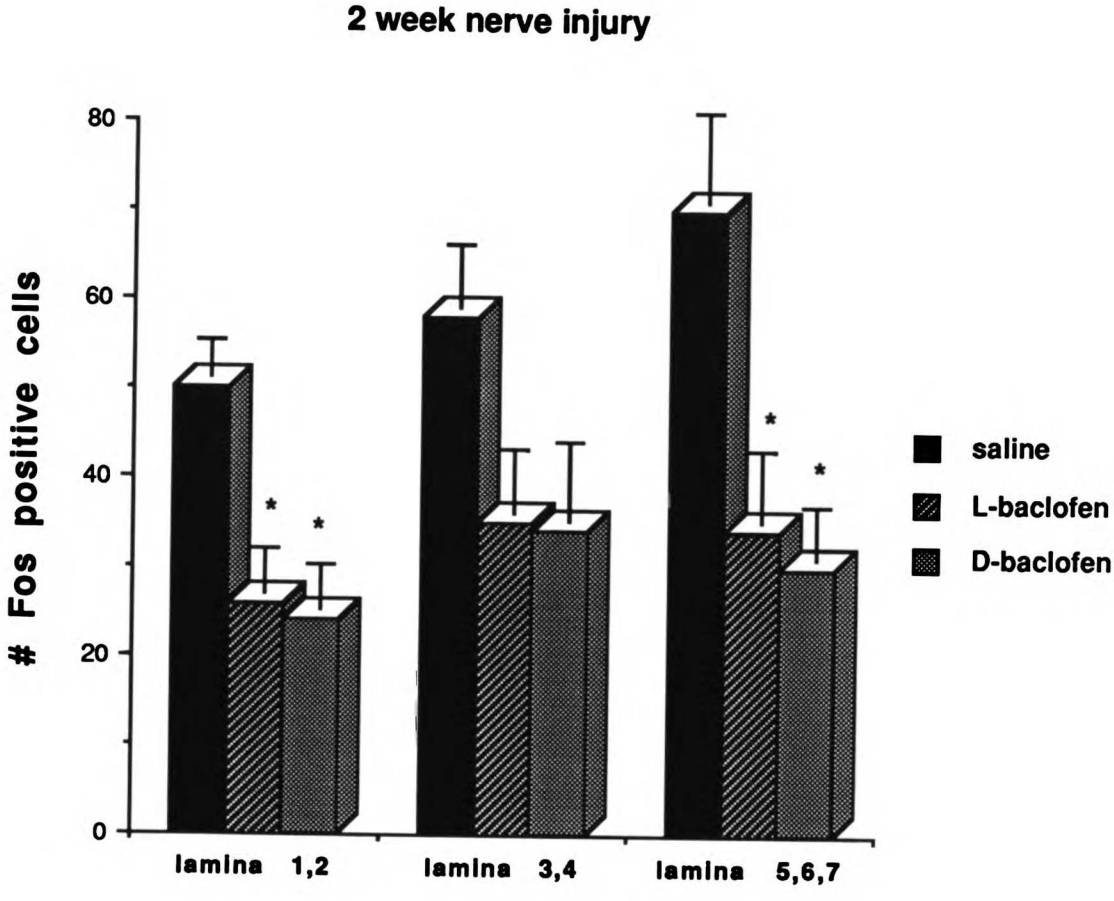
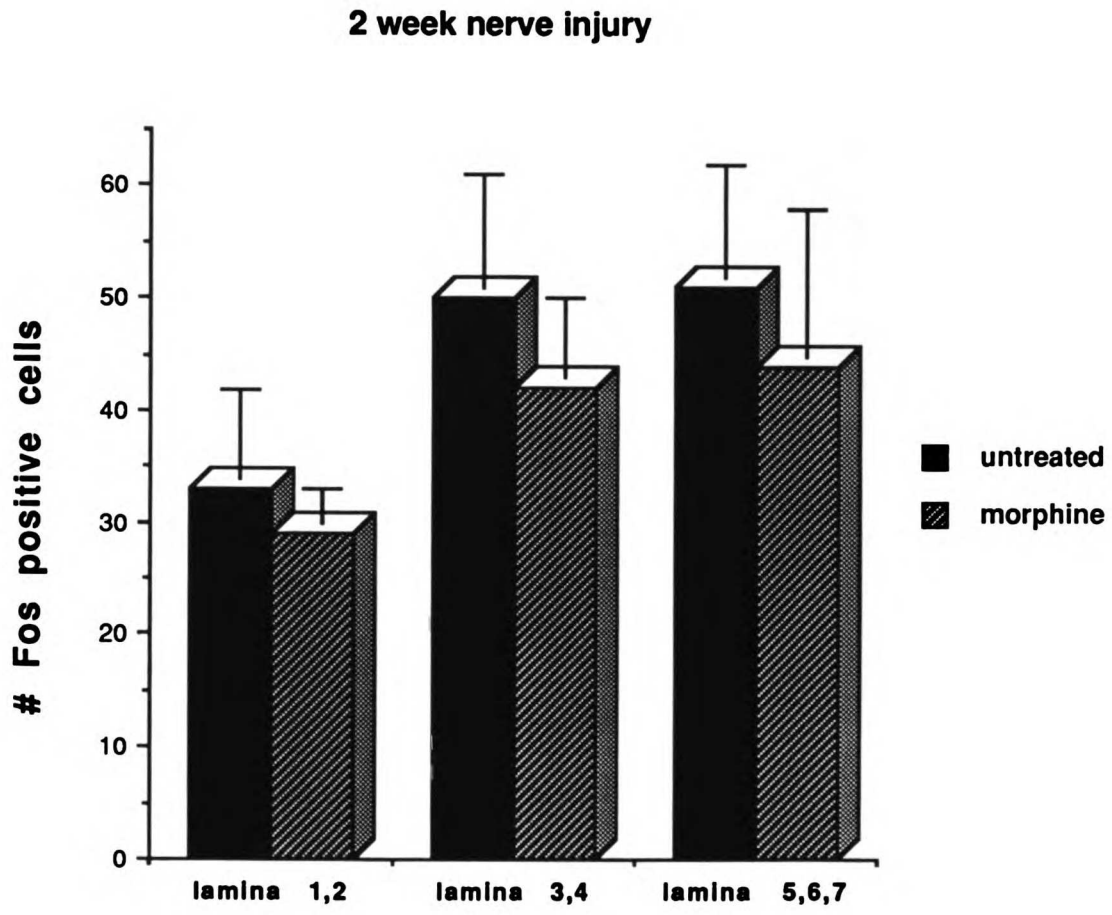

2 week nerve injury
Fos positive cells
80
60
40
20
0
*
*
*
*
lamina 1,2
lamina 3,4
lamina 5,6,7
saline
L-baclofen
D-baclofen

Fig. 3. The quantitative effect of morphine on spinal Fos expression at 2 weeks after nerve section (mean±S.E.M.). Morphine (5mg/kg, 2x, s.c) had no effect on the chronic Fos expression ($p>0.1$, $n=4$).


2 week nerve injury
Fos positive cells
60
50
40
30
20
10
0
lamina 1,2
lamina 3,4
lamina 5,6,7
untreated
morphine

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CHAPTER IV

Systemic and Intrathecal Baclofen Suppress Formalin-Evoked Nociceptive Behavior and Fos Expression in Rat Spinal Cord

Abstract

Fos, the nuclear protein of protooncogene c-fos product which can be induced by noxious and non-noxious stimulation. The neuronal expression of Fos in the spinal cord has shown to correlate with physiological properties of neurons. Here we study the effect of GABA-b agonist, baclofen, on pain behavior and Fos expression in a tonic pain model (formalin stimulation). Formalin (50ul,1%) is injected subcutaneously into the plantar surface of the hindpaw. Both systemic and intrathecal L-baclofen produced a dose dependent reduction of both pain behavior and Fos expression in the spinal cord. The degree of Fos expression in three regions (laminae 1,2; laminae 5,6 and laminae 7,8) which are known to associate with pain transmission and modulation, but not in the nucleus proprius (laminae 3, 4), is correlated with intensity of pain behavior. The results indicate that the noxious stimulation-evoked both pain behavior and Fos expression can be regulated by the GABA-b system in the spinal cord. The data further support that Fos expression in the pain-related region of spinal cord is not only a function of inputs of stimulation but also a function of output pain signals.

INTRODUCTION

Recent studies have documented the usefulness of following the expression of the fos protein product of the c-fos proto-oncogene to monitor the activity of populations of CNS neurons (Morgan, et al, 1987; Sagar, et al; 1988; Sharp, et al; 1990). In particular, it has been demonstrated that noxious stimulation induces expression of this gene in regions of the spinal cord which contain neurons responsive predominantly to noxious stimulation (Hunt, et al 1987). Importantly, since the neural activity is produced in awake, freely moving animals, it has also been possible to determine correlations between the number of Fos-immunoreactive neurons and pain behavior. For example, we have established that systemic or intracerebroventricular administration of opioids inhibits the noxious stimulus-evoked expression of Fos in a dose-dependent and naloxone reversible manner (presley, et al, 1990), as well as producing behavioral analgesia (Gogas,et al, 1991). Those studies provide strong evidence in favor of the hypothesis that supraspinally administered opioids exert their analgesic action via activation of descending controls which inhibit the firing of spinal cord nociresponsive neurons.

In addition to the opioids, there is evidence that the GABA -b ligand, baclofen , exerts a profound analgesic effect by systemic (Dickenson, et al; 1985, Dickenson and Sawynok, 1985; Sawynok, 1986), intracerebroventricular (Levy and Proudfit, 1979), or intrathecal injection (Wilson and Yarksh, 1978) in various animal models of pain, including tail flick and hot plate. The present study the effect of non-opiate analgesic drug baclofen on noxious stimulus-evoked expression of the Fos in spinal cord neurons. We also compared the patterns of Fos inhibition with the inhibition of pain behavior.

METHODS

Male Sprague-Dawley rats (350-500gm; Bantin-Kingman, Fremont, CA) were used in this study. Under halothane anesthesia (2.5%), 50 ul of 1% formalin was injected subcutaneously into the plantar surface of the hindpaw. The animals recovered from the halothane within four minutes and then were placed into a plexiglass chamber (30cm x 30cm) equipped with a 45° angled mirror below the floor. This made it possible to view without obstruction the animals' movements and to evaluate the extent of the contact between the plantar surface of the injected hindpaw and the floor.

Drug administration:

In the systemic baclofen study, the drugs were administered 30 minutes before the formalin. Both L- and D- baclofen were dissolved in 0.9% saline and administered in doses of 2.5-7.5mg/kg, i.p. For the intrathecal study, the rats were first anesthetized with halothane, as above, and injected by lumbar puncture in a dose range of 0.1-1.0μg, 15 minutes before injection of the formalin. Controls in both groups received an equivalent volume of saline administered by the appropriate route.

Behavioral testing:

A different pain scoring method was used for the systemic and intrathecal baclofen studies. In the systemic study, we measured the amount of time that the rat spent licking the injected hind paw. The licking time was determined for the one-hour period after injection. In the intrathecal baclofen study, the more traditional formalin pain score was calculated. Specifically, during the first hour after injection of the formalin, we scored the rat's tendency to favor the injected paw as a weighted average of the time spent in each of the following categories: 0, weight bearing such that there is detectable compression of the foot pad; 1, the hindpaw rests lightly on the floor, but there is no compression of the foot pad and the toes are not spread; 2, the injected paw is clearly elevated above the floor; 3,

the rat licks or shakes the injected paw. To calculate a weighted average pain score, we multiplied the time spent in each category by the category weight for each 5-minute block of the test period.

Immunostaining procedure:

Ninety minutes after injection of the formalin, the rats were anesthetized with pentobarbital (60mg/kg) and perfused intracardially with 150ml of 0.1M phosphate buffered saline (PBS) followed by 300ml of a fixative solution containing 0.1M phosphate-buffered 4% paraformaldehyde. The lumbar spinal cord was removed, postfixed for 6 hours, cryo-protected overnight and then 50 micron transverse frozen sections were cut. The sections were immunostained for the Fos protein using the avidin-biotin method (Hsu et al, 1981) and a rabbit polyclonal antiserum directed against the product of an *in vitro* translated c-fos gene (kindly provided by Dr. Dennis Slamon at UCLA). Details of the antiserum have been described previously (Menetrey, etal, 1989).

Data analysis:

To plot the distribution of immunoreactive neurons, we first sketched outlines of sections through the L 4-5 segment with a camera lucida attachment under dark field illumination. The sections were then examined with bright field illumination and the distribution of Fos-like immunoreactive (FLI) neurons plotted by camera lucida. Only those cells with nuclear staining which was easily discernible from background at 4x and 10x were included in the drawing. Labelled cells from three representative sections in each rat were counted and averaged. To quantify changes in different laminae of the spinal cord, we divided the cord into the following 4 regions; superficial laminae 1,2; laminae 3,4 (nucleus proprius); laminae 5,6 and laminae 7,8. The investigator who plotted the distribution of FLI neurons was blinded to the treatment received. Statistical tests were

performed by one way analysis of variance followed by Scheffe-F test for post hoc comparisons.

RESULTS

Although we used a lower dose of formalin in this study compared to our previous analysis of the effects of opioids on noxious stimulus evoked Fos expression, the pattern of pain behavior recorded was comparable. Thus 50µl of 1% formalin injected into the plantar surface of the hindpaw elicited two phases of pain behavior. The first phase of licking began 3-6 minutes after the formalin injection and lasted for 2-7 minutes. The second phase of licking began 13-30 minutes after the formalin injection and lasted for 30-45 minutes. Approximately one hour after the formalin injection the intensity and frequency of licking decreased considerably; most rats went to sleep. One half hour later, i.e., at 90 minutes after injection of the formalin, the rats were anesthetized with pentobarbital, perfused and the spinal cord tissue stained immunocytochemically.

Distribution of Fos-like immunoreactive neurons

As described previously, the pattern of FLI neurons in untreated rats (i.e., no formalin) was limited to scattered, lightly labelled cells in the nucleus proprius. Formalin injection, in saline pretreated rats, evoked a widespread expression of FLI neurons. The majority of labelled cells were located in the medial half of the superficial dorsal horn, ipsilateral to the injected paw. This distribution corresponds to the termination site of the primary afferent input from the hindpaw. The remaining cells were distributed through the spinal gray. Approximately equal numbers were located in laminae 3,4 and in laminae 5,6 The remainder of the cells were located in the laminae 7,8 (approximately 8% of the total number of labelled cells). The major difference between the 1% dose used here and the 5% dose used in our previous study was less cells in the ventral horn.

Effects of systemic baclofen on FLI neurons

Systemic L-baclofen produced a dose dependent reduction of both pain behavior (i.e.,time spent licking, data not shown) and FLI in all regions of the ipsilateral spinal cord. At lower dose, the % of inhibition of Fos expression was more in the ventral horn than in the dorsal horn (details see Figure). However, the absulutely number of reduction was greater in the dorsal horn than in the ventral horn, particularly in the superficial laminae. At dose 5-7.5 mg, three normal walking rats without pain behavior, their total Fos cells was decreased by 70%.

Effect of intrathecal baclofen on FLI neurons

Since the more detailed pain behavior measures were performed in this group of animals, we evaluated the relationship between the numbers of labelled cells and pain behavior in these rats. We found a significant positive linear relationship between the pain behavior score and the numbers of Fos labelled cells in the superficial laminae 1,2 ($r=0.83$); laminae 5,6 ($r=0.85$) and ventral laminae 7,8 ($r=0.71$)), but not in the laminae 3,4 .

Intrathecal L-baclofen also produced a dose-dependent reduction of both pain behavior and the expression of Fos (Figure). The highest dose tested (1.0μg) completely inhibited pain behavior and reduced Fos expression by greater than 70% in both the superficial laminae 1,2 and laminae 5,6. There was a reduction of the number of Fos-immunoreactive neurons in the laminae 3,4 and ventral horn, however, this was not significantly different from saline controls.

Effect of D-baclofen on Fos-like immunoreactivity

Neither systemic D-baclofen (10mg/kg), nor intrathecal D-baclofen (1.0-9.0μg) affected pain behavior or the numbers of formalin-evoked FLI neurons. On the other hand, the highest dose of intrathecal D-baclofen (9.0μg), produced a flaccid paralysis of the hind limb.

DISCUSSION

In previous studies we described the pattern of expression of the protein product of the c-fos proto-oncogene produced by injection of the formalin in to the plantar surface of the hindpaw of the rat and demonstrated that opioid analgesics, morphine (administered systemically, intrathecally, or intracerebroventricularly) or the mu selective opioid peptide agonist, DAMGO (i.c.v), dose dependently inhibited Fos expression (Presley, et al, 1990; Gogas, et al, 1991, Cho, et al, 1990; Botchkina, et al; 1990). In the present report we have extended those studies and demonstrated that the GABA-b agonist, baclofen, whether administered intraperitoneally or intrathecally, also inhibits Fos expression in a dose-dependent fashion. As with the opioid analgesics, we found that the inhibition of noxious stimulus-evoked Fos expression by baclofen was most pronounced in regions of the spinal cord which contain neurons responsive predominantly to noxious inputs, namely the superficial dorsal horn (laminae 1 and 2) and laminae 5 and 6. Importantly, since D-baclofen was without effect, we conclude that the effect of L-baclofen was indeed via interaction with the GABA-b receptor.

Although systemic administration of baclofen could have exerted its analgesic and Fos suppressive effects, via supraspinal and/or spinal routes, the profound effect of intrathecal baclofen, indicates that a spinal route is most likely involved. That conclusion is consistent with the cytochemical studies which have demonstrated high concentration of

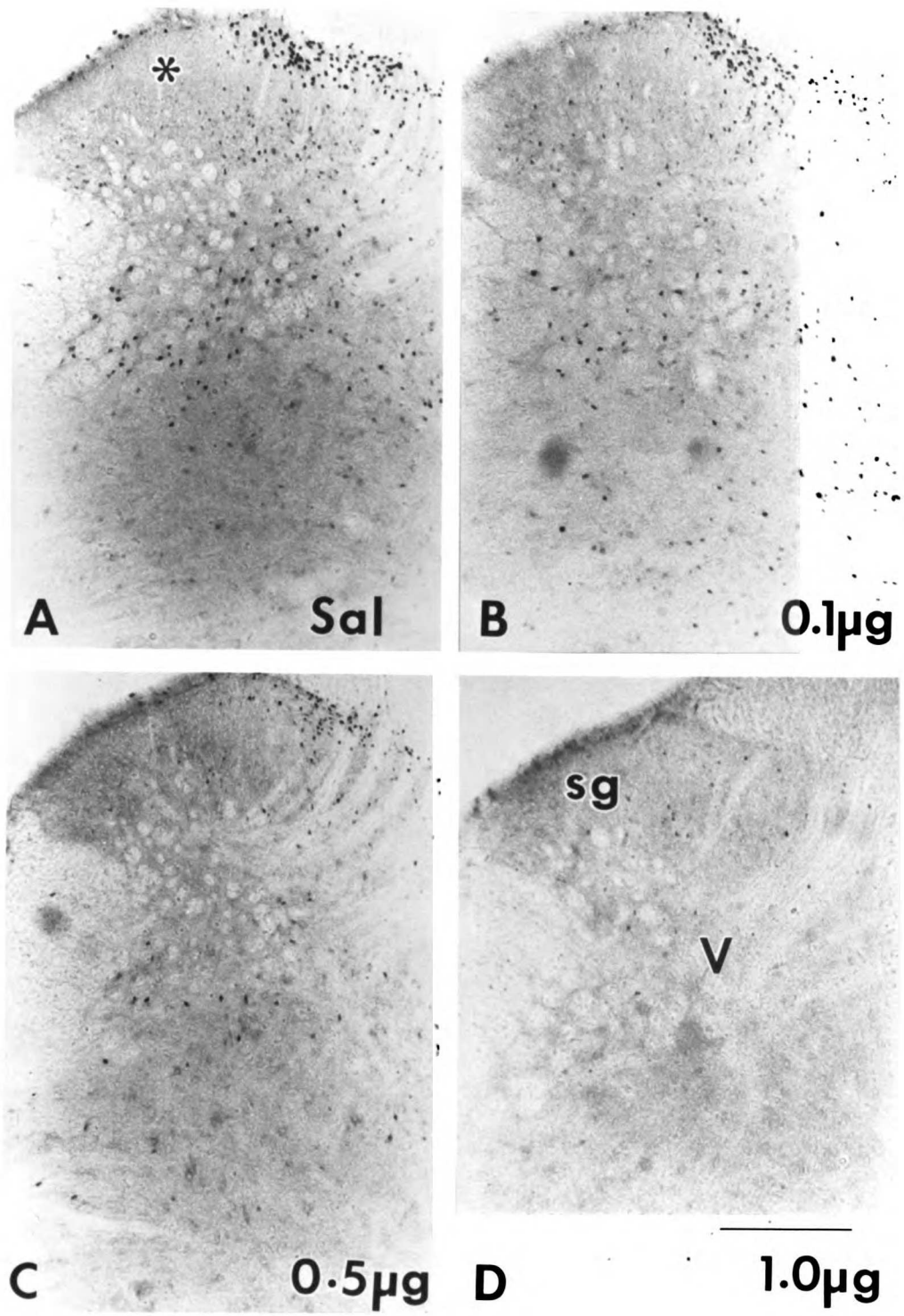
GABA-b (i.e. baclofen) binding sites in the superficial dorsal horn (Price, et al, 1984, 1987). Moreover, many of these binding sites are absent in rats treated neonatally with the C-fiber neurotoxin capsaicin, indicating that the binding sites are, in part, located on the unmyelinated primary afferents (Price,et al; 1984). This further indicates that the inhibitory effect of baclofen on nociceptive transmission involves presynaptic inhibitory mechanisms (Dickenson et al, 1985). This conclusion is consistent with results of Henry et al.(1982) which demonstrated that baclofen profoundly inhibits the firing of spinal nocicresponsive neurons to a peripheral stimulus, although it had a more limited effect on the excitation provoked glutamate iontophoesis.

As previously described, we found a high positive correlation between the numbers of Fos immunoreactive neurons and the expression of pain behavior in the formalin test. That result further validates the use of Fos expression to monitor the "activity" of large population of neurons in response to a noxious stimulus.

Our results demonstrate that baclofen is also effective in a test of persistent pain, the formalin test. Due to the difficulty in achieving effective doses without motor impairment, the therapeutic value of baclofen for the treatment of pain in humans is limited. However, a recent clinical report that intrathecal baclofen reduces chronic pain in patients with spinal lesion (Herman and D'Luzansky, 1990). Furthermore, we have demonstrated that the chronic Fos expression observed two weeks after sciatic nerve injury can be blocked by baclofen, but is unresponsive to the opioid agonist, morphine. Taken together with evidence for a profound modulation of Fos expression and pain behavior by baclofen, these studies emphasize the value which this combined approach offers to the analysis of the neural mechanisms underlying the control of pain.

Figure legend

Fig. 1. Effect of intrathecal baclofen on formalin-evoked Fos expression at L5 level. (A) saline (i.p), (B) 0.1ug, (C) 0.5ug, (D) 1ug. Scale bar, 200 um.


*
A
Sal
B
0.1µg
C
0.5µg
sg
V
D
1.0µg

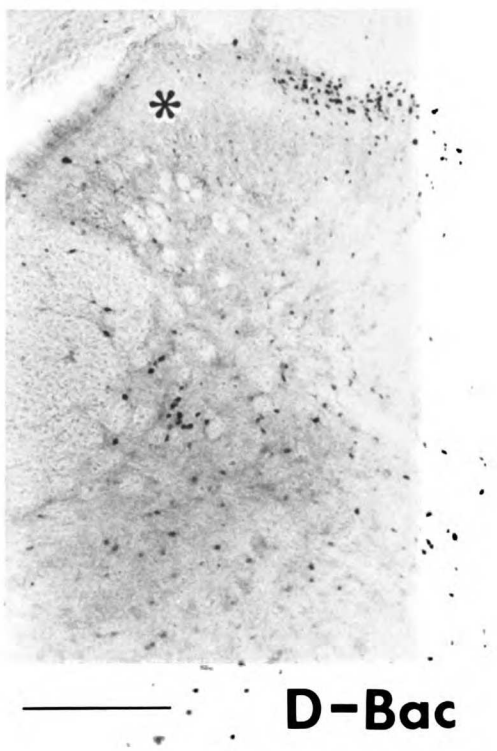

*
D-Bac

Fig. 3. The dose dependent inhibition of systemic baclofen on Fos expression in different regions of spinal cord as a % of each region's own control (n=4), (mean± SEM). There was suppressive effect of baclofen at lower dose of 2.5 mg/kg (n=3). However, the effect was not significant by Scheffe F-test. At dose 5mg/ kg (n=4), the significant inhibition were: 44% in laminae 1,2 (significant only in Fisher PLSD test).; 66% in laminae 3,4; 55% in laminae 5,6 and 74% in ventral horn. At high dose 7.5 mg/kg (n=3): the inhibition has plateaued except in the laminae 5,6.

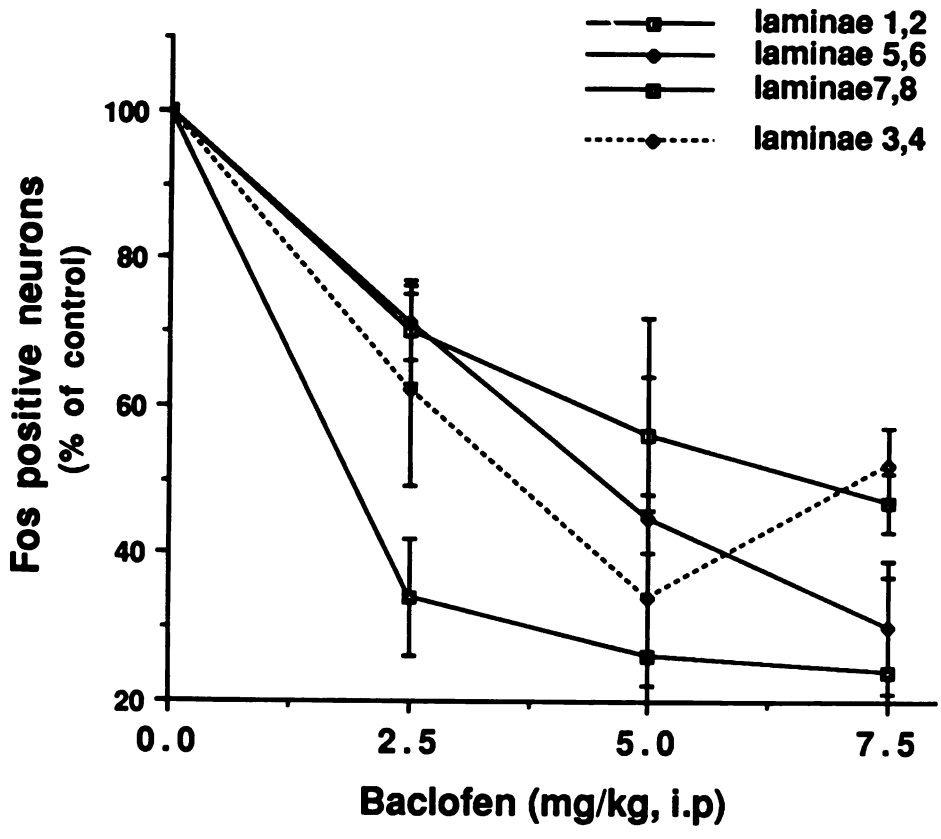
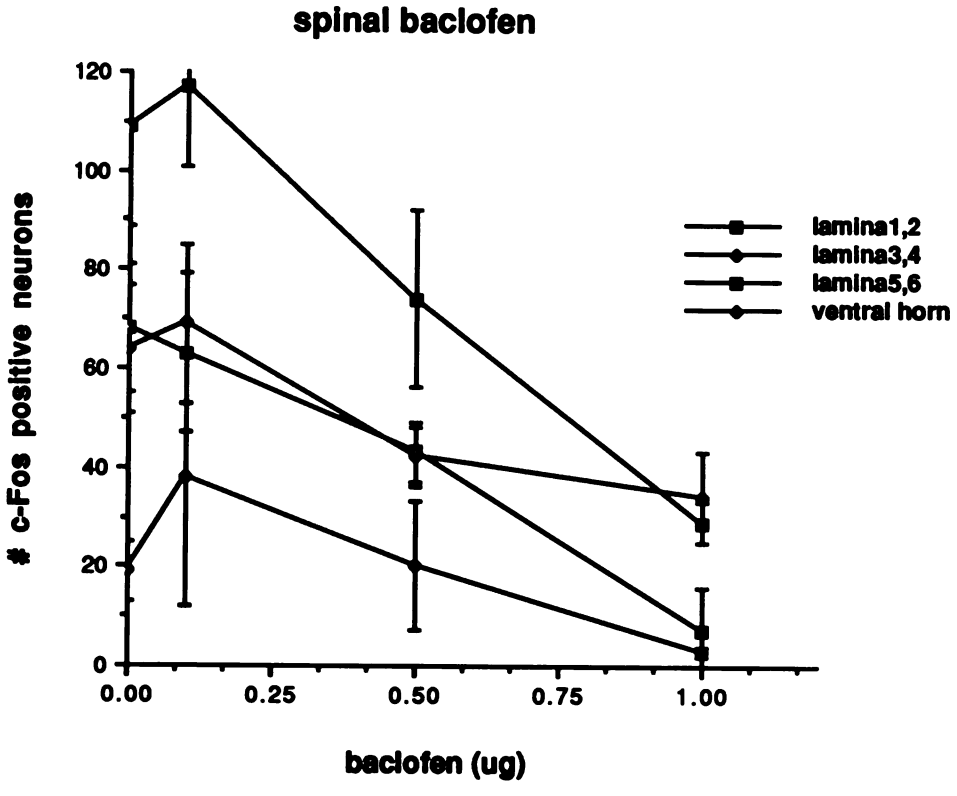

laminae 1,2
laminae 5,6
laminae7,8
laminae 3,4
Fos positive neurons
(% of control)
100
80
60
40
20
0.0
2.5
5.0
7.5
Baclofen (mg/kg, i.p)

Fig. 4. Effect of intrathecal baclofen on pain behavior and Fos expression in different regions of spinal cord (mean± SEM).The dose-response curve among laminae 1,2; laminae 5,6 and ventral horn were similar. However, at dose of 5ug-1 ug, a significant inhibition of Fos expression only in the laminae 1,2 and laminae 5,6 ($p<0.05$). At 1ug, there was no pain behavior and the Fos cells were much reduced.


spinal baclofen
c-Fos positive neurons
120
100
80
60
40
20
0
0.00
0.25
0.50
0.75
1.00
baclofen (ug)
lamina1,2
lamina3,4
lamina5,6
ventral horn

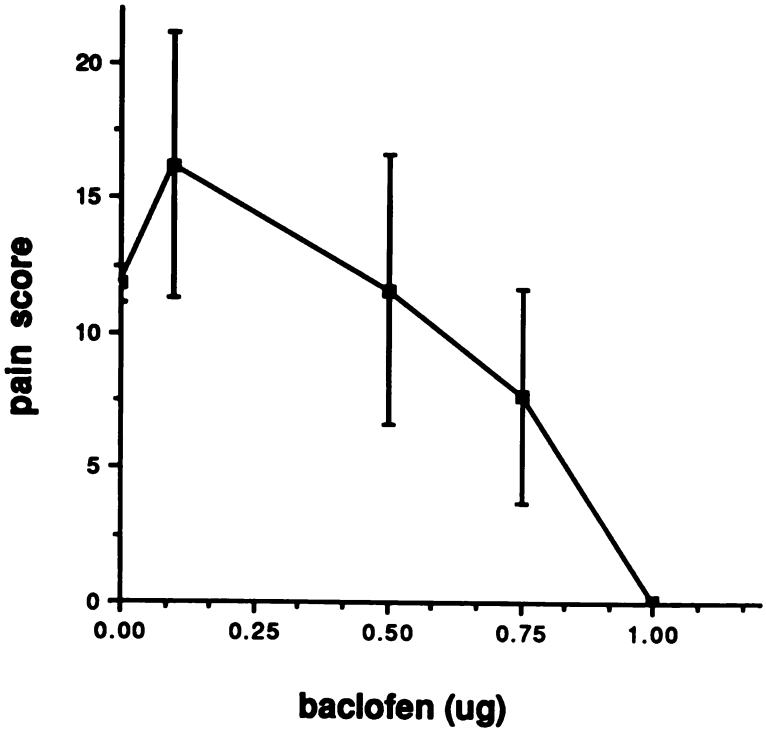
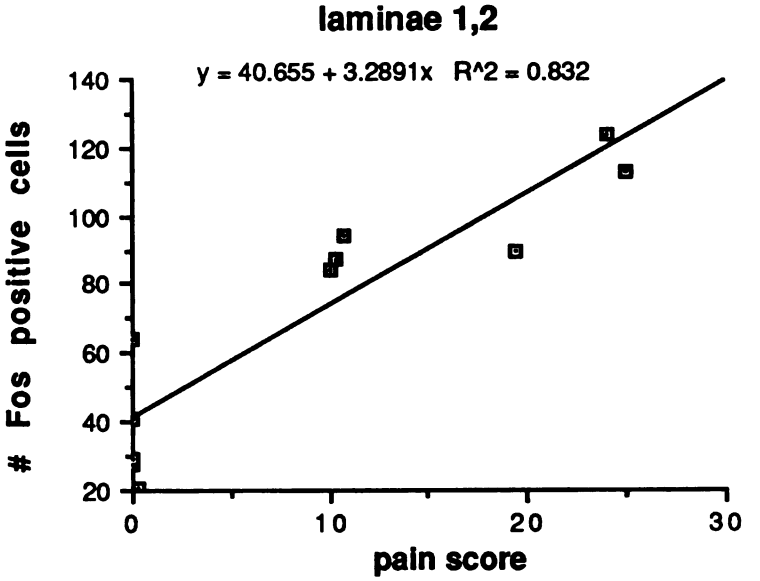
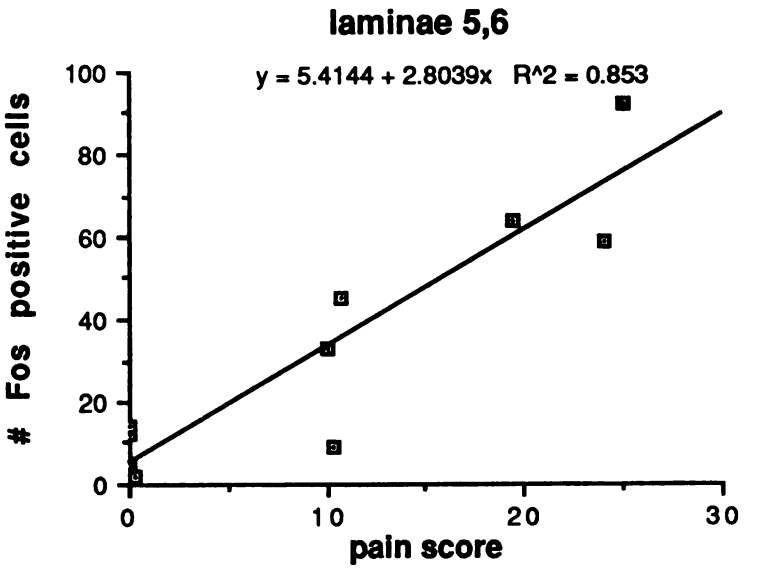
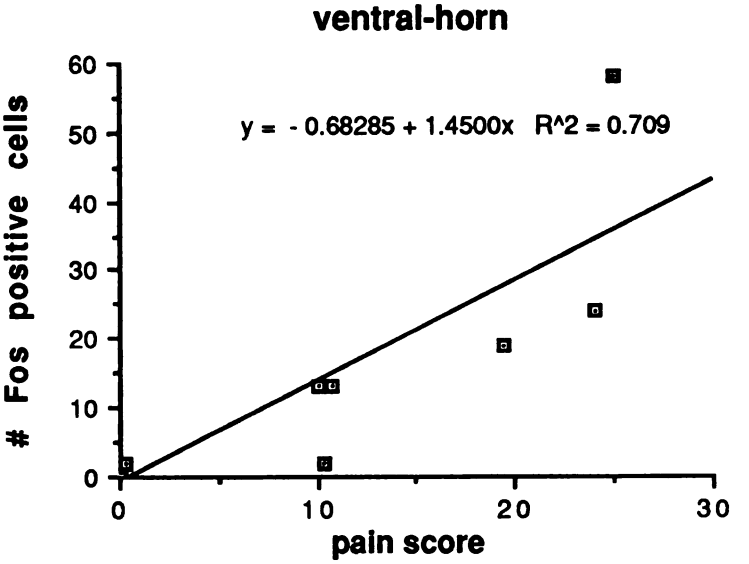

pain score
20
15
10
5
0
0.00
0.25
0.50
0.75
1.00
baclofen (ug)

Fig. 5. The positive linear relationship between pain behavior and the degree of Fos expression in the 3 different region of spinal cord but not in the laminae 3,4.


laminae 1,2
y = 40.655 + 3.2891x R^2 = 0.832
# Fos positive cells
pain score
0
10
20
30
40
60
80
100
120
140


laminae 5,6
y = 5.4144 + 2.8039x R^2 = 0.853
# Fos positive cells
pain score
0
10
20
30
40
60
80
100


ventral-horn
y = - 0.68285 + 1.4500x R^2 = 0.709
# Fos positive cells
pain score
0
10
20
30
40
50
60

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