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Comparison of the Distribution of *C-fos* With Primary Afferent Markers

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Abstract: The neuropeptides Substance P (SP) and Calcitonin gene related peptide (CGRP) were used as markers for peptidergic primary afferent fibres. A lectine, *Griffonia simplicifolia (Bandeiraea simplicifolia)* Isolectin-B4 (GSA I-B4), was used as a marker for non-peptidergic primary afferent fibres. C-Fos immunoreactivity was determined in the spinal cord of rats which had received 100% mustard oil application in the right hind limb. Darkly labelled c-Fos immunoreactive cells were concentrated in laminae I-IIo of the dorsal horn. SP and GSA I-B4 were distributed almost in the same regions. CGRP was more abundant in laminae I-IIo with some immunoreactive fibres in lamina IIi. These data suggest that a population of peptidergic and non-peptide primary afferents may play an important function in the induction of *c-fos* by mustard oil.

Key Words: *c-fos*, neuropeptides, lectin, primary afferent fibres, spinal cord.

Primer Afferent Marker'larla *C-fos*'un Dağılımının Karşılaştırılması

Özet: Neuropeptit olan substance P (SP) ve Calcitonin gene related peptid (CGRP) peptidergic afferent sinir telleri için marker olarak kullanıldı. Bir lectin olan *Griffonia Simplicifolia (Bandeiraea simplicifolia)* İsolectin-B4 non-pepdidergic primer afferent sinir telleri için marker olarak kullanıldı. Hardal yağının (%100) ratların sağ arka bacağına sürülmesinin sonucu medulla spinalislerinde c-Fos immunoreactivitesi tesbit edildi. Koyu c-Fos immunoreactiv sinir hücreleri kornu dorsalenin lamina I-IIo'de gözlendi. SP ve GSA I-B4 hemen hemen aynı laminalara dağıldığı görüldü. CGRP lamina I-IIo'da daha fazla miktarda idi. Bazı CGRP immunoreactiv fibers lamina IIi'de de görüldü. Bu bulgular hardal yağı tarafından aktive edilen c-fos'un uyarılmasında peptidergic ve non-peptidergic primer afferentlerin önemli rol oynadığını akla getirir.

Anahtar Sözcükler: *c-fos*, neuropeptidler, lectin, primer afferent neuronlar, medulla spinalis

Introduction

The heterogeneity of primary afferents has been clearly established from their morphology, biochemical characteristics and physiology. Small dorsal root ganglion cells which give rise to unmyelinated C-fibres and thinly myelinated Aδ fibres (1) contain a range of neurochemical markers. One major group of afferents contain peptides such as SP(2,3) and CGRP (4). Numerous studies demonstrate that these peptidergic afferents terminate most densely in lamina I and the outer part of lamina II (for SP (3,5), for CGRP (4,6). A second group of "non-peptide" (7) afferents contain GSA I-B4 binding sites or FRAP and terminate most densely in the deeper part of lamina II (5,8).

Physiological stimulation of the primary sensory neurones results in activation of the proto-oncogene *c-fos* in neurones of the dorsal horn in the corresponding topographic areas (9). In view of the differential distribution of primary afferents in the spinal dorsal horn the question arises as to whether a particular biochemically coded group of primary afferent C-fibres would activate *c-fos* expression in the spinal dorsal horn following mustard oil stimulation, which predominantly activates C-fibres polymodal with minimal activation of Aδ myelinated nociceptive afferents (10). Therefore a comparison of the distribution of c-Fos protein-positive neurones was carried out with small diameter primary afferent markers such as SP, CGRP and GSA I-B4.

Materials and Methods

Tissue preparation and sectioning:

Tissue for immunohistochemistry studies was obtained from adult rats of either sex (Wistar 300-450 body weight). Anaesthesia was induced with halothane

(Fluothane) 4% in air and then long-term anaesthesia was established with ethyl carbamate (Urethane) at a dose of 1000 mg/kg IP. The right hind limbs of the rats were then stimulated with noxious chemical stimuli which were produced by an application of 100% mustard oil to the plantar and dorsal surface of the limb with a paint brush. Three applications of the same concentration of mustard oil were made at 30-minute intervals. Two hours after the first application of mustard oil the animals were deeply anaesthetized with sodium-pentobarbitone (50-60 mg/kg IP) and heparinized (1000 U/rat intracardially), then, perfused via the left ventricle with 100-200 ml Kreb's solution. The vascular rinse was followed by fixation with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) with a volume of 300-500 ml.

The lumbar segments (L2-L5) of the spinal cord were removed from the vertebral column, postfixed in the same fixative for 2-3 h and cryoprotected with sucrose in PB at 4°C overnight. The tissue was transversally cut into 40 µm thick sections on a freezing microtome and collected in phosphate buffered saline (PBS). Due to loss of neuropeptide immunostaining in the rat superficial dorsal horn following stimulation of sciatic nerve at C-fibre intensity (11), sample blocks were taken from the same region of the spinal cord of the control animals which had received no stimulation, for the immunohistochemistry of peptides and non-peptides. All sections were processed free-floating.

Immunostaining:

Immunohistochemical analysis for c-Fos protein was carried out using an antibody to c-Fos protein (1:1000) (kindly provided by Dr. G. Evan) overnight at 4°C. This and subsequent antisera were diluted in PBS containing 0.5% Triton X-100 and 2.5% Bovine Serume Albumine. The sections were washed in PBS, pH 7.2, for 1 hour (four changes of buffer) following each incubation. They were then incubated in biotinilated anti-species antisera for 1-2 h at room temparature and in streptavidin conjugated horseradish peroxidase (HRP) (1:100) (Amersham) for 1 h at room temparature. A chromogen protocol (12) was used to reveal the distribution of HRP. Following staining, the sections were washed in PBS and distilled water and mounted on Chrome Alum Gelatine coded slides. Finally they were air dried, dehydrated and mounted in DPX. Omission of the primary antibody was used as a control in the staining procedure.

Immunohistochemistry for peptides and non-peptide was carried out using a similar protocol to that used for c-Fos histochemistry. The method was only modified for the lectin GSA I-B4, which was purhased already biotinilated. In this case following incubation with the lectine (5-10 mg/ml) (Sigma), the second-step incubation was with streptavidine HRP followed by the chromogen procedure. The working dilution for anti-SP (a gift from D.P.Keen) and anti-CGRP (a gift from Dr. P.K.Mulderry) was 1:1000.

Results

SP and CGRP immunoreactive fibres formed a dense network in the superficial laminae, in particular laminae I and II at the lumbar level of the spinal cord (Fig 1; A, B). SP immunoreactivity showed a very distinct band in laminae I-II. CGRP, similarly, was strongest in laminae I, II_o with some immunoreactive fibres in lamina II_i. GSA I-B4 binding was concentrated mainly in lamina II_o, although there was sparse staining in lamina I at the lumbar level of the spinal cord (Fig 1; C). Both the peptides and the lectin staining had the appearance of a dense band in these laminae. Application of 100% mustard oil to the left hindpaw produced many more labelled cells in laminae I-II_o with apparently fewer stained nuclei in lamina II_i. Some labelling in the deeper laminae of the dorsal horn (Fig 1; D) was also present. These data demonstrate that *c-fos* is expressed mainly in neurons in the region receiving the greatest density of small diameter primary afferent fibres following application of mustard oil to the skin.

Discussion

In agreement with a previous report (3), substance P immunoreactivity was found in lamina I and the outer part of lamina II. Bundles of fibres run through laminae I and II to innervate deeper parts of the dorsal horn. Additionally, many fibres were observed in the ventral horn and around the central canal. Substance P is also known to be present in some intrinsic neurons and in the descending fibres. The release of immunoreactive substance P in the spinal dorsal horn, particularly in laminae I and II, following peripheral noxious electrical and thermal stimuli (13,14) or following noxious mechanical stimuli (15) also indicates the importance of these laminae as a target for substance P. In view of the strong activation of *c-fos* following mustard oil stimulation in laminae I and II, and the activation of *c-fos* by intrathecal application of substance P, it might seem reasonable to assume that this peptide was mediating this response. However, from the distribution of NK-1 receptor, the principle target for substance P, it is apparent that the probable target neurons for this

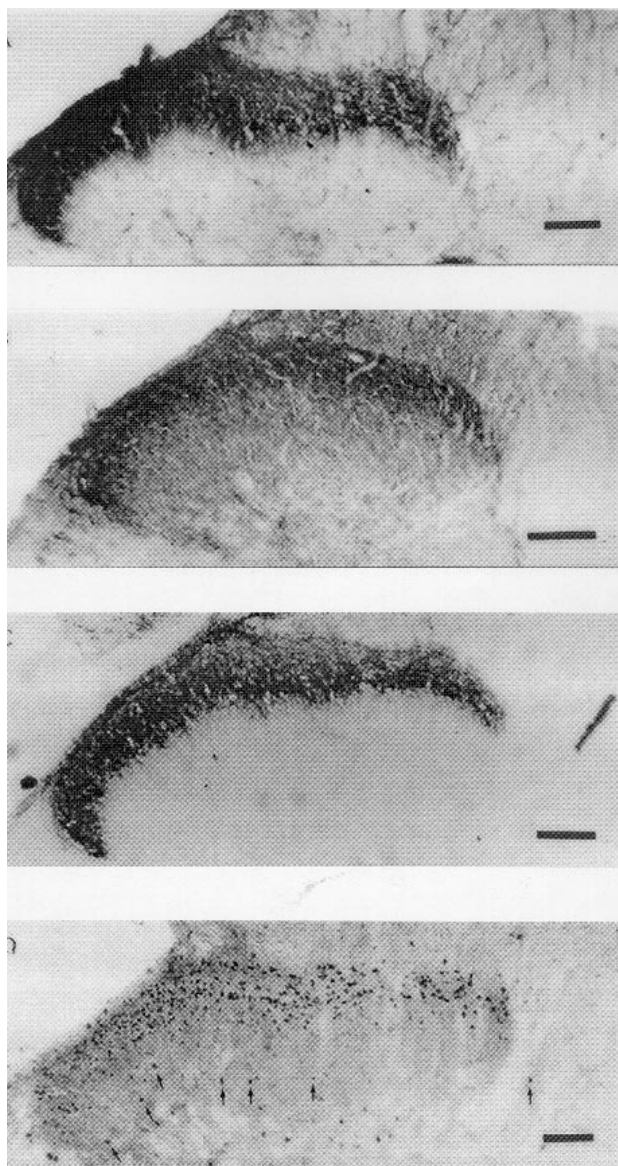

Figure 1. A comparison of the distribution of staining for c-fos-positive cells, SP, CGRP and GSA I-B4 lectin in the lumbar spinal cord. A: Sections from the lumbar cord were stained with antisera to SP, B: to CGRP and C: with GSA I-B4 lectin. D: the photograph illustrates the laminar pattern of c-Fos immunoreactivity in superficial dorsal horn of the lumbar cord produced by mustard oil (100%) ipsilateral to stimulation. C-Fos was concentrated mainly in laminae I-II_o. However, scatter-labelled cells can be seen in lamina IIi (arrowheads). SP and GSA I-B4 were found almost in the same regions. Scale bars: A= 100 µm, B= 100 µm, C= 100 µm, D= 100 µm.

peptide in the outer dorsal horn are in lamina I and deeper in laminae III-IV (16,17,18). Indeed very few neurons in lamina II respond to the application of neurokinin receptor agonists (16,19). This apparent mismatch between the distribution of substance P and its receptors has been studied in more detail (17). It appears that volume transmission probably accounts for some of the activation by substance P.

This raises the question of what mediates the induction of *c-fos* in lamina II cells following the peripheral application of mustard oil. In this study, again confirming previous reports (4,5,6), CGRP was found to be most dense in laminae I-II_o. CGRP is much more restricted in its distribution and is generally considered a good marker in the dorsal horn of the afferent terminals containing this peptide. However, most CGRP containing afferents also contain substance P and it would be tempting to conclude that this peptide has the same target cells as substance P. Radioligand CGRP binding in the trigeminal nucleus caudalis suggests that this is the case as the highest density of binding is found in laminae I and III-IV with little staining in lamina II (5).

The immunochemical co-existence of SP and glutamate in dorsal root ganglion cells (20) and the enhanced release of glutamate in the spinal cord following primary afferent stimulation produced by SP and CGRP (21) suggest an interaction between these peptides and glutamate ,which may be important in the activation of *c-fos*. SP also influences inward currents induced in dorsal horn neurons by activation of NMDA receptors (22). However, the absence of NK-1 receptors on lamina II neurons again suggests that the interaction of SP and glutamate is unlikely to be involved in this response. In lamina II neurons, NK-1 receptor agonists were also shown to have no effect on primary afferent evoked EPSPs mediated by AMPA and NMDA receptors (16). In conclusion, although the apparent correlation between peptidergic afferent and *c-fos* activation by noxious stimuli suggest the involvement of neuropeptides, more recent observations point to other mechanisms as well.

Lectins are constituted from proteins or glycoproteins and the isolated from plants and tissues of animals. Due to their selective affinity for carbohydrate residues, they have been widely used for identifying the expression of glucoconjugates in the nervous system as well as in other tissues. It has been observed that several lectins specifically bind to different subpopulation of neurons and glia (23). Isolectin GSA I-B4, a lectin which binds specifically to a terminal α-D-galactose, has been found to selectively bind to a subpopulation of small diameter primary afferents and their central terminals in the superficial layer of the spinal dorsal horn (24,25). Moreover, it is essentially a useful marker for the "non-

peptide" group (7) of the sensory C-fibres (26). Indeed, the present study demonstrated that the lectin staining was mainly restricted to lamina II, although there was sparse staining in lamina I. GSA I-B4 histochemical labelling of dorsal root ganglia neurons has revealed that the lectin localizes mainly to a subpopulation of small dark neurons (27) which have C-fibres (1). This may suggest that the GSA I-B4 containing afferents are nociceptive and could be reasonable candidates for inducing *c-fos*. However, many questions, such as what stimuli activate them? What transmitter do they release? What does IB4 label? Can they be selectively damaged?, concerned with their specific involvement in nociception still need to be answered. Afferents marked by GSA IB-4 and other markers such as FRAP terminate as glomerular terminals mainly in lamina II. Studies of the pharmacology of the C-fibre input to lamina II strongly suggest that these terminals release glutamate. This is further supported by the high level of the glutamate in most dorsal root ganglia neurons. Further evidence for this suggestion comes from the distribution of glutamate receptors in lamina II. In view of this, it seems probable that the activation of *c-fos* in lamina II neurons following mustard oil stimulation is due to the action of glutamate acting at one or more of its receptors.

References

1. Lawson, S.N: Morphological and biochemical cell types of sensory neurons. Sensory neurons, Diversity, Development, and Plasticity. Oxford University, New York-Oxford, 1992, pp. 27-59.
2. Dalsgaard, C.J., Haegerstrant, A., Theodorsson-Norheim, E., Brodin, E. Hökfelt, T. Neurokinin A-like immunoreactivity in rat primary sensory neurons: coexistence with substance P. Histochemistry 83: 1985, 37-39.
3. Hökfelt, T., Elde, R., Johansson, O., Luft, R., Nilsson, G. & Arimura, A. Immunohistochemical evidence for separate populations of somatostatin-containing and substance P-containing primary afferent neurons in the rat. Neuroscience 1: 1976, 131-136.
4. Rosenfelt, M.G., Mermod, J.J., Amara, S.G., Swanson, L.W., Rivier, J., Vale, W.W. & Evans, R.M. Production of a novel neuropeptide encoded by calcitonin gene via tissue specific mRNA processing. Nature 304: 1983, 129-135.
5. Hunt, S.P., Mantyh, P.W. & Priestley, J.V: The organisation of biochemically characterized sensory neurons. Sensory neurons, Diversity, Development, and Plasticity, Oxford University, New York-Oxford. 1992, pp. 60-76.
6. Kruger, L., Manthy, P.W., Sternini, C., Brecha, N.C. & Manthy, C.R. Calcitonin gene-related peptide (CGRP) in the rat central nervous system: patterns of immunoreactivity and receptor binding sites. Brain Res 463: 1988, 223-244.
7. Hunt, S.P. & Rossi, Peptide- and non-peptide-containing unmyelinated primary afferents: The parallel processing of nociceptive information. Phil Trans Roy Soc Biol Sci 308: 1985, 283-289.
8. Scott, S.A., Patel, N. & Levine, J.M. Lectin binding identifies a subpopulation of neurons in chick dorsal root ganglia. J Neurosci 10 (1): 1990, 336-345.
9. Hunt, S.P., Pini, A. & Evan, G. Induction of c-fos-like protein in spinal neurons following sensory stimulation. Nature 328: 1987, 632-634.
10. Woolf, C.J. & Wall, P.D. Relative effectiveness of C-primary afferent fibres of different origins in evoking a prolonged facilitation of the flexor reflex in the rat. J Neurosci 6 (5): 1986, 1433-1442.
11. Klein, C.M., Coggeshall, R.E., Carlton, S.M. & Sorkin, L.S. The effects of A- and C-fibre stimulation on patterns of neuropeptide immunostaining in the rat superficial dorsal horn. Brain Res. 580: 1992, 121-128.
12. Shu, S., Ju, G. & Fan, L. The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. Neurosci Lett 85: 1988, 169-171.
13. Duggan, A.W., Morton, C.R., Zhao, Z.Q. & Hendry, I.A. Noxious heating of the skin releases immunoreactive substance P in the substantia gelatinosa of the cat: a study with antibody microprobes. Brain Res 403: 1987, 345-349.
14. Duggan, A.W. & Hendry, I.A. Laminar localization of the sites of the release of immunoreactive substance P in the dorsal horn with antibody-coated microelectrodes. Neurosci Lett 68: 1986, 134-140.
15. Kuraishi, Y., Hirota, N., Sato, Y., Hino, Y., Satoh, M. & Takagi, H. Evidence that substance P and somatostatin transmit separate information related to pain in the spinal dorsal horn. Brain Res 325: 1985, 294-298.
16. Bleazard L., Hill G. & Morris R. The correlation between the distribution of the NK-1 receptor and the action of tachykinin agonist in the dorsal horn of the rat indicates that SP does not have a functional role on substantia gelatinosa (lamina II) neurons. J Neurosci 14 (12): 1994, 7655-7664.

17. Liu, H., Brown, J.C., Jasmin, L., Maggio, J.E., Vigna, S.R., Manthy, P.W. & Basbaum, A.I. Synaptic relationship between SP and the substance P receptors: Light and electron microscopic characterization of the mismatch between neuropeptides and their receptors. Proc Natl Acad Sci 91: 1994, 1009-1013.

18. Moussaoui, S.M., Le Prado, N., Bonici, B., Faucher, D.C., Cuine, F., Laduron, P.M. & Garret,C. Distribution neurokinin B in rat spinal cord and pheripheral tissues: comparison with neurokinin A and substance P and effects of neonatal capcaicin treatment. Neuroscience 48: 1992, 969-978.

19. Yoshimura, M., Shimizi, T., Yajiri, Y., Inokuchi, Y. & Nishi, S. Primary afferent-evoked slow EPSPs and responses to substance P of dorsal horn neurons in the adult rat spinal cord slices. Reg Peptides 46: 1993, 407-409.

20. Battaglia, C.G. & Rustioni, A. Coexistence of glutamate and substance P in dorsal root ganglion neurons of the rat and monkey. J Comp Neurol 277: 1988 302-312.

21. Kangrga, I. & Randic, M. Tachykinin and calcitonin gene related peptide enhance release of endogenous glutamate and aspartate from the rat spinal dorsal horn slice. J Neurosci 10: 1990, 2026-2038.

22. Randic, M., Hecimovic, H. Ryu, P.D. Substance P modulates glutamate-induced currents in acutely isolated rat spinal dorsal horn neurons. Neurosci Lett 117: 1990 74-80.

23. Ambalavanar, R: Lectin binding to primary afferents of the rat trigeminal system. Ph. D. thesis, Department of Veterinary Preclinical Sciences, University of Liverpool, U.K. 1991.

24. Silvermann, J.D. & Kruger, L. Lectin and neuropeptide labelling of separate populations of dorsal root ganglion neurons and associated "nociceptor" thin axons in rat testis and cornea whole-mount preparations. Somatosensory Res 5 (3): 1988, 259-267.

25. Streit, W.J., Schulte, B.A., Spicer, S.S. & Balentina, J.D. Lectin histochemistry of the rat spinal cord. J Histochem Cytochem 32: 1984, 909.

26. Ambalavanar, R. & Morris, R. An ultrastructural study of the binding of an á-D-Galactose specific lectin from Griffonia Simplicifolia to trigeminal ganglion neurons and the trigeminal nucleus caudalis in the rat. Neuroscience 52: 1993, 699-709.

27. Lynn, B. & Carpenter, S.E. Primary afferent units from the hairy skin of the rat hind limb. Brain Res 238: 1982, 29-43.