Immunohistochemical Studies of Peptidergic Neurons in the Dorsal Horn of the Spinal Cord

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The indirect immunofluorescence technique was used to localize substance P, somatostatin, methionine-enkephalin, neurotensin, and oxytocin in the dorsal horn of the rat spinal cord. The unique distribution of each peptide is described and the relative amount of each peptide in laminae I–III of the dorsal horn and the dorsal part of the lateral funiculus qualitatively assessed. Colchicine treatment and dorsal rhizotomy were used to determine, in part, the origin of immunoreactive fibers and terminals observed in the dorsal horn. KEY WORDS: Neuropeptides; Dorsal horn; Spinal cord.

Introduction

The chemical characterization of biologically active peptides from brain extracts has led to the discovery of neural circuitry in many regions of the central and peripheral nervous system that may utilize such peptides to mediate interneuronal communication. The dorsal horn of the spinal cord has proved to be an area rich in peptidergic components. Immunohistochemical and physiological analysis of peptides in the dorsal horn has led to new hypotheses concerning neural processing of noxious stimuli. These hypotheses propose an important role for substance P, somatostatin, and enkephalin (6-8) as well as neurotensin (13) in nociceptive primary afferent neurons and spinal circuits. The purpose of the present report is to describe and compare the distribution of several peptides in the dorsal horn of the spinal cord of the rat.

Materials and Methods

Animals and treatments. Male albino rats (250-300 g body weight) were used in this survey. Four untreated animals were studied as controls. Four animals received 50 μg colchicine (free base) in 20 μl of phosphate-buffered saline (PBS) by a cannula inserted through the cisterna magna into the subarachnoid space (14). The cannula reached to the lumbar region of the cord after insertion. Spinal laminectomies were performed on eight rats at the lumbar region of the cord with insertion of the cisterna magna. The vascular system was rinsed with 50 ml of calcium-free Tyrodes solution before perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 min. The spinal cord and dorsal root ganglia were then removed, postfixed by immersion in the same fixative for 90 min, and rinsed in 5% sucrose in 0.1 M phosphate buffer for 24 hr. All solutions in this procedure were ice-cold. Ten-micron transverse and longitudinal sections were cut through lumbar, thoracic, and cervical portions of the spinal cord with a cryostat.

Immunofluorescence. Tissue sections were processed for indirect immunofluorescence microscopy according to Coons (1). Sections were incubated with the primary antisera overnight at 4°C in a humid atmosphere. After rinsing in PBS, the sections were then incubated with fluorescein-isothiocyanate (FITC)-conjugated sheep anti-rabbit serum (diluted 1:8) at 37°C for 30 min, rinsed with PBS, cover-slipped with a mixture of PBS and glycerine (1:3) and examined in a Zeiss fluorescence microscope.

Antisera. Antisera to the following peptides were used in this survey in the stated dilutions: substance P (1:100), somatostatin (1:100), methionine–enkephalin (1:100), neurotensin (1:50), and oxytocin (1:100). All antisera were raised in rabbits and diluted in PBS containing 0.3% Triton X-100. Specificity of antisera was confirmed by preabsorption of binding with 50 μg homologous peptide/ml of diluted serum.

Results

This survey is limited to the distribution of peptides in the dorsal horn of the spinal cord. In the following text lamina I is equivalent to the marginal zone and laminae II and III are equivalent to substantia gelatinosa.

Substance P (SP)

In transverse sections SP immunoreactivity appears densely distributed in cross sections of fibers and terminals in lamina I through III of the dorsal horn (Figure 1A). Staining in this region was intense throughout the longitudinal extent of the cord, but was noticeably stronger in the lumbar portion. SP positive fibers were also apparent...
in cross section in the lateral part of the dorsal root entry zone. Bands of immunoreactive transverse fibers were consistently observed in lamina I, while single fibers were seen projecting toward deeper layers of the dorsal horn and running along its medial aspect. Loosely interwoven bundles of SP positive fibers were found in the dorsal part of the lateral funiculus (dLF). Interestingly, this pattern appeared much the same in longitudinal sections. SP positive fibers in laminae I through III of the dorsal horn appeared short and varicose in longitudinal sections.

**Somatostatin (SOM)**

In comparison to SP, SOM immunoreactivity occurred in lamina II and III, but was lacking in lamina I (Figure 1C). Furthermore, the density of terminals observed with the SOM antiserum was much less than that observed for SP. As with SP, SOM immunoreactivity in the dorsal horn was strongest at the lumbar level of the spinal cord. SOM immunoreactivity in the dLF was as intense as that of SP and occurred in the same pattern.
Methionine-Enkephalin (M-ENK)

The intensity of M-ENK staining in the dorsal horn appeared equal throughout the three levels of the spinal cord surveyed. Within the dorsal horn, the densest distribution of M-ENK immunoreactivity occurred over the marginal zone where the staining of transverse fiber bundles enhances the apparent density of this zone (Figure 1B). M-ENK immunoreactivity in laminae II and III is sparse and less than that of SOM. However, M-ENK immunoreactivity in the dLF appeared equal to that of SOM and SP.

Neurotensin

In transverse sections, neurotensin has a similar distribution to SOM in the dorsal horn of the spinal cord in that it is concentrated in laminae II and III (Figure 1F). However, the apparent density of neurotensin terminals was less than that of SOM. Interestingly, neurotensin immunoreactivity was more obvious in longitudinal sections where short varicose fibers appeared concentrated in lamina II (Figure 1E). Unlike the above-discussed peptides, neurotensin positive fibers were not present in the dLF.

Oxytocin (OT)

This peptide was present in only a few transversely oriented fibers, making it difficult to see in transverse sections. In longitudinal sections, however, very scattered, fine OT positive fibers were observed in the dLF, with collaterals projecting into deeper layers of the dorsal horn (Figure 1D).

Effect of Dorsal Rhizotomy and Proximal Transection

Whereas the distribution of SP, SOM, M-ENK, and neurotensin in the dorsal horn did not appear to be affected by proximal transection of the spinal cord, dorsal rhizotomy resulted in near depletion of SP and SOM from laminae I through III. Neurotensin and M-ENK immunoreactivity did not appear altered.

Effect of Colchicine Treatment

Cell bodies of peptidergic neurons in the spinal cord and dorsal root ganglia were not seen in untreated animals. After colchicine treatment, though, sparsely distributed immunofluorescent cell bodies were observed in the dorsal horn and dLF. M-ENK positive cell bodies were consistently seen in the marginal zone and in deeper layers of the dorsal horn; neurotensin cell bodies were observed in lamina II and the dLF. Occasionally, SOM immunoreactive cell bodies were observed in lamina II, and SP cell bodies appeared in deeper layers of the dorsal horn. Interestingly, SOM, SP, and M-ENK immunoreactivity in terminals in the dorsal horn and dLF was markedly decreased after colchicine treatment. Furthermore, the intensity of staining for SP was altered more in lamina I than in laminae II and III.

Dorsal root ganglia studied from colchicine-treated animals revealed small SP and SOM positive cell bodies. M-ENK and neurotensin cell bodies were not observed in dorsal root ganglia.

Discussion

The distribution of substance P, somatostatin, methionine–enkephalin, and neurotensin reported in this study largely confirms the work of Hökfelt and co-workers (4–6) and others (2, 13). One aspect of the significance of these peptides in laminae I through III of the dorsal horn is that they can serve as cell markers that will identify subpopulations of primary afferent fibers and interneurons and allow a more detailed appreciation of the interconnections in the dorsal horn that process nociception and analgesia.

Localization of substance P and somatostatin in dorsal root ganglion cells and the disappearance of these substances from the spinal cord after dorsal rhizotomy are evidence that substance P and somatostatin are contained in primary afferent neurons. In addition, there are reports that suggest these peptides may have a transmitter role. Electrophysiological studies have shown that substance P and somatostatin are released from terminals in the spinal cord after stimulation of dorsal roots (8, 10), and iontophoretic application of these peptides onto neurons that respond to noxious peripheral stimulation has revealed that substance P is excitatory (3, 11) while somatostatin is inhibitory (12) to these cells. The significance of somatostatin’s inhibitory action in nociception is not understood at this time.

The occurrence of met-enkephalin cell bodies and terminals within the dorsal horn suggests that neuronal circuitry capable of producing analgesia exists at the level of the spinal cord (6). Results of lesion studies and treatment with colchicine are evidence that met-enkephalin is contained in interneurons within the dorsal horn. The possible analgesic role of endogenous met-enkephalin at the level of the spinal cord is supported by the work of Yaksh and Rudy (15), which demonstrated that intrathecal administration of morphine into the lumbar level of the spinal cord produced dose-dependent elevation in nociceptive thresholds in rats. However, neurochemical substances which regulate met-enkephalin release at the level of the spinal cord remain to be elucidated.

One site of action of met-enkephalin within the dorsal horn may be primary afferent nerve terminals. The existence of opiate receptors on these terminals has been demonstrated by two different approaches. Using an opiate receptor binding assay, LaMorte and co-workers (9) showed that dorsal rhizotomy results in a significant reduction in the number of opiate receptors in laminae I through III of the dorsal horn where small diameter primary afferent neurons terminate. In addition, met-enkephalin has been reported to specifically block potassium-stimulated substance P release from synaptosomes prepared from the spinal trigeminal nucleus (7).

Thus, peptides are emerging as potential transmitters in nociceptive circuitry at the level of the spinal cord, both in the transmission of painful stimuli and in modulation of neuronal activity in primary afferent neurons to regulate nociception. Where neurotensin and oxytocin will fit into this circuitry remains to be resolved, but it is certain that immunohistochemistry will be a valuable tool in elucidating and confirming the interconnections processing nociception and analgesia.

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Literature Cited
