Immunohistochemical analysis of peptide pathways possibly related to pain and analgesia: Enkephalin and substance P

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ABSTRACT The distribution of Met-enkephalin- and substance P-immunoreactive neurons was studied by indirect immunofluorescence in some areas related to pain and analgesia. Met-enkephalin- and substance P-positive cell bodies and nerve terminals were observed in the peri-aqueductal central gray, the nucleus raphe magnus, the marginal layer and substantia gelatinosa of the spinal trigeminal nucleus, and the dorsal horn of the spinal cord. Lesion experiments suggest that Met-enkephalin neurons in the dorsal horn and possibly in the spinal trigeminal nucleus are interneurons or propriospinal neurons with nerve terminals in the laminae I and II of the cord and in the superficial layers of the spinal trigeminal nucleus, respectively. These areas are also very rich in substance P-positive nerve terminals, mainly representing central branches of primary afferent neurons.

The present immunohistochemical-anatomical findings support the hypothesis that stimulation-produced analgesia is related to activation of spinal and spinal trigeminal enkephalin interneurons forming axo-axonic synapses with (substance P) pain afferents in the superficial laminae of the dorsal horn and the spinal trigeminal nucleus. These interneurons may be activated by sensory fibers and by descending fibers from medullary stimulation sites. Transmitter substances in these descending fibers may be 5-hydroxytryptamine and substance P.

Morphine is a classical agent for the relief of severe, chronic pain. Strong analgesia can also be produced by electric stimulation of mesencephalic and medullary structures (refs. 1-4, see ref. 10), by acupuncture, or by “electroacupuncture” (11, 12), a modification of the classical Chinese procedure. Several observations suggest a common element in these approaches for pain relief. For instance, narcotic antagonists, such as naloxone (13), block analgesia induced by electrical stimulation (6-9, 14) and by morphine injections (15). Furthermore, tolerance develops to electrostimulation analgesia and to morphine analgesia and cross tolerance can be observed (16).

Recently, Hughes and collaborators (17) have identified two pentapeptides, Leu-enkephalin and Met-enkephalin, that may represent endogenous ligands for opiate receptors. Available evidence suggests that the activity profile of endogenous opioids is similar to that of the opiates. For instance, enkephalin has analgesic activity in vivo and the narcotic antagonist naloxone will also counteract enkephalin-induced elevation of pain thresholds (18, 19). It is therefore conceivable that these ligands may be involved in stimulation-produced analgesia.

Using the immunohistochemical approach, we have been able to outline the gross distribution of enkephalin-positive structures in the central nervous system (refs. 20 and 21, see also ref. 22). Enkephalin neurons were found in many areas of the brain and spinal cord, indicating involvement in many different types of central nervous system functions. Here, however, we focus attention strictly on areas assumed to be of importance.

MATERIAL AND METHODS

Male albino rats (Sprague-Dawley, body weight 150 g) were divided into three groups: (i) untreated rats, (ii) rats receiving an injection of 10 or 25 μg of colchicine dissolved in 0.9% (wt/vol) saline (1 μg/μl) into the lateral ventricle or the cisterna magna, and (iii) rats receiving an intraspinal injection of 20 μg of colchicine dissolved in 0.9% saline (10 μg/μl) at the lumbar-sacral level. The colchicine-treated animals were sacrificed 24-48 hr after the injection.

We have previously observed widespread endorphin-positive nerve terminals whereas no immunoreactive cell bodies could be detected (20). Opioid peptides have been used in other studies to increase cell body levels of catecholamines (27), probably due to inhibition of axonal transport.

Antiserum to Met-enkephalin was raised in rabbits as described elsewhere (20). Crossreactivity was about 10% with Leu-enkephalin and <0.1% with α-, β-, and γ-endorphins, substance P, and somatostatin (L. Tenerius, unpublished results). Furthermore, because Met-enkephalin occurs in amounts 3-4 times greater than those of Leu-enkephalin in rat brain (28), we ascribe most of the immunofluorescence to Met-enkephalin. Antiserum against substance P was raised in rabbits as described previously (29).

The rats were perfused with formalin. After being rinsed, the brain and spinal cord were cut on a cryostat (Dittes, Heidelberg) and processed for the indirect immunofluorescence technique (see ref. 50). Series of four consecutive sections at different levels of the mesencephalon, lower brain stem, and spinal cord were incubated for 30 min in a humid atmosphere at 37° with antiserum to Met-enkephalin (diluted 1:10 or 1:20), substance P (diluted 1:40), and the two control sera, respectively. The control sera, in which the Met-enkephalin and substance P antibodies were blocked with the respective peptide (50 μg/ml of serum diluted 1:10), were included to establish the specificity of the staining. After a rinse in phosphate-buffered saline, the sections were incubated with fluorescein isothiocyanate-conjugated antibodies (SBL, Stockholm; diluted 1:4) under the same conditions as described above, rinsed in...
phosphate-buffered saline, mounted in phosphate-buffered saline/glycerol, 1:3 (vol/vol), and examined in a Zeiss fluorescence microscope. The anatomical nomenclature is according to Palkovits and Jacobowitz (31).

**RESULTS**

In untreated rats, extensive networks of Met-enkephalin- and substance P-positive nerve terminals were observed in the periaqueductal central gray and in the marginal layer and central canal. (Bar indicates 50 μm.) Anatomic sites: dorsal horn of the spinal cord (A, B); nucleus raphe magnus (C); intermediate gray matter of the sacral spinal cord (D–F). Treatments: untreated (A, B); colchicine-treated (C–F). Sera: antiserum to substance P (A, C, E); antiserum to Met-enkephalin (B, D); enkephalin control serum (F). A and B and D, E, and F are consecutive sections, respectively. A high density of substance P-positive nerve terminals was seen in laminae I and II of the dorsal horn (A). The rectangle in A indicates approximately the area shown in B. The enkephalin-positive nerve terminals had a parallel but less dense distribution as seen at the higher magnification (B). Note lack of enkephalin-positive fibers in the zone of Lissauer (arrow in B) whereas numerous substance P-positive fibers are present in this area (arrow in A). Numerous substance P-immunoreactive cell bodies were seen in the nucleus raphe magnus (C) after colchicine treatment. At the sacral level both enkephalin- (arrows in D) and substance P-positive (arrows in E) cell bodies were seen after intraspinal colchicine injections. Note the networks of enkephalin- (D) and substance P-positive (E) nerve terminals. No fluorescent neurons or nerve terminals were seen after incubation with control serum (F).
sustantia gelatinosa of the spinal trigeminal nucleus. It is important to note that both enkephalin- and substance P-positive fibers were present only in the caudal parts of the spinal trigeminal nucleus and that there was a striking overlap between the distribution patterns of the nerve terminals containing these two peptides. In the medullary raphe nuclei (nucleus raphe magnus and nucleus raphe pallidus), low to moderate concentrations of substance P-positive and Met-enkephalin-positive nerve terminals were observed. In the spinal cord, the highest concentrations were observed in laminae I and II (32) (Fig. 1 A and B) with moderately dense networks in laminae IV–VII, the ventral horn, and the area around the central canal. In the first two laminae, there were considerably more substance SP-positive than Met-enkephalin-positive fibers. Whereas the zone of Lissauer contained numerous cross-sectioned substance P-positive fibers, no immunoreaction with Met-enkephalin antiserum could be observed. No Met-enkephalin- or substance P-immunoreactive cell bodies could be observed in any of these areas.

In colchicine-treated rats, numerous Met-enkephalin- and substance P-positive cells were seen in addition to nerve terminals. Generally, the immunofluorescence of nerve terminals, especially the enkephalin-positive ones, appeared less strong and distinct in colchicine-treated rats compared to untreated rats.

Met-enkephalin- and substance P-immunoreactive cell bodies were observed in the ventrolateral parts of the periaqueductal central gray and in the raphe magnus (Fig. 1C) and raphe pallidus nuclei. In the spinal trigeminal nucleus, many Met-enkephalin-positive cell bodies were observed in the marginal layer of its caudal part whereas substance P-positive cells were observed only occasionally. Numerous Met-enkephalin- and substance P-immunoreactive cell bodies were observed in the spinal cord (Figs. 1 D and E and 2). They were present mainly in laminae I–V. There were more Met-enkephalin-positive than substance P-positive cells in the dorsal horn of the spinal cord. Strongly fluorescent substance P-positive axons were present in the caudal medulla oblongata ventromedially to the decussatio pyramids. In this area, substance P-positive cells were also observed. The results described above are summarized in Fig. 3.

Met-enkephalin- and substance P-positive cell bodies, axons, and nerve terminals were observed also in many other areas of the central nervous system. A full account of the localization of these neurons will be given elsewhere.

None of the immunoreactive structures described above were observed after incubation with control sera (Fig. 1F).

DISCUSSION

There is evidence that morphine and electrical stimulations of some brain loci and of peripheral nerves produce analgesia via the same type of receptors and that the enkephalins represent important endogenous ligands for these receptors. The present findings demonstrate enkephalin-positive nerve terminals in all areas where morphine is known to produce behavioral analgesia, including the periaqueductal gray, the medullary raphe nuclei, the caudal trigeminal nucleus, and the spinal cord. It has been inferred from several studies that the ultimate site of action of midbrain stimulation would in fact be the spinal trigeminal nucleus or the spinal cord (refs. 5 and 33–36, see ref. 10). It is possible that, in electroacupuncture, activation of endorphin systems also occurs at the segmental level because this increases spinal cerebrospinal fluid levels of endorphins (37).

Our findings indicate that at the spinal level the enkephalin system is either interneuronal or propriospinal. Both enkephalin-positive terminals and cell bodies were found in the dorsal horn. Neither transection of the cord at the thoracic level nor unilateral dorsal rhizotomy at the lumbar and sacral level produced any marked changes in the concentration of enkephalin-positive nerve terminals in the lumbar cord (38). The presence of enkephalin nerve terminals and enkephalin cell bodies in the marginal layers and substantia gelatinosa of the spinal trigeminal nucleus may indicate the existence of enkephalin interneurons also in this nucleus. It is suggested that activation of enkephalin interneurons in the spinal trigeminal nucleus and in the dorsal horn is the last and critical step in stimulation-produced analgesia. This finding supports con-
Conclusions drawn by LaMotte et al. (39). They demonstrated high opiate receptor binding in the superficial laminae of the dorsal horn and a marked decrease in the binding after dorsal rhizotomy. They suggested presynaptic localization of the opiate receptors in primary sensory afferents. Enkephalin neurons may therefore form axo-axonic synapses (see refs. 26 and 40) on primary afferents, possibly on substance P afferents. The remarkable overlap of enkephalin and substance P nerve terminal fields in the superficial layers of the spinal trigeminal nucleus and in laminae I and II of the dorsal horn is striking.

Which central pathways may be involved in the activation of spinal enkephalnergic neurons? Both the periaqueductal central gray and medullary raphe nuclei are effective stimulation sites for producing analgesia, suggesting that descending systems (41) must be involved. From the periaqueductal central gray, descending projections to the spinal cord have been described in the cat (42) but so far not in the rat. From the medullary raphe nucleus of the rat, on the other hand, Brodal et al. (43) have demonstrated direct projections to the cord. In a combined neuroanatomical and electrophysiological study, Basbaum et al. (35, 36) analyzed the descending system involved in stimulation-produced analgesia (see also ref. 13). The cell bodies are located in the nucleus raphe magnus and the axons appear to run in the dorsolateral part of the lateral funiculus, terminating in the dorsal horn. Basbaum et al. (35, 36) suggested, on the basis of preliminary experimental evidence, that there is a circuitry from the periaqueductal central gray to the spinal cord via the nucleus raphe magnus.

With regard to the neurohumoral substrate of the medullary descending system, both morphological evidence (44) and pharmacological analysis (45, 46) suggest that a 5-hydroxytryptamine projection may represent one link between the stimulation site and the spinal cord. The present study demonstrates both enkephalin- and substance P-positive cell bodies in these nuclei. Whereas no evidence for descending enkephalin neurons exist, descending substance P pathways have in fact been demonstrated (38) and it may be that substance P pathways represent part of the system related to stimulation-produced analgesia described by Basbaum et al. (35, 36). Because substance P appears to exert an excitatory action (47), it could activate the enkephalin interneurons of the dorsal horn. Interestingly, intraventricular or systemic administration of substance P has in fact been found to produce naloxone-reversible analgesia (48, 49). 5-Hydroxytryptamine, on the other hand, appears mainly to be inhibitory and the analgesic effect mediated by descending 5-hydroxytryptamine systems has been ascribed to an axo-axonic influence on primary afferent nerve terminals in the dorsal horn (50).

Primary afferent neurons may also be involved in activation of enkephalin interneurons. However, the type of primary afferent neurons activated in classical acupuncture or electroacupuncture is not known, and we can only speculate about the primary afferent fibers that might activate the enkephalin neurons. There is now strong biochemical (51), neurophysiological (47), and immunohistochemical (25) evidence that substance P is present in some primary sensory neurons. Other primary afferent neurons may contain a somatostatin-like peptide (52). The substance P neurons have small cell bodies and fine-caliber axons with peripheral branches in most tissues. Their central branches terminate mainly in laminae I and II but probably also in deeper laminae of the dorsal horn. This distribution and these morphological characteristics are interesting in view of the results of Christensen and Perl (53) who found that cells responding to noxious stimuli are located mainly in lamina I. Furthermore, they may be considered to add further support for the view expressed by Henry (54) that substance P "may have an excitatory role in central transmission in spinal pain pathways acting...possibly at the first afferent synapse."
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