Opiates and Pain Pathways: Demonstration of Enkephalin Synapses on Dorsal Horn Projection Neurons

Abstract. *The participation of the opiate peptide enkephalin in the neural circuitry of the dorsal horn was examined at the light and ultrastructural level through the use of the combined techniques of immunocytochemistry and retrograde transport of horseradish peroxidase. Enkephalin immunoreactive axonal endings made direct synaptic contact with the soma and proximal dendrites of dorsal horn thalamic projection neurons. This observation demonstrates that one major synaptic site of enkephalin modulation of the transfer of nociceptive information in the dorsal horn is on the projection neurons themselves.*

Enkephalin, an opiate peptide that may represent one of the natural ligands of opiate receptors, can be localized to several different laminae in the medullary and spinal dorsal horns (MDH and SDH) (*1–3*). Several studies have demonstrated that enkephalin mediates inhibition of the response of neurons to noxious stimulation as well as inhibition of the behavioral response to painful stimuli (*4, 5*). An understanding of the neural circuitry accessed by enkephalin is thus critical to the elucidation of the mechanisms of pain and analgesia. Furthermore, demonstration of the site of action of enkephalin in the dorsal horn will provide insights into the localization of opiate receptors.

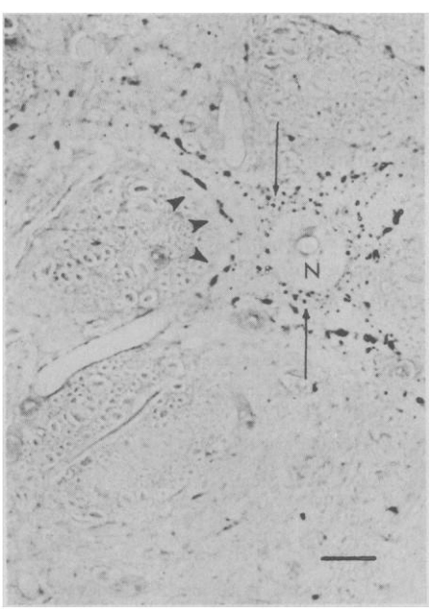

Fig. 1. Phase contrast photomicrograph of a 1-μm toluidene blue stained plastic section of a retrogradely labeled MDH neuron from lamina V. The section plane passes through the cell soma at a point where a prominent nucleolus is seen in the nucleus (*N*). The granular retrograde HRP reaction product (arrows) is found in the cytoplasm, but not in the nucleus. This thalamic projection neuron is surrounded along its soma and proximal dendrites by enkephalin immunoreactive varicosities (arrowheads). Since the section plane passes through the cell, the labeled endings line the edge of the cell, and the retrograde HRP is clearly within the cell. Bar represents 10 μm.

Biochemical evidence suggests a presynaptic site of opiate receptors on primary afferent axons (*6*), while physiological studies present evidence for a postsynaptic site of action of opiates in the dorsal horn (*5, 7*). Studies of cell cultures provide evidence for both a presynaptic and postsynaptic action of opiates (*8*). Ultrastructural studies of enkephalin localization in the dorsal horn demonstrate that enkephalin axons synapse primarily on intrinsic dorsal horn neurons (*9*). This study examines potential synaptic interactions between thalamic projection neurons (TPN's) and enkephalin-containing axonal endings by combining the techniques of immunocytochemistry for enkephalin and retrograde transport of horseradish peroxidase (HRP) to label TPN's. The TPN's represent a distinct population of dorsal horn neurons (*10*), most of which respond to noxious stimulation (*11*). The relationship of TPN's to the opiate system is thus important in that these neurons are responsible for the transmission of noxious input from the dorsal horn to higher centers of the neuraxis.

Several large injections of 50 percent solution of HRP were made into the thalamus of four adult cats. After a survival time of 68 to 72 hours, the brain and spinal cord were fixed by vascular perfusion with 4 percent paraformaldehyde and 0.2 percent glutaraldehyde in 0.1*M* phosphate buffer, *p*H 7.4. Transverse sections of the medulla and spinal cord were cut with a vibratome. The thalamus was sectioned on a freezing microtome. To identify the injection site and the retrogradely labeled neurons, the tissue was processed with cobalt chloride intensified diaminobenzidine (*12*), which produces a black reaction product. The injection sites centered around the ventral posterior complex of the thalamus and did not spread across the midline or caudal to the magnocellular part of the medial geniculate body. Sections containing the retrogradely labeled neurons were processed for enkephalin by the peroxidase-antiperoxidase (PAP) immunocytochemical staining method of Sternberger (*13*) to produce red-brown labeled profiles. The antiserum to Leu-enkephalin shows appreciable cross-reactivity with Met-enkephalin, but no cross-reactivity with β-endorphin (Immunonuclear). As a control, the primary antiserum was serially diluted and some sections were incubated with antiserum to enkephalin that had been absorbed with an excess of antigen (Boehringer-Mannheim). Results of all control procedures were negative.

After the immunocytochemical procedure was completed, sections from the MDH and cervical and lumbar SDH were cleared in glycerin and examined with the light microscope for the presence of red-brown immunocytochemically labeled profiles adjacent to neurons retrogradely labeled with blackened HRP granules. For electron microscopy, selected examples were fixed in osmium tetroxide, dehydrated, and embedded in Araldite resin (Polysciences) on a plastic slide. Serial 0.5-μm sections were cut until the retrogradely labeled neuron was located. The tissue was then trimmed to the size of an electron microscope block face and serial thin sections were cut, collected on Formvar-coated slot grids,

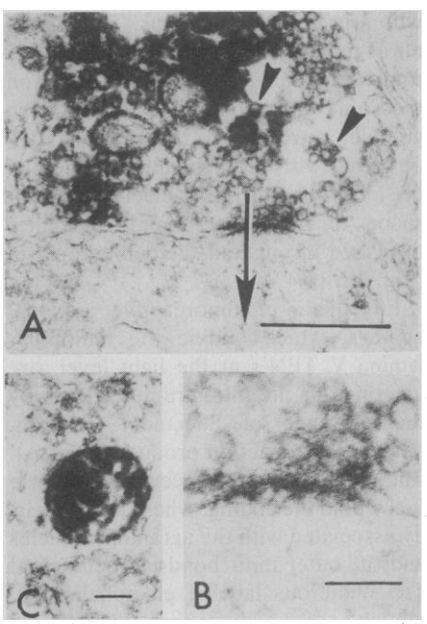

Fig. 2. (A) At the ultrastructural level, the enkephalin immunoreactive endings that synapse (arrow) on thalamic projection neurons contain primarily round agranular synaptic vesicles, which are frequently associated with the PAP reaction product (arrowheads). Bar represents 0.5 μm. (B) The synaptic contact is asymmetrical. Bar represents 0.1 μm. (C) At the ultrastructural level, the granular retrogradely transported HRP can be localized to vesicular structures. Bar represents 0.1 μm.

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and examined with an electron microscope (Zeiss EM 10C). Several ultrathin sections were scanned before being counterstained with lead citrate to unequivocally localize the electron-opaque peroxidase reaction product. In the MDH, four retrogradely labeled neurons in lamina V were examined in more than 50 semiserial sections per cell at the ultrastructural level.

The retrogradely labeled TPN's are readily identified at the light microscopic level by the presence of blackened HRP granules throughout the cell soma and proximal dendrites (Fig. 1). In lamina V, one morphological type of TPN is densely surrounded along its soma and proximal dendrites with enkephalin immunoreactive profiles. The large number of labeled enkephalin varicosities delineate the contours of the projection neuron, outlining the large caliber dendritic shafts for several micrometers. These heavily enkephalin-innervated neurons may represent a distinct population of TPN's that have a large multipolar cell soma in the transverse plane and that are located in the middle or lateral part of lamina V.

At the ultrastructural level, lamina V multipolar TPN's are characterized by a moderately dense cytoplasm and a large centrally placed, unindented nucleus with a prominent nucleolus. The diameter of the cell soma ranges from 25 to 35 μm. A few short, stubby spines occur on the soma and proximal dendrites. The granular retrogradely transported HRP observed with the light microscope can be localized with the electron microscope to membrane-bound organelles dispersed throughout the cell cytoplasm (Fig. 2C) (*14*).

Enkephalin-immunoreactive axonal endings, which outline the multipolar lamina V TPN's at the light level, are identified at the ultrastructural level by the presence of flocculent electron-opaque PAP reaction product (Fig. 2A). The reaction product occurs in discrete foci within the ending, where it is primarily associated with the agranular vesicles and the outer mitochondrial membrane. The numerous labeled enkephalin-containing axonal endings that are observed electron microscopically surrounding the lamina V TPN's are dome-shaped with long axes ranging from 0.8 to 2.8 μm. They contain mainly round agranular synaptic vesicles (Fig. 2A). An occasional dense-core vesicle is also present. All labeled enkephalin-containing axonal endings adjacent to the retrogradely labeled cell form asymmetrical synapses on the cell (Fig. 2B). At least ten enkephalin endings synapsed on each of the four neurons examined ultrastructurally. The neuron illustrated in Fig. 3 was examined in more than 66 semiserial sections, where it was contacted by 31 enkephalin immunoreactive axonal endings.

The enkephalin immunoreactive axonal endings that synapse on lamina V TPN's may originate from either intrinsic enkephalin-containing dorsal horn neurons or those at more rostral levels of the neuraxis (*3, 15*). Although recent evidence has identified a population of enkephalin-containing neurons in the brainstem that project to the spinal cord, three observations suggest that the endings identified in this study originate, at least in part, from intrinsic dorsal horn neurons. (i) Studies of enkephalin concentrations after spinal cord transection have shown only a small decrease, suggesting that most of the enkephalin in the cord originates from intrinsic neurons (*2*). (ii) Both enkephalin-containing neurons and projection neurons are found in the same laminae of the dorsal horn so that the axonal plexus of the enkephalin-containing neurons are strategically situated to ramify within the area of the projection neurons. (iii) Studies of dispersed spinal cord cell cultures have demonstrated a population of neurons that receive along their soma and proximal dendrites a dense plexus of enkephalin-containing axonal endings that originate from enkephalin-containing neurons in the dissociated cell culture (*16*).

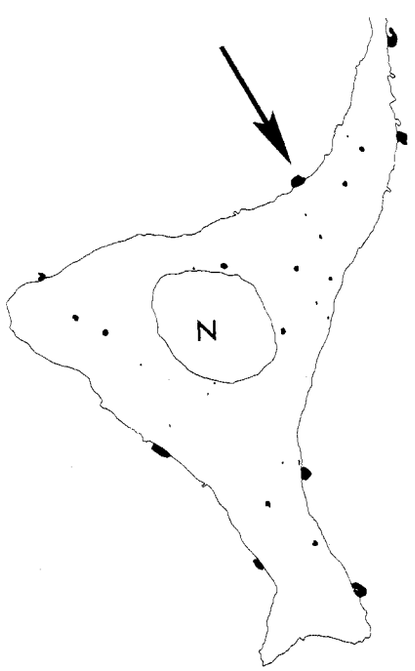

Fig. 3. At the ultrastructural level, the retrogradely labeled neurons were examined in semiserial sections. This line drawing represents a tracing of a photomontage reconstruction of a single ultrathin 400-Å section drawn at a final magnification of ×24,000. The section plane passes through the nucleus (*N*) of the cell. Numerous retrogradely transported HRP granules (drawn actual size) are dispersed in the cytoplasm. In this individual section, eight enkephalin profiles surround the neuron. The position of the enkephalin-containing axonal ending illustrated in Fig. 2 is indicated by an arrow. Bar represents 10.0 μm.

The presence of somatic enkephalin synapses is consistent with observations after the iontophoretic application of enkephalin onto lamina V neurons in the dorsal horn. The response of the lamina V neurons to both noxious and nonnoxious stimulation was inhibited by enkephalin (*5*). Enkephalin-containing axonal endings on the cell soma are strategically situated to modulate the total response repertoire of a neuron.

The observation that second-order projection neurons are the recipients of enkephalin-mediated input implies that opiates play a role in the transmission of nociceptive information from the periphery to higher centers of the neuraxis by directly modulating the response of TPN's in the dorsal horn to primary afferent input. Moreover, the presence of enkephalin-mediated synapses on projection neurons suggests that opiates act, at least in part, on postsynaptic receptors located on dorsal horn thalamic projection neurons.

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References and Notes

1. T. Hökfelt, A. Ljungdahl, L. Terenius, R. Elde, G. Nilsson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3081 (1977).
2. V. Seybold and R. Elde, *J. Histochem. Cytochem.* **28**, 367 (1980).
3. M. Sar, W. E. Stumpf, R. J. Miller, K. J. Chang, P. Cuatrecasa, *J. Comp. Neurol.* **182**, 17 (1978); E. J. Glazer and A. I. Basbaum, *ibid.* **196**, 377 (1981); G. R. Uhl, R. R. Goodman, M. J. Kuhar, S. R. Childers, S. H. Snyder, *Brain Res.* **166**, 75 (1979).
4. T. L. Yaksh, S. P. Huang, T. A. Rudy, *Neuroscience* **2**, 593 (1977); M. Randić and V. Miletić, *Brain Res.* **152**, 196 (1978); M. Satoh, S. Kawajiri, Y. Ukai, M. Yamamoto, *ibid.* **177**, 384 (1979).
5. A. W. Duggan, J. G. Hall, P. M. Headley, *Br. J. Pharmacol.* **61**, 399 (1977); W. Zieglgänsberger and I. F. Tulloch, *Brain Res.* **167**, 53 (1979).
6. C. LaMotte, C. B. Pert, S. H. Snyder, *Brain Res.* **112**, 407 (1976); T. M. Jessel and L. L. Iverson, *Nature (London)* **268**, 549 (1977); H. L. Fields, P. C. Emson, B. K. Leigh, R. F. T. Gilbert, L. L. Iverson, *ibid.* **284**, 351 (1980).
7. A. W. Duggan, B. T. Griersmith, P. M. Headley, J. G. Hall, *Neurosci. Lett.* **12**, 313 (1979).
8. R. L. Macdonald and P. G. Nelson, *Science* **199**, 1449 (1978); J. L. Barker, T. G. Smith, Jr., J. H. Neale, *Brain Res.* **154**, 153 (1978); A. W. Mudge, S. E. Leeman, G. D. Fischback, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 526 (1979).
9. S. P. Hunt, J. S. Kelly, P. C. Emson, *Neuroscience* **5**, 1871 (1980); K. K. Sumal, V. M. Pickel,

R. J. Miller, D. J. Reis, *Soc. Neurosci. Abstr.* **6**, 353 (1980); N. Aronin, M. DiFiglia, A. Liotta, J. B. Martin, *ibid.*, p. 353; E. J. Glazer and A. I. Basbaum, *ibid.*, p. 523.
10. E. Carstens and D. L. Trevino, *J. Comp. Neurol.* **182**, 151 (1978); G. J. Giesler, Jr., D. Menétrey, A. I. Basbaum, *ibid.* **184**, 107 (1979); W. D. Willis, D. R. Kenshalo, Jr., R. B. Leonard, *ibid.* **188**, 543 (1979); S. Hockfield and S. Gobel, *Brain Res.* **139**, 333 (1978); H. Burton and A. D. Craig, Jr., *ibid.* **161**, 515 (1979).
11. D. L. Trevino, R. A. Maunz, R. N. Bryan, W. D. Willis, *Exp. Neurol.* **34**, 64 (1972); W. D. Willis, D. L. Trevino, J. D. Coulter, R. A. Maunz, *J. Neurophysiol.* **37**, 358 (1974); D. D. Price, R. Dubner, J. W. Hu, *ibid.* **39**, 936 (1976); D. D. Price, R. L. Hayes, M. Ruda, R. Dubner, *ibid.* **41**, 933 (1978); D. R. Kenshalo, Jr., R. B. Leonard, J. M. Chung, W. D. Willis, *ibid.* **42**, 1370 (1979); D. D. Price, H. Hayashi, R. Dubner, M. A. Ruda, *ibid.*, p. 1590; G. J. Giesler, Jr., D. Menétrey, G. Guilbaud, J.-M. Besson, *Brain Res.* **118**, 320 (1976).
12. J. C. Adams, *Neuroscience* **2**, 141 (1977).
13. L. A. Sternberger, *Immunocytochemistry* (Wiley, New York, ed. 2, 1979).
14. J. H. LaVail and M. M. LaVail, *J. Comp. Neurol.* **157**, 303 (1975).
15. T. Hokfelt, L. Terenius, H. G. J. M. Kuypers, O. Dann, *Neurosci. Lett.* **14**, 55 (1979); R. M. Bowker, H. W. M. Steinbusch, J. D. Coulter, *Brain Res.* **211**, 412 (1981).
16. J. H. Neale, J. L. Barker, G. R. Uhl, S. H. Snyder, *Science* **201**, 467 (1978).
17. I thank J. Coffield and E. Humphrey for their expert technical assistance; L. Ostby for light microscopic photography; M. Abdelmoumene, G. Bennett, P. J. P. Brickfield, R. Dubner, S. Gobel, and M. Hoffert for reading the manuscript; and E. Welty for typing it.

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The Origin of Man: A Chromosomal Pictorial Legacy

Abstract. *Man, gorilla, and chimpanzee likely shared an ancestor in whom the fine genetic organization of chromosomes was similar to that of present man. A comparative analysis of high-resolution chromosomes from orangutan, gorilla, chimpanzee, and man suggests that 18 of 23 pairs of chromosomes of modern man are virtually identical to those of our "common hominoid ancestor," with the remaining pairs slightly different. From this lineage, gorilla separated first, and three major chromosomal rearrangements presumably occurred in a progenitor of chimpanzee and man before the final divergence of these two species. A precursor of the hominoid ancestor and orangutan is also assumed.*

Comparisons of banded metaphase chromosomes (320 to 500 bands per haploid set) of man, chimpanzee, gorilla, and orangutan have revealed a general homology of chromosomal bands in the four species and suggested a common ancestor for chimpanzee, gorilla, and man (*1, 2*). Using high-resolution G-banded chromosomes from late prophase (1000 bands per haploid set) (*3*), we can now account for every nonheterochromatic G-positive and G-negative band in the four primates. Furthermore, by comparing chromosomes of humans, apes, and some Old World monkeys, we have been able to work backward in evolution to suggest likely karyotypes for three presumed common ancestors of apes and man. This study was based on the remarkable similarity of chromosomes of man, chimpanzee, gorilla, and orangutan, the few changes needed to explain their differences, and the use of ancestral chromosomal patterns to derive the general sequence of events that might have taken place in primate evolution prior to man's emergence. Such an approach suggests (i) the existence of a precursor to orangutan and a hominoid ancestor of gorilla, chimpanzee, and man; (ii) the emergence of the hominoid ancestor; and (iii) the existence of a progenitor of chimpanzee and man after the divergence of gorilla.

Cultured lymphocytes from two male and three female orangutans (*Pongo pygmaeus*), one male and four female gorillas (*Gorilla gorilla*), four male and five female chimpanzees (*Pan troglodytes*), and ten women and 21 men (*Homo sapiens*) were examined by use of a high-resolution chromosome-methotrexate cell synchronization technique (*3*). To test for equivalence between chromosomes and bands in the four species, we photographed 20 relatively straight, G-banded, late-prophase examples of each chromosome from the four species (×1600). The photographs were enlarged twice and matched side by side for a detailed analysis of reproducibility of banding patterns, band thickness, and staining intensity (Fig. 1). Additional chromosome preparations were stained with the C-banding technique (*4*) to determine to what extent the banding patterns observed were related to heterochromatin. Descriptions were simplified by ascribing the new international human high-resolution chromosome nomenclature (*5*) to the chromosomes of the great apes. Occasionally, when a question arose regarding chromosomal ancestry of the four species, individual chromosomes from more primitive species were studied. These included two male and two female rhesus monkeys (*Macaca mulatta*) and one male baboon (*Papio papio*). The results illustrate a remarkable similarity in the banding patterns of most chromosomes. Except for differences in nongenic constitutive heterochromatin (*6*), chromosomes 6, 13, 19, 21, 22, and X appear to be identical in all four species; chromosomes 3, 11, 14, 15, 18, 20, and Y look the same in three species; and chromosomes 1, 2p, 2q (*7*), 5, 7 to 10, 12, and 16 are alike in two species (Figs. 1 and 2).

Most chromosomal differences in the four species consist of inversions of chromosomal segments and variations in constitutive heterochromatin. The most common inversions are of the pericentric type, although a few are paracentric. Chromosomes 4, 5, 9, 12, 15, and 16 of man and chimpanzee differ by a pericentric inversion, whereas chromosome 7 of chimpanzee and gorilla differ by a paracentric inversion. Occasionally, both peri- and paracentric inversions appear to be involved, as shown by comparisons of chromosome 16 of man and gorilla and chromosomes 3 and 17 of man and orangutan.

Differences in constitutive heterochromatin among the four species are caused by (i) variations in amount for the centromeric and paracentromeric regions (particularly in chromosomes 1, 9, 16, and the short arm of acrocentric chromosomes 13 to 15, 21, and 22) (*6*); (ii) the presence of intercalary bands in chimpanzee (added to subbands 7q22.2 and 13q14.2) and orangutan (additional to the distal end of band 4q12); and (iii) differences in size of the Y chromosome, which tend to obscure the basic homology of the nonheterochromatic segment (p11.32q11.23) in the four species (Fig. 2). In addition, telomeric or terminal bands are found in approximately half of all the chromosome arms from chimpanzee and in nearly all of those from gorilla, but they are conspicuously absent in chromosomes from man and orangutan (Fig. 2). Polymorphic variations of heterochromatin were commonly found in the four species and were particularly dramatic in the telomere of the short arm of gorilla chromosomes 2q, 13 to 15, and 18.

In addition to inversions and variations in heterochromatin, a few chromosomes of the four species showed reciprocal translocation (5; 17 in gorilla), band insertion (terminal band 20p13 on centromeric band 8q11.2 in orangutan), differences in the number and position of nucleolar organizers (*8*), and telomeric fusion (chromosomes 2p and 2q, with inactivation of the 2q centromere in man) (*1, 2*). Humans have nucleolar organizers on chromosomes 13 to 15, 21, and 22; in chimpanzee, they are on chromosomes 13, 14, 18, 21, and 22; in gorilla on chromosomes 13, 21, 22; and in orang-

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