Rossier, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5139 (1983).
7. H. Gainer, Y. Sarne, M. J. Brownstein, *J. Cell Biol.* **73**, 366 (1977).
8. R. V. Lewis, A. S. Stern, S. Kimura, J. Rossier, S. Udenfriend, *Science* **208**, 1459 (1980).
9. D. R. Liston, J. J. Vanderhaeghen, J. Rossier, *Nature (London)* **302**, 62 (1983); D. Liston, P. Bohlen, J. Rossier, *J. Neurochem.*, in press.
10. J. Rossier, Y. Audigier, N. Ling, J. Cros, S. Udenfriend, *Nature (London)* **288**, 88 (1980).
11. P. Giraud, E. Castanas, G. Patey, C. Oliver, J. Rossier, *J. Neurochem.* **41**, 154 (1983); G. Patey, A. Cupo, M. Chaminade, J.-L. Morgat, J. Rossier, *Life Sci.* **33**, 117 (1983). The antisera to the heptapeptide and octapeptide were directed specifically toward the carboxyl terminal part of the corresponding peptides and displayed less than 0.1 percent cross-reactivity with peptides that did not have the corresponding sequence at their carboxyl terminal.
12. S. L. Sabol, C. M. Liang, S. Dandekar, L. S. Kranzler, *J. Biol. Chem.* **258**, 2697 (1983).
13. M. Comb, personal communication.
14. A. J. Mason, B. A. Evans, D. R. Cox, J. Shine, R. I. Richards, *Nature (London)* **303**, 300 (1983).
15. E. S. Prado *et al.*, *Biochem. Biophys. Res. Commun.* **112**, 366 (1983).
16. Portions of the column fractions were lyophilized and resuspended in 250 μl of 50 m*M* tris-HCl, *p*H 7.8, containing *Staphylococcus aureus* V8 protease (40 μg/ml; 550 unit/mg). The amounts of heptapeptide and octapeptide immunoreactivities in the digests were determined by RIA (*11*). This proteolytic treatment releases small peptides ending with the sequence of the heptapeptide or octapeptide from the postulated high molecular weight carboxyl-terminal fragments of proenkephalin. Pilot experiments indicated that the immunoreactivity of these high molecular weight fragments was increased 50 to 80 percent by this digestion procedure, while the immunoreactivities of synthetic heptapeptide and octapeptide were not affected by this treatment.
17. G. R. Drapeau, Y. Boily, J. Houmard, *J. Biol. Chem.* **247**, 6720 (1972); J. Houmard and G. R. Drapeau, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3506 (1972).
18. Supported by the Esther A. and Joseph Klingenstein Fund, Centre National de la Recherche Scientifique, and Institut National de la Santé et de la Recherche Médicale (CRL 81 60 25). We thank A. Cupo for donating the octapeptide antiserum used in this study.
* To whom requests for reprints should be addressed at Department of Chemistry, University of Oregon, Eugene 97403.

20 March 1984; accepted 16 May 1984

Spinal Sympathetic Pathway: An Enkephalin Ladder

Abstract. *Enkephalin distribution was examined in autonomic areas of the rat thoracic spinal cord. The localization of enkephalin fibers coincided with nuclear regions containing sympathetic preganglionic neurons. Horizontal sections revealed a pattern for enkephalin fibers resembling Laruelle's description of the localization of sympathetic preganglionic neurons as rungs of a ladder.*

Laboratory and clinical findings indicate that the opioid peptides, methionine- and leucine-enkephalin, exert regulatory influences on the sympathetic nervous system. Peripherally, enkephalin-containing fibers and cell bodies are present in paravertebral and prevertebral ganglia (*1*), where enkephalin may serve as a presynaptic inhibitory transmitter, inhibiting the cholinergic fast excitatory postsynaptic potential (*2*). Centrally, enkephalin appears to depress the firing rate of spinal sympathetic preganglionic neurons and may do so along two separate pathways: an intraspinal excitatory pathway and a spinal reflex pathway (*3*). These pathways may participate in the augmented sympathetic activity that attends opiate withdrawal (*4*); the resultant clinical alterations provide the most dramatic evidence in man for enkephalin modulation of sympathetic nervous system activity.

The anatomical substrate for the interactions of enkephalin and the sympathetic nervous system in the spinal cord remains to be elucidated. We examined the morphological relations between preganglionic spinal sympathetic neurons and the neurotransmitter-neuromodulator enkephalin and found that enkephalin localization mimics Laruelle's (*5*) original description of the distribution of preganglionic sympathetic neurons as rungs of a ladder; thus, spinal sympathetic pathways include an enkephalin ladder.

Earlier immunocytochemical studies in the rat and cat spinal cord revealed enkephalin cells and fibers in the dorsal horn, in particular in laminae I and II (*6–12*). Enkephalin-containing fibers were found in the dorsolateral funiculus and ventral horn (*8–10, 13, 14*), and enkephalin cells were observed in the sacral preganglionic parasympathetic nucleus (*11*). Enkephalin cells and fibers were also located in lamina VII and around the central canal (lamina X) (*6–12, 14, 15*). At thoracolumbar levels in rat and guinea pig, laminae VII and X contain preganglionic sympathetic nuclear groups (*16*) that may provide enkephalin fibers to sympathetic ganglia (*1*). These earlier morphological studies showed that enkephalin neurons exist in spinal autonomic areas, but their extent, character, and interrelationships with spinal sympathetic areas were unknown.

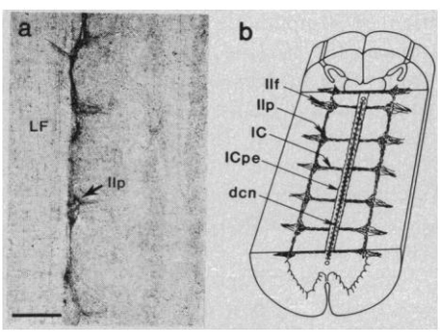

Fig. 1. (a) Low-power photomicrograph of a horizontal section of rat thoracic spinal cord showing enkephalin immunoreactivity in the nucleus intermediolateralis, pars principalis (IIp), and along the gray-white border of the lateral funiculus (LF) connecting adjacent IIp cell nests. Scale bar, 250 μm. (b) Summary diagram illustrating the location of enkephalin-immunoreactive fibers in the thoracic spinal cord of the rat. There is a coincidence of enkephalin fibers and nuclear regions containing sympathetic preganglionic neurons. Abbreviations: dcn, dorsal commissural nucleus; IC, nucleus intercalatus spinalis; ICpe, nucleus intercalatus, pars paraependymalis; IIf, nucleus intermediolateralis, pars funicularis; and IIp, nucleus intermediolateralis, pars principalis. Drawing adapted from figure 2 in Petras and Cummings (*16*).

We examined the distribution of enkephalin in the spinal cord of adult male and female Sprague-Dawley rats. Eight normal and eight colchicine-treated rats were used. Colchicine was administered either intracisternally (50 μg per 10 μl of distilled water) or by exposing the spinal cord (T_6 to T_7), opening a dural flap, and placing a Gelfoam pledget soaked in colchicine (50, 100, or 250 μg per 10 μl of distilled water) on the dorsal surface of the cord. Colchicine-treated animals were allowed to survive 24 to 48 hours after surgery. All animals were perfused with Zamboni's fixative (*17*). After perfusion the entire spinal cord was removed, postfixed overnight at 4°C in the same fixative, and transversely sectioned into four blocks: cervical, upper thoracic, lower thoracic, and lumbar-sacral. Each block was cut serially on a Vibratome in horizontal 40-μm sections. The unlabeled antibody method was used to test for the presence of enkephalin (*17*). Sections were incubated for 48 to 72 hours at 4°C in the primary antiserum at a dilution of 1:1000 (Immuno Nuclear) or 1:2000 (Sundberg) (*18*). Every sixth section was counterstained with cresyl violet to determine more accurately the location of labeled neurons and fibers. Nomenclature and definition of nuclear groups containing sympathetic preganglionic neurons are as given by Petras and Cummings (*16*).

Radioimmunoassay results indicated that the Sundberg antiserum cross-reacts twice as well with leucine-enkephalin as with methionine-enkephalin (*19*). The two antiserums identified similar distribution patterns of immunoreactivity. Control absorption studies consisted of preincubation of 1 ml of diluted (1:1000) primary antiserum with 10 μg of synthetic methionine- or leucine-enkephalin (Peninsula) or 5 μg each of both methionine- and leucine-enkephalin. No immunostaining was observed in sections preincubated with either or both peptides.

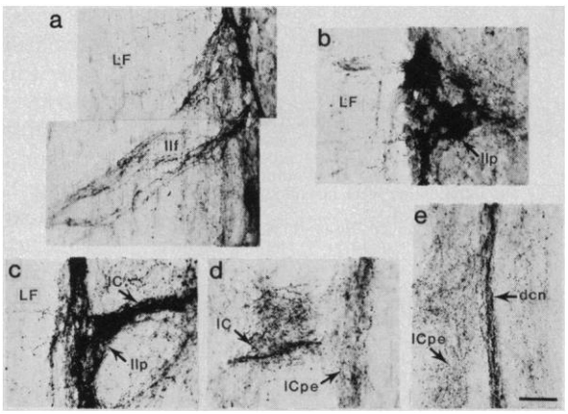

Fig. 2. Immunocytochemical localization of enkephalin-immunoreactive fibers on horizontal sections of rat thoracic cord. (a) Labeled fibers in the Ilf extend from the gray-white border of the LF toward the pia surface. (b) Enkephalin fibers in a single Ilp cell cluster. (c) Enkephalin fibers in the nucleus IC extend as a series of bridges from the Ilp medially toward central autonomic areas. (d) Continuation of the medially coursing IC fibers as they join the nucleus ICpe, located dorsolateral to the central canal. (e) Immediately dorsal to the central canal, enkephalin fibers are found in a band in the dcn. Abbreviations are as in Fig. 1. Scale bar, 60 μm.

Enkephalin-immunoreactive fibers appear as brown-beaded processes located within discrete nuclear regions of the thoracolumbar spinal cord. In particular, the results of our study demonstrate a coincidence between the location of enkephalin-containing fibers and nuclear regions containing sympathetic preganglionic neurons. Enkephalin fibers within the intermediate zone (lamina VII) of spinal cord gray matter are found throughout the entire rostrocaudal extent of thoracolumbar sympathetic outflow.

Preganglionic sympathetic neurons in the intermediate zone of the spinal cord are localized in the following cell groups: nucleus intermediolateralis thoracolumbalis, pars principalis (Ilp), nucleus intermediolateralis thoracolumbalis, pars funicularis (Ilf), nucleus intercalatus spinalis (IC), nucleus intercalatus, pars paraependymalis (ICpe), and the dorsal commissural nucleus (dcn) (*16, 20*). The Ilp neurons are grouped into cell clusters or nests located approximately 300 to 400 μm apart in a rostrocaudal direction and are situated on the border between the intermediate gray zone and the lateral funiculus. Dense accumulations of enkephalin fibers are found outlining or interspersed between cells of the Ilp (Figs. 1a and 2b). Horizontal sections reveal large numbers of enkephalin fibers in individual Ilp clusters forming a triangle with the base on the lateral funiculus (Figs. 1a and 2b). Connecting adjacent cell nests or bases of Ilp triangles is a column of densely labeled enkephalin fibers located along the gray-white border of the lateral funiculus (Figs. 1a and 2c).

The nucleus intermediolateralis, pars funicularis (Ilf), is found in the lateral funiculus at the same dorsal-ventral plane as Ilp. Sympathetic preganglionic neurons in this nucleus are scattered between Ilp and the pia surface (*16*). Moderate numbers of enkephalin fibers are seen in the Ilf extending laterally from individual Ilp cell clusters to the pia surface. Enkephalin fibers in the Ilf travel in a direction perpendicular to the fibers of the lateral funiculus (Fig. 2a).

Sympathetic preganglionic neurons in the nucleus intercalatus spinalis (IC) are found in the intermediate zone forming a series of bridges connecting the Ilp cell nests with more medial central autonomic areas (*16*). Immunoreactive enkephalin fibers in moderate numbers are found in intermediate zones forming distinct bands in a pattern identical to the location of sympathetic preganglionic neurons in the IC (Fig. 2, c and d). These transverse bridges of enkephalin fibers are located laterally at the same dorsal-ventral plane as Ilp but medially are found in a slightly more ventral plane, as the labeled fibers connect with the autonomic area located dorsolateral to the central canal.

The nucleus intercalatus, pars paraependymalis (ICpe), located dorsolateral to the central canal, between the nucleus of Clarke and the central canal, contains spinal autonomic neurons. Enkephalin fibers in the ICpe form a series of arches connecting adjacent IC nuclear groups (Fig. 2, d and e).

Dense accumulations of enkephalin fibers are found in the dorsal commissural nucleus (dcn) located immediately dorsal to the central canal in a distinct band running the entire length of the thoracolumbar spinal cord (Fig. 2e). This region corresponds to a central autonomic area located in the dorsal gray commissure (*16, 20*).

The distribution pattern of enkephalin fibers within preganglionic sympathetic nuclear groups is maintained throughout all levels of the thoracolumbar spinal cord. However, autonomic nuclear boundaries are more clearly delineated and heavily populated by enkephalin fibers in upper thoracic and upper lumbar levels than in lower thoracic levels. The dorsal commissural nucleus at L_1 and L_2 levels contains abundant enkephalin fibers.

We examined the distribution of enkephalin in the dorsal and ventral gray horns of thoracolumbar spinal cord and at cervical, lower lumbar, and sacral levels, and our findings are in agreement with earlier reports (*7, 9, 10, 12, 13, 21–23*).

To determine whether this intricate pattern of fibers originated from spinal sympathetic enkephalin neurons, we reexamined the thoracolumbar autonomic areas after colchicine treatment. An occasional enkephalin-labeled neuron is found in nucleus intercalatus spinalis and nucleus intercalatus, pars paraependymalis, in rats treated with colchicine. Enkephalin-containing neurons are observed throughout the dorsal horn of thoracolumbar spinal cord and are especially prevalent in lamina II and at junction of laminae II and III.

Our study shows an elaborate distribution pattern of enkephalin localization within laminae VII and X of the spinal cord: enkephalin fibers coincide with those regions of thoracolumbar spinal cord that contain sympathetic preganglionic neurons (*16*). Laruelle (*5*) described the distribution of preganglionic sympathetic neurons in laminae VII and X as resembling the rungs of a ladder. In our study, the use of horizontal sections of thoracolumbar spinal cord enabled us to observe a similar ladder-like arrangement of enkephalin fibers (Fig. 1b). At thoracic spinal cord levels, enkephalin fibers have been described within the intermediolateral cell column (*23–25*) and as a single band of fibers in lamina VII between the intermediolateral cell column and the central canal (*12*). These observations in spinal sympathetic areas extend the work of others (*6–9, 11, 14, 23, 25, 26*) who observed enkephalin fibers within sensory and motor zones of spinal cord gray matter and in the dorsolateral funiculus. These investigators described a dense distribution of enkephalin fibers in laminae I and II and moderate numbers of immunoreactive fibers throughout the ventral horn and surrounding the central canal in X.

Although the origin of this enkephalin network within sympathetic nuclear regions remains to be determined, there is

evidence for both intraspinal and supraspinal sources. We observed enkephalin cells after colchicine treatment throughout the dorsal horn, especially in the substantia gelatinosa and at the junction of laminae II and III. A small number of enkephalin-labeled cells were found in the sympathetic nuclei intercalatus spinalis and pars paraependymalis. Previous studies (*7, 8, 10, 11, 21*) showed enkephalin-labeled cells in the dorsal horn, intermediate zone, and dorsal to the central canal, although the majority of immunoreactive cells were found in the substantia gelatinosa (*7, 8, 10*). Transection of the spinal cord at thoracic levels or unilateral dorsal rhizotomy at lumbar or sacral levels does not produce changes in the distribution of enkephalin within the spinal cord (*8*), and this suggests an intraspinal origin for enkephalin fibers. However, the results of a study (*27*) in which a combined retrograde transport–immunocytochemical technique was used in the rat showed enkephalin projections from the medulla to the lower thoracic–upper lumbar regions. In corroboration, no enkephalin-immunoreactive staining was found in the thoracic cord after a C_7 spinal cord transection (*25*). Therefore, both intraspinal and descending enkephalin projections may exist, although the relative contribution of each to laminae VII and X is unknown.

Our results demonstrating that enkephalin fibers and sympathetic preganglionic nuclear regions are in the same place, provide an anatomical substrate at a spinal level for a functional relation between the enkephalin peptide and the sympathetic nervous system. More specifically, our findings may provide a morphological basis for the hypothesis that the augmented sympathetic activity during opiate withdrawal occurs at a spinal level. In a more general sense, our observations indicate that the integration of autonomic reflexes at a spinal level is mediated, at least in part, by an enkephalin system.

M. A. ROMAGNANO
R. W. HAMILL

Neurology Unit, Monroe Community Hospital, and *Departments of Neurology* and *Medicine,* and *Center for Brain Research, University of Rochester, School of Medicine and Dentistry, Rochester, New York 14603*

References and Notes

1. H. Kondo and R. Yui, *Brain Res.* **242**, 142 (1982); M. Schultzberg *et al.*, *Neuroscience* **4**, 249 (1979).
2. S. Konishi, A. Tsunoo, M. Otsuka, *Nature (London)* **282**, 515 (1979); S. Konishi, A. Tsunoo, N. Yanaihara, M. Otsuka, *Biomed. Res.* **1**, 528 (1980).
3. D. N. Franz, B. D. Hare, K. L. McCloskey, *Science* **215**, 1643 (1982).
4. M. S. Gold, D. E. Redmond, Jr., H. D. Kleber, *Lancet* **1978-II**, 599 (1978); M. S. Gold, D. E. Redmond, Jr., H. D. Kleber, *Am. J. Psychiatry* **136**, 100 (1979).
5. L. Laruelle, *C.R. Assoc. Anat.* **31**, 210 (1936).
6. R. Elde, T. Hokfelt, O. Johansson, L. Terenius, *Neuroscience* **1**, 349 (1976).
7. T. Hokfelt, R. Elde, O. Johansson, L. Terenius, L. Stein, *Neurosci. Lett.* **5**, 25 (1977).
8. T. Hokfelt, A. Ljungdahl, L. Terenius, R. Elde, G. Nilsson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3081 (1977).
9. S. J. Watson, H. Akil, S. Sullivan, J. D. Barchas, *Life Sci.* **21**, 733 (1977).
10. J. C. W. Finley, J. L. Maderdrut, P. Petrusz, *J. Comp. Neurol.* **198**, 541 (1981).
11. E. J. Glazer and A. I. Basbaum, *ibid.* **196**, 377 (1981).
12. E. Senba, S. Shiosaka, Y. Hara, S. Inagaki, M. Sakanaka, M. Tohyama, *ibid.* **208**, 54 (1982).
13. R. Simantov, M. J. Kuhar, G. R. Uhl, S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2167 (1977).
14. O. Johansson, T. Hokfelt, R. P. Elde, M. Schultzberg, L. Terenius, in *Advances in Biochemical Psychopharmacology*, E. Costa and M. Trabucchi, Eds. (Raven, New York, 1978), p. 51.
15. G. J. Bennett, M. A. Ruda, S. Gobel, R. Dubner, *Brain Res.* **240**, 162 (1982).
16. J. M. Petras and J. F. Cummings, *J. Comp Neurol.* **146**, 189 (1972).
17. L. Zamboni and C. DeMarbino, *J. Cell Biol.* **35**, 148A (1967); L. A. Sternberger, *Immunocytochemistry* (Wiley, New York, 1976); C. A. Bennett-Clarke, M. A. Romagnano, S. A. Joseph, *Brain Res.* **188**, 473 (1980).
18. Antiserum to enkephalin was obtained from two sources: Immuno Nuclear Corporation (Stillwater, Minn.) and D. K. Sundberg. The antiserum to methionine-enkephalin (Immuno Nuclear lot 36511) was generated in rabbit against glutaraldehyde-hemocyanin and ethyl-EDI-hemocyanin conjugates. The antiserum provided by Sundberg was generated in rabbit with a methionine-enkephalin–thyroglobulin conjugate prepared by the dimethylaminopropyl carbodiimide method. [D. K. Sundberg, L. Pittman, S. Deadwyler, *Endocr. Abstr.* **106**, 153 (1980)].
19. D. K. Sundberg, personal communication. Although the Sundberg antiserum has a higher affinity for leucine enkephalin than for methionine enkephalin, it does not immunocytochemically distinguish between the two. There is no significant cross-reactivity with β-endorphin and dynorphin-(1-13). Radioimmunoassay studies indicate that 100 μl of the Sundberg antiserum diluted 1:1000 binds 330 pg of methionine-enkephalin.
20. J. F. Cummings, *Acta Anat.* **73**, 27 (169); M. D. Hancock and C. A. Peveto, *J. Comp. Neurol.* **183**, 65 (1979).
21. R. Elde, T. Hokfelt, O. Johansson, A. Ljungdahl, G. Nilsson, S. J. Jeffcoate, in *Centrally Acting Peptides*, J. Hughes, Ed. (Macmillan, Hampshire, England, 1978), p. 587.
22. E. J. Glazer and A. I. Basbaum, *Science* **208**, 1479 (1980).
23. N. C. deLanerolle and C. C LaMotte, *J. Neurosci.* **2**, 1369 (1982).
24. F. J. Ditirro, R. H. Ho, G. F. Martin, *J. Comp. Neurol.* **198**, 351 (1981).
25. V. Holets and R. Elde, *Neuroscience* **7**, 1155 (1982).
26. M. Sar, W. E. Stumpf, R. J. Miller, K.-J. Chang, P. Cuatrecasas, *J. Comp. Neurol.* **182**, 17 (1978).
27. T. Hokfelt, L. Terenius, H. G. J. M. Kuypers, O. Dann, *Neurosci. Lett.* **14**, 55 (1979).
28. We thank D. K. Sundberg for the antiserum to enkephalin, M. Loomis for technical assistance, and J. Van Loan for secretarial help. This work was supported by Biological Research Support grant 5-27238 and by the University of Rochester–Monroe Community Hospital Research Fund.

1 March 1984; accepted 26 April 1984

Sex Ratio of Sea Turtles: Seasonal Changes

Abstract. *Sex ratios of hatchling loggerhead turtles* Caretta caretta *taken from South Carolina and Georgia ranged from no females in nests laid in late May to 80 percent females in those laid in early July; the sex ratio decreased to 10 percent females in nests laid in early August. These seasonal changes are consistent with the role of temperature in directing sexual differentiation in various reptiles. The data have implications for understanding the demography of sea turtles and for their conservation.*

Sexual differentiation in sea turtles, as in a number of reptiles, depends on the ambient temperature during incubation of the eggs (*1–3*). Therefore the sex ratio of offspring should differ at different times of year. This is especially likely to happen in species of sea turtles that lay several clutches over an extended nesting season. This idea has been discussed (*2, 3*), but conclusive data are lacking. We now report that seasonal changes occur in the sex ratio of loggerhead turtles (*Caretta caretta*) nesting in the southeastern United States. The effects are large and have implications for conservation programs and for the study of sea turtle demography.

Hatchling loggerhead turtles were collected from 1979 to 1982 from various barrier islands in South Carolina and Georgia. The nesting beaches frequented by loggerhead turtles in these regions are predominantly primary dune, either devoid of cover or sparsely covered with sea oats (*Uniola paniculata*). The lack of dense vegetation and the associated shade, along with the relative openness and homogeneity of the barrier island beaches, reduces the importance of spatial variables. From each clutch sampled, ten hatchlings were taken at random (*4*). Sex was determined histologically (*5*).

Sex ratio ranged from 10 percent female or less during the cooler ends of the season to 80 percent female in the middle of the summer (Fig. 1). Although variability occurred among clutches, none was less than 40 percent female between 12 June and 14 July, and most were 75 percent female or more. Not all the data came from the same year. When the results for 1982, the year with most available data, are considered separately, the seasonal trends are essentially the same. Also, some nests had been transplanted to protected sites soon after laying. However, there is no evidence that these