pretraining baseline ($P = 0.75$). As a final verification that the histamine release on the first test trial was not a generalized effect from the preceding antigen-paired CS+ training trial, we compared the first CS+ training trial with the next CS− trial. The histamine release in response to the first CS+ training trial was greater in all eight animals ($t = 2.94$; $P < 0.025$). Generalized sensitivity to the previous histamine release does not explain our result. An additional comparison of data from the first experience with an antigen to data from the first test trial—the first test trial was CS+ with no antigen present—showed an increase in plasma histamine comparable to that experienced from an allergen (means of 140 and 142 ng/ml, respectively).

These results indicate that the animals had experienced a significant increase in plasma histamine as a function of associative learning. We believe we have conditioned a histamine release of a magnitude similar to that found in a physiologic (*7*) reaction for our animals. The difference in the levels of histamine between the first and second CS+ trials also indicates that this learned response may be extinguished through repeated unpaired exposures to the stimulus odor, as would be expected of a classically conditioned response.

Through learned associations between allergic reactions and environmental stimuli, a specific allergic response may be generalized to a number of environmental elements. Associative learning should be included in understanding the development and treatment of allergies.

MICHAEL RUSSELL
KATHLEEN A. DARK
ROBERT W. CUMMINS
GEORGE ELLMAN
ENOCH CALLAWAY
HARMAN V. S. PEEKE

Brain-Behavior Research Center, Sonoma Developmental Center, University of California, Eldridge 95431

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Processing of Proenkephalin Is Tissue-Specific

Abstract. *Most neuropeptides are synthesized as large precursor proteins. These precursors undergo a maturation process involving several proteolytic events that generate the biologically active peptides. The enzymatic mechanisms underlying this processing are still largely unknown. The processing of the precursor protein proenkephalin was studied in two different bovine tissues, the hypothalamus and adrenal medulla. The high molecular weight enkephalin-containing peptides that accumulate in these two tissues were found to be different, indicating the existence of two processing pathways for this neuropeptide precursor.*

Neuropeptides are widely distributed throughout the central and peripheral nervous systems and participate in diverse neuronal functions. The major events underlying the biosynthesis of neuropeptides are not yet fully understood. Most neuropeptides appear to be synthesized initially as large precursor proteins, which undergo proteolytic processing to produce the active peptides (*1*). One example of such a precursor is proenkephalin, a 27.3-kD protein that contains four copies of [Met]enkephalin and one copy each of [Leu]enkephalin, the heptapeptide [Met]enkephalin-Arg^6-Phe^7, and the octapeptide [Met]enkephalin-Arg^6-Gly^7-Leu^8 (*2*) (see Fig. 1).

Several large enkephalin-containing peptides (ECP's) thought to represent intermediates in the processing of proenkephalin have been purified from the bovine adrenal medulla (*3*). The characterization of these intermediates has led to the proposal that the processing of proenkephalin in the adrenal medulla involves several proteolytic cleavages that start in the carboxyl-terminal region of this precursor (*4*).

The processing pathway of proenkephalin in brain tissue has not yet been described. The major obstacle in characterizing such a pathway has been the lack of a brain region in which the processing intermediates accumulate as they do in the adrenal gland. We observed that high molecular weight ECP's are present in the bovine hypothalamus (*5*), where the enkephalins and oxytocin are localized in the magnocellular neurons of the supraoptic nucleus (*6*).

The well-characterized anatomy of the hypothalamic magnocellular neuronal system projecting to the neurohypophysis provides a convenient model to study the axonal transport and processing of neuropeptide precursors (*7*). Dissection of the cell bodies located in the supraoptic nucleus, the axons traveling through the pituitary stalk, and the nerve terminals located in the neurohypophysis has allowed a separation of the different stages in the maturation of the precursor of the pituitary hormone vasopressin (*7*). By exploiting these anatomical features, we found that proenkephalin is processed during axonal transport through the hypothalamo-neurohypophysial system. Furthermore, the processing intermediates that accumulate in the supraoptic nucleus are different from those found in the adrenal medulla, indicating different processing pathways in these two tissues.

The amounts of free [Met]enkephalin and total [Met]enkephalin-containing material were determined in the supraoptic nucleus, pituitary stalk, and neurohypophysis (Table 1). The [Met]enkephalin content of each tissue was measured by radioimmunoassay (RIA) both before and after sequential digestion with trypsin and carboxypeptidase B. This proteolytic treatment releases [Met]enkephalin sequences from larger precursors in which they are flanked by basic amino acid residues (*8*). In the cell bodies of the supraoptic nucleus, the [Met]enkephalin immunoreactivity released by this enzymatic treatment represents 75 percent of the total [Met]enkephalin content. In the axons passing through the pituitary stalk the proportion of [Met]enkephalin immunoreactivity in ECP's is reduced to 56 percent of the total. The neuronal terminals of the neurohypophysis contain almost exclusively free [Met]enkephalin, with an increase of only 11 percent after digestion. Thus, the amount of [Met]enkephalin present in larger peptides decreases with increasing distance from the cell body.

The molecular size of the various [Met]enkephalin-containing peptides throughout the hypothalamo-neurohypophysial system was determined by gel

filtration chromatography (Fig. 2). In the supraoptic nucleus, 51 percent of the [Met]enkephalin immunoreactivity appeared in peptides with apparent molecular sizes greater than approximately 5 kD, whereas in the stalk only 26 percent of the [Met]enkephalin immunoreactive material eluted in this range. In the neurohypophysis only 8.5 percent of the [Met]enkephalin immunoreactivity was present in peptides larger than 5 kD, indicating that processing is nearly complete in the neuronal terminals.

The column fractions were also assayed for synenkephalin immunoreactivity. Synenkephalin, which comprises 70 residues at the amino terminal of proenkephalin, has been isolated recently from bovine brain (*9*). Antiserum to synenkephalin recognizes all of the adrenal intermediates that contain the amino terminal sequence of proenkephalin, but does not recognize enkephalins or large ECP's that do not have this portion of proenkephalin. In all three regions of the hypothalamo-neurohypophysial system, synenkephalin immunoreactive material eluted predominantly as a single peak (Fig. 2). Although a large amount of [Met]enkephalin immunoreactivity in the supraoptic nucleus eluted as high molecular weight material, only a very small amount of synenkephalin immunoreactivity was found in the corresponding fractions. This observation is not consistent with the existence of the same processing intermediates that have been characterized in the adrenal gland (*3, 4*).

In the adrenal medulla the initial cleavage of proenkephalin is thought to occur near the carboxyl terminal of the molecule, liberating large intermediates (8.6 to 18.2 kD) that remain attached to synenkephalin (*3, 4, 9*) (see Fig. 1). In the supraoptic nucleus the small amount of synenkephalin immunoreactivity present in the high molecular weight ECP's suggested that a proteolytic cleavage may remove the amino terminal fragment at an early stage in the processing of the precursor, producing in turn a high molecular weight carboxyl terminal fragment. This proposed fragment would contain up to seven copies of the enkephalin sequence and terminate with the sequence of the heptapeptide [Met]-enkephalin-Arg^6-Phe^7, which comprises the seven amino acid residues at the carboxyl terminal of proenkephalin (*2, 10*). Subsequent cleavage of this fragment could produce an intermediate that contains the sequence of the octapeptide, [Met]enkephalin-Arg^6-Gly^7-Leu^8, at the carboxyl terminal but does not contain the amino terminal of the precursor, synenkephalin.

To test for the presence of such proposed intermediates in the supraoptic nucleus, we used RIA's specific for the carboxyl-terminal regions of the heptapeptide and the octapeptide in conjunction with gel filtration chromatography of tissue extracts (*11*). Portions of each column fraction were also assayed for total [Met]enkephalin immunoreactivity after digestion with trypsin and carboxypeptidase B and for synenkephalin immunoreactivity without digestion.

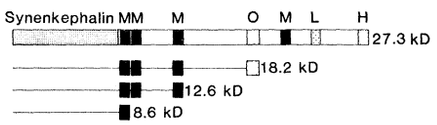

Fig. 1. Schematic representation of proenkephalin and the major high molecular weight ECP's present in the bovine adrenal medulla. All of the large ECP's isolated to date from this gland contain synenkephalin, the amino-terminal part of proenkephalin (*2, 3, 9*). This observation has led to the proposal of a processing pathway involving several successive proteolytic events that begin near the carboxyl-terminal of proenkephalin (*4*). M, [Met]enkephalin; O, octapeptide; L, [Leu]enkephalin; H, heptapeptide.

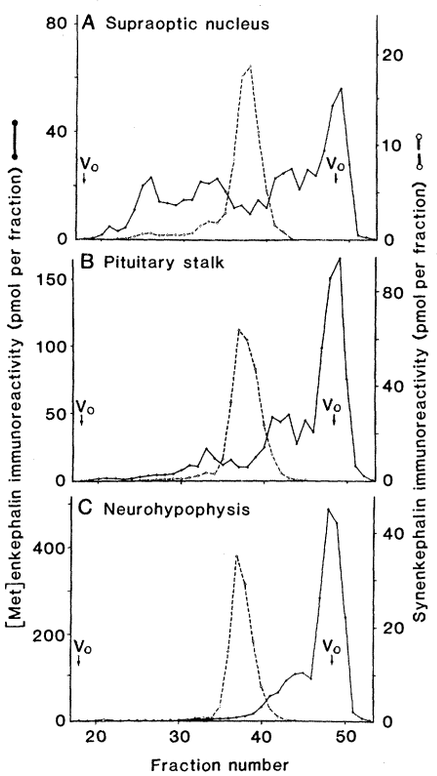

Fig. 2. The molecular forms of [Met]enkephalin immunoreactivity and synenkephalin immunoreactivity in the bovine hypothalamo-neurohypophysial system. The supraoptic nucleus, pituitary stalk, and neurohypophysis were dissected from six to eight brains and homogenized in four volumes of 1*M* acetic acid adjusted to *p*H 1.9 with HCl. Supernatants were fractionated on a column (1.6 by 90 cm) of Sephadex G-100 equilibrated with 1*M* acetic acid (*p*H 1.9). Portions of each fraction were lyophilized and assayed for [Met]enkephalin immunoreactivity (solid line) after enzymatic digestion, as described in Table 1, and for synenkephalin immunoreactivity (broken line). The relative proportion of high molecular weight ECP's decreased as the distance from the cell body increased.

The elution profiles of [Met]enkephalin immunoreactive material extracted from the supraoptic nucleus and adrenal were qualitatively similar, with several peaks of activity eluting together (Fig. 3A). A small amount of free [Met]enkephalin was present in the extract of the adrenal medulla, as has been reported by others (*4*). However, the elution profiles of the three other immunoreactivities tested showed striking differences between the two tissues. Synenkephalin immunoreactive material from the supraoptic nucleus eluted as a single peak, whereas at least three peaks of synenkephalin immunoreactive material were present in the adrenal extract (Fig. 3B). Heptapeptide immunoreactivity in the adrenal was restricted to peptides smaller than 10 kD, whereas the supraoptic nucleus contained a high molecular weight heptapeptide immunoreactive species that corresponded to the first peak of [Met]enkephalin immunoreactivity (Fig. 3C). The octapeptide immunoreactivity in the adrenal was predominantly in the form of a peptide eluting in the range 5 to 10 kD, with smaller amounts of activity residing in a high molecular weight species and the free octapeptide. In contrast, the supraoptic nucleus contained much greater relative amounts of the high molecular weight octapeptide immunoreactive proteins and free octapeptide, with only a small peak of immunoreactivity eluting in the 5- to 10-kD range of the column (Fig. 3D).

Unlike the large ECP's in the adrenal medulla, the high molecular weight heptapeptide and octapeptide immunoreactive species from the supraoptic nucleus were not associated with synenkephalin immunoreactivity, indicating that these peptides from the supraoptic nucleus have lost the amino-terminal region of the precursor. Furthermore, the presence of heptapeptide immunoreactivity in the largest ECP of the supraoptic nucleus demonstrates that this high molecular weight fragment is derived from the carboxyl-terminal portion of proenkephalin. The largest ECP's that accumulate in the supraoptic nucleus are thus different from those present in the adrenal medulla. Removal of the amino terminal fragment of proenkephalin apparently occurs at an early stage of processing in the supraoptic nucleus, giving rise to the high molecular weight heptapep-

tide- and octapeptide-containing proteins. This cleavage appears to be a much later processing event in the adrenal, occurring after cleavage within the carboxyl-terminal region of proenkephalin. However, rigorous determination of the order of appearance of the various processing intermediates will require pulse-chase experiments.

The observed differences in the ECP's that accumulate in the two tissues may result from differences in either the molecular form of the precursor or in the proteolytic reactions taking place in the processing of the precursor. Differences in the precursor would most likely arise from posttranslational modification of proenkephalin, as cell-free translation of bovine striatal and adrenal proenkephalin messenger RNA's appears to produce identical protein products (*12*). In addition, Southern hybridization analysis of bovine genomic DNA indicates the existence of a single proenkephalin gene (*13*).

Alternatively, tissue-specific expression of particular proteolytic activities could account for the distinct ECP's observed in the two tissues. Such differences may arise from identical processing enzymes operating in dissimilar environments (that is, different *p*H, ionic strength, or ionic composition) or from the presence of distinct processing enzymes. The recent discovery of a family of murine genes coding for kallikrein-related proteases (*14*) provides sufficient genetic potential for the production of a large number of such endoproteases, and enzymes of the kallikrein family have been suggested as candidates for the processing of proenkephalin (*15*). However, more direct characterization of the proteolytic enzymes involved in processing will be necessary to determine if tissue-specific expression of the enzymes is responsible for the differences observed in the processing intermediates.

Table 1. [Met]enkephalin immunoreactivity in the different parts of the bovine hypothalamo-neurohypophysial system. Tissues were homogenized in 4 volumes of 1*M* acetic acid which had been adjusted to *p*H 1.9 with HCl. After centrifugation at 50,000*g* for 30 minutes, the supernatants were lyophilized and suspended in 50 m*M* tris-HCl (*p*H 8.4) containing 2 m*M* $CaCl_2$. A portion was incubated with trypsin (trypsin TPCK, Worthington; 10 μg/ml) for 16 hours at 37°C, boiled for 15 minutes, incubated with carboxypeptidase B (Boehringer-Mannheim; final concentration 0.1 μg/ml) for 1 hour at 37°C, and again boiled for 15 minutes. Another portion was incubated in parallel in the presence of buffer without enzymes. [Met]enkephalin immunoreactivity was measured in the two portions by RIA. Values are the means ± standard error of the mean of extracts from six or seven animals.

Region	[Met]enkephalin immunoreactivity (pmol/mg)		Percent increase
	Before digest	After digest	
Supraoptic nucleus	0.565 ± 0.103	2.25 ± 0.44	298
Pituitary stalk	4.56 ± 1.01	10.26 ± 2.09	125
Neurohypophysis	28.78 ± 7.77	32.32 ± 10.12	12

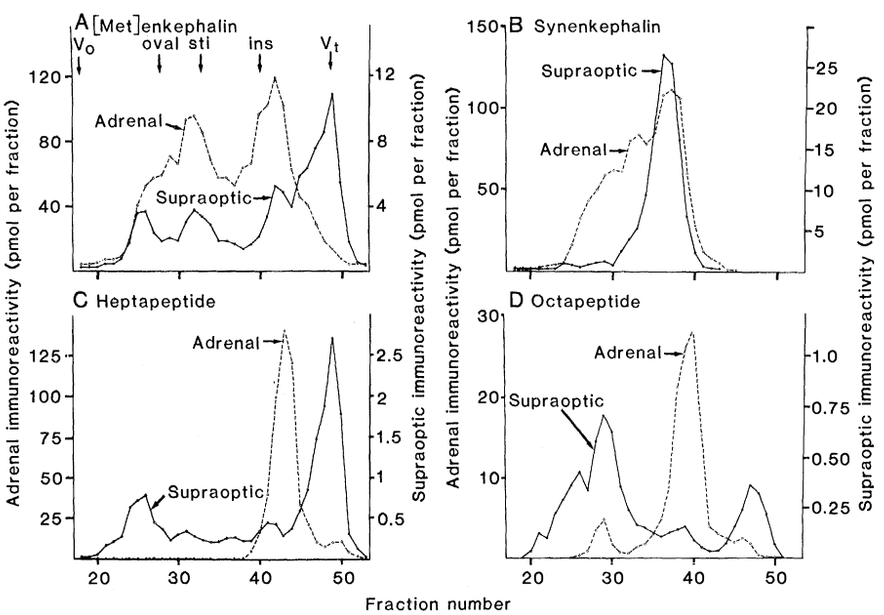

Fig. 3. Comparison of the ECP's present in extracts of bovine supraoptic nucleus and adrenal medulla. Tissues were prepared and chromatographed as described in the legend to Fig. 2. Portions of each fraction were assayed for [Met]enkephalin immunoreactivity after digestion with trypsin and carboxypeptidase B and for synenkephalin immunoreactivity (*9*) without digestion. Heptapeptide and octapeptide immunoreactivities were assayed in the same fractions by RIA's (*11*) after digestion with *Staphylococcus aureus* V8 protease (Miles Laboratories), which specifically cleaves peptide bonds at the carboxyl side of glutamic and aspartic acid residues (*16, 17*). (A) The elution profile of [Met]enkephalin immunoreactivity was qualitatively similar in the two tissue extracts, with several peaks of immunoreactivity eluting together. (B) Synenkephalin immunoreactivity from the adrenal medulla eluted as three main peaks but appeared as a single predominant peak in the supraoptic nucleus extract. This peak, as well as the third peak from the adrenal extract, eluted with pure synenkephalin prepared from bovine caudate nucleus. (C) Heptapeptide immunoreactivity in the adrenal extract was restricted to proteins of low molecular weight (<10 kD), but the supraoptic nucleus contained a high molecular weight heptapeptide immunoreactive species that eluted with the first peak of [Met]enkephalin immunoreactivity in this tissue. (D) The supraoptic nucleus also contained a large proportion of high molecular weight octapeptide immunoreactive material, whereas the predominant form of octapeptide immunoreactivity present in the adrenal gland eluted in the range 5 to 10 kD. The two peaks of octapeptide immunoreactive substance eluting in fraction 29 cannot be due to identical processing intermediates, since only the species from the adrenal medulla is also associated with synenkephalin immunoreactivity. The column markers used were V_o, blue dextran; oval, ovalbumin; sti, soybean trypsin inhibitor; ins, insulin; V_t, tyrosine.

DANE LISTON*
GILLES PATEY
JEAN ROSSIER
Laboratoire de Physiologie Nerveuse, Centre National de la Recherche Scientifique, 91190 Gif-sur-Yvette, France

PAUL VERBANCK
JEAN-JACQUES VANDERHAEGHEN
Erasme Hospital, Université Libre de Bruxelles, Queen Elisabeth Medical Foundation, Brussels, Belgium

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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.
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* To whom requests for reprints should be addressed at Department of Chemistry, University of Oregon, Eugene 97403.

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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.

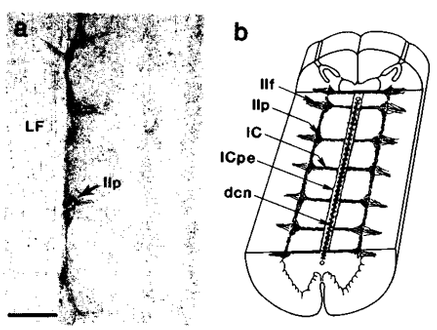

Fig. 1. (a) Low-power photomicrograph of a horizontal section of rat thoracic spinal cord showing enkephalin immunoreactivity in the nucleus intermediolateralis, pars principalis (IIp), and along the gray-white border of the lateral funiculus (LF) connecting adjacent IIp cell nests. Scale bar, 250 μm. (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.