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Enkephalin Systems in Diencephalon and Brainstem of the Rat

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ABSTRACT

The immunocytochemical distribution of [Leu]enkephalin and an adrenal enkephalin precursor fragment (BAM-22P) immunoreactivity was investigated in the diencephalon and brainstem of rats pretreated with relatively high doses of colchicine (300-400 μg/10 μl intracerebroventricularly). The higher ranges of colchicine pretreatment allowed the visualization of extensive enkephalin-containing systems in these brain regions, some of which are reported for the first time. Immunoreactive perikarya were found in many hypothalamic and thalamic nuclei, interpeduncular nucleus, substantia nigra, the colliculi, periaqueductal gray, parabrachial nuclei, trigeminal motor and spinal nuclei, nucleus raphe magnus and other raphe nuclei, nucleus reticularis paragigantocellularis, vestibular nuclei, several noradrenergic cell groups, nucleus tractus solitarius, as well as in the spinal cord dorsal horn. In addition to the above regions, immunoreactive fibers were also noted in the habenular nuclei, trigeminal sensory nuclei, locus coeruleus, motor facial nucleus, cochlear nuclei, dorsal motor nucleus of the vagus, and hypoglossal nucleus. When adjacent sections to those stained for [Leu]enkephalin were processed for BAM-22P immunoreactivity, it was found that these two immunoreactivities were distributed identically at almost all anatomical locations. BAM-22P immunoreactivity was generally less pronounced and was preferentially localized to neuronal perikarya. The results of the present as well as the preceding studies (Khachaturian et al., '83) strongly suggest substantial structural similarity between the adrenal proenkephalin precursor and that which occurs in the brain. Also discussed are some differences and parallels between the distribution of [Leu]enkephalin and dynorphin immunoreactivities.

Key words: [Leu]enkephalin, BAM-22P, proenkephalin immunocytochemistry, colchicine

Methionine [Met]- and leucine [Leu]enkephalin are opioid pentapeptides that occur in both the brain (Hughes et al., '75) and adrenal gland (cf. Kimura et al., '80). In the adrenal medulla, molecular cloning of cDNA from proenkephalin mRNA has revealed the structure of the enkephalin precursor (Comb et al., '82; Gubler et al., '82; Noda et al., '82). The precursor contains fours copies of [Met]enkephalin, and single copies of [Leu]enkephalin, [Met]enkephalin-Arg^6-Phe^7, and [Met]enkephalin-Arg^6-Gly^7-Leu^8, which are contained within longer peptide sequences, such as BAM-22P and peptides I, F, E, and B. The biosynthesis of the enkephalins is distinct from that of either β-endorphin (Mains et al., '77; Roberts and Herbert, '77; Nakanishi et al., '79) or dynorphin (Kakidani et al., '82).

The enkephalins have a wide distribution within the central nervous system, with highest radioimmunoassayable levels occurring in the striatum, and next the diencephalon, brainstem, and cortical-limbic areas (Rossier et al., '77; Yang et al., '77; Kobayashi et al., '78; Miller et al., '78). [Met]- and [Leu]enkephalin have also been immunocytochemically localized in many brain sites (Elde et al., '76; Watson et al., '77a, '78; Hökfelt et al., '77a,b, '79; Simantov et al., '77; Bloom et al., '78; Sar et al., '78; Uhl et al., '78, '79; Rossier et al., '79; Jacobowitz et al., '79; Wamsley et al., '80; Micevych and Elde, '80; Pickel et al., '80; Finley et al., '81; Gall et al., '81; Khachaturian et al., '83). The concentrations of the enkephalins in some brain sites, such as the cerebral cortex and some limbic regions, are apparently too low to be detected immunocytochemically without colchicine pretreatment. Studies using varying doses of colchicine have shown enkephalin-positive fibers (Sar et al., '78; Wamsley et al., '80) as well as perikarya (Gall et al., '81; Finley et al., '81) in the cerebral cortex and certain limbic structures. More recently, using relatively high doses of colchicine

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(300–400 μg, intracerebroventricularly), we have been able to visualize extensive cortical-limbic and other telencephalic enkephalinergic systems (Khachaturian et al., '83). Furthermore, in that study, we also used antisera against BAM-22P, a bovine adrenal medullary enkephalin precursor fragment (Mizuno et al., '80), to demonstrate an anatomical similarity between the distributions of [Leu]-enkephalin and BAM-22P in the rat telencephalon, thus providing evidence for the existence in rat brain of an enkephalin biosynthetic mechanism similar to that found in the adrenal gland. We now report on the codistribution of [Leu]enkephalin and BAM-22P immunoreactivities in diencephalic and other brainstem sites as well as in the spinal cord.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats were anesthetized with sodium pentobarbital and given colchicine (300–400 μg/10 μl normal saline) intracerebroventricularly, approximately 48 hours prior to perfusion. The rats were reanesthetized with sodium pentobarbital (50 mg/ml injected intraperitoneally), and the cardiovascular system was flushed with 50 ml of ice-cold 0.9% saline through the left ventricle, with the right atrium cut open. The ventricles were excised immediately and a cannula was inserted into the aorta, through which the animal was perfusion-fixed with ice-cold 0.1 M phosphate-buffered 4% formaldehyde (pH 7.4) at 140 mm Hg for 30 minutes. The brains from these rats were blocked and refrigerated overnight in phosphate-buffered 15% sucrose, and then frozen by immersion in −40°C isopentane for 30–40 seconds, and stored at −70°C. Sections were cut at 20-μm thickness in a cryostat (−20°C) and mounted onto subbed slides.

For peroxidase-antiperoxidase immunocytochemistry (Watson et al., '77b), frozen tissue sections were allowed to air-dry at room temperature and were then incubated (37°C) with normal goat serum (NGS, from GIBCO) at a dilution of 1/30 for approximately 5–10 minutes, followed by primary rabbit antisera to specific substances diluted with 0.02 M phosphate-buffered saline (PBS) and 0.3% Triton X-100, for 1 hour, and overnight at 4°C. Control sections were incubated with antiserum preadsorbed with 1–20 μM concentrations of peptide against which the antiserum was raised, as well as with other peptides having amino acid sequences similar to the peptide in question. For these cross-blocking studies, [Leu]enkephalin and BAM-22P antisera were preadsorbed with [Leu]enkephalin, [Met]enkephalin, BAM-22P, BAM-12P, peptide E, dynorphin A, or β-endorphin. The following day, the tissues were washed in PBS and incubated with NGS for 5–10 minutes at 37°C, and then goat-antirabbit serum (Sternberger-Meyer) at 1/100 dilution for 30 minutes, and overnight at 4°C. The next day, the tissues were washed in PBS, incubated at 37°C with NGS for 5–10 minutes, followed by antihorseradish peroxidase (HRP) serum at 1/200 dilution for 40 minutes. Again, the tissues were washed in PBS and incubated at 37°C with 4 μg/ml HRP enzyme (Sigma, type VI) for another 40 minutes. The peroxidase reaction was initiated by the immersion of the tissues in a solution of 0.03% H_2O_2 and 0.125 mg/ml diaminobenzidine (Sigma) for 15 minutes at room temperature, with constant stirring. The tissues were washed in distilled water, osmicated (2% OsO_4) briefly, washed, dehydrated through graded ethanols and xylenes, and coverslipped with Permount. Observations and photographs were made with a Leitz Orthoplan microscope.

[Leu]enkephalin antiserum was purified by affinity chromatography (March et al., '74). The amino terminus of the peptide was covalently linked to a cyanogen bromide-activated Sepharose-4B column and the crude anti[Leu]enkephalin serum was passed through the column in order to absorb and concentrate specific IgG molecules with affinity toward [Leu]enkephalin. The resultant purified, carboxyl terminus-directed antiserum yields a much improved immunocytochemical signal with greatly reduced background (Watson and Akil, '81). Also, for comparative purposes, we have employed antisera generated against BAM-22P (antiserum 'Tristan,' Hollt et al., '81), serotonin (our own), and tyrosine hydroxylase (supplied by Dr. T. Joh, Cornell University).

RESULTS

[Leu]enkephalin immunoreactivity is localized to perikarya and processes of neurons in many diencephalic, brainstem, and spinal cord structures (see Fig. 1). [Leu]enkephalin-positive perikarya are found in many hypothalamic and thalamic nuclei, interpeduncular nucleus, substantia nigra and other dopaminergic cell group areas, the colliculi, periaqueductal gray, nucleus raphe dorsalis (mostly lateral to), raphe magnus and other serotonergic raphe nuclei, parabrachial nuclei, motor trigeminal nucleus, spinal trigeminal nucleus, nucleus reticularis gigantocellularis and paragigantocellularis, several vestibular nuclei, several noradrenergic cell group areas, nucleus tractus solitarius, and spinal cord dorsal horn. Additionally [Leu]enkephalin-positive fibers or terminals are seen in all of the above regions, and further in habenular nuclei, locus coeruleus, main sensory trigeminal nucleus, motor facial nucleus, cochlear nuclei, dorsal motor nucleus of vagus, and hypoglossal nucleus. When sections adjacent to those processed for [Leu]enkephalin immunoreactivity were analyzed for BAM-22P immunoreactivity, a very similar anatomical patterning was noted between the distribution of [Leu]enkephalin and the precursor fragment immunoreactivities (see Fig. 6A,B). However, BAM-22P immunoreactivity was generally less pronounced than [Leu]enkephalin immunoreactivity and tended to be localized preferentially to neuronal perikarya. Thus, although most regions which showed [Leu]enkephalin-positive perikarya and fibers also contained BAM-22P immunoreactivity, certain areas did not demonstrate BAM-22P-positive fibers or terminals. These regions include the supraoptic and anterior hypothalamic nuclei, periventricular and lateral geniculate nuclei of thalamus, inferior colliculus, motor trigeminal nucleus, nucleus reticularis gigantocellularis, lateral, superior, and spinal vestibular nuclei, and the cochlear nuclei.

The immunoreactive signal obtained by using either [Leu]enkephalin or BAM-22P was much enhanced in colchicine-pretreated animals compared to untreated rats. Perikaryal staining, especially of smaller classes of neurons, was best demonstrated after relatively high doses of colchicine (300–400 μg). These doses of colchicine also enhanced the visualization of dendritic and axonal processes. The immunoreactive signal obtained by using [Leu]enkephalin or BAM-22P antisera was completely abolished by the addition of 1 μM [Leu]enkephalin or BAM-22P, respectively, to the primary rabbit antiserum prior to incubation. Cross-blocking paradigms further demonstrated

the specificity of each antiserum: [Leu]enkephalin immunoreactivity was not blocked by 20 μM BAM-22P, peptide E, dynorphin A, or β-endorphin. Likewise, the BAM-22P signal was not blocked by 20 μM [Met]- or [Leu]enkephalin, BAM-12P, peptide E, dynorphin A, or β-endorphin.

In the preoptic region, small [Leu]enkephalin immunoreactive perikarya are densely packed in the medial preoptic area and scattered in the lateral preoptic area. Within the hypothalamus proper, [Leu]enkephalin-positive perikarya are scattered throughout the anterior hypothalamic area, periventricular, dorsomedial, and arcuate nuclei, lateral and posterior hypothalamic areas, as well as medial regions of the medial mammillary (Fig. 2B) and lateral mammillary nuclei. In the region of the paraventricular nucleus, scattered perikarya are seen in the parvicellular but not magnocellular part of this nucleus (Fig. 2A), as well as more laterally surrounding the columns of the fornix (Fig. 2A). The ventromedial nucleus also contains scattered positive perikarya. However, in the supraoptic nucleus, no magnocellular perikarya exhibit [Leu]enkephalin immunoreactivity, although many positive varicose fibers are seen scattered in this nucleus, as well as throughout the hypothalamus, in dorsomedial and mammillary nuclei, and in lateral and posterior hypothalamic areas. A moderately denser accumulation of immunoreactive [Leu]enkephalin fibers or terminals appears in anterior hypothalamic area, as well as parvicellular paraventricular, periventricular, ventromedial, and arcuate nuclei. From the mammillary

Abbreviations

III	Oculomotor nucleus
X	Dorsal motor nucleus, vagus
XII	Hypoglossal nucleus
A 5	Area of noradrenergic cell group A5
AAA	Amygdala, anterior area
ABL	Amygdala, basolateral nucleus
AC	Anterior commissure
ACB	Nucleus accumbens
ACE	Amygdala, central nucleus
ACO	Amygdala, cortical nucleus
AD	Anterodorsal nucleus, thalamus
AHA	Anterior hypothalamic area
AM	Anteromedial nucleus, thalamus
AMB	Nucleus ambiguus
AME	Amygdala, medial nucleus
AON	Anterior olfactory nucleus
ARC	Arcuate nucleus
AV	Anteroventral nucleus, thalamus
BST	Bed nucleus, stria terminalis
CC	Corpus callosum
CG	Cingulate cortex
CI	Internal capsule
CM	Central-medial nucleus, thalamus
COCH	*Cochlear nuclear complex*
CPU	Caudate-putamen
CST	Corticospinal tract
CTN	Central tegmental nucleus
DFU	Dorsal funiculus, spinal cord
DH	Dorsal horn, spinal cord
DG	Dentate gyrus
DLL	Dorsal nucleus lateral lemniscus
DM	Dorsomedial nucleus, thalamus
DMH	Dorsomedial nucleus, hypothalamus
DTN	Dorsal tegmental nucleus
ENT	Entorhinal cortex
FN	Fastigial nucleus, cerebellum
FRT	Frontal cortex
FX	Fornix
GL	Glomerular layer, olfactory bulb
GP	Globus pallidus
HL	Lateral habenular nucleus
HM	Medial habenular nucleus
HPC	Hippocampus
IC	Inferior colliculus
IP	Interpeduncular nuclear complex
LC	Nucleus locus coeruleus
LFU	Lateral funiculus, spinal cord
LG	Lateral geniculate nucleus
LHA	Lateral hypothalamic area
LM	Medial lemniscus
LRN	Lateral reticular nucleus
LTN	Lateral tegmental nucleus
MF	Mossy fibers, hippocampus
MFN	Motor facial nucleus
MG	Medial geniculate nucleus
ML	Lateral mammillary nucleus
MLF	Medial longitudinal fasciculus
MM	Medial mammillary nucleus
MNT	Mesencephalic nucleus, trigeminal
MTT	Mammillothalamic tract
NCU	Nucleus cuneatus
NDB	Nucleus diagonal band (Broca)
NGR	Nucleus gracilis
NPH	Nucleus prepositus hypoglossi
NRGC	Nucleus reticularis gigantocellularis
NRPG	Nucleus reticularis paragigantocellularis
NSTT	Nucleus spinal tract, trigeminal
NTS	Nucleus tractus solitarii
NVL	Lateral vestibular nucleus
NVM	Medial vestibular nucleus
NVS	Superior vestibular nucleus
OB	Olfactory bulb
OCC	Occipital cortex
OT	Optic tract
OTU	Olfactory tubercle
P	Pons
PAG	Periaqueductal gray
PBL	Lateral parabrachial nucleus
PMB	Medial parabrachial nucleus
PC	Posterior commissure
PHA	Posterior hypothalamic area
PIR	Piriform cortex
POA	Preoptic area
PP	Perforant path
PV	Periventricular nucleus, thalamus
PVN	Paraventricular nucleus
RD	Nucleus raphe dorsalis
RE	Nucleus reuniens, thalamus
RF	Reticular formation
RH	Nucleus rhomboideus, thalamus
RM	Nucleus raphe magnus
RME	Nucleus raphe medianus
RPO	Nucleus raphe pontis
SC	Superior colliculus
SM	Stria medullaris thalami
SNC	Substantia nigra, pars compacta
SNR	Substantia nigra, pars reticulata
SON	Supraoptic nucleus
SPT	Septal nuclei
ST	Stria terminalis
STT	Spinal tract of trigeminal
TMN	Trigeminal motor nucleus
TSN	Trigeminal sensory nucleus (main)
V	Ventricle
VA	Ventral nucleus of thalamus, anterior
VD	Ventral nucleus of thalamus, dorsal
VE	Ventral nucleus of thalamus
VFU	Ventral funiculus, spinal cord
VH	Ventral horn, spinal cord
VLL	Ventral nucleus, lateral lemniscus
VMH	Ventromedial nucleus, hypothalamus
VTA	Ventral tegmental area
ZI	Zona incerta

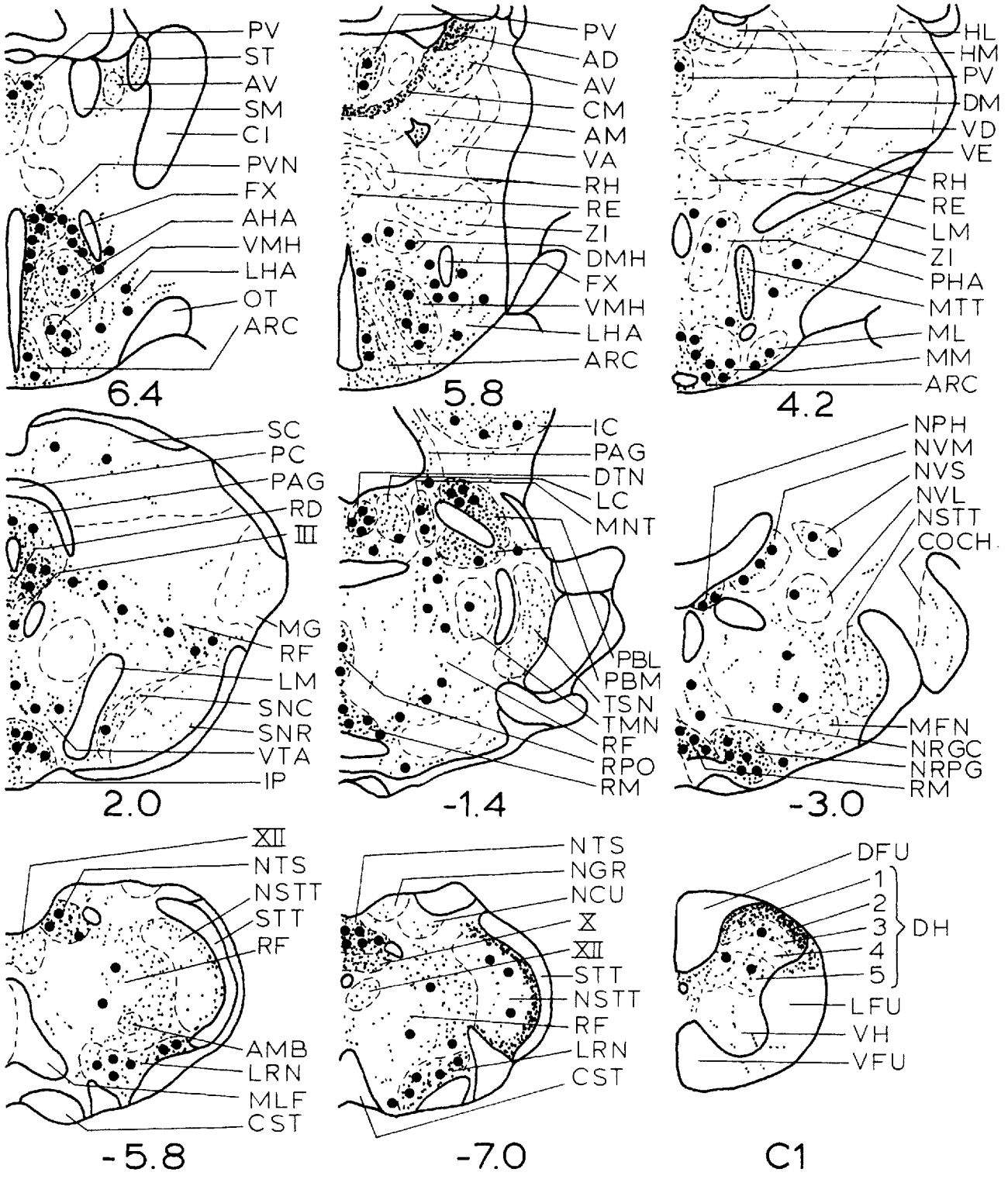


Fig. 1. These schematic frontal sections show the distribution of [Leu]-enkephalin perikarya (solid circles) and processes or terminals (dots) in nine selected levels through the diencephalon, brainstem, and spinal cord (cervical level 1, or C1). The numbers of perikarya shown in each nucleus or region are not meant to be quantitative. The number in the lower center of each drawing indicates the distance (mm) of that section from the vertical zero plane (modified from Pellegrino et al., '79; Paxinos and Watson, '82). See text for details.

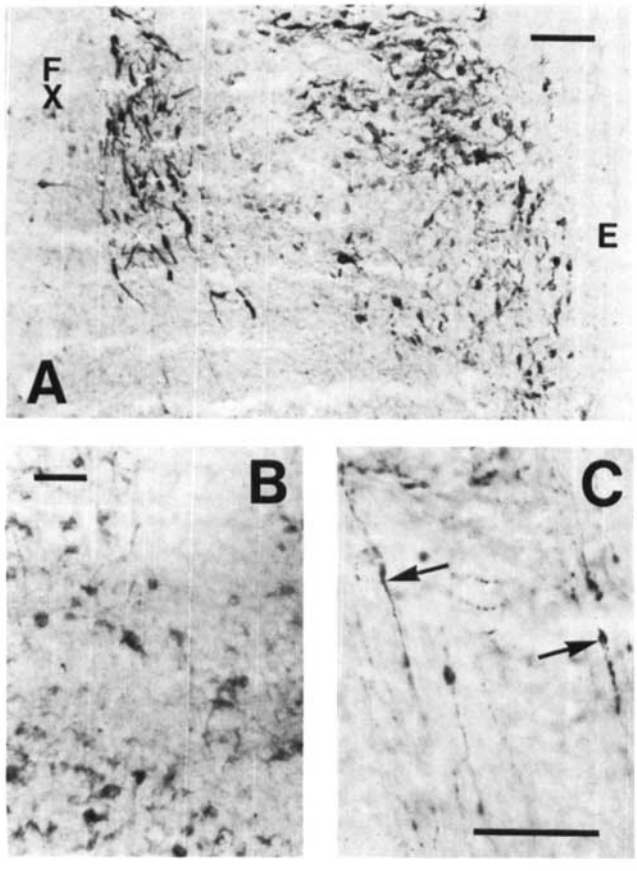


Fig. 2. *Hypothalamus.* Panel A shows immunoreactive [Leu]enkephalin perikarya in the parvocellular paraventricular nucleus and to the left of this panel many perifornical perikarya also exhibit positive immunoreactivity. [Leu]enkephalin immunoreactive perikarya can be seen in the medial aspect of the medial mammillary nucleus (panel B), as well as fibers in the memmillothalamic tract (arrows, panel C). E, Third ventricular ependyma. Bar A = 100 μm. Bar B,C = 50 μm.

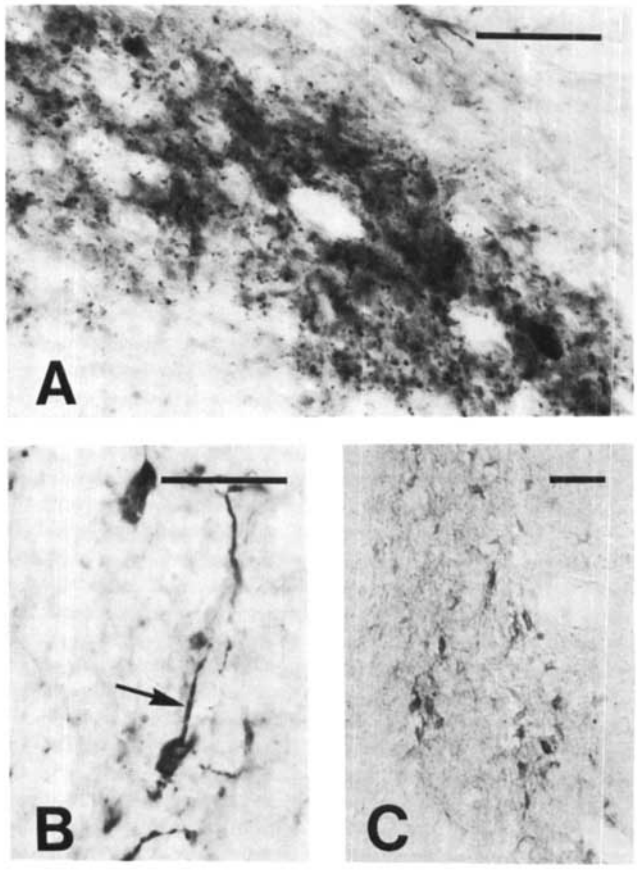
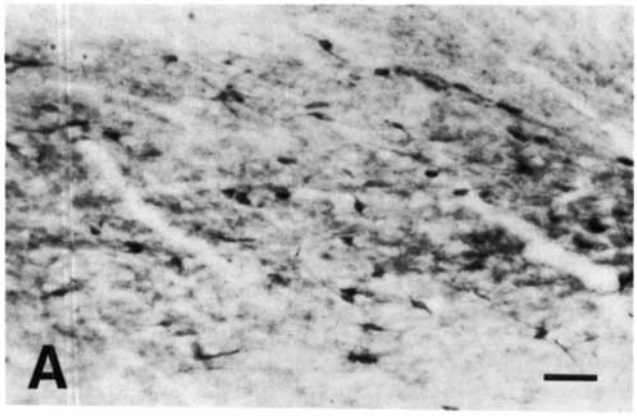


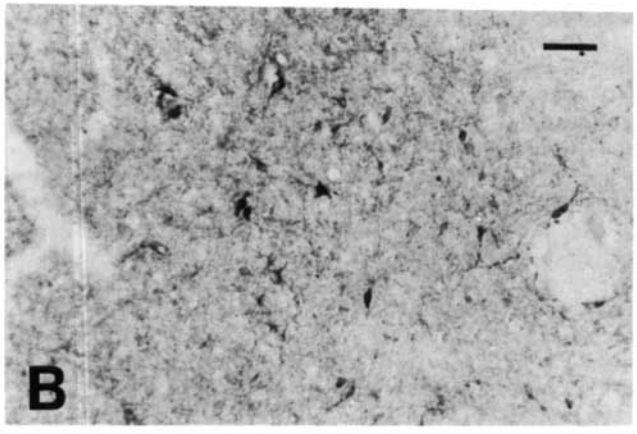
Fig. 3. *Thalamus.* Panel A shows a dense accumulation of [Leu]enkephalin immunoreactive terminals in the anterodorsal nucleus. Panel B shows immunoreactive perikarya and fibers (arrow) in periventricular nucleus. Panel C depicts [Leu]enkephalin-positive perikarya in the ventral region of the lateral geniculate nucleus. Bar = 50 μm.

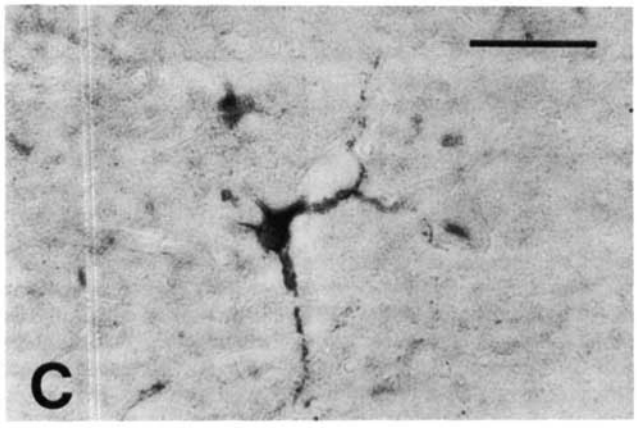
nuclei, immunoreactive fibers are seen to enter the mammillothalamic tract, which itself demonstrates positive fiber immunoreactivity throughout its entire course (Fig. 2C) to the anterior nucleus of the thalamus (Fig. 3A). Other hypothalamic fiber tracts with immunoreactive [Leu]enkephalin fibers include the ventral amygdalofugal tract, the medial forebrain bundle, and the external and internal (scattered) zones of the median eminence. Many reactive terminals end in the vicinity of hypothalamohypophysial portal vessels in the external zone of the median eminence.

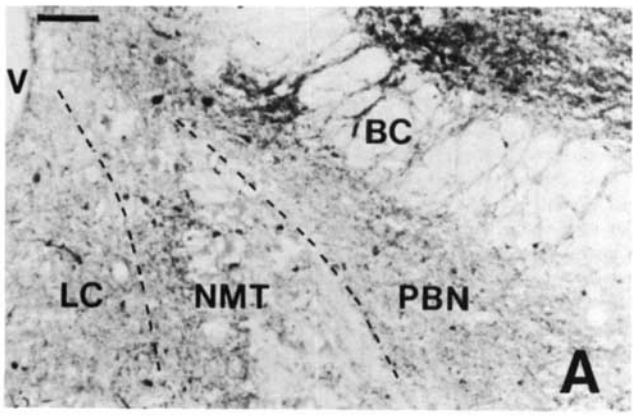
In the thalamus, scattered immunoreactive [Leu]enkephalin perikarya are localized in the periventricular nucleus throughout its rostrocaudal extent (Fig. 3B), and in the ventral regions of the lateral geniculate nucleus (Fig. 3C). These areas also contain [Leu]enkephalin-positive fibers, as do anterior thalamic nuclei (see below; Fig. 3A), medial geniculate nucleus, nucleus reuniens, nucleus rhomboideus, medial nucleus (see below), and lateral and medial habenular nuclei. Most of these nuclei contain scattered reactive fibers and varicosities. However, the periventricular and anteroventral thalamic nuclei contain a moderately dense distribution of immunoreactive fibers. The densest accumulation of [Leu]enkephalin-positive terminals in the thalamus appear in the anterodorsal (Fig. 3A) and centromedian nuclei.

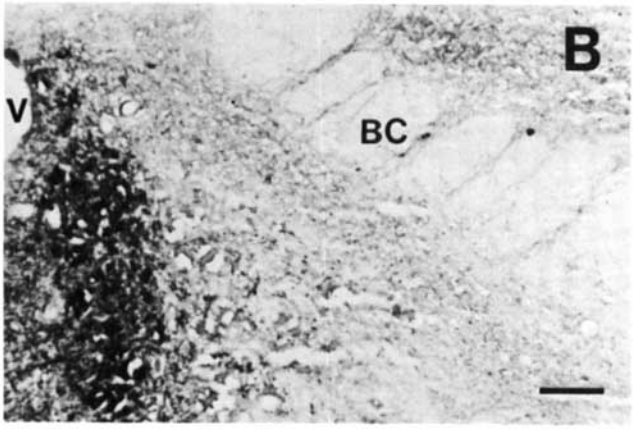
In the midbrain region, a dense accumulation of small immunoreactive [Leu]enkephalin perikarya is seen in the central and lateral subdivisions of the interpeduncular nucleus (Fig. 4A), and dorsally in the dorsal tegmental nucleus. Scattered positive perikarya also appear in the ventral, lateral, and central tegmental nuclei, dorsal and ventral nuclei of the lateral lemniscus, substantia nigra (only occasional positive perikarya in pars compacta), ventral tegmental area, both superior and inferior colliculi (Fig. 4C), medial nucleus of the optic tract, periaqueductal gray (Fig. 4B), oculomotor (occasional), and mesencephalic trigeminal nuclei (Fig. 5A). Other scattered [Leu]enkephalin perikarya are seen within the mesencephalic reticular formation, forming a band of cells that stretches medially and dorsally from a position just dorsal to the lateralmost regions of the cerebral peduncles, toward the periaqueductal gray. Scattered [Leu]enkephalin-positive fibers and terminals are also noted in aforementioned areas which contain immunoreactive perikarya, except in the following areas which contained a moderate distribution of fibers or terminals: all regions of the interpeduncular nucleus, sub-











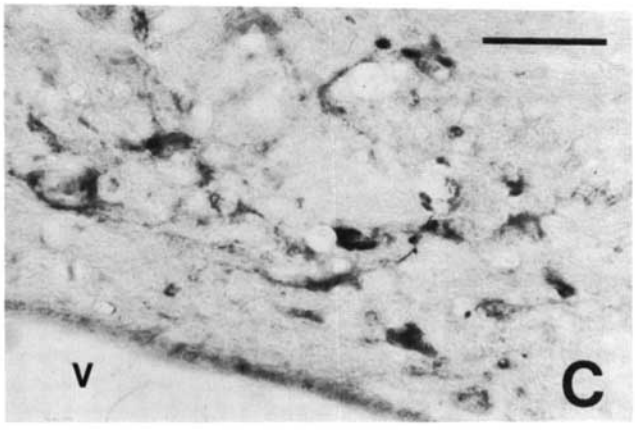


Fig. 4. *Mesencephalon.* Panel A shows immunoreactive [Leu]enkephalin perikarya in the central subdivision of the interpeduncular nucleus. Scattered [Leu]enkephalin-positive perikarya can be seen in the periaqueductal gray (panel B). Panel C depicts a [Leu]enkephalin immunoreactive perikaryon within the inferior colliculus. Bar = 50 μm.

Fig. 5. *Pons and cerebellum.* Panels A and B are adjacent tissue sections through the nucleus locus coeruleus (LC), mesencephalic nucleus of the trigeminal nerve (NMT), and parabrachial nucleus (PBN), showing [Leu]enkephalin (A) and tyrosine hydroxylase (B) immunoreactivity. Note that the concentration of [Leu]enkephalin-positive perikarya and fibers is relatively more dense in the lateral parabrachial nucleus, dorsal to the brachium conjunctivum (BC). Panel C shows [Leu]enkephalin-positive perikarya in the cerebellar fastigial nucleus. V, fourth ventricle. Bar A,B = 100 μm. Bar C = 50 μm.

stantia nigra pars compacta (and scattered within pars reticulata), ventral tegmental area, inferior colliculus, and periaqueductal gray.

In the pons, [Leu]enkephalin-positive perikarya are seen within the periaqueductal gray in the vicinity of the nucleus raphe dorsalis (but mainly concentrated lateral to this serotonergic nucleus), nucleus locus coeruleus (occasional; Fig. 5A) and subcoeruleus, lateral and medial parabrachial nuclei (Fig. 5A), motor trigeminal nucleus (occasional), serotonergic nuclei raphe pontis, raphe magnus (Fig. 6A,B), and widely scattered throughout the pontine reticular formation. Immunoreactive [Leu]enkephalin fibers are also noted in all of these nuclei and regions, some

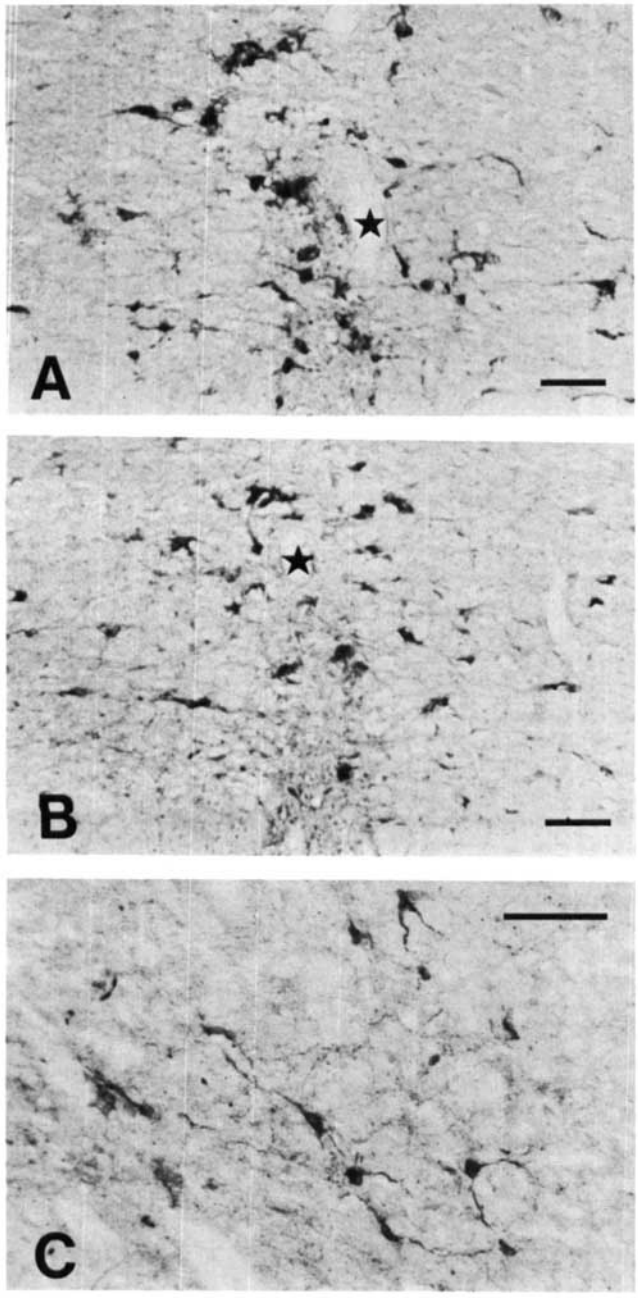


Fig. 6. *Rostral medulla.* Panels A and B are adjacent tissue sections through the nucleus raphe magnus showing [Leu]enkephalin (A) and BAM-22P (B) perikaryal immunoreactivities in similar anatomical position (note the position of the capillary lumen (star) situated at the midline in both panels A and B). Panel C shows immunoreactive [Leu]enkephalin perikarya within the nucleus reticularis paragigantocellularis. Bar = 100 μm.

containing relatively greater amounts of positive varicosities, including periaqueductal gray, locus coeruleus, lateral and medial parabrachial nuclei, and nuclei raphe pontis and raphe magnus. Immunoreactive [Leu]enkephalin fibers also occur in the main sensory nucleus of the trigeminal, motor facial nucleus, as well as in dorsal and ventral cochlear nuclei.

In the medulla, as in the pons, many [Leu]enkephalin-containing perikarya and fibers are scattered throughout the reticular formation. Many reactive perikarya are concentrated in the nucleus raphe magnus (Fig. 6A), nucleus reticularis paragigantocellularis (Fig. 6C), lateral reticular nucleus, caudal regions of nucleus tractus solitarius (Fig. 7C), among cells of the A5 noradrenergic cell group (Fig. 7A,B), and nucleus prepositus hypoglossi. Other scattered immunoreactive [Leu]enkephalin perikarya are present in medial aspects of the nucleus reticularis gigantocellularis, substantia gelatinosa of spinal trigeminal nucleus, medial, superior, and lateral vestibular nuclei, the cerebellar fastigial nucleus, and the parvicellular part of the lateral reticular nucleus. Immunoreactive [Leu]enkephalin fibers or terminals are present in moderate density in the above-mentioned nuclei, with the exception of nucleus reticularis gigantocellularis and the vestibular nuclei, which have scattered varicosities. Some areas contained moderate-to-dense accumulations of [Leu]enkephalin-positive terminals, including the spinal nucleus of the trigeminal, the nucleus tractus solitarii area, and extending ventrally into the dorsal motor nucleus of the vagus, hypoglossal nucleus, and laterally into the reticular formation, forming a band continuous with more ventrally located structures, including nucleus ambiguus, and lateral reticular nucleus.

Within the cervical spinal cord, scattered [Leu]enkephalin-containing perikarya are concentrated mainly in the deeper laminae of the dorsal horn (laminae III and V; Fig. 8B). A dense accumulation of immunoreactive fibers or terminals is located in the dorsal horn, especially within laminae I and II, and to a lesser extent in laminae III–V (Fig. 8A). Other scattered positively staining fibers are distributed around the central canal as well as in the ventral gray horn in the vicinity of the large spinal motor neurons. [Leu]enkephalin-positive varicosities are also seen in the dorsal aspects of the lateral funiculus near the dorsal gray horn (Fig. 8A).

A schematic summary of the findings of the present study and those of a previous study on telencephalic enkephalinergic systems (Khachaturian et al., '83) is shown in Figure 9.

DISCUSSION

Rats given relatively high doses of colchicine (300–400 μg/animal) have been used in this study of the distribution of [Leu]enkephalin and BAM-22P immunoreactivities in diencephalic, brainstem, and spinal cord structures. Colchicine pretreatment was found to enhance the immunocytochemical signal obtained using antisera generated against these peptides. In particular, perikarya, especially those of parvicellular nuclei, exhibited darker staining with the highest doses used (300–400 μg), presumably because of the inhibitory action of colchicine upon microtubule formation (Dahlström, '68; Kreutzberg, '69; Norstrom et al., '71; Fink et al., '73), which results in a diminution of axonal transport and an accumulation of material synthesized in the perikaryon. Perhaps because of the same action of colchicine, both dendrites and axons also exhibited an enhanced visual immunoreactive signal. Despite reports of neuronal damage following direct intracerebral injections of colchicine (Goldschmidt and Steward, '82), we did not detect any signs of neuronal damage in Nissl-stained sections from rats injected intraventricularly with colchicine, although tissue damage did occur in the immediate vicinity of the cannula injection track.

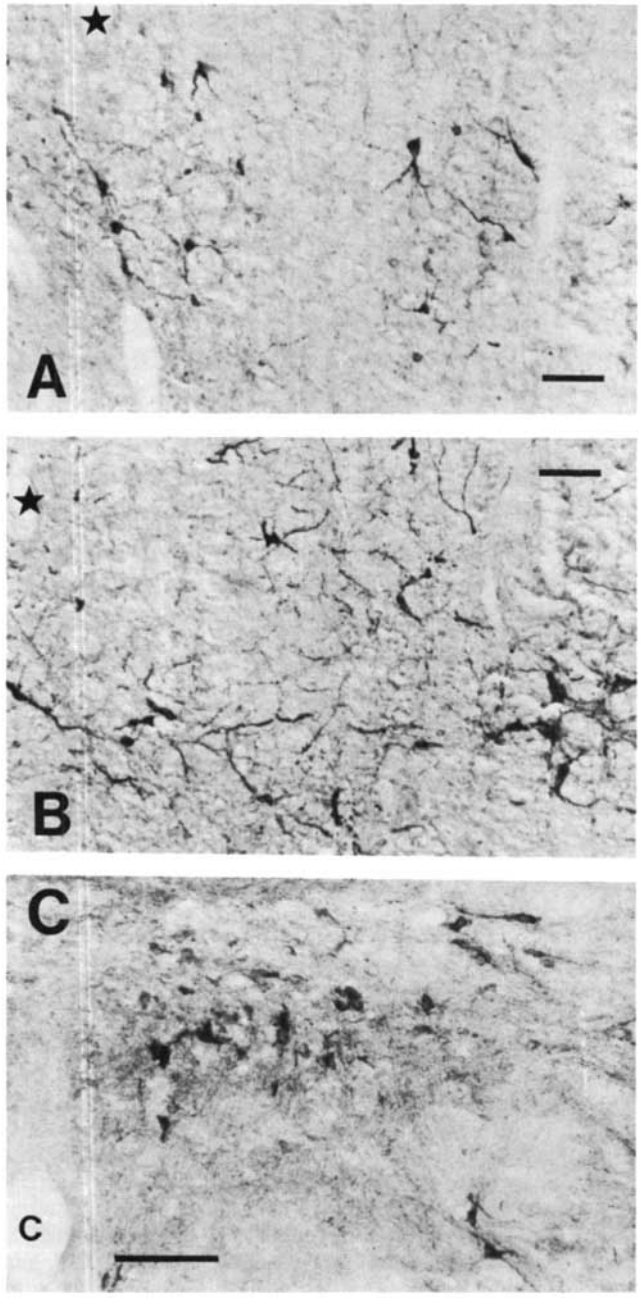


Fig. 7. *Caudal medulla.* Panels A and B are adjacent tissue sections through the A5 noradrenergic cell group showing positive perikarya to both [Leu]enkephalin (A) and tyrosine hydroxylase (B). Note the dissimilar distribution of [Leu]enkephalin- and tyrosine hydroxylase-containing perikarya. Also in A and B, note position of capillary lumen (stars). Panel C shows positive [Leu]enkephalin-containing perikarya in the caudal nucleus tractus solitarius (C, central canal). Bar = 100 μm.

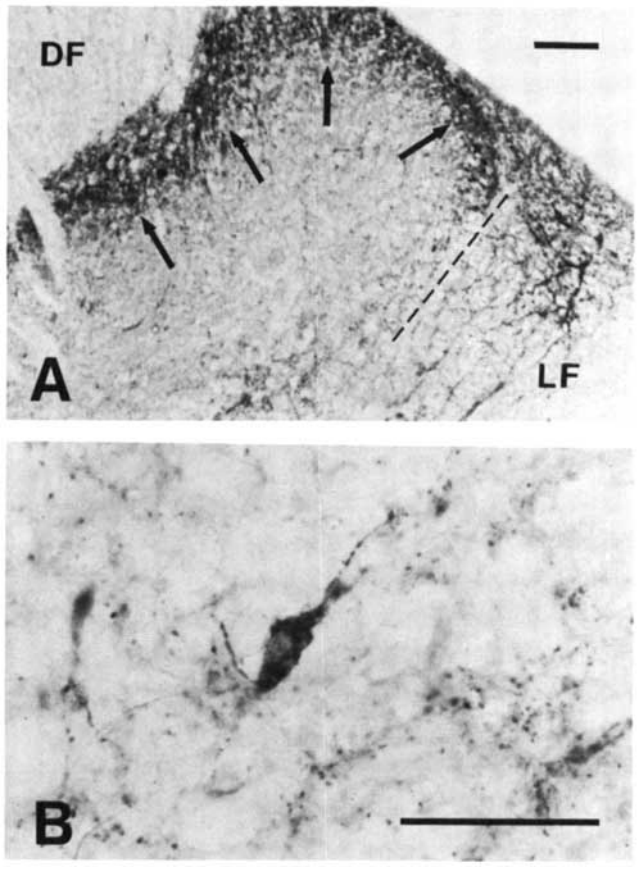


Fig. 8. *Spinal cord.* Panel A shows the dorsal horn of the spinal cord gray where immunoreactive [Leu]enkephalin terminals are concentrated within laminae I and II (arrows), dorsolateral funiculus (lateral to the dashed line), and scattered throughout the deeper laminae as well. A lamina V [Leu]enkephalin-containing perikaryon is shown in panel B. LF, lateral funiculus; DF, dorsal funiculus. Bar A = 100 μm. Bar B = 50 μm.

[Leu]enkephalin and BAM-22P immunoreactivities were found to be distributed similarly in neuronal perikarya, fibers, and terminals in most diencephalic, brainstem and spinal cord sites, thus suggesting substantial structural similarity between the adrenal pro-enkephalin precursor (Comb et al., '82; Gubler et al., '82; Noda et al., '82) and the presently unidentified precursor which occurs in brain (Kojima et al., '82). Adjacent-section analysis revealed that in the majority of brain sites observed in the present study, as well as the preceding study (Khachaturian et al., '83), BAM-22P immunoreactivity occurred in areas that also contained [Leu]enkephalin immunoreactivity, and further, the distributional patterns of these two immunoreactivities within these areas were identical. Since 20-μm sections were used in these studies, the question of colocalization within the same neurons was not approached. However, using serial 4-μm section analysis, we have recently demonstrated the existence of [Leu]enkephalin and BAM-22P immunoreactivities in the same brain neurons, reinforcing the hypothesis that the brain and adrenal enkephalin precursors are similar (Khachaturian et al., in press). Both of these immunoreactivities were shown to be specific for their respective peptides, since [Met]- or [Leu]-enkephalin, BAM-12P, peptide E, dynorphin A, and β-endorphin did not block the immunoreactive signal obtained using sera generated against BAM-22P (blocked by 1 μM BAM-22P), and further, BAM-22P, dynorphin A, and β-endorphin did not block the immunoreactive signal of anti[Leu]enkephalin serum (blocked by 1 μM [Leu]enkephalin).

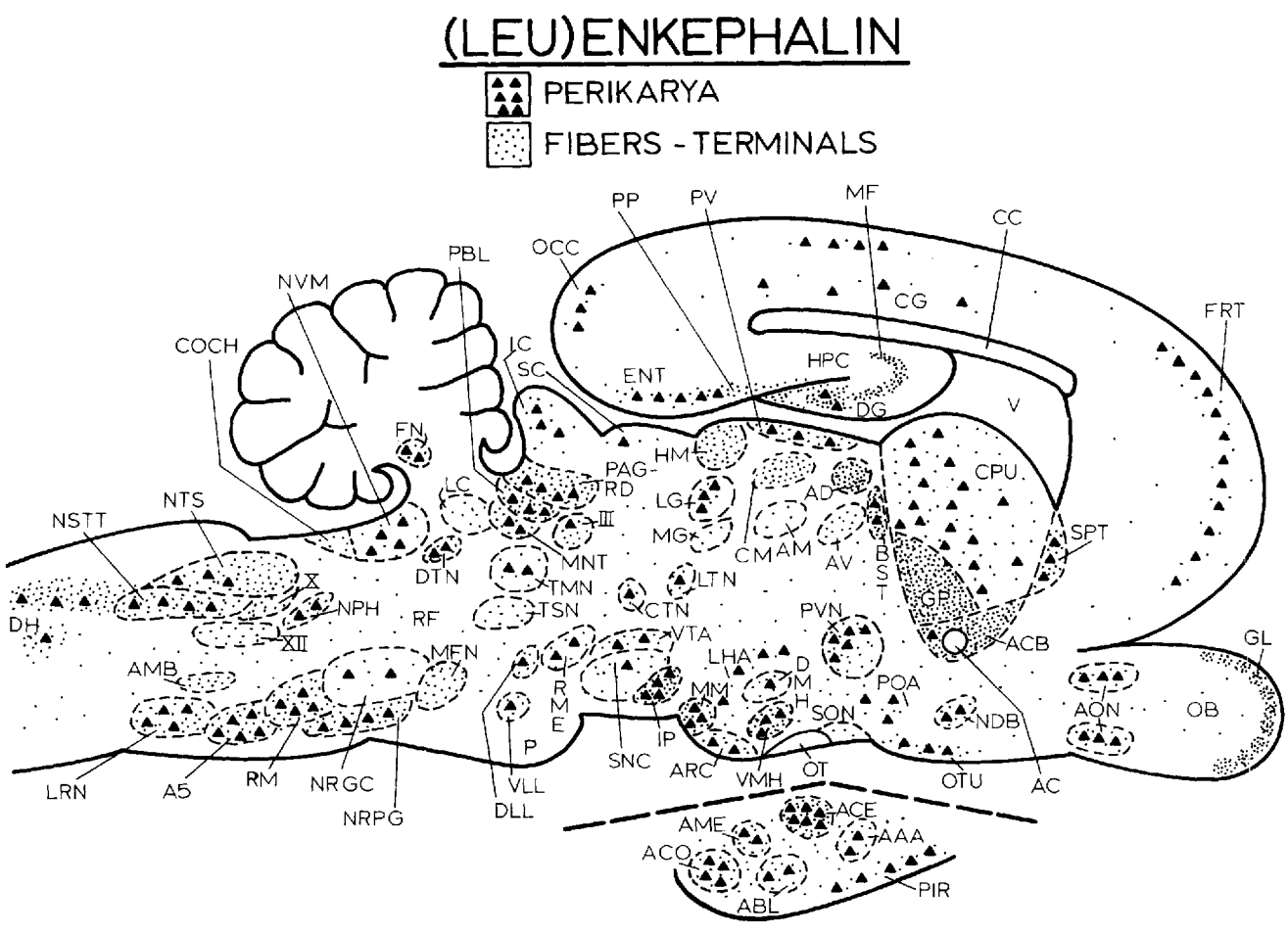


Fig. 9. Schematic summary of [Leu]enkephalin distribution in the rat central nervous system, shown in parasagittal view. Neuronal perikarya are represented by solid triangles, while fibers and terminals are shown as dots. See list of abbreviations and text for details.

[Leu]enkephalin-positive perikarya, fibers, and terminals are distributed widely throughout the neuraxis. In a previous study (Khachaturian et al., '83), we found more widespread and extensive enkephalinergic systems in cortical and limbic structures of the rat brain than had been recognized before (Sar et al., '78; Wamsley et al., '80; Finley et al., '81). However, the latter investigators, using relatively high doses of colchicine, were able to visualize enkephalinlike immunoreactivity in many central nervous system sites not previously reported. In the preceding report (Khachaturian et al., '83), we discussed some of the similarities and differences between our findings and those of others (Finley et al., '81; Gall et al., '81) pertaining to telencephalic enkephalinergic systems. Below, we discuss similarities and differences found with respect to other studies of diencephalic, brainstem, and spinal cord enkephalin-containing neurons. Several discrepancies exist between the present study and that reported by Finley et al. ('81): (1) We were unable to demonstrate magnocellular hypothalamic perikarya positive for [Leu]enkephalin, or a dense enkephalinergic pathway through the internal zone of the median eminence, as reported; (2) we were also unable to visualize immunoreactive perikarya in the parate-nial thalamic nucleus, cochlear nuclei, or granular layer of the cerebellar cortex, as reported; (3) in contrast, we have noted perikarya in motor trigeminal, mesencephalic trigeminal, occulomotor, and prepositus hypoglossi nuclei. At least part of the discrepancy between the present study and that of Finley et al. ('81) may be attributable to differences in the antienkephalin sera used in each study. We have used an affinity-purified anti[Leu]enkephalin serum which has been shown to be highly specific for that molecule. In contrast, Finley et al. ('81) used antisera that detects "not only authentic enkephalin but also other molecule(s) containing either the enkephalin sequence or unrelated, but cross-reactive, amino acid sequences." Since the [Leu]enkephalin sequence (Tyr-Gly-Gly-Phe-Leu) is incorporated into the NH_2-terminus of dynorphin A, antisera generated against either peptide might cross-react with both molecules. To address this issue, we have tested antisera raised against several dynorphin A fragments (1-13, 1-17, 7-17) and [Leu]enkephalin, in a serial-section analysis with cross-blocking controls, to demonstrate that dynorphin and enkephalin immunoreactivities are indeed separable in the central nervous system (Watson et al., '82b). For example, the hypothalamic magnocellular supraoptic

and paraventricular nuclei contain dynorphin-positive perikarya but not enkephalin-positive perikarya (Watson et al., '81, '82a). This finding may explain the previous observations of enkephalinlike immunoreactivity in both the cat and rat magnocellular neurons (Micevyeh and Elde, '80; Finley et al., '81). Our observation of previously undetected perikarya in several brainstem nuclei can perhaps be attributed to the technical improvement achieved by the use of high doses of colchicine.

The presence of enkephalin-positive perikarya in the motor nucleus of the trigeminal indicates a possible role of enkephalin in the control of mastication by way of the special visceral efferent component of the trigeminal nerve. This possibility is also supported by the finding of immunoreactive perikarya in the mesencephalic nucleus of the trigeminal nerve, which receives proprioceptive input relating to mastication. A role of enkephalin in the regulation of eye movements is suggested by the finding of immunoreactive perikarya in the oculomotor nucleus and the prepositus hypoglossal nucleus, an important "preoculomotor" area as indicated by anatomical (Graybiel and Hartwieg, '74) and physiological (Baker et al., '75, '77) studies. Our anatomical observations thus indicate possible roles of enkephalin which have not previously been considered and should be investigated further. Among the nuclei mentioned above, dynorphin immunoreactive perikarya have been detected only in the mesencephalic nucleus of the trigeminal nerve (Khachaturian et al., '82).

Further differences and parallels have been noted between the distribution of [Leu]enkephalin and dynorphin immunoreactivities. For example, in the substantia nigra, [Leu]enkephalin-positive varicosities are mainly concentrated in the pars compacta, among dopaminergic neurons, whereas the major dynorphin component occurs in the form of diffuse immunoreactivity in the pars reticulata (Khachaturian et al., '82). Other areas where such parallels have been found between the distribution of [Leu]enkephalin and dynorphin immunoreactivities include perikarya in the periaqueductal gray, parabrachial nuclei, and other brainstem nuclei where dynorphin-containing and enkephalin-containing neurons occur in distinctly separate populations. However, in areas such as the nucleus caudate-putamen, globus pallidus, nucleus accumbens, as well as in the spinal cord, the separation of dynorphin and [Leu]enkephalin immunoreactivities has proved to be difficult (Khachaturian et al., '82). On the basis of findings showing the separate biosynthetic origins of the enkephalins (Comb et al., '82; Gubler et al., '82; Noda et al., '82) and dynorphin (Kakidani et al., '82), and our own anatomical observations (Watson et al., '82b; Khachaturian et al., in press), it can be concluded that dynorphin and the enkephalins form separate opioid systems. Nevertheless, the spatial contiguity of these two systems in many areas of the central nervous system raises the question of the postsynaptic differentiation of the "messages" of these peptides. One possibility is that enkephalin and dynorphin interact preferentially with different opiate receptor subtypes in vivo, as indicated by the different affinities of these peptides for δ and κ receptors in vitro (Lord et al., '77; Huidobro-Toro et al., '81; Chavkin et al., '82). Another possibility, which does not exclude the first, is that enkephalin and dynorphin interact differentially at the same receptor, possibly in different conformational states (Bowen et al., '81; Quirion and Pert, '81). A third possibility is the interaction of dynorphin with a nonopiate receptor (Walker et al., '82a,b) which may be functionally antagonistic to opiate receptors mediating morphine analgesia (Tulunay et al., '81), e.g., the μ_1 class of sites, which appear to bind opiate alkaloids and enkephalins equally well with subnanomolar affinities (Wolozin and Pasternak, '81). Further analysis of this problem will depend upon anatomical studies of opioid peptide-receptor relationships (Lewis et al., '82), as well as functional studies of enkephalin-dynorphin interactions.

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