Anatomy of the CNS opioid systems

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The amino acid sequences of the three endogenous opioid peptide precursors are known, and the anatomical distribution of the opioid peptides has been studied extensively. This report summarizes these anatomical studies and looks at the problems that result from the biochemical relatedness of the precursors. We also discuss the relationship of opioid systems to opioid receptors, and the use of anatomical studies to derive new hypotheses of opioid function and provide dynamic measures of opioid neural activity, especially via specific mRNA quantitation.

The anatomical distribution of endogenous opioid peptides in the brain has been studied extensively, and has often preceded the complete structural characterization of the precursors from which these peptides are derived. In fact, immunocytochemical studies in all three cases established the cellular co-localization of opioid and related peptides either prior to or simultaneously with the biochemical and molecular biological establishment of their co-synthesis from a single precursor molecule. The structures of all three opioid precursors, as determined from these approaches, are shown in Fig. 1. Due to necessary limitations in the space available for describing three major, widely distributed neuronal systems, we have focused on the most salient features of these systems and have necessarily limited the citations of a wide body of important literature on these peptides. We have also restricted our anatomical descriptions (except where necessary) to observations made in the rat CNS and pituitary. Suffice it to say that significant species differences have been noted in the distribution of CNS opioids. For further detail, and a more complete list of references, the reader is referred to recent reviews.

Pro-opiomelanocortin

Peptides derived from pro-opiomelanocortin (POMC) include the opioid β-endorphin, and the non-opioid hormones ACTH and α-MSH (Fig. 1). The POMC precursor is synthesized in both the pituitary gland as well as the brain (Figs 2 and 3).

The pituitary gland is a major site of POMC biosynthesis. Although the existence of the pituitary peptides ACTH and α-MSH was already well established, the isolation and characterization of β-endorphin as an opiate-active peptide in 1976 by several laboratories[3] prompted extensive immunohistochemical studies beginning with the localization of β-endorphin, α-endorphin and β-lipotropin (a POMC fragment containing β-endorphin) immunoreactivities in the anterior lobe corticotrophs and intermediate lobe melanotrophs[4]. Electron-microscopic immunocytochemistry further demonstrated the existence of β-lipotropin and ACTH in the same pituitary granules[5], thus raising the possibility of a biosynthetic link between these peptides. Further biochemical studies[6] finally elucidated the protein structure of the POMC precursor which is processed to yield ACTH and β-endorphin in the anterior lobe, but α-MSH and β-endorphin in the intermediate lobe. The full structure of the POMC precursor was later deduced from the sequence of cDNA clones of POMC mRNA (Ref. 1).

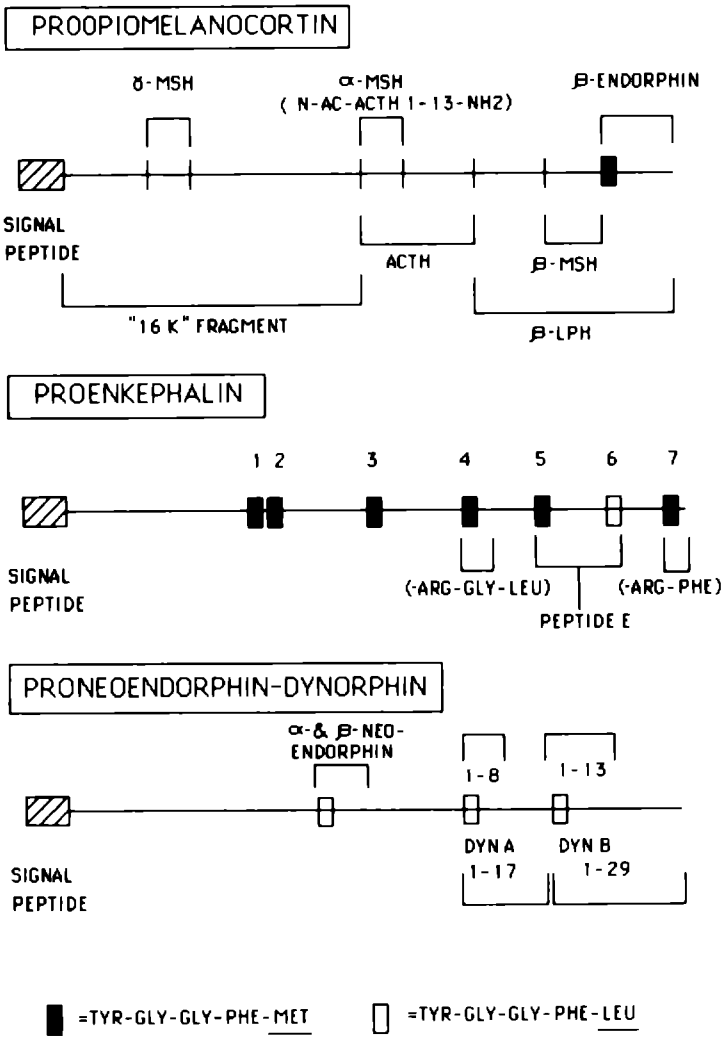

Fig. 1. *Schematic representation of the structure of the three opioid peptide precursors. Note that the opiate-active core sequence Tyr-Gly-Gly-Phe-Met ([Met]enkephalin) appears in both pro-opiomelanocortin and proenkephalin, while the opiate-active sequence Tyr-Gly-Gly-Phe-Leu ([Leu]enkephalin) is common to both proenkephalin and pro-neoendorphin-dynorphin.*

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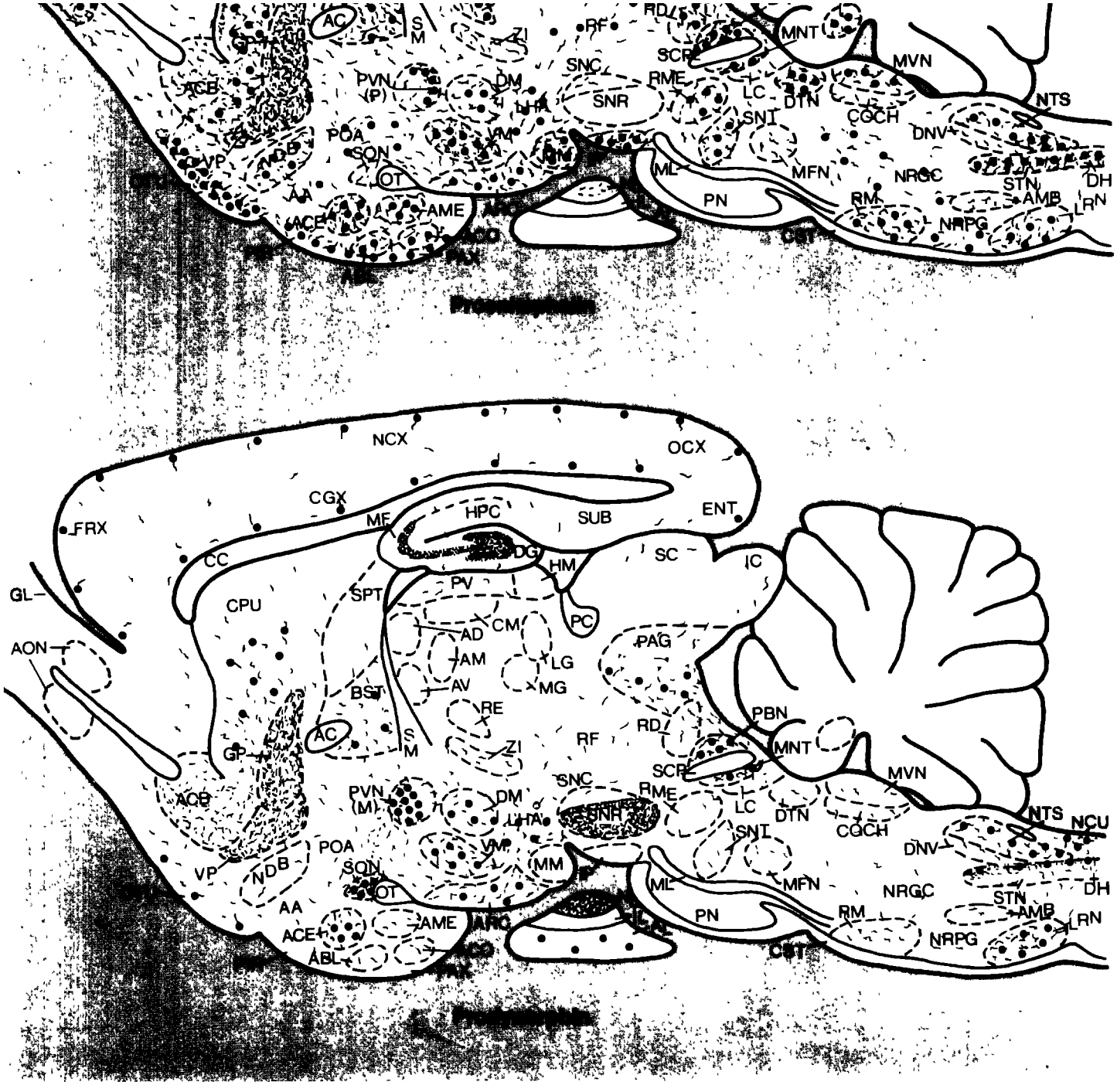

corpus callosum, CGX: cingulate cortex, CM: central-medial nucleus of thalamus, COCH: cochlear nuclear complex, CPU: caudate-putamen, CST: corticospinal tract, DH: dorsal horn of spinal cord, DG: dentate gyrus, DM: dorsomedial nucleus of hypothalamus, DNV: dorsal motor nucleus of vagus, DTN: dorsal tegmental nucleus, ENT: entorhinal cortex, FN: fastigial nucleus of cerebellum, FRX: frontal cortex, GL: glomerular layer of olfactory bulb, GP: globus pallidus, HM: medial habenular nucleus, HPC: hippocampus, IC: inferior colliculus, IL: intermediate lobe of pituitary, IP: interpeduncular nuclear complex, LC: nucleus locus coeruleus, LG: lateral geniculate nucleus, LHA: lateral hypothalamic area, LRN: lateral reticular nucleus, MF: mossy fibers of hippocampus, MFN: motor facial nucleus, MG: medial geniculate nucleus, ML: medial lemniscus, MM: medial mammillary nucleus, MNT: mesencephalic nucleus of trigeminal, MVN: medial vestibular nucleus, NCU: nucleus cuneatus, NCX: neocortex, NDB: nucleus of diagonal band, NL: neural lobe of pituitary, NRGC: nucleus reticularis gigantocellularis, NRPG: nucleus reticularis paragigantocellularis, NTS: nucleus tractus solitarius, OCX: occipital cortex, OT: optic tract, OTU: olfactory tubercle, PAG: periaqueductal gray, PAX: periamygdaloid cortex, PBN: parabrachial nucleus, PC: posterior commisure, PIR: piriform cortex, PN: pons, POA: preoptic area, PP: perforant path, PV: periventricular nucleus of thalamus, PVN(M): paraventricular nucleus (pars magnocellularis), PVN(P): paraventricular nucleus (pars parvocellularis), RD: nucleus raphé dorsalis, RE: nucleus reuniens of thalamus, RF: reticular formation, RM: nucleus raphé magnus, RME: nucleus raphé medianus, SC: superior colliculus, SCP: superior cerebellar peduncle, SM: stria medullaris thalami, SNC: substantia nigra (pars compacta), SNR: substantia nigra (pars reticulata), SNI: sensory nucleus of trigeminal (main), SON: supraoptic nucleus, SPT: septal nuclei, STN: spinal nucleus of trigeminal, SUB: subiculum, VM: ventromedial nucleus of hypothalamus, VP: ventral pallidum, ZI: zona incerta.

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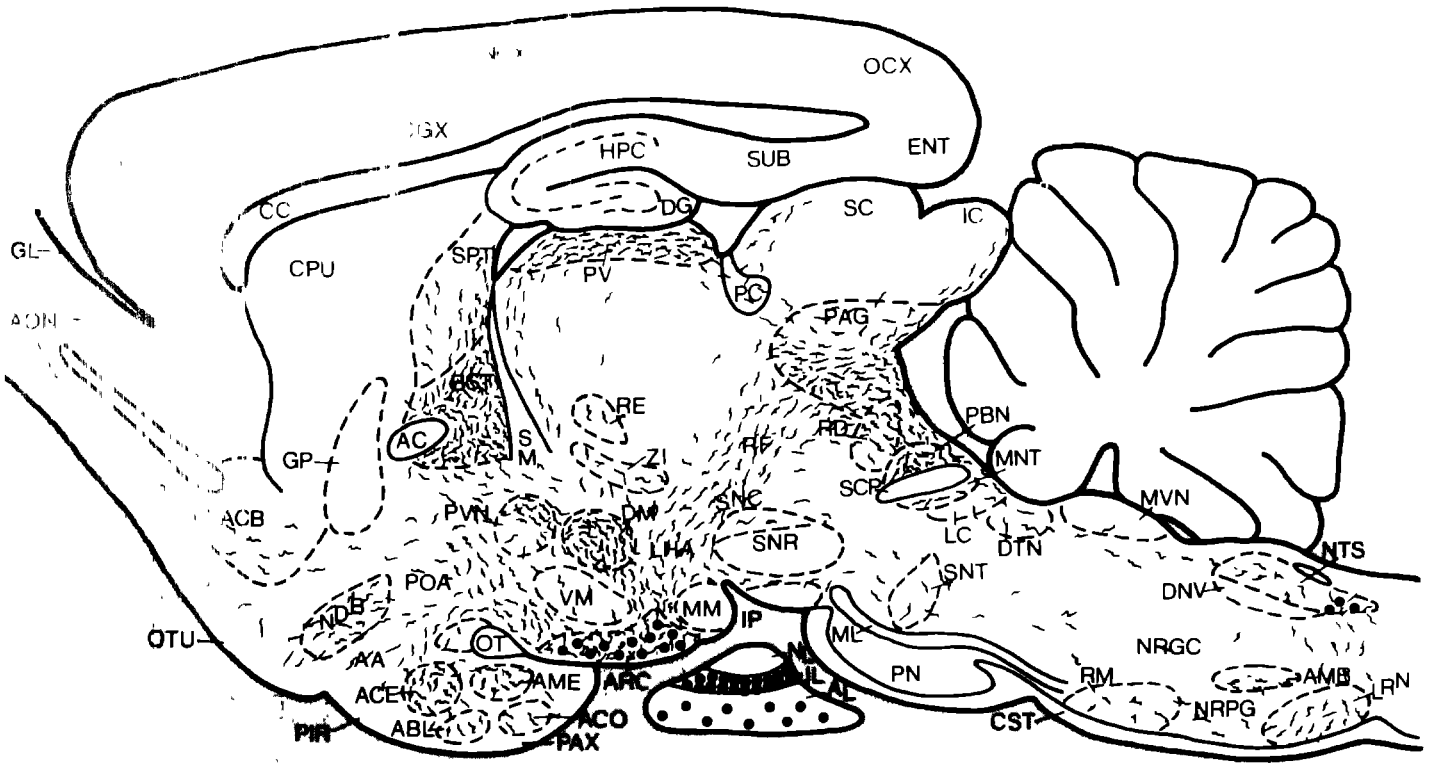
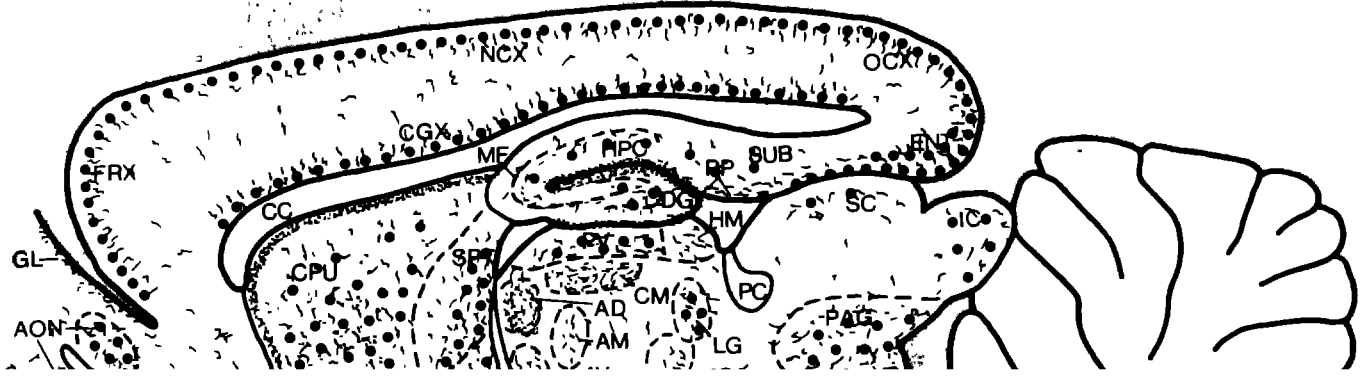


Pro-opiomelanocortin



Schematic representation of the distribution of pro-opiomelanocortin, proenkephalin, and prodynorphin derived peptides in the rat CNS, as determined from immunohistochemical studies.

1. Pro-opiomelanocortin contains one opioid peptide (β-endorphin), one copy of ACTH, and potentially three copies of MSH, namely α-, β- and γ-MSH. In the brain, the major POMC neuronal population resides in the arcuate nucleus, with projections to many limbic and brain stem nuclei.

2. Proenkephalin codes for several peptides containing the opioid-active core Tyr-Gly-Gly-Phe-Met (or -Leu). These include one copy of [Leu]enkephalin, four copies of [Met]enkephalin and one copy each of [Met]enkephalin-Arg-Phe and [Met]enkephalin-Arg-Gly-Leu. Peptides derived from proenkephalin are found in neuronal systems throughout the CNS, from the olfactory bulb to the spinal cord. These neurons form both local circuits and long-tract projections.

3. Prodynorphin also codes for several active opioid peptides containing the sequence of [Leu]enkephalin. These include dynorphin A, dynorphin B, and α-neoendorphin. This precursor is distributed in neuronal systems found at all levels of the neuraxis. Like their proenkephalin counterparts, the prodynorphin neurons form both short- and long-tract projections –often found in parallel with the proenkephalin systems.

In these three parasagittal maps, neuronal perikarya are shown as solid circles, and fibers-terminals as short curved lines and dots. Each map represents multiple parasagittal levels through the rat brain and was reconstructed using the rat brain atlas of G. Paxinos and C. Watson (1982, The Rat Brain in Stereotaxic Coordinates, Academic Press).

Key

AA: anterior amygdala, ABL: basolateral nucleus of amygdala, AC: anterior commissure, ACB: nucleus accumbens, ACE: central nucleus of amygdala, ACO: cortical nucleus of amygdala, AD: anterodorsal nucleus of thalamus, AL: anterior lobe of pituitary, AM: anteromedial nucleus of thalamus, AMB: nucleus ambiguus, AME: medial nucleus of amygdala, AON: anterior olfactory nucleus, ARC: arcuate nucleus, AV:

In the brain, there are two distinct cell groups which contain POMC-derived peptides. The first is located in the arcuate nucleus and with some cells scattered along the periarcuate medial-basal hypothalamus (Fig 3A) The second is found in the caudal nucleus tractus solitarius (Fig 3B) Bloom *et al.*[7] and Watson *et al*[8] demonstrated ACTH, β-lipotropin, and β-endorphin immunoreactivities to be localized in neurons of the arcuate nucleus, and showed that these perikarya are distinct from those containing the enkephalins Later, β-endorphin, β-lipotropin, and ACTH immunoreactivities were all found to be co-localized in the same arcuate neurons[9], an observation which was confirmed by other investigators (Ref 2) Similar co-localization studies were also carried out using α-MSH antisera[10], raising the possibility of a brain POMC processing mechanism similar to that shown to occur in the intermediate lobe Watson and Akil[10] further demonstrated a second, widespread, extra-arcuate hypothalamic α-MSH-immunoreactive cell group which did not exhibit immunoreactivity for any other POMC peptides Although the exact chemical nature of the α-MSH immunoreactive product of these neurons is still unknown, these neurones apparently project heavily to the striatum, hippocampus, and cerebral cortex Later, at the annual meeting of the Society for Neuroscience in 1981, Schwartzberg and Nakane[11] demonstrated another group of neurons which contained β-endorphin, ACTH and 16K (*N*-terminal fragment of POMC) immunoreactive material (Fig 3B) This group of small neurons resides within the commissural nucleus and caudal nucleus tractus solitarius, and exhibits projections that extend laterally and may innervate the lateral reticular nucleus

The POMC neurons located in the arcuate nucleus have extensive projections throughout the brain (Figs 2 and 4), with the possible exception of the striatum, hippocampus, and cerebral cortex[12] Rostrally projecting fibers course through periventricular diencephalic and telencephalic areas, innervating many hypothalamic and limbic structures, including preoptic area, septum, and the bed nucleus of stria terminalis. Lateral projections of the arcuate POMC neurons extend through the medial-basal hypothalamus ventrally and enter the amygdaloid region of the temporal cortex Dorso-caudally projecting fibers course through the dorsal diencephalon to enter the mesencephalon and brainstem, innervating many areas associated with nociceptive and other sensory integration These areas include the periventricular thalamus (Fig 4A) and periaqueductal gray (Fig 4B) Other caudal projections enter the brainstem ventrally to innervate numerous areas of the reticular formation, such as nucleus reticularis gigantocellularis, reticularis lateralis, and raphé magnus. Further brainstem sites containing POMC immunoreactivity include the nuclei parabrachialis and ambiguus, nucleus tractus solitarius, and dorsal motor nucleus of vagus, areas that are involved in respiratory and cardiovascular regulation

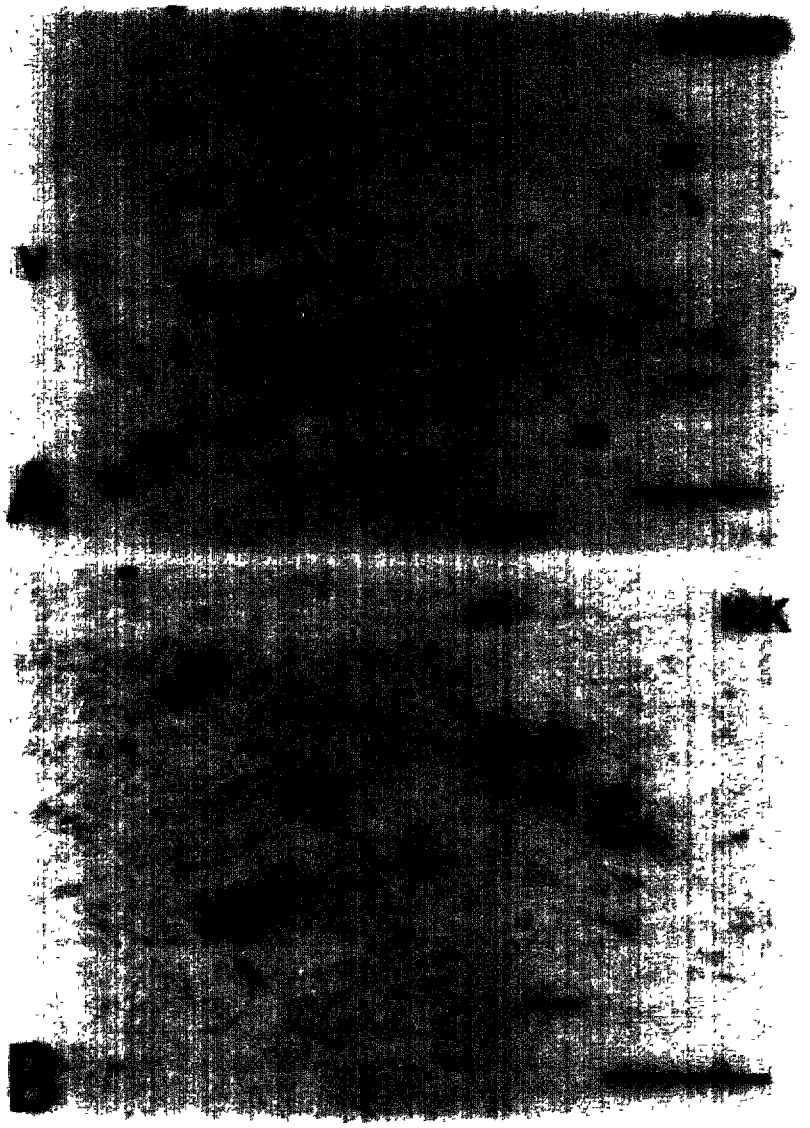


Fig. 3. (**A**) *shows β-endorphin (β-END) immunoreactive neurons in the arcuate nucleus of rat hypothalamus (V third ventricle, calibration bar = 100 μm) In* (**B**) *16K immunoreactive perikarya are seen in the nucleus tractus solitarius, pars commissuralis of a one-day-old postnatal rat (calibration bar = 50 μm)*

Proenkephalin

Unlike the POMC precursor which contains only one opioid peptide (β-endorphin), several opiate-active peptides are derived from proenkephalin (Fig. 1) These are [Leu]enkephalin, [Met]enkephalin, [Met]enkephalin-Arg-Phe, [Met]enkephalin-Arg-Gly-Leu, and potentially several larger opioids (e.g BAM-22P, peptides E and F) The proenkephalin precursor

is synthesized in many neuronal systems throughout the CNS, from the cerebral cortex down to the spinal cord (Figs 2 and 5A).

[Leu]- and [Met]-enkephalin were first isolated from the brain by Hughes *et al.*[13] in 1975 and were shown at that time to be opiate active. The first immunocytochemical studies by Elde *et al*[14] and others[2] demonstrated a very similar distribution pattern for both [Leu]- and [Met]-enkephalin in brain neurons, which was apparently different from that seen for β-endorphin and related peptides[7,9] These preliminary studies were followed by many immunohistochemical studies describing enkephalinergic neuronal systems, some forming local circuits, and others with long-tract projections[15–19] It is of course now known that [Met]- and [Leu]-enkephalin along with certain other *C*-terminally extended enkephalin peptides are all derived from a single precursor, proenkephalin, the structure of which was deduced from molecular cloning and sequencing of cDNA from proenkephalin mRNA (Ref. 1).

To date, most immunocytochemical studies of CNS enkephalin-containing neuronal systems are in good agreement regarding the widespread nature of these peptides More recently, the use of relatively higher doses of the neurotoxin colchicine, which inhibits microtubular axonal transport of nerve cell products, has enabled investigators to detect many more enkephalin perikarya Thus, in addition to the many brainstem, mesencephalic and diencephalic enkephalinergic neuronal systems described previously, we and other investigators have been able to detect extensive hippocampal, amygdaloid, cortical and other telencephalic neuronal circuits which exhibit enkephalin immunoreactivity[18,19] In these and numerous other studies of proenkephalin distribution in CNS, it was consistently noted that enkephalin-containing neurons often existed in many brain areas alongside dynorphin-containing neurons (Fig 5) Whether or not some CNS neuronal perikarya co-store these precursors is currently not known.

Neurons containing proenkephalin peptides are found at virtually all levels of the neuraxis (Fig 2). Immunoreactive perikarya have been noted in most regions of the telencephalon, including the cerebral cortex, olfactory tubercle, amygdala, hippocampus, striatum, septum, bed nucleus of stria terminalis and preoptic area. In diencephalon, perikarya are seen in most hypothalamic nuclei (Fig. 5), and in the periventricular and lateral geniculate nucleus of thalamus. In the midbrain, enkephalinergic cells are localized in the colliculi, periaqueductal gray, and interpeduncular nucleus In the pons and medulla, perikarya are seen in the parabrachial, dorsal tegmental, vestibular and raphé nuclei, nuclei reticularis gigantocellularis and paragigantocellularis, nucleus tractus solitarius, lateral reticular nucleus, spinal trigeminal nucleus and spinal cord dorsal gray In addition to these areas which contain both enkephalin perikarya and fibers, numerous other brain regions exhibit varying densities of fiber and terminal-like immunoreactivity (Figs 2 and 6). From the foregoing, it should be apparent that the enkephalins have the potential to influence a wide variety of CNS functions[1,2]

Prodynorphin

Prodynorphin (or pro-neoendorphin-dynorphin), like proenkephalin, contains several opiate-active peptides, including dynorphin A, dynorphin B (rimorphin), and α- or β-neoendorphin (Fig. 1) This precursor is also synthesized throughout the

Fig. 4. *β-Endorphin (β-END) immunoreactive fibers in parasagittal sections through the periventricular nucleus of thalamus* **(A)** *and the periaqueductal gray of mesencephalon* **(B)** *Compare with Fig 2 POMC projections. Micrograph made using darkfield optics AQ cerebral aqueduct, V lateral ventricle Calibration bar = 200 μm*

CNS in a wide variety of neuronal systems (Figs 2 and 5B).

In 1979, Goldstein *et al* [20] extracted from pituitary a 13 amino acid peptide which contained the [Leu]enkephalin sequence at its *N*-terminus. The full 17 amino acid structure of this peptide, now called dynorphin A, was later elucidated. The second [Leu]enkephalin-containing fragment of the precursor to be isolated was α-neoendorphin, which was extracted from the hypothalamus and fully sequenced. A third peptide, shown to be part of the *C*-terminus extension of dynorphin A, was also identified and termed dynorphin B or rimorphin[1]. Watson *et al.*[21] demonstrated dynorphin A(1–13) immunoreactivity in the posterior lobe of pituitary, and in hypothalamic supraoptic and paraventricular nuclei, and further showed this peptide to be co-localized with arginine-vasopressin in some magnocellular perikarya[22]. Similar observations were also made using antisera raised against dynorphin B and α-neoendorphin, showing these peptides and dynorphin A to be co-localized within the same neurons in several regions of the brain[23,24]. These results were in good agreement with the cloning and sequence analysis of prodynorphin cDNA demonstrating the co-synthesis of these peptides from a single precursor[1].

Further immunocytochemical studies of dynorphin distribution in the CNS soon followed the initial observations, and, as was the case with the enkephalins, the application of colchicine enabled the detection of increasing numbers of smaller dynorphin perikarya throughout the brain and spinal cord[19,24–26]. Our observations are summarized in Fig. 2. Immunoreactive dynorphin perikarya are distributed in several cerebral cortical areas, striatum, amygdala, hippocampus, several hypothalamic nuclei (including the supraoptic and paraventricular), midbrain periaqueductal gray, and numerous brainstem areas, such as the parabrachial and spinal trigeminal nuclei, nucleus tractus solitarius, lateral reticular nucleus, and in the spinal cord dorsal horn. Additionally, fiber immunoreactivity is seen in many other areas of the brain as depicted in Figs 2 and 7. Mention should also be made of dynorphin immunoreactivity in some as yet unidentified cells of the pituitary anterior lobe (Fig. 7B). However, these cells are distinct from the corticotrophs which synthesize POMC. When comparing the CNS distribution of the dynorphins and the enkephalins, it becomes obvious that these two systems are often anatomically contiguous (see Fig. 5), and thus may participate (possibly via different receptors) in several related CNS functions.

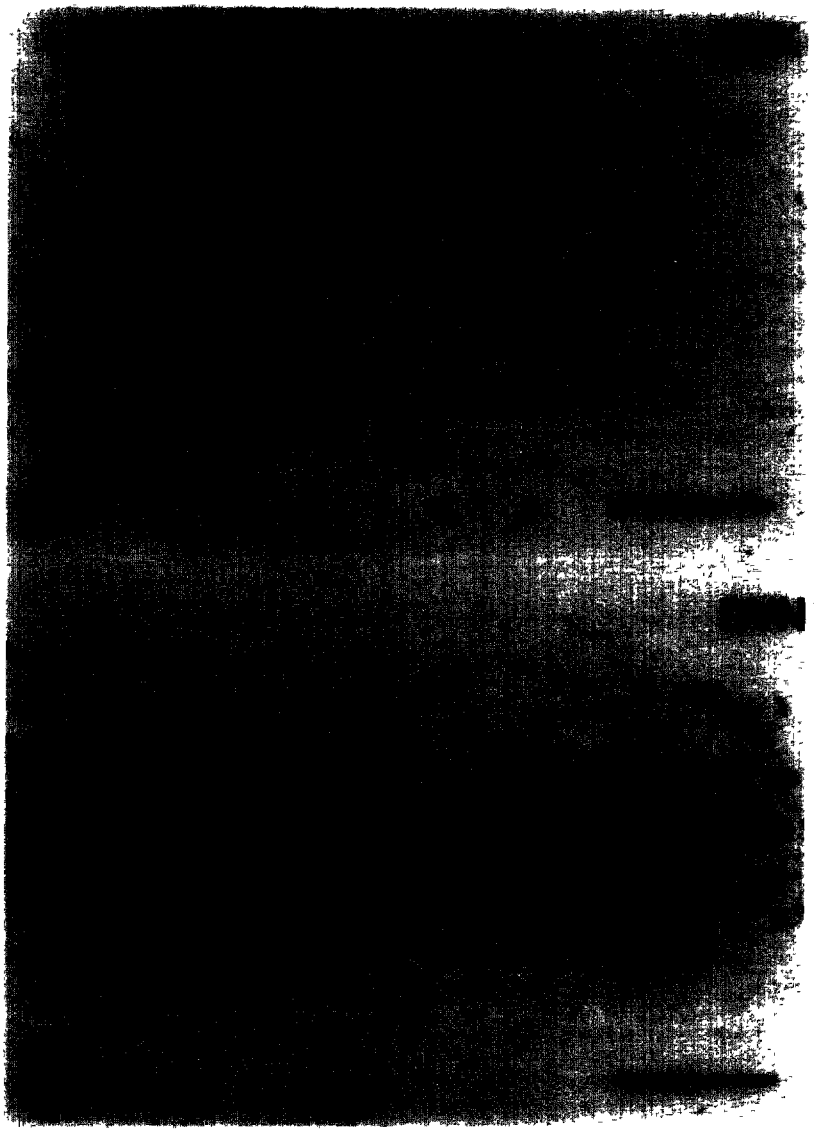


Fig. 5. *Adjacent 4 μm sections through the hypothalamic paraventricular nucleus (left is medial) immunostained for [Leu]enkephalin (ENK)*(**A**) *and dynorphin B (DYN)*(**B**). *Stars indicate a common vessel. Note that the majority of [Leu]enkephalin cells are concentrated in the parvocellular subdivision (dorsal and medial to the vessel), while most dynorphin B cells is in the magnocellular part (lateral to the vessel). Calibration bar = 100 μm.*

Problems of sequence homologies among the opioids

Since the opiate-active core sequence of all opioid peptides consists of either [Met]enkephalin or [Leu]enkephalin (Fig. 1), there has been considerable difficulty and confusion in the immunocytochemical as well as biochemical separation of these peptides. For example, the earlier observations of [Met]enkephalin-like immunoreactivity in the anterior lobe corticotrophs and intermediate lobe cells were obviously due to significant antibody cross-reactivity and/or enzymatic degradation that resulted in the detection of the *N*-terminal [Met]-enkephalin sequence of β-endorphin in these cells. In the case of [Leu]enkephalin, however, we are faced with a considerably more difficult task. The amino acid sequence of this pentapeptide is found in two opioid precursors

proenkephalin, which contains one copy, and prodynorphin, which contains three copies as part of the *N*-terminal sequence of α-neoendorphin, dynorphin A and dynorphin B. As noted before, proenkephalin and prodynorphin neurons are often found in the same regions of the brain (Fig. 5), complicating the separation of the two precursor systems. One of the most extensively investigated systems is the hypothalamic magnocellular paraventricular and supraoptic nuclei which synthesize the neuropeptides oxytocin and vasopressin. These cells project to the neural lobe of the pituitary. Initially, [Leu]enkephalin was extracted from the neurointermediate lobe of rat pituitary and was shown to be localized in the magnocellular nuclei[27]. Rossier *et al.*[27] first suggested this peptide to be involved in the regulation of vasopressin and oxytocin. Martin and Voigt[28] demonstrated the coexistence of [Met]enkephalin with oxytocin and [Leu]enkephalin with vasopressin in neurosecretory terminals of the rat neurohypophysis. However, the [Leu]enkephalin antiserum used was blockable by dynorphin A. Subsequently, Watson *et al.*[22,29], using highly purified antisera, showed that prodynorphin is associated with vasopressin in magnocellular neurons and that [Leu]enkephalin and other proenkephalin peptides are found in the parvocellular part of the paraventricular nucleus (Fig. 5). However, significant species differences appear to exist in the processing of prodynorphin in the magnocellular nuclei. For example, in the cat, substantial amounts of [Leu]enkephalin can be seen in the supraoptic nucleus[30]. We have also made similar observations in the rhesus monkey magnocellular system. Recently, indirect evidence has also been presented for the processing of prodynorphin of striatal origin into [Leu]enkephalin in the rat substantia nigra[31]. However, we have previously shown in the rat that [Leu]enkephalin in substantia nigra is concentrated primarily in the pars compacta while dynorphin A is found mainly in the pars reticulata[29]. In fact, through the use of antisera directed against various proenkephalin and prodynorphin peptides, we have seen the peptides derived from each precursor co-localized within distinct enkephalin-containing and dynorphin-containing neurons[23,29,32]. Of course, these observations do not necessarily exclude processing of some prodynorphin to [Leu]enkephalin in terminal fields. Nevertheless, because of the close anatomical proximity of the enkephalins and dynorphins, biochemical data should also be regarded with caution since it is difficult to obtain tissue samples which contain one but not the other precursor.

Opioid peptide-receptor relationships

Given the anatomical complexity and relatedness of the multiple opioid systems, it is worth asking how the 'messages' of these systems are differentiated at the receptor level. Although there is abundant pharmacological and biochemical evidence for multiple subtypes of opioid receptors[33], the relationship of these receptors to the multiple opioid systems is not well understood. However, there is some rationale for making predictions. *in vitro*, the enkephalins bind preferentially to δ-opioid receptors, while dynorphin-related peptides bind preferentially to κ-opioid receptors, and β-endorphin binds to μ- and δ-, but not κ-, opioid receptors[33]. Do these *in-vitro* associations predict anatomical relationships? To begin to explore this question, receptor autoradiographic and immunocytochemical studies were carried out on adjacent sections of formaldehyde-perfused rat and rhesus

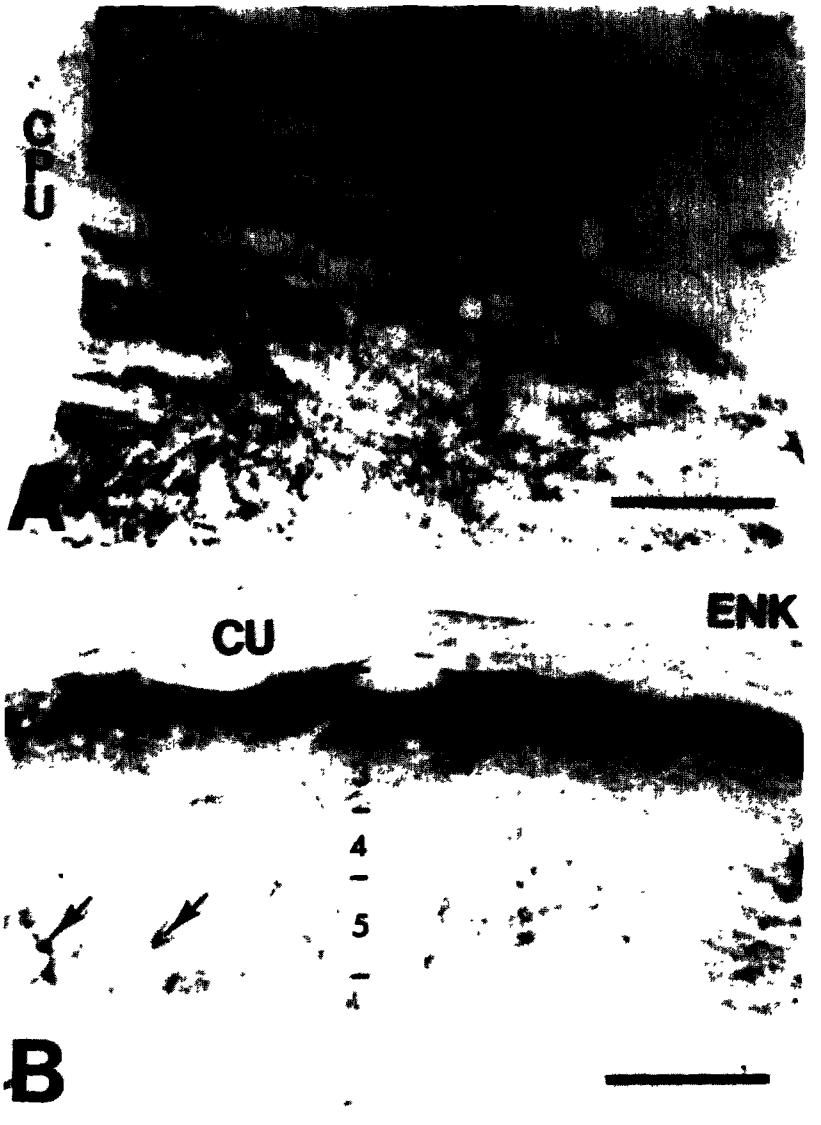


Fig. 6. (**A**) *and* (**B**) *are parasagittal sections showing the distribution of [Leu]enkephalin (ENK) immunoreactivity in the globus pallidus (A) and the dorsal horn of the spinal cord (B). In* (**B**) *(cervical cord), note the dense immunoreactivity in laminae 1 and 2, and scattered immunoreactive perikarya (arrows) in lamina 5. Compare with Fig. 2 proenkephalin projections. CI, internal capsule, CPU caudate-putamen, CU, fasciculus cuneatus. Calibration bar = 200 μm.*

monkey brains[34–36] Apparent μ- and κ-receptors are extensively co-localized in rat brain[37], and are distributed similarly to enkephalin immunoreactivity in many (but not all) CNS areas, e.g some cerebral cortical areas, habenula, interpeduncular nuclear complex, parabrachial nuclei, nucleus tractus solitarius, spinal trigeminal nucleus, and dorsal horn of the spinal cord Such findings indicate that the ligand-receptor anatomical associations predicted by *in-vitro* studies (e g enkephalin-δ) appear incorrect Instead, since it is now known that some extended proenkephalin peptides have substantial κ and μ properties[38], while dynorphin A loses κ selectivity upon *C*-terminal cleavage[38,39], the apparent availability of multiple opioid receptors to each opioid system may signify that differential processing of the opioid precursor is a biological strategy for yielding peptide products which act at the different receptors

Derivation of hypotheses of opioid function from anatomy

Whenever neuroanatomical studies are carried out, there is always the hope that some aspect of neural function will be illuminated. The study of the anatomical distribution of opioid peptides and receptors has not been disappointing in this regard; for example, opioid neurons and receptors are clearly part of the neural apparatus involved in nociception and analgesia Given the classical pharmacology of opiates, this association is not surprising. However, the study of opioid anatomy has given rise to less obvious possibilities. For example, the study of opioid receptor distribution has generated hypotheses concerning the role of opioids in multimodal sensory processing[40,41] Based upon a sensory corticolimbic gradient of [^{3}H]-naloxone binding sites in primate cerebral cortex, a possible role of opioids in selective attention was proposed[42] and supported by electrophysiological studies[43] Immunocytochemical studies of opioid peptide systems have also been useful not only in providing an anatomical 'rationale' for the actions of opioids on autonomic, neuroendocrine, and behavioral functions[1,2,12], but also for predicting previously unconsidered physiological roles of these peptides For example, our observation of enkephalin perikarya in the motor and mesencephalic nuclei of the trigeminal may indicate a role for this opioid in the control of mastication[17] Likewise, the presence of enkephalin perikarya in the oculomotor and prepositus hypoglossal nuclei suggests a possible role in the regulation of eye movements[17] These examples show how anatomical studies can be useful in the generation of new hypotheses of opioid function in the CNS

Toward a dynamic opioid anatomy

The anatomical localization of opioid peptides has revealed exceedingly complex multiple systems. We are now faced with the task of unraveling the myriad of cell groups and projection systems in order to be able to derive further clues as to their functional significance Some of these projections have been determined with the use of combined immunocytochemical and tract-tracing techniques Numerous other projections are yet to be elucidated. For example, in the case of POMC, it has been assumed that all brain projections emanate from a single neuronal population residing in the hypothalamic arcuate nucleus However, the discovery of additional POMC neurons in

Fig. 7. *Dynorphin A (DYN) immunoreactivity is shown in two parasagittal levels through the hippocampal formation (A) and the pituitary gland (B) In* (**A**) *the dentate gyrus mossy fiber projections into areas CA2 and CA3 is DYN-positive (calibration bar = 400 μm) In* (**B**) *the neural lobe contains DYN immunoreactivity Note also several immunoreactive cells (arrows) in the anterior lobe (calibration bar = 200 μm) Compare with Fig. 2 prodynorphin projections AL anterior lobe CA1,2,3 cornu ammonis fields 1, 2, and 3 IL intermediate lobe NL neural lobe SG stratum granulosum SM stratum moleculare SR stratum radiatum*

the nucleus tractus solitarius, and the fact that this nucleus (but not necessarily POMC neurons) projects to many POMC-rich brainstem and forebrain areas, makes it imperative to employ such combined immunocytochemical and tract-tracing techniques to begin to sort out the differential projections of these two POMC-containing neuronal groups. From this example, it should be apparent that a considerably more difficult and extensive task awaits us in the case of the proenkephalin and prodynorphin systems. Given this complexity, it would also be useful to be able to study the dynamics of these systems in an anatomical context *In-situ* cDNA–mRNA hybridization histochemistry has already proven useful for the localization of POMC-synthesizing cells in pituitary and brain[44], and recent studies have shown that this procedure can be used to detect the effects of physiological treatments on POMC mRNA levels[45], which appear to provide an index of cellular biosynthetic activity. Thus, two major future directions of opioid anatomical studies will be to conduct detailed regional immunocytochemical analyses of opioid neuronal projections in combination with tract-tracing methods, as well as *in-situ* hybridization of opioid mRNA, to begin to probe the functional significance of the multiple systems described in this review

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