Comparison of the Distribution of Dynorphin Systems and Enkephalin Systems in Brain

Abstract. *A study of the anatomical distribution of the endogenous opioid dynorphin in rat brain showed that the peptide is localized in a widespread system with multiple cell groups and projections. This network is revealed by the use of multiple antiserums against dynorphin and can be distinguished from the system containing methionine-enkephalin and leucine-enkephalin, which is mapped by the use of antiserums against the enkephalins and biosynthetically related peptides in the adrenal. It thus appears that the brain contains at least three separate opioid neuronal networks: an enkephalin family with components similar to those found in the adrenal, a β-endorphin family, and a dynorphin family.*

Several endogenous opioids contain the opioid active core Tyr-Gly-Gly-Phe-Met or Tyr-Gly-Gly-Phe-Leu (*1*). This has led to a number of problems in elucidating their biosynthetic relationships, their anatomical distributions, and their distinct functions. It is clear that the enkephalin system is entirely separable from the β-endorphin system in brain (*2*). However, the relation between methionine-enkephalin ([Met]enkephalin) and leucine-enkephalin ([Leu]enkephalin), or between [Leu]enkephalin and dynorphin has proved more difficult to establish. There is good evidence that dynorphin (which contains [Leu]enkephalin at the NH_2-terminus) is at least partially separable from [Leu]enkephalin. We have shown immunohistochemically that dynorphin occurs in neurons where enkephalin does not (*3*). Moreover, investigators working on bovine adrenal glands and on human pheochromocytomas have elucidated the structure of the enkephalin precursor in adrenal glands, using complementary DNA to the messenger RNA coding for the prohormone (*4*). This work demonstrates that one copy of [Leu]enkephalin and six [Met]enkephalin-containing structures all appear within the same precursor. The full dynorphin structure is not part of this adrenal proenkephalin molecule. It is therefore evident that adrenal [Leu]enkephalin can have a biosynthetic origin unrelated to dynorphin, and that dynorphin is derived from a precursor that is distinct from the proenkephalin precursor characterized in the adrenal (*5*). However, one cannot eliminate the possibility that dynorphin can also be cleaved into [Leu]enkephalin, since it contains a pair of basic residues immediately adjacent to the Tyr-Gly-Gly-Phe-Leu structure. Thus, dynorphin could be another source of [Leu]enkephalin, perhaps arising by degradation at sites of dynorphin release.

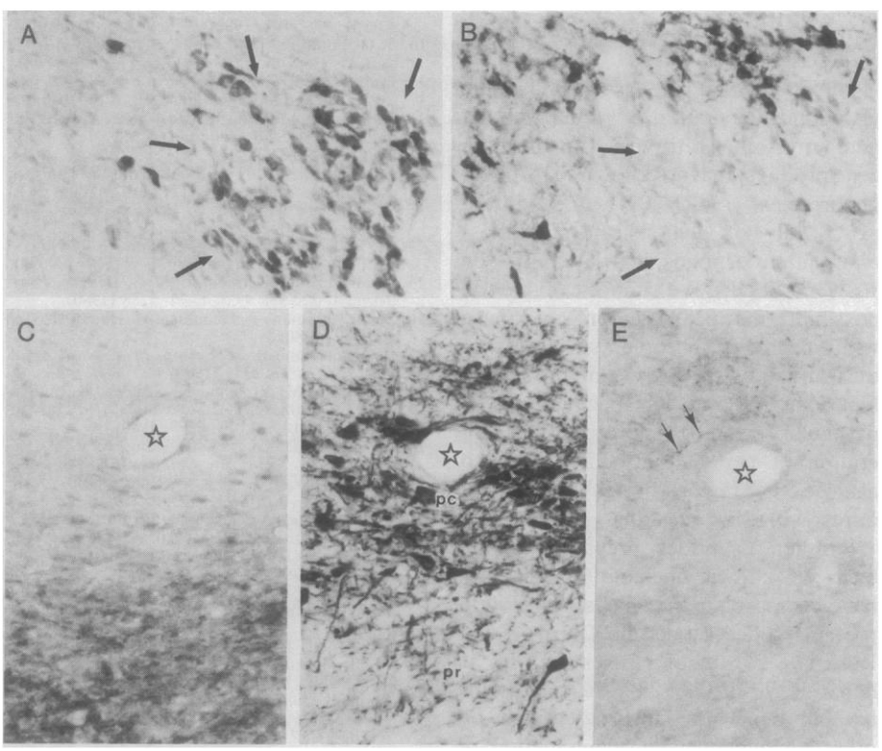

Fig. 1. Comparative distribution of dynorphin and [Leu]enkephalin perikarya and terminals in serial sections through the hypothalamic paraventricular nucleus (PVN) (A and B) and substantia nigra (SN) of the mesencephalon (C to E). (A) Immunoreactive dynorphin perikarya within the lateral magnocellular subnucleus of PVN (the vasopressin-rich region) (large arrows). (B) [Leu]Enkephalin-positive perikarya concentrated mainly in the parvocellular PVN. The large arrows in (A and B) demarcate the magnocellular PVN. (C) The diffuse dynorphin-immunoreactivity within the pars reticulata. (D) The SN with tyrosine hydroxylase–immunoreactive perikarya concentrated within the pars compacta (*pc*) but not pars reticulata (*pr*) of this dopaminergic nucleus. (E) Immunoreactive [Leu]enkephalin fibers (small arrows) and terminals are located more dorsally within pars compacta. It is of interest that 400 μg of colchicine can reveal cells and some large fibers. The size and nature of this immunoreactivity has not been determined, but the pattern of fibers is that usually seen with lower doses of colchicine and in untreated animals. Note the capillary lumen through sections (C to E) (stars) (× 234).

The biosynthetic origin of [Leu]enkephalin and [Met]enkephalin in brain remains to be fully established. Our own work, in which we have used antiserums to multiple regions of adrenal proenkephalin, suggests that the usual [Met]-enkephalin and [Leu]enkephalin pathways in brain include several distinctive peptide-containing regions, other than the regions containing enkephalin sequences that occur in adrenal proenkephalin (*6*). This suggests that the adrenal biosynthetic route for enkephalin is also present in brain. However, the relation between this enkephalinergic neuronal system and the dynorphinergic neuronal system remains unclear.

Immunohistochemical techniques have been useful in the past for separating the enkephalins from β-endorphin (*2*). The power of this approach is severely limited, however, by the potential for cross-reactivity between antiserums to the various opioid peptides, since there are substantial structural homologies between them. For example, an antiserum to the NH_2-terminus of dynorphin could easily cross-react with either [Leu]enkephalin itself or with the [Leu]enkephalin sequence present within a larger molecule. In order to eliminate this problem we have used a convergent approach, whereby we have raised multiple antiserums to various known regions of the dynorphin structure, particularly those not containing [Leu]enkephalin. The staining with these dynorphin antiserums was contrasted with the staining with antiserums to enkephalin and with antiserums to the other regions of the adrenal proenkephalin structure.

On the basis of results obtained with the systematic use of these multiple anti-

0036-8075/82/1210-1134$01.00/0

serums in serial sections, and the use of cross-blocking controls, we can distinguish with good reliability between dynorphin pathways on the one hand and enkephalin pathways that contain the adrenal enkephalin peptides on the other. We now report that there are indeed two anatomically distinct systems in brain, one that contains [Met]enkephalin and [Leu]enkephalin as well as other proenkephalin peptides of the adrenal type, and one that contains dynorphin. We focus here on the overall distribution of dynorphin-like immunoreactivity in contradistinction to the enkephalin immunoreactivity that derives from a precursor of the adrenal type.

The dynorphin antiserums were made against dynorphin-(1–13), the full dynorphin heptadecapeptide (dynorphin-17), or the non-enkephalin–containing fragment dynorphin-(7–17). This last antiserum was particularly important, since its recognition site must clearly be other than the NH_2-terminal [Leu]enkephalin-containing region of the full dynorphin molecule.

The three antiserums to dynorphin were raised in New Zealand White rabbits as described elsewhere (*3*). Their immunocytochemical titers varied between 1/300 and 1/1000. Their specificity was checked with peptide-blocking experiments wherein 50 μmole of the relevant peptide was added to the antiserum prior to the incubation. Dynorphin-(1–13) and dynorphin-17 were purchased from Peninsula Laboratories and dynorphin-(7–17) was synthesized in our laboratories. Further specificity tests were carried out with [Met]enkephalin and [Leu]enkephalin, human β-endorphin, and the adrenal peptides F and BAM-22P.

The antiserums to [Leu]enkephalin and [Met]enkephalin were characterized as described elsewhere (*7*). We also used antiserums against the midportion of the F peptide and against the COOH-terminus of BAM-22P. Both are parts of the adrenal enkephalin precursor. The use of the antiserums to adrenal proenkephalin and the results obtained with them are reported elsewhere (*6*). In that study, we used the enkephalin and BAM-22P antiserums to identify the enkephalin systems of adrenal type, whereas in the current study the three dynorphin antiserums were used to identify the dynorphin systems.

For the immunohistochemical staining we used the peroxidase-antiperoxidase immunocytochemical method (*3*) in formaldehyde-perfused Sprague-Dawley rats that had been treated with colchicine (300 to 400 μg in 10 μl administered by intracerebroventricular injection 24 to 48 hours before the rats were killed).

The results are summarized in Table 1. Both the enkephalin system (as stained by antiserums to [Leu]enkephalin and BAM-22P) and the dynorphin system [as stained by antiserums to dynorphin-(1–13), dynorphin-17, and dynorphin-(7–17)] occur in many of the same areas. Examples are the caudate-putamen, nucleus accumbens, globus pallidus, hippocampus, amygdala, periaqueductal central gray, nucleus locus coeruleus, parabrachial nuclei, nucleus tractus solitarius, and the spinal cord. The enkephalin system (Fig. 1, B and E), but not the dynorphin system, occurs in a large number of areas (for example, habenular nuclei, interpeduncular nucleus, periventricular nucleus of thalamus, nucleus raphe magnus, nucleus reticularis paragigantocellularis, and cingulate and pyriform cortex). In contrast, a few areas or nuclei contain dynorphin but not enkephalin [for example, the magnocellular neurosecretory nuclei of hypothalamus (Fig. 1A) and pars reticulata of the substantia nigra (Fig. 1C)]. It is clear from the consistency of the results obtained with the groups of antiserums used in this study (enkephalin and BAM-22P compared with the three dynorphin antiserums), and from the anatomical differences indicated above, that enkephalin and dynorphin neuronal systems are separable. Recent biochemical studies (*5*) and the current anatomical study indicate that the opioid peptide systems in brain can be divided into the enkephalin, dynorphin, and β-endorphin systems. The opioid peptide α-neo-endorphin has been immunocytochemically identified in dynorphin-containing cells of hypothalamus (*8*). The results of our study support the view that there is a biosynthetic link between dynorphin and α-neo-endorphin (*5*). As is evident from the work with adrenal proenkephalin, a single precursor can give rise to multiple copies and structural variants of opioid

Table 1. Comparative distribution of dynorphin and enkephalin systems in the rat central nervous system. Note that many of the dynorphin-containing areas in the brain also contain enkephalin, but that the opposite is not necessarily true. However, in many areas that contain both peptides, the fibers and perikarya of each system are differentially localized. For example, in hypothalamic magnocellular nuclei (*), dynorphin immunoreactivity can be located in vasopressin-positive perikarya, whereas [Leul]enkephalin-positive perikarya are primarily seen in parvocellular regions. Also, in periaqueductal gray (**), dynorphin-positive perikarya are situated more ventrally, whereas immunoreactive [Leu]enkephalin perikarya are distributed more dorsally.

Region	Dynorphin systems		Enkephalin systems (adrenal type)	
	Perikarya	Fibers, terminals	Perikarya	Fibers, terminals
Olfactory bulb				
Glomeruli		+		+
Anterior olfactory nucleus			+	+
Accumbens, candate-putamen nuclei		+	+	+
Globus pallidus		+		+
Hypothalamus				
Magnocellular nuclei	+ (*)	+		+
Arcuate nucleus	+	+	+	+
Thalamus				
Periventricular nucleus			+	+
Anterior nucleus				+
Substantia nigra				
Pars compacta				+
Pars reticulata		+		
Interpeduncular nucleus			+	+
Inferior colliculus			+	+
Periaqueductal gray	+ (**)	+	+	+
Nucleus locus coeruleus		+		+
Trigeminal sensory nuclei (all)		+		+
Raphe nuclei (some)		+	+	+
Nucleus reticularis paragigantocellularis			+	+
Lateral reticular nucleus			+	+
Nucleus tractus solitarius	+	+	+	+
Amygdala, central nucleus	+	+	+	+
Hippocampal formation				
Dentate gyrus	+	+	+	+
Mossy fibers		+		+
Paleocortex, pyriform, entorhinal			+	+
Spinal cord, dorsal horn	+	+	+	+

peptides. These variants may possess unique pharmacological profiles and perform different biological functions.

STANLEY J. WATSON
HENRY KHACHATURIAN
HUDA AKIL

Mental Health Research Institute, University of Michigan, Ann Arbor 48109

DAVID H. COY

Department of Medicine, Tulane University School of Medicine, New Orleans, Louisiana 70196

AVRAM GOLDSTEIN

Addiction Research Foundation and *Stanford University Medical Center, Palo Alto, California 94304*

References and Notes

1. A. F. Bradbury, W. F. Feldberg, D. G. Smyth, C. R. Snell, in *Opiates and Endogenous Opioid Peptides*, H. W. Kosterlitz, Ed. (Elsevier/North Holland, Amsterdam, 1976), pp. 9–17; M. Chretien, S. Benjannet, N. Dragon, N. Seidah, M. Lis, *Biochem. Biophys. Res. Commun.* **72**, 472 (1976); A. Goldstein, S. Tachibana, L. I. Lowney, M. Hunkapiller, L. Hood, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 6666 (1979); A. Goldstein, W. Fischli, L. I. Lowney, M. Hunkapiller, L. Hood, *ibid.* **78**, 7219 (1981); S. Tachibana, K. Araki, S. Ohya, S. Yoshida, *Nature (London)* **295**, 339 (1982); C. H. Li and D. Chung, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1145 (1976); J. Hughes, T. W. Smith, H. W. Kosterlitz, L. A. Fothergill, B. A. Morgan, H. R. Morris, *Nature (London)* **258**, 577 (1975).
2. F. E. Bloom, E. Battenberg, J. Rossier, N. Ling, R. Guillemin, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1591 (1978); R. Elde, T. Holfelt, O. Johansson, L. Terenius, *Neuroscience* **1**, 349 (1976); S. J. Watson, H. Akil, C. W. Richard, J. D. Barchas, *Nature (London)* **275**, 226 (1978).
3. S. J. Watson, H. Akil, V. E. Ghazarossian, A. Goldstein, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1260 (1981); S. J. Watson, H. Akil, W. Fischli, A. Goldstein, E. Zimmerman, G. Nilaver, T. B. van Wimersma Greidanus, *Science* **216**, 85 (1982).
4. M. Comb, P. H. Seeburg, J. Adelman, L. Eiden, E. Herbert, *Nature (London)* **295**, 663 (1982); U. Gubler, P. Seeburg, B. J. Hoffman, L. P. Gage, S. Udenfriend, *ibid.*, p. 206; M. Noda, Y. Furutani, H. Takahashi, M. Toyosato, T. Hirose, S. Inayama, S. Nakanishi, S. Numa, *ibid.*, p. 202.
5. H. Kakidani, Y. Furutani, H. Takahashi, M. Noda, Y. Morimoto, T. Hirose, M. Asai, S. Inayama, Sh. Nakanishi, S. Numa, *Nature (London)* **298**, 245 (1982).
6. H. Khachaturian, M. E. Lewis, S. J. Watson, *Life Sci.* **31**, 1879 (1982); H. Khachaturian, M. E. Lewis, V. Hollt, S. J. Watson, in preparation.
7. S. J. Watson, H. Akil, S. Sullivan, J. D. Barchas, *Life Sci.* **25**, 733 (1977).
8. E. Weber, K. A. Roth, J. D. Barchas, *Biochem. Biophys. Res. Commun.* **103**, 951 (1981); *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2062 (1982); S. J. Watson, H. Khachaturian, D. Coy, L. Taylor, H. Akil, *Life Sci.* **31**, 1773 (1982).
9. We thank A. Herz and V. Hollt for antiserum to BAM-22P and T. Joh for antiserum to tyrosine hydroxylase. This work was supported by NIDA grant DA02265, NIDA Center grant DA00154, and the Theophile Raphael Fund to H.A. and S.J.W.; NIMH training grant MH15794 to H.K.; and NIDA grant DA1199 to A.G.

4 June 1982; revised 23 August 1982

Nonopiate Effects of Dynorphin and Des-Tyr-Dynorphin

Abstract. *Intracerebroventricular administration of dynorphin produced potent and long-lasting effects on motor function and the electroencephalogram in rats. In addition, local iontophoretic or pressure ejection of dynorphin consistently inhibited hippocampal unit activity. None of these effects were significantly affected by naloxone even at high doses. Moreover, a fragment of dynorphin that failed to displace any of a number of tritiated narcotics from rat brain homogenates produced similar effects on these physiological measures in vivo. On the basis of a variety of criteria for "opiate action," the results suggest that a second biologically active site within the dynorphin sequence is capable of quite potent but nonopiate effects.*

Dynorphin was originally described as an "extraordinarily potent" opioid peptide (*1*). Indeed, data from smooth muscle bioassays as well as radioactive ligand binding assays of brain tissue show that dynorphin has potent agonistic effects at opiate receptors, especially at κ and μ receptors (*2*). However, when dynorphin-(1–13) is injected into the brain, it produces a wide range of motor and behavioral effects that differ significantly from those produced by alkaloid narcotics or other enkephalin-containing endorphins (*3*). Whereas dynorphin-(1–13) is capable of producing long-lasting motor changes, its analgesic properties have been more difficult to establish and require much higher doses. Moreover, many of the dramatic motor and behavioral changes that occur upon administration of dynorphin-(1–13) cannot be prevented by prior treatment with naloxone (*3*), even when the antagonist is administered in doses high enough to reverse the effects of κ-binding drugs. This feature of dynorphin pharmacology stands in marked contrast to β-endorphin, enkephalin, and stabilized enkephalin analogs that produce naloxone-reversible effects almost exclusively.

These novel and apparently naloxone-insensitive effects suggest that the dynorphin molecule may have two biologically active sites, an opiate site (*4*) and a nonopiate site. Conceivably, the intact molecule is capable of producing both sets of effects, depending on the targets that it encounters. Alternatively, it seems possible that dynorphin-(1–13) is rapidly hydrolized into some nonopiate but highly potent substance.

When the full molecule was sequenced (*5*) we reexamined these issues. We now show that dynorphin-(1–17) produces marked motor and electroencephalographic (EEG) changes but fails to produce reliable analgesia in the tail-flick assay, a test that tends to select for narcotic analgesia. In addition, administration of dynorphin-(1–17), either by iontophoresis or pressure ejection, consistently depresses the firing rate of single hippocampal (CA1 and CA3) neurons in contrast to the effects observed with other opioid agents (*6*). None of these effects of dynorphin-(1–17) on motor function, the EEG, or single-unit firing are significantly affected by even high doses of naloxone. We further show that a fragment of dynorphin, des-Tyr-dynorphin [dynorphin-(2–17)] (*7*), fails to bind to opiate receptors in vitro, yet produces a profile of pharmacological activity that is similar to that of its parent molecule. When taken together, these results strongly suggest that dynorphin has an active site that produces robust motor and electrophysiological effects but lacks the usual opiate properties.

We conducted three types of experiments: electrophysiological, behavioral, and binding, using male Sprague-Dawley rats throughout. In the electrophysiological experiments we recorded the effects of locally applied dynorphin and des-Tyr-dynorphin on single-unit activity in CA1 and CA3 hippocampus. In the behavioral experiments we determined the analgesic and EEG effects of intraventricularly (ICV) injected dynorphin using four groups of animals for studying (i) the dose-response curve for the production of EEG changes by dynorphin; (ii) the reversibility of EEG changes in animals treated with naloxone, and parallels to ethylketocyclazocine (EKC)-induced seizures; (iii) parallels between dynorphin and des-Tyr-dynorphin in the production of EEG changes; and (iv) the analgesic properties of ICV-administered dynorphin. In the binding experiments we used radioactively labeled ligands to compare the opiate-like activity of des-Tyr-dynorphin to dynorphin-(1–17).

Electrodes for the measurement of EEG from cortex (area 6, motor) and dorsal hippocampus were stereotaxically placed (coordinates: nosepiece + 5.0 AP, 1.5 lat, 1.5–2.5 D.V.) in 19 rats that were put under deep barbiturate anesthesia by methods described by Robinson (*8*). A stainless steel cannula (24 gauge) aimed for the left lateral ventricle and leads for monitoring the electrocardiogram were implanted at the same time. One group of rats ($N = 8$) received an ICV injection of 5, 10, or 20 μg of dynorphin or a control solution of artificial cerebrospinal fluid (CSF) (5 μl) during the recording of these responses. A

0036-8075/82/1210-1136$01.00/0