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Immunohistochemical distribution of α-neo-endorphin/dynorphin neuronal systems in rat brain: Evidence for colocalization

(opioid peptide neurons/brain distribution/immunofluorescence)

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ABSTRACT **α-Neo-endorphin and dynorphin immunoreactivities in rat brain were visualized by double antibody immunofluorescence of frozen sections. Antibodies were used that were specific for their respective antigens. The pattern of neuronal immunostaining produced by α-neo-endorphin and dynorphin antisera in adjacent serial sections was completely superimposible. No areas were found in which α-neo-endorphin or dynorphin immunoreactivities existed alone. The following brain regions contained α-neo-endorphin/dynorphin-immunoreactive fibers and terminals: the median forebrain bundle, the internal capsule, the substantia nigra, the hypothalamus, the nucleus accumbens, the hippocampus, and the medulla oblongata. A few fibers were seen in the cerebral cortex and in the corpus striatum. In many regions, this neuronal fiber system seems to overlap the neuronal system previously described to contain [Met]-/[Leu]enkephalin-immunoreactive material. In brains from colchicine-treated animals, numerous α-neo-endorphin/dynorphin-immunoreactive neuronal cell bodies were seen in the supraoptic, retrochiasmatic supraoptic, paraventricular, and magnocellular accessory nuclei of the hypothalamus. It is concluded that α-neo-endorphin-like and dynorphin-like immunoreactivities are part of the same neuronal system.**

Extracts from brain and posterior pituitary contain a number of opioid peptides (1–6) that are different in amino acid sequence from the classical opiate-like peptides [Met]- and [Leu]enkephalin (7) and β-endorphin (8, 9). Two of these are α-neo-endorphin (3, 4) and dynorphin (1, 6, *), whose complete primary structure was elucidated recently (4, 6, *). Both peptides are related to the enkephalins inasmuch as they contain an NH_2-terminal [Leu]enkephalin sequence attached to a series of more or less basic amino acid residues at the COOH terminus. The regional brain distributions of α-neo-endorphin and dynorphin were established by radioimmunoassay of extracts from dissected brain regions (10, 11). A preliminary immunohistochemical study of the localization of dynorphin-immunoreactive material in rat brain has been published (12). In that study, antibodies to an NH_2-terminal fragment of dynorphin [dynorphin-(1–13)] (1) were used.

Previously, we have shown that α-neo-endorphin and dynorphin immunoreactivity coexist in the neurons of the rat hypothalamus (13). Here, we report the distribution of α-neo-endorphin- and dynorphin-immunoreactive nerve fibers, terminals, and cell bodies in brains from normal and colchicine-treated rats. The patterns of neuronal immunostaining produced by the α-neo-endorphin and dynorphin antisera were completely identical. No areas in the brain were detected in which α-neo-endorphin or dynorphin immunoreactivity occurred alone.

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MATERIALS AND METHODS

Antibodies. Antisera were raised against synthetic dynorphin-(1–17) (6, *) and against synthetic α-neo-endorphin (4). All peptides used in this study were synthesized by J.-K. Chang at Peninsula Laboratories (San Carlos, CA). Antibodies were generated by injecting a carbodiimide-treated peptide/thyroglobulin mixture into rabbits as described for the production of antibodies to N^{α}-acetyl-β-endorphin (14). Two dynorphin antisera (R3-1 and R2-2) and two α-neo-endorphin antisera (R1-3 and R2-4) were used for the immunohistochemical studies. The specificity of all four antisera was tested extensively by radioimmunoassay (RIA), double antibody immunoprecipitation, and immunohistochemical blocking controls.

In RIA using ^{125}I-labeled dynorphin-(1–17) as tracer, dynorphin-(1–13) was 50% crossreactive with the dynorphin antibodies. The crossreactivity of dynorphin-(1–9) was <0.01%. Dynorphin-(6–13) and α-neo-endorphin did not crossreact. In the α-neo-endorphin RIA using ^{125}I-labeled α-neo-endorphin as tracer, the two α-neo-endorphin antibodies had a crossreactivity of <0.01% with β-neo-endorphin [i.e., α-neo-endorphin-(1–9)]. Dynorphin-(1–9), -(1–13), -(1–17), and -(6–13) were not crossreactive. [Leu]Enkephalin, [Met]enkephalin, α-neo-endorphin-(1–8), [Arg^8]vasopressin, oxytocin, and substance P were not crossreactive with either antiserum. Because the antisera dilutions used in the immunohistochemical studies were much less than those used in the RIA, we also tested the α-neo-endorphin and the dynorphin antibodies by double antibody immunoprecipitation at a dilution of 1:100 for binding of ^{125}I-labeled dynorphin and ^{125}I-labeled α-neo-endorphin. Under these conditions, all four antisera immunoprecipitated only their own antigens: the dynorphin antisera did not immunoprecipitate ^{125}I-labeled α-neo-endorphin and the α-neo-endorphin antisera did not precipitate ^{125}I-labeled dynorphin.

All four antisera were used throughout for visualizing α-neo-endorphin/dynorphin-immunoreactive neurons in the brain. The immunostaining patterns produced by the four antisera were essentially identical; however, antisera R2-4 and R2-2 showed a somewhat brighter fluorescence of the α-neo-endorphin/dynorphin neuronal system.

Immunohistochemistry. Serial sections (13 μm thick) through 12 brains from normal male rats (Sprague–Dawley; 150–250 g) and through 6 brains from rats that had been injected intraventricularly with 50 μg of colchicine in 25 μl of water 48 hr prior to death were immunofluorescently stained with α-neo-endorphin and dynorphin antisera. The fixation and double antibody

Abbreviations: P_i/NaCl, phosphate-buffered saline; RIA, radioimmunoassay.

* Tachibana, S., Araki, K., Ohya, S. & Yoshida, S. (1981) in *Proceedings of the International Narcotics Research Conference*, Kyoto, Japan, p. 20 (abstr.).

immunofluorescence procedures have been described (13, 15, 16). Briefly, animals were perfused with ice-cold 4% freshly depolymerized paraformaldehyde in 0.13 M isoosmotic sodium phosphate buffer (pH 7.4). Brains were dissected and further fixed for 2 hr at 4°C. After soaking in 0.01 M sodium phosphate/0.15 M NaCl, pH 7.4 (P_i/NaCl)/5% sucrose, brains were rapidly frozen and 13-μm thick coronal sections were cut in a cryostat. The sections were melted onto gelatin-coated slides, air dried, rehydrated with P_i/NaCl/0.3% Triton X-100, and then incubated for 12–16 hr at 4°C with primary antiserum diluted 1:400 in P_i/NaCl/Triton X-100/2.5% bovine serum albumin.

The sections, after washing, were fluorescently stained with fluorescein-conjugated sheep antirabbit IgG (Cappel, Cochranville, PA) diluted 1:40 in P_i/NaCl/Triton X-100 for 2 hr at room temperature. The fluorescein-labeled second antibody had been preabsorbed on rat liver acetone powder to reduce nonspecific background staining. After washing, the sections were attached to coverslips with glycerol/P_i/NaCl (1:1) and examined and photographed using a Leitz Orthoplan microscope equipped with an epifluorescence attachment. Specificity of immunofluorescent staining was controlled by incubating adjacent serial sections with either primary antibody alone or primary antibody in the presence of 10–100 μM dynorphin or α-neo-endorphin. In addition, control experiments were carried out in which both the α-neo-endorphin and the dynorphin antisera were absorbed with 10–100 μM dynorphin-(1–9), -(1–13), or -(6–17) or [Leu]enkephalin.

Immunohistochemical mapping of α-neo-endorphin/dynorphin neurons was carried out by incubating alternate serial sections throughout the brain with either α-neo-endorphin antiserum or dynorphin antiserum. Immunofluorescent staining was correlated with coronal sections from the stereotaxic atlases of Koenig and Klippel (17) and Pellegrino *et al.* (18).

RESULTS

General Observations. Serial sections through rat brains immunofluorescently stained with α-neo-endorphin and dynorphin antisera showed widely distributed immunoreactive neuronal fiber systems and terminals in various areas of the brain (Fig. 1). The overall intensity of the α-neo-endorphin/dynorphin-like immunofluorescence sometimes varied from brain to brain. It is not known whether this reflected slight variations in the fixation conditions or intrinsic differences among the animals. In general, the intensity of the α-neo-endorphin/dynorphin-like immunofluorescence in neuronal fibers appeared weaker than the fluorescence observed in enkephalin fibers when antisera to [Met]- or [Leu]enkephalin are used (16). This probably reflects the relatively low brain concentrations of α-neo-endorphin and dynorphin compared with those of the enkephalins (10, 11).

In a previous study, we found that α-neo-endorphin immunoreactivity was colocalized with dynorphin immunoreactivity in magnocellular neurons of the hypothalamus (13). In that study, in which brains from colchicine-treated animals were used, we observed a close overlap of the neuronal fiber systems that were labeled by the α-neo-endorphin and dynorphin antisera. This finding was confirmed in the present study: Serial sections throughout the brain immunostained alternately with the two α-neo-endorphin antisera and the two dynorphin antisera showed that the immunostaining patterns produced by the four antisera were completely identical (Fig. 2 *C–K*). In no area of the brain was dynorphin or α-neo-endorphin immunoreactivity observed to occur independently. The following descriptions of the distribution of immunoreactive neuronal elements are taken primarily from sections incubated with α-neo-endorphin antiserum R2-4 or dynorphin antiserum R2-2. These two antisera gave a somewhat brighter immunofluorescence than antisera R1-3 and R3-1.

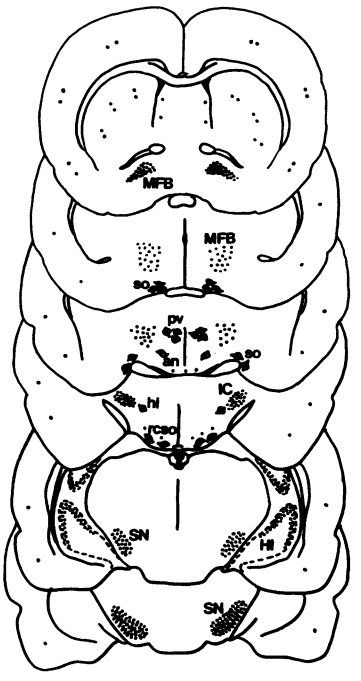

FIG. 1. Schematic distribution of the major α-neo-endorphin/dynorphin-immunoreactive neuronal systems in rat brain regions. Immunoreactive neuronal cell bodies (colchicine-treated animals) were seen in the supraoptic (so), retrochiasmatic supraoptic (rcso), paraventricular (pv), and magnocellular accessory nuclei (an). A few immunoreactive cell bodies were observed in the lateral hypothalamic nucleus (hl) just medial to the pars retrolenticularis of the internal capsule (IC). Fibers or (and) terminals (dots; normal and colchicine-treated animals) were seen in the median forebrain bundle (MFB), in the internal capsule (IC; pars retrolenticularis), and in the substantia nigra (SN). Fewer neuronal fibers were observed in the hypothalamus. A homogenously fluorescent band was seen along the pyramidal cell layer of the hippocampus (HI). Occasional fibers were observed in the cerebral cortex, in the lateral septal nucleus, and in the corpus striatum. Additional brain regions containing α-neo-endorphin/dynorphin-immunoreactive structures are discussed in *Results*.

Distribution of Neuronal Fibers and Terminals. The distribution of neuronal structures that were immunoreactive for the two peptides is described below in order of the relative intensity of the observed immunostaining rather than anatomically. The brain regions that were found to contain the highest densities of α-neo-endorphin/dynorphin fibers or terminals were the substantia nigra (Figs. 1 and 2 *A*, *C*, and *D*) and the median forebrain bundle (Fig. 2 *E* and *F*). The substantia nigra showed a granular terminal-like immunofluorescence with a slightly stronger staining in the pars reticularis than in the pars compacta. The median forebrain bundle showed fairly thick fiber-like structures that seemed to form a reticulum-like pattern. A rather dense immunofluorescence was also seen in the pars retrolenticularis of the internal capsule (Fig. 2*L*). This staining was also of the reticulum pattern type.

A broad band of fluorescence was found along the pyramidal cell layer of the caudal parts of the hippocampus (Figs. 1 and 2 *G* and *H*). This fluorescent band was more pronounced along the lateral pyramidal cell layer than along the medial layer. However, the intensity of the immunostaining in this region was in general considerably weaker than that in the median forebrain bundle or in the substantia nigra. No individual fibers or terminals could be identified in this band; the fluorescence seemed rather homogenous. The distribution and the morpho-

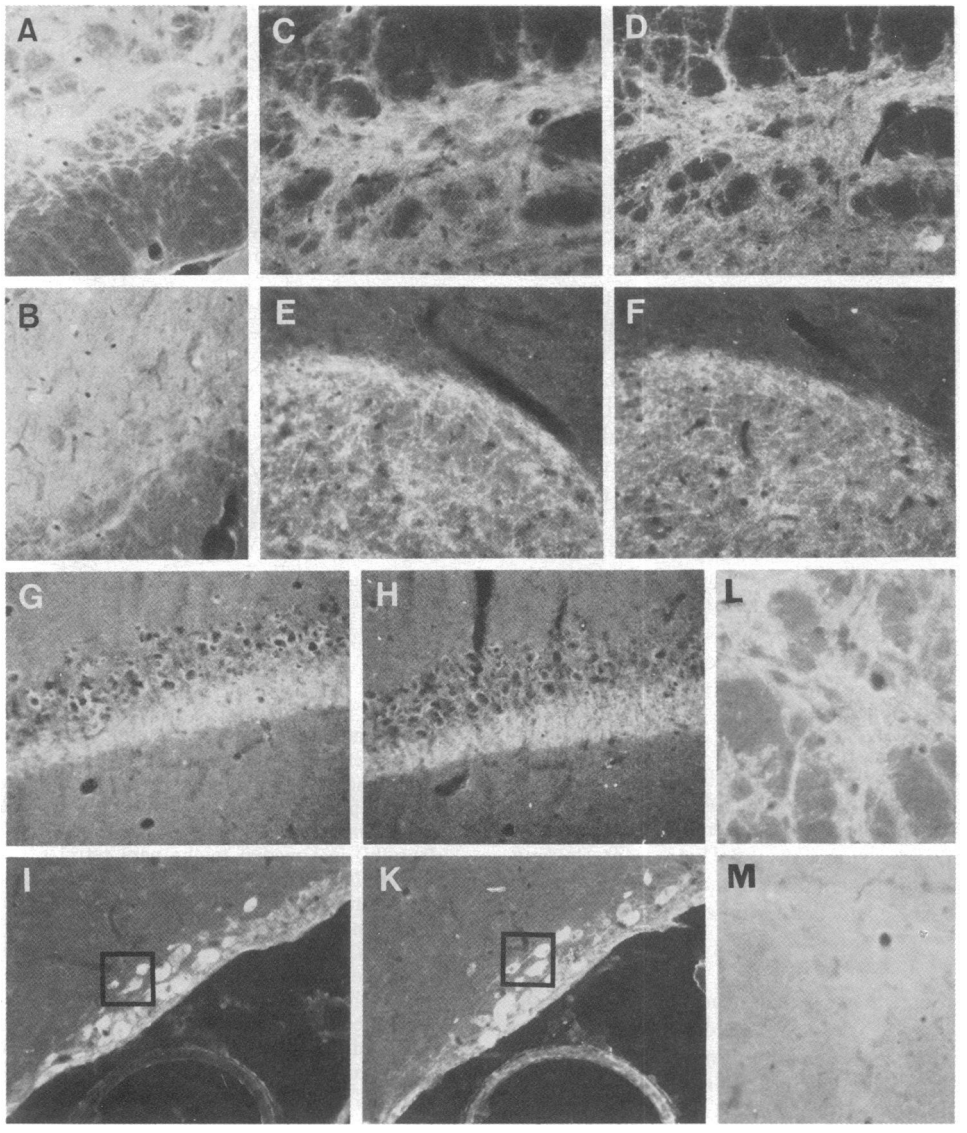

FIG. 2. Photomicrographs of α-neo-endorphin/dynorphin-immunoreactive neuronal elements in rat brain. Micrographs were taken from tissue sections that had been incubated with dynorphin antiserum R2-2 or α-neo-endorphin antiserum R2-4. (*A* and *B*) Specificity of the dynorphin-like immunostaining in the substantia nigra. The section in *A* was immunofluorescently stained with dynorphin antiserum and that in *B* is a blocking control in which excess (10 μM) synthetic dynorphin was added to the antiserum. (*L* and *M*) Specificity of the α-neo-endorphin-like staining in the IC (pars retrolenticularis). The section in *L* was stained with α-neo-endorphin antiserum and that in *M* was incubated with the same antiserum and excess (10 μM) synthetic α-neo-endorphin. (*C–K*) Paired serial sections through the pars reticularis of the substantia nigra (*C* and *D*), the median forebrain bundle (*E* and *F*), the hippocampus (*G* and *H*), and the retrochiasmatic supraoptic nucleus (*I* and *K*). The left section of each pair was immunostained with α-neo-endorphin antiserum and the right was stained with dynorphin antiserum. The immunostaining patterns produced by the two antisera are virtually identical. In *I* and *K*, three neuronal cell bodies can be identified twice in the two adjacent sections stained for α-neo-endorphin and dynorphin, demonstrating directly a neuronal colocalization of immunoreactivities for the two peptides.

logical characteristics of this hippocampal immunostaining seems to coincide with the hippocampal mossy fiber projection (19–21). Recently, this fiber system has been shown to be immunoreactive to antibodies raised against [Met]enkephalin and [Leu]enkephalin (22). Occasionally, a beaded axon was seen in the molecular layer of the hippocampus in addition to the fluorescent band along the pyramidal cells.

Less intense α-neo-endorphin/dynorphin fiber systems were found in the hypothalamus. There, beaded axons were identified mainly in the ventral part of the hypothalamus. In adjacent serial sections, these fibers were observed to converge toward the median eminence. The inner layer of the median eminence contained a pattern of cross-sectioned axons that closely resembled the fiber pattern observed when antisera to vasopressin or neurophysin are used. These fibers are part of the neurosecretory system that originates in the hypothalamic magnocellular neurons and terminates in the neurosecretory endings of the posterior pituitary. A moderate density of fibers and terminals was found in the nucleus accumbens. There, short beaded axons were the predominant immunoreactive structures.

Areas in which occasional immunoreactive structures—mainly of the beaded axon type—were found included the cerebral cortex (Fig. 2*L*), the lateral septal nucleus, and the corpus striatum. In the medulla oblongata, numerous extremely thin fibers and terminals were found in the nucleus tractus spinalis trigeminalis. These thin fibers and terminals were also diffusely distributed in an area dorsal and lateral to the central canal.

Distribution of Neuronal Cell Bodies. In brain sections from colchicine-treated animals, a high density of α-neo-endorphin/dynorphin-like immunostaining of neuronal cell bodies was

found in several hypothalamic nuclei (Fig. 1). The largest number of strongly immunostained cell bodies was found in the supraoptic nucleus and in the retrochiasmatic supraoptic nucleus (Fig. 2 *I* and *K*). The retrochiasmatic supraoptic nucleus belongs to the group of magnocellular neurons in the hypothalamus and it probably represents a caudal continuation of the supraoptic nucleus. Fewer α-neo-endorphin/dynorphin immunoreactive cell bodies were found in the magnocellular part of the paraventricular nucleus. In specificity controls, immunostaining of the paraventricular neurons by both α-neo-endorphin and dynorphin antisera was greatly reduced but not completely eliminated by absorption of the antisera with their own synthetic antigens. Several cell groups in the anterior hypothalamic nucleus were also immunoreactive to α-neo-endorphin and dynorphin antisera. These cell groups are probably part of the accessory nuclei of the magnocellular system. A small α-neo-endorphin/dynorphin cell group was found in the hypothalamic lateral nucleus near the pars retrolenticularis of the internal capsule.

In magnocellular neuronal cell bodies, we have already shown that α-neo-endorphin and dynorphin immunoreactivities are colocalized (13). In this study, serial sections through these nuclei were immunostained alternately with α-neo-endorphin antiserum and dynorphin antiserum. In these serial sections, the same neurons could sometimes be recognized in adjacent sections stained with the two antisera (Fig. 2 *I* and *K*). This confirmed our earlier observation that, in hypothalamus, immunoreactivities for the two peptides coexist in the same magnocellular neuronal perikarya (13).

Specificity Controls. The immunostaining specificities of the four antisera used were tested by immunohistochemical blocking controls on all brain regions in which positive immunofluorescence was observed. Concentrations of 10–100 μM of synthetic peptide were used in the blocking controls. All immunostaining reported above was specific in that it was blocked by incubation of the tissue sections with α-neo-endorphin antiserum in the presence of 10 μM synthetic α-neo-endorphin or with dynorphin antiserum in the presence of 10 μM dynorphin (Fig. 2 *A*, *B*, *L*, and *M*). α-Neo-endorphin (up to 50 μM) did not block dynorphin-like immunostaining and 50 μM dynorphin did not block α-neo-endorphin-like immunostaining (13). [Leu]Enkephalin (up to 100 μM) did not block staining of either antiserum. Dynorphin-like immunostaining was not blocked by 50 μM dynorphin-(1–13) or -(1–9); 10 μM dynorphin-(6–17) greatly reduced dynorphin-like immunostaining but did not eliminate it completely, indicating that the COOH-terminal tetrapeptide fragment of dynorphin was the major—but not the only—antigenic determinant for the antibodies that caused the dynorphin-like immunofluorescence.

DISCUSSION

We have demonstrated by immunohistofluorescence the distribution in rat brain of α-neo-endorphin- and dynorphin-immunoreactive neuronal systems. The patterns of neuronal fiber and terminal systems specifically labeled by the two antisera in adjacent serial sections throughout the brain were completely superimposible (Fig. 2 *C–K*). No areas were seen that contained immunoreactive neurons for one peptide but not for the other. In brain areas such as the substantia nigra or the median forebrain bundle, in which immunostaining for the two peptides was particularly dense, the identity of the staining patterns produced by the two antisera was particularly obvious (Fig. 2 *C–F*). In regions such as cerebral cortex and hypothalamus, in which fibers and terminals were not so dense, the identical morphology of the immunostained neuronal structures suggested that the two antisera labeled the same set of neurons. These findings, together with our earlier demonstration that the same neuronal cell bodies in the hypothalamic magnocellular nuclei contain both α-neo-endorphin- and dynorphin-immunoreactive material (13) suggest that α-neo-endorphin immunoreactivity and dynorphin immunoreactivity are both part of the same neuronal system in rat brain. However, direct proof of colocalization of α-neo-endorphin and dynorphin immunoreactivities in axons and terminals must await electron microscopic immunocytochemical studies. Colocalization of two or more peptides in the same cells often—but not invariably—reflects a common origin from a single precursor molecule. Immunohistochemical studies cannot clarify this question. If, however, such a precursor exists, it may be that part of the observed α-neo-endorphin/dynorphin immunostaining is due to this (hypothetic) peptide.

The distribution of α-neo-endorphin/dynorphin neuronal systems that we have described here partly confirms and extends preliminary observations on the immunohistochemical localization of dynorphin-immunoreactive neurons (12). In that earlier study, antisera were used that had been raised against dynorphin-(1–13) (1), an NH_2-terminal fragment of authentic dynorphin that has 17 amino acid residues (6, *). Although these antibodies revealed dynorphin immunoreactivity in the magnocellular neurons, the wide fiber distribution of α-neo-endorphin/dynorphin-immunoreactive neurons reported here was not found in the previous study (12). Apparently, it is crucial to use antibodies raised against dynorphin-(1–17) to obtain effective immunostaining of dynorphin neurons. This notion was substantiated by comparison of immunohistochemical blocking controls on the dynorphin antisera and their RIA specificity: Whereas dynorphin-(1–13) was strongly crossreactive (50%) in RIA, it did not block dynorphin-like immunostaining. Only dynorphin-(1–17) fully blocked the immunostaining produced by the dynorphin antisera, indicating that amino acid residues Trp^{14}–Gln^{17} in dynorphin represent a major antigenic site of the dynorphin-immunoreactive material in formaldehyde-fixed brain tissue sections used in immunohistochemistry.

The distribution of α-neo-endorphin/dynorphin-immunoreactive neurons in rat brain reported here confirms and substantiates distribution studies of the dynorphin (10) and α-neo-endorphin (11) content detectable by RIA in extracts of dissected brain regions. The authors of those studies suggested that regional concentrations of the two peptides are different from the reported regional distribution of the other major opioid peptide neuronal systems—i.e., the β-endorphin system and the [Met]/[Leu]enkephalin system (23–28). The immunocytochemical distribution of the α-neo-endorphin/dynorphin neuronal system described here indeed has little similarity with that of the β-endorphin neuronal system. β-Endorphin neurons have their cells of origin in the arcuate nucleus and project mainly to midbrain areas such as the periventricular grey (23, 24). Neither in the arcuate nucleus nor in the periventricular grey were significant numbers of α-neo-endorphin/dynorphin neurons observed.

It is not certain whether the α-neo-endorphin/dynorphin neuronal system is separate from the enkephalin/pentapeptide systems. There seems to be a significant overlap of the α-neo-endorphin/dynorphin neuronal system with the [Met]/[Leu]enkephalin systems. Areas such as the nucleus accumbens and the median forebrain bundle are commonly agreed to contain extensive [Met]/[Leu]enkephalin fibers (16, 25–28). Some other regions, like the hippocampus and the hypothalamic magnocellular nuclei, have been shown to be enkephalin immunoreactive in some reports only (22, 28). All these areas—especially the median forebrain bundle—are also dense in α-neo-endorphin/dynorphin neurons (Fig. 2 *E* and *F*). On the other hand, others areas rich in α-neo-endorphin/dynorphin-

immunoreactive neurons, such as the internal capsule and the substantia nigra, have not been regularly associated with [Met]/[Leu]enkephalin neurons. And vice versa, α-neo-endorphin/dynorphin-immunoreactive neurons have not been found in regions such as the interpeduncular nucleus and the central nucleus of the amygdala that contain extensive systems of [Met]/[Leu]enkephalin neurons.

Studies of the relationship of the enkephalin/pentapeptide neurons to α-neo-endorphin/dynorphin neurons are complicated by the fact that some [Leu]enkephalin antibodies seem to crossreact with dynorphin (1) and, therefore, some of the neuronal systems previously described as containing [Leu]enkephalin immunoreactivity may actually contain dynorphin and α-neo-endorphin immunoreactivity.

Much more work is needed to determine how the α-neo-endorphin/dynorphin neuronal system and the [Met]/[Leu]enkephalin system are related and whether in those regions in which the two systems overlap, [Met]/[Leu]enkephalin immunoreactivity can coexist with α-neo-endorphin/dynorphin immunoreactivity in the same neurons. The results described here do not exclude the possibility that—at least in some regions—dynorphin and α-neo-endorphin can serve as precursors to [Leu]enkephalin.

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