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Neurons containing β-endorphin in rat brain exist separately from those containing enkephalin: Immunocytochemical studies

(peptides/hypothalamus/thalamus/pituitary)

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ABSTRACT **Well-characterized antisera to porcine β-endorphin were used to localize immunoreactive sites in cryostat sections of formaldehyde-fixed rat brain by indirect immunohistochemistry. Specificity was established by absorption of immune sera with synthetic peptide fragments. Specific immunoreactivity was localized to neuronal perikarya in the basal tuberal hypothalamus, and to varicose nerve fibers which were distributed to midline nuclear areas throughout the diencephalon and anterior pons. These patterns of reactivity were unaffected by preabsorption of the immune sera with millimolar concentrations of Met^5- or Leu^5-enkephalin or α-endorphin. The β-endorphin immunoreactive structures were morphologically separate from those cells and fibers reported to react with antisera to the enkephalins. One anti-β-endorphin serum gave additional immunoreactivity with myelinated axons in limbic cortical zones; when absorbed with purified rat myelin basic protein, only the specific patterns of immunoreactivity remained. Thus, discrete β-endorphin-containing neuronal circuits exist in rat brain and are anatomically distinguishable from enkephalin-containing nerve cell and fiber pathways.**

The naturally occurring opioid peptides, the endorphins (see refs. 1 and 2), share common NH_2-terminal sequences with the COOH-terminal fragment of β-lipotropin (β-LPH) (3–7). The 31-amino acid COOH-terminal peptide of β-LPH, termed β-endorphin (8), is an extremely potent opioid agonist (8–11). β-Endorphin was isolated and purified from extracts of ovine and procine pituitary (3, 4) as was α-endorphin [β-LPH 61-76 (6)] and γ-endorphin [β-LPH 61-77 (7)]. Specific COOH-terminally directed antisera raised against synthetic β-endorphin and α-endorphin have permitted selective radioimmunoassays for these peptides, which do not read enkephalin pentapeptides (12, 13). These immune antisera have already been used to reveal the immunocytochemical location of α-endorphin- and β-endorphin-reactive cells in the rat intermediate lobe and adenohypophysis (14).

By radioimmunoassay, rat brain contains significant amounts of β-endorphin (15), and these levels are unchanged at 6–9 months after hypophysectomy (15). The regional distribution of β-endorphin in rat brain shows no fixed relationship to radioimmunoassayable enkephalins (15), and many areas reported to be rich in enkephalin immunoreactivity (16–22) are devoid of detectable β-endorphin. We now report that immunocytochemical localization studies reveal β-endorphin-containing neuronal perikarya and fiber tracts throughout the diencephalon that can be clearly distinguished cytologically and immunologically from those cells and fibers observed by us and reported by others (16–23) to be enkephalin-containing neurons.

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MATERIALS AND METHODS

Antiserum Preparation and Characterization. As described in greater detail elsewhere (12, 13) antisera were raised in rabbits to synthetic β-endorphin that had been coupled to bovine serum albumin with bis-diazotized benzidine. The antisera used here for cellular localization of β-endorphin immunoreactivity were those used by us for the radioimmunoassay procedures already described (12, 13, 15). The majority of immunocytochemical results reported here were obtained with antiserum RB 100-10/27; this antiserum recognizes a segment of the COOH-terminal portion of β-endorphin between asparagine-20 and histidine-27 (12). RB 100-10/27 does not recognize either enkephalin pentapeptide or α- or γ-endorphin, but it shows parallel competition for binding of ovine β-LPH and β-endorphin. Similarly, this antiserum also shows parallel displacement for binding of β-endorphin with the purified 31,000 molecular weight precursor peptide 31k described by Mains *et al.* (24) which contains β-LPH as its COOH-terminal peptide. Additional immunocytochemical localizations of β-endorphin-reactive material were also surveyed with five other antisera raised in separate animals to the same immunogen conjugate; in one case, RB 263-11/76, the rabbit immunoglobulin fraction obtained by ammonium sulfate fractionation was further purified by passage over a Sepharose column to which β-endorphin had been NH_2-terminally conjugated according to the procedures of Axen *et al.* (25)

Specificity Studies. Because the conditions for immunocytochemical staining differ significantly from those under which the radioimmunoassays for specificity are done (26), we used the following procedures to verify specificity of staining on our tissue sections. Specificity of primary antisera were evaluated by blocking experiments in which antisera diluted for tissue reactions (see below) were exposed overnight to solutions of synthetic peptides at concentrations of 1 mM to 1 μM; unreacted diluted primary antisera stored under the same conditions without added peptide served as controls for the absorption procedure. Specific staining described below indicates that all β-endorphin-reactive staining patterns were eliminated by prior absorption with 1 μM β-endorphin or with the fragment (β-LPH_{66-91}) bearing the recognition site as determined in radioimmunoassay studies (12) but not by any other enkephalin or endorphin peptides.

Tissue Preparation. After systematic tests of various fixation and sectioning procedures, detailed mapping and specificity studies were accomplished on material prepared according to the following protocol: rats were anesthetized with chloral hydrate (350 mg/kg, intraperitoneally) and perfused tran-

Abbreviations: β-LPH, β-lipotropin; P_i/NaCl, phosphate-buffered saline; FITC, fluorescein isothiocyanate.

scardially with freshly depolymerized 5% formaldehyde in phosphate buffer (0.3 M, pH 7.4); the perfusate was chilled to 5° and 200–400 ml of it was perfused under pressure over 90–120 sec. The brains and pituitaries were quickly removed and dissected into 1- to 3-mm coronal or sagittal slabs which were immersed in the same cold fixative for an additional 3 hr. The tissues were then washed in phosphate buffer briefly and passed through a series of increasing concentrations of sucrose [12–18% in phosphate-buffered saline (P_i/NaCl)] and left in the highest sucrose concentration until used. For sectioning, tissue slabs were frozen on brass chucks immersed in pulverized dry ice, equilibrated in a Harris cryostat (−18°), and sectioned serially at thicknesses of 10–20 μm, mounted on gelatin-coated chilled slides, and briefly air-dried.

Immunocytochemical Staining Procedures. Three types of procedures were used with antisera (whole or Ig fractions) diluted in P_i/NaCl containing 0.3% Triton X-100 (Sigma) and bovine serum albumin at 1 mg/ml (McAllister–Bicknell).

1. The indirect immunofluorescence method was used as described for studies on pituitary (14); primary antisera at dilutions of 1:10–1:50 were used in incubations for 30–500 min at 4°, 20°, or 37°. After incubation, the tissues were washed repetitively with P_i/NaCl and incubated with fluorescein isothiocyanate (FITC)-conjugated goat Ig raised to rabbit Ig (Cappel Labs, Cochranville, PA) at 1:50 dilutions for 30 min, rinsed in P_i/NaCl as above, coverslipped in Entellan, and examined in a Zeiss universal microscope with epi-illumination and the FITC filter pack.

2. The unlabeled antibody method was applied according to Sternberger (27). The primary antisera at dilutions of 1:1000–1:1500 were used in incubations for 24–48 hr at 4°. The samples were rinsed with P_i/NaCl (three times, 10 min each), incubated for 30 min at 20° with unlabeled goat IgG raised against rabbit Ig (Cappel Labs) diluted 1:100, and again rinsed (three times, 10 min each). The sections were then incubated for 30 min at 20° with rabbit peroxidase-antiperoxidase complex (Cappel Labs), diluted 1:50, and rinsed with P_i/NaCl (three times, 10 min each). The horseradish peroxidase marker was then allowed to react with freshly prepared 0.05% 3′,3′-diaminobenzidine tetrahydrochloride (Sigma) in phosphate (pH 7.4) or acetate (pH 5.0) buffer containing 0.002% H_2O_2 for 10–20 min, rinsed with P_i/NaCl and distilled water, coverslipped with paraffin oil, and examined under bright-field or dark-field optics.

3. The indirect peroxidase-labeled antibody method was applied in a few cases. Goat IgG raised against rabbit IgG was conjugated directly to horseradish peroxase (28) and incubated with tissue sections at dilutions and times comparable to the first two incubation steps in the unlabeled antibody method (27).

Distribution of immunoreactive structures was defined neuroanatomically according to Gurdjian (29), Krieg (30), and Konig and Klippel (31). All results were replicated independently in at least four different animals.

RESULTS

General Methodologic Observations. Specific immunoreactivity for β-endorphin was found in cells and cell processes within the basal hypothalamus as described in detail below. Using this basic reactivity, we examined both fixation and sectioning methods and then verified specificity of immunoreactivity. Optimal reactivity was obtained with formaldehyde solutions of 4–5%; lower concentration gave tissue sections that survived the long incubations poorly, and higher concentrations yielded generally lower immunoreactivity. No immunoreactivity was detected in tissues fixed with glutaraldehyde at 1% or more or in formaldehyde-fixed tissues that were sectioned after dehydration and embedding in paraffin. Optimal reactivity was obtained with either of the immunoperoxidase techniques applied for long incubation periods in the cold at high primary antisera dilutions. The indirect immunoperoxidase method gave slightly cleaner reactivity, in the same specific patterns, than did the unlabeled peroxidase-antiperoxidase method. For photographic purposes and for conservation of the antisera, the peroxidase methods were both highly preferable to the indirect immunofluorescence method.

Table 1. Immunocytochemical specificity tests

Antiserum	Absorbed with	Reactivity
RB 100	None	+++
	β-Endorphin 10^{-6}–10^{-4} M	0
	α-Endorphin 10^{-3} M	+++
	β-LPH_{66-91}	±
	Met^5- or Leu^5-enkephalin 10^{-3} M	+++
RB 263	None	+++ (plus myelin)
	β-Endorphin 10^{-6}–10^{-4} M	0 (plus myelin)
	Purified rat myelin basic protein	+++ (no myelin)

Specificity tests with RB 100-10/27 indicated (Table 1) that only β-endorphin or β-LPH_{66-91} interfered with immunoreactivity. Equivalent cytological staining patterns were obtained with RB 100-10/27 at dilutions of 1:1000 in 1 mM Met^5-enkephalin.

Specific immunoreactivity to β-endorphin could be seen in neurons and neuronal processes within the diencephalon and anterior pons. Two groups of closely adjacent β-endorphin reactive neurons were observed (Fig. 1*A*) within the tuberobasal hypothalamic region. One group was within and beyond the dorsolateral portion of the middle to posterior thirds of the arcuate nucleus. The second group was continuous with the first but extended anterolaterally beneath the ventromedial nucleus, almost to the lateral border of the hypothalamus. In addition, some reactive cells formed a continuous band across the floor of the third ventricle in the premamillary zone. The reactive cells tended to be fusiform with one or two large processes extending from the narrowed ends; overall sizes ranged between 10 and 20 μm. Prominent clusters of Golgi-like patches exhibited intense intracytoplasmic reactivity, but no reactivity was observed over the large, centrally placed nuclei (Fig. 1*B*). No other immunoreactive cells have been seen in any other portions of the rat brain.

Surrounding the reactive cell bodies and extending into the neuropil away from them were large, long, and relatively thick varicose processes (Fig. 1*C*) which we interpret as preterminal and terminal axons. These processes, which are generally confined to midline nuclei of the diencephalon, could be followed in serial sections, leading to the following overall assessment.

Hypothalamus. Fibers were most dense in the anterior hypothalamic area and were especially prominent at the level of the decussation of the anterior commissure, extending into the stria terminalis and its bed nucleus and diminishing in density when extending into the lateral septum and nucleus accumbens septi. The supraoptic, periventricular, paraventricular, and

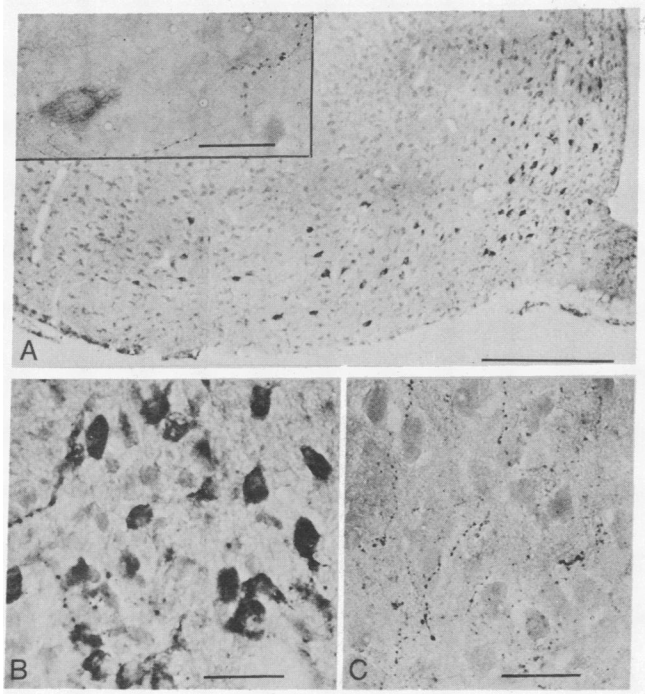

FIG. 1. (*A*) Localization of β-endorphin-reactive neurons in basal hypothalamus by immunoperoxidase histochemistry with antiserum RB-100. Small round and fusiform cells can be seen to lie at the perimeter of the arcuate nucleus at the base of the third ventricle (far right) and to extend laterally along the base of the hypothalamus. (Calibration bar = 100 μm.) (*Inset*) High magnification of one neuron exhibiting immunoreactivity with antiserum RB 100 to β-endorphin. Note intracytoplasmic granular reactivity and nonreactivity of the centrally placed nucleus. Immunoreactive nerve fibers with varicose thickenings can also be seen in the surrounding neuropil. (Calibration bar = 25 μm.) (*B*) A cluster of neurons exhibiting immunoreactivity with antiserum RB 100 to β-endorphin. The fusiform nature of the reactive cells and reactive neural processes can be seen; note the large, thick varicose fibers within the neuropil between the reactive neurons. Antiserum had been absorbed with 1 mM Met^5-enkephalin. (Calibration bar = 25 μm.) (*C*) Immunoreactive fibers within the paraventricular nucleus of the thalamus after reaction with antiserum RB 100 to β-endorphin. Several long varicose fibers can be seen entwining nonreactive neurons in the background. (Calibration bar = 25 μm.)

FIG. 2. (*A*) Immunoreactive varicose fibers within the periventricular nucleus of the hypothalamus after staining with antiserum RB 100 to β-endorphin that had been incubated overnight with 1 mM Leu^5-enkephalin. Note that some fibers lie just beneath the ependymal cells lining the third ventricle. (Calibration bar = 25 μm.) (*B*) Immunoreactivity of myelinated axons in hippocampus after staining with antiserum RB 263 to β-endorphin. After absorption with purified rat myelin basic protein (1 mg/ml), all reactivity in hippocampus was eliminated. (Calibration bar = 25 μm. (*C*) Schematic sagittal view of β-endorphin-reactive neurons and fibers in the rat brain. The neuronal perikarya in the basal hypothalamic region give rise to fibers that sweep forward along the routes indicated to enter the preoptic area and then to course within the periaqueductal region of the diencephalon and pons.

suprachiasmatic nuclei of the hypothalamus all showed these thick long fibers in abundance as did the lateral anterior hypothalamic area and median eminence. Reactive fibers were found within the immediate subependymal zone but were never observed penetrating the ependymal cell layer. A sparse but definite innervation of the medial cortical amygdala represented the most lateral extent of these fibers.

Thalamus-Pons. The fibers appeared to enter the pons with the stria medullaris and to remain mainly ventral to the aqueduct in midline structures, especially the paraventricular nucleus of the anterior midthalamus (Fig. 2*A*). At the level of the dorsal raphe nucleus, which showed considerable although less reactivity, the fibers moved into the dorsal and lateral regions of the peri-aqueductal gray in the immediate juxta-ependymal zone and coursed toward the middle third of the nucleus locus ceruleus from its medial surface. Caudal to the level of the locus ceruleus, β-endorphin reactive fibers were extremely scarce, and none have been seen in medulla or spinal cord.

By using the unlabeled antibody peroxidase procedure, staining patterns were investigated in some detail with a second antiserum to β-endorphin that had been purified to the Ig fraction and then further purified by affinity chromatography on a Sepharose column to which β-endorphin had been conjugated. Although somewhat decreased in titer in the β-endorphin radioimmunoassay (results not shown), the derived antiserum product showed good affinity to β-endorphin and gave immunocytochemical results on cell bodies and processes as just described. However, in addition, the affinity-purified antiserum also revealed profuse staining of myelinated axons that was especially prominent in prepyriform and rhinecephalic cortex, hippocampus, cingulate gyrus of the cerebral cortex, and cerebellum (Fig. 2*B*). This added myelinated fiber immunoreactivity was totally abolished by preincubation of this antiserum product with well-purified myelin basic protein (1 mg/ml; supplied by F. Westall and J. Salk). After preabsorption of this antiserum with β-endorphin (1–10 μm), all reactivity (i.e., cell bodies, cell processes, and myelinated axons) was completely suppressed. When the antiserum product was absorbed only with myelin basic protein, the same immunoreactive cell bodies and processes could be detected as with RB 100-10/27.

These latter observations coupled with previously reported results (15) of radioimmunoassays on materials within brain extracts separated by gel filtration suggest that some common reactive component may exist between the immune recognition segment of β-endorphin and some component of myelin. In separate experiments we observed that myelin basic protein can give parallel displacement with β-endorphin but at 3–4 orders of magnitude higher concentration. Nevertheless, because in most brain regions there is considerably more myelin present

than endorphin peptide, this unexpected crossreactivity could confuse results with radioimmunoassay.

Others have reported that globus pallidus, central nucleus of the amygdala, and substantia gelatinosa all show intense immunoreactivity with antisera to the enkephalin pentapeptides (16–23) which we have confirmed (not illustrated) in the present studies using antisera to enkephalin supplied by R. J. Miller (University of Chicago). However, serial sections of these enkephalin-rich regions showed no immunoreactivity with the anti-β-endorphin antisera. Serial section staining of diencephalic fields allowed direct comparison of fibers that were immunoreactive with the antisera to enkephalins and to β-endorphin. A detailed nucleus-by-nucleus analysis will be required for complete comparisons; however, in most cases the neuropil patterns and morphology of the reactive fibers permitted clear-cut separations. β-Endorphin-reactive fibers could be traced for hundreds of micra within a given section and exhibited thick, round varicosities of 3–5 μm (Figs. 1*C* and 2*A*). Enkephalin-reactive fibers have been reported (16–23) as mainly isolated reactive dots, and we found that these structures were much finer and rarely appeared to show connectedness for more than 100 μm. Finally, limited observations were made with a series of anti-corticotropin antisera provided by Dorothy Krieger (Mt. Sinai Medical School). With an antiserum directed against $ACTH_{1\text{-}39}$, we observed fibers within the hypothalamus that bore close morphological similarities to those reactive with the anti-β-endorphin antisera. These fibers persisted after hypophysectomy (not shown).

Fig. 3 summarizes the distribution of β-endorphin-reactive neurons and fibers within the rat brain.

CONCLUSIONS

The present observations demonstrate that β-endorphin-reactive neurons and nerve fibers exist within rat brain and are anatomically separable from those pituitary cells containing the same peptide and from those central neurons reported by others to contain the enkephalin pentapeptides. These observations support the view (15) that enkephalins and β-endorphins are contained within separate cellular systems in brain. Moreover, these observations suggest that the functional roles of these separate systems may be more diverse than the general term "opioid peptide" has been taken to imply until now.

The cellular pattern and restricted distribution of the β-endorphin-containing cells described here bear some similarities to the luteininzing hormone releasing factor-containing cells (32): both types of cells are clustered in the vicinity of the arcuate nucleus and basal tuberal area, both project through the anterior hypothalamic area into the dorsal midline thalamus, and both innervate the median eminence and both may innervate other circumventricular organs. Nevertheless, the systems are separate because the β-endorphin-containing cells appear to be somewhat more posterior and innervate different thalamic nuclei than those described for luteininzing hormone releasing factor-reactive fibers. The distribution of these immunoreactivity patterns within the ventral hypothalamus suggests that classic cytoarchitectonic definitions of nuclear groupings are inadequate to define the functional and chemical properties of these cell groups and their possible interrelationships (also see ref. 32). The fact that the immunoreactivity of cells does not fall within atlas definitions of nuclear groupings may also pose problems for neurochemical estimates based upon microdissections according to those atlases. The cells described here may well fall into that large class of multiprocess neurons of the basal hypothalamus described in electrophysiologic studies by Renaud (33, 34) as projecting to the medial preoptic

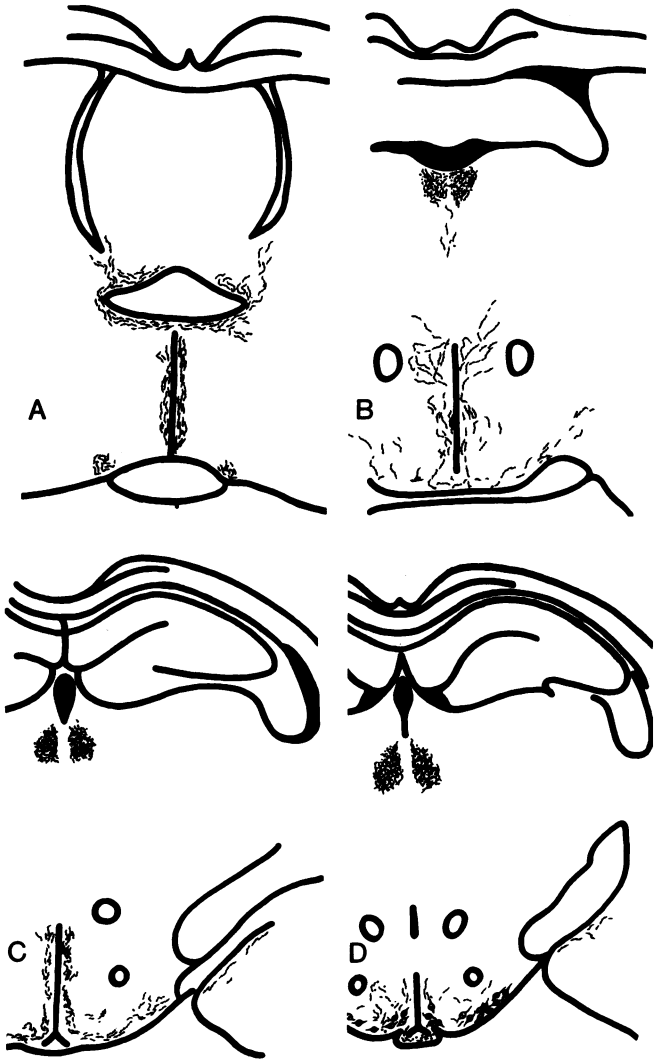

FIG. 3. Schematic representation of β-endorphin-reactive neurons and fibers at four frontal levels based upon the Konig and Klippel atlas (31). Approximate frontal levels are: (*A*) 6790; (*B*) 5780; (*C*) 4110; (*D*) 3750. Note that reactive fibers tend to lie very close to the ventricular surfaces of the diencephalic structures that they innervate.

area, median eminence, medial dorsal thalamus, and midbrain periaqueductal gray. These cells may function to integrate these various brain regions in some as yet undefined way.

Although the antisera used here to define sites that are immunoreactive for β-endorphin also recognize β-LPH (12), we have not been able to detect, in rat brain, any fiber or cell staining with antisera raised by others to porcine or ovine β-LPH (M. du Bois; D. Krieger), although patterns of staining similar to those observed here have been recently described in rat brain with antisera to human β-LPH (35). We have previously reported (12, 15) that the β-endorphin radioimmunoassay detects at least two molecular forms in brain extracts: one—representing about 60–70% of the material—closely coincides with the molecular properties of β-endorphin; the other is a higher molecular weight substance in the range of the proposed β-endorphin precursors β-LPH and the 31k material of Mains *et al.* (24). The most conservative explanation at present is that β-LPH, β-endorphin, and probably corticotropin all may coexist within the cells and fibers of the rat central nervous system as they do in reactive cells of the intermediate lobe and adenohypophysis. If such is the case, it will be im-

portant to determine whether both active peptides are released concomitantly within the central nervous system as has been demonstrated for pituitary secretion patterns (36) and whether corticotropin may modify the cellular (37) and behavioral (11, 38) actions already reported for β-endorphin. Finally, the proposal that β-endorphin- and enkephalin-containing central neurons are anatomically separable systems must now be pursued in terms of specific functional roles and distinctive receptive properties.

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