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# Regional dissociation of β-endorphin and enkephalin contents in rat brain and pituitary

(opiates/radioimmunoassay/neurotransmitter/adrenalectomy and hypophysectomy/myelin basic protein)

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ABSTRACT **β-Endorphin and enkephalin in extracts of whole brain, various brain regions, adenohypophysis, and combined pars intermedia and neurohypophysis of the rat were measured by radioimmunoassay. In brain extracts, the immunoreactive substances were further separated according to molecular size by gel filtration. β-Endorphin was found in the diencephalon but not in the hippocampus, cerebral cortex, cerebellum, and striatum. Enkephalin was found predominantly in the striatum and diencephalon. Attention is called to possible artifactual interference by myelin basic protein in the immunoassays for β-endorphin in some regions of the brain. In the pituitary, enkephalin was mainly restricted to the pars intermedia-neurohypophysis. Neither adrenalectomy nor hypophysectomy significantly altered levels of β-endorphin in brain extracts. Adrenalectomy increased the levels of β-endorphin in adenohypophysis and pars intermedia-neurohypophysis; after adrenalectomy, enkephalin was also increased in the adenohypophysis but less so in the pars intermedia-neurohypophysis. These results show that brain endorphin levels are independent of pituitary endorphin levels; they suggest that β-endorphin-containing neurons and those containing enkephalin constitute two separate groups of brain cells.**

Peptides with opiate-like properties have been isolated from brain and pituitary, and their sequences and structures have been confirmed by complete synthesis (1–10). The naturally occurring enkephalins and endorphins share common $NH_2$-terminal sequences with COOH-terminal fragments of β-lipotropin (β-LPH). The various bioassays or receptor-binding assays available (1–6, 11, 12) recognize the various opiate-like peptides as a class, although with different potencies; none of these methods permits the quantitative assessment specifically of any one of the opiate-like peptides. Thus, the nonspecific approaches have left unanswered such questions as whether Met$^5$-enkephalin could be no more than a breakdown product of β-endorphin.

We have developed specific antisera that can distinguish the enkephalin pentapeptides from α- and β-endorphin (13). Using these antisera, we found that significant amounts of β-endorphin exist in brain and that the regional content of β-endorphin bears no fixed relationship to the enkephalin content of the same brain regions. We conclude that, in brain and in pituitary, β-endorphin and enkephalin may be stored within different cells and thus likely are independent entities physiologically.

## METHODS

**Preparation of Extracts for Radioimmunoassays.** Rats (Sprague–Dawley, male, 150–200 g) were killed by decapitation. Pituitary, pineal, and various regions of the brain were dissected rapidly, frozen on dry ice, weighed, and placed in 1 M acetic acid preheated to 95° (2 ml for pituitary, pineal, brain regions; 16 ml for whole brain). After 15 min in the hot bath, samples were chilled in ice and homogenized (Polytron setting 6, 10 sec) and centrifuged (1000 × *g*, 1 hr). The supernatant was frozen overnight, neutralized to pH 7.5 with 1 M NaOH supplemented with 0.2 M $Na_2HPO_4$, and refrozen overnight. Centrifugation (1000 × *g*, 1 hr) after thawing yielded a clear supernatant that was then used in the radioimmunoassays.

**Radioimmunoassays.** The radioimmunoassay for β-endorphin was used as described (13). A double-antibody radioimmunoassay for Leu$^5$- enkephalin was performed as described here.

A conjugate was prepared by coupling Leu$^5$-enkephalin to bovine serum albumin with bis-diazotized benzidine. Rabbits were immunized with 2 mg of this conjugate plus 5 mg of dried killed *Mycobacterium tuberculosis* emulsified with complete Freund's adjuvant, by intradermal injections at multiple sites on the back. At 30-day intervals, booster injections with 0.5 mg of conjugate emulsified in incomplete Freund's adjuvant were given. After the second boost, one rabbit of four showed significant immunoreactivity (RB 92-12/76).

Leu$^5$-enkephalin was labeled with $^{125}I$ by the chloramine-T method, the reaction being stopped with Na metabisulfite. The labeled peptide was purified by chromatography on Sephadex G-25.

Standard doses of peptide or unknown samples were incubated with the antiserum at a final dilution of 1:4900 and with the trace (10,000 cpm of $^{125}I$-labeled Leu$^5$-enkephalin) for 25 hr at 4° in a final volume of 0.7 ml of 0.02 M Na phosphate buffer (pH 7.5) containing 145 mM NaCl and 0.1% gelatin. Goat anti-rabbit gamma globulin was used to precipitate antibody-bound trace.

## RESULTS AND DISCUSSION

**Immunospecificity of the Radioimmunoassay for Enkephalin.** As illustrated in Fig. 1, 16 pg of Leu$^5$-enkephalin produced 50% displacement of the bound trace; neither α-endorphin nor β-endorphin showed any crossreactivity. However, parallel displacement of the Leu$^5$-enkephalin trace was obtained with Met$^5$-enkephalin at molar levels 30-fold higher (crossreactivity, 3.3%).

In view of this crossreactivity, results of radioimmunoassays with this antiserum could be expressed in terms of either pentapeptide. However, because the ratio of Leu$^5$-enkephalin to

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Abbreviations: β-LPH, β-lipotropin; U-Enk, unit of enkephalin; $M_r$, molecular weight; ACTH, corticotropin.

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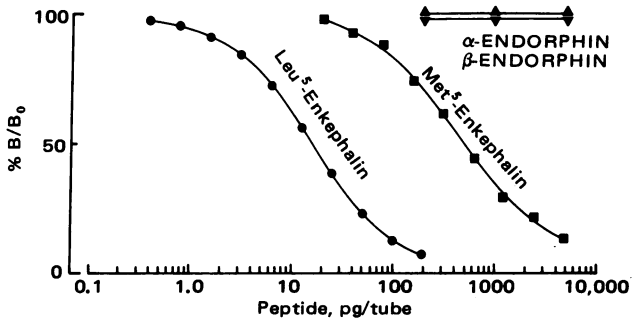


FIG. 1. Leu[5]-enkephalin radioimmunoassay. Crossreactivity with Met[5]-enkephalin. Synthetic α-endorphin and β-endorphin do not show any crossreactivity at doses up to 1 ng per tube.

Met[5]-enkephalin may vary from one brain region to another, as it reportedly does from species to species (1, 12, 14), there is at present no certain way of deducing, from the results obtained with such a radioimmunoassay, the exact amount of each pentapeptide. Therefore, in order to avoid misleading expressions of pentapeptide concentration, we have chosen to express the results of this radioimmunoassay in terms of arbitrary enkephalin units, from which extrapolated molar values of either enkephalin could be calculated by assuming that all reactive material is either Met[5]-enkephalin or Leu[5]-enkephalin.

If rat brain contains no Met[5]-enkephalin, 1 unit of enkephalin (U-Enk) would represent 1 ng of Leu[5]-enkephalin (or 1 mU-Enk would represent 1 pg of Leu[5]-enkephalin). If rat brain contains no Leu[5]-enkephalin but only Met[5]-enkephalin, 1 U-Enk would represent 30 ng of Met[5]-enkephalin.

**Characterization of β-Endorphin-Like Immunoreactive Substances.** As previously reported (13), the radioimmunoassay system used for measurement of β-endorphin (Fig. 2) is specific for the Leu[14]–His[27] segment of the molecule. Because β-endorphin is the COOH-terminal 31 amino acid fragment of β-LPH, this antiserum also binds on an equimolar ratio β-endorphin, β-LPH, and the 31,000 molecular weight ($M_r$) prohormone (13, 15). Therefore, before proceeding with detailed analysis of the β-endorphin-like immunoreactive substances present in brain and pituitary extracts, attempts were made to separate the immunoreactive components by gel filtration. Extracts of whole rat brains were passed through a Bio-Gel P-60 column equilibrated and eluted with 4 M guanidine. Consistently, two peaks of immunoreactivity (β-endorphin radioimmunoassay) were resolved (Fig. 3). One peak coincided precisely with the location of $^{125}$I-labeled synthetic β-endorphin;

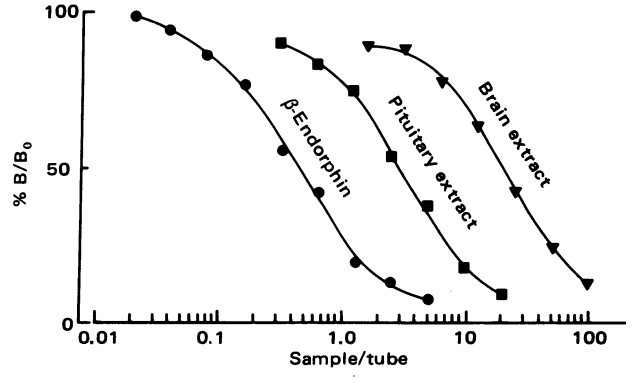


FIG. 2. β-Endorphin radioimmunoassay. Dose-response curves for synthetic porcine β-endorphin (●, mg peptide/tube), pituitary extract (■, μg wet tissue/tube), and whole brain extract (▲, mg × $10^{-1}$ wet tissue/tube).

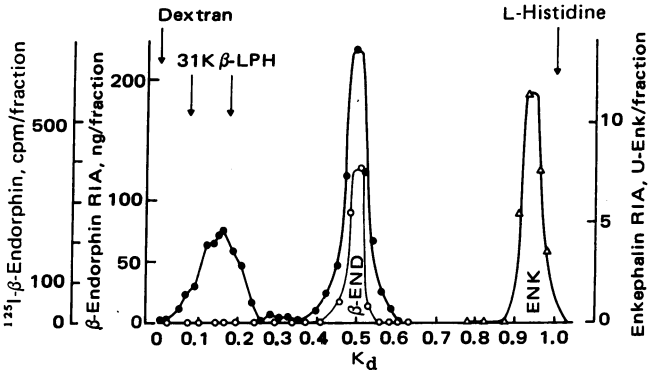


FIG. 3. Gel filtration of rat brain extract. Two rat brains were boiled and homogenized in 32 ml of 1 M acetic acid as described in *Methods*. The supernatant (10,000 × *g*, 30 min) was lyophilized. Two hundred microliters of 4 M guanidine HCl containing 0.02% crystalline bovine serum albumin was added to the dry residue. After boiling for 5 min, the supernatant was applied to the column (45 × 0.7 cm, Bio-Gel P-60) with blue dextran and L-histidine. The column was eluted with 4 M guanidine; 0.3-ml fractions were collected. $K_d = (V_e - V_0)/(V_f - V_0)$ in which $V_e$ is the elution volume of the fraction, $V_0$ is the void volume (blue dextran peak: 5 ml), and $V_f$ is the elution volume of the smallest molecule (L-histidine peak: 17 ml). β-Endorphin and enkephalin immunoreactivity were assayed by radioimmunoassay (RIA). In another run, $^{125}$I-labeled β-endorphin was applied to the column and the radioactivity was monitored. The elution peaks of $M_r$ 31,000 prohormone (31 K) and of β-LPH are indicated.

the other peak (amounting to 37% of the total β-endorphin-like immunoreactive substance) was eluted in a broad zone of larger $M_r$ (10,000–30,000) which did not coincide closely with the elution pattern of either β-LPH or the $M_r$ 31,000 prohormone. Moreover, the β-endorphin-like immunoreactive substance corresponding to labeled β-endorphin (63% of the total immunoreactive material) was clearly separable from the enkephalin peak detected by the radioimmunoassay for enkephalin.

When similar gel filtration was performed with extracts of specific brain regions (Table 1), striatum and cerebral, cerebellar, and hippocampal cortices were shown to contain only high $M_r$ substances. In hypothalamus, septum, pons, medulla, and mesencephalon the fraction of total β-endorphin-like immunoreactive substances attributable to the larger $M_r$ substances was considerably lower. In areas other than the hypothalamus, the major immunoreactive component was of high $M_r$. Values obtained with β-endorphin radioimmunoassays in extracts of striatal and cortical regions may be due to an as yet uncharacterized crossreacting larger molecule, which may or may not be either β-LPH (19) or the $M_r$ 31,000 common precursor of β-LPH and corticotropin (ACTH) (15). Indeed, we have recently found by immunocytochemistry that the β-endorphin antiserum (RB100-10/76) used here stains myelinated fibers, especially in cortical regions (cerebral, hippocampal, and cerebellar cortex). By radioimmunoassay, the degree of crossreactivity with purified myelin basic protein ($M_r$ 18,500) was determined to be 0.001% of β-endorphin on a molar basis. Large quantities of myelin basic protein are expected to be present in the brain extracts assayed here for β-endorphin; myelin basic protein is therefore another possible candidate for the crossreacting larger molecules separated by gel filtration.

**Regional Separation of β-Endorphin and Enkephalin.** When extracts of all brain regions are corrected for radioimmunoassay values due to the uncharacterized large $M_r$ material, significant amounts of β-endorphin were found in extracts of whole brain, hypothalamus, septum, midbrain and pons/

Table 1. Distribution of immunoassayable opioid peptides in brain and pituitary gland

| | β-Endorphin, ng/mg tissue | | Enkephalin, mU-Enk/mg tissue | |
|---|---|---|---|---|
| Pituitary | | | | |
| Whole | 269 ± 20 | (11) | 72 ± 4 | (6) |
| Adenohypophysis | 128 ± 9 | ( 3) | 3.7 ± 0.7 | (3) |
| Neurohypophysis and pars intermedia | 1500 ± 600 | ( 3) | 740 ± 47 | (3) |
| Pineal | 4.8 ± 0.8 | (10) | 19 ± 2 | (7) |
| | ng/g tissue | | U-Enk/g tissue | |
| Brain | | | | |
| Whole | 108 ± 8 | (10) | 25 ± 2 | (6) |
| Hypothalamus | 490 ± 30 | ( 5) | 120 ± 7 | (6) |
| Septum | 234 ± 34 | ( 3) | 85 ± 7 | (6) |
| Midbrain | 207 ± 15 | ( 5) | 32 ± 1 | (6) |
| Medulla and pons | 179 ± 5 | ( 5) | 30 ± 4 | (6) |
| Striatum | None | ( 5) | 112 ± 11 | (6) |
| Hippocampus | None | ( 5) | 13 ± 1 | (6) |
| Cortex | None | ( 5) | 15 ± 2 | (6) |
| Cerebellum | None | ( 5) | 5 ± 1 | (6) |

Rat brains were dissected as outlined by Glowinski and Iversen (22). Data are means ± SEM; numbers of animals are shown in parentheses. One unit of immunoreactive enkephalin corresponds to 1 ng of $Leu^5$-enkephalin or 30 ng of $Met^5$-enkephalin.

Table 3. Effect of hypophysectomy and adrenalectomy on brain and pituitary content of immunoassayable opioid peptides

| | Control | Adrenalectomy | Hypophysectomy |
|---|---|---|---|
| Whole brain | | | |
| β-Endorphin, ng/g | 129 ± 18 (10) | 107 ± 15 (7) | 96 ± 5 (7) |
| Adenohypophysis | | | |
| β-Endorphin, μg/tissue | 1.1 ± 0.2 (3) | 5.4 ± 0.7* (3) | |
| Enkephalin, U-Enk/tissue | 0.031 ± 0.006 (3) | 0.177 ± 0.070† (3) | |
| Neurohypophysis and pars intermedia | | | |
| β-Endorphin, μg/tissue | 2.2 ± 0.7 (3) | 5.4 ± 0.8† (3) | |
| Enkephalin, U-Enk/tissue | 1.11 ± 0.07 (3) | 1.41 ± 0.023 (3) | |

Adrenalectomy and hypophysectomy were performed 2 months before sacrifice; similar results were obtained 9 months after hypophysectomy. Data shown as means ± SEM; numbers of animals in parentheses. *, $P < 0.01$; †, $P < 0.05$; statistical analysis by analysis of variance and Duncan's multiple comparison test. Other information as in legend of Table 1.

medulla (Table 1). However, no material attributable to the specific β-endorphin component could be found in extracts of neostriatum (caudate/globus pallidus/putamen) or of the cerebral, cerebellar, or hippocampal cortex. These latter regions all contain significant amounts of enkephalin according to others (12, 14, 16, 17) and as confirmed here by our own radioimmunoassay for enkephalin. When the same regions were assayed for β-endorphin and enkephalin, there was a clear-cut independent variation, from region to region, of the two classes of opioid peptides. Furthermore, when the diencephalon was dissected in accordance with the distribution of immunocytochemically detected β-endorphin neurons and fibers, the ratio between β-endorphin and enkephalin values was found to vary from 1.6 in hypothalamus to 9.1 in periaqueductal thalamus (Table 2). In addition, globus pallidus and caudate nucleus, which contain large numbers of immunocytochemically detected enkephalin fibers, contained virtually no β-endorphin. Thus, these data strongly suggest that β-endorphin and enkephalin are found in the brain within different neuronal systems.

**Relationships between Endorphin and Enkephalin Immunoassayable Material in Pituitary and Brain.** As already reported for rat pituitary (18) and as seen recently in mouse, kitten, pig, and frog pituitary, immunocytochemical and radioimmunoassay studies indicate that α- and β-endorphin are found in every cell of the intermediate lobe and in discrete cells—corresponding to those reactive to antisera against ACTH—in the adenohypophysis; neither α- nor β-endorphin is present in neurohypophysis (ref. 18; unpublished data). With the radioimmunoassay for enkephalin, immunoreactive material was primarily found in the intermediate lobe-neurohypophysis and was almost absent from the adenohypophysis (Table 1).

To pursue further the relationships between β-endorphin and enkephalin in brain and pituitary, radioimmunoassays for each were performed on tissues from rats after long-term hypophysectomy and adrenalectomy. In brain, neither treatment altered significantly the amount of β-endorphin immunoreactive substance attributable to authentic β-endorphin (Table 3). In the adenohypophysis, there was a significant increase of both β-endorphin and enkephalin immunoassayable materials

Table 2. Distribution of immunoassayable opioid peptides in the diencephalon

| | Tissue weight, mg | β-Endorphin, ng/g | Enkephalin, U-Enk/g | Ratio: β-endorphin/enkephalin |
|---|---|---|---|---|
| Thalamus | 55 | 329 ± 19 | 36 ± 5 | 9.1 |
| Dorsal preoptic | 35 | 742 ± 156 | 140 ± 22 | 5.2 |
| Ventral preoptic | 57 | 987 ± 127 | 260 ± 31 | 3.8 |
| Hypothalamus | 31 | 217 ± 32 | 134 ± 17 | 1.7 |

Means (±SEM) for 12 rat brains dissected as follows. With the brain placed on the dorsal surface, two parasagittal slices were made at the lateral borders of the hypothalamic recess and extended from anterior to posterior. The resultant midsagittal slice (4–5 mm thick) was laid on its sagittal surface, and dissecting cuts were made vertically anterior and posterior to the septum and posterior to the mammillary bodies. Horizontal cuts were made at the level of the anterior commissure (yielding an anterior or ventral preoptic segment and a posterior or hypothalamic segment) and at the level of a line between the dorsal aspect of the septum and the aqueduct [yielding anterior the septal nuclei (or dorsal preoptic) and thalamic segments].

after adrenalectomy; in the intermediate lobe-neurohypophysis, there was a greater increase in the content of immunoassayable β-endorphin than of enkephalin. These data indicate that the pituitary contents of β-endorphin and enkephalin are regulated separately from the content of the brain and that, within pituitary or brain, enkephalin content need not be viewed exclusively as an epiphenomenon of β-endorphin breakdown.

## CONCLUSIONS

The present experiments have demonstrated that the contents of β-endorphin and enkephalin vary independently from one brain region to another, that brain β-endorphin content does not depend upon the integrity of the pituitary, and that hormonal manipulation can alter the pituitary content of β-endorphin without necessarily modifying the content of enkephalin. All of these observations support the view that β-endorphin and enkephalin are contained within separate cellular systems in brain and neurohypophysis. As a result, the proposal that enkephalin might represent exclusively an artifact of β-endorphin breakdown is difficult to maintain.

With the β-endorphin radioimmunoassay used here, the brain content of immunoreactive material appears to be of at least two molecular forms. One form, which coincides closely in $M_r$ with that of synthetic β-endorphin, constitutes the majority of the material present in extracts of whole brain and almost all of the material present in extracts of brain regions most rich in cell bodies and nerve fibers identified by immunocytochemistry with the antiserum used in the radioimmunoassay (unpublished data). A second form of immunoreactive β-endorphin-like material is present in cortical brain regions; this material has a $M_r$ in the range 10,000–30,000 which would include the proposed precursors of β-endorphin, β-LPH, and the $M_r$ 31,000 precursor (15). Indeed, β-LPH has been reported to be present in bovine brain (19, 20) in striatal and cortical regions (19) which, in the rat, show exclusively the $M_r$ 10,000–30,000 components. However, as mentioned above, the larger material found in striatal and cortical regions could also be myelin basic protein (unpublished data). Results of the radioimmunoassays must therefore be interpreted with caution; it is pertinent to add that highly purified myelin basic protein (ovine or porcine origin) has no opiate-like activity in the myenteric plexus/longitudinal muscle of the guinea pig's ileum.

Finally, the data offered here speak more broadly to the issue of the so-called opiate-receptors, the characterization of which began the effort that led to the isolation of the enkephalins and endorphins. If β-endorphin- and enkephalin-containing cell systems are anatomically separable, as well as biochemically distinct, the logical expectation might be that postsynaptic receptors for the two systems would also show possible pharmacological separation. In this regard it will be important to probe areas rich in one opioid peptide and poor in another for discriminable patterns of cellular responsivity (21) to the gamut of synthetic morphinomimetic peptides now available.

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