Characterization of the Prodynorphin and Proenkephalin Neuropeptide Systems in Rat Hippocampus¹

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Abstract

Opioid peptides derived from prodynorphin were localized immunocytochemically to dentate granule cells and mossy fibers of the rat hippocampus with antisera against dynorphin A(1-17) and dynorphin B. Extracts of microdissected hippocampal regions were resolved by reverse phase and molecular exclusion chromatography to identify the molecular forms of the dynorphin A immunoreactivity and to quantify regional contents. Results demonstrated that the relative concentration of dynorphin A within each dissected region of hippocampus agreed well with the distribution of dynorphin A detected by immunocytochemical methods. Immunostaining of proenkephalin-derived opioid peptides, [Leu⁵]enkephalin and bovine adrenal medullary peptide-22P, was concentrated in cell bodies of the entorhinal cortex, nerve fibers in the perforant pathway, and terminals in the outer molecular layer of the dentate gyrus. Light immunostaining of granule cells and mossy fibers with these antisera was also found. The relative concentration of [Leu⁵]enkephalin immunoreactivity in each microdissected region of the hippocampus also agreed well with the distribution of [Leu⁵]enkephalin immunostaining. Chromatography of hippocampal regional extracts demonstrated that the immunoreactivity measured was due to the presence of authentic [Leu⁵]enkephalin. The probable neurotransmitter function of both [Leu⁵]enkephalin and dynorphin A was shown by their calcium-dependent release after *in vitro* depolarization of hippocampal tissue. The reported presence of β -endorphin in hippocampus was not verified. Comparison of the hippocampal distribution and content of prodynorphin and proenkephalin-derived opioids suggests that separate populations of neurons containing

these two peptide families form distinct neurotransmitter systems of roughly equal concentration.

Currently, three families of endogenous opioid peptides are known, each derived from a separate precursor protein and encoded by separate genes (for review, see Cox, 1982; Bloom, 1983). β -Endorphin is derived from pro-opiomelanocortin that also is a precursor for the non-opioid peptides adrenocorticotrophic hormone and melanotropin-stimulating hormone (Nakanishi et al., 1979). The proenkephalin family consists of the opioids [Met⁵]enkephalin, [Leu⁵]enkephalin, and other carboxy-terminally extended enkephalin forms (Comb et al., 1982; Gubler et al., 1982; Noda et al., 1982). The prodynorphin family (Kakidani et al., 1982) includes at least five opioids: dynorphin A(1-17), dynorphin A(1-8), dynorphin B, α -neo-endorphin, and β -neo-endorphin.

Interest in the role of these opioids in the hippocampus was spurred by their unique excitatory actions there (Nicoll et al., 1977). Subsequent studies identified the cellular mechanisms for the opioid action and suggested that an endogenous opioid system may normally act on hippocampal circuits to control cellular function (Martinez et al., 1979; Zieglansberger et al., 1979; Dunwiddie et al., 1980; Lee et al., 1980). Initial immunocytochemical reports of opioid peptide distribution within the hippocampus (Elde et al., 1976; Hokfelt et al., 1977; Simantov et al., 1977; Watson et al., 1977; Sar et al., 1978) produced conflicting results. However, later studies of the distribution of [Met⁵]enkephalin immunoreactivity in hippocampal regions by radioimmunoassay (RIA) (Hong et al., 1980; Hong and Schmid, 1981; Hoffman et al., 1983) and by immunocytochemistry (Gall et al., 1981) provided a clearer image of enkephalin immunoreactivity distribution. Two major hippocampal projection systems were observed (Gall et al., 1981): the intrinsic dentate granule cell-mossy fiber pathway that innervates CA3/CA4 pyramidal cells (Blackstad and Kjaerheim, 1961) and the extrinsic lateral perforant/temperoammonic pathway which originates in entorhinal cortex and innervates both granule and pyramidal cells (Steward and Scoville, 1976). In addition, scattered, presumably local, circuit neurons throughout hippocampus demonstrated enkephalin immunoreactivity. Recently, the presence of prodynorphin-derived opioids has also been detected in hippocampus (Goldstein and Ghazarossian, 1980; Minamino et al., 1981; Weber et al., 1982; Weber and Barchas, 1983). McGinty et al. (1983, 1984) demonstrated that granule cells and mossy fibers stain much more intensely with dynorphin A antiserum than with antisera directed against proenkephalin-derived peptides. In contrast, hippocampal interneurons and cells and fibers of the perforant pathway stain only with antisera directed against proenkephalin-derived peptides (McGinty et al., 1983, 1984). The possible presence of β -endorphin immunoreactivity in hippocampus is still controversial. Although none was reported in initial studies (Rossier et al., 1977; Bloom et al., 1978), hippocampal β -endorphin immunoreactivity was subsequently described with immunocyto-

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chemical (Zakarian and Smyth, 1979) and chromatographic methods (Kreiger et al., 1977; Zakarian and Smyth, 1982).

The defined anatomical circuits of the rat hippocampus provide an ideal preparation to compare and contrast the opioid peptide systems present there. Toward our ultimate goal of determining whether these peptides have neurotransmitter functions in this CNS structure, we have now (a) used immunocytochemistry to further define their distribution in the regions of the hippocampus, (b) identified the molecular forms and quantified the relative contents of the immunoreactive species, and (c) begun to characterize the molecular forms that are released upon stimulation of neural circuits.

Materials and Methods

Immunocytochemistry. Colchicine (100 μ g in 10 μ l of saline) was infused into the lateral ventricles of male CD albino rats (200 to 300 gm) of the Sprague-Dawley strain (Charles River Breeding Laboratories, Wilmington, MA) 48 hr before perfusion. Colchicine-treated and naive rats were perfused with 5% paraformaldehyde in 0.2 M phosphate buffer and postfixed for 2 to 3 hr as described previously (McGinty et al., 1983, 1984). The brains were stored in 15% sucrose until they were frozen in dry ice and cut on a sledge microtome. Free-floating sections (50 μ m) were preincubated with 1% H₂O₂ for 5 min to eliminate endogenous peroxidase, rinsed, and then incubated with rabbit antiserum raised against dynorphin A (84C, donated by L. Terenius, Uppsala, Sweden) diluted 1:1000, dynorphin B antiserum (R2-4, donated by E. Weber, Stanford University, Stanford, CA) diluted 1:1000, or bovine medullary peptide (BAM-22P) antiserum (B6, donated by A. Baird, The Salk Institute, San Diego, CA) diluted 1:2000. The characteristics of each antiserum have been described (Miller et al., 1978; Bloch et al., 1983; McGinty et al., 1983, 1984; Weber and Barchas, 1983). Control sections were incubated with each antiserum adsorbed for 24 hr at 4°C with 1 to 100 μ M of the peptide against which the antiserum was raised or related opioid peptides. The secondary immunoreagents were goat anti-rabbit IgG-biotin followed by an avidin-biotin-horseradish peroxidase (HRP) complex (Vectastain, Vector Laboratories, Burlingame, CA). The HRP staining procedure and diluents have been described (McGinty et al., 1983).

RIAs. [¹²⁵I]Enkephalin and dynorphin A RIAs were performed on tissue extracts and column fractions using specific antisera previously described (Rossier et al., 1977; Ghazarossian et al., 1980). Peptides were iodinated as described previously (Ghazarossian et al., 1980) and then purified by reverse phase C18 high pressure liquid chromatography (C18-HPLC). Rabbit antisera Lucia (provided by A. Goldstein, Stanford University) is directed to the dynorphin A(2-11) segment and recognizes both amino- and carboxy-terminally extended forms of the peptide (Ghazarossian et al., 1980). Characterization of the dynorphin A and [¹²⁵I]enkephalin antisera specificity is summarized in Table I. β -Endorphin RIA was performed as described previously

(Bayon et al., 1979a). Peptides were synthesized and purified by N. Ling (Salk Institute).

Preparation of tissue extracts. Hippocampal extracts were prepared from tissue freshly dissected from male CD albino rats of the Sprague-Dawley strain (180 to 220 gm). Hippocampi were suspended in 10 vol of hot (90°C) 1 M acetic acid, heated in a boiling water bath for 30 min, homogenized for 10 sec (Polytron, 12-mm-diameter probe, setting 5), and then centrifuged for 60 min at 20,000 $\times$ g (4°C). The pellets remaining after final centrifugation were resuspended in 1 N NaOH and assayed for protein content by the method of Lowry et al. (1951). Total hippocampal tissue protein was found to be twice the protein content in the acetic acid-insoluble fraction; thus, pellet values were multiplied to obtain tissue protein content. Acid-soluble tissue extracts were lyophilized and then resuspended in a minimal volume of methanol:0.1 M HCl (1:1, v/v). To microdissect hippocampal fragments, the hippocampus was removed from each hemisphere and placed on an ice-cold stainless steel plate in such a way that it could be cut perpendicular to its longitudinal axis. Using gross anatomical markers under a dissecting microscope, razor-blade cuts were made as shown in Figure 1A to produce tissue pieces each containing chiefly one of the following regions: dentate/CA4; CA2/CA3; CA1; subiculum. Representative pieces taken by this procedure were fixed and stained to verify the accuracy of the dissection (Fig. 1B). For entorhinal cortex pieces, cuts were made caudal to the amygdaloid fissure and ventral to the rhinal sulcus. Entorhinal cortex was included because immunocytochemical evidence suggested that cells containing enkephalin immunoreactivity project from that region to the hippocampal formation. Typically, tissue pieces from 10 to 20 brains were pooled for extraction as described above.

For release experiments, hippocampal tissue was rapidly dissected and then sliced in two perpendicular planes at 250 μ m thickness using a Mclwain tissue chopper. The resulting slices were washed three times with isotonic bicarbonate solution containing: 127 mM NaCl, 3.85 mM KCl, 1.8 mM CaCl₂, 1.8 mM KH₂PO₄, 1.8 mM MgSO₄, 20 mM NaHCO₃, 11 mM D-glucose, 1 mg/ml of bovine serum albumin (Miles Laboratories, Elkhart, IN; crystalline), and bubbled with carbon dioxide/oxygen (5/95, v/v) for at least 30 min (pH 7.4). In addition, the buffer contained the peptidase inhibitors thiophan (0.3 μ M; Schering Corp., Kenilworth, NJ and B. Roques, University of Rene Descartes), bestatin (20 μ M; Sigma Chemical Co., St. Louis, MO), captopril (10 μ M; E. R. Squibb & Sons, Inc., Princeton, NJ) and polylysine (20 μ M; Sigma) used to protect the released opioids from proteolysis (Chavkin et al., 1983b). For potassium stimulation, the bicarbonate buffer ion concentrations were adjusted to 50 mM KCl and 87 mM NaCl. During experiments in the absence of calcium ions, CaCl₂ was replaced by 1 mM CoCl₂ in both the normal and 50 mM KCl bicarbonate solutions.

Slices were placed in a 1-inch diameter superfusion chamber having a 0.45- μ m filter (Gelman Sciences, Inc., Ann Arbor, MI); chamber volume was 0.3 ml. Each chamber contained the tissue equivalent of six hippocampi (200 to 300 mg of tissue/chamber) taken from a pool of hippocampal slices. Slices were superfused with isotonic bicarbonate buffer at 0.4 ml/min at 24°C. After an initial wash period of 10 min, superfusate samples were collected at 2-min intervals, immediately boiled for 15 min, then frozen and lyophilized. After 30 min of superfusion with normal bicarbonate, the superfusion buffer was changed to 50 mM KCl, Krebs-bicarbonate for 10 min; later the slices were again superfused with normal bicarbonate. To correct for the high salt concentration in the lyophilized superfusate samples, 0.8-ml aliquots of each superfusion buffer were boiled and lyophilized as above, and then included in the RIA as blanks that were subtracted from the release fraction values. Appropriate peptides were also diluted in buffer containing ion concentrations equivalent to the release fractions and included in the RIAs as standards.

Chromatographic methods. To resolve hippocampal extracts by molecular size, Protein Analysis Columns (PACs, Waters, Inc., Bedford, MA) were used as described by Rivier (1980). Two I-125 PACs connected in series were run using triethylamine phosphate (TEAP)/acetonitrile (2:1, v/v) at 1 ml/min at 24°C. TEAP buffer was made by bringing the pH of 0.25 M phosphoric acid to 2.25 with redistilled triethylamine. Tissue extracts were clarified by centrifugation (5 min, 9000 $\times$ g in a Brinkmann microfuge) prior to injection. The volume of injection (100 μ l) and the concentrations of protein in the sample were adjusted to be similar for each region. Fractions were collected at 0.4-min intervals, frozen, and assayed as described. Absorbance at 254 nm was measured. The HPLC column system was calibrated by determining the elution position of molecular weight standards prior to resolution of extracts. Buffer blank injections were run immediately before extracts and assayed in RIAs as described to ensure the lack of peptide contamination in the HPLC system.

Hippocampal extracts were also resolved by HPLC on a reverse phase

TABLE I
Percentage of molar cross-reactivities

Dilutions of the opioid peptides were assayed in the two RIAs as described. Percentages of molar cross-reactivities were determined by comparing the concentrations able to displace 50% of the radiolabeled tracer binding. For peptides unable to displace half of the binding, the maximum concentration tested is listed. The antisera for the RIAs are directed predominantly against the nominal antigens, although there is some degree of cross-reactivity toward similar molecular species, as shown below. For example, it is evident from this table that the [¹²⁵I]enkephalin RIA will also detect [¹²⁵I]enkephalin present in hippocampal extracts. Similarly, the dynorphin A RIA will detect both COOH- and NH₂-terminally extended forms of that molecule (Ghazarossian et al., 1980).

	Dynorphin A RIA	[¹²⁵ I]Enkephalin RIA
Dynorphin A(1-17)	100	0.19
[¹²⁵ I]Enkephalin	<1.0 $\times$ 10 ^{-6a}	100
[¹²⁵ I]Met-enkephalin	<1.0 $\times$ 10 ^{-3a}	3.0
Dynorphin B	6.7 $\times$ 10 ⁻⁴	0.22
Dynorphin A(1-8)	0.25 ^a	0.19
α -Neo-endorphin	1.6 $\times$ 10 ⁻⁴	0.08
β -Endorphin (human)	<1.0 $\times$ 10 ^{-4a}	<1.0 $\times$ 10 ⁻³
β -Endorphin (porcine)	<1.0 $\times$ 10 ⁻³	<1.0 $\times$ 10 ⁻³

^a Values taken from Ghazarossian et al. (1980).

A **B**

Figure 1. A, Horizontal section of the dorsal hippocampus illustrating the orientation of the tissue for the knife cut placement. The black lines indicate the planes of knife cuts. B, Histological verification of the regional dissection using representative pieces taken during dissection of hippocampus as described under "Materials and Methods." Sections (20 μ m) of formaldehyde-fixed tissue were stained with cresyl violet.

Figure 2. Immunocytochemical localization of prodynorphin- and proenkephalin-derived peptides in the hippocampal formation of colchicine ($100 \mu\text{g}$ in $10 \mu\text{l}$)-pretreated rats. **A to E**, Tissue stained with anti-dynorphin B. **A** shows dynorphin B-immunoreactive mossy fibers and cells in the ventral dentate gyrus. Magnification $\times 131$. **Inset**, Dynorphin B-immunoreactive dentate granule cell. Magnification $\times 512$. **B to D** show dynorphin B-immunoreactive multipolar cells in the inner dentate molecular layer. **D** contains two overlapping immunoreactive cells. **E** shows a dynorphin B-immunoreactive varicose fiber in CA1 stratum lacunosum. Magnification: **B**, $\times 578$; **C**, $\times 336$; **D**, $\times 660$; **E**, $\times 938$. **F to K**, Tissue stained with anti-BAM-22P. **F**, BAM-22P-immunoreactive mossy fibers and cells in the ventral dentate gyrus. Magnification $\times 70$. **Inset**, BAM-22P-immunoreactive dentate granule cells. Magnification $\times 304$. **G**, BAM-22P-immunoreactive cell at the base of the granular layer. Magnification $\times 563$. **H**, BAM-22P-immunoreactive hilar cells shown boxed in **F**. **I** and **J**, BAM-22P-immunoreactive cells in stratum lacunosum moleculare of CA1. Magnification: **I**, $\times 308$; **J**, $\times 311$. **K**, BAM-22P-immunoreactive cells in layer II of entorhinal cortex. Magnification $\times 435$.

$\mu\text{Bondapak C18}$ (Waters, Inc.) $3.9 \times 300 \text{ mm}$ column. Extracts were clarified by centrifugation; then, 50 to $100 \mu\text{l}$ (0.6 to 1.2 mg of protein) were injected. Material was eluted at a flow rate of 1.5 ml/min by a nonlinear gradient of acetonitrile (Burdick and Jackson Laboratories, Inc., Muskegon, MI) in 5 mM trifluoroacetic acid. Absorbance was monitored at 254 nm . Eluate was collected at 1 min/fraction and lyophilized. Lyophilized column fractions were redissolved in 1% Triton X-100, 0.1 M HCl, and then assayed at several dilutions to bring the immunoreactivity content within the linear portion of the appropriate standard curves.

Whole hippocampal extracts and other brain regions were also resolved on controlled pore glass (G 200 glycoPhase, Pierce Chemical Co., Rockford, IL) columns and eluted with 2 N acetic acid, 0.01% Triton X-100 (Bayon et al., 1979b). Fractions from this chromatographic system, which separates according to molecular size, were assayed using the RIA for β -endorphin immunoreactivity as previously described (Bayon et al., 1979a).

Results

Cells and fibers of the hippocampus from colchicine-treated rats were stained with anti-dynorphin B (Fig. 2, **A to E**), with anti-BAM-22P (Fig. 2, **F to K**), and with anti-dynorphin A (not shown). The rabbit antiserum raised against BAM-22P, an intermediate precursor derived from proenkephalin (Mizumo et al., 1980), had negligible cross-reactivity to $[\text{Met}^5]\text{enkephalin}$, $[\text{Leu}^5]\text{enkephalin}$, dynorphin A (1-17), or dynorphin B; BAM-22P immunostaining was blocked by preabsorption with BAM-22P but not with the other opioids. Neither dynorphin antiserum cross-reacted with the other dynorphin peptides or with $[\text{Leu}^5]\text{enkephalin}$; immunostaining was blocked by preabsorption with the immunizing opioid but not with other opioids.

TABLE II
Regional concentration of immunoreactivities^a

Region	Dynorphin A Immunoreactivity	[Leu ⁵]Enkephalin Immunoreactivity	Total Protein
	fmol/mg of protein		mg/region
CA2/CA3	328 ± 32	435 ± 91	27
Dentate/CA4	385 ± 115	609 ± 204	46
CA1	30 ± 1.4	143 ± 37	27
Subiculum	31 ± 2.8	138 ± 20	44
Entorhinal	45 ± 5.3	570 ± 63	20

^a Data are means and SE of four independent determinations. Brain regions were dissected as described in the legend to Figure 1.

Dynorphin A immunoreactivity was concentrated in the mossy fiber projection from the dentate granule cells to the apical dendrites of the CA3/CA4 pyramidal cells in the hippocampus as previously reported (McGinty et al., 1983). Dynorphin B immunoreactivity, like dynorphin A immunoreactivity, was found to be prominent in dentate granule cells and their axons, the mossy fibers (Fig. 2A), as well as in multipolar neurons scattered in the dentate granular and molecular layers (Fig. 2, B to D). No cells containing dynorphin A or dynorphin B immunoreactivity were observed in the dentate hilus or any of the CA fields. However, isolated, long, varicose fibers were stained with anti-dynorphin B in the CA1 region (Fig. 2E).

When hippocampal sections were stained with antisera raised against BAM-22P, light staining was detected in mossy fibers and a few granule cells (Fig. 2F). In addition, stained multipolar cell bodies were scattered throughout all layers of the dentate hilus (Fig. 2, G and H) and hippocampal fields (Fig. 2, I and J). In the entorhinal cortex, BAM-22P immunostaining was concentrated in layers II and III of the lateral entorhinal cortex but was most prominent in layer II cells from which perforant path fibers emanated (Fig. 2K).

The concentrations of dynorphin A and [Leu⁵]enkephalin immunoreactivities were quantitated by RIA in the major hippocampal fields and entorhinal cortex (Table II). As predicted by immunocytochemical results, dynorphin A immunoreactivity was highest in the dentate-CA4 region and in the combined CA2/CA3 regions; its concentration was 10 times lower in CA1, subiculum, and entorhinal cortex. [Leu⁵]Enkephalin immunoreactivity was more evenly distributed than dynorphin A immunoreactivity throughout the hippocampal formation, although dentate/CA4, CA2/CA3, and entorhinal cortex contained 4 to 5 times higher concentration of [Leu⁵]enkephalin immunoreactivity than did subiculum and CA1. Since there are cross-reacting molecules for each of these antisera (Table I), and since one of our objectives was to determine the proportion of immunoreactive substances in the nominal form, we resolved each regional extract using two different chromatographic systems.

Resolution by molecular size using HPLC PACs of extracts made from each region is shown in Figure 3. In each of the five regions studied, the molecular size of the dynorphin A immunoreactivity was heterogeneous. At least two forms were present: one migrating at approximately the same molecular weight as dynorphin A(1-17) (2100 to 2200), the second form being of apparently higher molecular weight (approximately 3500 to 4000). The relative amounts of these two molecular forms vary in the different regions. The higher molecular weight form was most prevalent in dentate/CA4. In the subiculum and CA2/CA3 regions, the contents of the two forms were nearly equal; in CA1 and entorhinal cortex, the lower molecular weight form predominated. The higher molecular weight form is probably dynorphin A(1-32) as described by Fischli et al. (1982), on the basis of its molecular weight and its cross-reactivity in both dynorphin A and dynorphin B RIA (Chavkin et al., 1983b). The identities of the less predominant forms of dynorphin A immunoreactivity were not determined. Consistent with the immunocytochemical data, dynorphin A immunoreactivity was most concentrated in dentate/CA4 and CA2/CA3 dissected regions. In contrast, [Leu⁵]enkephalin immunoreactivity was of nearly homogeneous mo-

Figure 3. Extracts of five hippocampal regions resolved by PAC-HPLC. Both dynorphin A and [Leu⁵]enkephalin immunoreactivities are plotted for each region. The PAC-HPLC columns separate the molecular forms strictly

lecular size and eluted with the same retention as [Leu⁵]enkephalin or [Met⁵]enkephalin standard. The regions showing the highest [Leu⁵]enkephalin immunostaining (entorhinal cortex and dentate) also had the greatest [Leu⁵]enkephalin content of the dissected regions.

Since low molecular weight peptides are not completely resolved by PAC-HPLC column, we also analyzed the extracts on C18 reverse phase HPLC. The two abundant forms of dynorphin A immunoreactivity seen with the molecular sizing PACs were also evident in this system (Fig. 4). In each region a peak of dynorphin A immunoreactivity eluted in the position that synthetic dynorphin A(1-17) appears, and a second peak eluted with a longer retention time (39 min) than that for dynorphin A(1-17) standard. This second peak is probably the higher molecular weight form as indicated by its retention and relative concentration in the hippocampal regions shown in Figure 3. The proportion of dynorphin A immunoreactivity eluting as the higher molecular weight form varied between regions but also slightly between extractions. The data shown in Figure 4 are from a single extraction but are different from the extract used for Figure 3. Comparison between the regions shown in Figure 4 shows that, again, the intermediate precursor form (having longer retention on C18-HPLC) predominated in the regions containing dynorphin-immunostained cell bodies.

The majority of the [Leu⁵]enkephalin immunoreactivity resolved by C18-HPLC had the same retention as synthetic [Leu⁵]enkephalin standard (Fig. 4). Less than 10% of the total immunoreactivity was due to other immunoreactive forms. The small peak having a retention time of 10 min was probably due to cross-reactive [Met⁵]enkephalin which elutes at that position. Each chromatogram shown represents the equivalent contents of 1 mg of wet tissue from the hippocampal regions; comparison of the regional contents shows good agreement with the [Leu⁵]enkephalin contents listed in Table III.

To compare hippocampal opioid peptide contents, whole hippocampal extracts were examined using specific RIAs. As shown in Table III (column 2), the dynorphin A immunoreactivity measured was roughly 2-fold greater than either [Leu⁵]enkephalin or β -endorphin immunoreactivity. When whole hippocampal extracts were chromatographed on controlled pore glass columns, β -endorphin immunoreactivity eluted in a small peak at a retention greater than that of synthetic β -endorphin(1-31) (Fig. 5). This immunoreactive β -endorphin elution position is consistent with low molecular weight substances (<300). In contrast, other brain regions produced clearly identifiable β -endorphin-immunoreactive peaks at the mobility of the standard (Fig. 5). From these data, the molecular identity of the β -endorphin immunoreactivity seen in hippocampal extracts was not established, but it is evidently not due to authentic β -endorphin or any of its known precursors.

Evidence that the proenkephalin and prodynorphin systems could have neurotransmitter roles in the hippocampus was provided by the *in vitro* release studies summarized in columns 3 and 4 of Table III. Both peptides showed low stable basal release rates. K⁺ stimulation dramatically increased the rate of [Leu⁵]enkephalin-immunoreactive efflux by 10-fold above the basal rate. The rate of dynorphin A-immunoreactive release was also increased by high K⁺ stimulation, but to a lesser extent (2- to 3-fold increase). When CoCl₂ was substituted for CaCl₂ in the Krebs-bicarbonate buffer, there was no further release of either peptide's immunoreactivity during the high K⁺ stimulation. Comparison of the release rates of the two opioid

according to size (see the standards at the bottom of the figure). Each chromatogram represents the content of 1.0 mg of tissue protein placed on the column; however, because of the range of immunoreactive contents of the two peptides in the five regions, the scales on the abscissa were adjusted to facilitate the comparisons of the peaks within each chromatogram. Note that the abscissa for the standards is the log₁₀ of molecular weight. Each molecular weight standard was injected at 20 ng/100- μ l injection and measured by absorbance at 254 nm. Points represent the mean retention of three to five determinations.

Figure 4. Extracts of five hippocampal regions were resolved on C18 μ Bondapak HPLC columns. Injections were made and figure scales of the abscissa were adjusted as described in the legend to Figure 3. The small dashed line represents the gradient of increasing acetonitrile (20 to 30% in 60 min) over the course of elution. The acetonitrile gradient was 20 to 30% for the first 60 min and 30 to 50% for the final 10 min. This column system separates peptides on the basis of peptide hydrophobicity, thus allowing a different separation of molecular forms than was seen in the PAC-HPLC columns. The calibration standards are shown as arrows at the top of the figure: A, [Met⁵]enkephalin; B, α -neo-endorphin; C, [Leu⁵]enkephalin; D, dynorphin B; E, dynorphin A(1-17).

TABLE III

Contents and release of opioids from whole hippocampus

Tissue content was determined in dilutions of acetic acid-soluble extracts and expressed in relation to tissue protein content as described below. Dilutions of whole hippocampal extracts were assayed by the three specific RIAs described under "Materials and Methods." Values are not corrected for possible cross-reactions with other opioids present. Freshly prepared hippocampal slices were superfused for release studies with oxygenated isotonic bicarbonate buffer at 24°C. Spontaneous release rates are the means and standard errors for the 15 min preceeding and the 10 min following the 50 mM potassium superfusion. Stimulation release rates are the means during the 10-min 50 mM K⁺ superfusion periods.

Opioid Peptide	Tissue Concentration fmol/mg of protein	Release Rate	
		Spontaneous	K ⁺ Stimulated
[Leu ⁵]Enkephalin	193 ± 21.8 (10) ^a	3.0 ± 1.41 (8)	15.6 ± 2.7 (8)
Dynorphin A	413 ± 110 (10)	1.3 ± 0.36 (6)	2.9 ± 0.63 (6)
β-Endorphin	188 ± 10.3 (10)	ND ^b	ND

^a Numbers in parentheses, numbers of independent replicates.
^b ND, not determined.

peptides indicates that, for the same stimulus, [Leu⁵]enkephalin was released at a 5-fold higher rate. This is in marked contrast to the 2-fold greater concentration of dynorphin A immunoreactivity in the tissue.

Discussion

This study extends the earlier immunocytochemical observations of opioid peptide distribution in the hippocampus by examining the proenkephalin distribution, using more selective antisera, and by determining the distribution of the prodynorphin product, dynorphin B, in more detail. [Leu⁵]Enkephalin antisera, which cross-react with both proenkephalin and prodynorphin-derived peptides, stained three major neuronal groups: the lateral perforant/temporoammonic pathway originating in lateral entorhinal cortex, the dentate granule cell mossy fiber pathway, and intrinsic neurons scattered throughout the dentate gyrus and hippocampal and subicular fields as described (Gall et al., 1981, 1984; McGinty et al., 1983, 1984). BAM-22P

antiserum stained layer II entorhinal cortex cells and their axons in the perforant/temporoammonic pathway, scattered neurons in layer III of entorhinal cortex and throughout the hippocampal and subicular fields, as well as mossy fibers (although the latter are stained very lightly). The BAM-22P antiserum used in this study is useful because it can distinguish between the products of the proenkephalin and prodynorphin families, unlike antisera to [Leu⁵]enkephalin. The staining pattern obtained with anti-BAM-22P was similar to that seen using anti-[Leu⁵]enkephalin in this study and in previous reports, strengthening the conclusion that cells stained by either antiserum contain proenkephalin products.

The distribution of dynorphin A(1-17) immunoreactivity paralleled that of dynorphin B immunostaining (data not shown). Dynorphin A(1-17) and dynorphin B antisera predominantly stained granule cells and mossy fibers as well as scattered multipolar neurons in the granular and supragranular layers of the dentate gyrus. The absence of dynorphin B-immunoreactive perikayria in CA fields confirms a similar observation made previously for cells containing dynorphin-A immunoreactivity (McGinty et al., 1983). The concordance between the dynorphin A and dynorphin B staining patterns reinforces the conclusion that these hippocampal cells contain prodynorphin products.

Although immunocytochemistry provides the better resolution of the two peptides' distribution, the dissection of the hippocampal formation into five discrete regions allowed a quantitative estimate of the peptide concentrations and also permitted the characterization of the molecular forms in each region. The RIA data of the dissected regions generally agreed with the immunocytochemical localization of the peptides. The immunocytochemical data predicted that the highest concentration of dynorphin immunoreactivity is contained in the dentate/CA4 and CA2/CA3 (mossy fiber distribution) regions, and, indeed, those two regions have a 10-fold higher concentration than the other regions (Table II). The enkephalin and BAM-22P immunostaining pattern positively correlates with high levels of [Leu⁵]enkephalin in the entorhinal cortex and in the entorhinal projection fields in the dentate gyrus, as well as in the CA3 region containing mossy fibers and proenkephalin-positive interneurons. Such interneurons in CA1 and subiculum, in addition to the proenkephalin-positive temporoammonic pathway, are most probably responsible

Figure 5. Chromatograms of whole hippocampal and spinal cord extracts placed on gravity columns (30 × 2.5 cm) packed with controlled pore glass glycochase (G-200, Pierce Chemical Co.) and eluted with a solution of 2 N acetic acid, 0.01% Triton X-100. Fractions of 230 μl were collected, lyophilized, and assayed in the β-endorphin assay as described. The choice of spinal cord as an appropriate comparison to the hippocampus was based on the similarities of the two regions regarding density of reported β-endorphin innervation and the absence of positive cell bodies. Because these two CNS regions were chromatographed on different days using columns of different lengths, the two chromatographs were normalized by converting fraction number to relative mobility (K_d) as described by Rossier et al. (1977). The resulting elution positions of ¹²⁵I-β-endorphin standard for the two runs were superimposable. Note the difference in scale values for the two tissues.

for the [Leu⁵]enkephalin immunoreactivity in these regions. The paucity of prodynorphin-positive neuronal structures (a few scattered, varicose fibers) in CA1 and subiculum corresponds to the lower dynorphin immunoreactivity there.

The demonstrated presence of [Leu⁵]enkephalin and dynorphin A satisfies only one of the criteria necessary to support the hypothesis that these opioid peptide systems have neurotransmitter roles in the hippocampus. The demonstration of calcium-dependent stimulated release of the two opioids satisfies another (Table I). We had shown previously (Chavkin et al., 1983a) that dynorphin A immunoreactivity released from hippocampal slices could also be stimulated by 75 μ M veratridine or 0.5 mM kainic acid in a calcium-dependent manner, supporting the hypothesis that the release measured is due to synaptic terminal release. In addition, we had shown previously that the dynorphin A immunoreactivity released from hippocampal slices by potassium stimulation co-migrates with synthetic dynorphin A(1-17) on C18-HPLC (Chavkin et al., 1983a). The absolute identity of the [Leu⁵]enkephalin immunoreactivity released was not determined, although the molecular homogeneity of the immunoreactivity closely matching authentic [Leu⁵]enkephalin suggests that the released immunoreactivity is probably [Leu⁵]enkephalin pentapeptide.

As to the relative significance of proenkephalin and prodynorphin systems in the hippocampus, one commonly used index is their relative abundance. The hippocampal content of the five known prodynorphin products has been determined (Chavkin et al., 1983b). Their sum equals 1.34 pmol/mg of protein. The [Met⁵]enkephalin concentration in rat hippocampus was determined by Hong and Schmid (1981) to be 0.96 pmol/mg of protein, and we found 0.19 pmol/mg of protein for [Leu⁵]enkephalin (cf. Table III). Thus, although the full details of the proenkephalin precursor processing are not yet known, the sum of the hippocampal proenkephalin-derived opioid concentration probably will be roughly equal to the prodynorphin-derived opioid concentration. Nevertheless, relative abundance alone is not enough to indicate the contribution of these systems to hippocampal circuitry or function; cellular location, fiber density on target neurons, peptide turnover rates, and receptor affinities are equally important functional indices.

The presence of BAM-22P immunoreactivity in the mossy fibers raises the question of whether the detected [Leu⁵]enkephalin arises from proenkephalin and/or prodynorphin precursor peptides in this hippocampal pathway. Preliminary data (McGinty, 1984) suggest that proenkephalin and prodynorphin-derived peptides are present in separate granule cells and parallel mossy fibers. However, in other brain regions, Zamir et al. (1983, 1984) have shown that prodynorphin can act as a precursor for [Leu⁵]enkephalin. In the hippocampus this is unlikely to be the case since the ratio of [Met⁵]enkephalin to [Leu⁵]enkephalin is 5:1 (cf. Table III and Hong and Schmid, 1981), a ratio similar to that found in the proenkephalin sequence (Noda et al., 1982). Thus, any contribution of prodynorphin to [Leu⁵]enkephalin content in hippocampus must be minor. The basis for the higher rate of [Leu⁵]enkephalin-immunoreactive efflux from slices during K⁺-stimulated release (Table III) may reflect a higher proportion of [Leu⁵]enkephalin as compared to dynorphin A in hippocampal neuron terminals rather than a precursor product relationship of the two peptides.

In summary, three different results were found regarding the three opioid peptide systems in this study of the hippocampus. (1) Authentic β -endorphin(1-31), representing pro-opiomelanocortin-derived peptides, appears not to be present, and the immunoreactivity detected may be artifactual. (2) The enkephalin in system yielded the most uncomplicated story; authentic [Leu⁵]enkephalin was found to be the sole molecular form responsible for [Leu⁵]enkephalin immunoreactivity present in extracts and was the major opioid peptide released upon stimulation. (3) Dynorphin A presented the most interesting case from a neurobiological perspective. Dynorphin A immunoreactivity was found to be represented by a variety of molecular weight forms. Thus, the amount of dynorphin A(1-17) present in hippocampus is much less than what is reported using

RIA values alone. In addition, the ratio of high to low molecular weight forms was different for different hippocampal regions and enabled us to ascribe a possible sequence of peptide processing from cell body regions to terminal fields.

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