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ON THE DISTRIBUTION OF [MET^5]- AND [LEU^5]-ENKEPHALINS IN THE BRAIN OF THE RAT, GUINEA-PIG AND CALF

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1 By means of a highly sensitive radioimmunoassay, the content of [met^5]-enkephalin (ME) and [leu^5]-enkephalin (LE) was measured in various regions of the rat, guinea-pig and calf brain. Provisions were made to differentiate ME from LE by the use of cyanogen bromide (CNBr) to destroy methionine, the carboxy terminal amino acid in the ME sequence. This allows for correction of possible errors due to the cross-reactivity of the ME to the LE antiserum. Evidence is presented to demonstrate that the specific radioimmunoassay combined with the CNBr technique is a valid method to measure LE and ME content in crude tissue extracts.

2 In all the species studied, enkephalins appeared to be highly concentrated in the striatum and hypothalamus while very low amounts were found in the cerebellum and hippocampus.

3 Although the ratio between ME and LE content varied from area to area, the ME content in every region of the rat, guinea-pig and calf brain was more than 4 fold greater than that of LE.

Introduction

Radioreceptor and radioimmunoassay studies of the [met^5]-enkephalin (ME) and [leu^5]-enkephalin (LE) content of various areas of the brain from different species have indicated that the ratio of these two peptides changes with the brain region and the species (Simantov & Snyder, 1976a; Smith, Hughes, Kosterlitz & Soja, 1976; Simantov, Childers & Snyder, 1977a; Wesche, Höllt & Herz, 1977; Hughes, Kosterlitz & Smith, 1977). Interestingly, some areas of the calf brain appear to contain more LE than ME (Simantov & Snyder, 1976b). Unfortunately the specificity and resolution of immunohistochemical methods does not allow one to decide whether or not the two pentapeptides are located in different neuronal pathways and whether the calf brain contains more LE-neurones (Elde, Hökfelt, Johansson & Terenius, 1976; Watson, Akil, Sullivan & Barchase, 1977; Simantov, Kuhar, Uhl & Snyder, 1977b). Previous work from this laboratory has shown that in every structure of the rat brain there is 5 to 10 fold more ME than LE and that when the ME content of specific brain regions increases, as a result of a drug treatment, there is a parallel selective increase of LE, as if the regulation of the two compounds is closely related (Yang, Hong & Costa, 1977; Hong, Yang, Fratta & Costa, 1978a; Gillin, Hong, Yang & Costa, 1978; Hong, Yang, Gillin, Di Giulio, Fratta & Costa, 1978b). We have also observed that ME cross reacts by roughly 10% with LE antiserum. Since ME content in all the rat brain areas studied is always higher than that of LE, this cross-reactivity cannot be disregarded since it hampers the understanding of the anatomical distribution and physiological role of both peptides in the brain.

In order to overcome this difficulty, we have developed a radioimmunoassay to differentiate LE from ME, by destroying ME with cyanogen bromide (CNBr). The ME and LE content of various areas of the guinea-pig, calf and rat brain has been examined by this radioimmunoassay.

Methods

Tissue extractions

Male Sprague Dawley rats (Zivic Miller, Allison Park, PA) were killed by exposure of their skulls for 4 s to a focussed beam of microwave radiation (2.0 kW, 2.45 GHx, 75 W/cm^2) (Guidotti, Cheney, Trabucchi, Doteuchi & Wang, 1974). After dissection, tissues were homogenized in 0.1 N HCl and centrifuged at 29,000 *g* for 20 min.

Male Hartley guinea-pigs were killed by decapitation, the brain was rapidly removed, dissected on an ice-cooled plate and immediately frozen. Calf brains from a local slaughter-house were removed from the skull immediately after decapitation and kept in ice until dissection was completed. Tissues were homogenized in 0.1 N HCl, heated at 100°C for

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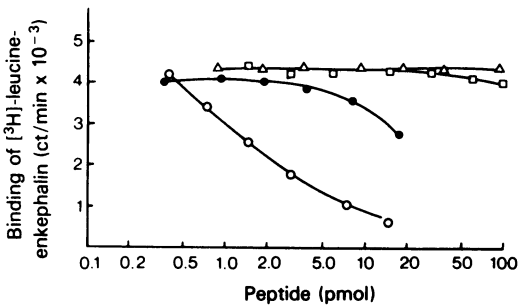

Figure 1 Specificity of leucine-enkephalin antiserum. Binding of [^{3}H]-leucine enkephalin (20 Ci/mmol) in the presence of leucine enkephalin (LE, ○); met-enkephalin (ME, ●); Phe-LE (□) or α-endorphin, β-endorphin or Ty-Gly-Gly-Phe (△).

3 to 5 min and centrifuged at 29,000 *g* for 20 min. The resulting supernatant was neutralized with 1 N NaOH and the precipitate formed again removed by centrifugation. The calf tissue extract was then directly radioimmunoassayed for enkephalins by the procedure previously described by Yang *et al.* (1977).

Cyanogen bromide treatment

In order to determine the optimal amount of CNBr required to eliminate the binding of ME to LE antiserum, synthetic enkephalins, 50 ng of LE and 100 ng of ME in 1 ml of 0.1 N HCl, were treated with different amounts of CNBr for 1 h at 50°C. The sample was then lyophilized, resuspended in distilled water and subjected to enkephalin radioimmunoassay. Control samples were treated by the same procedure as the other samples except that CNBr was omitted.

Rat and guinea-pig tissue extracts prepared as stated above, were subjected to CNBr treatment. The clear supernatant was treated with CNBr, 20 mg/ml of tissue extract, for 1 h at 50°C. The sample was lyophilized, dissolved in H_2O and then assayed for enkephalins as previously described.

Chromatography

Nucleus caudatus (1.5 g) from calf brain was homogenized in 1 N CH_3COOH and then heated at 100°C for 5 min. After centrifugation at 2.9×10^4 *g*, the supernatant was set aside and the precipitate was re-extracted with 1 N CH_3COOH. The combined supernatants were lyophilized, dissolved in 1 N CH_3COOH and subjected to chromatography with a Biogel P-2 column (0.9 × 60 cm) pre-equilibrated with 1 N CH_3COOH. The void volume was determined by blue dextran. The fractions collected were lyophilized, dissolved in water and radioimmunoassayed for ME and LE.

Protein measurement

Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as standard.

Materials

Tritiated ME and LE ([tyrosyl-3,5-^{3}H]-enkephalin, 5-L-methionine, 43 Ci/mmol and [tyrosyl-3,5-^{3}H]-enkephalin, 5-L-leucine, 20 Ci/mmol) were purchased from Amersham. ME and LE were purchased from Peninsula Laboratories, cyanogen bromide from Eastman Chemicals and Biogel P-2 from BioRad Laboratories.

Results

Specificity of leu-enkephalin antiserum

The specificity of the antiserum directed toward LE was examined by the displacement of [^{3}H]-LE bind-

Table 1 Cynogen bromide (CNBr) treatment of synthetic enkephalins

Enkephalin (*ng*)	*Cyanogen bromide* (*mg*)	*Leu-enkephalin** (*ng*)	*Met-enkephalin*** (*ng*)
Leu-enkephalin (50)	0	47.0	
	20	47.8	
Met-enkephalin (100)	0	6.9	98.5
	10	< 1.0	7.4

Leu-enkephalin (LE) 50 ng or met-enkephalin (ME) 100 ng was dissolved in 1 ml of 0.1 N HCl; these solutions were treated for 1 h at 50°C with the indicated amounts of CNBr. The incubation mixture was then lyophilized, suspended in H_2O and immunoassayed for enkephalins. * Antiserum directed toward LE was used for the radioimmunoassay. ** Antiserum directed toward ME was used for the radioimmunoassay.

ing by several related peptides. The ME antiserum specificity has been tested previously (Yang, Hong, Fratta & Costa, 1978). α-Endorphin, β-endorphin and other structurally related compounds in a concentration range of 1 to 100 pmol failed to change the [^{3}H]-LE binding to the antiserum (Figure 1). However, ME displaced [^{3}H]-LE with 10% of the affinity of LE (Figure 1).

Cyanogen bromide treatment

The affinity of LE (50 ng) for the antibody is unaffected following incubation with 20 mg of CNBr at 50°C for 1 h; in contrast, a lower dose (10 mg) of CNBr, incubated with 100 ng of ME in the same conditions destroyed 90% of the ME, thereby eliminating the cross-immunoreactivity of ME to the LE antibody (Table 1).

Table 2 shows the effect of CNBr treatment on the quantitative values yielded by ME and LE radioimmunoassay in rat cortical and striatal tissue extract. With LE antiserum, the immunoreactivity of LE in both tissues was reduced by less than 30% after treatment with various concentrations of CNBr, whereas with ME antiserum the immunoreactivity was reduced by more than 90%, indicating that ME was rendered unreactive to the antiserum by CNBr treatment. Since the tissue immunoreactivity for LE was reduced by only 30% even when the CNBr concentration was increased 4 fold, it is concluded that in both cortex and striatum the error created by the ME present was not more than 30% of the LE value measured by LE radioimmunoassay without CNBr treatment.

Regional distribution of enkephalins in guinea-pig and calf brain

The distribution of ME and LE in various areas of the guinea-pig brain is shown in Table 3. The highest concentration of enkephalins was found in striatum and hypothalamus, a result similar to that of Hughes *et al.* (1977). The treatment of the tissue extract with CNBr before radioimmunoassay again resulted in a 30% decrease in the LE value as was found in rat tissues (Table 2).

In the calf, the highest concentrations of LE and ME were found in the caudate nucleus and in the globus pallidus, followed by putamen, septum and hypothalamus (Table 4). The concentrations of the two peptides were below the sensitivity limit of the assay in cerebellum or hippocampus. In the areas where both peptides were detected, the ratio between ME and LE varied between 4 and 7.

The reliability of the values reported has been tested using different dilutions of the calf caudate tissue extract for the assay. There was a linear relationship between the amount of extract utilized for the radioimmunoassay (1.8 to 9.0 mg for ME and 9 to 54 mg for LE) and the quantity of LE or ME measured.

Identification of met- and leu-enkephalin

In order to examine whether there are other enkephalin-like peptides in calf brain, the enkephalin immunoreactivities in crude calf striatal extract was characterized by gel filtration chromatography.

As shown in Figure 2, in the Biogel P-2 column chromatography, the main immunoreactive material emerged at the same elution volume as the synthetic enkephalins.

Discussion

Various studies have indicated that LE, ME and β-endorphin are located intraneuronally (Elde *et al.*, 1976; Simantov *et al.*, 1977b). However, it is not yet

Table 2 Cyanogen bromide (CNBr) treatment of rat striatal and cortical tissue extracts

Tissue	*Cyanogen bromide* (*mg*)	*Met-enkephalin* (*ng*)	(*% control*)	*Leu-enkephalin* (*ng*)	(*% control*)
Striatum	0	98 ± 2.8	100	14 ± 0.6	100
	5	6.0 ± 0.5	6.1	10.6 ± 0.1	74.1
	10	4.8 ± 0.4	4.4	9.8 ± 0.1	69.0
	20	5.4 ± 0.5	5.1	10.5 ± 0.3	73.9
Cortex	0	60 ± 4.4	100	6.1 ± 0.3	100
	5	2.9 ± 0.5	4.6	4.4 ± 0.4	72.1
	10	2.6 ± 0.3	4.3	4.3 ± 0.2	70.5
	20	3.1 ± 0.2	5.1	4.4 ± 0.3	72.1

Values (ng) ± s.e.mean are given for met-enkephalin and leu-enkephalin. Tissues from 1 rat killed by microwave irradiation were extracted with 1 ml 0.1 N HCl and then treated with various amounts of CNBr as indicated in the table.

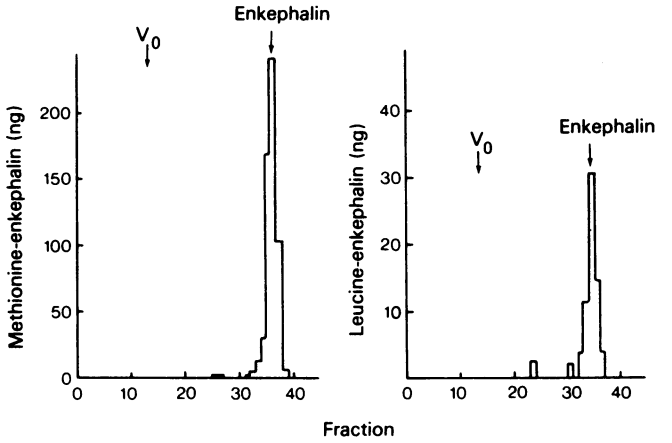

Figure 2 Enkephalin radioimmunoassay after separation by biogel P-2 column chromatography. The extract from 1.5 g of calf caudate in 1 N CH_3COOH was fractionated by Biogel P-2 column (0.9 × 60 cm) pre-equilibrated with 1 N CH_3COOH; 1 ml fractions were collected. The void volume was determined to be at fraction 14 by blue dextran.

Table 3 Regional distribution of enkephalins in guinea-pig brain

Region	*Leu-enkephalin (ng/mg protein)* *Uncorrected**	*Corrected***	*Met-enkephalin (ng/mg protein)*
Striatum	0.90 ± 0.08	0.59 ± 0.05	7.13 ± 0.40
Hypothalamus	1.04 ± 0.05	0.69 ± 0.06	7.39 ± 0.24
Hippocampus	0.15 ± 0.02	0.09 ± 0.01	0.90 ± 0.01
Cortex	0.13 ± 0.03	0.09 ± 0.01	0.76 ± 0.09

Values are mean ± s.e. mean. * Leu-enkephalin (LE) measured by radioimmunoassay; ** LE remeasured by radioimmunoassay after CNBr treatment (see text for details).

Table 4 Regional distribution of enkephalins in calf brain

Region	*Met-enkephalin* (ng/mg protein)	*Leu-enkephalin*	*ME/LE*
Caudate	6.0 ± 0.9 (8)	0.88 ± 0.06 (8)	6.8
Putamen	2.6 ± 0.4 (8)	0.56 ± 0.07 (8)	4.6
Globus pallidus	5.8 ± 1.6 (6)	1.2 ± 0.2 (6)	4.8
Septum	2.0 ± 0.3 (7)	0.51 ± 0.07 (7)	3.9
Hippocampus	<0.3	<0.25	
Hypothalamus	2.0 ± 0.2 (8)	0.45 ± 0.02 (8)	4.4
Cerebellum	<0.07	<0.13	
Medulla	1.0 ± 0.2 (6)	0.21 ± 0.05 (5)	4.8

Values are mean ± s.e. mean. Leu-enkephalin (LE) was determined by LE-radioimmunoassay without cyanogen-bromide treatment.

clear whether LE and ME are stored in the same or in different neurones. This uncertainty is compounded by a number of reports showing a wide variability in the ME/LE ratio in different brain areas of guinea-pig, rat and calf. Suspecting that differences in extraction procedures, unaccounted presence of endogenous ligands in radioreceptor and bioassay procedures and antisera specificity might account for this variability, we have re-examined the content of ME and LE in various brain areas of the rat, guinea-pig and calf. In our radioimmunoassay for LE, the cross reactivity of ME to LE antiserum was eliminated by modification of ME by CNBr treatment and taking the immunoreactivity of the LE present in CNBr-treated samples as the measurement of the LE present in the sample. Moreover, the correction required for the LE value after CNBr treatment was not very large (less than 30%).

The present results demonstrate that LE and ME are distributed in the various parts of calf and guinea-pig brain in a similar pattern. The concentrations of both pentapeptides are highest in caudate or globus pallidus and lowest in hippocampus. Although the ratio between ME/LE content varied from area to area, the ME content in every area of the calf and guinea-pig brain was more than 4 fold greater than that of LE. The reasons for our failure to confirm a previous report (Simantov & Snyder, 1976b) showing that in calf brain, the LE content exceeds that of ME, are not clear. The validity of our data is supported by the chromatographic isolation of the two pentapeptides and the selective destruction of methionine in ME sequence by CNBr. Our data on guinea-pig brain are in general agreement with an earlier paper (Smith *et al.*, 1976) and the small discrepancies might be explicable on the basis of the different types of assays: bioassay in their experiments and immunoassay in our work.

In conclusion, in the three species we have studied, there is more ME than LE in various brain regions. By treatment of the sample with CNBr, cross reactivity of ME to the LE antiserum is eliminated, thereby making it possible to radioimmunoassay LE selectively in the crude tissue extract.

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