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REGIONAL DISTRIBUTION OF α-NEO-ENDORPHIN IN RAT BRAIN AND PITUITARY

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SUMMARY : α-Neo-endorphin was isolated as the first form of "big" Leu-enkephalin and its complete amino acid sequence has recently been established. Using an antiserum raised against synthetic α-neo-endorphin, a highly sentitive and specific radioimmunoassay was developed. The antiserum practically possesses no cross-reactivity to Leu-enkephalin, dynorphin[1-13] and PH-8P, and very little to β-neo-endorphin. Distribution of α-neo-endorphin has been determined in rat brain and pituitary by the use of the highly specific antiserum. The highest concentration was observed at posterior lobe of pituitary. Furthermore, immunoreactive α-neo-endorphin was characterized by gel-filtration and high performance liquid chromatography, and shown to be identical with authentic α-neo-endorphin.

α-Neo-endorphin is a "big" Leu-enkephalin isolated as the first form from porcine hypothalamic extracts (1). Three other "big" Leu-enkephalins, i.e., β-neo-endorphin and PH-8P in hypothalamus as well as dynorphin in pituitary have been identified (2,3,4). Recently, the complete structures and chemical syntheses of α- and β-neo-endorphin and PH-8P have been reported in our previous papers (2,3,5,6), although dynorphin is not fully sequenced as yet. Alpha-, and β-neo-endorphin structurally belong to one family, while PH-8P and dynorphin to another one, as follows;

α-neo-endorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys
β-neo-endorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro
dynorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys---
PH-8P(dynorphin[1-8])	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile

Natural occurrence of two families in brain has led to the realization that there exist two different biosynthetic pathways to Leu-enkephalin in brain. Moreover, their potent opioid activities in in vitro assay suggest that they may possess their own physiologic roles in brain. To respect with these, the knowledge of the regional distribution of "big" Leu-enkephalins in brain should provide information of significance.

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The present paper describes the preparation of a highly sensitive antiserum against α-neo-endorphin. Cross-reactivity of the antiserum was examined with ten kinds of enkephalin related peptides, and the antiserum was revealed to be highly specific to α-neo-endorphin and suitable for determining the content of α-neo-endorphin in the tissue extracts. The radioimmunoassay (RIA) by utilizing the antiserum allowed us to demonstrate regional distribution of immunoreactive (ir-) α-neo-endorphin in rat brain and pituitary. Further characterization of ir-α-neo-endorphin was performed by gel-filtration and high performance liquid chromatography (HPLC).

MATERIALS AND METHODS

MATERIALS : Synthetic peptides used in this work were synthesized by Dr. Sakakibara's group, Protein Research Foundation, Minoh, Osaka, Japan, to whom our deep gratitudes are due. Syntheses of α-neo-endorphin [2-10], α-neo-endorphin-NH_2 and α-neo-endorphin[1-8] will be reported elsewhere, otherwise have been described (2,5,6,7,8). Homogeneity of the synthetic peptides was confirmed by TLC and HPLC on a reverse phase column as well as amino acid analyses. Bovine serum albumin (BSA : fraction V) purchased from Miles was twice recrystallized before use. Bovine γ-globulin was purified from bovine serum in our laboratory. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide·HCl (water soluble carbodiimide) was a product of Protein Research Foundation.

Conjugation of α-neo-endorphin to bovine serum albumin : The antigenic conjugate of α-neo-endorphin was prepared in a similar method as reported in our previous paper (9). Synthetic α-neo-endorphin (11.25 mg) and BSA (13.45 mg) were dissolved in 1 ml of water. To the solution, was added 50 mg each of water soluble carbodiimide five times every two hours under stirring at room temperature. The resulted mixture was further left standing overnight at 4 °C and then dialyzed five times against 500 ml of saline and twice against 500 ml of phosphate buffer for RIA use at 4 °C for every 12 hr. The dialysate thus obtained was diluted to the final volume of 14 ml by adding the above buffer containing 0.1 % NaN_3. The conjugate was proved to contain about 6 molecules of α-neo-endorphin per one BSA molecule on the basis of amino acid analysis data.

Immunization procedure : The antigenic conjugate solution (1 ml) prepared as above was emulsified with an equal volume of Freund's complete adjuvant and used immediately for immunizing male New Zealand White rabbit by subcutaneous injection at multiple sites in the interscapulo-vertebral region Rabbits were boosted every 20 days with the half quantity of a conjugate and bled 10 days after every booster. Sera were separated and stored frozen at -20 °C after adding NaN_3 (0.1%).

Radioiodination : α-Neo-endorphin was radioiodinated by the lactoperoxidase method (10). The ^{125}I-labeled peptide was purified on a CM-cellulose (CM-52) column and stored at -20 °C after adding BSA (0.25%).

Radioimmunoassay procedure : Assay was performed in a way similar to the case of [Arg^6]-Leu-enkephalin as reported in our previous paper (9). Standard buffer used was 0.05M phosphate buffer (pH 7.4), containing 0.25% BSA, 0.08M NaCl, 0.025M EDTA·2Na and 0.05% NaN_3.

A 500 µl radioimmunoassay mixture consists of;

1) 100 µl of standard solution of the peptide or 100 µl of neutralized tissue extract.
2) 100 µl of ^{125}I-labeled ligand (ca. 15,000 cpm) in standard buffer.
3) 100 µl of antiserum at a dilution of 1:9,000 in standard buffer.
4) 200 µl of standard buffer.

The above mixture was placed in an acryl tube (10 x 78 mm), mixed well and then incubated to equilibrate at 4 °C for 40-50 hr. The incubation was stopped by the addition of 100 µl of 1% bovine γ-globulin in standard buffer and 1 ml of 25% polyethyleneglycol (#6,000) in the buffer. After vigorous shaking, the mixture was left standing at 4 °C for 20 min. The resulted mixture was centrifuged at 3,000 rpm at 4 °C for 15 min. After removal of a supernatant by aspiration, radioactivity of the pellets thus obtained was counted in a gamma counter. Assays were performed routinely in duplicate.

Tissue extraction : Of male Wistar rats (ca. 300g), brains were dissected on ice at 4 °C immediately after decapitation, in the described way according to Glowinski and Iversen (11). After weighing, tissues were homogenized with a Polytron mixer for 60 sec in 10 volumes (v/w) of 1 M acetic acid containing 20 mM HCl, 0.01% phenylmethylsulfonyl fluoride and 0.1% β-mercaptoethanol. Immediately, the homogenates were heated at 95 °C in a boiling water bath for 7 min under shaking. After cooling to 0 °C, the homogenates were centrifuged at 16,000 rpm for 20 min. An aliquot of the supernatant was taken and neutralized by adding an equal volume of 1.3 M Tris solution. The solution thus prepared was used as a sample solution for radioimmunoassay.

Characterization of ir-α-neo-endorphin : Three rat hypothalami (357 mg) were extracted as described above and the extract was loaded on a Sephadex G-75 gel-filtration column (1.2 x 105 cm) and eluted with 1M CH_3COOH (Fig. 1). Aliquots (25 µl) of fractions were submitted to RIA for α-neo-endorphin. Immunoreactive fractions were collected and one-tenth of them was submitted to HPLC on a LS-410 ODS SIL column (C-18, 4.0 x 250 mm, Toyosoda Co. Ltd.) and eluted at a flow rate of 2.0 ml/min with 50 mM KH_2PO_4 (pH 2.0) using a linear gradient of CH_3CN from 10% to 50%. The immunoreactivity of α-neo-endorphin was also measured after neutralizing the aliquots of fractions.

RESULTS AND DISCUSSION

Specificity of the antiserum to α-neo-endorphin : Highly specific antiserum against α-neo-endorphin was raised in rabbits by immunizing with α-neo-endorphin-BSA conjugate, prepared by the action of carbodiimide. The antiserum bound 40% of ^{125}I-labeled α-neo-endorphin at a final dilution of 1:45,000 under the incubation conditions described in Materials and Methods. As seen from Fig. 1, increase of cold α-neo-endorphin added induced a dose-dependent decrease in the percentage of bound labeled ligand. Half-maximum inhibition by α-neo-endorphin was observed at 22 pg (ca. 18 fmole). Accordingly, α-neo-endorphin was found to be quantitatively measurable in a range of 1-200 pg/tube by the RIA, and to be detectable as little as 0.5 pg/tube. The specificity of the antiserum was verified by determining its cross-reactivity to ten kinds of peptides, as shown in Fig 1. The antiserum possessed no cross-reactivity with Leu-enkephalin (α-neo-endorphin[1-5]), which corresponds to the first five residues of α-neo-endorphin, even at the concentration as high as 1 µg. Elongation of the peptide from Leu-enkephalin to α-neo-endorphin[1-7] did not show any increase in the immunoreactivity of the peptides and α-neo-endorphin[1-8] also had practically no immunoreactivity. It should be emphasized that α-neo-endorphin[1-9], i.e., β-neo-endorphin had very

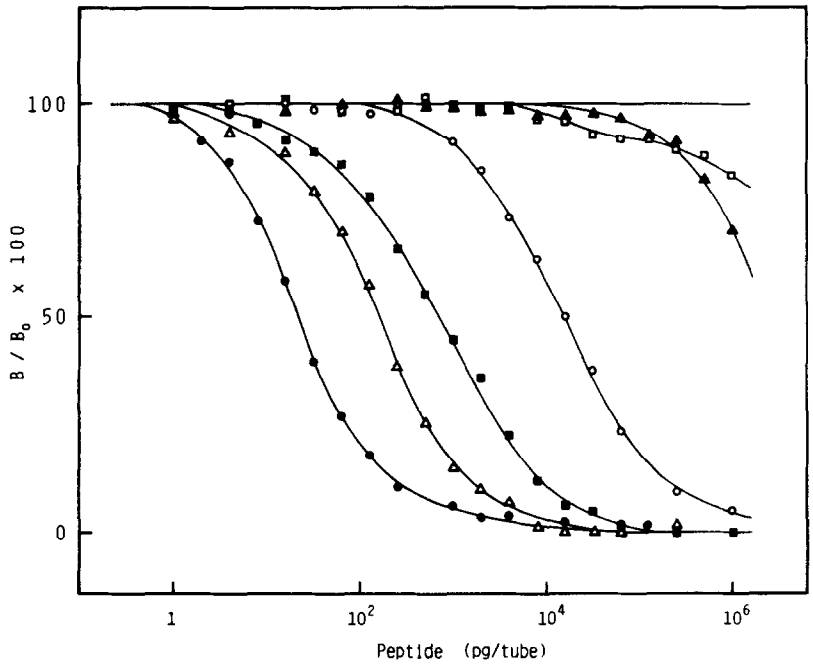

Fig. 1. Inhibition of binding of ^{125}I-α-neo-endorphin to the antiserum (dilution 1 : 45,000) by serial dilution of unlabeled ligands ; α-neo-endorphin (—●—), α-neo-endorphin[2-10] (—△—), α-neo-endorphin-NH_2 (—■—), β-neo-endorphin (—○—), α-neo-endorphin[1-8] (—▲—), dynorphin[1-13] (—□—). α-Neo-endorphin[1-7], [Arg^6]-Leu-enkephalin, Leu-enkephalin, PH-8P and β_h-endorphin were also tested, but did not show any inhibition of binding below 1 pg/tube.

little (0.15%) cross-reactivity, even when its concentration was raised up to 10^4 pg/tube. Moreover, α-neo-endorphin-NH_2 showed only slight cross-reactivity (2.9%) in this RIA system. Thus, the antiserum was proved to recognize predominantly C-terminal -Pro^9-Lys^{10}-OH sequence of α-neo-endorphin. On the other hand, α-neo-endorphin[2-10], lacking N-terminal Tyr of α-neo endorphin, showed 13.3% of cross-reactivity at the concentration of 10^2 pg/tube. This result suggests that N-terminal extended form of α-neo-endorphin may be detectable, if it exists in rather high concentration. The cross-reactivity of dynorphin[1-13] and PH-8P (dynorphin[1-8]), which belong to another family of "big" Leu-enkephalin, was also either not detectable or neglisible. Incidentally, Met-enkephalin and β_h-endorphin did not show any cross-reactivity to the antiserum. Consequently, the present RIA was found to be virtually specific to α-neo-endorphin by discriminating other endogenous opioid ligands.

ir-α-Neo-endorphin in rat brain and pituitary : The high specificity and

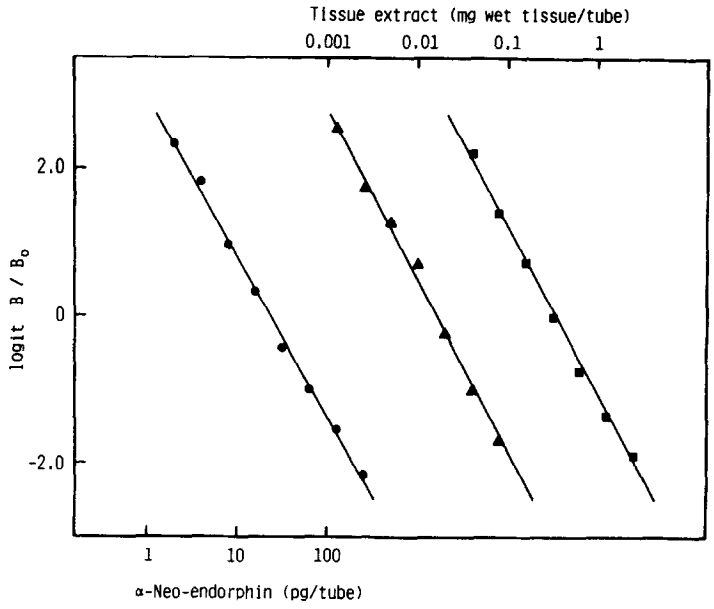

Fig. 2. Log probit plots of inhibition of ^{125}I-α-neo-endorphin binding to the antiserum by serial dilution of acid extracts from rat posterior pituitary (—▲—) and hypothalamus (—■—). The dilution curves of posterior pituitary and hypothalmus are quite parallel with that of standard α-neo-endorphin (—●—).

sensitivity of the antiserum against α-neo-endorphin prepared above enabled us to determine its regional distribution in rat brain and pituitary. Dilutions of tissue extracts from all brain regions yielded competition curves that were parallel to the standard curve of α-neo-endorphin. Typical runs with the extracts of hypothalamus and posterior lobe of pituitary were shown in Fig. 2.

Prior to the measurements of α-neo-endorphin in individual brain regions, efficiency of extraction, and reliability and reproducibility of RIA procedures were validated by following experiments with rat whole brain; 1) ^{125}I-α-neo-endorphin, which had been added to the tissue before homogenization, was found to be completely extractable within experimental error. 2) An appropriate amount of cold synthetic α-neo-endorphin, which had been added to the brain homogenate, was proved to be precisely determined by the present RIA.

Figure 3 shows a gel-filtration chromatogram on Sephadex G-75 of an acid extract of rat hypothalamus, which was prepared under the identical conditions to those in the RIA. There was observed one major peak of ir-α-neo-endorphin, which possessed more than 95% of the total immunoreactivity,

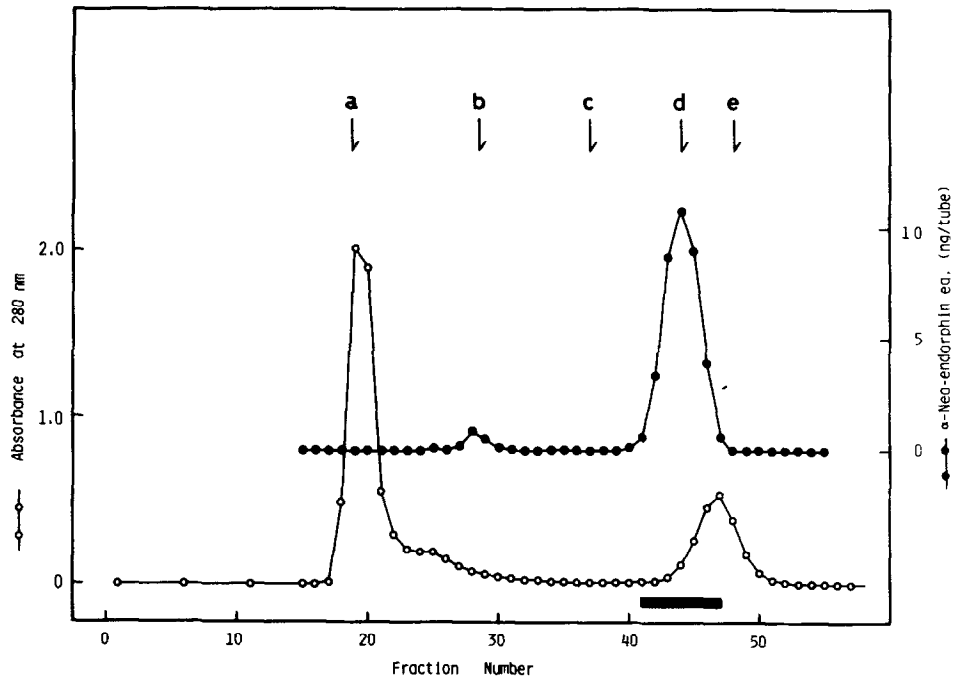

Fig. 3. Sephadex G-75 gel-filtration of the acid extract of rat hypothalamus (357 mg).
Column size : 1.2 x 105 cm. Fraction size : 2.5 ml/tube.
Elution buffer : 1 M CH_3COOH.
The column was calibrated with (a) blue dextran, (b) cytochrome C, (c) ^{125}I-β_h-endorphin, (d) α-neo-endorphin and (e) NaCl.

along with one minor immunoreactive peak, eluted earlier than the former. The major immunoreactive peak obtained above was found to consist exclusively of α-neo-endorphin, which was identified with authentic specimen by the comparison on the reverse phase HPLC, as shown in Fig. 4. The minor immunoreactive peak, presumably of 15,000 daltons, is likely a precursor or an intermediate of α-neo-endorphin, which is thought to have α-neo-endorphin sequence at its C-terminal on the immunological basis. Extracts from other dissected regions also gave patterns similar to that of hypothalamic extract, both in gel-filtration and in HPLC. Consequently, ir-α-neo-endorphin values determined in the present RIA was evidenced to substantially represent the immunoreactivity due to α-neo-endorphin itself. It was also revealed that α-neo-endorphin exists really as an endogenous ligand in brain and pituitary . In addition, α-neo-endorphin in rat brain was confirmed to be identical with that of porcine origin.

Table 1 summarizes the regional distribution in rat brain and pituitary of ir-α-neo-endorphin thus determined. α-Neo-endorphin was found predomi-

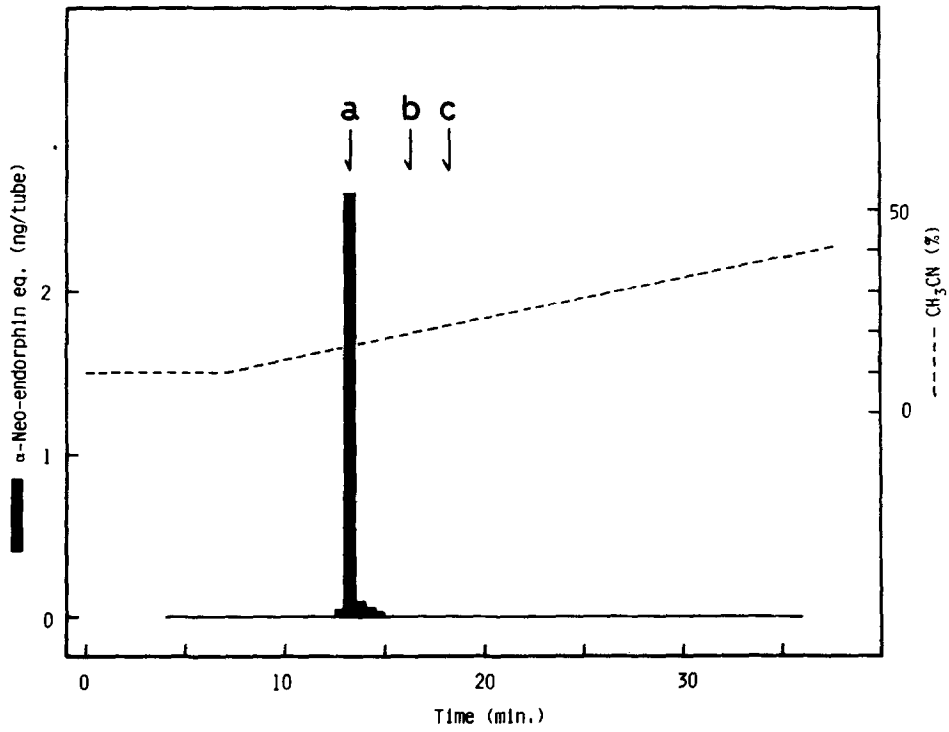

Fig. 4. Reverse phase HPLC of immunoreactive α-neo-endorphin obtained by Sephadex G-75 gel-filtration of rat hypothalamic extract.
Sample : Black bar area in Fig. 3. Flow rate : 2.0 ml/min.
Column : 4.0 x 250 mm, LS-410 ODS SIL (C-18, Toyosoda Co. Ltd.)
Solvent system : A linear gradient elution from (A) to (B) (40 min).
(A) 50 mM KH_2PO_4 (pH 2.0) : CH_3CN = 90 : 10 (v/v)
(B) 50 mM KH_2PO_4 (pH 2.0) : CH_3CN = 50 : 50 (v/v)
The arrows indicate the elution positions of (a) α-neo-endorphin, (b) β-neo-endorphin and (c) PH-8P, respectively.

nantly in pituitary, especially much concentrated in posterior lobe. Of the total immunoreactivity in pituitary, which corresponded to about 9.4% of that in the whole brain, 77.5% was found in posterior lobe. In brain, α-neo-endorphin is widely distributed with rather low concentration to compare with that in pituitary but significant. Of the total ir-α-neo-endorphin in brain, 22.9% is in cortex. However, the order of ir-α-neo-endorphin concentration is: hypothalamus >> striatum = hippocampus > midbrain = medulla-pons > cortex > olfactory bulbs > cerebellum. The highest concentration in brain was found in hypothalamus. It has been reported that dynorphin is also most concentrated in posterior lobe of pituitary, while it is distributed in brain with lower concentrations (12). However, it should be noted that the content of ir-α-neo-endorphin in the whole brain is about five times as high as that of

Table 1. Distribution of immunoreactive α-neo-endorphin in rat brain and pituitary.

Region	Wet tissue weight (mg)	pmol/g wet tissue	pmol/region	% of total ir-α-neo-endorphin
Brain total	2004.5 ± 47.3	24.6 ± 1.6	49.37	100.0
Olfactory bulbs	89.3 ± 5.7	1.3 ± 0.8	0.12	0.2
Hippocampus	97.3 ± 5.8	45.7 ± 4.5	4.45	9.0
Striatum	85.3 ± 4.8	50.2 ± 5.3	4.28	8.7
Cortex	810.3 ± 100.1	13.9 ± 3.0	11.30	22.9
Cerebellum	285.3 ± 6.7	0.5 ± 0.3	0.15	0.3
Midbrain-thalamus	218.3 ± 10.0	28.3 ± 5.6	6.17	12.5
Medulla-pons	269.0 ± 8.2	22.3 ± 2.6	5.99	12.1
Hypothalamus	149.7 ± 31.6	113.0 ± 13.2	16.91	34.3
Pituitary total	9.2 ± 0.1	500.5 ± 55.1	4.62	100.0
Anterior lobe	7.7 ± 0.1	135.0 ± 58.1	1.04	22.5
Posterior lobe	1.5 ± 0.2	2386.0 ± 316.0	3.58	77.5

Data are mean values ± standard deviation expressed as α-neo-endorphin equivalents (pmol) in five Wistar rats (mean body weight: 317 ± 27 g).

ir-dynorphin. Especially, hypothalamus contains α-neo-endorphin about ten times more than dynorphin. Additionally, the order of concentration in brain reported for dynorphin, i.e., hypothalamus >> medulla-pons = midbrain > hippocampus > striatum > cortex > cerebellum, is different from the order of α-neo-endorphin concentration described above. Furthermore, distribution of ir-α-neo-endorphin in brain is also different from that reported for Leu-enkephalin (13), which is most concentrated in striatum and hypothalamus and least in hippocampus, cortex and cerebellum.

α-Neo-endorphin and dynorphin, both of which have a Leu-enkephalin sequence, followed by a characteristic basic amino acid linkage such as Arg-Lys and Arg-Arg, are presumed to be intermediates to Leu-enkephalin from the structural basis. However, questions as to whether Leu-enkephalin originates from these peptides, and as to whether a common precursor to α-neo-endorphin and dynorphin exists in brain, are still to be solved.

In relation to Leu-enkephalin biosynthesis, further characterization of ir-α-neo-endorphin of 15,000 daltons, observed as a minor peak in gel-filtration, is now going on.

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