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Differential processing of prodynorphin and proenkephalin in specific regions of the rat brain

(dynorphins/neo-endorphins/[Leu]enkephalin/[Met]enkephalin-Arg^6-Gly^7-Leu^8/rat brain nuclei)

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ABSTRACT **Prodynorphin-derived peptides [dynorphin A (Dyn A)-(1–17), Dyn A-(1–8), Dyn B, α-neo-endorphin, and β-neo-endorphin] and proenkephalin-derived peptides {[Leu]enkephalin ([Leu]Enk) and [Met]enkephalin-Arg^6-Gly^7-Leu^8 ([Met]Enk-Arg-Gly-Leu)} in selected brain areas of the rat were measured by specific radioimmunoassays. We report here that different regions of rat brain contain strikingly different proportions of the prodynorphin and proenkephalin-derived peptides. There is a molar excess of α-neo-endorphin-derived peptides over Dyn B and Dyn A-derived peptides in many brain areas. [Leu]Enk concentrations exceed those of [Met]Enk-Arg-Gly-Leu in certain brain areas such as the substantia nigra, dentate gyrus, globus pallidus, and median eminence (areas rich in dynorphin-related peptides). These results indicated that (*i*) there is differential processing of prodynorphin in different brain regions and (*ii*) [Leu]Enk may be derived from Dyn A or Dyn B (or both). In certain brain regions [Leu]Enk may derive from two separate precursors (prodynorphin and proenkephalin) in two distinct neuronal systems.**

The enkephalins are present in brain not only as the free pentapeptides but also as parts of larger polypeptides of varying sizes (1, 2). Enkephalin sequences are found in at least three distinct precursors (3–6). Prodynorphin contains three copies of [Leu]enkephalin ([Leu]Enk), one each in α-neo-endorphin (7), dynorphin A (Dyn A)-(1–17) (8), and dynorphin B (Dyn B) (6, 9). Proenkephalin contains four copies of [Met]enkephalin ([Met]Enk) and single copies of [Leu]Enk, [Met]Enk-Arg^6-Gly^7-Leu^8 ([Met]Enk-Arg-Gly-Leu), and [Met]Enk-Arg^6-Phe^7, which are contained within larger peptide sequences such as BAM 22P and peptides I, F, E, and B (1, 4, 5). It is clear that the enkephalin- and dynorphin-containing neuronal systems in brain are anatomically distinct from each other and from the β-endorphin-containing neuronal systems (10–20). However, because of the existence of overlapping enkephalin and dynorphin/neo-endorphin circuits in the hypothalamus, hippocampus, and presumably other brain regions, the relationship between these two neuronal systems needs further clarification. In the present study, prodynorphin and proenkephalin-derived peptides were measured, by specific RIAs, in selected areas of the rat brain. These two polypeptide families are differentially distributed throughout the brain. [Leu]Enk probably derives from proenkephalin in some brain areas and from prodynorphin in others, or from both precursors.

MATERIALS AND METHODS

Preparation of Extracts for RIAs. Rats (Sprague–Dawley, male, 220–250 g) were killed by decapitation (between 08:00 and 10:00 hr). The brains were quickly removed and frozen on dry ice. Brain regions were removed by the micropunch technique, from 300-μm thick frozen coronal sections cut in a cryostat at −10°C (21). Tissue samples were placed in Eppendorf tubes containing 200 μl of 0.1 M HCl and transferred to a boiling water bath for 10 min. Samples were chilled in ice and then homogenized by sonication, and 20-μl aliquots of the homogenates were removed for protein determination (22). The extracts were centrifuged at 2000 × *g* for 10 min at 4°C. The supernatants were transferred to 12 × 75 mm polypropylene or polystyrene tubes (the latter for RIAs of [Leu]Enk and [Met]Enk-Arg-Gly-Leu) and evaporated to dryness in a vacuum centrifuge.

RIAs. Samples were rehydrated in phosphate-buffered saline (pH 7.6) containing 0.1% gelatin, 0.1% bovine serum albumin, 0.1% Triton X-100, and 0.01% merthiolate. Antisera were used at final dilutions of 1:100,000 for Dyn A, 1:120,000 for Dyn A-(1–8) and Dyn B, 1:60,000 for α-neo-endorphin, 1:10,000 for β-neo-endorphin, 1:100,000 for [Leu]Enk, and 1:75,000 for [Met]Enk-Arg-Gly-Leu, which resulted in 30–45% uncompeted binding of the trace. The RIA for Dyn A, Dyn A-(1–8), Dyn B, α-neo-endorphin, and β-neo-endorphin was used as described (12–16). A double-antibody RIA for [Leu]Enk and [Met]Enk-Arg-Gly-Leu was performed as described here. Each sample was incubated in a 500-μl volume that contained 300 μl of sample in assay buffer and 100 μl of ^{125}I-labeled opioid peptide (about 6000 cpm). Normal rabbit serum (NRS) was also added to the first antibody solution to give a total rabbit serum concentration of 1%; 100 μl of this solution was added to all except "nonspecific binding" tubes, which received 100 μl of 1% NRS in assay buffer. Reagents were mixed and the tubes were incubated for 16–24 hr. One hundred microliters of goat anti-rabbit gamma globulin diluted with assay buffer to a concentration sufficient for maximal precipitation was added to all tubes. The reagents were mixed and the tubes were incubated for 16–24 hr. After incubation with the second antibody, all tubes were centrifuged for 20 min at 2000 × *g* at 4°C. The supernatant was aspirated and pellets were assayed for radioactivity (γ-counter). The RIA could detect <4 pg per tube for all opioid peptides except β-neo-endorphin. The sensitivity for β-neo-endorphin was <8 pg per tube.

Specificity. The specificities of the antisera used in this study have been described. Briefly, Dyn A antiserum ["Lucia"; ref. 23 (a generous gift of A. Goldstein)] was raised against Dyn A-(1–13). It does not crossreact with Dyn A-(1–8), [Leu]Enk, α-neo-endorphin, β-neo-endorphin, Dyn B, Dyn B-(1–29), [Met]Enk, [Met]Enk-Arg-Gly-Leu, β-endorphin, or α-endorphin. It recognizes Dyn A-(1–13) or its methyl esther and Dyn A equally well, but the molar crossreactivity of Dyn-(1–32) is only 36%. Dyn B antiserum (19) does not crossreact with [Leu]Enk, [Met]Enk, Dyn A, α-neo-endorphin, β-neo-endorphin, or Dyn A-(1–8). Dyn B antiserum shows partial crossreactivity towards Dyn B-(1–29). Dyn B-

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Abbreviations: Dyn A, dynorphin A; Dyn B, dynorphin B; [Leu]Enk, [Leu]enkephalin; [Met]Enk, [Met]enkephalin.

(1–29) exhibits a nonparallel displacement curve in the RIA. At ED_{80}, Dyn B antiserum exhibits 16% crossreactivity towards Dyn B-(1–29) and, at ED_{50}, it exhibits <0.007% crossreactivity towards Dyn B-(1–29). Antisera raised against Dyn A-(1–8), α-neo-endorphin, and β-neo-endorphin are directed against the COOH-terminal portion of each peptide and do not tolerate COOH-terminal extension. Dyn A-(1–8) antiserum (24) does not recognize Dyn A, Dyn A-(1–13), [Leu]Enk, α-neo-endorphin, or β-neo-endorphin. The crossreactivity of Dyn A-(1–9) is only 1%. It does not crossreact with [Met]Enk-Arg-Gly-Leu. The crossreactivity of β-neo-endorphin with α-neo-endorphin and α-neo-endorphin with β-neo-endorphin is <1%. The neo-endorphin antisera (25) do not recognize the enkephalins or dynorphins. The [Leu]Enk antiserum (26) shows 0.35% crossreactivity towards [Met]-Enk, <0.01% towards Dyn A-(1–13) and Dyn A-(1–8), <0.02% towards Dyn A-(1–6), <0.04% towards Dyn A-(1–7), and <0.001% towards Dyn A, α-neo-endorphin, and [Met]Enk-Arg-Gly-Leu. The antiserum showed no crossreactivity with β-neo-endorphin, Dyn A-(6–17), and Dyn A-(1–9) when the highest concentration of the unlabeled peptide tested was 1 μM. The [Met]Enk-Arg-Gly-Leu antiserum (27) crossreacts <0.005% with [Leu]Enk, [Met]Enk, [Met]Enk-Arg, [Met]Enk-Arg-Arg, [Met]Enk-Arg-Phe, BAM 12P, Dyn A-(1–8), β-neo-endorphin, and human β-endorphin at a concentration of 1 μM.

Peptides. All peptides were purchased from Peninsula Laboratories (San Carlos, CA).

Iodination. All peptides were labeled with ^{125}I by the chloramine-T method (28). The reaction was stopped with sodium metabisulfite. Labeled peptides were purified by chromatography on Sep-Pak C_{18} cartridges (Waters Associates), with an increasing gradient of methanol in 0.01 M HCl/0.1 M acetic acid solution.

RESULTS

The distributions of prodynorphin- and proenkephalin-derived peptides in selected areas of the rat brain measured by RIA are shown in Table 1. Both prodynorphin- and proenkephalin-derived peptides are widely but unevenly distributed in brain. The pattern of distribution of α-neo-endorphin correlated well with that of Dyn B. [Leu]Enk and [Met]Enk-Arg-Gly-Leu have similar distribution patterns. The highest concentrations of prodynorphin-derived peptides in the rat brain are in the substantia nigra [α-neo-endorphin, Dyn B, and Dyn A-(1–8)] and median eminence (Dyn A and β-neo-endorphin). The lateral preoptic area is relatively rich in all prodynorphin-derived peptides measured. The highest concentrations of [Leu]Enk and [Met]Enk-Arg-Gly-Leu are in the globus pallidus, followed by the central amygdaloid nucleus. The frontal cortex has low concentrations of prodynorphin- and proenkephalin-derived peptides.

DISCUSSION

In this study we determined the distribution of five peptides derived from prodynorphin and one peptide derived from proenkephalin. In addition, we examined the distribution of [Leu]Enk, a peptide that could potentially come from either prodynorphin or proenkephalin.

Processing of Proenkephalin. The fragments of proenkephalin that arise from its proteolytic processing are depicted

Table 1. Comparative distribution of immunoreactive prodynorphin- and proenkephalin-derived peptides in rat central nervous system

Region	Opioid peptide, fmol/mg of protein						
	α-Neo-endorphin	β-Neo-endorphin	Dyn A	Dyn A-(1–8)	Dyn B	[Leu]Enk	[Met]Enk-Arg-Gly-Leu
Frontal cortex	53.8 ± 6.3 (6)	72.2 ± 4.6 (5)	42.1 ± 11.0 (3)	101.1 ± 23.1 (5)	82.1 ± 13.2 (6)	126.7 ± 30.4 (6)	96.6 ± 18.0 (6)
Caudate putamen	235.6 ± 19.0 (6)	70.4 ± 12.3 (6)	54.2 ± 6.4 (6)	106.2 ± 16.3 (6)	140.4 ± 21.0 (6)	470.6 ± 68.4 (6)	549.9 ± 80.8 (6)
Globus pallidus	624.5 ± 101.7 (6)	104.7 ± 12.8 (6)	75.7 ± 14.6 (6)	154.9 ± 37.5 (5)	250.5 ± 17.6 (6)	5190.0 ± 952.0 (6)	4378.8 ± 351.1 (6)
Central amygdaloid nucleus	424.5 ± 65.3 (6)	226.1 ± 28.9 (6)	98.4 ± 13.7 (6)	ND	290.2 ± 32.4 (6)	1213.1 ± 302.7 (6)	2525.7 ± 435.0 (6)
Bed nucleus of stria terminalis	454.1 ± 53.3 (6)	160.5 ± 25.5 (6)	107.2 ± 12.5 (6)	146.7 ± 26.3 (5)	307.9 ± 27.8 (6)	875.1 ± 238.4 (6)	1669.0 ± 248.1 (6)
Dentate gyrus	676.3 ± 131.8 (6)	126.2 ± 26.6 (6)	80.4 ± 6.7 (4)	ND	448.8 ± 97.3 (6)	126.9 ± 29.0 (6)	58.9 ± 12.3 (6)
Lateral preoptic area	1002.3 ± 260.6 (6)	278.8 ± 49.4 (6)	169.2 ± 16.0 (5)	565.1 ± 64.6 (6)	581.3 ± 148.1 (6)	936.5 ± 116.8 (6)	1545.3 ± 211.7 (6)
Supraoptic nucleus	186.7 ± 15.2 (6)	176.2 ± 21.4 (6)	76.5 ± 6.8 (12)	170.4 ± 24.5 (5)	152.6 ± 12.5 (6)	354.1 ± 54.0 (5)	383.2 ± 42.9 (6)
Paraventricular nucleus	453.0 ± 43.1 (6)	266.3 ± 29.9 (6)	128.6 ± 8.0 (28)	178.3 ± 31.7 (6)	310.4 ± 24.0 (6)	689.8 ± 137.3 (6)	818.0 ± 106.0 (6)
Median eminence	521.2 ± 56.7 (5)	341.4 ± 30.7 (5)	186.1 ± 15.9 (22)	197.6 ± 24.7 (5)	386.1 ± 30.6 (5)	591.1 ± 70.4 (6)	463.4 ± 66.8 (6)
Substantia nigra	1692.1 ± 430.6 (6)	312.9 ± 47.1 (6)	98.4 ± 7.5 (27)	673.8 ± 164.2 (6)	1106.2 ± 229.2 (6)	539.9 ± 100.2 (6)	244.2 ± 22.0 (6)
Ventral tegmental area	152.9 ± 29.5 (6)	133.5 ± 26.7 (6)	52.3 ± 7.8 (5)	177.2 ± 15.8 (5)	133.9 ± 23.4 (6)	334.7 ± 36.7 (6)	526.8 ± 56.8 (5)
Periaqueductal central gray	420.3 ± 42.5 (6)	288.6 ± 30.5 (6)	90.4 ± 19.6 (6)	216.7 ± 74.9 (6)	388.0 ± 44.3 (6)	808.2 ± 143.6 (6)	1033.1 ± 115.0 (6)
Parabrachial nuclei	665.7 ± 68.5 (6)	294.3 ± 34.0 (6)	116.4 ± 18.1 (12)	133.1 ± 29.9 (6)	452.4 ± 34.3 (6)	822.0 ± 143.6 (6)	1068.1 ± 120.9 (6)
Locus coeruleus	301.0 ± 40.4 (6)	170.5 ± 22.7 (6)	100.7 ± 12.5 (12)	181.6 ± 31.4 (5)	217.6 ± 23.3 (6)	657.0 ± 133.9 (6)	854.7 ± 123.0 (6)
Nucleus of the solitary tract (medial part)	447.4 ± 43.4 (6)	278.5 ± 60.8 (6)	92.1 ± 13.6 (12)	159.7 ± 14.5 (5)	289.9 ± 36.4 (6)	890.2 ± 168.3 (6)	1298.2 ± 166.0 (6)

Data are means ± SEM. *n* values are given in parentheses. ND, not determined.

in Fig. 1*A*, which shows that [Leu]Enk and [Met]Enk-Arg-Gly-Leu are found in the precursor itself in equimolar amounts (1, 4, 5), and one would predict that in the steady state these two peptides would be present in the cell in equimolar concentrations. [Met]Enk-Arg-Gly-Leu, which contains the fourth [Met]Enk sequence in proenkephalin, seems to be derived from a 5.3 kDa species. [Leu]Enk forms a part of two intermediates, peptide I and peptide E, which are 4.9 and 3.2 kDa, respectively (1).

The ratio of [Met]Enk-Arg-Gly-Leu to [Leu]Enk varies from 0.5 to 2.1 (the average figure is about 1.2). Variations in the steady-state concentrations of these two peptides could result from differences in their rate of release, generation, or intracellular breakdown (2). It is possible that peptides residing in the same granule might be secreted selectively and that the intracellular breakdown of various enkephalins may pro-

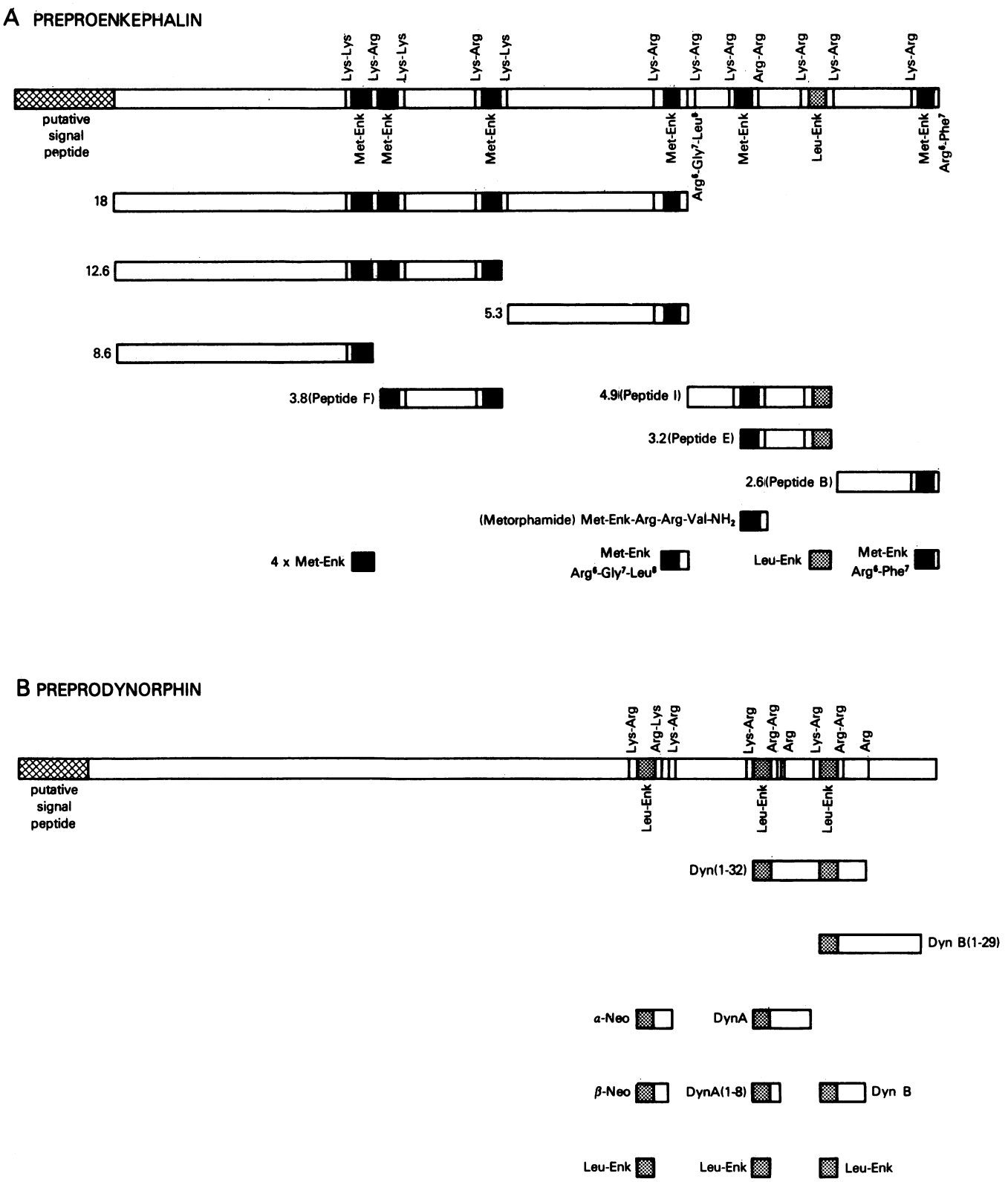

FIG. 1. Processing of enkephalin precursors (given in kDa).

ceed at different rates, but these phenomena have not been demonstrated. We feel that the most likely explanation for the regional differences observed is that processing rates vary. If so, one would predict that the ratios of the concentrations of peptides I and E + "pro-[Met]Enk-Arg-Gly-Leu" would also vary from region to region and that these ratios would bear an inverse relationship to the [Leu]Enk to [Met]-Enk-Arg-Gly-Leu ratio. The above statement would be true if prodynorphin contributed little or no [Leu]Enk to a brain region; but this may not be the case.

Processing of Prodynorphin. The prodynorphin precursor (Fig. 1*B*) contains three [Leu]Enk sequences that are parts of α-neo-endorphin, Dyn A, and Dyn B, respectively (6–9). It is evident that the neo-endorphins, Dyn A, and Dyn B should be made in equimolar amounts and that the sums of the concentrations of each of these peptides and their respective products should be equal. Typically, α-neo-endorphin + β-neo-endorphin concentrations are greater than Dyn A + Dyn A-(1–8) or Dyn B concentrations. This suggests that Dyn A and Dyn B may give rise to peptides other than Dyn A-(1–8), specifically [Leu]Enk.

In certain regions, such as the substantia nigra, the ratio of [Leu]Enk to [Met]Enk-Arg-Gly-Leu is especially high. The levels of prodynorphin derivatives in the substantia nigra are also high. It seems likely that a significant portion of the [Leu]Enk in the substantia nigra is in prodynorphin-containing processes. Indeed, using deafferentation experiments isolating globus pallidus from caudate putamen, we have suggested that [Leu]Enk in the striato-nigral pathway is derived from prodynorphin-containing neurons, and in the striato-pallidal pathway [Leu]Enk is derived mainly from neurons containing proenkephalin (29). Thus, [Leu]Enk in the brain can be derived from either proenkephalin or prodynorphin.

Prodynorphin-derived peptides are potent κ-opiate receptor agonists, whereas [Leu]Enk is a δ-opiate receptor agonist (30). Thus, the same precursor (prodynorphin) may yield ligands for different opiate receptor subtypes that can exert different actions.

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