Enkephalin convertase localization by [³H]guanidinoethylmercaptosuccinic acid autoradiography: Selective association with enkephalin-containing neurons

(peptide processing/carboxypeptidase/magnocellular hypothalamus/hippocampus/proenkephalin)

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ABSTRACT Enkephalin convertase, an enkephalin-forming carboxypeptidase, is potentially inhibited by guanidinoethylmercaptosuccinic acid (GEMSA). We have localized enkephalin convertase in rat brain by *in vitro* autoradiography with [³H]GEMSA. [³H]GEMSA-associated silver grains are highly concentrated in the median eminence, bed nucleus of the stria terminalis, lateral septum, dentate gyrus, hippocampus, central nucleus of the amygdala, preoptic hypothalamus, magnocellular nuclei of the hypothalamus, interpeduncular nucleus, dorsal parabrachial nucleus, locus coeruleus, nucleus of the solitary tract, and the substantia gelatinosa of the spinal trigeminal tract. This distribution corresponds closely with immunocytochemical localizations of enkephalin-containing cells and axons, indicating that enkephalin convertase is selectively involved in enkephalin biosynthesis.

Most biologically active peptides are derived from large protein precursors in which they are flanked by pairs of basic amino acids (1). The successive actions of a trypsin-like enzyme and a carboxypeptidase B-like enzyme can yield the biologically active peptide. The opioid peptides, enkephalins, are produced in this manner from proenkephalin A and proenkephalin B (2–5). Numerous carboxypeptidases can generate hormonal and neurotransmitter peptides, including enkephalins, *in vitro* (6, 7). Whether each of these peptides is formed physiologically by highly selective and discretely localized carboxypeptidases or by ubiquitous, more generalized enzymes heretofore has been unclear. We described a carboxypeptidase B-like enzyme, designated enkephalin convertase, and purified it to homogeneity from brain, adrenal, and pituitary (8, 9). Its distribution within the brain and adrenal corresponds to the distribution of enkephalins (8–11). We identified inhibitors up to 1000-fold more potent in inhibiting enkephalin convertase than other carboxypeptidases (12). The tritiated form of one of these inhibitors, guanidinoethylmercaptosuccinic acid (GEMSA) binds selectively to membrane bound and soluble enkephalin convertase (13). We now have localized enkephalin convertase in rat brain by autoradiography with [³H]GEMSA. The localization of [³H]GEMSA binding sites corresponds closely to the distribution of enkephalinergic neurons, indicating that enkephalin convertase is selectively associated with enkephalin biosynthesis.

METHODS

[³H]GEMSA was obtained from Steve Hurt (New England Nuclear) and carboxypeptidase inhibitors were the generous gift of Thomas Plummer, Jr. Male Sprague–Dawley rats (7 weeks old, 150–200 g) were anesthetized, perfused, and sec-

tioned by the method of Young and Kuhar (14) as modified by Strittmatter *et al.* (15). For autoradiography, sections were incubated at 4°C in 0.05 M sodium acetate (pH 5.6) for 5 min and then in the same buffer with 4 nM [³H]GEMSA and any inhibitors for 30 min. Nonspecific binding was determined in the presence of 10 μ M unlabeled GEMSA. The slides were washed twice for 1 min in sodium acetate (pH 5.6) at 4°C, dipped in water, and dried rapidly under a stream of air. The slides were desiccated overnight and applied to LKB Ultrafilm or photographic emulsion-coated coverslips for 12 days at 4°C. Silver grain densities in the films were quantified with single beam densitometry or with a computer-assisted image analysis system (Loats Associates, Westminster, MD) and converted to fmol of [³H]GEMSA bound per mg of protein, using standards (16, 17). The sections were stained with 0.1% toluidine blue.

In saturation experiments, serial 8- μ m sections were incubated with 20 nM, 10 nM, 5 nM, 2.5 nM, 1.2 nM, or 0.6 nM [³H]GEMSA as described above. Binding varied by <20% in two sections of the lateral septum.

RESULTS

Binding Properties of [³H]GEMSA to Rat Brain Tissue Sections. Binding of [³H]GEMSA to rat brain tissue sections is saturable and specific. Nonspecific binding in the presence of 10 μ M unlabeled GEMSA is negligible (Fig. 1D), so that all binding is specific. Saturation analysis yields a K_d of 4.6×10^{-9} M and a B_{max} of 3.7 pmol per mg of protein in the lateral septum. This K_d value agrees with the K_d values of $3\text{--}5 \times 10^{-9}$ M found in binding to homogenates and the K_i value of 8×10^{-9} M for enkephalin convertase activity (10, 13).

Carboxypeptidase inhibitors have similar potencies at inhibiting enkephalin convertase and [³H]GEMSA binding to tissue sections or homogenates (Table 1). Guanidinopropylsuccinic acid and 2-mercaptomethyl-3-guanidinoethylthiopropionic acid are extremely potent inhibitors of enkephalin convertase with K_i values in the low nanomolar range (12, 13). Enkephalin convertase activity is inhibited by 1,10-phenanthroline because of its metal dependence (11). Similar inhibition is observed in binding to tissue sections and to homogenates (13). Together with the saturation analysis, these results show that [³H]GEMSA binds selectively to enkephalin convertase under autoradiography conditions.

Regional Localization of [³H]GEMSA Binding. Within the telencephalon, [³H]GEMSA binding is high in the lateral septum, the bed nucleus of the stria terminalis, the diagonal band, the amygdala, and the hippocampal formation (Fig. 1). Although all of the amygdaloid nuclei display binding, the central nucleus is most densely labeled (Fig. 1E). In the cerebral cortex, the piriform cortex has 3 times as much labeling

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Abbreviation: GEMSA, guanidinoethylmercaptosuccinic acid.
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FIG. 1. Distribution of [^3H]GEMSA binding in rat brain. Rat brain sections were incubated in 4 nM [^3H]GEMSA and apposed to LKB-Ultrafilm. Pictures were printed directly from Ultrafilm, so white areas have high levels of [^3H]GEMSA binding. (A) The most densely labeled areas are the lateral septum (S) and the piriform cortex ($\rightarrow$). The caudate putamen (C) and the frontal cortex are less densely labeled. (B) The densely labeled areas are the bed nucleus of the stria terminalis (b), olfactory tubercle (t), and the rostral hippocampus ($\blacktriangleright$); the globus pallidus (g) is moderately labeled. The anterior commissure (ac) is unlabeled. (C) Labeling in the hippocampus is high in both the dentate gyrus and the area around pyramidal cell region CA3-4 ($\blacktriangleright$). Labeling in the stria terminalis is also visible ($\rightarrow$). The preoptic hypothalamus (h) is labeled much more densely than the thalamus (th). In the thalamus, labeling is highest in the periventricular nucleus. (D) Binding in the presence of 10 μM unlabeled GEMSA. Nonspecific binding is negligible. (E) The area of highest binding is the median eminence ($\blacktriangleright$). Several nuclei of the amygdala are labeled, but labeling in the central nucleus (ca) is the most intense. The habenula (hb) is also labeled. (F) Labeling is present in the dorsal parabrachial nucleus (p) and the nucleus of the solitary tract (st). The dentate gyrus ($\blacktriangleright$) and hippocampus ($\rightarrow$) are distinctly labeled. Labeling is low in the cerebellum.

as the frontal cortex (Fig. 1 A and B). Binding in the dentate gyrus is higher in the granule cell layer than in the molecular layer. In the hippocampus, region CA3-4 is labeled more than CA1 or CA2 (Fig. 1 C and E). Under higher magnification, more silver grains are seen over the mossy fibers of the stratum lucidum surrounding CA3-4 than over the pyramidal cells (data not shown).

In the diencephalon, [^3H]GEMSA binding is generally greater in the hypothalamus than in the thalamus (Fig. 1C). Of the hypothalamic nuclei, labeling is densest in the supraoptic nucleus and the magnocellular portion of the paraventricular nucleus (Fig. 2). Neurons in the magnocellular

portions of these nuclei project to the posterior pituitary, where enkephalin convertase activity is enriched (11). The medial basal region of the hypothalamus, including the arcuate nucleus, and the preoptic hypothalamus are also labeled with [^3H]GEMSA. Binding in the thalamus is greatest in the periventricular nucleus and the nucleus reuniens.

Labeling is also present in various midbrain and brainstem regions, including the periaqueductal grey matter, the substantia nigra, the interpeduncular nucleus, the dorsal parabrachial nucleus, the locus coeruleus, and the nucleus of the solitary tract (Fig. 1F). However, binding is low in both the pontine nuclei and the cerebellar cortex. Although

Table 1. Effect of carboxypeptidase inhibitors on [³H]GEMSA binding and enkephalin convertase activity

Inhibitor	[³ H]GEMSA binding		Enkephalin convertase activity
	Autoradiography	Homogenate	
<i>K_i</i> , ×10 ⁻⁹ M			
Exp. A			
GPSA	5	2	8
GEMSA	14	6	8
MGTA	22	5	44
APMSA	330	1000	400
% control activity			
Exp. B			
1,10-Phe- nanthroline	12	9	5

For Exp. A, sections were incubated in 4 nM [³H]GEMSA with inhibitor concentrations varying by factors of 5. The inhibitors used were guanidinopropylsuccinic acid (GPSA), GEMSA, 2-mercaptomethyl-3-guanidinoethylthiopropionic acid (MGTA), and aminopropylmercaptosuccinic acid (APMSA). The sections were apposed to Ultrafilm. Microdensitometry was measured in the central nucleus of amygdala. Concentrations producing 50% inhibition were determined graphically, and the *K_i* values were computed using a *K_d* value of 5 × 10⁻⁹ M for [³H]GEMSA. For Exp. B, 1 mM 1,10-phenanthroline was included in the incubation with 4 nM [³H]GEMSA. Values for homogenate binding and enkephalin convertase activity are taken from refs. 9, 11, and 13.

[³H]GEMSA-associated grains occur throughout the grey matter of the spinal cord, labeling is greatest in the substantia gelatinosa (Fig. 3). The analogous region of the nucleus of the spinal tract of the trigeminal nerve also displays a high concentration of grains (Fig. 3).

FIG. 2. [³H]GEMSA binding in the hypothalamic magnocellular nuclei. (A) [³H]GEMSA labeling of the hypothalamus. (×19.) Extremely dense labeling is present bilaterally in the paraventricular (upper arrow) and supraoptic (lower arrow) nuclei, but no labeling is found in the fornix (f) or the optic tract (ot). In the paraventricular nucleus, binding is higher in the lateral magnocellular portion of the nucleus as compared to the medial parvocellular portion of the nucleus. (B) Photomicrograph of the same section stained with 0.1% toluidine blue. (×19.)

FIG. 3. [³H]GEMSA binding in the substantia gelatinosa. Sections of rat medulla and spinal cord are incubated and exposed. In the caudal medulla (A), labeling is concentrated in the substantia gelatinosa of the spinal trigeminal tract. In the spinal cord (B), labeling is found throughout the grey matter, but it is highest in the dorsal horn containing the substantia gelatinosa.

DISCUSSION

In vitro autoradiography with [³H]GEMSA localizes enkephalin convertase to enkephalin-containing regions of the brain (Table 2). All regions containing enkephalin convertase have detectable amounts of peptides derived from at least one enkephalin precursor, and all areas with high levels of enkephalin-like immunoreactivity (18–21) except the corpus striatum contain enkephalin convertase. Very little enkephalin convertase is detected in areas such as the pontine nuclei and the cerebellum, which have low levels of enkephalin.

Several enkephalinergic pathways elegantly illustrate the colocalization of enkephalin convertase and enkephalins. One enkephalinergic pathway projects from the central nucleus of the amygdala through the stria terminalis to the bed nucleus of the stria terminalis (22). By autoradiography, we find high levels of enkephalin convertase in the central nucleus of the amygdala, the bed nucleus of the stria terminalis, and the stria terminalis itself. Another pathway expressing proenkephalin B-derived peptides projects from the granule cells of the dentate gyrus through the mossy fibers to the striatum lucidum of CA3–4 of the hippocampus (23–25). [³H]GEMSA binding is concentrated in both the dentate gyrus and striatum lucidum of CA3–4, but it is much lower in CA1–2 of the hippocampus.

Only in the corpus striatum does the localization of enkephalin convertase not correlate with enkephalin localization. While enkephalin convertase is present in low levels in the caudate and in moderate levels in the globus pallidus, enkephalin-like immunoreactivity is present in both of these regions. Enkephalin-containing cells are diffusely distributed in the caudate and project into the globus pallidus, which contains very high enkephalin levels (26, 27). Some studies find only faint staining of enkephalin-containing cells in the caudate, consistent with the lower levels of enkephalin convertase in this region (18, 20, 28, 29).

Other neuropeptides, such as substance P and neurotensin, possess regional localizations similar to those of enkephalin. A review of the comparative distribution of enkephalin, substance P, and neurotensin indicates that [³H]GEMSA binding corresponds much more closely to localizations of enkephalin than substance P or neurotensin (30, 31). For instance, the hippocampus is highly enriched in enkephalin convertase and enkephalinergic neurons, but it contains very little neurotensin or substance P.

Two distinct protein precursors of enkephalin have been described: proenkephalin A and B (2–5). In many areas of the brain, proenkephalin A- and B-derived peptides occur together. However, in several areas their distributions can be discriminated (Table 2). [³H]GEMSA binding is observed both in proenkephalin A-expressing regions, such as the interpeduncular nucleus, the habenula, the periventricular nucleus of the thalamus, and the piriform cortex, and in proenkephalin B-expressing regions, such as the striatum lucidum of the hippocampus and the hypothalamic magnocellular nuclei. This indicates that enkephalin convertase processes

Table 2. Regional distribution of [³H]GEMSA binding and enkephalin precursors

Region	[³ H]GEMSA, pmol/mg of protein	Pro. A	Pro. B	Region	[³ H]GEMSA, pmol/mg of protein	Pro. A	Pro. B
Telencephalon				Mesencephalon			
Lateral septum	2.25	+	+	Interpeduncular nucleus	1.38	+	0
Medial septum	0.72	+	NR	Substantia nigra, pars compacta	0.75	+	0
Caudate nucleus	0.36	+	+	Periaqueductal grey	0.81	+	+
Nucleus accumbens	0.59	+	+	Superior colliculus	0.65	+	NR
Globus pallidus	0.71	+	+	Inferior colliculus	0.93	+	0
Nucleus of diagonal band	1.51	+	NR	Metencephalon			
Bed nucleus stria terminalis	1.97	+	+	Dorsal tegmental nucleus	0.65	+	NR
Olfactory tubercle	0.85	+	+	Locus coeruleus	0.88	+	+
Piriform cortex, layer II	1.47	+	0	Dorsal parabrachial nucleus	1.21	+	+
Frontal cortex	0.47	Weak	+	Pontine nuclei	0.41	0	NR
Amygdala				Cerebellum			
Anterior	1.01	+	NR	Cortex	0.23	+	NR
Medial	1.02	+	NR	White matter	0.12	0	NR
Lateral	1.03	+	NR	Myelencephalon			
Central	1.89	+	+	Nucleus of solitary tract	1.15	+	+
Hippocampus				Substantia gelatinosa of trigeminal nerve	0.88	+	+
CA3-4 (including stratum lucidum)	1.77	0	+	Spinal cord			
CA1-2	0.75	+	NR	Grey matter	0.45	+	+
Dentate gyrus	1.22	+	+	White matter			
Diencephalon				Stria terminalis	0.73	+	NR
Thalamus				Corpus callosum	0.20	0	NR
Periventricular	0.85	+	0	Anterior commissure	0.21	0	NR
Anterior	0.37	+	0				
Reuniens	1.09	+	NR				
Habenula	0.96	+	0				
Hypothalamus							
Preoptic	1.56	+	NR				
Anterior	0.96	+	+				
Paraventricular (magnocellular)	1.36	0	+				
Supraoptic	2.05	0	+				
Medial basal (including arcuate nucleus)	0.88	+	+				
Median eminence	3.10	+	NR				

Sections were incubated with 4 nM [³H]GEMSA and exposed. Densitometry was measured with a computer-assisted image analysis system. Localization of proenkephalin A-derived peptides (Pro. A) is from ref. 20, and localization of proenkephalin B-derived peptides (Pro. B) is from refs. 18 and 19. NR, not reported.

peptides derived from both proenkephalin A and proenkephalin B.

Since enkephalin convertase is present in both membrane and soluble forms, one may ask which form we have localized with autoradiography. Soluble angiotensin-converting enzyme is not detected by autoradiography with [³H]captopril (32), and autoradiographic conditions that solubilize the benzodiazepine receptor eliminate binding to tissue sections (33). This suggests that autoradiography reveals only the membrane-bound form of enkephalin convertase.

Taken together, our findings indicate that enkephalin convertase is selectively associated with the processing of enkephalin precursors to enkephalin. This does not mean that the enzyme serves no other functions. The anterior pituitary possesses high levels of enkephalin convertase activity but no detectable enkephalin (34). In brain regions containing enkephalins and other peptides, enkephalin convertase might also process other non-enkephalin peptide precursors.

Although it has been known for years that biologically active peptides are synthesized from large protein precursors, the question of whether certain processing enzymes are specific for individual peptides has been largely unanswered.

The regional correspondence of enkephalin convertase with enkephalins indicates that this enzyme primarily processes proenkephalin A and proenkephalin B. Similar selective processing enzymes may also exist for other neurotransmitter and hormonal peptides.

Enkephalin convertase is unique in its chemical properties as well as in its localization. Thus, GEMSA and other compounds are up to 1000-fold more potent inhibitors of enkephalin convertase than other carboxypeptidases (12). Such selectivity suggests that inhibitors of specific processing enzymes may block the biosynthesis of individual neuropeptides. The polar properties of GEMSA impede its entry into cells in culture (unpublished observations). Lipophilic inhibitors of enkephalin convertase may penetrate the brain and other organs to prevent enkephalin formation, clarifying mechanisms of enkephalin synthesis and facilitating development of therapeutic agents.

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