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# Calcitonin: Regional distribution of the hormone and its binding sites in the human brain and pituitary

(calcitonin fragments/hypothalamus)

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ABSTRACT **Immunoreactive calcitonin (CT), indistinguishable from human CT-(1–32) and its sulfoxide, has been identified in extracts of the hypothalamus, the pituitary, and the thyroid obtained from human subjects at autopsy. CT concentrations were highest in a region encompassing the posterior hypothalamus, the median eminence, and the pituitary; intermediate in the substantia nigra, the anterior hypothalamus, the globus pallidus, and the inferior colliculus; and low in the caudate nucleus, the hippocampus, the amygdala, and the cerebral and cerebellar cortices. Specific CT binding measured with $^{125}$I-labeled salmon CT was highest in homogenates of the posterior hypothalamus and the median eminence, shown to contain the highest concentrations of endogenous CT in the brain; CT binding was less than 12% of hypothalamic binding in all of the other regions of the brain examined and was negligible in the pituitary. Half-maximal binding was achieved with 0.1 nM nonradioactive salmon CT-(1–32), and the binding was directed to structural or conformational sites, or both, in the COOH-terminal half of salmon CT. The rank order of the inhibition of the binding by CT from different species and analogues of the human hormone was the same as in receptors on a human lymphoid cell line (Moran, J., Hunziker, W. & Fischer, J. A. (1978) *Proc. Natl. Acad. Sci. USA* 75, 3984–3988). The functional role of CT and of its binding sites in the brain remains to be elucidated.**

Calcitonin (CT) is a single chain polypeptide hormone of 32 amino acid residues that causes hypocalcemia by inhibition of the release of calcium from bone and stimulation of renal calcium excretion (1, 2). Human CT was isolated and its amino acid sequence was derived from tumor tissue of patients with medullary carcinoma of the thyroid (1). The origin of the CT-producing C cells in the neural crest (3) may suggest a possible function of CT in the central nervous system. Indeed, immunoreactive CT has been demonstrated in the nervous system of a primitive ascidian, the sea-squirt, primitive chordates, the lizard, and the pigeon, as well as in the pituitary and the adrenal gland (4–11). Among human neural tissues, immunoreactive CT has been detected in the cerebrospinal fluid and in extracts of the hypothalamus, pheochromocytoma, and a mucosal neuroma (12–16).

The present report describes measurement of immunoreactive CT concentration and distribution of CT binding sites in various regions of the human brain. Both endogenous CT and CT binding were found to be highest in the posterior hypothalamus and the median eminence. Immunoreactive CT in the brain, the pituitary, and the thyroid from normal subjects was eluted in high-performance liquid chromatography (HPLC) at the same positions as the synthetic monomeric CT-(1–32) and its sulfoxide.

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## MATERIALS AND METHODS

**Peptides and Reagents.** Synthetic salmon CT and fragments thereof were donated by W. Doepfner and S. Guttmann (Sandoz AG, Basel, Switzerland); synthetic human CT-(1–32) and fragments and analogues thereof and corticotropin-(1–39) [ACTH-(1–39); adrenocorticotropic hormone] and fragments thereof, by A. Jöhl and W. Rittel (Ciba–Geigy AG, Basel, Switzerland); human [$^3$H]CT-(1–32) (17), by R. Wade (Ciba–Geigy, Horsham, England); synthetic human CT-(1–32) sulfone, the extracted amino-terminal 16,000-dalton fragment of the rat ACTH-$\beta$-lipotropin precursor and synthetic bovine fragments-(1–36), -(2–36), -(9–36) thereof, and extracted bovine parathyroid hormone-(1–84), by H. Keutmann (Boston, MA); porcine CT-(1–32), by Armour Pharmaceutical (Phoenix, AZ); carcinoembryonic antigen, by H. Karmann (Hoffmann–La Roche AG, Basel, Switzerland); and naloxone, by Eli Lilly. Synthetic angiotensin I, $\beta$-endorphin, vasopressin, oxytocin, and substance P were purchased from Beckman Instruments (Geneva, Switzerland); angiotensin II, bradykinin, [Leu$^5$]- and [Met$^5$]enkephalin, lutropin- and thyrotropin-releasing factors (luliberin and thyroliberin, respectively), and neurotensin, from Bachem Fine Chemicals (Torrance, CA); synthetic porcine vasointestinal peptide-(1–28), from Peninsula Laboratories (San Carlos, CA); synthetic somatostatin, extracted bovine and porcine glucagon, and insulin, from Sigma; human growth hormone (somatotropin), from Kabi Diagnostica (Stockholm, Sweden); and morphine sulfate, from Pentax (Lyndhurst, NJ).

**Preparation of Tissue Fragments.** Human brains and pituitaries obtained at autopsy were dissected as described (18) not later than 20 hr post mortem and, after determination of wet weight, were frozen at −80°C for time periods not exceeding 3 wk. Patients with malignant tumors and chronic renal insufficiency were excluded from the study. None of the patients had a recorded history of previous neurological or psychiatric illness. A total of 12 autopsies each have been used for CT determination and evaluation of $^{125}$I-labeled salmon CT ($^{125}$I-CT) binding sites, respectively. Except for the characterization of hypophyseal CT, which required 10 pituitaries, CT was analyzed in tissues obtained from individual subjects.

**Extraction of CT from Tissue.** The frozen fragments were placed in 10–20 vol of ice-cold 2 M acetic acid containing in

Abbreviations: ACTH, corticotropin (adrenocorticotropic hormone); CT, calcitonin; $^{125}$I-CT, $^{125}$I-labeled salmon CT; HPLC, high-performance liquid chromatography.

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some extractions 40,000 dpm (1 dpm = 16.7 mBq) of human [$^3$H]CT-(1–32) and its sulfoxide. The tubes were subsequently transferred to a boiling water bath for 5 min. The fragments were homogenized with an Ultra-Turrax (18K pestle) homogenizer (Ika-Werk, Staufen, Federal Republic of Germany). The homogenates were frozen at −20°C overnight and, after thawing at room temperature, were refrozen, rethawed, and centrifuged at 48,000 × *g* at 4°C for 30 min. The clear supernatants either were lyophilized, stored at −20°C, and reconstituted in 0.5–1 ml of radioimmunoassay diluent for subsequent determination of immunoreactive CT or were passed 20 times through Sep-Pac $C_{18}$ cartridges (Waters Associates), activated with 10 ml of methanol, and equilibrated in 20 ml of 0.1% trifluoroacetic acid. The cartridges were washed with 40 ml of 0.1% trifluoroacetic acid, and the CT was eluted in 15 ml of 80% (vol/vol) methanol in water containing 0.1% trifluoroacetic acid. The methanol was evaporated, and the samples were lyophilized after the addition of 10 ml of 0.1 M acetic acid. The precipitates were dissolved in 1 ml of 0.1 M acetic acid, and CT was analyzed on a reversed-phase HPLC system (Altex, Berkeley, CA) with Nucleosil C-18 (Machery–Nagel GmbH, Düren, Federal Republic of Germany) (10 μm; 250 × 4.6 mm) columns under isocratic conditions with methanol/$H_2O$/trifluoroacetic acid, 65:34:1 (vol/vol), as eluant (19). Fractions (0.5 ml) were collected in tubes containing 300 μl of 1% human serum albumin in 0.1 M acetic acid and analyzed for immunoreactive CT and for $^3$H by liquid scintillation spectroscopy (model MR 300, Kontron AG, Zurich, Switzerland) in Rotiszint 22 (Carl Roth KG, Karlsruhe, Federal Republic of Germany). Overall recovery of human [$^3$H]CT ranged from 50% to 75%. Less than 15% of the human [$^3$H]CT-(1–32) was converted into the [$^3$H]CT-(1–32) sulfoxide during the extractions.

**Radioimmunoassay.** CT was determined in a homologous human CT-(1–32) assay (20). The antibodies (goat 6A obtained on day 143) used were predominantly directed to determinants located in the COOH-terminal parts of the CT-(1–32) molecule. A 50% reduction of the binding of human $^{125}$I-CT to its antibodies was obtained with 13.6 ± 1.0 fmol, whereas a similar inhibition was seen with 5.8 × $10^5$, 1.4 × $10^5$, and 1.5 × $10^5$ times higher amounts (on a molar basis), respectively, of ACTH-(1–13), -(1–24), and -(1–39). β-Endorphin, oxytocin, and vasopressin showed a minimal inhibition of the immunological reaction only at 20 μg/ml, whereas all other substances examined—ACTH-(11–24), -(11–39), and -(25–39); [Leu$^5$]- and [Met$^5$]enkephalin; the amino-terminal 16,000-dalton fragment of the ACTH-β-lipotropin precursor and amino-terminal fragments thereof; angiotensin I and II; bradykinin; carcinoembryonic antigen; glucagon; growth hormone; insulin; lutropin- and thyrotropin-releasing factors; neurotensin; parathyroid hormone; somatostatin; substance P; vasointestinal peptide-(10–28); morphine; and naloxone—did not affect the immunological reaction in amounts of as high as 20 μg/ml.

**Preparation of Brain Homogenates.** The tissue fragments were placed in 20 vol of ice-cold 0.025 M Hepes·Tris (pH 7.4) buffer, homogenized for 30–45 sec, and centrifuged at 48,000 × *g* at 4°C for 20 min. The pellets were then resuspended in the initial buffer volume, briefly homogenized, and recentrifuged for 20 min. The pellets were resuspended by trituration in the same buffer volume, centrifuged in the same manner for 20 min, and finally resuspended and stored at −20°C until used.

**Binding Studies.** Salmon CT-(1–32) was iodinated to a specific activity of 600 Ci/mmol (1 Ci = 3.7 × $10^{10}$ becquerels), and homogenates were derived from 2.5 mg of fresh tissue incubated with $^{125}$I-CT (10,000 cpm) for 4 hr at 6°C (unless otherwise stated) as described (20, 21). Values are representative means ± SEM or duplicate determinations differing by less than 10% from at least four independent experiments.

## RESULTS

**Biochemical Characterization of CT in Tissue Extracts.** The predominant immunoreactive CT forms identified in extracts of the human brain, pituitary, and thyroid included peaks coeluting with synthetic monomeric CT-(1–32) (peak III) and its sulfoxide (peak II) in HPLC (Fig. 1). The earliest eluting component (peak I) could not be identified. The amounts of monomeric CT extracted from the hypothalamus and the pituitary were similar but were less in the cerebellum. Normal thyroid glands contained ≈100 times higher concentrations of CT than the hypothalamus contained.

**Binding Studies.** $^{125}$I-CT bound specifically to human brain homogenates: incubation with 0.7 μM salmon CT-(1–32) inhibited 75–85% of the total binding. This fraction is referred to as specific binding (Fig. 2), which was abolished by placing the membranes in a boiling water bath for 5 min. The rate of dissociation was negligible compared to the rate of association. Comparable results were obtained after diluting with a 27-fold excess of medium (with and without unlabeled CT), washing the homogenates, and reincubating in fresh media (not shown). The specific binding of $^{125}$I-CT was linear in the range of concentrations of homogenates used.

The binding of $^{125}$I-CT was decreased after the addition to the incubation medium of unlabeled salmon and human CT-(1–32) and fragments thereof, porcine CT-(1–32), and several analogues of the human hormone (Fig. 3). The amounts of inhibitor required to prevent 50% of the binding of salmon $^{125}$I-CT to the membranes was lowest with salmon CT-(1–32) (0.1 nM). Fifty-six, 90, 4 × $10^3$, and 1.8 × $10^5$ times higher amounts of salmon CT-(10–32), -(17–32), -(24–32), and -(1–9), respectively, were required to obtain a similar inhibition of the binding of $^{125}$I-CT to the brain homogenates. Furthermore, 45, 285, 425, and 1370 times higher amounts of human [Leu$^{12,16,19}$, Tyr$^{22}$]CT-(1–32), porcine CT-(1–32) or human [Arg$^{24}$]CT-(1–32), human [Tyr$^{22}$]CT-(1–32), and native human CT-(1–32), respectively, were required to obtain comparable inhibition of the binding of $^{125}$I-CT to the homogenates. Human CT-(11–32) showed a minimal inhibition. The salmon fragments CT-(1–9), -(10–16), and -(18–23); the free acid of human CT; and human CT-(1–32) sulfone did not affect $^{125}$I-CT binding. ACTH-(1–24) showed minimal inhibitory properties (not shown). All other peptides examined, morphine, and naloxone did not affect the binding of $^{125}$I-CT in amounts of up to 30 μg/ml.

**Regional Distribution of CT and CT Binding Sites in the Human Brain and Pituitary.** CT concentrations were highest in the posterior hypothalamus, the median eminence, and the pituitary, followed by the substantia nigra, the anterior hypothalamus, the globus pallidus, and the inferior colliculus (Fig. 4). Mean levels of CT were at least a factor of 7 lower in the caudate nucleus, the hippocampus, the amygdala, and the cerebral and cerebellar cortices as compared with the posterior hypothalamus and the pituitary. Specific binding of $^{125}$I-CT was highest to homogenates of the posterior hypothalamus and median eminence. $^{125}$I-CT binding to homogenates of the substantia nigra, the amygdala, and the inferior colliculus was lower by factors of 8.7, 9.4, and 13.4, respectively, than in the posterior hypothalamus and at least a factor of 21 lower in all the other regions of the brain examined and in the pituitary.

## DISCUSSION

Immunoreactive CT has been detected in the hypothalamus of man and of thyroidectomized monkeys (12, 22), and evidence

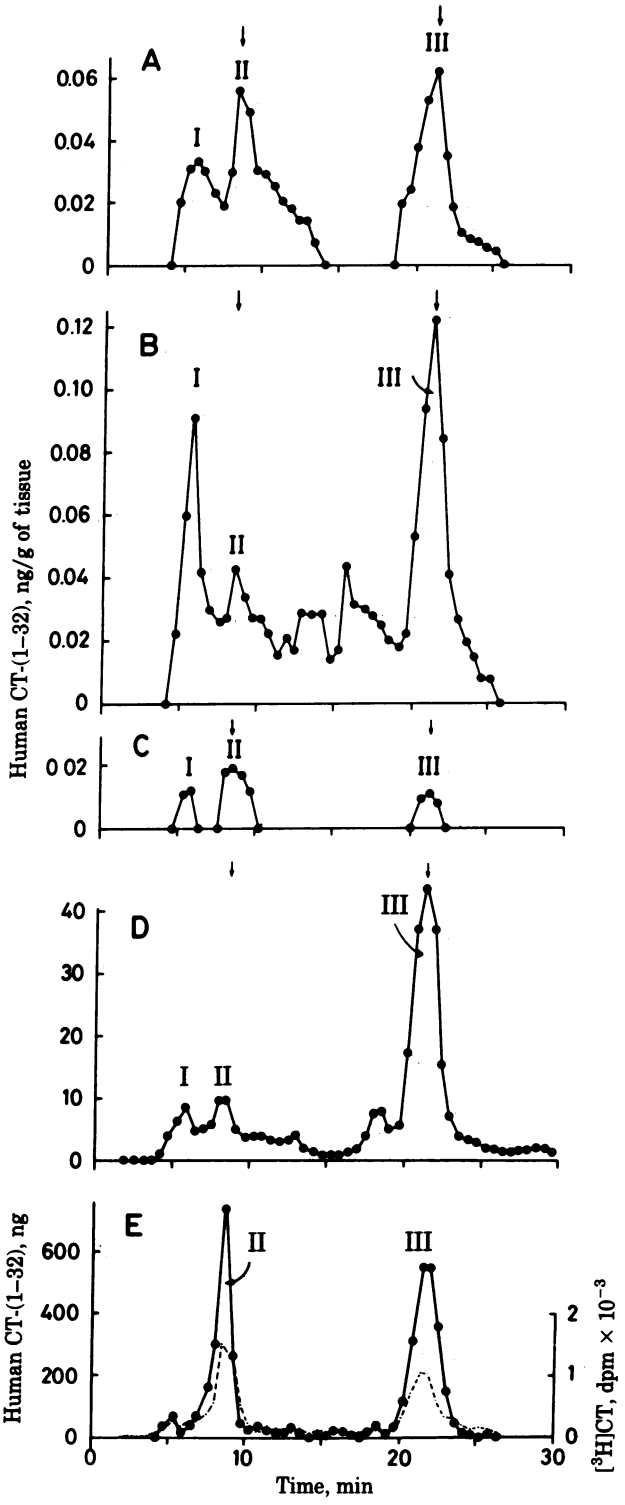


FIG. 1. Characterization of CT from human tissue extracts by reversed-phase HPLC on Nucleosil C-18. Human [$^3$H]CT-(1–32)sulfoxide (peak II) and [$^3$H]CT-(1–32) (peak III) were added to fresh tissues (4–6 g) as calibration substances. Effluent fractions were analyzed for immunoreactive CT (●——●) and for $^3$H-radioactivity (---- and arrows). (*A*) Hypothalamus. (*B*) Pituitary. (*C*) Cerebellum. (*D*) Thyroid. (*E*) Synthetic human CT-(1–32)sulfoxide (peak II) and CT-(1–32) (peak III).

has been reported for the presence of hypocalcemic factors in the hypothalamus of guinea pigs and cattle (23, 24). Immunoreactive CT-like components have been demonstrated on gel filtration analysis of human cerebrospinal fluid, pheochromocytoma, and porcine pituitaries (5–7, 9, 12, 14–16, 24). With

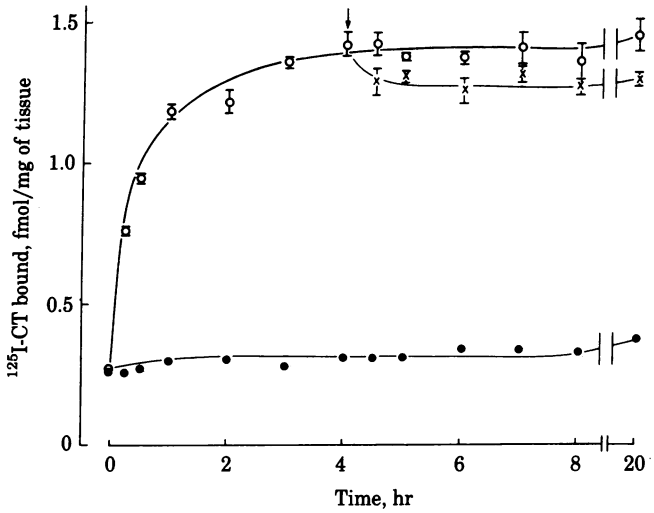


FIG. 2. Time course of $^{125}$I-CT binding in the human hypothalamus. Homogenates were incubated in the absence (○) and in the presence (●, ×) of unlabeled salmon CT. Nonspecific binding was determined in the presence of 0.7 μM salmon CT (○), and dissociation was determined with the addition of 0.7 μM unlabeled salmon CT (arrow) after 4 hr (×).

HPLC, CT has been found in the nervous systems of protochordates, the lizard, and the pigeon (5–7). We identified by HPLC components indistinguishable from human CT-(1–32) and its sulfoxide in extracts of the human hypothalamus, the pituitary, and the thyroid. In a previous communication, the positions of two immunoreactive CT forms of normal human thyroids, moreover, were found to coincide with synthetic monomeric CT-(1–32) and its sulfoxide on thin-layer chromatography (25). The former had comparable biological activity in the hypocalcemic rat assay to synthetic monomeric CT-(1–32), and the latter was biologically inactive. In the present report, CT concentrations in the different regions of the central nervous system and the pituitary were, on the average, at least 10–100 times higher than in plasma (26) and cerebrospinal fluid (not shown).

Specific binding of $^{125}$I-CT to rat (21, 27, 28) and human brain homogenates and membranes has been demonstrated and is reported to be highest in the hypothalamus. A feature of the binding sites in human and rat brains, as well as in the kidney and cultured bronchial carcinoma cells, has been the slow and incomplete reversibility of the binding of $^{125}$I-CT (21, 29, 30). In the present experiments, we have demonstrated rebinding of $^{125}$I-CT to fresh membranes after extraction from brain membrane preparations. Similarly, rebinding has been shown by $^{125}$I-CT in the supernatant fluid after sedimentation of the membranes, indicating preservation of binding activity. Furthermore, $^{125}$I-CT used in the rebinding experiments remained essentially undegraded as analyzed by gel permeation chromatography (21) (not shown).

In the present and in a previous study (21), amounts as low as 0.1 nM unlabeled salmon CT-(1–32) inhibited the binding of $^{125}$I-CT to human brain homogenates and rat brain membranes. Porcine and human CT-(1–32) and analogues of the human hormone, all with less activity than salmon CT-(1–32) in the hypocalcemic rat bioassay, displayed the same rank order of inhibition of the binding of $^{125}$I-CT to cultured human lymphocytes (31) as to human brain homogenates. Similar findings with respect to the inhibition of the binding of $^{125}$I-CT and stimulation of adenylate cyclase activity by human CT and analogues thereof have been obtained in human breast cancer cell lines (30, 32).

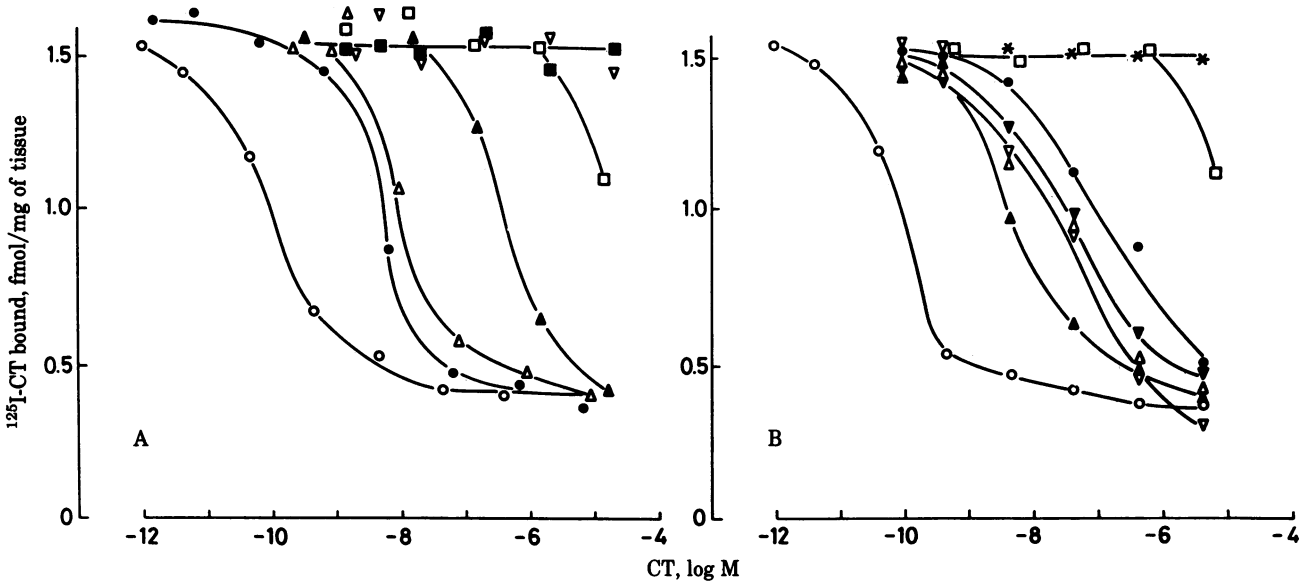


FIG. 3. Displacement of $^{125}$I-CT in the human hypothalamus. Values are means of duplicate determinations. (*A*) Salmon CT inhibitors: ○, CT-(1–32); ●, CT-(10–32); △, CT-(17–32); ▲, CT-(24–32); □, CT-(1–9); ■, CT-(10–16); ▽, CT-(17–23). (*B*) Human and other inhibitors: ○, salmon CT-(1–32); ▲, human [Leu$^{12,16,19}$Tyr$^{22}$]CT-(1–32); △, porcine CT-(1–32); ▽, human [Arg$^{24}$]CT-(1–32); ▼, human [Tyr$^{22}$]CT-(1–32); ●, human CT-(1–32); □, human CT-(11–32); *, human CT-(1–32)OH.

Antibodies to human CT-(1–32) predominantly recognize COOH-terminal parts of the human CT molecule (5, 20). Moreover, the specific binding of salmon $^{125}$I-CT to brain membranes and of an eel $^{125}$I-CT analogue to kidney membranes was directed to structural or conformational features, or both, in the COOH-terminal half of salmon CT-(1–32) (21, 33). Interestingly, both antibodies and receptors appear to be directed to determinants located in the COOH-terminal region of the CT-(1–32) molecule. In view of the remote resemblance, not unexpectedly the CT-like structure of the ACTH-β-lipotropin precursor (34) was neither recognized radioimmunologically nor by the rat and human brain membranes. Moreover, ACTH-(1–24) was only detected in at least $10^5$ times higher amounts than human CT-(1–32) was in the homologous human CT-(1–32) radioimmunoassay system and also by the brain membranes.

ACTH-(1–13), -(1–24) and -(1–39), β-endorphin, oxytocin, and vasopressin inhibit the immunological reaction only in at least $10^5$-fold higher concentrations than that of human CT-(1–32); all have shorter retention times than monomeric CT-(1–32) and its sulfoxide have in the HPLC system used in the present study (not shown) and, therefore, do not interfere with our radioimmunological CT measurements. Similar findings have been obtained with ACTH and β-endorphin (35).

The concentration of endogenous CT was highest in a region encompassing the posterior hypothalamus, the median eminence and the pituitary. Similarly, of all the regions of the brain

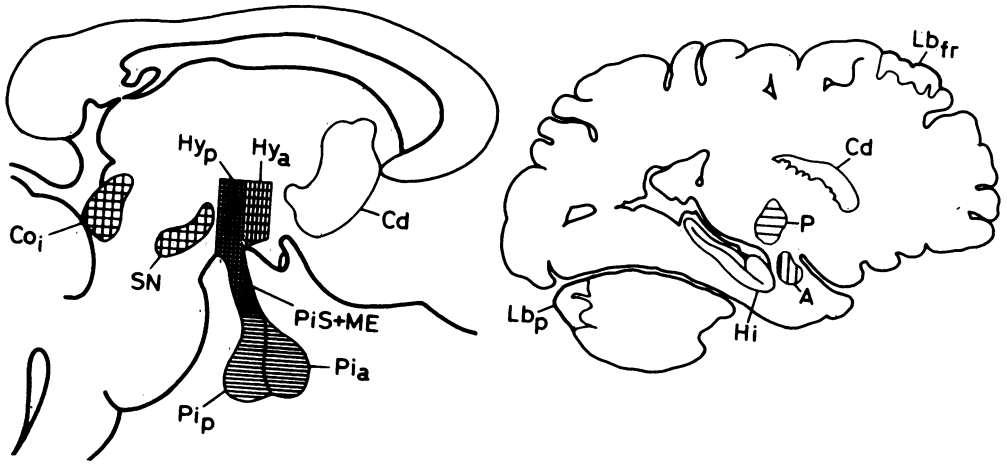


FIG. 4. Regional distribution of human CT and specific binding sites of $^{125}$I-CT in the human brain and pituitary (parasagittal sections). CT content in fmol/mg of tissue ($n$ = 7 experiments): >0.7 (▤), 0.2–0.5 (▭), <0.1 (□). $^{125}$I-CT binding in fmol/mg of tissue ($n$ = 5 experiments): homogenates were derived from 0.63, 2.5, and 10 mg of fresh tissue; >0.7 (▥), 0.08–0.12 (▯), <0.05 (□). In the following, the first values represent CT contents and the second values CT binding, both in fmol/mg of tissue: posterior hypothalamus (Hy$_p$) 0.79 ± 0.23, 1.07 ± 0.35; anterior hypothalamus (Hy$_a$) 0.35 ± 0.12, 0.73 ± 0.08; pituitary stalk (PiS) and median eminence (ME) 0.71 ± 0.29, 0.98 ± 0.34; posterior pituitary (Pi$_p$) 0.81 ± 0.18, 0.03 ± 0.01; anterior pituitary (Pi$_a$) 0.81 ± 0.16, 0.03 ± 0.01; substantia nigra (SN) 0.45 ± 0.19, 0.12 ± 0.02; inferior colliculus (Co$_i$) 0.21 ± 0.01, 0.08 ± 0.03; caudate nucleus (Cd) 0.12 ± 0.04, 0.04 ± 0.02; globus pallidus (P) 0.25 ± 0.08, 0.01 ± 0.005; amygdala (A) 0.09 ± 0.03, 0.11 ± 0.03; hippocampus (Hi) 0.07 ± 0.01, 0.05 ± 0.01; frontal cerebral cortex (Lb$_{fr}$) 0.09 ± 0.02, 0.04 ± 0.02; parietal cerebellar cortex (Lb$_p$) 0.09 ± 0.02, 0.02 ± 0.01.

examined with the same extracts, somatostatin and neurotensin were found to be highest in the hypothalamus and the median eminence (18). The question as to whether these peptide hormones are synthesized locally or transported from other areas remains to be answered. CT binding sites are concentrated in the brain regions shown to contain the highest amounts of endogenous CT, but binding of $^{125}$I-CT was negligible in the pituitary. It can be speculated that CT is transported from the pituitary and delivered directly to the brain as has been shown for the transport of ACTH (36). Studies in hypophysectomized animals would help to answer this question. Moreover, $^{125}$I-CT injected intracardially was shown to be concentrated in circumventricular organs of the rat brain, such as the median eminence; $^{125}$I-CT binding was subnormal in rats with hereditary diabetes insipidus and, thus, might be implicated in the metabolic alterations of the disease (37). Intracerebroventricular but not intravenous injections of CT stimulated prolactin secretion in rats (38). Furthermore, intracerebroventricular administration of CT resulted in analgesia and blocking of evoked potentials to painful stimuli, an effect which was not suppressed by the opiate antagonist naloxone (39–41). In rat brain membranes (21) and human brain homogenates, naloxone and morphine failed to inhibit $^{125}$I-CT binding. Finally, pituitaries of obese rats contained higher amounts of CT than those of lean rats contained, and intracerebroventricular administration of CT inhibited feeding in rats (42, 43).

In conclusion, the presence of a CT form which is indistinguishable from human CT-(1–32) and of specific $^{125}$I-CT binding sites in the hypothalamus may suggest CT as a possible neurotransmitter or modulator whose physiological role remains to be evaluated in more detail.

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