L-Triiodothyronine: Is this Peripheral Hormone a Central Neurotransmitter?

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L-triiodothyronine (T_3) has previously been shown to enhance fast-phase, depolarization-induced ^{45}Ca uptake and ^3H-gamma-aminobutyric acid release by rat brain synaptosomes at low nanomolar concentrations comparable to those reported for whole brain. Nevertheless, the physiologic importance of these nonnuclear-mediated effects of T_3 has remained uncertain, in part because specific mechanisms and the presence of T_3 at presumptive sites of action have not been demonstrated.

Isotopic studies showing that L-tetraiodothyronine (thyroxine, T_4) and T_3 are concentrated in synaptosomes, and that T_4 is deiodinated to T_3 suggested that endogenous levels of T_3 in nerve terminals are probably much higher than in other compartments of the brain. In the present study we confirmed that endogenous levels of T_3 in nerve terminals are at least eightfold higher, and may be as much as 60-fold higher, than in whole brain. More importantly, we showed that both ^{125}I-labeled T_3 and endogenous T_3, but not ^{125}I-T_4 or endogenous T_4, are released from depolarized synaptosomes, primarily by a Ca^{2+}-dependent process. This demonstrates a mechanism for raising the level of T_3 within the synapse, where the hormone may interact with pre- and postsynaptic binding (or uptake) sites, and suggests that the peripheral hormone T_3 may be a neurotransmitter.

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KEY WORDS: *Synaptosome; Thyroid hormones; Release; Superfusion; Rat*

Most of the known effects of thyroid hormones, particularly on growth, development, and structural maintenance of the central nervous system, are thought to be initiated by the binding of L-triiodothyronine (T_3) to nuclear receptors, which regulate protein synthesis (Oppenheimer 1979; Sterling 1979). However, in the synaptosome model of the nerve terminal, thyroid hormones also have been shown to alter some of the processes that mediate synaptic transmission (Mason et al. 1987, 1990; Hashimoto et al. 1991). Radiolabeled thyroid hormones that enter the brain are concentrated in synaptosomes (Dratman et al. 1976; Dratman and Crutchfield 1978), where L-tetraiodothyronine (thyroxine, T_4) is converted to T_3, a more active metabolite (Dratman and Crutchfield 1978), and T_3 is degraded to T_2 (Tanaka et al. 1981).

Recent work indicates that T_3 is taken up by two kinetically distinct active transport systems, whereas T_4 enters the synaptosome by diffusion (Kastellakis and Valcana 1989). Specific binding sites for T_3 on synaptosomes and synaptic membranes from rat brain have also been described (Mashio et al. 1982, 1983). The clinical observation that small concurrent doses of T_3 augment the therapeutic actions of tricyclic antidepressants in apparently euthyroid depressed patients (Prange et al. 1969; Goodwin et al. 1982; Schwarcz et al. 1984) is likewise consistent with the concept of the nonnuclear brain effects of thyroid hormones because the nuclear T_3 receptors in the brains of these patients would be expected to be fully occupied (Crantz et al. 1982) before T_3 was given.

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Although the evidence supporting direct actions of thyroid hormones on synaptic processes is compelling, there has been no clear, comprehensive conceptual framework within which to evaluate its physiologic relevance. It has been shown that concentrations of thyroid hormones in rat whole brain are in the low nanomolar range (Dratman et al. 1983), but hormone concentrations in presynaptic terminals and the synaptic cleft are uncertain, and the dynamics of thyroid hormone levels in this complex anatomic area are poorly understood.

In the present study we confirmed earlier isotopic studies indicating that T_3 levels in rat brain synaptosomes are much higher than whole brain levels. Perhaps more importantly, we show that endogenous T_3, but not T_4, can be released from depolarized synaptosomes by a Ca^{2+}-dependent process like the classic neurotransmitters. This demonstrates a mechanism for raising the levels of T_3 within the synapse where the hormone can potentially interact with pre- and postsynaptic binding (or uptake) sites.

MATERIALS AND METHODS

Animals

All experiments were performed with brain tissue preparations from adult male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) weighing at least 200 g. A total of 22 rats was used in this study: four for determinations of hormone levels in whole brain, four for determinations of hormone levels in synaptosomes, eight for determinations of the release experiments with radiolabeled hormones, and six for determinations of endogenous hormone release (see below). They were housed two to a cage and given free access to laboratory chow and water. Animal quarters were kept at temperature of between 22 and 25°C, and the light-dark cycle was 12 hours.

Preparation of Tissue Homogenates

The brains of experimental animals killed by decapitation were removed immediately and kept on ice while the cerebral cortex was dissected and weighed. Cortical tissue was homogenized in 10 volumes of 0.32 mol/L sucrose containing 3 μmol/L iopanoic acid to inhibit thyroid hormone metabolism, and centrifuged for 10 minutes at 800 × *g* to remove nuclei and cell debris. The synaptosome-containing supernatant (protein concentration, about 700 μg/100 μl) was used in the release assay.

Preparation of Synaptosomes

Synaptosomes were prepared from the cerebral cortex by the procedure of Dodd et al. (1981). The cortex was homogenized in 10 volumes of 0.32 mol/L sucrose immediately after dissection and centrifuged for 10 minutes at 800 × *g*. The resulting supernatant was then layered onto 1.2 mol/L sucrose and centrifuged at about 178,000 × *g* for 30 minutes using a Beckman 60 Ti fixed-angle rotor. Synaptosomes were collected at the interface between the 0.32 mol/L and 1.2 mol/L sucrose, diluted 1:2.5 with 0.32 mol/L sucrose and layered onto 1 volume (original homogenate) of 0.8 mol/L sucrose. The pellet from the first spin containing cell debris and nuclear material, the mitochondrial pellet from the first high-speed spin, and the synaptosomal pellet from a second 30-minute centrifugation at 178,000 × *g* were subjected to thyroid hormone extraction described below. In some experiments, the sucrose solutions contained 1 nmol/L T_3, the approximate concentration in whole brain, and only the synaptosomal pellet was extracted. This was done to simulate the in vivo condition in which additional hormone is available to replace that lost from the synaptosomes by diffusion or other processes. Protein concentrations were later determined by the method of Lowry et al. (1951).

Superfusion System

Thyroid hormone release was studied with a two-channel or chamber superfusion system previously used to study neurotransmitter release (Hashimoto et al. 1991). A superfusion technique was chosen over a vacuum filtration or centrifugation method to avoid the potential problems of interpretation associated with the reuptake of hormones. The superfusion apparatus consisted of two Acrodisc disposable filter assemblies (0.45-μ pore size, Gelman Sciences Inc., Ann Arbor, MI), which served as superfusion chambers. All buffer vessels and the two superfusion chambers, which were used simultaneously, were submerged in a 37°C constant-temperature water bath during each run. The chamber inlets were connected to an adjustable flow ISCO WIZ multichannel peristaltic pump (Gilson Inc., Middleton, WI). Three-way valves (American Pharmaseal Co., American Hospital Corp., Valencia, CA) were used to switch from wash to release buffer (see below) and vice versa without disrupting flow or introducing air bubbles into the closed system. Buffers were pumped through the chambers at a flow rate of 1 ml/min, and fractions of the superfusate from the chambers were collected at 1-minute intervals, using an ISCO Retriever II fractionator, into either borosilicate glass tubes or 12 × 75-mm antibody-coated radioimmunoassay (RIA) tubes (see below). The ^{125}I-T_3 and ^{125}I-T_4 were counted in an LKB 1272 Clinigamma gamma counter at an efficiency of 70%.

For some experiments, fresh synaptosome-containing homogenates were preloaded with ^{125}I-T_3 or ^{125}I-T_4 (3 nmol/L) by incubation for 25 minutes at 37°C with superfusion wash buffer (135 mmol/L NaCl, 5

mmol/L KCl, 1.4 mmol/L $MgSO_4$, 25 mmol/L glucose, 3 mmol/L $CaCl_2$, pH 7.4). Preparations preloaded with ^{125}I-T_3 or ^{125}I-T_4 were simultaneously drawn by the peristaltic pump into both superfusion chambers and superfused with wash buffer for 4 minutes at a flow rate of 2 ml/min, and then for 3 minutes at a rate of 1 ml/min. The collection of 1 ml portions of the perfusate was begun at this time and continued throughout the release assay. Homogenates that were not preloaded with ^{125}I-labeled hormones were not superfused with wash before collection of fractions began.

In the first set of experiments, ^{125}I-T_3 or ^{125}I-T_4-loaded tissues in the two chambers were exposed to high K^+ release buffer in the presence or absence of Ca^{2+}. The Ca^{2+} was used as a variable because depolarization-induced release of neurotransmitters from nerve terminals is generally thought to be dependent upon the rapid entry of Ca^{2+} through voltage-sensitive channels (Augustine et al. 1987). The release buffer was identical to the wash buffer except that it contained 80 mmol/L NaCl, 55 mmol/L KCl, and no $MgSO_4$. In the second set of experiments, depolarization-induced release of endogenous T_3 and T_4 in the presence and absence of Ca was measured by RIA.

Radioimmunoassay of T_3 and T_4

Following extraction as described by Nejad et al. (1975), T_3 and T_4 in homogenates of whole brain or synaptosomes purified from cerebral cortex were measured using commercially available reagent kits (Becton-Dickinson Immunodiagnostics, Orangeburg, NY). Extracts were dried down with N_2 and diluted with "0" standard, and standards were diluted 1:1 with blank extracts (containing no T_3 or T_4); otherwise, assays were performed according to kit protocols. The efficiency of the extraction was greater than 75% for both hormones. The sensitivities and the interassay coefficients of variation for the T_3 and T_4 assays were 4.9 pg/tube and 95 pg/tube, and 8% and 7%, respectively. Whole brains and synaptosomes from cerebral cortex were obtained from different animals in three separate experiments. The concentrations of T_3 in whole brain and purified synaptosomes are expressed as the means of four experiments ± the standard errors of the means.

Assay of T_3 and T_4 was performed without extraction by collecting fractions of the superfusates directly into antibody-coated tubes from the free-T_3 and free-T_4 assay kits. The fractions were then concentrated by drying under N_2 and diluted with "0" standard from the kit. Standards from the kits were diluted 1:1 with superfusion wash buffer. Assays were then performed according to kit protocols. The sensitivities and interassay coefficients of variation for the free-T_3 and free-T_4 assays were 15 fg/tube and 23 fg/tube, and 7% and 8%, respectively.

Reagents

Reagents ^{125}I-T_3 (1080 to 1320 μCi/μg) and ^{125}I-T_4 (1080 to 1320 μCi/μg) were purchased from New England Nuclear Corp. (Boston, MA), and thyroid hormones and general chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific Co. (Raleigh, NC).

RESULTS

To validate our methods of measuring thyroid hormone levels in brain tissue, we homogenized whole brains from healthy adult male Sprague-Dawley rats assumed to be euthyroid and determined that the concentration of extracted T_3 was 1.64 ± 0.16 nmol/L (mean ± SEM, $n = 4$), which is in agreement with previous findings (Dratman et al. 1983). Then, we determined the concentration of T_3 after extraction of the synaptosomal fraction from cerebral cortex obtained by ultracentrifugation. In this fraction, T_3 was also present at a concentration of 14.6 ± 1.4 nmol/L (mean ± SEM, $n = 4$). In addition, T_3 was present in the nuclear pellet containing cell debris and nuclear material and in the mitochondrial pellet at concentrations of approximately 1.4 nmol/L and 2.9 nmol/L, respectively.

Because the purification of synaptosomes takes about 2 hours, during which synaptosomes are diluted many fold with fresh buffers several times, we were concerned that some endogenous T_3 might be lost by diffusion (^{125}I-T_3-loaded synaptosomes suspended in 20 volumes of superfusion wash buffer lost about 35% of their radioactivity after an incubation of only 15 minutes at 22°C). Therefore, to simulate presumptive in vivo conditions, where central T_3 is constantly replenished from peripheral stores of T_4 and T_3, we added T_3 (1 nmol/L) to buffers used in the synaptosome purification. Under this condition, we then found that the concentration of T_3 extracted from these synaptosome preparations was 63.5 ± 11.4 nmol/L (mean ± SEM, $n = 4$.).

In the first set of superfusion experiments, we observed depolarization-induced release of radioactivity from tissues preloaded with ^{125}I-T_3 (Fig. 1). A statistical analysis comparing the mean areas under the curves by a two-tailed Student's t-test showed that significantly more ^{125}I-T_3 was released when Ca^{2+} was present in the 55-mmol/L K^+ release buffer than when it was omitted ($t = 2.92$, $p < 0.05$). These data suggested that the release was initiated by a Ca^{2+}-dependent process. Then, we showed that 85.1 ± 3.9% (mean ± SEM, $n = 3$) of the released radioactivity was bound by T_3 antibody–coated RIA tubes, confirming that it was predominantly ^{125}I-T_3 rather than a metabolite of ^{125}I-T_3 or free-^{125}I. We found no depolarization-induced release

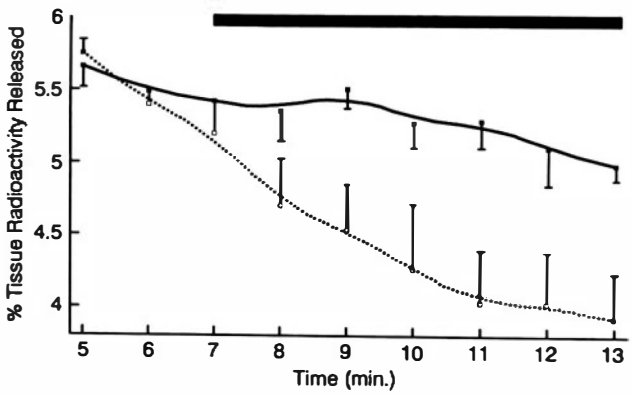

Figure 1. Depolarization-induced release of ^{125}I-T_3 from synaptosome-containing homogenates of rat cerebral cortex in the presence (■) and absence (□) of Ca^{2+} at 37°C. Homogenates were superfused with low (5 mmol/L) K^+ buffer, then superfused with high (55 mmol/L) K^+ depolarization (release) buffer (▬). This figure represents four separate experiments that showed ^{125}I-T_3 was released primarily by a Ca^{2+}-dependent process. Each point represents a mean with standard error bars. See text for experimental details.

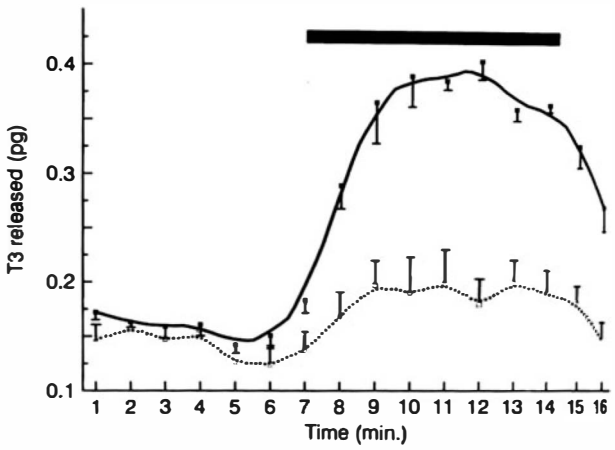

Figure 2. Depolarization-induced release of endogenous T_3 from synaptosome-containing homogenates of rat cerebral cortex in the presence (■) and absence (□) of Ca^{2+} at 37°C. Homogenates were first superfused with low (5 mmol/L) K^+ buffer, then superfused with high (55 mmol/L) K^+ depolarization (release) buffer (▬). This figure represents three separate experiments that showed T_3 was released primarily by a Ca^{2+}-dependent process. Each point represents a mean with standard error bars. See text for experimental details.

of radioactivity from tissues that had been preloaded with ^{125}I-T_4.

Next, we demonstrated that endogenous T_3, like ^{125}I-T_3, was also released from depolarized synaptosomes primarily by a Ca^{2+}-dependent process (Fig. 2). A statistical comparison of the mean areas under the curves by a two-tailed Student's *t*-test confirmed that more endogenous T_3 was released when Ca^{2+} was present in the release buffer ($t = 6.75$, $p < 0.01$). Only about 20% of the amount of endogenous T_3 released in the presence of Ca^{2+} was released when Ca^{2+} was omitted from the release buffer (Fig. 2). During the 7-minute depolarization, 4.8% ± 0.5% (mean ± SEM, $n = 3$) of total endogenous T_3 was released in the presence of Ca^{2+}.

DISCUSSION

Based in part on the structural similarity between iodothyronines and the catecholamines, Dratman (1974) hypothesized that thyroid hormones may act as neurotransmitters. Dratman and associates (Dratman et al. 1976; Dratman and Crutchfield 1978) subsequently showed that radiolabeled T_4 and T_3 intravenously administered to live animals were preferentially taken up into the synaptosomal fraction obtained from rat brain, where T_4 was also converted to T_3. In the present study we have demonstrated what was suggested by these earlier isotopic studies: that concentrations of endogenous T_3 are much higher in synaptosomes than in whole brain.

Kastellakis and Valcana (1989) reported that T_3 was actively transported into rat brain synaptosomes by a sodium-dependent process, whereas T_4 entered primarily by diffusion. They also showed that ^{125}I-T_3 could be released by homoexchange with nonphysiologic (10 μmol/L) concentrations of unlabeled T_3; however, they did not report on depolarization-induced release. In the present study we demonstrated Ca^{2+}-dependent, depolarization-induced release of ^{125}I-T_3 by synaptosome-containing homogenates of rat cerebral cortex using a superfusion system. Using highly sensitive commercial RIAs, we measured T_3 and T_4, in superfusates, thereby showing that endogenous T_3, but not T_4 was released by the same process. It is of interest that a much smaller amount of T_3 was released when Ca^{2+} was deleted from the high K^+ depolarization buffer. This release may have been triggered by a process involving intracellular rather than extracellular Ca^{2+}, or by reversal of the proposed Na^+-coupled T_3 uptake transporter to release T_3 from the cytoplasm (Erecinska 1987; Nicholls 1989).

We observed no release of ^{125}I-T_4 or endogenous T_4 from depolarized synaptosomes. Although our free-T_4 RIA is extremely sensitive, it is possible that T_4 was released in minute amounts that we were unable to detect. However, such small amounts would probably not be of physiologic importance. Perhaps a more plausible explanation is that T_4 was not released because it was not taken up by putative release vesicles, or it was taken up but converted to T_3. Some T_3 may have been released from cytoplasmic stores by transporter rever-

sal following depolarization; however, we would not expect T_4 to be released in this way because it does not enter the synaptosome by a Na^+-coupled transporter (Kastellakis and Valcana 1989).

Our group showed previously that low nanomolar concentrations of T_3 will enhance depolarization-induced Ca^{2+} uptake (Mason et al. 1990) and release of the neurotransmitter gamma-aminobutyric acid (Hashimoto et al. 1991). However, the physiologic relevance of these actions has remained equivocal, in part because the presence of T_3 at presumptive sites of actions has not been demonstrated. Although we still are unable to directly measure endogenous T_3 concentrations in the synapse, the present study demonstrates a mechanism whereby T_3 concentrations in the synapse can reach levels that can enhance Ca^{2+} uptake and release of a typical neurotransmitter.

We have shown that endogenous T_3 is concentrated in presynaptic nerve terminals where the cellular apparatus mediating its active uptake, its synthesis from T_4, and its degradation are also located. The release of T_3, a peripheral hormone, from synaptosomes by Ca^{2+}-dependent depolarization satisfies yet another criterion (Erulkar 1989) for a central neurotransmitter. What remains to be shown is a specific, receptor-mediated, postsynaptic effect of T_3. If T_3 does indeed prove to be a central neurotransmitter, it is surely an unusual one; it is readily absorbed by the gut and as readily enters the brain. Thus, given the value of T_3 as an adjunct to tricyclic antidepressants, the iodothyronine molecule may provide a model for the construction of useful drugs.

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REFERENCES

Augustine GJ, Charlton MP, Smith SJ (1987): Calcium action in synaptic transmitter release. Annu Rev Neurosci 10:633–693

Crantz FR, Silva JE, Larsen PR (1982): An analysis of the sources and quantity of 3,5,3′-triiodothyronine specifically bound to nuclear receptors in rat cerebral cortex and cerebellum. Endocrinology 110:367–375

Dodd PR, Hardy JA, Oakley AE, Edwardson JA, Perry EK, Delaunoy J-P (1981): A rapid method for preparing synaptosomes: Comparison with alternative procedures. Brain Res 226:107–118

Dratman MB (1974): On the mechanism of action of thyroxin, an amino acid analog of tyrosine. J Theor Biol 46:225–270

Dratman MB, Crutchfield FL (1978): Synaptosomal ^{125}I-triiodothyronine following intravenous ^{125}I-thyroxine. Am J Physiol 235:E638–E647

Dratman MB, Crutchfield FL, Axelrod J, Colburn RW, Thoa N (1976): Localization of triiodothyronine in nerve ending fractions of rat brain. Proc Natl Acad Sci USA 73:941–944

Dratman MD, Crutchfield FL, Gordon JT, Jennings AS (1983): Iodothyronine homeostasis in rat brain during hypo- and hyperthyroidism. Am J Physiol 245:E185–E193

Erecinska M (1987): The neurotransmitter aminoacid transport systems. Biochem Pharmacol 36:3547–3555

Erulkar SD (1989): Chemically mediated synaptic transmission: an overview. In Siegel G, Agranoff B, Albers RW, Molinoff P (eds). Basic Neurochemistry 4th Ed. New York, Raven Press, pp 151–182

Goodwin FK, Prange AJ Jr, Post RM, Muscettola G, Lipton MA (1982): Potentiation of antidepressant effects by L-triiodothyronine in tricyclic nonresponders. Am J Psychiatry 139:34–38

Hashimoto H, Walker CH, Prange AJ Jr, Mason GA (1991): The effects of thyroid hormones on potassium-stimulated release of ^{3}H-GABA by synaptosomes of rat cerebral cortex. Neuropsychopharmacology 5:49–54

Kastellakis A, Valcana T (1989): Characterization of thyroid hormone transport in synaptosomes from rat brain. Mol Cell Endocrinol 67:231–241

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951): Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Mashio Y, Inada M, Tanaka K, Ishii H, Naito K, Nishikawa M, Takahashi K, Imura H (1982): High affinity 3,5,3′-L-triiodothyronine binding to synaptosomes in rat cerebral cortex. Endocrinology 110:1257–1261

Mashio Y, Inada M, Tanaka K, Ishii H, Naito K, Nishikawa M, Takahashi K, Imura H (1983): Synaptosomal T_3 binding sites in rat brain: Their localization on synaptic membrane and regional distribution. Acta Endocrinol 104: 134–138

Mason GA, Walker CH, Prange AJ Jr (1987): Modulation of GABA uptake of rat brain synaptosomes by thyroid hormones. Neuropsychopharmacology 1:63–69

Mason GA, Walker CH, Prange AJ Jr (1990): Depolarization-dependent ^{45}Ca uptake by synaptosomes of rat cerebral cortex is enhanced by L-triiodothyronine. Neuropsychopharmacology 3:291–295

Nejad I, Bollinger J, Mitnick MA, Sullivan P, Reichlin S (1985): Measurement of plasma and tissue triiodothyronine concentration in the rat by radioimmunoassay. Endocrinology 96:773–779

Nicholls DG (1989): Release of glutamate, aspartate, and γ-aminobutyric acid from isolated nerve terminals. J Neurochem 52:331–341

Oppenheimer JH (1989): Thyroid hormone action at the cellular level. Science 203:971–979

Prange AJ Jr, Wilson IC, Rabon AM, Lipton MA (1969): Enhancement of imipramine antidepressant activity by thyroid hormone. Am J Psychiatry 126:457–469

Schwarcz G, Halaris A, Baxter L, Escobar J, Thompson M, Young M (1984): Normal thyroid function in desipramine

nonresponders converted to responders by the addition of L-triiodothyronine. Am J Psychiatry 141:1614–1616

Sterling KN (1979): Thyroid hormone action at the cell level. N Engl J Med 300:117–123, 173–177

Tanaka K, Inada M, Ishii H, Naito K, Nishikawa M, Mashio Y, Imura H (1981): Inner ring monodeiodination of thyroxine and 3,5,3′-L-triiodothyronine in rat brain. Endocrinology 109:1619–1624