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6. Microelectrodes (60 to 130 megohms), filled with 3*M* potassium acetate, 2*M* $KMeSO_4$, or 3*M* KCl, were used for intracellular recordings. Data were stored on tape and plotted on a d-c chart recorder or photographed from the oscilloscope. The stratum radiatum was stimulated by a monopolar tungsten electrode at 0.2 Hz (0.1 msec, 10- to 120-μA constant-current pulses) to evoke orthodromic EPSP's, spikes, and IPSP's. Antidromic spikes and IPSP's were similarly evoked from alveus stimulation (Fig. 1D). To block Na^+ action potentials and to bring out Ca^{2+} spikes, we applied TTX (2×10^{-5} *M*) in large drops to the strata radiatum, pyramidale, and oriens.
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* Address reprint requests to 33 Russell Street, Toronto, Ontario, Canada M5S 2S1.

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Iodine-125–Labeled Triiodothyronine in Rat Brain: Evidence for Localization in Discrete Neural Systems

Abstract. *Autoradiograms prepared from adult rat brains demonstrate that nerve cells and neuropil in different brain regions selectively concentrate and retain intravenously administered triiodothyronine, by mechanisms susceptible to saturation with excess triiodothyronine. A neuroregulatory role for thyroid hormones, strongly supported by the observations, may account for their marked effects on behavior and the activity of the autonomic nervous system.*

Marked changes in nervous system functions which develop in hypothyroid and hyperthyroid individuals are generally attributed to hormone-mediated events originating outside the nervous system (*1*). Recently, however, new observations have again raised the possibility that thyroxine (T_4) and its metabolites may function directly within the mature brain. These observations demonstrate that intravenously administered labeled T_4 slowly enters selected regions of rat brain by a saturable mechanism and becomes progressively more concentrated in the nerve terminal fraction. There it gives rise, through monodeiodination, to triiodothyronine (T_3), a metabolite with severalfold greater activity than its fully iodinated precursor. When T_4 supplies are low, as in hypothyroidism, the fractional conversion of T_4 to T_3 is unchanged or even reduced in most T_3-forming tissues, whereas it increases markedly in the brain. Intracerebral labeled T_3 (T_3*), whether generated in situ or taken up as such from the systematic circulation, remains concentrated in an osmotically sensitive compartment of the nerve terminal fraction long after the injected T_3* has largely disappeared from the circulation (*2*).

The foregoing evidence was obtained exclusively from biochemical studies requiring disruption of cytoarchitecture during tissue homogenization and extraction. To avoid the potential artifacts inherent in these methods, we reexamined the problem of the T_3 distribution in brain by using autoradiography. Earlier efforts to do so had been hampered by the low specific activity of labeled thyroid hormone preparations then available (in the range of 80 μCi/μg). In spite of this limitation, a number of different studies have provided preliminary evidence in support of a differential regional and subcellular distribution of iodothyronines in the brains of guinea pigs, rats, and *Rana pipiens* tadpoles (*3*).

In our experiments in which we used high specific activity T_3*, we sought first to identify the histologic correlates of previously observed gross anatomical differences in the regional distribution of thyroid hormones. For this purpose, two 150-g male Sprague-Dawley rats (Zivic-Miller Laboratories), surgically thyroidectomized 1 week earlier (*4*), received 1 mCi of ^{125}I-outer (phenolic) ring–labeled T_3 (specific activity, ~ 3000 μCi/μg; from P. Shadden, Abbott Laboratories) given under light ether anesthesia into the jugular vein. Rats were decapitated 3 hours after the injection, and the brains were processed for thaw-mount autoradiography (*5*). Parallel biochemical analyses confirmed earlier findings that radioactivity in brain homogenates at both 3- and 10-hour intervals after intravenous T_3* administration was more than 80 percent due to T_3 itself, whereas iodide never accounted for more than 8 percent.

To visualize labeling patterns at low magnification without the use of tinctorial stains, we left the brain sections exposed to the photographic emulsion for 7 months. The autoradiograms revealed clear-cut differences in the distribution of T_3* and its labeled metabolites in different brain regions and emphasized the selectivity of neuronal cell and neuropil labeling within regions (Fig. 1). For example, in the cerebellum (Fig. 1d), strong labeling of Purkinje cells contrasted with lesser labeling over basket cells. On the other hand the intensity in neuropil was different in different regions of the cortex and caudate, whereas in general neuropil labeling contrasted with the lower silver-grain density in white matter of the corpus callosum (Fig. 1, a to c). It therefore seems evident that gross dissections can provide only a hint

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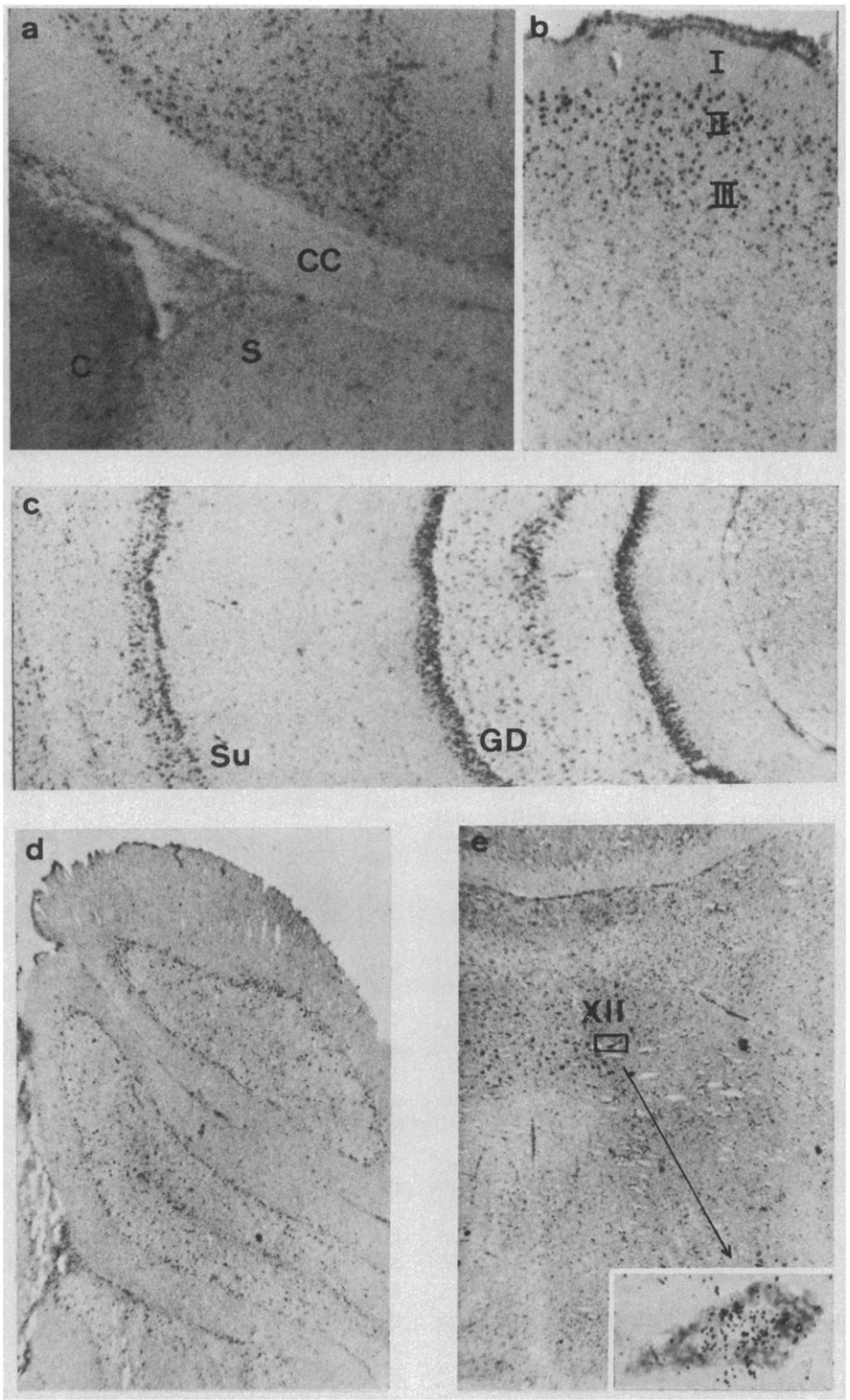


Fig. 1. Unstained thaw-mount autoradiograms (a to e) (4-μm sections) of the rat brain, showing selective concentration of radioactivity in cell bodies and neuropil 3 hours after intravenous injection of [^{125}I]triiodothyronine. In (a), note the contrast of neuropil labeling between the caudate nucleus (*C*) and septum (*S*), with the lowest radioactivity in the corpus callosum (*CC*). In the outer parietal cortex (b), neuronal labeling is strongest in laminae II and III. In (c), high intensity of labeling is apparent in pyramidal cells of the hippocampus, including subiculum (*Su*), and in granule cells of the dentate gyrus (*GD*). Strong labeling of Purkinje cells in (d) and neurons of the nucleus of the hypoglossal nerve (*XII*) in (e) can be recognized. The inset to (e) (×880) is from a stained section (methylene blue basic fuchsin), showing the nuclear concentration of radioactivity in the neuron of the hypoglossal nucleus. Magnifications: a, b, and d (×40); c and e (×70). In preparing the autoradiograms, brains were mounted on tissue holders and frozen in −180°C liquid propane; serial frozen sections were cut in a wide-range cryostat (Harris Manufacturing Company, North Billerica, Massachusetts) and thaw-mounted on Kodak NTB-3 photographic emulsion-coated slides; slides were photographically processed after exposure at −15°C for 210 days.

of the marked discreteness of the T_3 distribution in neuronal elements of the rat brain.

To determine the effects of T_3 competition on T_3* localization, two thyroidectomized animals received either T_3* only or T_3* plus a 1000-fold concentration of unlabeled T_3 (T_3* + T_3) intravenously; the brains obtained 3 hours later were processed for thaw-mount autoradiography. In these experiments we used dark-field illumination to distinguish tissue labeling at low magnification without unduly prolonging the exposure times. An example of the marked effect of T_3 competition on cellular labeling patterns is shown in Fig. 2, where the concentration of T_3 in cells of laminae II and III of the cingulate cortex (Fig. 2a) was completely obscured in the presence of excess T_3 (Fig. 2b), even though the radioactivity in the brain was, at that time, only 10 percent less than in the control (T_3* only) rats (*6*).

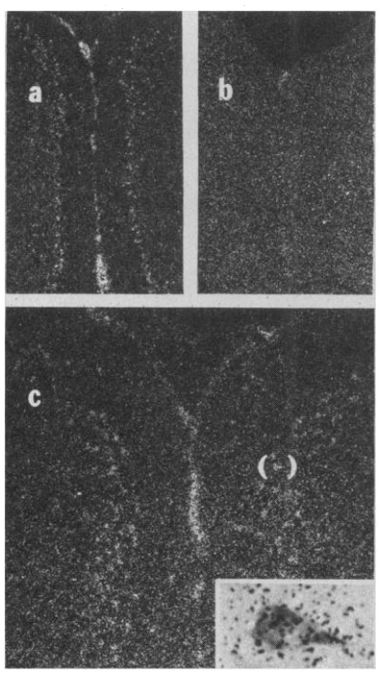
At least two different saturable cellular labeling patterns were observed in higher magnification views of the thaw-mount autoradiograms. In some cell groups, the radioactivity appeared to be concentrated over the entire cell body and, to a lesser extent, over the surrounding neuropil (Fig. 2c). On the other hand, in many cell populations, labeling was predominantly over nucleoplasm (Fig. 1e). The latter observation conforms with previous analytical evidence demonstrating the presence of saturable binding sites for T_3 in cell nuclei obtained from homogenates of different brain regions (*7*). Although saturable cytoplasmic binding sites in the brain have not been described, such sites have been noted in other tissues (*8*) and may be inferred tentatively from the evidence provided by these autoradiograms.

We used the technique of thaw-mount autoradiography initially because it assures optimum retention and minimum redistribution of the compounds of interest and is therefore notably reliable for localizing soluble and diffusible substances such as the steroid hormones or 2-deoxyglucose (*5*, *9*). However, conventional autoradiography requiring extensive solvent extractions and other disruptive manipulations is nevertheless usually satisfactory, and is normally used, for studying the distribution of many synaptically active small molecules, such as biogenic amines or amino acid neurotransmitters. Presumably, this class of compounds resists displacement and loss from the sections as a result of tight binding to membranes or sequestration within nerve ending vesicles (*10*).

In view of theoretical considerations which have raised the possibility that the amino acid T_3 or its metabolites act as neuromodulators, or alternate neurotransmitters (*11*), within the domain of the autonomic nervous system (*12*), we decided to evaluate the autoradiographic results after prolonged perfusion-fixation and alcohol dehydration of labeled rat brains (*13*). The results (Fig. 3) reveal that, in spite of the extensive preparative procedures used, radioactivity in the final Epon-embedded sections was distributed in highly distinctive patterns. For example, there was clear-cut evidence of hormone localization in relation to hippocampal and cortical gray matter and in the choroid plexus, whereas white matter of the corpus callosum gave a negative autoradiographic reaction (Fig. 3a). Moreover, these distribution patterns, seen at the 3-hour time interval, were maintained for at least 10 hours, although at that time the reaction in the choroid plexus was markedly reduced (Fig. 3b). After T_3* + T_3 treatment, on the other hand, the distribution of radioactivity was no longer so discrete, and evidence for differential localization in relation to well-defined regions of gray matter was effaced, while the reaction over the choroid plexus was still prominent (Fig. 3c).

In spite of general similarities in the regional distribution of T_3* in thaw-mount and Epon-embedded sections, higher magnification views of the labeled regions revealed striking differences. Selective cellular labeling evidenced in thaw-mount autoradiograms was no longer visible in the Epon sections; this result suggests that a large fraction of the cellular T_3 was in a readily diffusible state. Loss of label from cellular elements in the perfused and fixed tissues highlighted the radioactivity remaining in the pericellular neuropil and revealed that the saturable component of this radioactivity was highly localized to the immediate cell surround (compare a-1 and c-1 in Fig. 3). These results imply that the hormone was concentrated in the short cell processes impinging upon or arising from neurons known from thaw-mount preparations to selectively take up T_3 in a readily mobilizable form.

It seems unlikely that the autoradiographic results obtained from these perfusion-fixation experiments reflect the presence of hormone covalently incorporated into nerve terminal proteins. Although rapid formation and turnover of (iodo)proteins is a feature of the early stages of thyroid hormone–induced brain development in *Rana pipiens* tadpoles, these proteins are detected in only very



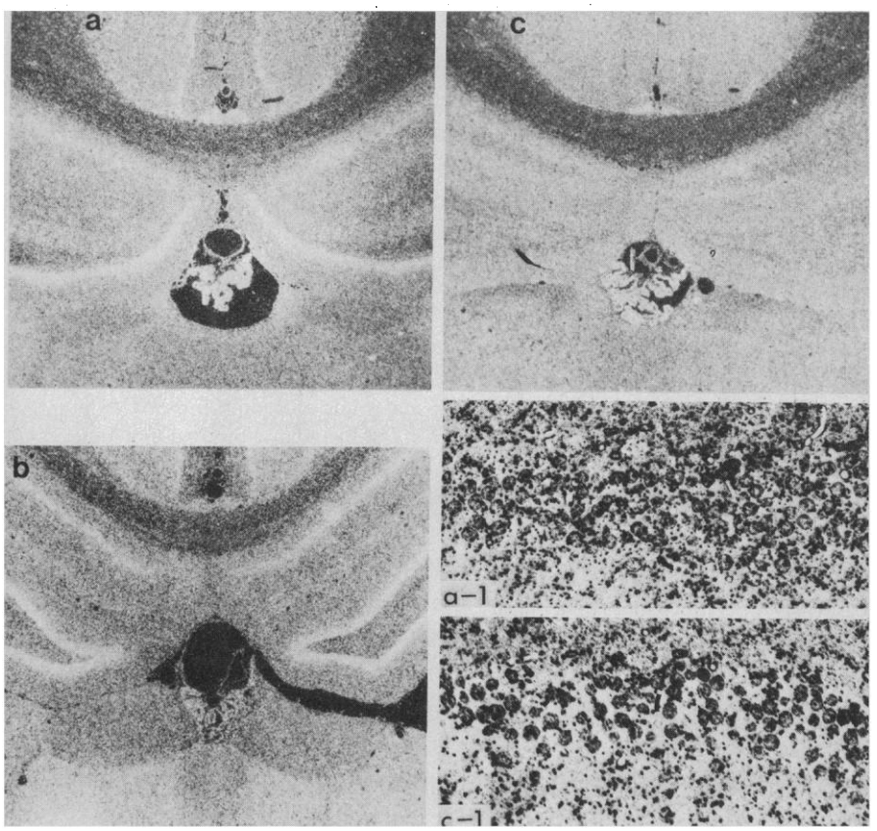


Fig. 2 (left). Dark-field views of thaw-mount autoradiograms illustrating the saturable T_3*-concentrating ability of neuronal cells. Note the T_3* localization in cells of laminae II and III in sections of the cingulate cortex prepared from rats receiving T_3* only (a), whereas parallel sections from the same region (b) show loss of differential labeling with T_3 competition (×26). In (c) (×65), parentheses indicate the location of the labeled cell in the inset, viewed under bright-field illumination (×880), showing the concentration of radioactivity over the cell body, extending into the surrounding neuropil. Sections were prepared from brains obtained 3 hours after intravenous injection of the isotope. Fig. 3 (right). Dark-field microphotographs of Epon-embedded 0.5-μm sections (×26), showing autoradiographically labeled bands of gray matter in the cortex and hippocampus 3 hours (a) and 10 hours (b) after intravenous T_3* administration, whereas during T_3 competition at 3 hours (c) the discrete labeling pattern is lost. Autoradiograms were exposed simultaneously and developed after 210 days. Higher magnification (×130) dark-field (negative reversed) views of methylene blue basic fuchsin–stained sections from brains obtained 3 hours after intravenous T_3* administration and developed after 67 days show heavy silver-grain accumulations obscuring the boundaries of granule cells of the dentate gyrus (a-1), whereas sharp cellular outlines and clear pericellular spaces are evident when labeling is reduced by T_3 competition (c-1). All the autoradiograms were prepared from the brains of ether-anesthetized rats perfused first with phosphate buffer containing 10 units of heparin per milliliter, then with 0.5 percent tannic acid and 2.5 percent glutaraldehyde in dilute Veronal acetate buffer, and finally with another phosphate buffer flush; brains were removed and then fixed in 2 percent osmium tetroxide and 2.5 percent glutaraldehyde in dilute Veronal acetate buffer containing 4 percent sucrose, rinsed in water, and dehydrated through 50, 70, 80, 90, 95, and 100 percent ethanol.

low concentrations in adult rat brain (*14*). More likely, the observations reflect sequestration of the hormone, or its metabolites, by membranes or vesicles within cell processes, in conformity with previous analytical evidence demonstrating early localization, osmotic releasability, and long retention of intravenously administered T_3* and its products in synaptosomal fractions of rat brain (*2*).

Certain conservative inferences may be drawn from these autoradiographic observations. The hormone T_3, which produces profound effects on behavior and on the autonomic nervous system, is taken up and concentrated in certain nerve cells and nerve cell aggregates of rat brain; the hormone is also differentially concentrated in the surrounding neuropil; the mechanisms involved are saturable with T_3. At 3- and 10-hour intervals after intravenous T_3* administration, there is little or no labeling of large fiber systems; further studies are required to determine whether fiber tracts may become labeled after 10 hours. The evidence is consistent with the uptake and retention of the hormone in specific neural systems and adds to a growing body of information indicating that iodothyronines serve as neuroregulators—that is, as neuromodulators, amino acid neurotransmitters, or neurotransmitter precursors (*15*) in the nervous system of the adult rat.

MARY B. DRATMAN
Medical Research Service, Philadelphia Veterans Administration Hospital, Philadelphia, Pennsylvania 19104, and *Department of Medicine, Medical College of Pennsylvania, Philadelphia 19129*
YATAKA FUTAESAKU
Laboratory of Cell Biology, Medical Research Institute, Tokyo Medical and Dental University, Yushima, Bunkyo-ku, Tokyo, Japan
FLOY L. CRUTCHFIELD
Medical Research Service, Philadelphia Veterans Administration Hospital, and *Department of Medicine, Medical College of Pennsylvania*
NANCY BERMAN
Departments of Anatomy, Physiology, and *Biochemistry, Medical College of Pennsylvania*
BERTRAM PAYNE
Departments of Physiology and *Biochemistry, Medical College of Pennsylvania*
MADHABANANDA SAR
WALTER E. STUMPF
Department of Anatomy, School of Medicine, University of North Carolina, Chapel Hill 27514

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Scatchard Plots

A Scatchard plot of the binding of [^{125}I]albumin to isolated hepatocytes in a recent study (*1*) is one of a number of such graphs of experimental measurements made under conditions that differ significantly from those for which Scatchard devised his analytic method (*2*). If, for example, measurements are not made at equilibrium, or if the molarity of ligand and binding molecules is not known, a Scatchard plot of the results may be of heuristic value, but it will not yield the number and association constants of the sites on the binding macromolecule. Since Scatchard's first treatment of the binding of small to large molecules appeared 32 years ago (*3*), it may be useful to reexamine the basis for determining under which experimental conditions the use of a Scatchard plot is rigorously correct, under which some useful information may be obtained, and under which its application is virtually meaningless.

In his original article Scatchard set forth the objectives of his treatment of results of protein-binding experiments: "We want to know of each molecule or ion which can combine with a protein molecule, 'How many? How tightly? Where? Why?' The answer to the first two questions, and sometimes to the third, can be furnished by the physical chemist . . . but the answers to both of the more complicated problems . . . may be the business of the physiologist or physiological chemist . . . [and] will depend on the answers to the simpler questions, 'How many?' and 'How tightly bound?' "

Scatchard made it practical and relatively easy to extract from suitable measurements the answers to both of these "simpler questions." If the initial probability of binding a molecule of A is the same at each of n sites or groups on a protein molecule (or other macromolecule) P, the change in free energy $(\Delta G)_\nu$ for the reaction $P + \nu A = PA_\nu$ is zero if, and only if, all the components and species of the reaction are at equilibrium when a measurement is made (*4*). If binding the first molecule of A to P has a negligible effect on the tightness of binding of the second molecule of A—and so on—then

$$kc_A = \bar{\nu}/(n-\bar{\nu}) \quad (1)$$

where k is the intrinsic association constant for the reaction at a single site, c_A is the concentration of free ligand molecules, and $\bar{\nu}$ is the average number of A molecules bound to each protein molecule.

Plotting $\bar{\nu}/c_A$ against $\bar{\nu}$ forms a Scatchard plot. If the points fall on a straight line the intercept on the ordinate, where c_A and $\bar{\nu}$ approach zero, is nk, and the intercept on the abscissa, where c_A is very large, is n. The straight

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