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Film Autoradiography Identifies Unique Features of [^{125}I]3,3′,5′-(Reverse) Triiodothyronine Transport From Blood to Brain

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SUMMARY AND CONCLUSIONS

1. Steady-state iodothyronine profiles in plasma are composed of thyroid gland-synthesized hormones (mainly thyroxine) and tissue iodothyronine metabolites (mainly triiodothyronine and reverse triiodothyronine) that have entered the bloodstream. The hormones circulate in noncovalently bound complexes with a panoply of carrier proteins. Transthyretin (TTR), the major high-affinity thyroid hormone binding protein in rat plasma, is formed in the liver. It is also actively and independently synthesized in choroid plexus, where its function as a chaperone of thyroid hormones from bloodstream to cerebrospinal fluid (CSF) is undergoing close scrutiny by several groups of investigators. Because TTR has high-affinity binding sites for both thyroxine and retinol binding protein, its potential role as a mediator of combined thyroid hormone and retinoic acid availability in brain is of further interest.

2. While they are in the free state relative to their binding proteins, iodothyronines in the cerebral circulation are putatively subject to transport across both the blood-brain barrier (BBB) and choroid plexus CSF barrier (CSFB) before entering the brain. Previous autoradiographic studies had already indicated that after intravenous administration the transport mechanisms governing thyroxine and triiodothyronine entry into brain were probably similar, whereas those for reverse triiodothyronine were very different, although the basis for the difference was not established at that time. Intense labeling seen over brain ventricles after intravenous administration of all three iodothyronines suggested that all were subject to transport across the CSFB.

3. To evaluate the role of the BBB and CSFB in determining iodothyronine access to brain parenchyma, autoradiograms prepared after intravenous administration of [^{125}I]-labeled hormones (revealing results of transport across both barriers) were compared with those prepared after intrathecal (icv) hormone injection (reflecting only their capacity to penetrate into the brain after successfully navigating the CSFB).

4. Those studies revealed that thyroxine and triiodothyronine were mainly transported across the BBB. They shared with reverse triiodothyronine a generally similar, limited pattern of penetration from CSF into the brain, with circumventricular organs likely to be the main recipients of iodothyronines (with or without retinol) transported across the CSFB.

5. Analysis of all of the images obtained after intravenous and icv hormone administration clarified the basis for the unique distribution of intravenously injected reverse triiodothyronine. The hormone is excluded by the BBB but may be subject to limited penetration into brain parenchyma via the CSF.

6. Overall the observations single out reverse triiodothyronine as the iodothyronine showing the most distinctive as well as the most limited pattern of transport from blood to brain. Although it is considered to be a largely inactive metabolic product formed in the service of thyroxine disposal, a number of considerations suggest that reverse triiodothyronine, actively formed from thyroxine within the brain at selected sites of inner ring monodeiodinase activity, may have as yet undiscovered functions. The present results raise the possibility that, as in the case of other known neuroactive molecules that are formed within the brain but excluded by the BBB, reverse triiodothyronine generated intracerebrally may exert important brain-specific and site-specific functional effects.

INTRODUCTION

The mechanisms of thyroid hormone actions in both developing and adult brain have not been elucidated despite considerable knowledge of the morphologic and metabolic fates and the nuclear receptor binding activities of these hormones in the CNS. In view of the obvious requirement for iodothyronines in developing brain (Schwartz 1983) and growing interest in the possibility that thyroid hormones may have a role in adult disorders of behavior, mood, and cognition (Bauer and Whybrow 1988), it is not surprising that a wide variety of approaches have been taken in the effort to shed light on this problem. One of the issues under consideration is the relative importance and the functional consequences of the different blood-to-brain hormone transport routes in determining thyroid hormone access to particular neuronal systems.

As in the case of other precursor amino acids used for product synthesis in brain, specific transport mechanisms govern the entry of iodothyroamino acids into the brain parenchyma (Pardridge 1990). Whether released from thyroid follicles or back-transported from tissues into the bloodstream, iodothyroamino acids join in the circulation, where they are bound to carrier proteins with varying affinities for the different iodothyronines (Bartalena 1990). The bound state is highly favored in the main circulation, but there is little certainty about the conditions that prevail in tissue capillary beds. Although disagreeing somewhat about the details, all agree that only when iodothyronines are in the free phase of the ligand-protein binding equilibrium are they subject to passage across either the blood-brain barrier (BBB) or the choroid plexus cerebrospinal fluid (CSF) barrier (CSFB) before entering the brain (Pardridge 1990).

Previous autoradiographic and parallel radiochemical studies in vivo have provided considerable evidence relevant to thyroid hormone entry and fate in brain. Although these studies showed that the thyroxine (T4) molecule itself

enters brain parenchyma, its localization in discrete neural elements requires its transformation to triiodothyronine (T3) (Dratman and Crutchfield 1989). This process is catalyzed by the active, low Km 5′deiodinase (type II) readily available in neuronal cells, yielding the bulk of T3 found in brain, especially during hypothyroidism (deEscobar et al. 1992; Dratman et al. 1983). However, and despite claims to the contrary based on biochemical studies alone (see Larsen 1988 for review), parallel imaging and biochemical studies have provided definitive evidence that T3 in the circulation of the hypothyroid rat enters and is long retained in the brain. Moreover, the transported hormone is noted to become highly localized over the nucleoplasm in neuronal cells of discrete neural systems (Dratman et al. 1982) in a pattern not discernibly different from that which develops when intracerebral T4 is the source of T3 (Dratman and Crutchfield 1989; Dratman et al. 1987). Additional autoradiographic studies have also revealed that T3 entering the brain from the bloodstream does so mainly through the BBB, whereas entry from CSF is markedly limited, with penetration from the ventricular system seemingly restricted to the circumventricular organs (Dratman et al. 1991).

Transthyretin (TTR), one of the major high-affinity thyroid hormone carrier proteins in serum, is synthesized in the liver. TTR is also separately synthesized within the choroid plexus epithelium. In that locale it facilitates the transport of thyroid hormones across the CSFB and binds them in the CSF (Dickson et al. 1987; Schreiber et al. 1990). Because TTR has a higher affinity for T4 and even for reverse T3 (revT3) than for T3, it is reasonable to question whether differential binding of these hormones by TTR in spinal fluid might differentially effect their rate of penetration from CSF to brain. Therefore autoradiographic studies were undertaken to evaluate the distribution of [^{125}I]T4 (T4*) and [^{125}I]revT3 (revT3*) in rat brain after transport across the BBB or CSFB, and to compare the results obtained with those of previous studies using [^{125}I]T3 (T3*) (Dratman et al. 1991).

The autoradiographic results, which could not have been predicted from biochemical data alone, provide clear-cut evidence bearing on an ongoing controversy regarding the "major" route of thyroid hormone entry from blood to brain. Imaging methods show that the transport route depends on the hormone under consideration. In the case of T3 and T4, most of the hormone entering brain parenchyma clearly arrives there via the BBB. On the other hand, the choroid plexus CSFB appears to transport virtually all the revT3 passing from blood to brain. Biochemical monitoring confirms that the autoradiographic images are due to the injected hormones or their organic iodinated metabolites.

The combined autoradiographic and biochemical results cast some new light on the potential functional importance of the different iodothyronines in brain. Considerable evidence points to a central role for brain T4 as the precursor and T3 as the active product. The ability of TTR to bind both T4 and retinol binding protein (RBP) increases the likelihood that it will facilitate transport of both thyroid hormone and retinol into the CSF (Van Jaarsveld et al. 1973). The active products of those ligands, T3 and retinoic acids, may thereby become coordinately available in brain, suggesting yet another basis for different effects of BBB-versus CSFB-transported T4.

Although a few studies tentatively suggest that revT3, actively formed from T4 in brain, may have a role there (Dubuis et al. 1992; Obregon et al. 1986), particularly during brain development, when it is the predominant iodothyronine in the fetus (Chopra and Crandall 1975), this T3 isomer is generally considered to be an inactive byproduct of T4 disposal. However, evidence that revT3, like some known neuroactive substances of considerable importance, is barred from entry across the BBB raises the possibility that this molecule may have specific functions in brain and that any actions that it may mediate on formation from T4 might be best expressed if intracerebral mechanisms direct the sites and rates of its production.

METHODS

Preparation of hormones and animals; intrathecal injection of labeled thyroid hormones

Surgically thyroparathyroidectomized (Tpx) Sprague-Dawley rats weighing ~200 g were received from Zivic-Miller Laboratories. They were treated thenceforth in accordance with institutionally approved protocols. Rats were housed in groups of three to four per cage and had free access to rat chow and 0.5% calcium chloride as drinking water and, as has been customary in previously reported studies (e.g., Dratman et al. 1987), autoradiographic and coordinated radiochemical studies were initiated within 7–10 days post-Tpx.

Methods for intrathecal (icv) injection of radioactive thyroid hormones into the lateral ventricle have been described previously (Dratman et al. 1991; Goldman et al. 1985). Briefly, T4* and revT3* dissolved in 50% 1-propanol were received from New England Nuclear [specific activity 4,400 μCi/μg (311 μCi/ml) and 815 μCi/μg (250 μCi/1.25 ml), respectively]. The isotopic preparations were made suitable for icv administration by *1*) dilution with 20 vol 10^{-3} M HCl; *2*) application to a C18 sample preparation column (treated in advance with a methanol wash followed by equilibration with 10^{-3} M HCl); *3*) removal of [^{125}I]iodide quantitatively by washing twice with 10^{-2} M HCl; *4*) elution of retained iodothyronines with 95% ethanol containing 10^{-3} M HCl; *5*) evaporation of the eluates with nitrogen to a volume of 20 μl; and finally, *6*) dilution 1:5 with saline.

Rats were anesthetized with Nembutal (40 mg/kg ip) and atropine (0.1 mg/kg) and when unresponsive to stimulation were placed in a stereotaxic apparatus. Using a Hamilton syringe, 5.0 μl of the purified and diluted labeled iodothyronine was injected slowly over a 10-min period through a burr hole into the right lateral cerebral ventricle, (coordinates 0.6 mm posterior to bregma, 1.5 mm lateral to saggital sinus, and 3.0 mm below dura); each rat received ~0.25 μCi (0.45 pmol) revT3* or 0.5 μCi (0.15 pmol) T4*. Rats were removed from the stereotaxic apparatus and were decapitated 1, 3, 5, 24, and 48 h after icv delivery of T4* and 10, 30, and 180 min after icv revT3* injection. Although trunk blood was routinely collected and serum was separated and counted, serum radioactivity was variable and generally too low to permit analysis of individual iodocompounds. Therefore no radiochemical data derived from the serum of icv-injected animals are reported here.

Preparation of autoradiograms

Methods for preparing film autoradiograms of rat brain after injection of labeled thyroid hormones have been described in de-

tail (Dratman and Crutchfield 1989; Dratman et al. 1987, 1991; Sokoloff et al. 1977). Briefly, after decapitation by guillotine, brains were rapidly removed and frozen in freon, or more recently in 2-methyl butane brought to −40°C by immersion in dry ice. The frozen brain was attached to a chuck with brain paste and serial 20-mm-thick coronal sections were cut in a cryostat maintained at −18°C. The sections were transferred to coverslips and dried briefly at 50°C, and in the darkroom under safelight the sections were apposed to tritium-sensitive nonscreen film for periods of time that were inversely proportional to the dose administered (~15 days per 1 mCi per 200-g rat in the case of icv administration). D-19 developer and Kodak rapid fix were used to develop the films. After drying in air the films were examined in a Jena microfilm enlarger to facilitate qualitative comparisons among different brain structures and regions.

Extraction and analysis of labeled brain iodocompounds

As a necessary adjunct to the interpretation of the brain autoradiograms, information about the nature of the iodocompounds found in brain tissues from rats prepared and injected with labeled thyroid hormones was gained through radiochemical analyses. Brains removed at 10 and 180 min after revT3* and 1, 3, 5, 24, and 48 h after T4* were dissected into three regions: cerebral hemispheres (Cx), cerebellum (C'bell), and the rest of the brain, designated subcortex (subCx), on the basis of observations indicating differences in distribution of hormone in these regions seen in autoradiograms previously prepared after icv administration of labeled iodothyronines (Dratman et al. 1991).

The biochemical methods used have been described in detail (Gordon et al. 1982). Briefly, as recently modified, dissected tissue was weighed; radioactivity was measured; the tissue was cut into pea-sized fragments and, to retard in vitro deiodination, treated with concentrated $Na_2S_2O_3$ to obtain a concentration of 1 mg per g tissue; tissue was immediately frozen in pulverized dry ice and transferred to a Waring Blender Mini-jar and homogenized. The brain homogenate was held at −15°C until the dry ice evaporated, radioactivity was again measured to account for nonselective losses during homogenization, and the homogenate was extracted four times with $CHCl_3$ and methanol in a ratio of 2:1. Radioactivity in the protein residues that had resisted extraction by $CHCl_3$:methanol was measured and designated "nonextractable iodocompounds." Iodide, iodothyronines, and other iodocompounds in the extract were partitioned into an aqueous phase by shaking four times with 0.2 parts 0.05% $CaCl_2$ containing 25 mg $Na_2S_2O_3$ per 100 ml; the aqueous supernatant phases were combined, lyophilized, recovered in methanol, and analyzed by reversed-phase high-performance liquid chromatography (HPLC). Concentrations of the separated iodocompounds, identified on the basis of their elution times, were calculated with the assistance of the Vision IV software program (Scientific Systems); the integrated radioactivity in each peak of the chromatogram, expressed as a fraction of the sum of integrated radioactivity above background in the eluates, specified the fractional contributions of each analyte to the total sample.

Data processing

In the autoradiographic components of these experiments, only the qualitatively evident differences in brain distribution patterns of T4* and revT3* were evaluated.

Unextractable radioactivity and labeled iodocompounds in extracts of three dissected brain regions from each of two animals obtained at each time point (10 and 180 min after icv revT3*; 1, 3, 5, 24, and 48 h after icv T4* injection) were corrected for decay and counting efficiency, and total disintegrations per minute (DPM) per milligrams tissue as well as DPM per milligrams tissue for each identified iodocompound were expressed as percentages of the injected dose. When applicable, results were compared with those previously obtained after intravenous injection of T4* and revT3* (Dratman and Crutchfield 1989) using a two-way analysis of variance (SAS Institute 1989).

RESULTS

Film autoradiography: overview

Film autoradiograms prepared at short and long intervals after hormone injection into the lateral cerebral ventricle revealed only minor differences in the distribution of T3*, T4*, and revT3* in brain. As formerly shown after icv-injected T3* and described in the case of icv-injected T4* (Dratman et al. 1991), activity over the brain after icv injection of revT3* was mainly limited to the ventricular system and, to a lesser extent, to the subarachnoid space or membranes. Although we regularly observed labeling in the ipsilateral hippocampal complex after icv T3* administration, there was no clear-cut autoradiographic evidence for penetration of the brain by T4* or revT3* after icv administration. However, even this apparent difference between T3 on the one hand and revT3 and T4 on the other may not be real. In the earlier experiments the rate of T3* injection into the ventricle may have been too rapid, possibly leading to overflow into brain tissue along the path of the needle. Subsequent experiments with revT3* and T4* were carried out more expertly and showed no evidence of ipsilateral infiltration into brain tissue.

Overall, the results after icv administration differ markedly from those obtained when the intravenous route was used to administer the labeled iodothyronines (Dratman and Crutchfield 1989; Dratman et al. 1987, 1991). Although differential quenching of radioactivity may have accentuated the differences between white and gray matter seen in the autoradiograms within 1–5 h after intravenous T4* (Alexander et al. 1981), wide distribution of label in selected regions of gray matter was characteristic of the patterns seen in autoradiograms prepared during that time interval. Between 3 and 48 h, labeling over selected fiber tracts increased and radioactivity became progressively more resolved within defined brain nuclei in a pattern not discernibly different from that seen after intravenous T3*. However, strong labeling within brain ventricles was noted to persist for ≥72 h after intravenous T4*, whereas it had dissipated by 24 h after intravenous T3*.

As shown in previous studies, the pattern of T4* distribution (but not of T4* entry) was entirely dependent on the intracerebral conversion of T4 to T3 (see Dratman and Crutchfield 1989, describing failure of T4* resolution in brain networks during treatment with the 5'deiodinase inhibitor sodium ipodate). Thus the distinctive autoradiographic patterns seen (after a lag time of ~3 h) after intravenous T4* reflect the distribution of T3*, whether T3* enters as such from the bloodstream or is derived intracerebrally from T4*. Moreover, comparison of results after intravenous and icv administration demonstrates that BBB transport accounts for the quantitatively major, although not necessarily the most important, fraction of T3 and T4 delivery from the blood to the brain.

A strikingly different pattern is seen in overviews of the film autoradiograms prepared after intravenous revT3* ad-

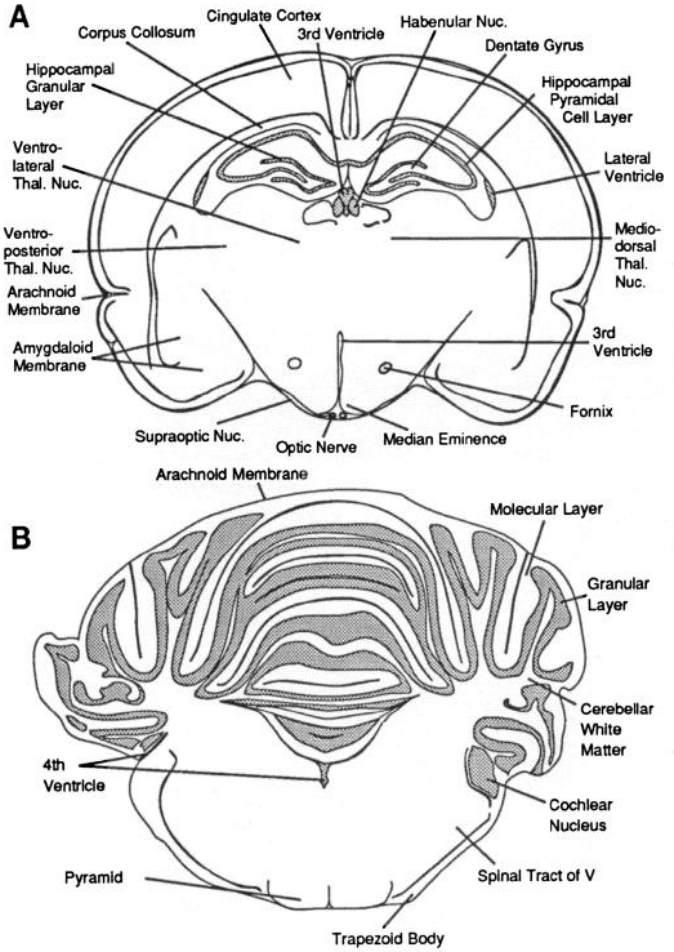


FIG. 1. Schematic representation of brain structures provided for general orientation to autoradiograms shown in Figs. 2 and 3. *A*: level of the lateral and 3rd ventricles showing cortex, hippocampal formation, thalamus, and hypothalamus (as in Plate 31, Paxinos and Watson 1986). *B*: level of the 4th ventricle showing cerebellum and brain stem (as in Plate 59, Paxinos and Watson 1986).

ministration. The differences between results of intravenous and icv administration of revT3* and T4* are clearly seen in autoradiograms of representative brain sections taken at the level of the hippocampus/thalamus/hypothalamus and C'bell/brain stem, which together reveal the reproducible patterns of lateral and third and fourth ventricle labeling and their periventricular environs, which are the focus of these studies. Schematic representations of brain sections from these regions are presented in Fig. 1; they provide a key to the range of labeled structures encountered in the autoradiograms shown in Figs. 2 and 3.

Details of autoradiographic results after revT3 administration*

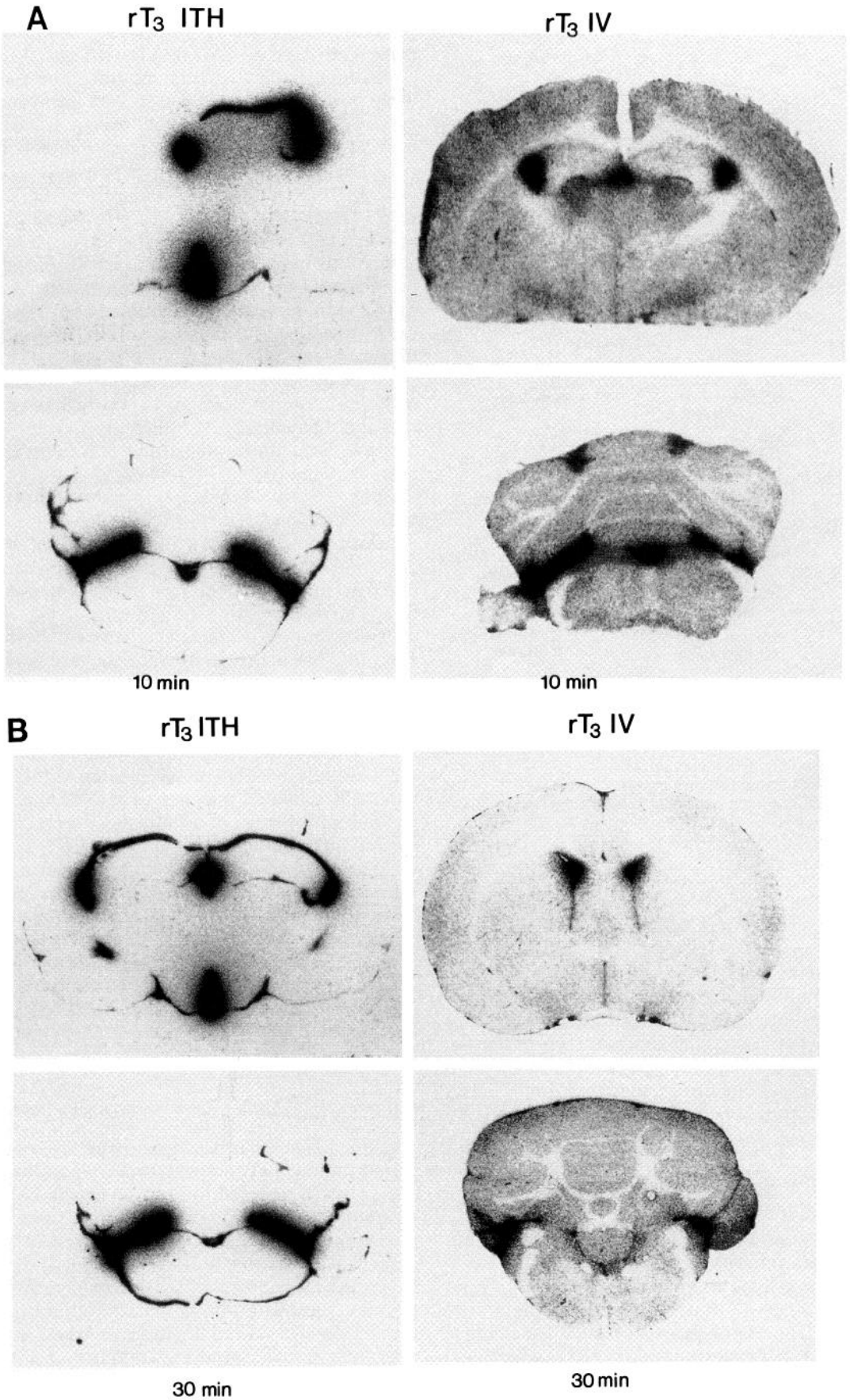
Film autoradiograms prepared from rat brains of animals given icv or intravenous revT3* are shown in Fig. 2. However, unlike the procedures followed in the case of T3* and T4*, the time of observation only extended for 3 h after revT3* injection. The use of this abbreviated time course was based on data of Obregon et al. (1985), which had shown that the plasma disappearance rate of the hormone was rapid (described as a double exponential with an initial rapid and later slower rate having slopes of −6.2 and −0.75 per hour, respectively). Moreover, the brain data derived from their studies (corrected for ^{125}I-revT3 in the cerebral circulation) indicated that the hormone accumulated in brain during the 10-min period after intravenous injection, followed by a rapid decline that soon reached a low plateau, paralleling the rapidly falling plasma levels of hormone.

Evaluation of autoradiograms prepared in our laboratory at 1, 3, 10, 30, and 180 min after intravenous injection of revT3* (only 10-, 30-, and 180-min autoradiograms shown in Fig. 2) demonstrated that the activity over brain parenchyma was in all respects characteristic of markers of cerebral blood flow (Reivich 1987) and behaved like such markers in that equilibration between blood and brain revT3* was rapid, occurring within 10 min after intravenous hormone injection (Obregon et al. 1985). These morphological and kinetic features strongly suggested that entry of blood-borne revT3 into brain through the BBB was minimal and that activity over the brain was due to label in the cerebral circulation rather than brain parenchyma. However, as noted in Fig. 2 (*right panels*), the autoradiograms did not rule out but in fact were highly indicative of passage of the hormone across the CSFB.

Information relevant to the fate of the hormone after entry into the CSF was provided by autoradiograms prepared at 10, 30, and 180 min after icv revT3* administration. The results of this study, shown in Fig. 2 (*left panels*), provided the data needed to help resolve the issue of revT3* entry into brain, especially when the results were compared with those obtained after intravenous revT3* administration (Fig. 2, *right panels*) and T4* administration by either route (seen in Fig. 3). It is noteworthy that, in the case of revT3*, and quite unlike the situation with T4* (or T3*), the distribution of hormone in brain autoradiograms prepared 180 min after injection of revT3* appears to be independent of the route of administration (compare results of icv and intravenous injection in Fig. 2*C*). Thus radioactivity is largely confined to the ventricles and arachnoid membranes in preparations from both the intravenously and the icv-injected rats. Moreover, patterns of labeling in autoradiograms prepared at both 10 min (Fig. 2*A*) and 30 min (Fig. 2*B*) in the revT3*-injected animals would also be considered independent of the route of administration if, in each instance, the blood levels of revT3* (and therefore differences in labeling of the cerebral circulation) are taken into account.

*Details of autoradiographic results after T4**

As already noted in describing overviews of the autoradiograms, radioactivity derived from icv-administered T4* appears largely confined to the ventricular system. Focusing on autoradiograms prepared from sections of hippocampus/hypothalamus and C'bell/brain stem provides convincing evidence that at both early (Fig. 3*A*) and late (Fig. 3*B*) times after receipt of the isotopic hormone, the results after icv administration are in striking contrast with those seen in parallel autoradiograms prepared at similar times after intravenous injection. Comparison of the autoradiograms in Fig. 2 with those in Fig. 3 shows that whereas the (rapidly decreasing) activity over the brain parenchyma is



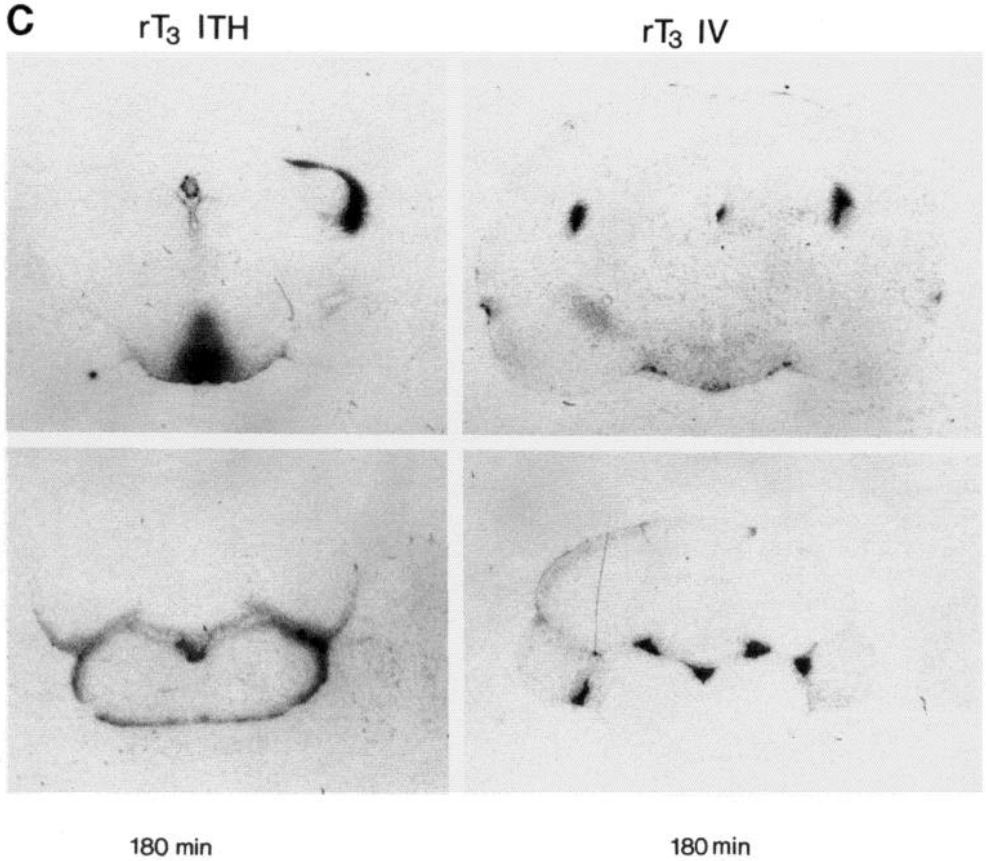


FIG. 2. Distribution of radioactivity in rat brain after injecting [^{125}I]reverse triiodothyronine (revT3*) by the intrathecal (icv) (*left panels*) vs. intravenous (*right panels*) route. Autoradiograms were prepared from coronal 20-μm-thick sections obtained from brains of injected animals at the level of the hippocampus/hypothalamus (*top panels*) and cerebellum/brain stem (*bottom panels*). ×4.4 magnification. Note that label is seen in all cerebral ventricles at all times studied after both intravenous and icv revT3* administration and in this regard resembles results after icv injection (but not intravenous injection) of [^{125}I]thyroxine (T4*), as shown in Fig. 3. However, note that intravenously delivered revT3* is distributed like markers of the cerebral circulation at 10 min (*A*, *right panel*) and rapidly dissipates over the next 20 min (*B*, *right panel*) in concert with rapidly diminishing blood levels of the hormone. Moreover, after 180 min (*C*, compare *right* and *left panels*) there are no evident differences in the distribution of label over the brain parenchyma; all autoradiograms in *C* show little activity in any brain region outside of the ventricular system, despite differences in routes of administration. This time course and distribution pattern are decidedly different from that exhibited by intravenously administered T4* (illustrated in Fig. 3).

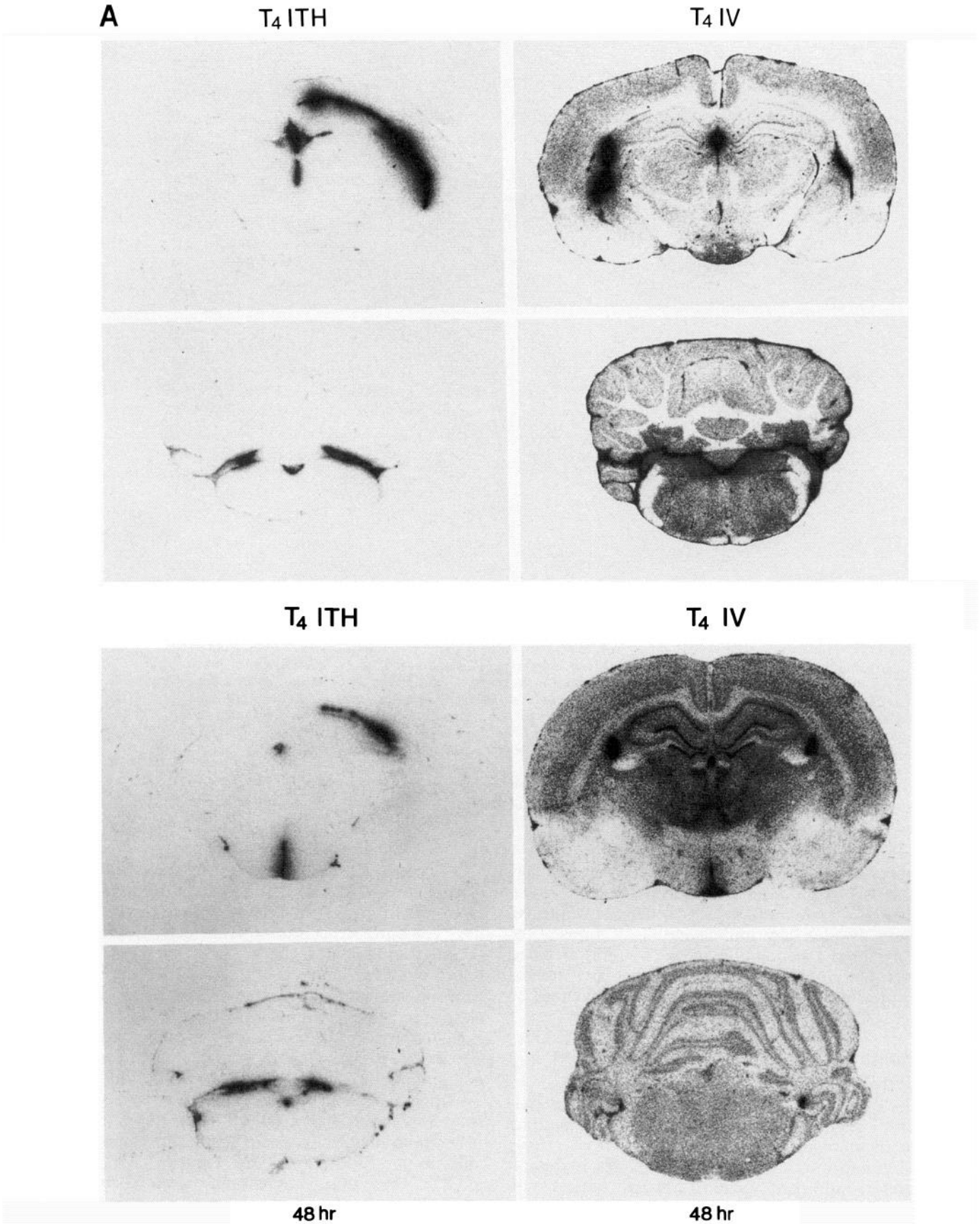
limited and poorly resolved after intravenous revT3* injection, it is noted to be widely but selectively localized in discrete neural systems when T4* is delivered intravenously. More detailed descriptions and illustrations of the distribution patterns seen in film autoradiograms prepared at intervals after intravenous hormone delivery are described in previous reports from our laboratory (Dratman and Crutchfield 1989; Dratman et al. 1987).

Radiochemical analyses after icv revT3 and T4* administration*

The fate of the icv-injected hormones was monitored by means of radiochemical analyses performed in conjunction with autoradiographic studies. As is usually the case, grossly dissected brain regions used for HPLC analyses, representing Cx, subCx, and C′bell, included not only brain parenchyma but also their contents of ventricular fluid, arachnoid membranes, and choroid plexuses. Therefore the radiochemical results have all the known limitations of bulk brain tissue analyses.

As early as 10 min after revT3* delivery into the lateral ventricle, only 20% of the radioactivity injected could be recovered from the brain, representing a loss of 8% per minute. The further decline of brain activity, measured between 10 and 180 min after icv delivery, was slower (loss of 0.25% per min), about half that reported during the same time interval after intravenous administration of revT3 (loss of 0.50% per minute). In the latter instance, as mentioned, decreasing levels of hormone in the cerebral circulation strictly parallel the loss of radioactivity from the peripheral circulation (Obregon et al. 1985). Although over the 3-h interval after icv injection the rate of revT3* disposal is more rapid than that of T4* (88% vs. 77%), the difference is less than might be expected from their reported relative affinities for TTR [T4: 100; revT3: 8.0; T3: 4.8 (Jorgensen 1978)]. These observations raise questions about the rate of equilibration of the injected hormones with TTR in CSF.

As expected from the autoradiographic results, considerably greater effects of the route of administration on rates of hormone disposal in brain were noted in the case of T4*. By



contrast with results after icv administration, levels of radioactivity in brain increased progressively over the course of 3 h after intravenous T4* injection, kinetic analyses indicating an approximate gain of 1.2% per minute during that period of time. However, in keeping with the known rapid rate of T4 replacement by T3 in the rat brain, T4* decreased by 40% whereas T3* derived from T4* in situ increased by 250% between 20 and 180 min after intravenous T4* injection (Dratman et al. 1983).

Figure 4 shows representative chromatograms prepared from extracts of subCx obtained 3 h after icv injection of revT3* (*top panel*) and T4* (*bottom panel*); similar but not identical results were seen in chromatograms prepared at other time intervals and in other brain regions. The major message conveyed by these chromatograms is that the injected iodocompounds were well separated from iodide, a problem, previously nettlesome, which is now resolved using HPLC. After T4*, the revT3 peak was reproducibly distinguished from the T4 peak but was usually not well separated from it. Therefore the mathematical ratio of activity of these iodothyronines after T4* administration, provided by VISION 4 software, (version 1.06, Scientific Systems, State College, PA), shown in Fig. 4, must be viewed with reservation. Diiodotyrosine was usually found after icv injection of both T4* and revT3*, although it is not detectable in the chromatogram shown in the *bottom panel* of Fig. 4. As noted, several T4 and even more revT3 metabolites are unidentified.

Results of HPLC analyses of brain extracts after icv revT3* injection demonstrated that overall processing of revT3* in Cx and subCx was similar, whereas results for C'bell were somewhat different. The mean fractional values for each identified iodocompound found in each region, analyzed at the 10- and 180-min time points, are shown in Table 1. Radiochemical studies after icvT4* injection suggest that the transport of the hormone from CSF to brain is governed by processes similar to those that, under comparable circumstances, prevail in the case of revT3*. Thus, as seen in Fig. 5, T3:T4 ratios, generally considered reflective of in situ T4 conversion to T3 in Tpx rat brain, are noted to mount rapidly over 3 h after intravenous T4* administration (to 1.5 ± 0.062, mean $\pm$ SE). These ratios also increase progressively, but more slowly, after icv T4* injection. By the same token, the ratio of revT3* to its major identified organic metabolite, 3,3'-diiodothyronine, also increased from the time of icv injection through the 3-h time of observation. Direct comparison of the rates of 5'deiodination mediating these metabolic events, based on ratios of T2* to revT3* or of T3* to T4*, is not possible because of different turnover times of the individual injected iodothyronines and their respective metabolites.

Unexpectedly nonextractable products were discovered to be present in brain homogenates at much higher levels after icv than after intravenous administration of T4*, at some time periods amounting to >25% of the total homogenate radioactivity. By contrast, the corresponding percent unextractable radioactivity in brain homogenates after intravenous hormone administration ranged between 5 and 10%. The extent of unextractable radioactivity was still higher after icv revT3* administration, despite the shorter time period of observation (Table 1).

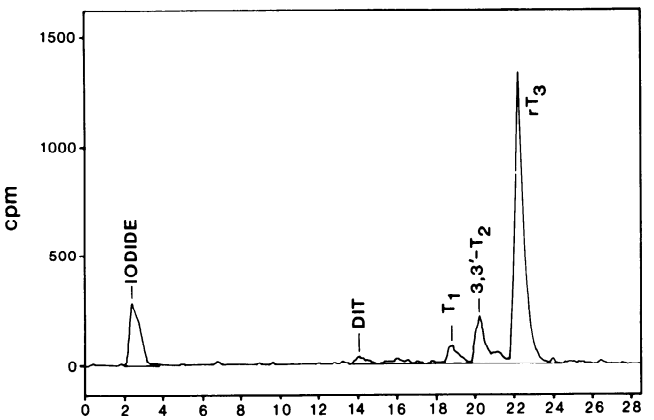
Because precursor-to-product ratios in brain were noted to increase with time after icv administration of both revT3* and T4*, it is likely that the hormones in the CSF, equilibrating with brain extracellular fluid, enter periventricular and even more remote regions of brain but at levels too low to be detected autoradiographically. Less likely is the possibility that the hormones do not move beyond the CSF compartment and are metabolized entirely in ependymal or arachnoid membrane cells lining the CSF compartment. Although some information regarding the rates and direction of movement between brain and CSF of conventional amino acids is reported (Hamberger et al. 1990), these data are not available for iodoamino acids.

DISCUSSION

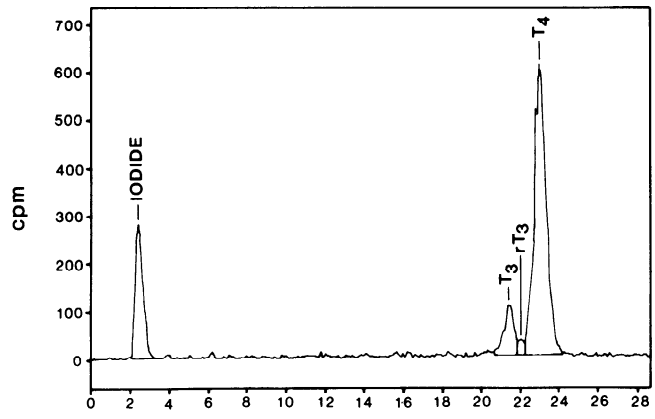
Although both T4 and T3 are known to enter the brain across the BBB (Pardridge 1979), interest has recently been focused on the choroid plexus CSFB as a potentially important, even a primary path (Schreiber et al. 1990) of thyroid hormone entry. The basis for this interest lies in a series of related but independently reported observations that may be summarized as follows:

Molecules passing through the choroid plexus CSFB enter the CSF, a fluid that is in equilibrium with the extracellular fluid compartment that surrounds, supports, and nourishes the cellular components of brain tissue (Davson et al. 1987). CSF has a higher concentration of free iodothyronines than serum (Hagen and Solberg 1974) and therefore, on transport across the CSFB and equilibration with brain extracellular fluid, iodothyronines should have access to brain cells and nerve terminals. Evidence that functional effects are promoted by thyroid hormones in the CSF is provided by experiments demonstrating that icv-adminis-

FIG. 3. Distribution of radioactivity in rat brains after icv (*left panels*) vs. intravenous (*right panels*) injection of T4*. Autoradiograms of coronal 20-μm-thick sections were obtained from brains of T4*-injected animals at the level of the hippocampus/hypothalamus (*top panels A* and *B*) and cerebellum/brain stem (*bottom panels A* and *B*). ×4.4 magnification. Note lack of penetration of the hormone into the contralateral ventricle and the apparent confinement of radioactivity to the rest of the ventricular system at both 3 h (*A*) and 48 h (*B*) after icv T4*. Markedly contrasting results at those time intervals are evident after intravenous hormone injection, viz: *A*: at 3 h, *right panels*: note the widespread distribution of radioactivity in gray matter, together with evidence of beginning resolution of label in discrete nuclei of thalamus, hypothalamus, and brain stem. Differences in self-absorption between white and gray matter may exaggerate but do not account for their evident differences in labeling intensity (Alexander et al. 1981). Also note that intravenously administered hormone labels the ventricular system, presumably as a result of passage of blood-borne T4* across the choroid plexus CSFB. *B*: at 48 h, *right panels*: contrasting results in intravenously injected rats are even more striking at this time interval in that radioactivity is highly and selectively resolved in the granular and pyramidal cell layers of the hippocampal formation; in discrete nuclei of the thalamus, hypothalamus, and brain stem; in the granular layer of cerebellum; and in some regions of white matter.



** Ret. Time (Min)	Area (uV Sec)	Height (uV)	Area %	Norm. Area	Area/Hgt (Sec)
2.470	5314.2	292.5	15.258	25.145	18.17
14.050	672.7	33.7	1.931	3.183	19.99
18.800	1697.7	90.2	4.874	8.033	18.82
20.200	3730.3	226.7	10.710	17.650	16.46
22.226	21134.0	1345.6	60.679	100.000	15.71



** Ret. Time (Min)	Area (uV Sec)	Height (uV)	Area %	Norm. Area	Area/Hgt (Sec)
2.427	7643.4	283.7	19.768	28.647	26.94
21.450	3642.2	103.6	9.420	13.651	35.17
22.000	698.7	32.5	1.807	2.619	21.52
23.029	26681.2	611.5	69.005	100.000	43.63

FIG. 4. Chromatographic separation of iodocompounds in brain extracts prepared after icv injection of revT3* (*top panel*) and T4* (*bottom panel*). Rats were injected and decapitated, and brain extracts were prepared and high-performance liquid chromatography (HPLC) analyses carried out as described in METHODS. Note that separation of the injected iodothyronine and its metabolites from iodide is excellent. Identification of labeled reverse triiodothyronine (revT3) formed after icv injection of T4* was reproducible but overlap of the ascending and descending limbs of the revT3 peak with the triiodothyronine (T3) and thyroxine (T4) peaks, respectively, makes quantitative estimation of labeled revT3*:T4* ratios unreliable. (Data provided by Vision 4-assisted analysis of the chromatograms.) Diiodotyrosine (DIT), shown as a peak in the chromatogram illustrated in the *top panel* (revT3* injection), was not seen in the chromatogram in the *bottom panel* (T4* injection) but was usually identified in chromatograms after icv injection of both revT3* and T4*.

tered T3 causes greater and earlier heart rate changes than T3 injected into the peripheral circulation (Goldman et al. 1985).

TTR, the major thyroid hormone binding protein in the

TABLE 1. *Proportions of labeled iodocompounds identified in rat brain after intrathecal injection of revT3*

	Time After revT3* Injection Into Lateral Ventricle	
	10 min	180 min
Cortex		
Iodide	36.5	12.1
3'3-T2	3.9	7.4
revT3	25.4	27.1
Unextractable†	27.5	51.5
T2/revT3	0.154	0.273
Subcortex		
Iodide	33	11.2
3'3-T2	4.3	5.6
revT3	28.4	27.2
Unextractable†	26.0	48.0
T2/revT3	0.151	0.206
Cerebellum		
Iodide	32.6	13.6
3'3-T2	2.0	19.6
revT3	43.9	36.0
Unextractable†	16.5	29.5
T2/revT3	0.046	0.544

Values are percentages of total radioactivity in each brain region. Four rats were injected; 2 were decapitated at each time intervals shown; brain regions were dissected and individually homogenized, extracted, and analyzed, and labeled iodocompounds identified as described in METHODS. Radioactivity in high-performance liquid chromatography-separated peaks was adjusted for unextractable radioactivity and expressed as a percent of the total DPM in each dissected brain region, as described in METHODS. Data represent mean values for each compound from each region for each of 2 rats. revT3*, [^{125}I]reverse triiodothyronine; 3'3-T2, diiodothyronine; revT3, reverse triiodothyronine. † Unextractable radioactive iodocompounds.

CSF and extracellular fluid space, constitutes >25% of the total protein in the CSF (Weisner and Roethig 1983). The source of TTR in CSF is the choroid plexus epithelium

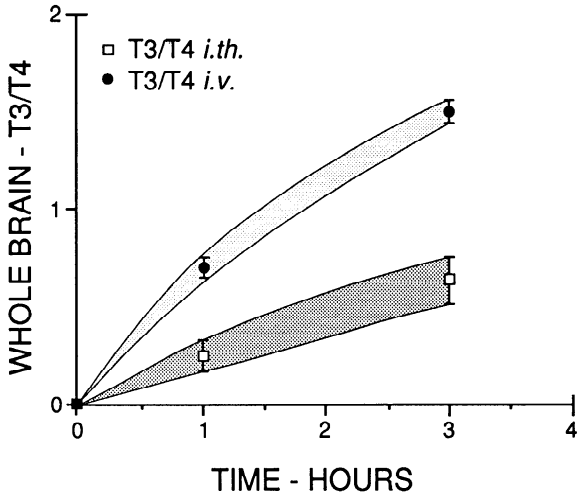


FIG. 5. Ratios of [^{125}I]T3 (T3*) to T4* after intravenous and icv injection of T4*. Rats were decapitated and whole brains were homogenized at 1 and 3 h after injection. T3* and T4* peaks in brain extracts were resolved by (HPLC) as described in METHODS. Data show mean ratios of T3* to T4* after intravenous T4* (● with light shading: SE) or after icv T4* (□ with dark shading: SE); $n = 4$ in each category. Analysis of variance (ANOVA) reveals that the brain T3:T4 ratios are significantly higher and increase more rapidly after intravenous compared with icv injection of T4* ($P < 0.001$). i.th., intrathecal.

(Aleshire et al. 1983; Soprano et al. 1985), where TTR mRNA is abundant and separately controlled [i.e., not responsive to conditions that markedly influence the process in liver cells (Dickson et al. 1986)]. Moreover, in addition to its well-known high-affinity binding capacity for T4, TTR specifically binds RBP (Van Jaarsveld et al. 1973). Because a large component of thyroid hormone transport across the choroid plexus CSFB probably involves an obligatory interaction of T4 with TTR of choroid plexus origin (Robbins and Lakshmanan 1992; Schreiber et al. 1990) it is likely that retinol, by virtue of its binding to RBP, also enjoys facilitated transport into the bulk CSF.

After entry into the CSF under the guidance of TTR, thyroid hormones, and presumably retinol, may be selectively delivered to the circumventricular organs of the CNS (Dratman et al. 1991), where hormone-dependent functions are reported to be particularly active (Gross 1987). Some evidence even suggests that TTR may facilitate transport of T4 (and retinol) into some brain cells, and may do so more actively with increasing CSF T4 concentrations (Divino and Schussler 1990a,b). T3 (Dratman and Crutchfield 1978) and various isomers of retinoic acid (MacDonald et al. 1990; anonymous review, 1991) are reported to be formed from their precursor molecules within many tissue cells, including brain cells. Therefore TTR may be seen as a conduit for selective delivery of these products into defined brain regions.

The combined T4 and retinol carrying properties of TTR are emphasized in this report because the potential functional implications of those properties has received little attention, despite that fact that the direct interactions of their active products, T3 and retinoic acids, have recently been the object of intensive investigation. Interest in the interactions of T3 and retinoic acids became active when it was noted that, on binding to their respective nuclear receptors, the complexes can form functionally active heterodimers capable of binding to thyroid hormone response elements in DNA (Forman et al. 1989). Further studies (e.g., Davis and Lazar 1992) emphasize the potentially important and complex links between retinoids and thyroid hormones. In view of the active rate of synthesis of TTR by choroid plexus cells, TTR may, through its activity in transporting its ligands into the CSF, serve a potentially important and still unexplored role in facilitating and maintaining certain region-specific aspects of combined T3 and retinoid-dependent brain functions.

The major routes by which iodothyronines in the circulation may have access to the brain are represented in the diagram in Fig. 6. Unfortunately, the kinetics of transport at most of the steps depicted in the scheme shown in the figure are largely unknown. Moreover, even the results derived from the limited efforts made in the present experiments to evaluate certain kinetic features of CSF-transported hormone turnover are fraught with uncertainties. For example, as early as 10 min after revT3* or T4* delivery into the lateral ventricle, only a very small fraction of the injected dose could be recovered from the brain. A similar result was noted in the case of icv-injected unlabeled T3 (Goldman et al. 1985). In the latter study, the early rapid rate of hormone clearance could be accounted for by rapid back-transport of T3 into the circulation from the CSF. However, the observations do not necessarily reflect the physiologic fate of hormone delivered into the CSF from the bloodstream via the choroid plexus. In the normal course of events, facilitation of hormone entry by TTR would, prima facie, favor entry of bound hormone into the CSF. Moreover, hormone or hormone products returned from brain cells to the CSF (whatever the original route of hormone entry into brain) would more likely be in the free state. Therefore iodocompounds entering the CSF from the brain might then be more readily subject to back-transport into the bloodstream through the arachnoid villae that penetrate into the dural sinuses (Davson et al. 1987). If the ratio of bound to free hormone in CSF differs according to the sources of hormone (exit from brain cells into CSF vs. entry into CSF from arterial capillaries in the choroid plexus), the rate of hormone disposal might also differ according to the source.

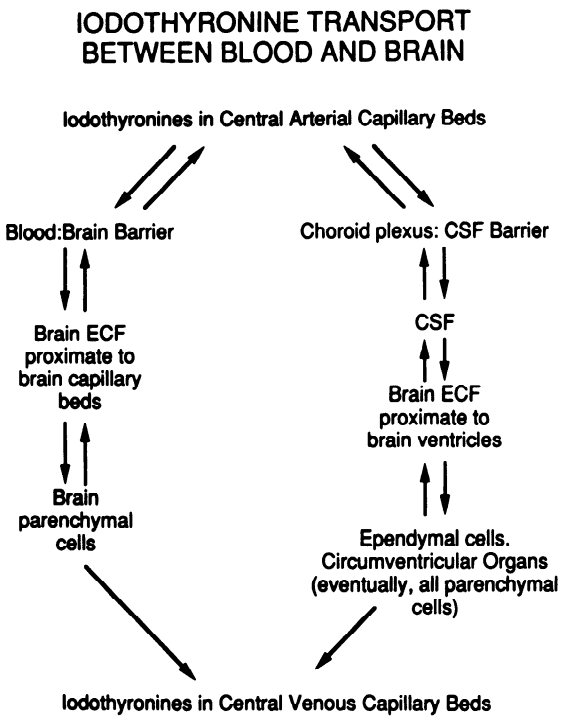


FIG. 6. Routes of iodothyronine transport between blood and brain. According to the autoradiographic results, revT3* in central arterial capillary beds does not cross the blood-brain barrier (BBB) (route shown at *left*) but probably crosses the cerebrospinal fluid barrier (CSFB) (route shown at *right*), whereas T4* crosses both the BBB and the CSFB.

Despite emphasis on a slow icv hormone injection rate in our experiments, it is likely that a large part of the injectate did not come into equilibrium with the TTR in the CSF and could therefore be in a mainly free state during that early period characterized by a very rapid rate of hormone disposal. Under those conditions, penetration of free hormone through the arachnoid villus membrane into the vascular sinuses in the dura would be expected, whereas passage of TTR-bound hormone through villus pores might be much more restricted. Modification of our present approach through simultaneous administration of TTR-bound T4 into both the lateral ventricle and peripheral vein in which each injectate carried different iodine isotopes might help to clarify some of these issues.

Routes of revT3 transport into the brain: implications for its role in the CNS

Using radiolabeled hormone and radiochemical analyses, Obregon et al. (1985) have demonstrated that after intravenous administration of revT3* there is an early (10 min) period of accumulation of the hormone in brain followed by a rapid and synchronous fall of brain and blood levels of revT3* between 10 and 180 min after injection. Preliminary autoradiographic studies of serially sectioned rat brains after intravenous administration of revT3* led to the suggestion that the hormone was either barred from entry across the BBB or that it was transported across this barrier but was so rapidly metabolized that it could not be seen by means of imaging techniques. To address this possibility, brain imaging was performed as early as 1, 3, 10, and 30 min after intravenous hormone administration (Dratman et al. 1991). However, the autoradiograms prepared at these time intervals, although uniformly showing strong choroid plexus activity, failed to demonstrate any brain activity that could be distinguished from labeling of the cerebral circulation.

The present autoradiographic results have now served to identify the morphologic correlates of the earlier biochemical observations of Obregon et al. (1985): labeling attributed to the brain is due to hormone within choroid plexus, ventricular CSF, and the cerebral circulation. As blood levels of hormone in blood fall, so do levels of hormone in the cerebral circulation. After 3 h, the net radioactivity measured over the brain is clearly due to activity over the ventricles. Taken together, these morphologic and kinetic features demonstrate that entry of blood-borne revT3 into brain through the BBB is minimal, whereas passage of the hormone across the CSFB provides its main, albeit limited, route of entry.

Restricted entry across the BBB is also reported to apply to the hormonally inactive T3 isomer D-T3 (Terasaki and Pardridge 1987). Thus evidence that revT3 does not cross the BBB may reinforce the idea that it is an inactive molecule for which no particular BBB transport mechanism into the brain has been developed. And so it may be. However, other conceptual approaches to the data may be relevant. For example, it is well known that the brain, through its barriers, rejects or minimally transports those intravenously borne neuroactive molecules whose rate of production it must control through mechanisms relevant to its own neural requirements. Thus blood-borne norepinephrine is reported to be excluded by the BBB, and neuroactive amino acids like glycine and glutamic acid penetrate very slowly (Oldendorf 1971). As a result, direct injection of these substances into the cerebral ventricles is often a ploy used to test their fate or function in the CNS.

The possibility that revT3 may also be excluded from the BBB on grounds of its importance as a tightly regulated neuroactive substance may therefore deserve consideration. In vitro data of Kaplan and Yaskoski (1980) suggest that as much as 34% of T4 reaching the brain may be converted to revT3. Evidence reviewed by Larsen (1988) amply attests to the fact that revT3 is derived intracerebrally from T4 in vivo under highly controlled conditions. Given the low levels of revT3* recovered in brain homogenates after intravenous T4* administration (Dratman and Crutchfield 1978; Obregon et al. 1985), it appears that this T4 product, unlike T3, is rapidly further metabolized. Because a rapid rate of metabolism and/or disposal is a feature characterizing the handling of many highly potent signaling molecules in the nervous system, the apparently rapid rate of revT3 disappearance might further stimulate interest in this T4 metabolite. Thus it appears that further information regarding the morphologic, biochemical, and functional fate of revT3 derived from T4 in brain may be of considerable importance for understanding the role of this molecule in the CNS.

Finally, it is evident that a decisive contribution to the issue of the major route of thyroid hormone entry into the brain has been made through the combined use of film autoradiography and radiochemical analysis. The data gathered in these and previous studies have demonstrated that T3 and T4 are mainly transported into the brain across the BBB, whereas revT3 is essentially barred from transport across that barrier. On the other hand, the ability of revT3 to traverse the CSFB is apparently not different from that of the other iodothyronines. According to the present evidence, all three iodothyronines studied share a limited ability to penetrate into the brain on entering the CSF. Despite the apparent quantitative limitations on entry, the possibility of a directed flow of hormone into circumventricular regions may, in terms of function, compliment significantly the widely disseminated supplies of T3 and T4 available via the BBB. When the unknown potential of codelivery of retinol and iodothyronines into the CSF through the agency of TTR is added to these considerations, the possible consequences of hormone delivery by the choroid plexus CSF route become even more intriguing.

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