CLINICAL STUDY

Evidence for a role of the type III-iodothyronine deiodinase in the regulation of 3,5,3′-triiodothyronine content in the human central nervous system

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Abstract

Objective: Thyroid hormone is essential for maintaining normal neurological functions both during development and in adult life. Type III-iodothyronine deiodinase (D3) degrades thyroid hormones by converting thyroxine and 3,5,3′-triiodothyronine (T3) to inactive metabolites. A regional expression of D3 activity has been observed in the human central nervous system (CNS), and a critical role for D3 has been suggested in the regulation of local T3 content in concert with other enzymes.

Design: This study was undertaken to further characterize D3 activity in human CNS and to understand its role in the local regulation of T3 content.

Methods: Autopic specimens from various areas of human CNS were obtained 6–27 h postmortem from 14 donors who died from cardiovascular accident, neoplastic disease or infectious disease. D3 was determined by measuring the conversion of T3 to 3,3′-diiodothyronine. The T3 content was measured by radioimmunoassay in ethanol extracts, using a specific antiserum.

Results: High levels of D3 activity were observed in hippocampus and temporal cortex, lower levels being found in the thalamus, hypothalamus, midbrain cerebellum, parietal and frontal cortex, and brain stem. An inverse relationship between D3 activity and T3 content in these areas was demonstrated.

Conclusions: We have concluded that D3 contributes to the local regulation of T3 content in the human CNS.

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Introduction

Thyroxine (T4) is the main secretory product of the thyroid gland. It needs to be converted to 3,5,3′-triiodothyronine (T3) to achieve hormonal activity. Only a minority of circulating T3 is secreted directly from the thyroid and most of it derives from peripheral deiodination of T4 (1). This reaction is catalyzed by 5′(outer ring)-iodothyronine deiodinases, named type I- (D1) and type II-deiodinase (D2), which are located in several tissues (2). These enzymes have a dual role of maintaining adequate amounts of T3 in the bloodstream and of regulating the T3 content in selected tissues (3, 4). Local production of T3 appears to be especially important in the central nervous system (CNS) because of the necessity of maintaining the concentration of T3 within narrow limits in face of wide variations in circulating levels of T4 (5). A distinct 5(inner ring)-deiodinase, named type III (D3), catalyzes the inactivation of thyroid hormones by converting T4 and T3 to inactive 3,3′,5′-triiodothyronine (rT3) and 3,3′- diiodothyronine (3,3′-T2) respectively. D3 is located mainly in the placenta, skin, intestine and CNS and is of particular relevance in the fetus in which it contributes to maintaining the concentration of serum T3 at a low level (6). Recently, a new syndrome of severe hypothyroidism caused by excess D3 activity in infantile hemangiomas has been described (7). The interplay of activating (D1 and D2) and inactivating (D3) pathways of thyroid hormones is responsible for the differential regulation of intracellular T3 levels in selected tissues. The best evidence for this phenomenon has been provided by studies in Rana catesbeiana (8) and the rat (9) while much less is known in humans. A regional expression of the D3 activity has been observed in human CNS, which was coincident with the distribution of rT3, suggesting that this iodothyronine is locally
generated by 5-monodeiodination of T4 (10). In the same study, the tissue concentrations of T3 in CNS were directly correlated with those of T4, indicating local 5’-monodeiodination of T4 as the main source of T3 in CNS. Our study was undertaken to further characterize D3 activity in human CNS and to understand its role in the local regulation of T3 content.

Materials and methods
Autopic specimens were obtained between 6 and 27 h postmortem from 14 donors (8 males, 6 females, age range 42–89 years) who died from cardiovascular accident (n = 8), neoplastic disease (n = 4) or infectious disease (n = 2). Samples were dissected from macroscopically normal areas of CNS, immediately frozen and kept at −70 °C until used in specific assays. Placental tissue was obtained immediately after delivery of a full-term newborn.

Assay of D3 activity in CNS
D3 activity was determined by measuring the conversion of T3 to 3,3’-T2 as previously described (11). Tissue samples were homogenized in 50 mmol/l phosphate buffer (pH 7.4) containing 10 mmol/l EDTA and 0.4 mmol/l phenylmethylsulfonylfluoride (Sigma Chemical Co., St Louis, MO, USA). Microsomes were prepared by ultra centrifugation and suspended in the same buffer by sonication. The protein concentration was determined by Bio-Rad micro assay reagent (Richmond, CA, USA). Microsomes (1–30 μg protein) were incubated with 0.6 nmol/l 125I-T3 (Amersham International, Milan, Italy) in the presence of dithiothreitol (10 mmol/l, Sigma), in 0.1 mol/l Tris buffer, pH 7.4, at 37 °C for 30–240 min (final volume 0.25 ml). The reaction was stopped by adding 0.1 ml 5% bovine serum albumin, followed by 2 vol. ethanol. The mixture was centrifuged and 125I-3,3’-T2 was quantified in an aliquot (75 μl) of the supernatant by antibody-bound 125I-3,3’-T2 was precipitated by adding a previously determined excess of goat anti-rabbit gamma-globulin. Data are means of duplicate determinations that differed from each other by less than 10%. When the effect of inhibitors of D3 activity was studied, they were added in various concentrations at the beginning of the incubation, and the results are expressed as the percentage of D3 activity observed in the absence of inhibitor.

Assay of T3 content in CNS and placenta
The T3 content in autopic specimens was measured by radioimmunoassay (RIA) using a specific rabbit antiserum (Sigma) with low cross-reactivity for other iodothyronines (cross-reactivity with T4, rT3, T3 sulfate, 3–5 diiodothyronine <1%). Tissue samples were homogenized in 5 vol. ethanol and an aliquot of the ethanol extract was used for T3 assay. The following reagents were added to disposable tubes in two replicates: (1) 0.075 mol/l barbital buffer (pH 8.6) containing 1 g/l sodium azide and 0.125% normal rabbit serum to a final volume of 1 ml; (2) 50 μl ethanol in standard curve tubes or an equal volume of an ethanol extract of test sample; (3) 100 μl of various solutions of cold T3 to achieve 9.6–614 fmol T3 in tubes for a seven-point standard curve; (4) 100 μl 1/150 000 diluted T3-binding rabbit antiserum; (5) approximately 12 000 c.p.m. 125I-T3 in 100 μl barbital buffer. The tubes were mixed after each addition and then incubated at 4 °C for 16 h. One hundred microliters of a previously titered goat antirabbit antibody and 0.6 ml 10% polyethylene glycol were then added. Subsequent steps for separation of bound from free radioactivity and calculation of results were undertaken as described for the RIA of T3 in serum (13). Results are expressed as pmol T3/g tissue.

Five specimens from different areas of placenta were collected immediately after delivery and placed in ice until homogenization in 5 vol. ethanol, about 30 min after collection. Placenta was then kept at room temperature for a time-period up to 6 h. At 1, 3 and 6 h, five specimens each time were again collected and homogenized. T3 content in ethanol extracts was measured as described for CNS.

In our hands, the detection threshold of the T3 RIA approximated 9.6 fmol which permitted the detection of 1.15 pmol T3/g tissue when 50 μl ethanol extract, representing 8.3 mg tissue, were assayed. The intra- and the interassay coefficients of variation approximated 8% and 12% respectively. The recovery of non-radioactive T3 (amounts ranging from 9.6 to 307 fmol/tube) added to 50 μl ethanol extract of brain tissue) averaged 99% (Table 1), indicating that ethanol extracts in these experimental conditions do not interfere with T3 measurement. Figure 1 shows the

Table 1 Recovery of non-radioactive T3 added to 50 μl pooled ethanol extracts from ten different brain areas. Results are the mean of duplicates and were obtained in one representative experiment out of three.

<table>
<thead>
<tr>
<th>T3 added to sample (fmol/tube)</th>
<th>T3 expected (fmol/tube)</th>
<th>T3 measured (fmol/tube)</th>
<th>Recovery (%)</th>
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<tr>
<td>0 — 44.9 —</td>
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<tr>
<td>9.6</td>
<td>54.5 —</td>
<td>48.5 —</td>
<td>89 —</td>
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<td>19.2</td>
<td>64.1 —</td>
<td>70.4 —</td>
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<td>38.4</td>
<td>83.9 —</td>
<td>85.4 —</td>
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<td>76.8</td>
<td>121.7 —</td>
<td>118.9 —</td>
<td>98 —</td>
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<td>153.6</td>
<td>198.5 —</td>
<td>196.0 —</td>
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<tr>
<td>307.2</td>
<td>352.1 —</td>
<td>327.9 —</td>
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(mean ± s.d.)
parallelism of the standard curve of T3 and the curves obtained with graded amounts of ethanol extracts from various CNS areas, indicating that what was measured by the RIA is real T3. No changes of T3 content were observed as long as 6 months after homogenization in ethanol.

Results
Characterization of D3 activity in human CNS
Before investigating the role of D3 in the regulation of T3 content in the CNS, we characterized the activity of this enzyme in autopic brain specimens. D3 activity was low to barely detectable in autopic CNS specimens obtained 12 or more hours after death. The following experiments were therefore performed using brain samples obtained in the earliest (6 h after death) autopsies. These autopic brains were identified as brains 1 and 2. D3 activity increased linearly with incubation time up to 120 min, and with protein concentration up to 40 µg microsomal protein/ml incubation mixture. Figure 2 shows the kinetic parameters of 5-monodeiodination of T3 as examined by Lineweaver–Burk analysis. The $K_m$ was 11.2 nmol/l and the $V_{max}$ was 6.6 pmol/mg microsomal protein per hour. To establish the effects of putative inhibitors on D3 activity, increasing concentrations of various substances were added to the incubation mixture. The results of these experiments are shown in Fig. 3. Among iodothyronines tested in experiments on inhibition of D3 activity, T3 was the most potent inhibitor, 3,5-T2 and T4 showed an intermediate potency, while $rT_3$ and T3 sulfate exhibited little or no effect up to 100 nmol/l. Iopanoic acid and aurothioglucose produced about 70% inhibition of D3 activity when tested at concentrations of 0.4 and 2 µmol/l respectively. More than 90% inhibition was achieved with 2 µmol/l iopanoic acid and with 10 µmol/l aurothioglucose.

Mapping of D3 activity and T3 concentration in different CNS areas
Figure 4 shows the distribution of D3 activity and the T3 content in different areas of brain 1 and brain 2.
The overall D3 activity was higher in brain 2 than in brain 1. D3 activity differed among various areas of the CNS. In both brains high levels of D3 activity were observed in the hippocampus and temporal cortex, lower levels being found in the thalamus, hypothalamus, midbrain, cerebellum, parietal and frontal cortex, and brain stem. D3 activity was nearly undetectable (0.27 fmol 3,3'-T2/µg per h) in the pituitary of brain 2 (data not shown because the residual pituitary specimen was insufficient for T3 measurement; pituitary from brain 1 was not available). In both brains there was an inverse relationship between the D3 activity and the T3 content in various areas of the CNS. To analyze this correlation in the two brains, D3 activity was normalized by expressing it as a percentage of the activity measured in the hippocampus of the corresponding brain. This allowed pooling of data obtained from the two brains. The results of this analysis are shown in Fig. 5. Figure 5A indicates that a T3 content >2.5 pmol/g was measured only when D3 activity was lower than 50%. When D3 was plotted on a log scale, a highly significant negative linear regression between D3 activity and T3 content was demonstrated (Fig. 5B).

Figure 6 shows the T3 content in several areas of CNS from all the 14 donors. The T3 content in pituitary, medulla oblongata, pons and cerebellum was higher than that observed in other CNS areas. The lowest concentrations were found in temporal cortex, hippocampus, choroid plexus and epiphysis. A remarkable variability of T3 content was found in each CNS area among different donors. This variability could not be attributed to postmortem degradation of T3, because there was no relationship between the T3 content in different CNS areas and the time elapsed from death to autopsy (data not shown).

**Time-course of T3 content in placenta after delivery**

To test whether T3 degradation by type III monodeiodination occurs in devitalized human tissues kept at room temperature, we measured the T3 content in human placenta at various times after delivery. Placenta was chosen because it contains high amounts...
of D3 and is easily available for experimental purposes. The mean (±S.D.) T3 content was 3.16 ± 1.14, 2.91 ± 2.13, 3.04 ± 2.24, 2.81 ± 0.44 pmol/g tissue at 0, 1, 3 and 6 h respectively. The T3 content at various time-periods did not differ significantly, as assessed by ANOVA test.

Discussion

The term D3 designates the enzyme(s) that selectively deiodinates the tyrosyl ring of T4 and T3, yielding the inactive metabolites rT3 and 3,3'-T2 respectively. Substrate specificity, kinetic parameters, response to inhibitors and tissue distribution differentiate D3 from D1 which is also capable of tyrosyl ring deiodination of iodothyronines (2). Studies on D3 have been mainly carried out in the rat and non-mammalian species. In humans, D3 activity has been detected in CNS (10, 14–16), liver (17) and placenta (11, 18–20), but most in vitro studies have been conducted in the latter tissue due to its availability. A molecular weight of 15–45 kDa was predicted by gel filtration of human placental microsomes (11), but further attempts to purify the D3 protein by conventional means were unsuccessful. Indeed, D3 is an intrinsic, membrane-bound enzyme, and loses its activity during solubilization with detergents because of disruption of essential phospholipid–protein interaction (11). Recently, the cDNA for a 32 kDa selenoprotein with the kinetic properties of D3 has been isolated in the human placenta, and the corresponding mRNA has been detected in placenta and lung but not in several other tissues including brain (21). Data from D3 in human CNS derive from studies in brain tumors (14, 16), neurosurgical and autopic specimens (10, 16). In the study by Campos-Barros et al. (10) D3 activity was demonstrated in samples from the frontal and temporal cortex, and from the hippocampus, but was undetectable in the cerebellum and pons. Our study confirms and extends these previous data, and provides strong evidence for an important role of D3 in the regulation of the local content of T3 in human CNS. We studied D3 activity in brains from two donors submitted to autopsy 6 h postmortem. Preliminary experiments indicated that D3 activity was low to barely detectable in autopic CNS specimens obtained 12 or more hours after death, indicating a postmortem degradation of the enzyme. Thus, a considerable loss of enzyme likely occurred even in the 6-h samples. Nevertheless, in line with previous observations (10), we found high D3 activity in hippocampus and temporal cortex. We also examined other CNS areas and were able to detect significant enzyme activity in midbrain, hypothalamus, thalamus, basal ganglia, parietal, frontal and occipital cortex. Pituitary, pons and cerebellum exhibited little or no activity. The kinetic characteristics of 5-monodeiodination of T3 and the pattern of inhibition by iodothyronines, iopanoic acid and aurothioglucose were similar to those previously reported for D3 activity in the placenta (22). These findings suggest that the same enzyme is responsible for D3 activity in human CNS and in placenta. The distribution of D3 activity in different CNS areas was slightly different between the two brains. This difference may depend on interindividual variations or might be due to the uneven distribution of D3 within each area. However, when the T3 content was measured in the same tissue...
samples, a highly significant inverse relationship between T3 concentrations and D3 activity was observed. The source of T3 in the CNS has been extensively investigated. Current evidence indicates that local deiodination of T4 by D2 is the main mechanism yielding T3 in the CNS. Availability of T4 is also important, and differential uptake of the hormone from the circulation may regulate the production of T3. Although our data are limited to two brains only, they strongly suggest that local inactivation of T3 by D3 is important for the regulation of thyroid hormone homeostasis in CNS by preventing excess accumulation of T3 derived from the circulation or locally generated by D2 deiodination of T4. Thus, our data suggest that local T3 production is most important in areas where D3 activity is low, while local production is less relevant in areas where D3 activity is elevated (Fig. 5). Nevertheless, we cannot rule out the alternative possibility that the low T3 concentration in some areas is due to low D2 expression more than high D3 expression.

It could be argued that the low T3 content found in areas exhibiting high D3 activity is the result of postmortem deiodination of T3. This argument is contradicted by our experiments in human placenta, strongly suggesting that after cessation of blood supply the T3 content remains stable in tissues containing D3. These data are in keeping with previous results indicating no major differences between the T3 content measured in human cortical samples obtained intraoperatively and that found in samples obtained by autopsy at 24–96 h postmortem (10). Even if some T3 degradation should occur immediately after death, this phenomenon would not argue against a role of D3 in the regulation of T3 content in brain tissues. Rather, it would indicate a continuation of the in vivo function of the enzyme.

The concentration of T3 that we measured in human CNS was comparable to that reported in previous studies, both in the human (10, 16, 23) and the rat (24, 25), but the pattern of distribution of T3 in different areas of the CNS showed wide variations among different subjects. This finding may suggest that the T3 content is not controlled as tightly as previously postulated. However, the variability in T3 content may result from many factors including: interindividual variability, circadian variations of T3 content (26), sampling from different spots within the same area, nature of the disease leading to death, and administration of drugs (27). Nevertheless, an overall analysis of T3 content in different CNS areas (Fig. 6) provides further support to the view that D3 is important for the regulation of T3 residency time in CNS. Indeed, an elevated content of T3 was found in those areas that others (10) and ourselves have shown to contain little or no D3 activity (medulla oblongata,pons and cerebellum). In particular, the highest T3 concentration was measured in the pituitary, which does not contain D3, while it has abundant 5′-monodeiodinase activity (28–30) and is equipped with an active system for the transport of thyroid hormone from blood (31). On the other hand, low T3 concentrations were measured in the hippocampus and temporal cortex, in which D3 activity is elevated. Low T3 levels where also found in the pineal gland and choroidal plexus, but we do not know whether this is associated with high D3 activity in humans.

Thyroid hormone receptors and T3-responsive gene products have been demonstrated in various brain regions and are critical for maintaining normal neurological functions both during development and in adult life (32, 33). The control of T3 concentrations in brain appears to be more important than in other tissues. In this regard, an important role is played by D2 which is up-regulated in the hypothyroid state (34–36), thus increasing the fractional T4 to T3 conversion in spite of reduced availability of substrate. The opposite role is clearly played by D3 which prevents excess accumulation of T3 in the areas of the CNS where the enzyme is expressed at high level (see Fig. 5). This regulatory role is further supported by the observation that D3 activity is decreased and mRNA is undetectable in experimental models of hypothyroidism (37–39) and iodine deficiency (15) in order to increase the residency time of T3 in tissues.

In conclusion: (1) D3 activity in human CNS displays the same kinetic and substrate-specificity as the placental enzyme, suggesting that a single enzyme is responsible for this activity in the two tissues; (2) different levels of D3 activity are found in different regions of CNS; (3) the content of T3 is inversely correlated to D3 activity, indicating that D3 contributes to the local regulation of T3 levels in human CNS.

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