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Iodothyronine Levels in the Human Developing Brain: Major Regulatory Roles of Iodothyronine Deiodinases in Different Areas

MONIQUE H. A. KESTER, RAQUEL MARTINEZ DE MENA, MARIA JESUS OBREGON, DANIJELA MARINKOVIC, ALLAN HOWATSON, THEO J. VISSER, ROBERT HUME, AND GABRIELLA MORREALE DE ESCOBAR

Department of Internal Medicine (M.H.A.K., D.M., T.J.V.), Erasmus University Medical Center, 3000 DR Rotterdam, The Netherlands; Departmento de Endocrinología (R.M.d.M., M.J.O., G.M.d.E.), Instituto de Investigaciones Biomédicas Albert Sols, Consejo Superior de Investigaciones Cientificas-UAM, 28029 Madrid, Spain; Pathology Department (A.H.), Royal Hospital for Sick Children, Yorkhill National Health Service Trust, Glasgow G3 8SJ, Scotland, United Kingdom; and Maternal and Child Health Sciences (R.H.), University of Dundee, Dundee DD1 9SY, Scotland, United Kingdom

Thyroid hormones are required for human brain development, but data on local regulation are limited. We describe the ontogenic changes in T_4, T_3, and rT_3 and in the activities of the types I, II, and III iodothyronine deiodinases (D1, D2, and D3) in different brain regions in normal fetuses (13–20 wk postmenstrual age) and premature infants (24–42 wk postmenstrual age). D1 activity was undetectable.

The developmental changes in the concentrations of the iodothyronines and D2 and D3 activities showed spatial and temporal specificity but with divergence in the cerebral cortex and cerebellum. T_3 increased in the cortex between 13 and 20 wk to levels higher than adults, unexpected given the low circulating T_3. Considerable D2 activity was found in the cortex, which correlated positively with T_4 ($r = 0.65$). Cortex D3 activity was very low, as was D3 activity in germinal eminence and choroid plexus. In contrast, cerebellar T_3 was very low and increased only after midgestation. Cerebellum D3 activities were the highest (64 fmol/min·mg) of the regions studied, decreasing after midgestation. Other regions with high D3 activities (midbrain, basal ganglia, brain stem, spinal cord, hippocampus) also had low T_3 until D3 started decreasing after midgestation. D3 was correlated with T_3 ($r = -0.682$) and rT_3/T_3 ($r = 0.812$) and rT_3/T_4 ($r = 0.889$).

Our data support the hypothesis that T_3 is required by the human cerebral cortex before midgestation, when mother is the only source of T_4. D2 and D3 play important roles in the local bioavailability of T_3. T_3 is produced from T_4 by D2, and D3 protects brain regions from excessive T_3 until differentiation is required. (*J Clin Endocrinol Metab* 89: 3117–3128, 2004)

THYROID HORMONE IS necessary for normal brain development. It becomes increasingly clear that the levels of thyroid hormones required at different stages of development are critical. Conditions relating thyroid hormones to poor brain development have recently been summarized in a review by Morreale de Escobar *et al.* (1). For instance, congenital hypothyroidism leads to severe mental retardation if it remains untreated. Maternal hypothyroxinemia caused by marked iodine deficiency during the first half of pregnancy is also causally related to neurological cretinism (2) and a decreased mental development of a large proportion of the noncretin population (3). Even undiagnosed early maternal hypothyroxinemia (4, 5) or hypothyroidism (6) has been suggested to adversely affect neurological development of the child. Not only maternal and/or fetal and neonatal hypothyroidism clearly affect brain development, but also excessive levels of thyroid hormones may lead to abnormal brain development (7).

Nuclear thyroid hormone receptors have been demonstrated in the brain from wk 10 (8). In the first trimester, the fetus is solely dependent on maternal thyroid hormones, which cross the human placenta (9–11). From midgestation, secretion of thyroid hormone by the fetal thyroid becomes increasingly important (12), although the maternal contribution of thyroid hormone persists until birth (13) and may still play a critical role in the preferential protection of the fetal brain from T_3 deficiency (11). Serum T_3 levels are very low during fetal development, ranging from less than 33 ng/dl (0.5 nM) at 13 wk postmenstrual age (PMA) (14) up to approximately 65 ng/dl (1.0 nM) at term (15). Despite these very low serum concentrations, Bernal and Pekonen (8) and Bernal and colleagues (16, 17) measured T_3 in several fetal tissues between 13 and 18 wk PMA. Their studies showed that the concentration of T_3 in the fetal cortex at 13 wk PMA may actually reach 50–60% of the values reported for adults (18), with 20–30% nuclear receptor occupancy. The concentrations both of the nuclear receptors and total T_3 in the cerebral cortex continue to increase rapidly up to 18 wk gestation. This does not occur during the same developmental period in other fetal tissues, such as the lung and liver, and

Abbreviations: BG, Basal ganglia; BS, brain stem; Cbl, cerebellum; CC, cerebral cortex; CP, choroid plexus; D1, D2, D3, types I, II, and III deiodinase; DTT, dithiothreitol; GE, germinal eminence; H, hippocampus; MB, midbrain; PMA, postmenstrual age; PTU, 6-n-propyl-2-thiouracil; SC, spinal cord.

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there is no information for brain areas other than the cortex. The T_3 levels of the human fetal cortex are much higher than would be expected from the serum T_3 levels, a finding that may be explained by the active transport of thyroid hormone through the plasma membrane, the difference in intracellular *vs.* extracellular thyroid hormone-protein binding, and mainly by local deiodination of iodothyronines (19, 20).

Deiodination is catalyzed by three deiodinases, *i.e.* types I, II, and III deiodinase (D1, D2, and D3). D1 is mainly expressed in the liver, kidney, and thyroid. Its main function is the production of serum T_3 and the clearance of serum rT_3 (20–22). D1 is not expressed in cells of the central nervous system. D2 is present in brain, pituitary, brown adipose tissue, human thyroid, and skeletal muscle (20, 23–25). It catalyzes the outer ring deiodination of T_4 to T_3 and is thus important for the local production of T_3. D2 expression in the different tissues is down-regulated in hyperthyroidism and up-regulated in hypothyroidism (23). In the rat, D2 has been demonstrated in astrocytes throughout the brain, in the median eminence and tanycytes lining the third ventricle (26, 27). D3 catalyzes the inner ring deiodination of T_4 to rT_3 and of T_3 to 3,3′-T_2 (20–22). It is expressed in brain, skin, fetal tissues, placenta, and uterus and at other sites of the maternal-fetal interface, such as the umbilical arteries and vein (28–33). Brain D3 activity is up-regulated in hyperthyroidism and down-regulated in hypothyroidism. D3 is predominantly present in neuronal cells (34, 35), which are the main cells that express thyroid hormone receptors (36, 37). It has been hypothesized (38, 39) that T_4 is taken up from the blood by glial cells and converted to T_3 in these cells. Subsequently, depending on the type of glial cells in which this has occurred, T_3 would be released from astrocytes to neurons by a paracrine route, whereas the T_3 generated in the tanycytes could be secreted into the cerebrospinal fluid and from there reach neural cells. Once T_3 reaches neurons, it would be available to the thyroid hormone receptors and exert its effects. The D3 expressed in the neurons would limit T_3 bioavailability for receptor binding. In such a model, a close ontogenic regulation of brain D2 and D3 expression seems crucial for providing T_3 to the brain in the amounts needed in different structures at different stages of development.

Different regions of the brain have specific temporal patterns of development, and thus may require a different regulation of T_3 bioavailability. Figure 1 shows the human brain roughly subdivided into different regions. Table 1 summarizes the main function of these regions in the adult brain (40). To study the importance of local control of T_3 synthesis and degradation for human brain development, we determined deiodinase activities (D1, D2, and D3) as well as T_3, T_4, and rT_3 concentrations in the brain regions shown in Fig. 1 at different stages of development.

Materials and Methods

Tissue samples

Brains were obtained from 28 fetuses of 13–20 wk PMA at termination of uncomplicated pregnancies for psychosocial reasons. Fetal tissue was collected within 1 h after termination of pregnancy using Misoprostol (Roussel, Uxbridge, UK) vaginal pessaries. Fetal developmental age was carefully estimated solely by one of us (R.H.) based on size, including crown-heel, crown-rump, and heel-toe measurements (41); menstrual history; and ultrasound dating of pregnancy. Normality of fetuses was confirmed by autopsy. Fetal lung organ cultures were routinely established as an indicator of tissue quality. Only tissues from fetuses in which lung airways dilated and the lining epithelium autodifferentiated in culture were used (42). Pregnancies were terminated in accord with the Abortion Act 1967 (United Kingdom) and fetal samples and data handled according to the recommendations of the United Kingdom Government: Review of the guidance on the research use of fetuses and fetal material (Polkinghorne Report) 1989 HMSO. The study was approved by the Multicenter Research Ethics Committee (Edinburgh), the Tayside Committee on Medical Research Ethics, and the Yorkhill Local Research Ethics Committee. In all cases written informed consent was obtained. The fetal brain samples were divided into two PMA age-matched groups, one of which was sent to the Rotterdam laboratory and the other to Madrid.

Brains were also available from nine premature infants who were born at 23–33 wk PMA and died between 24 and 42 wk PMA with

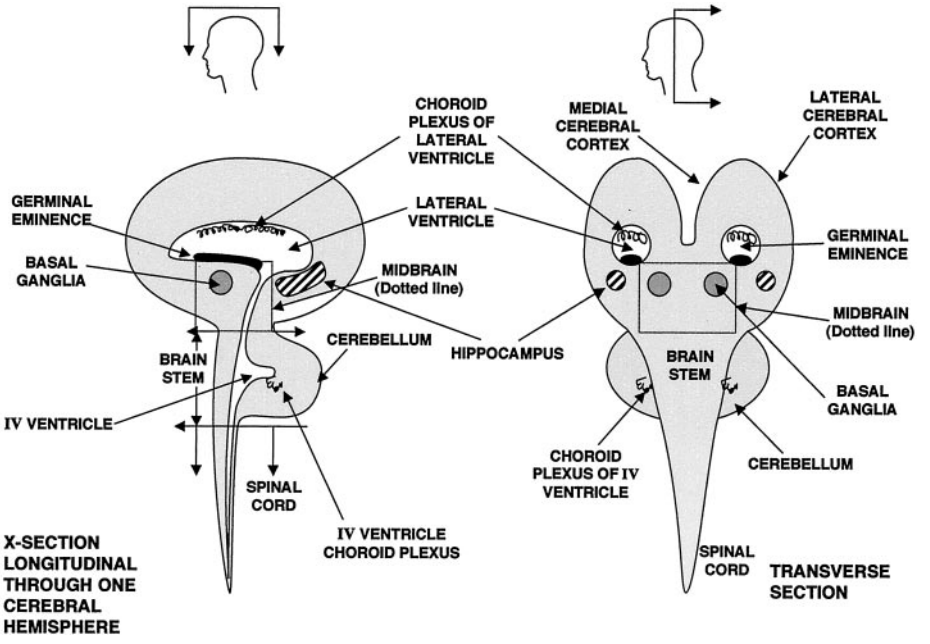

FIG. 1. Cartoon roughly illustrating the different areas from which brain samples were obtained. In fetuses, cortex samples were obtained from the region separating the two hemispheres (medial cortex) and the parietal region (lateral cortex). The CP used in the present study was that of the lateral ventricles. GE samples were no longer available after 28 wk PMA, whereas samples of the H were obtained only from the premature infants. Samples from the MB were obtained up to 20 wk PMA, after which the BG could be identified and dissected out.

TABLE 1. Functions of the different brain regions in adults (40)

Region	Function
SC	Controls movement of the limbs and the trunk; receives and processes sensory information from the skin, joints, and muscles of the limbs and trunk
BS	Receives sensory information from and provides motor output to head, face, neck, and eyes and receives information from special senses as hearing, balance, and taste. In addition, conveys information from the brain to the SC and vice versa
MB	Controls many sensory and motor functions, including eye movement and coordination of visual and auditory reflexes
Cbl	Modulates the force and range of movements and is involved in the learning of motor skills
CC	Is involved in cognitive functions such as language
BG	Participates in regulating motor performance
H	Is involved in memory storage
GE	Fetal brain structure from which cortical interneurons migrate; this brain structure disappears during late fetal brain development
CP	Secretes cerebral spinal fluid, maintains chemical stability of the central nervous system

TABLE 2. Medical histories of premature infants

Cause of death	Additional history	Weight (g)	PMA (wk)		Survival	Sex
			Birth	Death		
Extreme prematurity	Emergency lower uterine segment cesarean section (LUSCS); hepatomegaly with cholestasis, focal myocardial ischemic injury, *Candida albicans* septicemia	580	23	26	22 d	F
Extreme prematurity	Normal pregnancy until assisted breech delivery	655	24	24	3 min	M
Extreme prematurity	Twin transfusion syndrome; sac with polyhydramnios	527	24	24	70 min	M
Extreme prematurity	Twin transfusion syndrome; mild pulmonary hypoplasia; sac with oligohydramnios; twin of baby 3	569	24	24	122 min	M
Extreme prematurity	*Pseudomonas aeruginosa* septicemia, endocarditis, intraventricular hemorrhage	740	26	27	8 d	F
Extreme prematurity	Bronchopneumonia (*P. aeruginosa*); twin of baby 5	590	26	27	8 d	F
Complications of prematurity	Intraventricular hemorrhage, necrotizing enterocolitis, bronchopulmonary dysplasia, pulmonary hypertension, recurrent sepsis; cerebellar hypoplasia	840	27	42	15 wk	M
Hydrops fetalis (congenital heart anomaly)	Emergency LUSCS at 31 wk; ventilator dependent throughout life	1970	31	33	15 d	F
Congenital cardiac anomaly	Hyaline membrane disease	1877	33	33	13 h	M

F, Female; M, male.

postnatal ages of 3 min to 15 wk (Table 2). Parental authorization for postmortem examinations including full organ histology and ancillary investigations was obtained, and examinations were performed by a pediatric pathologist (A.H.). The major postmortem findings and diagnoses are given in Table 2. The samples from each hemisphere of the brains of the premature infants were frozen separately for shipment to the Madrid and Rotterdam laboratories. The tissues were divided and frozen in liquid nitrogen and stored at −80 C until use.

Different areas from fetal and premature infants' brains were dissected fresh and snap frozen immediately: choroid plexus (CP), medial and lateral cerebral cortex (CC), germinal eminence (GE), cerebellum (Cbl), brain stem (BS), spinal cord (SC), midbrain (MB), basal ganglia (BG), and hippocampus (H), as illustrated in Fig. 1. Exact dissection of the fetal brain areas was done as follows. The fetal brain was exposed by partial removal of the calvaria after incisions were made through the lambdoid, sagittal, and metopic sutures. In the now partially exposed fetal brain, a longitudinal incision was made through the superior aspect of the left CC, and this was extended into the lateral ventricle. The lateral ventricle CP was lifted out, exposing the tissue ridge of the GE, which was removed by careful dissection. The tissue between the ventricular wall and the surface of the CC on the medial and lateral aspects was removed, thereafter called the medial and lateral CC. A similar dissection procedure was carried out on the right cerebral hemisphere and the tissue retained. The remaining cerebral cortical tissue was then removed, exposing the superior aspect of the MB region. A horizontal incision was made just superior to the upper border of the Cbl to define the superior border of the BS and the inferior aspect of the MB region, which was then dissected free. The fourth ventricle CP was carefully lifted free, and the cerebellar hemispheres dissected from the BS tissue. The inferior border of the BS was defined as the level of the pyramidal decussation. The entire SC was removed by dissection and retained. In all brain regions the pia-arachnoid membrane was removed.

The nine premature brains available for sampling ranged from 23 to 33 wk. At these stages of development, readily identifiable landmarks and anatomical relationships allowed accurate sampling of brain areas of interest. The Cbl and BS were detached from the upper MB by a transverse incision. The brain was subjected to coronal sectioning, with the first incision made at the level of the mamillary bodies, and if these were not readily identified, the incision was made immediately behind the optic chiasma. Serial coronal sections, at intervals of 0.5 cm, were then performed. The coronal sections of brain were then examined to permit accurate orientation for sampling. The medial cortical sample represented a block of cortex lining the interhemispheric sulcus between the cerebral hemispheres. This cortical sample was made to a maximum depth of 1 cm. The lateral cortical sample was of similar depth and was from the parietal cortex. CP was taken from the lateral ventricles. The BG mass was identifiable adjacent to and below the lateral ventricles. The GE was identifiable in the cases between 24 and 27 wk gestation as

a subependymal protuberance over the head of the caudate nucleus on the lateral wall of the lateral ventricle. The H was identified as a gyral structure seen in the coronal section taken immediately posterior to the aqueduct identified in the cut surface of the MB. Cbl, BS, and SC samples were taken from these readily identifiable structures. The BS samples comprise lower pons and medulla.

Materials

[3′-^{125}I]T_3 was obtained from Amersham (Amersham, UK) for the determination of D3; T_4, T_3, rT_3, and 3,3′-T_2 were purchased from Henning Berlin GmbH (Berlin, Germany). High specific activity [3′,5′-^{131}I]T_4, [3′,5′-^{125}I]T_4, [3′-^{125}I]T_3, and [3′,5′-^{125}I]rT_3 (~3000 μCi/μg) were prepared by radioiodination of T_3, 3,5-T_2, and 3,3′-T_2, respectively, as previously described (43), and used for the determinations of T_4, T_3, and rT_3 concentrations and D2 activities. [3,5-^{125}I]T_3 was obtained from Formula GmbH (Berlin, Germany). Dithiothreitol (DTT) and 6-n-propyl-2-thiouracil (PTU) were obtained from Sigma (St. Louis, MO); Sephadex LH-20 from Pharmacia (Woerden, The Netherlands); and Dowex-50W-X2 and AG 1 × 2 resins from Bio-Rad Laboratories (Richmond, CA).

Determination of T_4, T_3, and rT_3 concentrations in human fetal brain

T_4, T_3, and rT_3 were determined by highly sensitive and specific RIAs after extensive extraction and purification of the iodothyronines from tissues, as described elsewhere (43, 44). In brief, the sample was homogenized directly in methanol, and [^{131}I]T_4 and [^{125}I]T_3 were added to each sample as internal tracers for recovery calculations. These tracers were added in amounts small enough to avoid interferences in the final RIAs. Appropriate volumes of chloroform were added to extract with chloroform/methanol (2:1), twice. The iodothyronines were then back-extracted into an aqueous phase and purified by passing this aqueous phase through Bio-Rad AG 1 × 2 resin columns. After a pH gradient, the iodothyronines were eluted with 70% acetic acid, which was then evaporated to dryness and the residue dissolved in RIA buffer. Each extract was extensively counted to determine the recovery of the [^{131}I]T_4 and [^{125}I]T_3 added to each sample during the initial homogenization process. Average recovery was 50–60% for [^{131}I]T_4 and 60–70% for [^{125}I]T_3. T_4 and T_3 contents were determined by RIAs in triplicate at two dilutions. For the determination of rT_3, we used the same procedure as for T_4 and T_3 but using [^{125}I]rT_3 as recovery tracer. The limits of detection are 3.3 fmol T_4, 1.1 fmol T_3, and 1.5 fmol rT_3/tube. The molar cross-reactivities for the RIAs and the inter- and intraassay variations (<10%) have been previously described (10, 14, 45, 46). Concentrations were then calculated using the amounts of T_4 and T_3 found in the respective RIAs, the individual recovery of the [^{131}I]T_4 and [^{125}I]T_3 added to each sample during the initial homogenization process, and the weight of the tissue sample submitted to extraction. The results are given throughout in picomoles per gram wet weight.

No corrections for the amounts iodothyronines contributed by the blood trapped in the tissue aliquot could be carried out due to lack of blood or serum from the fetuses or premature infants studied.

Determination of D1 and D3 activity

Tissues were homogenized on ice in 5 volumes 0.1 M phosphate (pH 7.2), 2 mM EDTA, containing 1 mM DTT, using a Polytron (Kinematica, Lucerne, Switzerland). The tissue homogenates were stored at −80 C until further analysis. Protein concentrations were determined using the method of Bradford (47), using BSA as standard.

D1 activities were determined by incubation of 0.1 μM [^{125}I]rT_3 (100,000 cpm) for 60 min at 37 C with 1 mg protein/ml tissue homogenate in the presence or absence of 0.1 mM PTU in 0.1 ml 0.1 M phosphate (pH 7.2), 2 mM EDTA, 10 mM DTT. Reactions were stopped by the addition of 0.1 ml 5% BSA. Protein-bound [^{125}I]iodothyronines were precipitated by addition of 0.5 ml 10% trichloroacetic acid. After centrifugation, the supernatants were analyzed for $^{125}I^-$ production on Sephadex LH-20 minicolumns (bed volume 0.25 ml), equilibrated, and eluted with 0.1 M HCl.

D3 activities were measured in the Rotterdam laboratory by incubation of 1 nM [^{125}I]T_3 (200,000 cpm) for 60 min at 37 C with 0.05 or 1 mg protein/ml tissue homogenate in 0.1 ml 0.1 M phosphate buffer (pH 7.2), 2 mM EDTA, and 50 mM DTT. Reactions were stopped by the addition of 0.1 ml ice-cold methanol. After centrifugation, 0.15 ml supernatant was mixed with 0.1 ml 0.02 M ammonium acetate (pH 4.0), and 0.1 ml of the mixture was applied to a 4.6 × 250 mm Symmetry C18 column connected to an Alliance HPLC system (Waters, Etten-Leur, The Netherlands), and eluted with a gradient of acetonitrile in 0.02 M ammonium acetate (pH 4.0) at a flow of 1.2 ml/min. The proportion of acetonitrile was increased linearly from 30 to 44% in 10 min. The radioactivity in the eluate was determined using a Radiomatic A-500 flow scintillation detector (Packard, Meriden, CT). D3 activities are expressed in femtomoles per minute per milligram protein.

D3 activities in a smaller number of samples were also determined in the Madrid laboratory by measuring the iodide released after incubation of tissue homogenates with 40,000 cpm of inner-ring labeled [3,5-^{125}I]T_3 (80 μCi/μg) at 37 C during 1 h. Assay final conditions were 25 nM T_3, 20 mM DTT, 1 mM PTU (pH 7.5), and 40–50 μg protein in a total volume of 100 μl. [^{125}I]iodide was separated from the rest of the reaction products using Dowex 50W X2 columns as described (48). The amount of iodide in the blanks was routinely less than 0.5% of the total radioactivity. Detection limits were 1.2–1.7 fmol/min·mg protein.

Determination of D2 activity

Brain samples were homogenized in buffer [0.32 M sucrose, 10 mM HEPES, and 10 mM DTT (pH 7.0)]. Before each assay [^{125}I]T_4 was purified by paper electrophoresis from contaminating iodide. D2 activity was assayed as previously described (49), incubating 80–100 μg protein with 80,000 cpm of [^{125}I]T_4, 2 nM T_4, 1 μM T_3, 20 mM DTT, and 1 mM PTU for 1 h at 37 C. The total volume was 100 μl. The ^{125}Iodide released was separated by ion-exchange chromatography on Dowex-5OW-X2 columns equilibrated in 10% acetic acid. The amount of iodide in the blanks was routinely less than 1% of the total radioactivity. Results were expressed in femtomoles per hour per milligram protein. Detection limits were 2–5 fmol/h·mg protein.

Statistical analysis

Unless data points are shown individually, results are given as means ± SE. These values, significance of differences between means (Student's *t* test), and Pearson's correlation coefficients, bivariate or partial (correcting for PMA), were calculated using the SPSS statistical package (SPSS Inc., Chicago, IL). $P \leq 0.05$ was considered significant. The regression coefficients r and *P* values shown in some panels of the figures (see Figs. 2, 3, 4, and 6) were calculated with the SPSS statistical package for curve estimation regression analysis, which evaluates the degree of fitting of the different variables (iodothyronine concentrations, T_3/T_4 ratios, D2 activities, *etc.*) as different functions of PMA. Eleven different functions were tested (linear, logarithmic, inverse, quadratic, cubic, power, compound, logistic, growth, exponential, S mode). Only when $P \leq 0.05$, the regression coefficients from the curve estimation analysis are shown in the corresponding panels, the type of function being indicated in the figure legend. Curves through data points shown in the same panels were obtained using the options provided by CA-Cricket Graph III for MacIntosh (Computer Associates International, Inc., Plaza Islandia, NY) for the type of function disclosed by the curve estimation regression analysis.

Results

Figure 2 shows the changes with increasing PMA of the concentrations of T_4, T_3, and rT_3 in different regions of the brain between 13 and 20 wk PMA. There were no statistically significant differences in the iodothyronine concentrations between medial *vs.* lateral cortex, and data were pooled as CC. Major differences are seen between some of the patterns corresponding to different brain areas. Thus, the T_4 concentrations were fitted to a quadratic function of PMA in the cortex, GE, and CP, with positive values of the coefficient r. The changes in T_4 *vs.* PMA in the Cbl also fitted a quadratic function of PMA, but it was a distinctly different one, considering the regression coefficient r is negative. The concen-

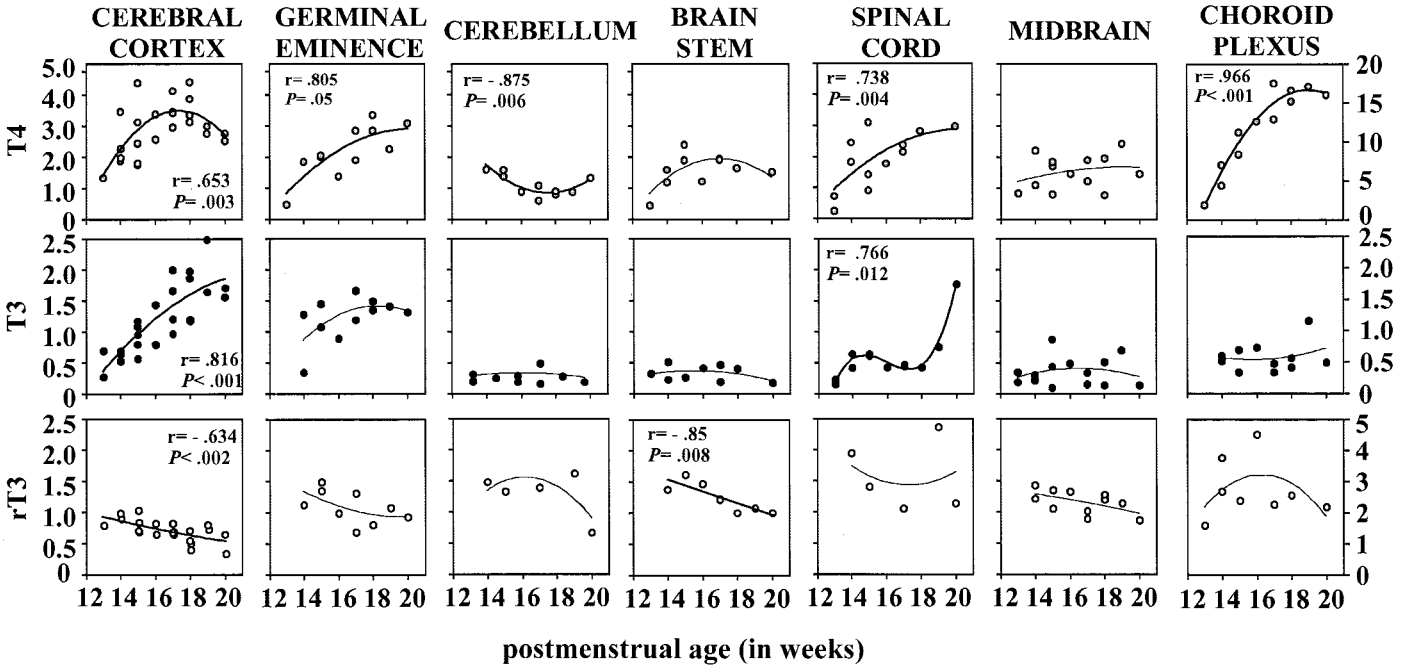

FIG. 2. Ontogenic changes of the concentrations of T_4, T_3, and rT_3 (in picomoles per gram wet weight), up to 20 wk PMA. To convert values for T_4 to nanograms per gram, divide by 1.287; to convert values for T_3 and rT_3 to nanograms per gram, divide by 1.54. The ordinate scales shown on the *left axis* are the same for all areas, with the exception of the CP, for which they are shown on the *right-hand axis*. In this and further figures, the regression coefficients r and the *P* values shown in the panels are those corresponding to the functions calculated by curve estimation regression analysis (as outlined in *Materials and Methods*). No r values are shown if $P > 0.05$. T_4 concentrations were fitted to a quadratic function of PMA in the cortex, GE, SC, CP, and Cbl, with positive regression coefficients. In contrast, it is negative for the Cbl. The concentrations of T_3 in the cortex and SC increased significantly with PMA, following a quadratic function but not in other areas, including the CP, despite the striking increase of the concentrations of T_4 in the latter area. The concentrations of rT_3 decreased linearly in the cortex and BS with increasing PMA.

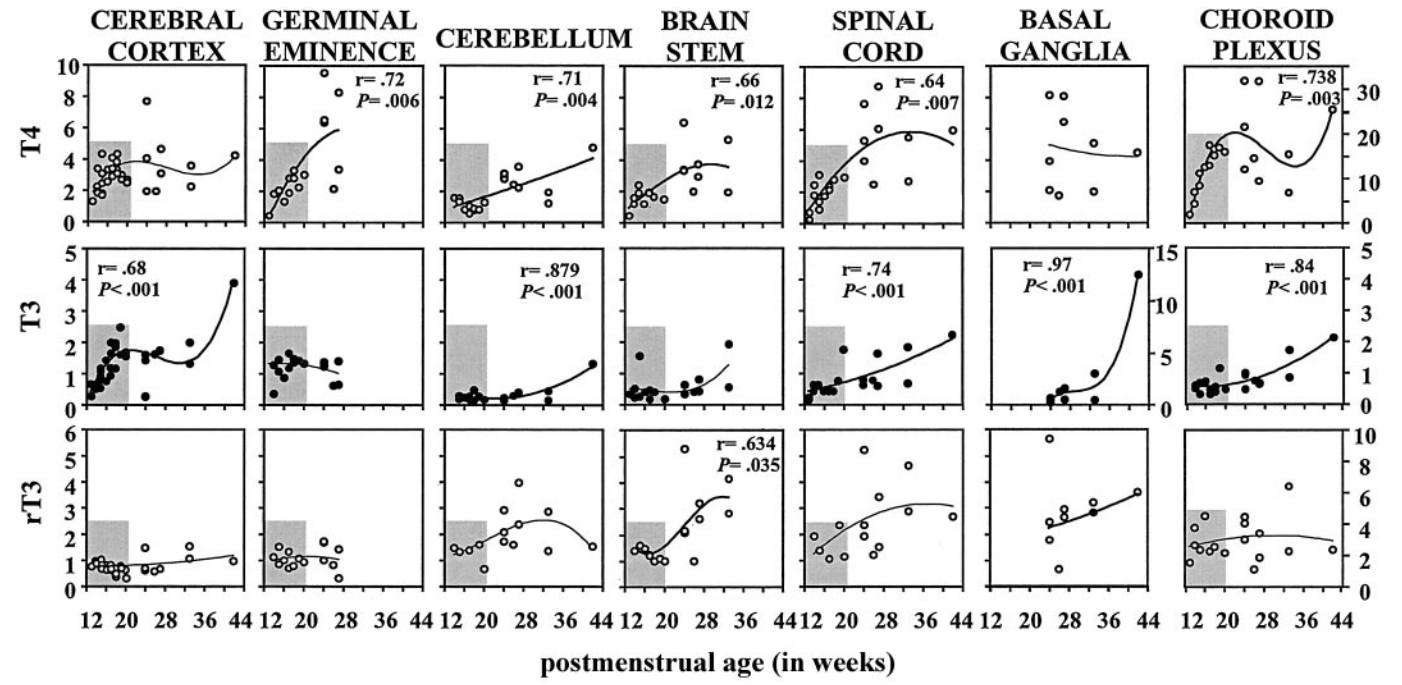

FIG. 3. Concentrations of T_4, T_3, and rT_3 (in picomoles per gram wet weight) in different areas of the brain of premature infants, as a function of their PMA at death (see Table 2), shown in continuation of data points (within the *shaded insets*) that correspond to the fetal samples of Fig. 2. To convert values for T_4 to nanograms per gram, divide by 1.287; to convert values for T_3 and rT_3 to nanograms per gram, divide by 1.54. For the meaning of the r and *P* values, see the legend to Fig. 2. They correspond to quadratic functions of PMA in all panels where r and *P* values are shown, except for CP T_4, CC, and BG T_3, and BS rT_3, which were cubic functions of PMA.

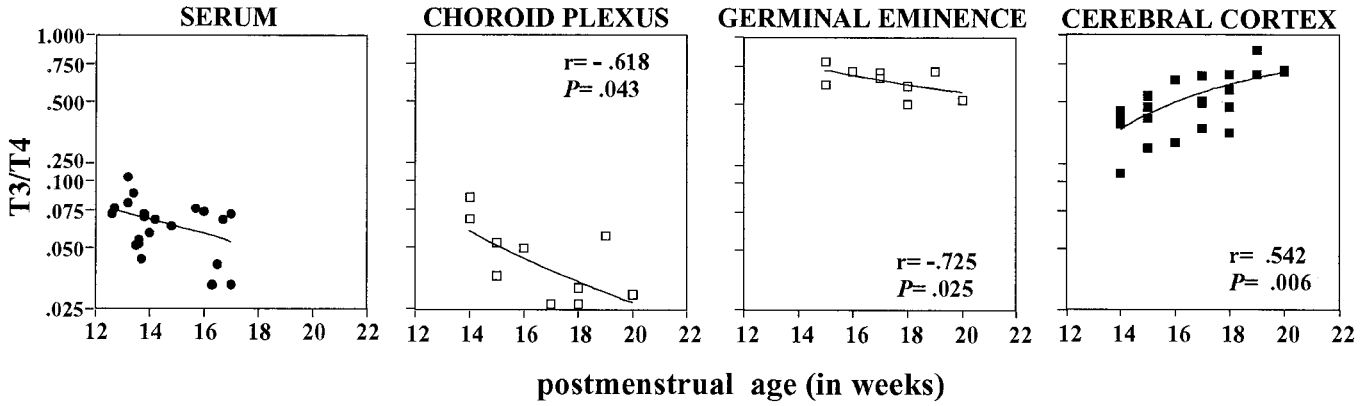

FIG. 4. Comparison of the changes in T_3 to T_4 ratios in fetal serum, CP, GE, and cortex, as linear functions of PMA. The serum T_3/T_4 ratio tended to decrease as a linear function of PMA but did not reach statistical significance ($r = -0.403$; $P = 0.070$). The ratios are plotted on a logarithmic scale to emphasize the differences among these three brain areas and between them and the serum. The functions fitting the CC data were distinctly different from the others because there was no overlap between the 95% confidence intervals of its positive regression coefficient and the negative ones of the other two areas. These coefficients did overlap when the CP and GE were compared.

tration of T_3 in the cortex and SC increased significantly with PMA, following quadratic functions, but not in the other areas, including the CP, despite the striking increase of the concentrations of T_4 in this area. The concentrations of rT_3 decreased linearly in the cortex and BS and tended to decrease in the GE and MB (not statistically significant).

The observed changes during this intrauterine period represent ontogenic profiles because the fetuses and their mothers were presumably normal. This may not be so for the data obtained from the brains of the premature infants because the illnesses suffered and the different causes of their death might affect the observed profiles. For this reason they are shown separately in Fig. 3 as a continuation of the data of the fetuses, and are referred to the PMA at death, to follow the same criterion as used for Fig. 2.

It appears that T_4 concentrations continue to increase in most areas, following quadratic or cubic functions of PMA (Fig. 3). T_3 concentrations also start increasing in areas in which they hardly changed before 20 wk PMA or in which they had actually been decreasing in fetuses (Cbl). The concentrations of rT_3 increase in the BS, in which they had been decreasing before 20 wk PMA, with no well-defined patterns of change being found in the remaining areas. In the H (not shown in Fig. 3), obtained only from the premature infants, no correlations were found with PMA at death, mean values being 3.54 ± 0.49 pmol T_4/g (2.75 ± 0.38 ng T_4/g, n = 9), 1.35 ± 0.50 pmol T_3/g (0.879 ± 0.33 ng T_3/g, n = 9), and 1.98 ± 0.57 pmol rT_3/g (1.289 ± 0.37 ng rT_3/g, n = 9). When data from all brain areas obtained between 13 and 42 wk PMA are considered as a whole, positive correlations were found *vs.* age for the three iodothyronines, with $P < 0.003$ for T_4 and $P < 0.001$ for T_3 and rT_3. Considering the data of the fetuses alone, the positive correlation for rT_3 was lost. When the premature babies alone were considered, only the positive correlation for T_3 persisted ($P < 0.001$).

Tissue iodothyronine levels depend on not only local iodothyronine deiodinase activities but also, among others, on the supply of thyroid hormone, in particular T_4, from the circulation. As a means to correct for changes in T_4 supply, the T_3/T_4, rT_3/T_4, and rT_3/T_3 ratios were calculated and plotted against PMA. Some correlations between the ratios and PMA were found. Thus, for instance, the T_3/T_4 ratio increased throughout the study period in the CC ($r = 0.482$; $P = 0.005$), whereas it tended to decrease in the GE ($r = -0.473$; $P = 0.053$). The rT_3/T_4 ratio decreased in the CC, but only in fetuses ($r = -0.721$; $P < 0.001$), and increased in the same area in premature infants ($r = 0.669$; $P = 0.049$). It decreased throughout the study period in the GE ($r = -0.807$; $P < 0.001$) and CP ($r = -0.485$; $P = 0.048$). The rT_3/T_3 ratios showed changes similar to those of the rT_3/T_4 ratios in the CC of the fetuses ($r = -0.861$; $P < 0.001$) and also decreased in the CP ($r = -0.804$; $P < 0.001$) throughout the study period and in the BG of the premature infants ($r = -0.620$; $P = 0.024$). The changes with PMA of these ratios in the human developing brain and of the concentrations of the iodothyronines shown in Figs. 2 and 3 clearly suggest that patterns are both area and age specific.

Therefore, they cannot be predicted from the circulating levels of the iodothyronines at different stages of development. This point is illustrated in Fig. 4, in which the T_3/T_4 ratios in the CP, GE, and CC up to 20 wk PMA are compared with those obtained in sera from developing fetuses. The latter are taken from a previous study (14), in which the same analytical procedures had been used as for the present brain areas, thus permitting the determination of the very low concentrations of T_3 in fetal serum. The T_3/T_4 ratios in serum tended to decrease with PMA, but the regression coefficient did not reach statistical significance. In the GE and CP, the T_3/T_4 ratios decrease linearly with PMA. In contrast, the T_3/T_4 ratio in the CC increased with PMA.

In addition to the iodothyronine levels, deiodinase activities were determined in the human developing brain samples. No detectable D1 activity was found in any brain sample (data not shown). D2 and D3 activities were determined in the Cbl, BS, SC, CP, and CC from both fetal and premature infants' samples (13–42 wk PMA), MB and GE from fetal samples (13–20 wk PMA), and BG and H from premature infants (23–42 wk PMA at death). Average D2 an D3 activities for the different brain regions are shown in Fig. 5 for fetuses and premature infants separately. D3 activity was highest in Cbl, but considerable D3 activities were also found in MB, BG, BS, SC, and H, whereas D3 activity was low in the

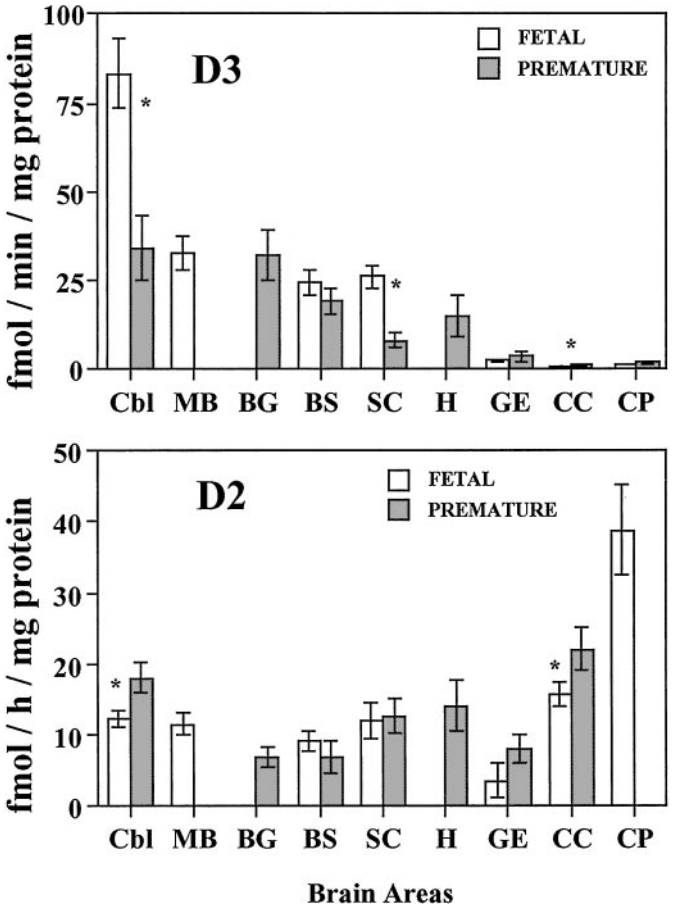

FIG. 5. Average D3 and D2 activities of the different brain regions. Results are the means ± SE of samples from fetuses (13–20 wk PMA) and premature infants at death (Table 2). *Asterisks* identify statistically significant differences between the mean values for fetuses *vs.* premature infants. D3 activities decreased from Cbl to CP and CC (Cbl > MB = BS = SC > GE = CC = CP for fetuses; Cbl = BG > BS = H = SC > GE = CP = CC for premature infants). D2 activities were highest in CP, followed by CC (CP > CC = Cbl = SC = MB > BS = GE for fetuses; CC = Cbl > H = SC = GE = BS = BG for premature infants). The > sign indicates a statistically significant difference between areas, whereas the = sign indicates that the differences were not statistically significant.

GE, CP, and CC. In some fetal samples, D3 activities were higher than in those of the same region obtained from the premature infants. D2 activities were highest in CP and CC, in which D3 activities were the lowest. Most D2 activities ranged between 8 and 20 fmol/h·mg protein. Although such values are about 100 times lower than the D3 activities, they are similar to, or higher than, those found in normal brain tissue from adults (18).

Figure 6 shows the D2 activities in different brain areas, plotted against PMA. D2 activity was detected in all regions and increased with PMA in the CC and Cbl ($P < 0.05$). After controlling for PMA and using all data from all regions throughout the fetal period as a whole, D2 activities were found to correlate positively with the concentrations of T_4 ($r = 0.65$, $P < 0.001$) and rT_3 ($r = 0.42$, $P = 0.001$) and negatively with the T_3/T_4 ratios in the CC ($r = -0.29$, $P = 0.008$). Except for the correlation between D2 activities and T_4 concentrations, the statistical significance disappeared when the data from the premature infants were included.

As shown in Fig. 7, D3 activities decreased with PMA in Cbl, BS, and SC ($P < 0.05$). At all stages, highest D3 activities were found in the Cbl. D3 activities were low throughout the study period in GE, CP, and CC.

Table 3 shows the correlations between the average D3 activities and the iodothyronine levels and ratios in the different brain regions. Figure 8A shows the correlation between the average D3 activities and the rT_3/T_3 and rT_3/T_4 ratios. T_4 levels tended to decrease and rT_3 levels tended to increase with D3 activity. A significant negative correlation was found between D3 activity and the T_3 level ($r = -0.682$). D3 activities were positively correlated with the rT_3/T_3 ratio ($r = 0.812$, $P = 0.008$) and the rT_3/T_4 ratio ($r = 0.889$, $P = 0.001$). Figure 8B depicts the average D3 activities and rT_3/T_3 and rT_3/T_4 ratios in the different brain regions. Except for the CP, the rT_3/T_3 and rT_3/T_4 ratios correlated positively with increasing D3 activities.

The D3 activities shown in Figs. 5, 7, and 8 and in Table 3 were measured in the Rotterdam laboratory. The fewer determinations performed in the Madrid laboratory with a different methodology fully supported these findings, including the different correlations that have been described here.

Discussion

Studies using the rat as an animal model have shown that fetal and neonatal hypothyroidism lead to multiple structural, functional, and biochemical alterations of brain development (see reviews in Refs. 38, 50, 51). These studies, together with clinical evidence for the effects of maternal hypothyroxinemia on brain development (for review see Ref. 1), indicate the importance of a tightly regulated thyroid hormone bioavailability during brain development. Iodothyronine deiodination contributes to this regulation. In this study we determined local iodothyronine levels and deiodinase activities in different brain regions at different stages of development to evaluate the possible contribution of the different deiodinases in controlling local T_3 availability in the human developing brain.

As already pointed out previously, it is quite likely that the changes in the concentrations of T_4, T_3, and rT_3 observed with the samples of the fetuses from normal mothers reflect the true ontogenic profile for different areas of the human brain. They are quite different for the different areas studied and cannot be predicted from the changes found in the fetal circulation. Obviously we cannot exclude that there are also differences within each area related to cellular heterogeneity and different timing of maturational events in each structure, but this point cannot yet be adequately resolved with the sensitivity of presently available techniques. A similar comment might also be pertinent for the D2 and D3 activities here reported.

The changes found in some areas before midgestation appear to merit closer attention. The highest T_4 and rT_3 concentrations were observed in the choroid plexus. T_4 increased significantly with PMA, despite little change in the low T_3 concentrations; indeed, the T_3/T_4 and rT_3/T_4 ratios

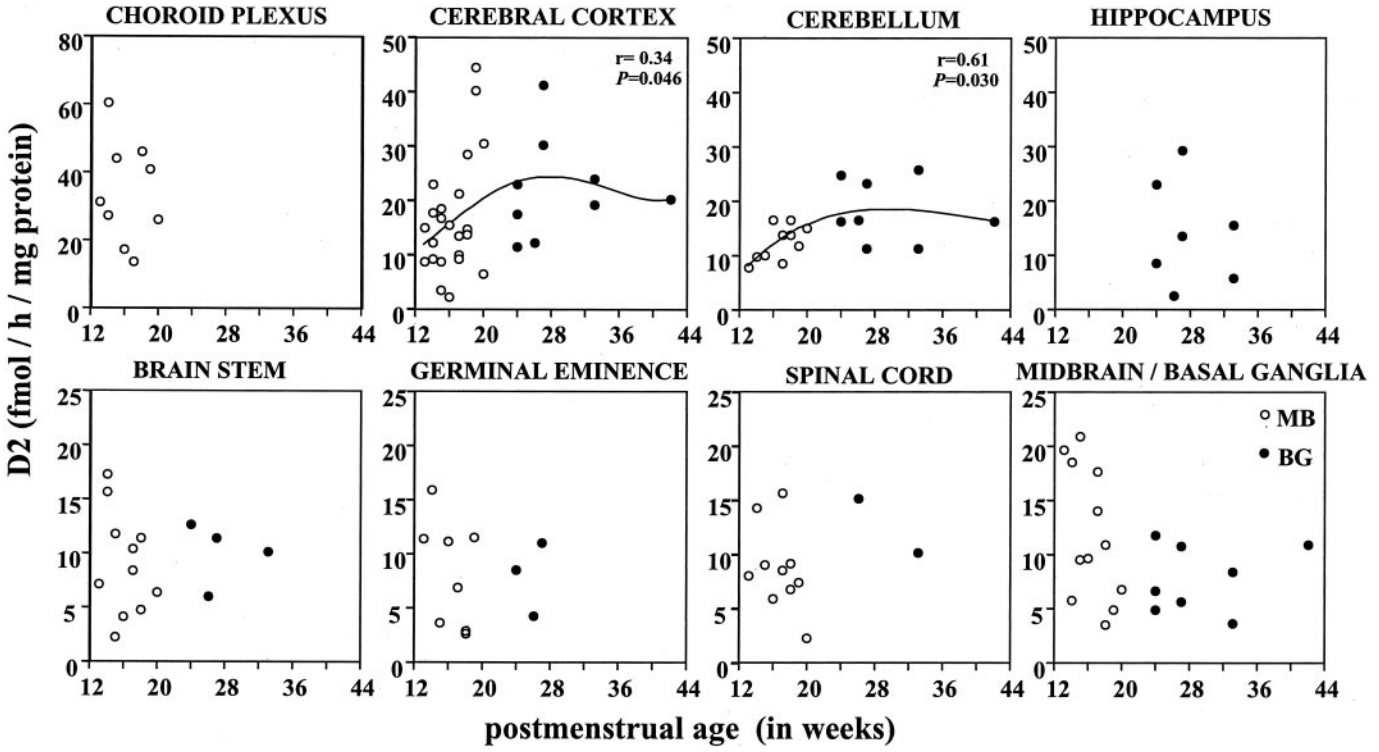

FIG. 6. D2 activities in samples from fetuses (○) and premature infants (●) as a function of PMA from CP, CC, Cbl, H, BS, GE, SC, MB, and BG. For the meaning of r and *P* values, see the legend to Fig. 2. D2 activities changed with increasing PMA, the CC and Cbl following cubic functions of PMA, but no well-defined patterns were found in the remaining areas.

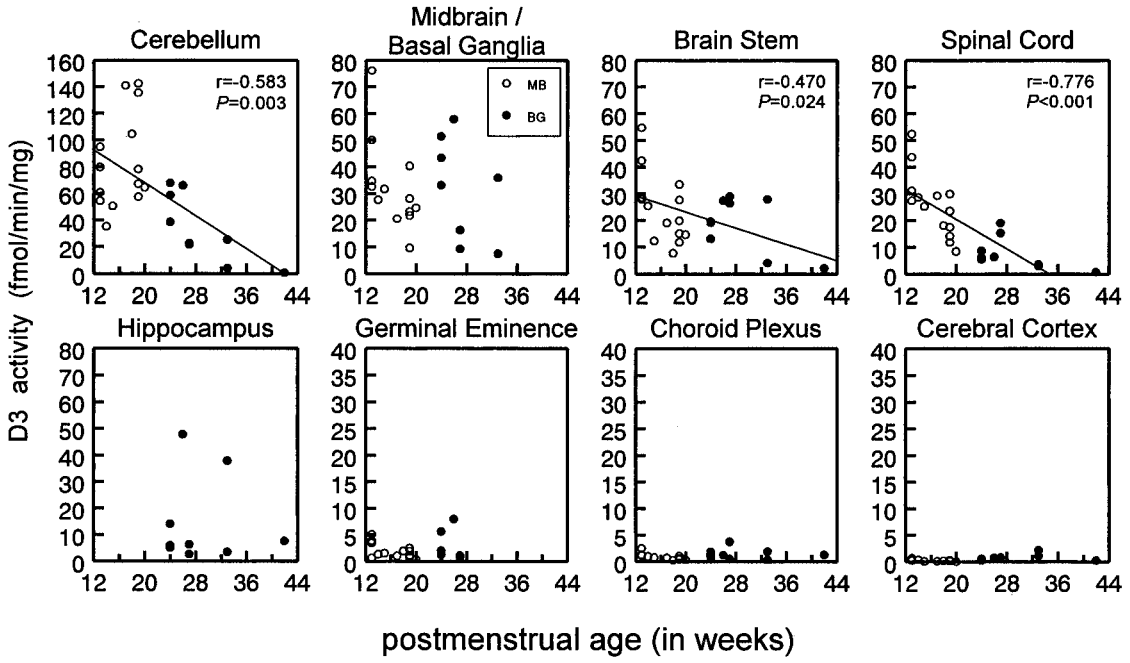

FIG. 7. D3 activities in fetuses (○) and premature infants (●) as a function of PMA from Cbl, MB, BG, BS, SC, H, GE, CP, and CC as a function of PMA. The r values show Pearson's correlation coefficient with PMA.

were decreasing with PMA. The lack of increase in the concentration of T_3 is also rather striking when we consider that the D2 activities were among the highest. This could not be attributed to high D3 activities because these were very low. However, it should be realized that the deiodination rates measured under *in vitro* conditions may not represent deiodination taking place *in vivo*. It is possible that only a small proportion of the total T_4 we have measured in the choroid plexus samples is actually available intracellularly for deiodination by D2 and D3: most of it is likely to be bound by

transthyretin, which is already synthesized in the human CP long before 13 wk PMA (52). Despite the fact that transport of T_4 from the plasma to the brain is normal in transthyretin-null mice (53), the CP is considered to be important for the transport of T_4 into the brain. The transthyretin that is synthesized in the CP epithelial cells would either transfer T_4 from the epithelial cells to the cerebrospinal fluid or facilitate its passage after the transthyretin is excreted into the cerebrospinal fluid (54). The amounts of substrate iodothyronines actually reaching D2 and D3 deiodination sites may well be much lower than expected from the total concentrations.

TABLE 3. Correlation of D3 activity with iodothyronine levels and ratios in different brain regions from fetuses and premature infants

	Iodothyronine levels			Iodothyronine ratios		
	rT_3	T_4	T_3	rT_3/T_4	rT_3/T_3	T_3/T_4
r	0.138	−0.454	−0.682	0.889	0.812	−0.236
P	0.723	0.219	0.043	0.001	0.008	0.539

Pearson's correlation coefficient r and *P* values were calculated using SPSS.

It is therefore likely that in this unique and morphologically heterogeneous structure mechanisms other than deiodination, such as thyroid hormone transport, are more important for the regulation of intracellular thyroid hormone levels.

The largest increase in T_3 concentration up to midgestation was observed in the CC, which appeared to continue even when the T_4 concentrations were no longer increasing. As a result, the cortex T_3/T_4 ratio increased throughout this period, in contrast to the serum T_3/T_4 ratio, which tended to decrease. On the contrary, rT_3 concentrations and rT_3/T_4 ratios were decreasing during the same developmental period. Thus, both the changes in T_3 and rT_3 concentrations were consistent with the findings that D2 activities were clearly detectable by 13 wk PMA, and D3 activities were the lowest found in the present study. We cannot exclude that other regulatory mechanisms are also involved in determining the concentration of T_3 in the CC. The changes described here for the CC up to midgestation are consistent with previous findings by others (8, 16, 17), showing that both T_3 and thyroid hormone receptor concentrations are increasing in the human brain between 8 and 18 wk PMA: at 13 wk ges-

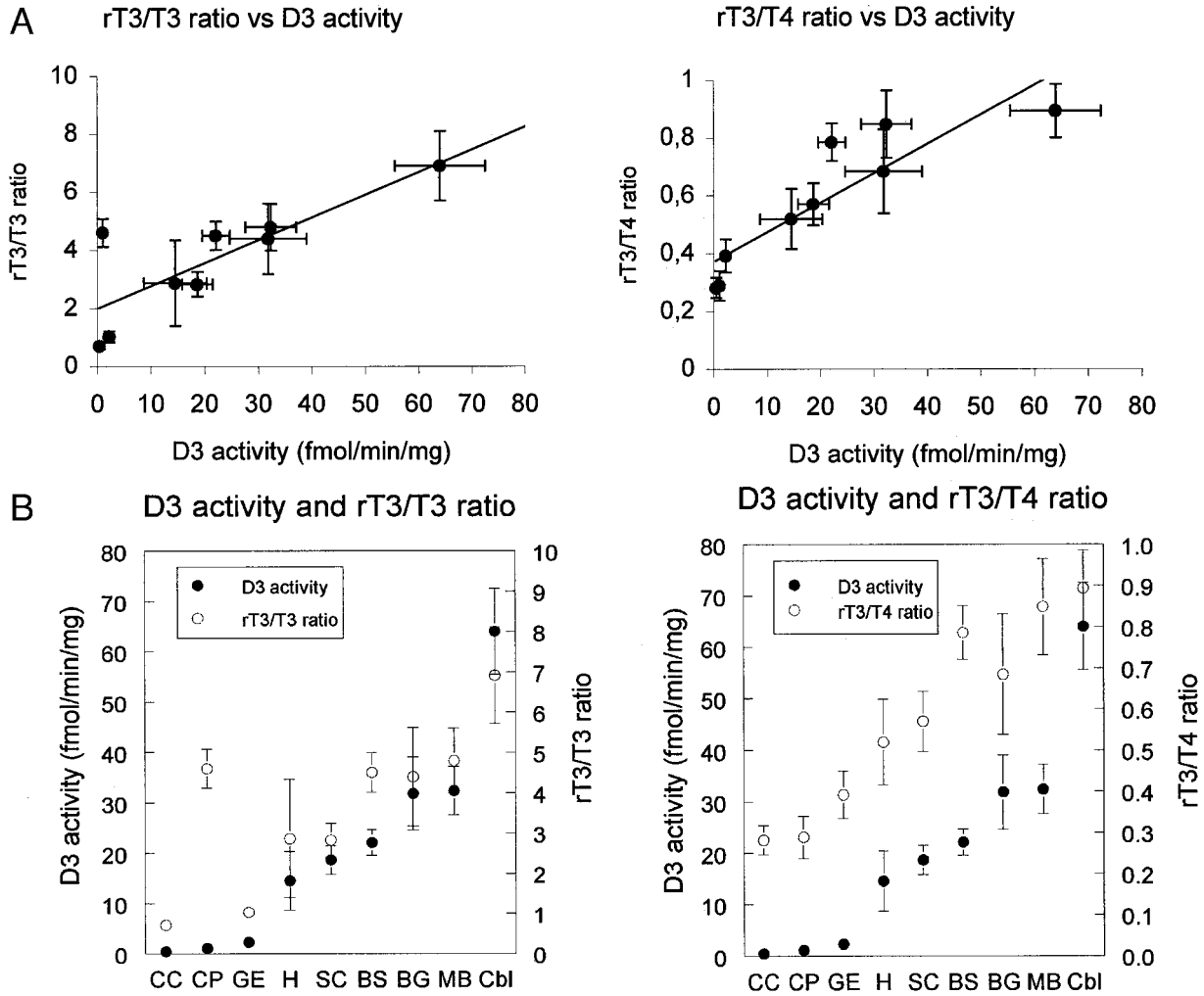

FIG. 8. Average rT_3/T_3 and rT_3/T_4 ratios as a function of D3 activities (A) and average D3 activities and rT_3/T_3 and rT_3/T_4 ratios in the different brain regions (B). Results are the means per brain region ± SE. The r value indicates the Pearson's correlation between D3 activity and the iodothyronine ratio (B).

tation T_3 concentrations in the cortex had already reached 60% of adult values. D2 and D3 activities have also been previously reported in the human CC by 11–14 wk PMA (55). We point out that the present T_3 concentrations in the human CC are comparable with those reported in adults: 1.5–2.2 pmol T_3/g (1.0–1.4 ng T_3/g) (18, 56). The fetal D2 activities are actually much higher than reported in the adult cortex (~8 fmol/h·mg protein) (18). The ontogenic changes of T_4, T_3, and D2 observed in the present study are in conceptual agreement with those reported for the rat brain (46, 49); between 18 and 22 d of gestation, there is a 4-fold increase in D2 activity, a 10-fold increase in T_4 concentrations, and an 18-fold increase in T_3 concentrations in rat CC.

The present results also indirectly support the hypothesis that T_3 is relevant for the development of the human CC from very early in gestation, possibly soon after completion of morphogenesis of the pros-encephalon (57). Although this hypothesis is supported by epidemiological and clinical findings (1), no direct proof is available for man. It has, however, been directly confirmed (58) in the rat for a developmental period corresponding to that occurring in man before midgestation. The tendency of T_3 concentrations to increase in the GE before midgestation suggests that this structure might already be thyroid hormone sensitive, but we are unaware of any studies regarding abnormalities in this structure related to thyroid hormone insufficiency.

The ontogenic changes in the developing human Cbl contrast with those described for the CC. The concentrations of T_4 and T_3 remained low, and especially T_3 was maintained at levels that were appreciably lower than those found during the same period in other areas, such as the cortex, GE, SC, and even CP. D2 activities were similar to those found in the cortex, but D3 activities were the highest found in any of the brain areas studied during this developmental period and are likely to be a very important factor in the maintenance of the low cerebellar T_3 concentrations. So are the high D3 activities found in MB, BS, and SC, all areas in which T_3 concentrations were low during most of the developmental period up to midgestation.

Some caution should be applied to the interpretation of data obtained in the postnatal brain samples, insofar as it is not excluded that they may to some extent be influenced by nonthyroidal illness, which is known to affect peripheral thyroid hormone metabolism (59, 60).

The present results confirm for the human developing brain the same principles that appear to modulate T_3 bioavailability in different developing structures, and in different species, in a temporally and spatially specific sequence of events, namely by the ontogenetically programed expression of the iodothyronine deiodinase isoenzymes, mainly D2 and D3 (29, 61, 62). D1 activity was not detected in any brain area. This is in agreement with previous studies of Campos-Barros *et al.* (63), who found D2 and D3 activity, but no D1 activity, in adult human brain. We have already discussed the D2 activities found in different areas, compared with those in adults. The activities of D3 during early development that we report here for different brain areas show very high levels in specific structures that, in general, tend to decrease with PMA. The highest D3 activities were found in Cbl and were higher than in the adult brain (64). The spatial distribution of D3, however, differs: in the adult brain, D3 activity is low in Cbl, MB, and BS, whereas higher levels are found in the H and CC (Visser, T. J., E. Kaptein, and E. Fliers, unpublished data, and Ref. 64). Santini *et al.* (64) found that T_3 levels are also negatively correlated with D3 activity in the adult brain, as described here for the developing human brain.

The D3 activities found here for the brain are only 2-fold lower than those reported in human placenta (31). D3 expression in the placenta is believed to protect the fetus from excessive maternal T_3 (12, 15, 28). Thyroid hormone induces neuronal differentiation such as dendritic and axonal growth, neuronal migration, and myelination (38). Strict regulation of thyroid hormone bioavailability is critical because neuronal development is affected in the hypothyroid and hyperthyroid brain. The high D3 activities we found in the brain, which tended to decrease with age, suggest that local D3 is important to limit T_3 in the various brain regions during critical stages of development. It is unclear whether D3 has an additional physiological role in the production of rT_3 and 3,3′-T_2. Because rT_3, but not T_3, has profound and acute effects on the cytoskeleton in brain cells (65), it is not excluded that rT_3 also has a function in brain development. 3,3′-T_2 has been shown to increase the basal metabolic rate in adult rat. This effect may be mediated by direct mitochondrial binding (66).

D2 and D3 are expressed in distinct cell types: D2 in astrocytes and tanycytes and D3 in neurons. The hypothesis has been put forward (38) that astrocytes and tanycytes take up T_4 from the circulation and convert it to T_3, which is delivered to neurons (that contain most of the nuclear receptors), in which D3 would limit T_3 availability according to the local temporal needs for thyroid hormone action. In addition, although still poorly studied, metabolic pathways other than deiodination, such as sulfation, may play regulatory roles in the developing brain.

A large number of cerebral genes are regulated by thyroid hormone (38). Although not much is known on the molecular basis for the specific timing of action on gene expression, it is known that the different regions of the brain have specific temporal patterns of development and thus require different regulation of T_3 bioavailability. In general, roughly, the cerebral cortex starts to develop in the second month of pregnancy, whereas major events in cerebellar development do not occur until wk 34 (51). In agreement with this, we found low D3 activity in the CC, which would require T_3 for differentiation early in development and high D3 activity in the later developing Cbl.

In this study, we also compared the average D3 activities with the average thyroid hormone levels and ratios in the different brain regions. Except for the CP, we observed that D3 activity was high in the regions with low T_3 and T_4 and high rT_3 levels and low in regions with high T_3 and T_4 and low rT_3 levels. We found a significant negative correlation between D3 activities and T_3 levels and significant positive correlations between D3 activity and the ratio of rT_3/T_3 and the ratio of rT_3/T_4. Because D3 catalyzes the degradation of T_3 and T_4 and the production of rT_3, our results suggest that D3 is also important in humans for the regulation of the intracellular thyroid hormone levels in the different brain regions. Furthermore, no D1 activity was found in any brain

region. In addition to the presence of D3 activity, the absence of D1 activity may contribute to the high tissue rT_3 levels.

In conclusion, by determining and correlating the ontogenic patterns of deiodinase activities and thyroid hormone levels in the human brain, we have shown that both D3- and D2-catalyzed deiodination are important pathways for the intracellular regulation of thyroid hormone in the different regions of the developing human brain, this regulation being region and time specific. Although D3 is expressed to a greater extent than D2, the latter is clearly important in thyroid hormone activation at the cellular level. Further *in situ* hybridization and immunohistochemistry studies are required to confirm the hypothesis that a close regulation of D2 and D3 activities is crucial for tailoring T_3 bioavailability to changing needs of human developing brain structures.

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Address all correspondence and requests for reprints to: Theo J. Visser, Department of Internal Medicine, Erasmus Medical Center, Room Ee 502, Dr Molewaterplein 50, 3015 GE Rotterdam, The Netherlands. E-mail: t.j.visser@erasmusmc.nl.

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