**Editorial: Local Control of the Timing of Thyroid Hormone Action in the Developing Human Brain**

Frog metamorphosis has long been a fascinating example of thyroid hormone actions on development (1), and insights gained from studies of frog metamorphosis are helping us understand the role of thyroid hormone in the development of a completely different tissue—the human brain. In frogs such as *Xenopus laevis*, thyroid hormone controls the dramatic transformation from the larval to the adult form (2, 3), in which many larval tissues are lost (e.g. gills and tail), adult structures formed (e.g. limbs), and other organs are remodeled to support adult functioning. Importantly, frog metamorphosis is characterized by an orderly sequence of events; thus, different tissues undergo thyroid hormone-dependent metamorphic changes at different times and at different rates, all in the face of elevated circulating levels of thyroid hormone. A seminal observation is that local metabolism of thyroid hormone is a major factor controlling the timing of tissue responsiveness to thyroid hormone during frog metamorphosis and thus the sequence of metamorphic events (4). In this issue of the JCEM, Kester et al. (5) describe results of a new study indicating that local metabolism of thyroid hormone in different regions of the developing human brain likely contributes to the timing of thyroid hormone-driven development.

Like frog metamorphosis, development of the mammalian brain is characterized by an orderly sequence of developmental events (6). Moreover, the relative timing of maturational events within the brain is quite similar among mammalian species (7). Recent work in both humans and experimental animals demonstrates that thyroid hormone exerts effects on the developing brain throughout a broad period of fetal and neonatal development (8) and that the developmental events and brain structures affected by thyroid hormone differ as development proceeds. Therefore, it is possible that the human brain uses a strategy for “timing” thyroid hormone sensitivity of different brain regions that is similar to that used by Xenopus. The work by Kester et al. represents a key observation suggesting that this is indeed the case.

Kester et al. (5) report that in several brain regions, especially the cerebral cortex, levels of T₃ increase during fetal development and this is correlated with an increase in the activity of type 2 deiodinase (D2), whereas the activity of the type 3 deiodinase (D3) is low to undetectable. D2 controls the conversion of T₄ to the hormonally active T₃, but D3 controls the conversion of T₄ to the hormonally inactive reverse T₃. Because T₃ levels in the fetal cerebral cortex increased to an extent that could not be accounted for simply on the basis of the age-dependent increase in T₄, it indicates that D2 is causing the age-dependent increase in T₃ from 14–20 wk (postmenstrual age). Importantly, during this same period, the fetal cerebellum has high levels of D3 and low levels of T₃. Finally, at later gestational ages, D3 activity in the cerebellum declines and T₃ levels increase.

These data further support the concepts that thyroid hormone plays a role in brain development during the fetal period, that different parts of the brain are differentially sensitive to thyroid hormone at any one time during development, and that the sensitivity to thyroid hormone is controlled, in part, by local control of hormone production. In turn, these observations imply that the consequences of thyroid hormone insufficiency during fetal development will differ from those of thyroid hormone insufficiency during postnatal development. In fact, this implication is supported by empirical evidence. For example, Smit et al. (9) studied a small group of infants of women with hypothyroidism diagnosed before pregnancy who were seemingly adequately treated. Although tests indicated that their children displayed normal neurophysiological and motor development, they had significantly lower mental development indices at 6 and 12 months. Importantly, Rovet et al. (10) followed a relatively large group of infants whose mothers had hypothyroidism diagnosed before or during pregnancy and found mild effects on specific cognitive abilities, particularly visual attention and visuospatial processing abilities. Compared with offspring of euthyroid women, these children showed poorer attention, slower and more variable reaction times to visual stimuli, and visual deficits, particularly reduced contrast sensitivity. Moreover, the specific types of visual deficits appeared to reflect the timing of thyroid hormone insufficiency during pregnancy (11).

The concept that the fetal brain is sensitive to thyroid hormone is of relatively recent origin. Early work indicated that thyroid hormone is not transferred from the mother to the fetus because the human placenta and fetal membranes contain high levels of D3 that degrade thyroid hormones and might prevent such transfer (12, 13). Thus, it was somewhat paradoxical that, in the 1960s and 1970s, Man et al. (14) published the results of a series of studies that found an association between “butenol-extractable iodine” in pregnant women and measures of cognitive function in the offspring, indicating that thyroid hormone may play a role in fetal brain development. This paradox was reconciled in part by Vulsma et al. (15) who reported that newborns with a genetic incapacity to synthesize thyroid hormones have T₄ levels that are nearly the same as normal neonates, indicating that the fetus obtains a considerable proportion if its T₃ from maternal circulation and this is likely to be true throughout gestation.

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**Abbreviations:** D2, Type 2 deiodinase; D3, type 3 deiodinase; TR, thyroid receptor.

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The observations of Vulsma et al. were pivotal because the fetus does not begin to produce its own thyroid hormone until around the end of the first trimester (16); therefore, if thyroid hormone acts on the fetal brain in the first trimester, the only source of hormone would be the mother. In fact, thyroid hormones are detected in human coelomic fluid as early as 8 wk gestation (17, 18), several weeks before the onset of thyroid function at 10–12 wk (16), and these levels of thyroid hormones are biologically relevant (19). In addition, all the major thyroid receptor (TR) isoforms are present in human cerebral cortex as early as 8 wk gestation, with immunostaining being reported for TR expression in cerebellar pyramidal cells and Purkinje cells at this time (20, 21). TRs in fetal brain appear to be occupied by thyroid hormone as early as 9 wk gestation (17) and the proportion of TR occupancy by thyroid hormone is in the range known to produce physiological effects.

More recent studies have confirmed that thyroid hormone of maternal origin exerts functional effects on the fetus. Pop et al. (22, 23) showed that levels of free T4 and the presence of circulating antibodies for thyroid peroxidase, were strong predictors of infant mental development and children’s IQ. In addition, these authors found that children of women with hypothyroxinemia at 12 wk gestation had delayed mental and motor function compared with controls; the two groups were different by 8–10 index points on the mental and motor scales at both 1 and 2 yr of age (24). Finally, Haddow and colleagues (25, 26) showed that the children of women with low circulating levels of thyroid hormone exhibit a number of measurable neurological deficits depending on the severity of the hormonal insufficiency. Thus, the literature supports the conclusion that thyroid hormone insufficiency in pregnancy can lead to cognitive deficits in the offspring, clearly indicating that thyroid hormone plays an important role in fetal brain development.

Recent authors discuss the relative merits of developing a routine screening program for thyroid function in pregnant women (27–31). The relative lack of information about the potential adverse consequences, to the mother or to the fetus, of T4 replacement therapy in pregnant women is one of the critical arguments that screening programs should not presently be implemented, although T4 replacement is recommended for pregnant women who are clinically hypothyroid (29) and an increase in the dose of T4 replacement is recommended for pregnant women currently on T4 replacement (32). Recent animal studies indicate that thyroid hormone insufficiency in the mother can influence cortical neuronal migration in the absence of effects on maternal TSH. Specifically, Ausò et al. (33) found that 3 d of methimazole treatment to pregnant rats could alter neuronal migration in the cerebral cortex without affecting maternal TSH. In combination with the observations of Kester et al. (5), it is important to recognize that, although the deiodinases may account for tissue differences in thyroid hormone sensitivity, they may not always compensate for changes in circulating levels of thyroid hormone.

Acknowledgments

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

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Thyroid hormones are required for human brain development, but data on local regulation are limited. We describe the ontogenic changes in $T_3$, $T_2$, and $rT_3$, and in the activities of the types I, II, and III iodothyronine deiodinases ($D_1$, $D_2$, and $D_3$) in different brain regions in normal fetuses (13–20 wk postmenstrual age) and premature infants (24–42 wk postmenstrual age). $D_1$ activity was undetectable.

The developmental changes in the concentrations of the iodothyronines and $D_2$ and $D_3$ 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 $D_2$ activity was found in the cortex, which correlated positively with $T_3$ ($r = 0.65$). Cortex $D_3$ activity was very low, as was $D_9$ activity in germinal eminence and choroid plexus. In contrast, cerebellar $T_3$ was very low and increased only after midgestation. Cerebellum $D_3$ activities were the highest (64 fmol/min/mg) of the regions studied, decreasing after midgestation. Other regions with high $D_3$ activities (midbrain, basal ganglia, brain stem, spinal cord, hippocampus) also had low $T_3$ until $D_3$ started decreasing after midgestation. $D_3$ 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 $T_3$. $D_2$ and $D_3$ play important roles in the local bioavailability of $T_3$. $T_3$ is produced from $T_2$ by $D_2$, and $D_3$ protects brain regions from excessive $T_3$ until differentiation is required. (*J Clin Endocrinol Metab* 89: 3117–3128, 2004)

**Abbreviations**: BG, Basal ganglia; BS, brain stem; Cbl, cerebellum; CC, cerebral cortex; CP, choroid plexus; $D_1$, $D_2$, $D_3$, 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 r$T_3$ (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 r$T_3$ and of $T_3$ to 3,3',5'-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 r$T_3$ 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)

<table>
<thead>
<tr>
<th>Region</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC</td>
<td>Controls movement of the limbs and the trunk; receives and processes sensory information from the skin, joints, and muscles of the limbs and trunk</td>
</tr>
<tr>
<td>BS</td>
<td>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</td>
</tr>
<tr>
<td>MB</td>
<td>Controls many sensory and motor functions, including eye movement and coordination of visual and auditory reflexes</td>
</tr>
<tr>
<td>Cbl</td>
<td>Modulates the force and range of movements and is involved in the learning of motor skills</td>
</tr>
<tr>
<td>CC</td>
<td>Is involved in cognitive functions such as language</td>
</tr>
<tr>
<td>BG</td>
<td>Participates in regulating motor performance</td>
</tr>
<tr>
<td>H</td>
<td>Is involved in memory storage</td>
</tr>
<tr>
<td>GE</td>
<td>Fetal brain structure from which cortical interneurons migrate; this brain structure disappears during late fetal brain development</td>
</tr>
<tr>
<td>CP</td>
<td>Secretes cerebral spinal fluid, maintains chemical stability of the central nervous system</td>
</tr>
</tbody>
</table>

TABLE 2. Medical histories of premature infants

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

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 BS 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 protruberance 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, B5, and SC samples were taken from these readily identifiable structures. The BS samples comprise lower pons and medulla.

**Materials**

\[3^{-125}I]\text{T}_4 \text{, obtained from Amersham (Amersham, UK) for the determination of D}_3 \text{, T}_3 \text{, and 3,3'-T}_3 \text{, were purchased from Henning Berlin GmbH (Berlin, Germany). High specific activity}[3',5'-131]I\text{T}_4 \text{, [3',5'-125]I}\text{T}_3 \text{, and [3',5'-125]I}\text{T}_2 \text{, (}300 \text{ mCi/}g\text{) were prepared by radioiodination of T}_3 \text{, T}_2 \text{, and T}_1 \text{, respectively, as previously described (43), and used for the determinations of T}_4 \text{, T}_3 \text{, and rT}_3 \text{. The molar cross-reactivities for the RIAs and the inter- and intraassay variations ([3',5'-125]I}\text{T}_4 \text{, [3',5'-125]I}\text{T}_3 \text{, and [3',5'-125]I}\text{T}_2 \text{, were added in amounts small enough to avoid interferences in the final RIAs. Appropriate volumes of chloroform were added to extract into an aqueous phase and purified by passing this aqueous phase through 2:1 chloroform/methanol, twice. The iodothyronines were then back-extracted with 0.1 ml 5% BSA. Protein-bound [125I]iodothyronines were prepared by radioiodination of T3, 3,5-T2, and 3,3'-T3, respectively, as previously described (43), and used for the determinations of T4, T3, and rT3 concentrations and D2 activities. [3,5-125I]T3 was obtained from Formula Gmbh (Berlin, Germany). Dithiothreitol (DTT) and 6-mercaptopropyl-2-ethanol (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\text{p}, T\text{q}, and rT\text{p} concentrations in human fetal brain**

T\text{p}, T\text{q}, and T\text{p} 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 [125I]T4 and [125I]T3 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 [125I]T4 and [125I]T3 added to each sample during the initial homogenization process. Average recovery was 50–60% for [125I]T4 and 60–70% for [125I]T3. T4 and T3 contents were determined by RIAs in triplicate at two dilutions. For the determination of T4, we used the same procedure as for T3 and Tp but using [125I]T4 as recovery tracer. The limits of detection are 3.3 fmol T4, 1.1 fmol T3, and 1.5 fmol Tp/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 T3 and Tp found in the respective RIAs, the individual recovery of the [125I]T4 and [125I]T3, 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 [125I]T3 (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 mM 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 [125I]iodothyronines were precipitated by addition of 0.5 ml 10% trichloroacetic acid. After centrifugation, the supernatants were analyzed by [125I]-production on Sephadex LH-20 microcolumns (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 [125I]T3 (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 0.63 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, Watertown, MA), and eluted with a gradient of acetonitrile in 0.2 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']I\text{T}_3 (80 μCi/g at 37°C during 1 h). Assay final conditions were 20 mM Tp, 20 mM DTT, 1 mM PTU (pH 7.5), and 40–50 μg protein in a total volume of 100 μl. [125I]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/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 ≤ 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, Tp/T3 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 ≤ 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 T4, Tp, and Tp 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 T4 concentrations were fitted to a quadratic function of 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. 3. Concentrations of T₄, T₃, and rT₃ (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 T₄ to nanograms per gram, divide by 1.287; to convert values for T₃ and rT₃ 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 T₄, CC, and BG T₃, and BS rT₃, which were cubic functions of PMA.

FIG. 2. Ontogenic changes of the concentrations of T₄, T₃, and rT₃ (in picomoles per gram wet weight), up to 20 wk PMA. To convert values for T₄ to nanograms per gram, divide by 1.287; to convert values for T₃ and rT₃ 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. T₄ 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 T₃ 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 T₄ in the latter area. The concentrations of rT₃ decreased linearly in the cortex and BS with increasing PMA.
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 $3.54 \pm 0.49 \text{ pmol} T_4/g$ ($2.75 \pm 0.38 \text{ ng} T_4/g$, $n = 9$), $1.35 \pm 0.50 \text{ pmol} T_3/g$ ($0.879 \pm 0.33 \text{ ng} T_3/g$, $n = 9$), and $1.98 \pm 0.57 \text{ pmol} rT_3/g$ ($1.289 \pm 0.37 \text{ ng} rT_3/g$, $n = 9$). 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 $T_3$, 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 CP and GE, the $T_3/T_4$ ratios decreased 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 and 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
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 T4 (r = 0.65, P < 0.001) and rT3 (r = 0.42, P = 0.001) and negatively with the T3/T4 ratios in the CC (r = −0.29, P = 0.008). Except for the correlation between D2 activities and T4 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 rT3/T4 and rT3/T3 ratios. T4 levels tended to decrease and rT3 levels tended to increase with D3 activity. A significant negative correlation was found between D3 activity and the T4 level (r = −0.682). D3 activities were positively correlated with the rT3/T3 ratio (r = 0.812, P = 0.008) and the rT3/T4 ratio (r = 0.889, P = 0.001). Figure 8B depicts the average D3 activities and rT3/T3 and rT3/T4 ratios in the different brain regions. Except for the CP, the rT3/T3 and rT3/T4 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 T3 availability in the human developing brain.

As already pointed out previously, it is quite likely that the changes in the concentrations of T4, T3, and rT3 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 T4 and rT3 concentrations were observed in the choroid plexus. T4 increased significantly with PMA, despite little change in the low T3 concentrations; indeed, the T3/T4 and rT3/T4 ratios
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. 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-

<table>
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<th>TABLE 3. Correlation of D3 activity with iodothyronine levels and ratios in different brain regions from fetuses and premature infants</th>
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Pearson’s correlation coefficient $r$ and $P$ values were calculated using SPSS.
tation T₃ 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 T₃ concentrations in the human CC are comparable with those reported in adults: 1.5–2.2 pmol T₃/g (1.0–1.4 ng T₃/g) (18, 56). The fetal D2 activities are actually much higher than reported in the adult cortex (∼8 nmol/h·mg protein) (18). The ontogenetic changes of T sucker, T₄, 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 T₃ concentrations, and an 18-fold increase in T₃ concentrations in rat CC.

The present results also indirectly support the hypothesis that T₃ 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 T₃ 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 ontogenetic changes in the developing human Cbl contrast with those described for the CC. The concentrations of T₄ and T₃ remained low, and especially T₃ 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 T₃ concentrations. So are the high D3 activities found in MB, BS, and SC, all areas in which T₃ 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 T₃ 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 T₃ 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 T₃ (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 hypothalam 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 T₃ in the various brain regions during critical stages of development. It is unclear whether D3 has an additional physiological role in the production of rT₃ and 3,3’-T₂. Because rT₃, but not T₃, has profound and acute effects on the cytoskeleton in brain cells (65), it is not excluded that rT₃ also has a function in brain development. 3,3’-T₂ has been shown to increase the basal metabolic rate in adult rats. This effect may be mediated by direct mitochondrial binding (66).

D2 and D3 are expressed in distinct cell types: D2 in astrocytes and tanyocytes and D3 in neurons. The hypothesis has been put forward (38) that astrocytes and tanyocytes take up T₄ from the circulation and convert it to T₃, which is delivered to neurons (that contain most of the nuclear receptors), in which D3 would limit T₃ 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 T₃ 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 T₃ 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 T₃ and T₄ and high rT₃ levels and low in regions with high T₃ and T₄ and low rT₃ levels. We found a significant negative correlation between D3 activities and T₃ levels and significant positive correlations between D3 activity and the ratio of rT₃/T₃ and the ratio of rT₃/T₄. Because D3 catalyzes the degradation of T₃ and T₄ and the production of rT₃, 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 area.
In conclusion, by determining and correlating the ontogenetic 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 T3 bioavailability to changing needs of human developing brain structures.

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