CLINICAL STUDY

Thyroid hormone transporters and deiodinases in the developing human hypothalamus

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Abstract

Objective: Thyroid hormone (TH) signaling in brain cells is dependent on transport of TH across the plasma membrane followed by intracellular deiodination and binding to the nuclear TH receptors. The aim of this study is to investigate the expression of the specific TH transporters monocarboxylate transporter 8 (MCT8 (SLC16A2)), MCT10, organic anion transporting polypeptide 1C1 (OATP1C1 (SLCO1C1)), and the types 2 and 3 deiodinases (D2 and D3) in the developing human hypothalamus. **Design:** Fifteen postmortem brain samples of fetuses and young children ranging between 17 weeks of gestation and 29 months of postnatal age including one child (28 months) with central congenital hypothyroidism were studied.

Methods: Sections of the different hypothalami were stained with polyclonal rabbit antisera against MCT8, MCT10, OATP1C1, D2, and D3.

Results: We found MCT8 and D3 but not D2 protein expression to be present in our earliest sample of 17 weeks of gestation, indicating triiodothyronine degradation, but not production at this time of development. At term, expression of TH transporters and D2 decreased and D3 expression increased, suggesting decreased TH signaling just before birth. The child with central congenital hypothyroidism showed higher MCT8 and D2 expression compared with the other children of similar age.

Conclusions: This study reports the developmental timing of expression of components crucial for central TH signaling in the human hypothalamus. In general, during fetal hypothalamic development, the coordinated expression of D2 and D3 in combination with the different TH transporters suggests that proper TH concentrations are regulated to prevent untimely maturation of brain cells.

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Introduction

Thyroid hormone (TH) is essential for proper development and function of the human brain including the hypothalamus (1). During pregnancy, the fetus is highly dependent on maternal TH supply, especially in the first trimester when the fetal thyroid is not yet functional, but when TH receptors (TRs) are already present (2, 3). Lack of TH during this crucial period in development leads to neurological damage and causes mental retardation. This has been shown in patients suffering from mental retardation due to maternal hypothyroxinemia caused by iodine deficiency during early gestation or due to insufficient TH in the postnatal period (4, 5).

Transport across the plasma membrane is the first step in the signaling pathway of TH as both deiodinases and nuclear TRs are located inside the cell. Recently, different TH transporters have been identified, including the organic anion transporting polypeptide 1C1

(OATP1C1 (SLCO1C1)), and the monocarboxylate transporter 8 (MCT8 (SLC16A2)) and MCT10 (6, 7, 8). The clinical importance of TH transporters is evident from patients with mutations in *MCT8* (9). These patients suffer from severe psychomotor retardation in combination with disturbed TH levels, referred to as the Allan–Herndon–Dudley syndrome (AHDS). In these patients, the cause of the mental retardation is not due to disturbances in TH supply from the mother, but due to the disturbed entry of TH into the developing brain.

Expression of OATP1C1 is restricted to specific areas of the adult brain, in particular in the capillary wall and in the choroid plexus, whereas MCT8 and MCT10 are widely expressed throughout the body including the brain (8, 10, 11, 12, 13, 14). From the three deiodinases, types 1, 2, and 3 (D1, D2, and D3), D2 and D3 are particularly important for the regulation of intracellular TH levels in the brain (15). Rodent studies have shown that during development, D2 expression is low, while D3 expression is high. This mechanism of low

triiodothyronine (T_3) during gestation has been proposed to prevent untimely maturation of brain cells (16). A similar mechanism of coordinated expression of D2 and D3 has been shown in cochlear development and during brown adipogenesis, where absence of either deiodinase leads to inappropriate levels of T_3 and results in deafness or impaired adaptive thermogenesis of brown adipose tissue respectively (17, 18, 19).

No data are yet available on the development of the functional neuroanatomy required for TH signaling in the human fetal and neonatal hypothalamus. As a first step, we describe here the expression of D2 and D3 together with MCT8, MCT10, and OATP1C1 to obtain more insight into the timing of TH signaling in the separate hypothalamic nuclei in fetuses and young children at different stages of development.

Materials and methods

Subjects

Postmortem hypothalamus specimens were obtained from 15 fetuses and young children ranging in age

between 17 weeks of gestation and 29 months of post-natal age. One of these children suffered from central congenital hypothyroidism in association with empty sella syndrome. Clinicopathological data of these subjects are summarized in Table 1. Brain material was obtained from The Netherlands Brain Bank at The Netherlands Institute for Neuroscience (Director Dr I Huitinga) in accordance with the formal permissions for brain autopsy and for the use of human brain material and clinical information for research purposes.

Histology

Hypothalami were fixed in 10% phosphate-buffered formalin at room temperature (RT) for 1–32 months. After dehydration in a series of graded ethanols, tissues were cleared in xylene and embedded in paraffin. Coronal serial sections (6 μ m) were cut over the entire rostrocaudal axis of the hypothalamus. Every 100th section was used for Nissl staining for anatomical orientation. D2, D3, MCT8, MCT10, and OATP1C1 staining was performed on every 100th section of the rostrocaudal axis of the hypothalamus.

Table 1 Clinicopathological data.

NBB	Sex	Age	Postmortem delay (h)	Fixation duration (days)	Brain weight (g)	Cause of death, clinical diagnosis
87-019	F	17 weeks of gestation	17	39	ND	No dysmorphic features
89-056	F	25 5/7 weeks of gestation, died 1 day <i>postpartum</i>	65	31	100	IRDS , cerebral hemorrhage
96-401	F	27 2/7 weeks of gestation	41	301	77	Dysmaturity, possibly congenital herpes infection, pulmonary hypoplasia
96-415	M	27 weeks of gestation, died 17 days <i>postpartum</i>	41	965	152	Respiratory insufficiency , pneumonia, IRDS
96-412	M	28 3/7 weeks of gestation, died 1 day <i>postpartum</i>	41	863	119	Respiratory insufficiency, IRDS
87-024	F	34 5/7 weeks of gestation, died 2 days <i>postpartum</i>	3	38	180	IRDS , Caesarean section for placental abruption, cerebral hemorrhage
96-266	M	35 weeks of gestation, died shortly after delivery	17	803	350	Respiratory failure , pulmonary and renal hypoplasia
96-282	F	At term	41	370	410	Intrauterine death
88-077	F	At term	65	56	350	Intrapartum asphyxia , unsuccessful resuscitation
96-235	M	At term	17	479	358	Intrauterine death, stillborn
96-203	F	2 months	ND	708	1150	Viral pneumonia
89-001	M	20 months	65	161	1140	Respiratory failure, due to acute epiglottitis
87-039	M	23 months	8	30	1430	Respiratory failure , virilizing congenital adrenal hyperplasia, pseudohermaphroditism, upper airway infection treated with hydrocortisone and fludrocortisone
91-003	M	28 months	15	33	1340	Status epilepticus , partial pituitary insufficiency (thyroid and adrenal failure), partial empty sella, treated with thyroxine and hydrocortisone
89-052	M	29 months	41	41	1230	Circulatory failure , gastroenteritis and diarrhea, Hirschsprung disease

F, female; M, male; ND, not determined; IRDS, infant respiratory distress syndrome.

Antibodies

For immunocytochemical staining, we used polyclonal rabbit antisera raised against synthetic peptides for human D3 (#676, amino acids 265–278 (10, 20, 21), MCT8 (#1306, amino acids 527–539 (20, 21)), MCT10 (#1758, amino acids 473–487 and 503–515 (6, 10)), and OATP1C1 (#3516, amino acids 696–711 (10)). The D2 antibody (#763, kindly provided by Dr J L Leonard, Worcester, MA, USA) was raised against the C-terminus (amino acids 247–266) of full-length rat D2: NH₂-YNLQEVRSWLEKNFSKRCILD-COOH where cysteine (C) replaces selenocysteine. This antibody has been extensively used and tested before in the immunohistochemical localization of rat and human D2 (20, 21, 22). It is specific for the D2 selenodeiodinase and does not cross-react with the thyroxine (T₄)-binding protein p29, which has been identified as DICKKOPF protein DKK3 (23, 24).

Antiserum from the final bleed was used without further purification. Antibody specificity has been described earlier and was supported using western blotting (D2, D3, MCT8, and MCT10), staining of transfected cells (OATP1C1), testing of pre-immune sera, and pre-adsorption with the immunizing peptides (7, 20, 21, 22).

Immunocytochemical procedures

All immunocytochemical procedures and antibody specificity have been described before (7, 10, 20, 21, 22). To exclude any variation in staining procedures, all samples were stained in the same run.

In short, sections were mounted on Superfrost plus slides and dried for at least 2 days at 37 °C. After deparaffinization in xylene and rehydration through graded ethanol series, sections were washed in TBS and antigen retrieval was performed using microwave treatment (10 min, 700 W) in TBS at pH 7.6 for MCT8 and OATP1C1 and in 0.05 M Tris-HCl at pH 9.0 for MCT10. After cooling down to RT, sections for OATP1C1 staining were first pre-incubated for 1 h at RT in TBS-1% nonfat milk (Campina, Eindhoven, The Netherlands, pH 7.6). Sections were incubated in the first antibody diluted 1:500 in SUMI (supermix, 0.05 M Tris, 0.15 M NaCl, 0.5% Triton X-100 (Sigma), and 0.25% gelatin (Merck; pH 7.6)) for MCT8, MCT10, or OATP1C1, 1:1250 for D2, and 1:900 in SUMI containing 1% BSA for D3 overnight at 4 °C in a humidified chamber. Sections were washed in TBS and incubated in the second antibody (biotinylated goat anti-rabbit, 1:400 in SUMI) for 1 h at RT. After washing in TBS, sections were incubated for 1 h at RT in avidin biotinylated complex (1:800 in SUMI; Vector Laboratories, Burlingame, CA, USA) and subsequently rinsed in TBS. Finally, sections were incubated in 0.5 mg/ml 3,3'-diaminobenzidine (Sigma) in TBS containing 0.2% ammonium nickel sulfate (BDH; Brunswick,

Amsterdam, The Netherlands) and 0.01% H₂O₂ (Merck) for ~15 min. The reaction was stopped in distilled water. The sections were dehydrated in graded ethanol series, cleared in xylene, and coverslipped using Entellan (Merck). Staining was visualized using a Zeiss Axioskop with Plan-NEOFLUAR Zeiss objectives (Carl Zeiss GmbH, Jena, Germany) and images were photographed using a CCD (JVC KY-F553CCD) or Axioplan2 (Zeiss) camera. Staining intensities of the different areas of the hypothalamus were scored by visual inspection by two researchers (A Alkemade, E C H Friesema).

Results

We studied the developmental expression of proteins involved in TH signaling in fetal and infant human hypothalamus. Overall staining intensities for the hypothalamus throughout the developmental period studied are summarized in Fig. 1. For D3, MCT8, MCT10, and OATP1C1, the developmental expression is shown for the paraventricular nucleus (PVN). No clear effects of postmortem delay or fixation duration were observed. Previously, we did not find D2 expression in neurons of the PVN in the adult brain (21); we also did not detect D2 expression in the fetal or infant human brain. Therefore, the developmental expression shown for D2 represents staining of the infundibular nucleus (IFN)/median eminence and periventricular area. All subjects showed staining of MCT8, MCT10, OATP1C1,

Figure 1 Schematic representation of (A) D2 expression in the infundibular nucleus/median eminence and periventricular area and D3 expression in the paraventricular nucleus (PVN) and (B) TH transporter expression throughout PVN development. The figure is based on 13 observations. Subjects #96-415 (possible relative hypothyroid state after birth) and #91-003 (central congenital hypothyroidism) were omitted. Note the opposite expression pattern of D3 vs TH transporters and D2 expression.

D2, and D3 in the choroid plexus, although staining intensities varied. Interestingly, MCT10 was observed only on the apical side of the choroid plexus cells, whereas MCT8 and OATP1C1 were present both on the basal and apical side throughout the choroid plexus (Fig. 2).

Previous studies by Koutcherov *et al.* (25) have shown that at 9–10 weeks of gestation, only minimal nuclear differentiation is present in the hypothalamus, although already the lateral hypothalamus (LH), supraoptic nucleus (SON), and IFN can be distinguished. Their studies have also shown that at 11–14 weeks, the fornix becomes visible and the anlage of the PVN can be distinguished, and at 15–17 weeks, the mammillary body becomes prominent. At our first sampling point (#87-019, 17 weeks of gestation), we observed some MCT8 expression surrounding blood vessels, whereas no MCT10 or OATP1C1 staining was observed. No D2 expression was found and a few D3-positive cells were observed only in the IFN.

Koutcherov *et al.* (25) described that at 18–23 weeks of gestation, the PVN resembles the postnatal structure and the perifornical area is formed as well as the LH. The suprachiasmatic nucleus (SCN) is visible at 23 weeks of gestation, and the first neuropeptide Y-positive cells are present in the IFN, and at weeks 24–33, the fetal

hypothalamus has an adult-like appearance (25). At 25 5/7 weeks of gestation (#89-056), staining for MCT8 was found in neurons of PVN and IFN, as well as in scattered neurons of other hypothalamic nuclei, tanycytes, and surrounding blood vessels. Neuronal MCT10 expression was present in SON, PVN, and IFN, and OATP1C1 was also expressed in IFN, just as D3. D2 expression was only observed surrounding blood vessels.

At 27 weeks of gestation (#96-415), MCT8 immunoreactivity was present in PVN and LH. MCT10 was present in PVN, LH, and SON and OATP1C1 was present in LH, PVN, and IFN. Hardly any D2 staining was present, whereas D3 was prominent in the PVN.

At 27 2/7 weeks of gestation (#96-401), MCT8 staining was present in neurons of the PVN, IFN, and LH, and in addition, MCT8 was expressed in tanycytes. MCT10 was strongly expressed in the LH. In addition, MCT10 was expressed in neurons of the IFN, PVN, and SON. OATP1C1 was present surrounding blood vessels of the organum vasculosum laminae terminalis (OVLt) and in LH, IFN, PVN, and SON neurons. D2 was also observed surrounding OVLt blood vessels; in addition, D2 staining was found along the lining of the third ventricle. However, D3 expression was not observed.

At 28 3/7 weeks of gestation (#96-412), MCT8 staining was present in the IFN, PVN, and LH. MCT10 and OATP1C1 also showed expression in the LH. In addition, OATP1C1 was also present in the IFN and in the tanycytes. D2 was found along the lining of the third ventricle and D3 showed almost no expression.

From 34 weeks of gestation to at term-born children, the nucleus tuberalis lateralis (NTL) and tubero-mammillary nucleus (TMN) can be distinguished (23). At 34 5/7 weeks of gestation (#87-024), we did not observe any transporter staining in SON or PVN. The caudal part of this hypothalamus was not available.

At 35 weeks of gestation (#96-266), only some MCT8 immunoreactivity was observed in tanycytes. MCT10 did not show any staining, and OATP1C1 showed only very weak staining in the IFN. Weak D2 immunoreactivity was present only surrounding blood vessels and strong D3 expression was present in the neurons of IFN, TMN, and LH.

At term #96-282 showed no staining, and #88-077 and #96-235 showed only weak staining in SON, PVN, and IFN for MCT8 and MCT10. OATP1C1 was present in tanycytes, PVN, and IFN neurons in patients #96-282 and #88-077, whereas #96-235 did not show any neuronal OATP1C1. D2 was present surrounding blood vessels. D3 staining was present in PVN, IFN, TMN, and LH. Overall, staining was weak in children born at term, except for D3.

At 2 months of age, #96-203 staining for MCT8 in SON, PVN, and in the lining of the third ventricle was found. MCT10 immunoreactivity was present in SON and PVN. Sporadic expression was found in the LH. OATP1C1 showed a more scattered pattern and increased staining was observed in SON, PVN, TMN,

Figure 2 Staining of choroid plexus for (A) Nissl, (B) OATP1C1, (C) MCT8, (D) MCT10, (E) D2, and (F) D3 in subject #89-056 (25 5/7 weeks of gestation). Note the expression of OATP1C1 and MCT8 in the basolateral and apical surface of the choroid plexus, whereas MCT10 was only expressed on the apical side of the choroid plexus. Bar represents 100 μ m.

IFN, and the ependymal layer. D2 expression was only present surrounding blood vessels. D3 showed expression in SON, PVN, IFN, TMN, and NTL (Fig. 3).

In children aged 20–29 months of age (#89-001, #87-039, and #89-052), we found moderate MCT8 staining in PVN, SON, IFN, and weak staining in the LH. MCT10 was also present in these areas and was strongest in #89-052 in which we also observed strong expression in LH. OATP1C1 was present in PVN, SON, IFN, and in some cells in the ependymal layer. In addition, scattered OATP1C1-positive cells were present and again staining was strongest in #89-052. In this patient, we also observed staining in NTL, TMN, SCN, and LH. D2 was expressed surrounding blood vessels especially in the IFN and in tanycytes.

In the 28-month-old child with central congenital hypothyroidism (#91-003), MCT8 expression was higher than in other children aged over 20 months (Fig. 4). The difference in MCT8 expression is shown in Fig. 5, where staining of MCT8 is compared in a representative area of the hypothalamus of this child with central congenital hypothyroidism and an age-matched control child of 29 months (#89-052). The child received supplementation therapy with hydrocortisone and T_4 (doses unknown). Staining was present in PVN and SON. Interestingly, we found strong staining surrounding the blood vessels located in the PVN. Strongest staining was observed in the LH. MCT10 was weak and present in LH and some staining was

Figure 3 Staining of the supraoptic nucleus for (A) Nissl, (B) OATP1C1, (C) MCT8, (D) MCT10, (E) D2, and (F) D3 in subject #96-203 (2 months of age). Bar represents 100 μ m.

Figure 4 Staining of the paraventricular nucleus for (A) Nissl, (B) OATP1C1, (C) MCT8, (D) MCT10, (E) D2, and (F) D3 in subject #91-003 suffering from central congenital hypothyroidism (28 months of age). Note the strong MCT8 staining in brain capillaries (high power insert). Asterisks indicate the third ventricle. Bar represents 100 μ m.

observed in IFN. Weak OATP1C1 staining in PVN, SON, IFN, TMN, and NTL was present. D2 showed very strong expression surrounding the blood vessels of the IFN, but not of the PVN (Fig. 6). D3 staining was almost absent.

Discussion

In this study, we report for the first time the expression of proteins involved in TH signaling in human fetal hypothalamic development using immunocytochemistry. We already found *MCT8* and *D3* expression in our earliest sample of 17 weeks of gestation, although *D2* was absent. During this period (15–17 weeks of gestation), the differentiation of the lateral hypothalamic zone takes place (25). At this time, both TRs and the ligand T_3 are present in the human brain and TR mRNA increases strongly from 10 to 18 weeks of gestation (3, 26). Our observations indicate T_3 degradation but not production in the fetal hypothalamus at this moment in development. This supports the notion that during early development, the brain is protected against high T_3 concentrations to prevent untimely maturation of brain cells (16). Earlier studies have suggested that at 18 weeks of gestation, all T_3 in the brain is produced via local deiodination (26). This notion is supported by increased *D2* activity and T_3 content in the cerebral

Figure 5 Staining of MCT8 in a representative area of the hypothalamus of a 28-month-old child with central congenital hypothyroidism (A, #91-003) and a 29-month-old age-matched control child (B, #89-052). F, fornix. Bar represents 100 μ m.

cortex during the second trimester (27). However, in this study, we did not observe the increase in D2 immunoreactivity in the hypothalamus at 17 weeks of gestation, neither in tanycytes nor in surrounding blood vessels. It is possible that timing differences exist between different brain areas.

During the second trimester, the hypothalamus develops further, and during the late second trimester (24–33 weeks of gestation), the hypothalamic structure takes on an adult-like appearance, although the development is not yet complete (28). In this study, we found that MCT8, MCT10, and OATP1C1 are expressed at this stage, although staining intensities vary between subjects. Subject #96-415 showed almost absent deiodinase immunoreactivity. This child survived 17 days after preterm delivery (27 weeks of gestation), a period during which a developing child would normally still be partially dependent on the mother's TH production *in utero* (29). Preterm children therefore suffer from transient hypothyroxinemia, which may affect hypothalamic TH signaling (30, 31, 32). In addition, we cannot exclude effects of non-thyroidal illness or glucocorticoid treatment on protein expression levels (33, 34). Strikingly, toward the end of gestation, TH transporters and D2 immunoreactivity decreased, whereas D3 expression increased. These data suggest a lower hypothalamic T_3 demand at the end of gestation. Whether this is of physiological relevance for the TSH surge present after birth is unknown. Studies have shown that the TSH surge in at least the first hours of postnatal life was markedly attenuated in infants born after 24–27 weeks of gestation (35). After at term birth, staining for TH transporters increased again, and in children aged 20–29 postnatal months, the staining patterns closely resemble our earlier observations in the adult hypothalamus, in which we performed double staining experiments to confirm the identity of the cell types that were stained (10, 21).

We studied 15 tissues from eight male and seven female brains, including one subject with virilizing congenital adrenal hyperplasia (#87-039) as human hypothalamic tissues from different stages of development are not readily available. To our knowledge, no sexual differences have been described for hypothalamic

components involved in TH signaling. It is therefore unlikely that the use of samples from both sexes has influenced the results of our studies.

In our studies, we had the unique opportunity to investigate the hypothalamus of one child with central congenital hypothyroidism as a result of a partial empty sella syndrome. In addition to the hypothalamus–pituitary–thyroid (HPT) axis, the hypothalamus–pituitary–adrenal axis was also affected, and this child received hydrocortisone supplementation in addition to TH treatment, which may have influenced the HPT axis, although we have no information on the dosage of hydrocortisone. In addition, we cannot exclude developmental hypothalamic adaptation to the defective pituitary development. Nevertheless, the staining pattern observed in this child fitted a hypothyroid state. Also in the other studied subjects, we cannot exclude effects of concomitant illness on protein expression levels, which is an inherent limitation when studying human brain and cannot be resolved for obvious reasons. Strikingly, MCT8, which is important for T_3 uptake in the cell, showed stronger expression than in children of similar age, especially in blood vessels. This may represent an adaptive mechanism to increase local T_3 concentrations in the hypothalamus. Although we describe only one case of central congenital hypothyroidism, the strong expression of D2 and the almost absent D3 staining in this patient support such an adaptive mechanism; whether this is a developmental adaptation or a response to an inadequate supplementation dose is unclear.

The importance of TH transporters in humans became evident for the first time in 2004 from patients in whom a mutation in MCT8 had been identified (36, 37). Defects in the human MCT8 gene lead to severe X-linked psychomotor retardation in combination with elevated serum T_3 concentrations, also known as AHDS (38). The striking differences in the neurological phenotype between *Mct8*-null mice and MCT8 patients have been proposed to result from

Figure 6 Intense D2 staining in blood vessels of the infundibular nucleus of subject #91-003 suffering from central congenital hypothyroidism (28 months of age). Arrowheads indicate blood vessels. Bar represents 100 μ m (A) and 50 μ m (B).

interspecies differences in the expression (or subset) of TH transporters in the central nervous system (39, 40). The clear expression of MCT10 and OATP1C1 already in the second trimester of pregnancy suggests that MCT10 and OATP1C1 cannot compensate for the MCT8 deletion in AHDS. Wirth *et al.* (41) suggest that the L-type amino acid transporter LAT2 might compensate in the mouse but not in the human brain for the lack of MCT8 as only a low LAT2 expression was found in developing neurons in the human brain. Also, the rat brain expresses the TH transporter variants OATP1A4 and OATP1A5, but to our knowledge, both transporters have no ortholog in the human brain (39).

This study provides a next step toward understanding the development of the central components of the human HPT axis. Recently, Chan *et al.* (42) have published that many TH transporters including MCT8, MCT10, and OATP1C1 are also expressed in the human fetal cerebral cortex, which could regulate cellular TH supply during early development (7–20 weeks of gestation). The number of observations in our study is still limited, and therefore, caution should be used when interpreting the results. Nevertheless, our data show that variations in deiodinase and TH transporter expression levels are present in the hypothalamus during fetal and neonatal development (17 weeks of gestation to 29 months of age). At present, it is unknown whether the variation serves a physiological purpose, but this is an interesting possibility requiring further studies, which should be extended to other brain areas as well.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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