EXPERIMENTAL STUDY

Novel neuroanatomical pathways for thyroid hormone action in the human anterior pituitary

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Abstract

Objective: An increasing number of proteins appear to be involved in thyroid hormone feedback action at the level of the anterior pituitary, but the cell types expressing these proteins are largely unknown. The aim of the present study was to identify cell types in the human anterior pituitary that express type II and type III deiodinase (D2 and D3), the recently described thyroid hormone transporter (MCT8) and thyroid hormone receptor (TR) isoforms by means of double-labeling immunocytochemistry.

Results: We found TR isoforms to be expressed most prominently in gonadotropes and – although to a lesser extent – in thyrotropes, corticotropes, lactotropes and somatotropes. D3 staining showed a distribution pattern that was remarkably similar. By contrast, D2 immunoreactivity was observed exclusively in folliculostellate (FS) cells showing coexpression with human leukocyte antigen (HLA), a marker of major histocompatibility complex (MHC)-class II. MCT8 immunostaining was present in FS cells without HLA coexpression.

Conclusions: From these results, we propose a novel neuroanatomical model for thyroid hormone feedback on the human pituitary, with a central role for FS cells in thyroid hormone activation, which thus play an important role in the suppression of TSH secretion by circulating thyroxine (T₄).

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Introduction

The importance of thyroid hormone feedback action at the level of the anterior pituitary has been clearly established. Thyroid hormones modulate thyroid-stimulating hormone (TSH) release from the anterior pituitary within the framework of a negative feedback loop, and an increasing number of proteins appear to be involved in this feedback action. About 50% of tri-iodothyronine (T₃), the biologically active form of thyroid hormone, is produced locally by conversion of thyroxine (T₄) (1). In the human pituitary, type II deiodinase (D2) converts T₄ into T₃, while type III deiodinase (D3) converts T₄ and T₃ into inactive metabolites (2, 3). We have recently described the presence of both D2 and D3 activity in the human anterior pituitary (4). Type I deiodinase activity does not appear to play a major role in thyroid hormone feedback in the human anterior pituitary (3). T₃ acts by binding to thyroid hormone receptor (TR) isoforms, which have been reported in both human and rat anterior pituitary (5, 6). In rats, TRβ2 is prominent in somatotropic

and thyrotropic cells (7). Monocarboxylate transporter 8 (MCT8), a thyroid hormone transporter with a higher affinity for T₃ than for T₄ in man, may play an important role in the anterior pituitary by providing cells with thyroid hormone (8). However, no information is available at present on cells expressing these proteins in the human anterior pituitary.

Animal studies support a functional role of D2 and TRs in the endocrine feedback regulation of thyroid hormone. During hypothyroidism, a large increase in D2 and TRβ2 expression occurs in the rat anterior pituitary (9, 10). Furthermore, a role for MCT8 in thyroid hormone feedback is suggested by the high serum TSH levels in the face of highly elevated serum T₃ levels recently reported in children with mutations or deletions in the MCT8 gene (11, 12). In addition to clear effects on TSH secretion, changes in the serum concentrations of other glycoprotein hormones from the anterior pituitary have been reported during altered thyroid hormone status. For example, serum follicle-stimulating hormone (FSH) is elevated in primary hypothyroidism, and the response of luteinizing

hormone (LH) to gonadotropin-releasing hormone (GnRH) is decreased (13).

We have recently reported the distribution of TR α 1, α 2, β 1 and β 2 in the human anterior pituitary, but the cell types expressing these TR isoforms remain unknown (5). Likewise, no data are available on cell types expressing deiodinases or MCT8 in the human anterior pituitary. In the human hypothalamus TR isoforms, D3 and MCT8 are all expressed in hypophysiotropic TRH neurons of the paraventricular nucleus (PVN), whereas D2 is expressed mainly in glial cells (4). These data suggest that hypothalamic T₃ production and action occur in different cell types. Whether similar anatomical routes are present in the anterior pituitary is unknown. In the present study, we identified cell types expressing D2, D3, MCT8 and TR isoforms in the human anterior pituitary by fluorescent double-labeling immunocytochemistry using polyclonal rabbit antisera directed against TR α 1, TR α 2, TR β 1, TR β 2, D2, D3 and MCT8 in combination with mouse monoclonal markers for pituitary hormones and folliculostellate (FS) cells. On the basis of the data obtained, we propose a novel neuroanatomical model for thyroid hormone feedback action in the human anterior pituitary.

Materials and methods

Pituitary material

We studied five human anterior pituitaries by immunocytochemistry. Tissues were obtained from the Netherlands Brain Bank at the Netherlands Institute for Brain Research (NIBR) in accordance with the formal permissions for brain autopsy and the use of human brain material and clinical information for research purposes. Clinicopathologic data are presented in Table 1. Tissues were obtained after a postmortem delay of 5–10 h. Our earlier studies have shown that in human pituitary tissue with a comparable postmortem delay both D2 and D3 enzyme activity can still be detected (4). Pituitary samples were fixed in 10% phosphate-buffered formalin at room temperature (RT) for 4 weeks (Table 1). Tissues were dehydrated in a graded

ethanol series, cleared in xylene and embedded in paraffin. Sections of 6 μ m were made on a microtome.

Antisera

For immunocytochemical staining, we used polyclonal rabbit antisera raised against synthetic peptides derived from rat D2 (nos. 763: 247–266 and 762: 247–262 (14, 15) (kindly provided by Dr J L Leonard, University of Massachusetts Medical Center, Worcester, MA, USA) and human D3 (no. 676: 265–278) and MCT8 (nos. 1305 and 1306: 527–539). Polyclonal antisera for D3 and MCT8 were raised in rabbits by Eurogentec SA (Herstal, Belgium) after conjugation of the synthetic peptide to keyhole limpet hemocyanin. Antiserum from the final bleed was used without further purification.

Specificity of the antisera was supported by absence of staining with the preimmune serum and after preadsorption with homologous peptides. Cross-reactivity of the antibodies was assessed by preadsorption with heterologous peptides. In addition, Western blotting on human anterior pituitary and, for TR antibodies, Western blotting of tissues of knockout mice were performed. These data have been published earlier (4, 5, 15–18). Mouse monoclonal pituitary markers were obtained from Biogenesis (Poole, UK) (FSH, LH, TSH, GH, prolactin (PRL) and adrenocorticotrophic hormone (ACTH)), and DAKO Diagnostics (Glostrup, Denmark) (human leukocyte antigen (HLA)-DR).

Immunocytochemical staining

Sections were mounted on Superfrost Plus slides and subsequently dried for at least 2 days at 37 °C. After deparaffinization in xylene and rehydration through a graded ethanol series, sections were rinsed in distilled water and in Tris-buffered saline (TBS, 3 $\times$ 10 min). Sections used for D3 or TR isoform staining were microwave-treated in TBS for 10 min at 700 W (19). Immunocytochemistry for morphologic studies of cell types was performed in the following steps:

1. incubation with the first antibody (D2 1:1250; MCT8 1:500 in Supermix (SUMI, 0.05 M Tris,

Table 1 Clinicopathological data.

Subject no.	Sex	Age (years)	PMD (hours)	Fix (days)	Cause of death and clinical diagnosis
95 101	F	73	6	31	Heart failure, type II diabetes, angina pectoris
96 082	M	74	8	27	Unknown, Parkinson's disease
95 102	M	53	10	31	Circulatory failure, unsuccessful resuscitation, cardiac tamponade after surgery for aorta dissection
96 081	F	63	5	28	Respiratory insufficiency, astrocytoma in the left hemisphere and brainstem
96 084	F	78	8	32	Respiratory insufficiency, emphysema, hypothyroidism (thyroxine treatment, daily dose 0.125 mg)

Fix: fixation duration; PMD: postmortem delay.

0.15 M NaCl, 0.5% Triton X-100 (Sigma) and 0.25% gelatin (Merck) (pH 7.6)), D3 1:900 in SUMI containing 1% BSA, TR α 1 1:1000, TR α 2 1:2000, TR β 1 1:1000, TR β 2 1:1000 in SUMI containing 5% milk) for 1 h at RT and subsequently overnight at 4°C

2. rinsing in TBS (3 × 10 min)
3. incubation in biotinylated goat-antirabbit (1:400 in SUMI), for 1 h at RT
4. rinsing in TBS (3 × 10 min)
5. incubation in avidine-biotinylated complex (1:800 in SUMI) for 1 h at RT
6. rinsing in TBS (3 × 10 min)
7. incubation in a solution of 0.5 mg/ml 3,3'-diaminobenzidine (DAB) (Sigma) in a total volume of 15 ml TBS containing 0.01% H₂O₂ (Merck) and 0.035 g/ml ammonium nickel sulfate (BDH, Poole, UK) for approximately 15 min at RT.

The reaction was stopped in distilled water. Subsequently, sections were dehydrated through a graded ethanol series, cleared in xylene, and coverslipped with Entellan (Merck).

Colocalization studies were performed by immunocytochemistry using immunofluorescence. This technique appeared to be less sensitive than the protocol with DAB as a chromogen; therefore, antibody concentrations were increased. The protocol consisted of the following steps:

1. incubation with first antibody (rabbit polyclonal anti-D2, D3, MCT8 or TR isoform 1:300), combined with a monoclonal mouse pituitary marker (TSH, FSH, LH, GH, PRL, HLA undiluted and ACTH 1:20 in SUMI for 1 h at RT and subsequently overnight at 4°C
2. rinsing with TBS (3 × 10 min)
3. incubation with biotinylated goat anti-rabbit (1:400 in SUMI) for 1 h at RT
4. rinsing with TBS (3 × 10 min)
5. incubation in avidin biotinylated complex (Vector, ABC, Burlingame, CA, USA 1:800 in SUMI) for 1 h at RT
6. rinsing with TBS (3 × 10 min)
7. incubation in biotinylated tyramide (1:750 in SUMI).

Staining was visualized with either Cy2 and streptavidin-coupled Cy3, or Cy3 and streptavidin-coupled Cy2 as fluorophore.

Results

D2, D3, TR α 1, TR β 1 and TR β 2 staining showed an inhomogeneous distribution in the anterior pituitary. Cells expressing these proteins were clustered, whereas cells showing MCT8 and TR α 2 immunoreactivity were more uniformly distributed over the anterior pituitary. Staining intensity for deiodinases, TR α 1 and TR β

showed strong interindividual variation, whereas TR α 2 and MCT8 staining was intense in all patients studied. Immunocytochemical staining for all antibodies with DAB-Ni for visualization is shown in Fig. 1. TR isoform and D3 immunoreactivity were present in the cytoplasm of granular cells showing prominent colocalization with FSH (Figs 2 and 3). TR α 2 and TR β 2 showed clear colocalization with TSH (Fig. 2). D3 showed sporadic coexpression with TSH. D2 and MCT8 immunoreactivity was prominent in FS cells showing no colocalization with pituitary hormones (Figs 1 and 3). Coexpression of D2 and HLA immunoreactivity was observed, although not all D2-expressing cells coexpressed HLA. MCT8 immunoreactivity, which was present in stellate-shaped cells, as we concluded from inspection of DAB-Ni-stained sections, did not colocalize with HLA immunoreactivity.

One patient (no. 96 084) diagnosed with hypothyroidism and treated with T₄ showed very low TSH immunoreactivity as compared with all other patients. The limited number of TSH-positive cells did not show coexpression with TRs. TR isoforms were expressed in other hormone-secreting cells in this patient. Deiodinase and MCT8 staining was not different from that in pituitaries of other subjects. Results are summarized in Table 2.

Discussion

In the present study, we have characterized cells in the anterior pituitary that express deiodinases, T₃ receptor isoforms and MCT8 thyroid hormone transporter. Specificity of the antisera has been described before and was supported by testing of the preimmune serum, preadsorption with the synthetic peptide and Western blotting on human anterior pituitary (4, 5). Specificity of the TR isoform antisera was further supported by Western blotting in knockout mice (4, 17, 18).

D2 and MCT8 immunoreactivity were observed in FS cells (Fig. 3). FS cells are agranular cells of the anterior pituitary with long cytoplasmic processes between endocrine cells and are able to modulate anterior pituitary hormone secretion (20–22). We found that HLA, a determinant of major histocompatibility complex (MHC)-class II, which is expressed in a subset of FS cells (10–20%) (21, 22), colocalized with D2 immunoreactivity. These results strongly suggest that a subset of FS cells produce T₃ from T₄ and may be capable of transporting T₃ to other cells expressing TR isoforms. MCT8 and a subset of D2-expressing FS cells did not coexpress HLA. Recently, expression of nestin, in rat anterior pituitary was described in nontypical FS cells (23). Whether these cells also express MCT8 and D2 remains to be clarified.

Our present findings in the human anterior pituitary gland may seem at variance with earlier experimental studies in rats by Koenig *et al.*, who attempted to

Figure 1 Immunostaining in human anterior pituitary visualized with DAB-NI. Bar: 50 μ m.

identify cell types expressing D2 in enriched cell pools obtained by gradient centrifugation of cultured rat anterior pituitary cells (24). The experiments pointed to somatotropes and mammotropes as main cell types

expressing T_3 -responsive D2 activity (25). However, these cell fractions contained both hormone-secreting cells and agranular cells of the anterior pituitary; therefore, the representation of FS cells in these

Figure 2 TR immunostaining combined with pituitary markers. Cells expressing TR isoforms are stained green, and cells expressing pituitary marker are stained red. Coexpression results in yellow staining. TR isoforms show colocalization with pituitary hormones, but not with HLA, indicating that changes in pituitary glycoprotein serum concentrations during thyroid disease may be, at least partly, based upon a direct action of thyroid hormone. Magnification = 63 × objective.

experiments cannot be excluded with certainty. More recent studies in rat pituitary tumor cells showed that D2 is expressed in hormone-secreting tumor cells (26). However, it remains to be established that D2 is also expressed in hormone-secreting cells of normal

rat anterior pituitary. It therefore remains unknown at present whether the finding of D2 expression in FS cells of the human anterior pituitary gland represents an interspecies difference, or, alternatively, whether D2 expression may also be present in rat FS cells.

Figure 3 Deiodinase and MCT8 immunostaining combined with pituitary markers. Cells expressing deiodinases are stained green, and cells expressing pituitary markers are stained red. Coexpression results in yellow staining. Note the colocalization of D2 with HLA. Magnification = 63 × objective.

Table 2 Expression of thyroid hormone-related proteins in pituitary cells.

	TSH	FSH	LH	GH	ACTH	PRL	MHC-II FS cells*	Other FS cells*
D2	—	—	—	—	—	—	+	+
D3	+/-	+	+	+	+	—	—	—
MCT8	—	—	—	—	—	—	—	+
TR α 1	—	+	+	—	—	+	—	—
TR α 2	+	+	+	+	+	—	—	—
TR β 1	—	+	+	+	+	—	—	—
TR β 2	+	+	+	+	+	+	—	—

FS cells: folliculostellate cells. *D2, D3 and MCT8 expression in MHC-II cells was determined by double-labeling studies; other expression in FS cells was determined by morphologic studies.

We have previously described both nuclear and cytoplasmic TR staining in the human anterior pituitary (5). Although TRs have been assumed to be largely confined to the nuclear compartment (27), we found mainly cytoplasmic staining. Cytoplasmic localization of TRs has also been described in rats with the antisera that were used in this study (17, 18). More recent studies have shown that TRs may shuttle rapidly between the nuclear and cytoplasmic compartment (28). Translocation of the TR is suggested to be ligand dependent (29) and to require multiple protein interaction with various cofactors (30).

Different TR isoforms may have different roles in thyroid hormone function. In somatotropes, T_3 increases TR α 1, while it decreases TR β 2 (7, 31). Serum TSH concentrations are not altered in mice lacking all TR α transcripts (32). This is in contrast with TR β knockout mice, which have increased TSH subunit mRNA content in the anterior pituitary. This suggests a specific role for TR β in the negative feedback of thyroid hormone on TSH (33, 34). A specific role for TR β 2 in TSH regulation is also supported by our data. TR β 2 shows clear coexpression with TSH, while TSH coexpression with the other ligand-binding TR isoforms was not found.

Earlier studies in rats showed the most prominent expression of TR β 2 in somatotropes and thyrotropes (7), whereas, in the present study on human postmortem material, most prominent TR α and β expression was observed in gonadotropes. The difference in expression may reflect an interspecies difference, or it may be related to the presence of severe illness in the patients in the present study. During severe illness, serum concentrations of T_3 (and eventually T_4) decrease without giving rise to elevated TSH levels. This phenomenon is known as non-thyroidal illness (NTI) (35).

The pathogenesis of NTI is still unclear, but decreased TRH expression at the level of the hypothalamus suggests a major change in central thyroid hormone feedback regulation, contributing to persistently low serum TSH (36). Whether sporadic D3 staining in thyrotropes, as found in the present study, reflects downregulation of D3 enzyme activity in the framework of NTI is unknown. One might speculate that decreased degradation of T_3 in thyrotropes represents an additional contribution to inappropriately low serum TSH concentrations during illness. However, to establish whether altered TR or deiodinase expression in the anterior pituitary plays a role in the pathogenesis of NTI requires quantitative studies in tissues of patients with a biochemically defined endocrine status before death. Although expression levels of proteins may have been affected by critical illness in these patients, it is unlikely that distribution patterns are influenced by NTI.

A number of endocrine changes have been described in patients with thyroid dysfunction. Hypomenorrhea has been reported in hyperthyroid women and

hypermenorrhea in hypothyroid women. LH, FSH and GH levels are often increased during hyperthyroidism (37, 38). CRH responsiveness of the corticotrope is increased in hypothyroid patients (39). The presence of TRs in somatotropes, corticotropes and gonadotropes indicates that changes in pituitary glycoproteins may be based, at least partly, upon a direct action of thyroid hormone on these hormone-secreting cells in the anterior pituitary. In the present study, a patient (no. 96 084) treated with T_4 for primary hypothyroidism appeared to have relatively low TSH expression. This may be related to the relative hyperthyroxinemia often present in patients on T_4 -supplementation therapy. We could not substantiate this in view of the lack of data on antemortem serum thyroid hormone concentrations in this patient. MCT8 and deiodinase staining were unremarkable. No colocalization was observed between TR isoforms and TSH in this patient, although TR isoforms were expressed in the anterior pituitary. The lack of colocalization between TR isoforms and TSH can possibly be explained by decreased TSH expression, but it may also be the result of downregulation of TR expression in thyrotropes in this patient.

This study reports the anatomical distribution of deiodinases, MCT8 and TRs in the human anterior pituitary for the first time. From the results, we propose a novel neuroanatomical route for thyroid hormone feedback action on hormone-secreting cells of the human anterior pituitary. In this model, the FS cell is central in local hypophyseal T_3 production (Fig. 4). The expression of the TSH receptor reported earlier in MHC-class II cells (40) and the increase in D2 activity that has been observed in TSH-producing tumors (41) suggests that D2 expression in the anterior pituitary can be influenced directly by TSH. This is further supported by a dose-dependent increase of D2 mRNA by TSH, which has been reported in human thyroid cells and the presence of a cAMP response element in the D2 promoter (42).

The absence of D2 from cell types that express thyroid hormone receptors indicates that production and action of T_3 in man occurs in separate cell types. A similar neuroanatomical separation has been reported in both rat and human hypothalamus, where D2 and TR isoforms are differentially expressed as well (4, 5, 43–45). Both TR isoforms and D3 expression appear to be present in hormone-secreting cells of the anterior pituitary. The overlap between TR isoform and D3 expression suggests that thyroid hormone action and degradation may occur in the same hormone-secreting cells, while T_4 conversion to T_3 occurs in a subset of FS cells of the anterior pituitary and may be stimulated by TSH via TSH-R binding. This fits very well with the ultrashort feedback loop regulation of TSH, as proposed by Prummel *et al.* (40).

MCT8 did not show colocalization with anterior pituitary hormones. Double labeling with HLA in human anterior pituitary and immunostaining in the

Figure 4 Proposed model for thyroid hormone action on hormone-secreting cells of the anterior pituitary. T₄ is taken up by FS cells (FS cells) and is converted by type II deiodinase (D2) into T₃ in HLA-positive (+ve) or -negative (-ve) FS cells. TSH can stimulate D2 expression via binding to the TSH receptor (TSHR) in HLA-positive cells. T₃ is subsequently transported to hormone-secreting cells that coexpress thyroid hormone receptor isoforms (TRs). T₃ can bind to the TR and alter gene expression, or may be degraded locally by D3. The role of MCT8 is unclear at present. MCT8 may be involved in T₄ transport into, or in T₃ release from, HLA-negative cells.

MHC-class II-expressing FS cell line were absent. Therefore, the subtype of FS cells expressing MCT8 remains to be identified. The absence of distribution overlap between MCT8 and deiodinases or TRs suggests that yet another thyroid hormone transporter may be involved in providing hormone-secreting cells of the anterior pituitary with T₃. At present, the role of MCT8 in the FS cells is unknown. Human MCT8 transports T₄, although not as well as T₃ (Friesema *et al.*, unpublished observations). No data have been published on the role of MCT8 in thyroid hormone efflux. In view of the involvement of other members of the MCT family in substrate efflux (46–48), a similar role for MCT8 in FS cells is an interesting possibility, requiring further investigation. Patients with a mutation in the MCT8 gene do not show a dramatic increase in TSH expression (12), as would be expected if T₃ was unable to exert its negative feedback due to an inability to enter the cell. These data support our finding that MCT8 is not expressed in thyrotropes.

The present study is purely qualitative and does not allow for comparison of protein expression levels between patients. Our future studies will aim to quantify expression levels of proteins involved in thyroid hormone feedback action in the human anterior pituitary, defining their roles in situations with altered set-point regulation such as NTI.

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