Type II iodothyronine deiodinase protein in chicken choroid plexus: additional perspectives on T₃ supply in the avian brain

C H J Verhoelst, V M Darras, S A Roelens, G M Artykbaeva¹ and S Van der Geyten

Laboratory of Comparative Endocrinology, Zoological Institute, Naamsestraat 61, B-3000 Leuven, Belgium

¹Laboratory of Hormone Biochemistry, Institute of Biochemistry, Academy of Sciences, Uzbekistan

(Requests for offprints should be addressed to C H J Verhoelst; Email: Carla.Verhoelst@bio.kuleuven.ac.be)

Abstract

It is widely accepted that type II iodothyronine deiodinase (D2) is mostly present in the brain, where it maintains the homeostasis of thyroid hormone (TH) levels. Although intensive studies have been performed on activity and mRNA levels of the deiodinases, very little is known about their expression at the protein level due to the lack of specific antisera. The current study reports the production of a specific D2 polyclonal antiserum and its use in the comparison of D2 protein distribution with that of type I (D1) and type III (D3) deiodinase protein in the choroid plexus at the blood–brain barrier level. Immunocytochemistry showed very high D2 protein expression in the choroid plexus, especially in the epithelial cells, whereas the D1 and D3 proteins were absent. Furthermore, dexamethasone treatment led to an up-regulation of the

D2 protein in the choroid plexus. The expression of D2 protein in the choroid plexus led to a novel insight into the working mechanism of the uptake and transport of thyroid hormones along the blood–brain barrier in birds. It is hypothesized that D2 allows the prohormone thyroxine (T₄) to be converted into the active 3,5,3'-triiodothyronine (T₃). Within the choroidal epithelial cells, T₃ is subsequently bound to its carrier protein, transthyretin (TTR), to allow transport through the cerebrospinal fluid. Neurons can thus not only be provided with a sufficient T₃ level via the aid of the astrocytes, as was hypothesized previously based on *in situ* hybridization data, but also by means of T₄ deiodination by D2, directly at the blood–brain barrier level.

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Introduction

It is well established that thyroid hormones play a crucial role in vertebrate development in general and in brain development and maturation in particular. The impact of thyroid hormones (THS) on the development of the central nervous system is shown in situations of clinical or induced TH deficit or excess. Under these circumstances, processes of cell migration and formation of cortical layers are affected in particular, as well as neuronal and glial cell differentiation (Bernal *et al.* 2003). Thyroxine (T₄), the prohormone produced by the thyroid gland, is considered to be inactive and needs to be converted into the active 3,5,3'-triiodothyronine (T₃). This peripheral deiodination reaction demands the interference of the so-called iodothyronine deiodinases. Three different deiodinases (type I iodothyronine deiodinase (D1), type II iodothyronine deiodinase (D2) and type III iodothyronine deiodinase (D3)) have been cloned and characterized for different species, including mammals, birds, amphibians and fish (Bianco *et al.* 2002). The three deiodinases differ in the reaction they catalyse, their substrate preferences and their

sensitivity to certain inhibitors. D1 catalyzes deiodination of both the outer and the inner ring deiodination (ORD and IRD) of iodothyronines. D2 and D3 deiodinase are responsible for the maintenance of TH homeostasis in the brain by their respective ORD and IRD activity (Leonard & Visser 1986). Of all the deiodinases, D2 is the one most highly expressed in vertebrate brain. To date, very little is known about the cellular distribution of D2 due to the lack of specific antisera. Diano *et al.* (2003) described for the first time immunocytochemical evidence that the D2 protein is localized in brain cells, more specifically in astrocytes and tanycytes in the rat hypothalamus. Their results were in agreement with previously reported data on the mRNA distribution of rat D2. Guadaño-Ferraz *et al.* (1997) reported *in situ* hybridization studies for D2 in neonatal rat brain. They found excessive D2 mRNA expression in different brain areas, but more importantly, mainly, if not exclusively, in glial cells. It was hypothesized that these support cells would provide a sufficient level of active TH for utilization by the neurons. That way a close coupling would exist between the glial cells and the neurons. Tu *et al.* (1997) also reported the expression of

D2 mRNA in rat hypothalamic tanycytes. Guadaño-Ferraz *et al.* (1999) confirmed their previous statement by demonstrating that the most TH dependent regions would be protected in a hypothyroid status by an increase of D2 expression in these areas.

In the current study, a specific polyclonal antiserum against chicken D2 was produced and used to compare the D2 protein distribution with that of D1 and D3 proteins in the choroid plexus, a structure which is of extreme importance for TH uptake in the brain via the blood–brain barrier. The main function of the choroid plexus, apart from its protective aspect, is the production of the cerebrospinal fluid by a process that requires the movement of Na^+ , Cl^- and HCO_3^- ions from the blood towards the ventricles. By means of this movement, an osmotic gradient is created and H_2O can be secreted. The epithelial cells of the choroid plexus are equipped with a number of transporter proteins and ion channels in order to fulfil their responsibility in maintaining a homeostasis in the chemical environment of the brain via cerebrospinal fluid production. The monocarboxylate transporter 8 (MCT8) just recently identified by Friesema *et al.* (2003) to be a very specific TH transporter, is highly expressed in the choroid plexus of mammalian brain (Simpson 2004). The latter indicates that the transport of thyroid hormones in and eventually out of the brain, via the choroid plexus, is indeed of significant importance.

The staining of D2 protein in the choroid plexus was further examined in this study by comparing dexamethasone (DEX) treated and control chicken embryos. DEX is a synthetic glucocorticoid that, upon administration to chicken embryos, increases both brain D2 activity and mRNA levels (Van der Geyten *et al.* 2001). This experiment allowed further validation of the antiserum. Taken together, the results obtained from the present study gave rise to a novel hypothesis concerning deiodination and transport of thyroid hormones along the blood–brain barrier, namely at the level of the choroid plexus.

Materials and Methods

Polyclonal antisera production

Based on the full-length amino acid D2 sequence of the chicken, described by Gereben *et al.* (1999), and taking into account the hydropathy profile of this enzyme and possible cross-reactivity with other deiodinases, a specific peptide was chosen for immunization purposes. The following peptide was synthesized and coupled to keyhole limpet hemocyanin: NH_2 –(164)PSDGWAAPGISPPS (179)– COOH . New Zealand White rabbits were injected every 3 weeks and blood collection occurred within 7–10 days after immunization (final bleeding after 8 injections). Serum was separated, snap frozen using a mixture of dry ice and ethanol and stored at -80°C . The same approach was used for the production of the antisera

against chicken D1 and D3, as was described in detail by Verhoelst *et al.* (2002, 2003). Possible cross-reactivity with the other deiodinases was checked by means of an immunospotting test, in which the three different peptides used for the generation of the respective antisera, namely peptide D1, D3a and the D2 peptide, were spotted on a nitrocellulose membrane. Binding of these peptides to the D2 antiserum was tested using the preimmune D2 antiserum as negative control.

Experiments and tissue preparation

Chicken embryos (Ross, Avibel, Aarschot, Belgium) were injected on day 18 of incubation (E18) with either saline or $50\text{ }\mu\text{g}$ dexamethasone. Four hours after injection the animals were sacrificed and brain tissue was fixed in Bouin–hollande solution for 24 h (Sigma). Tissues were washed thereafter for 24 h using running tap water. Before paraffin embedding, the following incubation steps were performed: 50% EtOH (2 h), 70% EtOH (2 h), 95% EtOH (2 h), 100% EtOH (overnight), 1:1 EtOH:xylol (4 h), 100% xylol (4 h), 100% xylol (overnight), 1:1 xylol:paraffin (4 h), 100% paraffin (4 h), 100% paraffin (overnight). Paraffin slices of $7\text{ }\mu\text{m}$ thickness were cut using a historange CBK microtome. The slices were always dried for a minimum 72 h prior to use in immunocytochemical applications.

Immunocytochemical staining

The paraffin slices were hydrated using 100% xylol ($2\times 15\text{ min}$), 100% EtOH (3 min), 96% EtOH (3 min), 80% EtOH (3 min), 70% EtOH (3 min) and washed in distilled water for 10 secs. Blocking buffer (TBS–Triton X-100 + 5% low fat milk) was applied for 1 h to block the non-specific binding sites. Thereafter, the tissue slices were incubated with a 1:150 dilution of the primary D2 antiserum in TBS–Triton X-100 for 1 h at room temperature and overnight at 4°C . For the D2 antiserum, both preimmune and exhausted primary antiserum (exhaustion overnight, 4°C , $200\text{ }\mu\text{g}$ D2 peptide) were tested as negative controls. For the D1 (1:200) and D3 (1:200) antisera, preimmune serum served as a negative control, since exhaustion experiments were already described before (Verhoelst *et al.* 2002, 2003). The slices were washed twice for 10 min with TBS–Triton X-100. Next, they were incubated with goat–anti–rabbit alkaline phosphatase linked IgG (GAR–AP) (1:250) for 1 h at room temperature. Again, they were washed twice with TBS for 10 min and twice with AP detection buffer ($2\times 10\text{ min}$). Thereafter, the substrate (NBT/BCIP) was applied (45–60 min). The slices were washed in methanol and enclosed with chromoglycerine.

In situ hybridization

Chicken embryos of 18 days old were perfused with 4% PFA. Subsequently, brains were removed and submitted

to the following preparative manipulations: 4% PFA (24 h), PBS (24 h), PBS-10% sucrose (24 h). Finally the brains were put in PBS-10% sucrose solution containing 10% gelatine for embedding and preserved at -80°C . Cryostat sections of $30\text{ }\mu\text{m}$ were made. Thereafter, the sections were prepared in 4% PFA (10 min), PBS ($3\times 5\text{ min}$), 0.2N HCl (10 min), TEA buffer (10 min), PBS-1% Triton X-100 (30 min) and PBS ($3\times 5\text{ min}$). Subsequently, the slices were prehybridized for 2 h at room temperature in hybridization buffer (50% formamide, 10% dextran sulphate, 5% Denhardt's, 0.625 M NaCl, 0.2 M Na-PIPES pH 6.8 in 50 ml DEPC-AD) containing 0.05 M DTT, $250\text{ }\mu\text{g}/\mu\text{l}$ denatured herring sperm and $250\text{ }\mu\text{g}/\mu\text{l}$ yeast tRNA. Afterwards, the sections were hybridized under coverslips overnight at 80°C in the same hybridization buffer containing a 1:1000 dilution of a digoxigenin-labeled cRNA probe. The probes were generated by performing PCR using forward primer: 5'(1027)-GCT GGT GGA AGA GTT CTC TGG AGT-(1050)3' and reverse primer: 5' (1262)-GCA CAC TCG CTC AAA TGA AAC CCC-(1285)3'. The thus obtained 260 bp PCR fragment was subsequently subcloned in a PCR II-TOPO plasmid vector (Invitrogen). The sense probe was digested with SacI, while the antisense probe was cut using ApaI, allowing amplification of the cRNA probes using the T7 and SP6 transcription kit (Roche) respectively and DIG-labelled dNTP (Roche). After hybridization, the sections were washed for 45 min at 72°C in $0.2\times$ sodium saline citrate (SSC), $2\times 5\text{ min}$ $0.2\times$ SSC at room temperature and $3\times 5\text{ min}$ in PBS- 0.1% Triton X-100 at room temperature. Thereafter, the sections were incubated for 1 h with PBS-BSA-Triton X-100 containing 0.1 M lysine to reduce background signal. Per section, 0.5 ml anti-DIG antibodies (1:5000, in PBS-BSA-Triton X-100) were added and incubation continued overnight at 4°C . The alkaline phosphatase signal was detected using the NBT/BCIP chromogen system (Roche) according to the manufacturer's guidelines.

All products used were of the highest quality commercially available. The K U Leuven Ethical Committee for Animal Experiments approved all experimental procedures used.

Results

An alkaline phosphatase staining with anti-D2 carried out on brain slices of 18-day-old control chicken embryos revealed a very intense staining of the cuboidal epithelial cells in the choroid plexus. The central vascular core of fenestrated capillaries also displayed a very light positive signal (Fig. 1A). However, incubation of the subsequent slice with preimmune serum reveals that this colouring is of a non-specific nature (Fig. 1B). Exhaustion of the primary antiserum using the D2-specific peptide led to the

loss of this strong signal in the choroid plexus (Fig. 1C,D).

Comparison of the D2 protein distribution (Fig. 1F) in the choroid plexus with that of D1 (Fig. 1G) and D3 (Fig. 1H) deiodinase proteins demonstrated that only D2 protein is expressed in the choroidal epithelial cells. Slices stained with the D1 and D3 antisera remained completely blank.

Since, ontogenetically, the epithelium cells of the choroid plexus originate from the ependymal cells lining the ventricle walls, and since at E18 the chicken brain is still developing, the D2 protein staining was monitored there as well. It can be seen in Fig. 1J-K that the cells circumscribing the lining of the lateral ventricle and those around the third ventricle stained positively using the D2 antiserum in comparison with the preimmune serum (Fig. 1I). These cells are called ependymal cells and tend to be arranged in monolayers of cuboidal to columnar epithelium upon differentiation.

In the dexamethasone injection experiment, a dilution of 1:200 was used for the primary D2 antiserum. Comparing the results of the control animals versus the DEX treated animals shows that DEX treatment causes the D2 protein staining to increase. The cuboidal cells are immuno positive in both cases but the intensity of the AP staining is clearly higher in the DEX treated section (Fig. 1L,M).

In situ hybridization shows the distribution of D2 mRNA in elongated cell clusters throughout the brain. Furthermore, it confirms the presence of D2 mRNA in the epithelial cells of the choroid plexus. Comparison of the negative control slice hybridized with the sense probe (Fig. 2A) with the slice hybridized with the antisense probe (Fig. 2B) reveals a clear D2 mRNA expression.

Based on the above mentioned results, the hypothesis concerning the T_3 supply to the neurons primarily by aid of the astrocytes should be revised. Therefore, a novel scheme was drawn describing the TH transport mechanism in birds at the blood-brain barrier level in comparison with the previously reported mammalian scheme (Fig. 3).

Discussion

The present study describes, for the first time, the production and application of a polyclonal chicken D2 antiserum. D2 protein expression is found in the epithelial cells of the choroid plexus. Because the selected peptide for immunization is situated in the iduronidase-like domain of the D2 molecule, which is common for all deiodinases (Callebaut *et al.* 2003), several control experiments were done to exclude cross reaction with D1 and D3. Negative controls such as incubation with the pre-immune serum and exhaustion of the primary antiserum remained blank in the epithelial cell layer. Comparison of the preimmune incubated slice with the anti-D2 incubated slice revealed the non-specific nature of the

Figure 1 Alkaline phosphatase staining of chicken choroid plexus (E18 SAL) with (A) anti-D2 antiserum, (B) preimmune serum, (C) anti-D2 antiserum, (D) exhausted anti-D2 antiserum, (E) schematic overview of the choroid plexus epithelium. (F-H) Alkaline phosphatase staining of chicken choroid plexus with (F) anti-D2, (G) anti-D1, (H) anti-D3. (I-K) Alkaline phosphatase staining of chicken lateral and third ventricle with (I) preimmune serum, (J-K) anti-D2 antiserum. Arrowheads indicate ependymal cells. (L-M) Comparison of alkaline phosphatase staining of chicken choroid plexus using anti-D2 in (L) control chicken embryos and (M) DEX treated chicken embryos. EpC, epithelial cell; CSF, cerebrospinal fluid; LV, lateral ventricle; IIIrdV, third ventricle; EpdC, ependymal cell.

Figure 2 *In situ* hybridization of the chicken choroid plexus with the (A) sense probe and (B) antisense probe. Closed arrowhead indicates choroid plexus epithelial cells; open arrowhead indicates elongated cell clusters.

staining of the vascular core of the choroid plexus. *In situ* hybridization of the chicken choroid plexus using a D2 cRNA probe clearly showed the expression of D2 mRNA in the epithelial cells. Furthermore, D2 mRNA was demonstrated in elongated cell clusters throughout the brain, comparable with the results recently described by Gereben *et al.* (2004). Comparison of the D2 protein distribution with that of D1 and D3 protein within the choroid plexus using earlier developed antisera (Verhoelst *et al.* 2002, 2003), led to the conclusion that neither of the two other deiodinase proteins are present in the choroid plexus. The staining in the cuboidal cells therefore solely originates from the D2 protein. The results of the DEX experiment confirm the specificity of the D2 staining. The D2 protein expression in the DEX-treated animals shows

the same up-regulation as does the D2 activity, as previously described (Van der Geyten *et al.* 2001). Nevertheless, the authors would like to point out that the up-regulation of the D2 activity levels in the DEX-treated animals can not at all be explained by merely looking at the D2 protein expression in the choroid plexus. Other, much larger brain areas are most likely responsible for this overshoot of D2 activity in the brain after DEX treatment. Looking at the staining of the overall brain sections (data not shown) confirms this, but further experiments need to be done to further elucidate this issue.

However, the data resulting from the current study, namely the expression of the D2 protein in the choroid plexus, implicates the introduction of a new hypothesis concerning TH transport and communication pathways of

Figure 3 Scheme illustrating the proposed hypothesis of TH transport along the blood-brain-barrier in birds, compared with the existing scheme in mammals, described by Southwell *et al.* (1993) (Copyright 1993. The Endocrine Society).

different neuronal and non-neuronal cells in chicken brain. To better understand this hypothesis, it is of extreme importance to be familiar with the structure and function of the choroid plexus in general. The choroid plexus has a lobulated appearance and consists of a single epithelial cell layer that surrounds the central vascular core. The choroidal epithelial cells originate from the ependymal lining of the ventricles, while the blood vessels and connective tissue that represent the core originate from a vascular fold of the pia mater, namely the tela choroidea. The central core of the choroid plexus contains an unusually large number of capillaries, with very thin endothelial walls that do not possess a blood–brain barrier due to the fact that these capillaries are fenestrated. The epithelial cells of the choroid plexus on the other hand are designed to form a barrier between the blood and the brain. The ependymal cells are polarized, as are many other secretory epithelia, and consist of an apical (ventricle facing) and basolateral (blood facing) membrane (Speake *et al.* 2001). In mammals, it is described that T_4 enters the epithelial cells of the choroid plexus from the blood via the basolateral membrane. These epithelial cells produce the binding protein TTR, which is known to be a carrier protein for thyroid hormones in general. In mammals, TTR has a higher affinity for T_4 in comparison to T_3 (Schreiber *et al.* 2002). This is why T_4 binds to the TTR either in the choroidal epithelium or in the cerebrospinal fluid, which allows the transport of the lipophilic TH through the CSF. Thyroxine can reach the subarachnoid space and can flow towards the rest of the brain coupled to TTR (Fig. 3). It is described that T_4 will be deiodinated by D2 mainly in the astroglial cells (Guadaño-Ferraz *et al.* 1997). The production of the active T_3 is thus mainly guaranteed by these neuron-supporting cells. Triiodothyronine is then supplied to the neurons, which mainly express D3, for further deiodination and inactivation of the thyroid hormones (Guadaño-Ferraz *et al.* 1997). In chicken, it has been shown that D3 protein is expressed in the Purkinje cells of the cerebellum, again a proof of a neuronal D3 expression (Verhoelst *et al.* 2002). However, the present study reports the presence of D2 in a non-neuronal cell type, namely the epithelial cells of the choroid plexus. If the avian blood–brain barrier mechanism is similar to that of mammals, the high expression of the D2 protein in the choroidal epithelial cells seems rather surprising. It has been proven, however, that chicken TTR shows little affinity for T_4 , while it binds T_3 very well (Chang *et al.* 1999). This would explain why the D2 protein is indeed present in the choroid plexus. The D2 protein deiodinates T_4 that enters the choroid plexus via the basolateral membrane in order to form T_3 , which can subsequently bind to TTR to allow further transport through the cerebrospinal fluid (Fig. 3). This would suggest that astrocytes are not the sole source for active neuronal TH and that both the choroid plexus and the astroglial cells might be responsible for the T_3 flow towards

the neurons. Further support for this similar function of the astrocytes and the choroidal epithelial cells comes from their developmental origin. As mentioned before, the epithelial cells of the choroid plexus are generated by fusion of the ependymal cells lining the ventricles and the pia mater. The ependymal cells lining the ventricular cavities, however, do not solely give rise to the choroidal epithelial cells but partially differentiate to form glial cells, like astrocytes, as well. This common origin of both types of cells may explain their similar TH-activating task. Furthermore, the immunohistochemical stainings performed in this study showed the staining of the cells migrating from the lining of the lateral ventricle, indicating that D2 protein is already present in these cells. Therefore, the authors propose this new hypothesis concerning the transport of thyroid hormones in the cerebrospinal fluid in chicken. A schedule based on the earlier published mammalian blood–brain barrier mechanism is introduced. Whether the expression of D2 protein in the embryonic chicken choroid plexus is a temporary, and thus a transient development related expression, should be elucidated further.

In conclusion, the present study reports, for the first time, the expression of D2 in the cuboidal epithelial cells of the choroid plexus. No expression of other deiodinase proteins could be found at the level of this blood–brain barrier. Dexamethasone treatment led to an up-regulation of the D2 protein in the cuboidal cells of the choroid plexus. This is the first evidence of the D2 enzyme in this vertebrate blood–brain barrier structure so far. The presence of D2 protein in the choroid plexus, along with the expression of D2 enzyme along the lining of the ventricular walls, leads to the proposal of a novel mechanism concerning the transport of thyroid hormones along the blood–brain barrier in birds in comparison with the previously established working mechanism for mammalian TH transport.

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