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Research paper

The affinity of transthyretin for T₃ or T₄ does not determine which form of the hormone accumulates in the choroid plexusSamantha J. Richardson^{a,*}, Stijn Van Herck^b, Joke Delbaere^b, Bronwyn M. McAllan^c, Veerle M. Darras^b^a School of Health and Biomedical Sciences, RMIT University, Bundoora 3083, Victoria, Australia^b Laboratory of Comparative Endocrinology, Department of Biology, KU Leuven, B-3000 Leuven, Belgium^c Physiology and Bosch Institute, School of Medical Sciences, University of Sydney, 2006 New South Wales, Australia

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ABSTRACT

Normal development of the brain is dependent on the required amounts of thyroid hormones (THs) reaching specific regions of the brain during each stage of ontogeny. Many proteins are involved with regulation of TH bioavailability in the brain: the TH distributor protein transthyretin (TTR), TH transmembrane transporters (e.g. MCT8, MCT10, LAT1, OATP1C1) and deiodinases (D1, D2 and D3) which either activate or inactivate THs. Previous studies revealed that in mammals, T₄, but not T₃, accumulated in the choroid plexus and then entered the cerebrospinal fluid. In all mammalian species studied so far, TTR binds T₄ with higher affinity than T₃, whereas TTR in non-mammalian vertebrates binds T₃ with higher affinity than T₄. We investigated if the form of TH preferentially bound by TTR influenced the form of the TH that accumulated in the choroid plexus and consequently other areas of the brain. We measured the mRNA levels corresponding to TTR, MCT8, MCT10, LAT1, OATP1C1, D1, D2 and D3 in the brains of chickens at 11 days post-hatching. TTR, D3 and OATP1C1 expression were found to be highly concentrated in the choroid plexus. D1, MCT8 and MCT10 mRNA levels were slightly greater in the choroid plexus than in other areas of the brain while D2 mRNA levels were lower. LAT1 mRNA was evenly expressed throughout the brain. Therefore, the choroid plexus appears to be a structure which exhibits sophisticated control of TH levels within the brain. We also measured the uptake of intravenously injected ¹²⁵I-T₃ and ¹²⁵I-T₄ into brains of chickens of the same age. ¹²⁵I-T₄ but not ¹²⁵I-T₃ accumulated in the choroid plexus and optic lobes. Therefore, the form of TH preferentially bound by TTR does not determine the form of TH that accumulates in the choroid plexus and other areas of the brain. As for mammals, T₃ present in the avian brain therefore seems mainly produced locally by conversion of T₄ into T₃ by D2.

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1. Introduction

Thyroid hormones (THs) are signal compounds that regulate transcription of specific genes, as well as having some non-genomic effects. Many of the genes regulated by THs are involved in growth and development of vertebrates, and the most spectacular example of this is amphibian metamorphosis. However, as THs are lipophilic and partition between the aqueous and lipid phases with a ratio of 1:20,000 (Dickson et al., 1987), specific thyroid hormone distributor proteins (THDPs) are required in the blood and cerebrospinal fluid (CSF) to ensure appropriate distribution of the

hormones from the site of synthesis (the thyroid gland) to the sites of action: cells throughout the body and brain (Mendel et al., 1987). In humans and many other mammals, the THDPs circulating in the blood are albumin, transthyretin (TTR) and thyroxine-binding globulin (TBG), whereas in birds the THDPs are albumin and TTR (Richardson et al., 1994) (for a review of the distribution of THDPs in various groups of vertebrates, see (Richardson, 2007)). Albumin has the lowest affinity and greatest capacity for binding THs, whereas TBG has highest affinity and lowest capacity for binding THs. TTR has intermediate affinity and capacity for TH binding, but the largest distribution volume, and is responsible for release of most THs in peripheral capillaries (see (Richardson, 2007)). There are two main forms of THs: 5',3',5,3-tetraiodo-L-thyronine (thyroxine, T₄) and 3',5,3-triiodo-L-thyronine (T₃). T₄ is the predominant form found in blood and T₃ is the form with higher affinity for the TH nuclear receptors. In mammals, TTR has a higher

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affinity for T_4 than for T_3 (Chang et al., 1999). However, among vertebrates, this situation appears to be the exception, as in fish (Santos and Power, 1999), amphibians (Yamauchi et al., 1993), reptiles and birds (Chang et al., 1999), TTR has a higher affinity for T_3 than it does for T_4 .

Precise amounts of THs are required in very specific temporal and spatial patterns for normal brain development to occur. Both deficiencies and excessive amounts of TH during development of the central nervous system (CNS) results in malformations and disease states (Braverman and Utiger, 2000a,b). It is critical that the required amounts of THs enter the brain at the requisite times during development. Furthermore, the correct amounts of THs are required by specific areas of the mature brain to maintain normal cerebral functioning during adulthood (Braverman and Utiger, 2000a,b). Thus, once the circulating THs have been carried to the brain barriers by the THDPs, they must cross the blood-brain barriers and the blood-CSF barrier to reach the brain and the CSF respectively.

The blood-CSF barrier is formed by the choroid plexi, which are located in the third, fourth and lateral ventricles of the brain, and produce most of the CSF. The protein concentration and composition of the CSF vary drastically from those of blood (Davson et al., 1987) because the blood-CSF barrier (i.e. the choroid plexus epithelial cell tight junctions) physically separates the blood from the CSF, and the epithelial cells of the choroid plexus synthesise and secrete proteins into the CSF (Cserr, 1971; Davson et al., 1987). Importantly, specialised transporters in the blood-brain barrier (BBB) selectively allow components from the blood into the brain (Saunders et al., 2013).

The main protein synthesised and secreted by the choroid plexus of mammals, birds and reptiles is TTR (Harms et al., 1991), whereas the main protein synthesised and secreted by the choroid plexus of amphibians is a lipocalin, prostaglandin D synthetase, also known as Cpl1 and β -trace (Achen et al., 1992; Beuckmann et al., 2000; Lepperdinger, 2000). In mammals, TTR synthesis by the choroid plexus and secretion into the CSF is involved with the transport of T_4 , but not T_3 , from the blood into the CSF (Chanoine et al., 1992; Dickson et al., 1987; Schreiber et al., 1990; Soprano et al., 1985; Southwell et al., 1993; Stauder et al., 1986). In rats, ^{125}I - T_4 that had been injected into the blood rapidly accumulated firstly into the choroid plexus then subsequently moved to striatum, cortex then cerebellum (Dickson et al., 1987). Studies of TTR null mice showed there was no accumulation of ^{125}I - T_4 into the choroid plexus and the choroid plexus had only 14% T_4 content when compared to wild type mice (Palha et al., 2000). Moreover, T_4 delivery to the subventricular zone of the brain is reduced in TTR null mice (Richardson et al., 2007). Thus, TTR synthesised by the choroid plexus is likely involved in the delivery of T_4 to the CSF and the brain. Humans can lack either of the other TH distributor proteins, albumin (see (Peters, 1992) or TBG (Bennhold, 1954) without an overtly different phenotype, but no description of a human lacking TTR is known. Thus, it was concluded that as TTR is the only THDP also synthesised in the brain (i.e. in the choroid plexus), it may be essential for human life in its role in the movement of T_4 from the blood into the CSF (Harms et al., 1991).

At least three classes of solute carrier transporters actively participate in the influx and efflux of THs into and out of cells: monocarboxylate transporters (MCTs) such as MCT8 and MCT10, organic anion transporting polypeptides (OATPs) such as OATP1C1 and I-type amino acid transporters (LATs) such as LAT1 and LAT2 (Visser et al., 2011). Representatives of these TH transmembrane transporters are found in both the blood-brain barrier and in the blood-CSF barrier (e.g. (Mayerl et al., 2012; Van Herck et al., 2015) and the distribution of specific transmembrane transporters differs between species (Darras et al., 2015; Wirth et al., 2014). For

example, LAT2 is found in the choroid plexus of mice but not in the choroid plexus of humans (Wirth et al., 2009) and appears to be completely absent in chickens (Darras et al., 2015).

Once inside the cell, THs can be activated or inactivated by a family of deiodinase enzymes (D1, D2 and D3). These activities include activation of T_4 to T_3 (which has higher affinity for the nuclear receptors) and inactivation of T_4 to rT_3 and of T_3 to T_2 (for review see (Darras et al., 2015)). Thus, the combination of THDPs in blood, TTR synthesized by the choroid plexus, THTTs in cell membranes, and intracellular deiodinases all contribute to determining the absolute amounts of T_4 and T_3 and ratios of T_4 to T_3 within specific regions of the brain. To the best of our knowledge, the proportional contribution of THs entering the brain via choroid plexus-derived TTR compared with entry via THTTs at the choroid plexus and at the blood-brain barrier is unknown.

In order to investigate whether the preference of ligand affinity (for T_4 or T_3) of TTR influences the form of TH that accumulates in the choroid plexus and then subsequently moves into other areas of the brain, we analysed the uptake of each of the two main forms of THs (T_4 and T_3) into the choroid plexus and various brain regions. Initially, we confirmed data generated by others in rats (Dickson et al., 1987) in a second mammal: mice. Then we performed a similar experiment in 1.5 week old chickens. We chose birds, as TTR from birds has higher affinity for T_3 than for T_4 (Chang et al., 1999), whereas TTRs from mammals has higher affinity for T_4 , and because Van Herck et al. (2015) hypothesised that chicken brains could have enhanced T_3 uptake compared to that seen for mammalian brains. The ontogenetic profiles of TTR, THTTs and deiodinases mRNAs in the choroid plexus and blood-brain barrier of chickens from embryonic until early post-natal developmental stages are known, as are the T_3 and T_4 content of various brain regions (Van Herck et al., 2015). However, the factors that could be involved in the regulation of TH availability and accumulation in the choroid plexus are not known. Thus, we analysed the mRNA expression pattern of TTR, the known TH transmembrane transporters (MCT8, MCT10, LAT1 and OATP1C1) and the deiodinases (D1, D2 and D3) in the choroid plexus and compared this with expression in the rest of the brain (telencephalon, diencephalon, mesencephalon, cerebellum and brain stem). We also tracked the uptake of ^{125}I - T_3 and ^{125}I - T_4 into the choroid plexus and other brain regions.

2. Materials and methods

2.1. Animals

2.1.1. Animals used for TH uptake assays

Adult BalbC mice weighing 23–30 g, adult Wistar rats weighing 325–366 g and Ross (broiler) chickens (from a commercial hatchery: Belgabroed, Merksplas Belgium) between 7 and 10 days post-hatch weighing 160–250 g were used. All animals were kept at room temperature in species appropriate cages and nesting materials with *ad libitum* access to food and water.

2.1.2. Animals used for qPCR analyses and TH extractions

Chicken eggs were obtained from a commercial hatchery (Belgabroed, Merksplas Belgium) and for each chick, the day of hatching was recorded. Chickens were euthanized on post-hatch day 11 (C11) as per KU Leuven ethics protocols. Blood samples were collected by carotid artery bleeding after sedation. After centrifugation, plasma was stored at -20°C until analysis. The choroid plexus was collected from the lateral ventricles in the telencephalon and the remaining brain was divided into 5 parts: telencephalon (forebrain), diencephalon, optic lobes and rhombencephalon which was then subdivided into cerebellum

and brain stem regions. All tissue samples were snap frozen in liquid nitrogen and stored at -80°C until analysis.

The Ethical Committee for animal experiments of the KU Leuven approved experimental protocols: P130/2012.

2.2. Materials

Bovine serum albumin was sourced from Applichem. Singly substituted ^{125}I -T₃ ($1200\ \mu\text{Ci}/\mu\text{g}$) and ^{125}I -T₄ ($1250\ \mu\text{Ci}/\mu\text{g}$) and Na ^{125}I iodide were purchased from Perkin Elmer.

2.3. Methods

2.3.1. Quantitation of TTR, deiodinases and TH transmembrane transporters in the choroid plexus and other regions of chicken brains by reverse transcription quantitative PCR

RNA isolation, cDNA production and qPCR procedures were performed as previously described (Van Herck et al., 2012). Primer pairs were as described previously (Van Herck et al., 2015). Data were normalised using β -actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and cyclophilin A as reference genes.

2.3.2. Measurement of total T₄ and T₃ levels in plasma, choroid plexus and other brain regions of chickens

Total T₄ and T₃ levels were measured in plasma and in brain tissue extracts as described before (Reyns et al., 2002; Van Herck et al., 2015). For choroid plexus, pools were used from 2 animals while all other samples were from individual animals ($n=6$). Results are expressed in pmol/ml for plasma, pmol/g tissue for all brain regions except choroid plexus. Due to its extremely low weight, accurate weighing was difficult and therefore levels were calculated as pmol per single choroid plexus.

2.3.3. Uptake of ^{125}I -T₄ and ^{125}I -T₃ into tissues

Adult mice ($n=6$) and rats ($n=2$) were anaesthetised with Nembutal prior to injection of approx. 5×10^6 cpm ^{125}I -T₄ diluted to $200\ \mu\text{l}$ with PBS in the jugular vein. After 10 min or 2 h, animals were killed and tissues were dissected. Brains were divided into choroid plexus, cerebellum, cortex and “rest of brain”. Visceral tissues collected were blood, liver and kidneys.

Chickens (between seven and ten days post-hatch) were weighed and anaesthetised with an intramuscular injection of Zoletil 100 at $30\ \text{mg/kg}$ body weight into a leg. A brachial artery was cannulated as described previously (Buyse et al., 1994), and animals were then placed under heating lamps. Approximately 10^6 cpm of either ^{125}I -T₄ or ^{125}I -T₃ diluted in PBS to $200\ \mu\text{l}$ was injected via the cannula of anaesthetised chickens. Intramuscular Zoletil 100 injections were used when required to keep the animals under anaesthesia. After 0.5, 1, 2, or 4 h, chickens ($n=6$ per time point for each hormone) were killed and tissues were dissected. Brains were divided into choroid plexus,

telencephalon, diencephalon, optic lobes and cerebellum. Visceral tissues dissected were blood, liver and kidneys. The amount of radioactivity (cpm) in each tissue sample was measured in a gamma counter (Wallac Wizzard, Perkin Elmer). After normalisation for tissue blood content, cpm data were converted to fmol hormone/g tissue.

2.3.4. Determination of blood content of tissues

Ten microgram of albumin was labelled with Na ^{125}I iodide in PBS using Iodogen. Approx. 10×10^6 cpm ^{125}I -albumin was injected into a brachial artery of 6 chickens. After 10 min, animals were killed and tissues (choroid plexus, telencephalon, diencephalon, optic lobes, cerebellum, liver, kidneys and $3 \times 100\ \mu\text{l}$ aliquots of whole blood) were dissected for determination of tissue blood content.

2.3.5. Determination of half-lives of ^{125}I -T₄ and ^{125}I -T₃ in chickens

Approximately 5×10^6 cpm ^{125}I -T₄ or ^{125}I -T₃ was injected into the cannulated brachial artery (see above) of 13 day post-hatch chickens ($n=3$ for each hormone). At 5, 10, 15, 20, 40, 60, 120 and 240 min after injection $3 \times 100\ \mu\text{l}$ aliquots of whole blood were collected via the cannula and analysed for radioactivity.

2.3.6. Statistical analyses

Data were analysed by one or two-way ANOVA, and if significant, the analysis was followed by Tukey's pairwise post hoc tests (Zar, 2010). Because of the large variance in the results at some time points non-parametric analyses were performed. Analysis was done using MINITAB software.

3. Results

3.1. Quantitation of TTR, TH transmembrane transporter and deiodinase expression in the choroid plexus and other regions of chicken brains

In the chicken, TTR, OATP1C1 and D3 mRNAs were expressed approximately 900, 300 and 50-fold higher in the choroid plexus compared to other brain regions (Table 1). D1, MCT8 and MCT10 mRNAs showed slightly but significantly higher expression in the choroid plexus than in other brain regions (Table 1). In contrast, D2 mRNA expression was lower in the choroid plexus compared to the rest of the brain (Table 1). LAT1 mRNA levels were more similar across all regions (Table 1).

These data clearly show that many genes that code for proteins involved with regulation of THs (distribution, cellular influx/efflux, activation/inactivation) show higher levels of expression in the choroid plexus, compared to other brain regions of C11 chickens. Next, we wanted to measure the functional implications of these

Table 1

qPCR results for TH distributor protein TTR, TH transporters OATP1C1, LAT1, MCT8 and MCT10 and deiodinases D1, D2 and D3. Data are means $\pm$ SEM ($n=6$). Asterisks indicate choroid plexus values significantly greater than found for all other brain regions at $P < 0.0001$ while ‡ indicates choroid plexus values significantly less than those of other brain regions at $P < 0.0001$. Other values are not significantly different from one another. Expression levels were normalised using mRNAs for GAPDH, β -actin and cyclophilin A as reference genes. The data from choroid plexus have been published before in an ontogenetic study shown as relative values compared to embryonic data (Van Herck et al., 2015). Original data have been included here for comparison with expression levels in all other brain regions derived from the same animals.

Tissue	Distributor protein	Thyroid hormone transmembrane transporter proteins				Deiodinases		
	Transthyretin	MCT10	MCT8	OATP1C1	LAT1	D1	D2	D3
Choroid plexus	932.8 $\pm$ 81.8*	1.68 $\pm$ 0.16*	3.18 $\pm$ 0.24*	323.2 $\pm$ 13.5*	1.23 $\pm$ 0.17	4.05 $\pm$ 0.87*	0.45 $\pm$ 0.09‡	53.5 $\pm$ 13.4*
Telencephalon	1.08 $\pm$ 0.58	0.83 $\pm$ 0.10	0.96 $\pm$ 0.03	0.50 $\pm$ 0.19	1.14 $\pm$ 0.11	1.03 $\pm$ 0.16	1.22 $\pm$ 0.03	0.48 $\pm$ 0.03
Diencephalon	2.55 $\pm$ 1.63	0.88 $\pm$ 0.08	0.69 $\pm$ 0.04	1.12 $\pm$ 0.55	0.54 $\pm$ 0.12	0.76 $\pm$ 0.09	1.20 $\pm$ 0.07	0.65 $\pm$ 0.18
Optic lobes	0.03 $\pm$ 0.01	0.99 $\pm$ 0.07	0.95 $\pm$ 0.18	0.12 $\pm$ 0.01	1.06 $\pm$ 0.13	0.86 $\pm$ 0.15	1.41 $\pm$ 0.16	0.52 $\pm$ 0.07
Cerebellum	0.33 $\pm$ 0.15	0.98 $\pm$ 0.09	0.68 $\pm$ 0.14	0.20 $\pm$ 0.05	0.97 $\pm$ 0.10	0.83 $\pm$ 0.17	1.07 $\pm$ 0.11	0.46 $\pm$ 0.09
Brain stem	2.79 $\pm$ 1.18	0.96 $\pm$ 0.09	1.55 $\pm$ 0.13	1.68 $\pm$ 0.29	0.96 $\pm$ 0.08	0.64 $\pm$ 0.08	1.30 $\pm$ 0.10	1.68 $\pm$ 0.29

Fig. 1. Uptake of $^{125}\text{I-T}_4$ into mouse tissues (as a percentage of T_4 in the blood 10 min after injection). Confirming data from Dickson et al., 1987 that $^{125}\text{I-T}_4$ specifically accumulates in the choroid plexus of rodents. Data are means $\pm$ SEM ($n = 3$).

proteins, by measuring the uptake of T_4 and T_3 into the brains of chickens and to compare this data with that previously published for rats (Dickson et al., 1987).

3.2. Uptake of T_4 and T_3 into brains of rats and mice

To ensure integrity of our techniques and that we could reproduce the data of Dickson et al. (Dickson et al., 1987), we initially measured $^{125}\text{I-T}_4$ and $^{125}\text{I-T}_3$ uptake into choroid plexus, cerebellum, cortex, “rest of brain”, blood, liver, kidneys and lungs of rats. Similar results were obtained (data not shown). We then repeated TH uptake experiments using mice and demonstrated that, in a second mammal where TTR has higher affinity for T_4 than T_3 , $^{125}\text{I-T}_4$ accumulated in the choroid plexus whereas $^{125}\text{I-T}_3$ did not (Fig. 1).

3.3. Uptake of T_4 and T_3 into various tissues in the body and brain of chickens

To investigate the uptake of $^{125}\text{I-T}_4$ and $^{125}\text{I-T}_3$ into chicken tissues, 0.5, 1, 2 and 4 h after injection, animals were killed and choroid plexus, telencephalon, diencephalon, optic lobes (left + right), cerebellum, blood, liver and kidneys (left + right) were analysed for $^{125}\text{I-T}_4$ or $^{125}\text{I-T}_3$ content, and subsequently normalised for blood content of tissues. In a separate set of experiments, the mean half-life of $^{125}\text{I-T}_4$ in the circulating blood was estimated as 2.48 h and the mean half-life of $^{125}\text{I-T}_3$ was estimated as 2.84 h.

Fig. 2. Uptake of $^{125}\text{I-T}_4$ and $^{125}\text{I-T}_3$ in chicken brain regions and control peripheral tissues (liver and kidney) for up to 4 h after injection. $^{125}\text{I-T}_4$ accumulates strongly in the choroid plexus. Note the different y-axis scales in the different figures (5–250 fmol/g tissue). Data are means $\pm$ SEM ($n = 6$).

3.3.1. Chicken choroid plexus and optic lobes accumulate more T_4 than T_3

Two-way ANOVA showed a significant difference between the total ^{125}I - T_4 and ^{125}I - T_3 content of the choroid plexus (Fig. 2A, $P = 0.027$), and there was not a significant difference across the time course of the experiment for choroid plexus tissue, nor was there a significant interaction between hormone and time. Note that the large variance at 2 h for T_4 obliterated the interaction between time and hormones, as T_4 had returned to lower amounts by 4 h while T_3 was rising at 4 h (interaction $P = 0.094$).

There was significantly more ^{125}I - T_4 than ^{125}I - T_3 present in the optic lobes (Fig. 2B, $P < 0.0001$), at all time points ($P = 0.018$) and there was a significant interaction between hormone and time ($P = 0.018$). The optic lobes contained significantly more thyroid hormones at 2 h than at 0.5 h (T -value = 2.840, $P = 0.0334$), and at 1 h the values were higher than at 0.5 h, but not significantly so (T -value = 2.238, $P = 0.1026$). Visual inspection of the graph suggests that ^{125}I - T_4 peaked at 2 h then declined, while ^{125}I - T_3 continued to rise to 4 h. Some of the statistical interpretation of the data was affected by the large variance.

3.3.2. No significant differences in accumulation of T_4 and T_3 in the telencephalon, diencephalon, cerebellum, liver or kidney of chickens

There was no significant difference in content of ^{125}I - T_4 and ^{125}I - T_3 in the telencephalon (Fig. 2C, $P = 0.899$), diencephalon (Fig. 2D, $P = 0.921$), cerebellum (Fig. 2E, $P = 0.454$), liver (Fig. 2F, $P = 0.538$) or kidney (Fig. 2G, $P = 0.137$). Nor was there a significant difference across the time course of the experiment for telencephalon tissue ($P = 0.316$), diencephalon ($P = 0.321$), cerebellar tissue ($P = 0.384$), for liver tissue ($P = 0.100$), nor kidney ($P = 0.260$). Non-parametric analyses of the non-normalised data did not alter the statistical outcome.

3.4. Total T_4 and T_3 levels in plasma, choroid plexus and other brain regions of chickens

Detailed results of total T_4 and T_3 levels are provided as supplementary data (Table S1). One way ANOVA between different brain regions showed some significant differences for T_4 ($F = 15.05$, $P = 0.002$) but no significant differences for T_3 ($F = 2.325$, $P = 0.169$) (analysis excluding choroid plexus and plasma). In order to compare with the uptake pattern of labelled THs, we calculated the T_4/T_3 ratios (Fig. 3). One way ANOVA ($F = 13.63$, $P < 0.000001$) followed by Tukey's pairwise post hoc test showed that the T_4/T_3

ratio was significantly higher in choroid plexus than in any other brain area or in plasma.

4. Discussion

The development of the brain in all vertebrates is partly controlled by THs. Previous studies have shown that in rodents, T_4 is the main form of TH that enters the CNS via the choroid plexus (Dickson et al., 1987). Moreover, TTR is the main protein synthesised and secreted by the choroid plexus (Harms et al., 1991) and this choroid-plexus derived TTR is secreted into the CSF and not into the blood (Schreiber et al., 1990) and is implicated in the movement of T_4 into the CSF (Chanoine et al., 1992; Dickson et al., 1987; Southwell et al., 1993). At the time these experiments were carried out, the TH transmembrane transporters had not yet been discovered. Later, it was shown that mammalian TTRs are the exception amongst vertebrate TTRs, as TTRs from all other vertebrates have higher affinity for T_3 than T_4 (Chang et al., 1999; Santos and Power, 1999; Yamauchi et al., 1993). We wanted to know whether the form of TH preferentially bound by TTR influenced the form of TH that entered the CSF via the choroid plexus. To answer this question, we used chickens, as TTR in birds has higher affinity for T_3 than for T_4 .

We took a twofold approach to investigating this question: (i) we analysed the mRNA levels of the main regulators of THs in various regions of the chicken brain as well as total T_4 and T_3 levels; and (ii) we performed functional assays measuring the uptake of ^{125}I - T_4 and ^{125}I - T_3 into specific regions of chicken brains.

The choroid plexus appeared to have significantly differing patterns of gene expression of TTR, deiodinases and THTTs than other regions of the brain, which had more consistent expression of these genes. The T_4/T_3 ratio was also considerably higher in the choroid plexus than in other regions of the brain or in plasma. This implies that the choroid plexus has a different function in TH regulation than do the other areas of the brain. Being one of the main entry points for peripherally sourced THs, it is plausible that control of TH regulation is more sophisticated in the choroid plexus than in other areas of the brain. The combination of high levels of OATP1C1 (specific T_4 transporter) and low levels of D2 (T_3 producing enzyme) could be working in concert to ensure high levels of T_4 in the chicken choroid plexus, however high levels of D3 (T_3 and T_4 inactivating enzyme) could counteract this outcome. It appears that the combination of high levels of OATP1C1 and D3 in combination with low levels of D2 could be acting in concert to finely control the levels of T_4 and T_3 in the choroid plexus.

We replicated the experiments of Dickson et al., 1987 in rats and in mice, where ^{125}I - T_4 (but not ^{125}I - T_3) accumulated more strongly in the choroid plexus than in other areas of the brain (cortex, cerebellum or "rest of brain") or in the lungs, kidneys or liver. We then performed similar experiments in chickens, where, despite chicken TTR having greater affinity for T_3 than for T_4 , ^{125}I - T_4 accumulated strongly in the choroid plexus (and to a lesser extent in the optic lobes) whereas ^{125}I - T_3 did not specifically accumulate in any tissue. Therefore, it is more likely that the specific combinations of THTTs result in the accumulation of T_4 in the choroid plexus, rather than the affinity TTR has for T_4 .

THs may enter the brain either via the choroid plexus (blood-CSF barrier) or via cerebral endothelial cells (BBB) (Dratman et al., 1991). Studies using EMD21388/F21388 (inhibitor of T_4 binding to TTR) implicated a role for TTR in the movement of T_4 across the choroid plexus (Chanoine et al., 1992; Southwell et al., 1993). Studies of TTR null mice revealed that TTR null mice have only 35% TH content in total brains compared with wild type mice (Palha et al., 1997) and only 14% of the TH content in their choroid plexus compared with that in wild type mice (Palha et al., 2000).

Fig. 3. T_4/T_3 ratio in chicken plasma, choroid plexus and other brain regions. The ratio is significantly higher in choroid plexus compared to all other ratios. Data are means ± SEM ($n = 6$ except for choroid plexus where $n = 3$).

These studies indicate that TTR does have a role in the movement of T_4 from the blood via the choroid plexus into the CSF. Furthermore, the role of TTR synthesised by the liver and secreted into the blood most likely has an impact on the bound/free ratios of THs in the blood, which would influence the uptake of THs via THTTs into cells, including the endothelial cells of all brain capillaries.

From analyses of knock out mice, it is apparent that as soon as one of the TH regulators e.g. TTR (Monk et al., 2013; Richardson et al., 2007), MCT8 (Dumitrescu et al., 2006), OATP1C1 (Mayerl et al., 2012) or MCT8+OATP1C1 (Mayerl et al., 2014) is absent, the content of T_4 , T_3 or both is drastically disturbed. While TTR null mice do not have TTR-mediated T_4 transport across the choroid plexus, surprisingly they were apparently viable and fertile and without an overtly different phenotype when compared to wild-type mice (Episkopou et al., 1993). However, TTR null mice have reduced TH delivery to the neural stem cells in the subventricular zone of the brain (Richardson et al., 2007) and delayed development of multiple TH-regulated processes, including several aspects of brain development (Richardson et al., 2007). While the present study on C11 chickens could not confirm the differences in T_3 levels between different brain regions found previously (Van Herck et al., 2015), we again found that T_4 levels were significantly greater in the brain stem (which includes the subventricular organs) than in other parts of the brain. The C11 chicken is still undergoing neurogenic events relating to behaviour and motor development, and thus the concentration of THs in these regions may well be important for these developing attributes in the chicken. The data also support those seen for TTR null mice, that is, that THs and intact transport mechanisms are essential for continuing development in post-natal/post-hatch brain tissues.

When comparing the data on ^{125}I - T_4 and ^{125}I - T_3 uptake with those of total T_4 and T_3 levels in chickens from the same strain and age, we detected some similarities but also some interesting differences. The rapid and strong accumulation of ^{125}I - T_4 in choroid plexus could be a major factor to explain the high T_4/T_3 ratio in this structure. The much lower and rather similar uptake of ^{125}I - T_4 and ^{125}I - T_3 in telencephalon, diencephalon and cerebellum could fit with the T_4/T_3 ratios around 1 to 2 in these areas. In contrast, the preferential accumulation of ^{125}I - T_4 in the optic lobes is not accompanied by a higher T_4/T_3 ratio since both TH levels and T_4/T_3 ratio are similar to those found in other brain regions. At this point we cannot explain this apparent discrepancy but we should take into account that the tracer injections and the extraction analyses provide different information. Both radiolabelled and endogenous THs enter the brain similarly via the blood CSF barrier and the BBB but while total TH levels following extraction represent a kind of steady state between TH entry, metabolism and efflux, the tracer studies provide a short term and dynamic image of hormone uptake, irrespective of local metabolism via deiodination as well as other pathways. As birds in general highly depend on visual detection, for instance for finding food, it is possible that TH uptake dynamics and metabolism in the optic lobes of post-hatch chickens is different from that in most other regions, although this remains to be investigated.

In summary, whilst choroid plexus derived TTR contributes to T_4 movement from the blood into the CSF, the affinity of TTR for T_3 or T_4 does not determine which form of the hormone accumulates in the choroid plexus. It appears that T_4 is the main form of TH that enters the brain, where it is deiodinated to T_3 in target cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ygcen.2017.09.012>.

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