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Expression of thyroid hormone transporters and deiodinases at the brain barriers in the embryonic chicken: Insights into the regulation of thyroid hormone availability during neurodevelopment

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ABSTRACT

Thyroid hormones (THs) are key regulators in the development of the vertebrate brain. Therefore, TH access to the developing brain needs to be strictly regulated. The brain barriers separate the central nervous system from the rest of the body and impose specific transport mechanisms on the exchange of molecules between the general circulation and the nervous system. As such they form ideal structures for regulating TH exchange between the blood and the brain. To investigate the mechanism by which the developing brain regulates TH availability, we investigated the ontogenetic expression profiles of TH transporters, deiodinases and the TH distributor protein transthyretin (TTR) at the brain barriers during embryonic and early postnatal development using the chicken as a model. *In situ* hybridisation revealed expression of the TH transporters monocarboxylate transporter 8 (*MCT8*) and 10 (*MCT10*), organic anion transporting polypeptide 1C1 (*OATP1C1*) and L-type amino acid transporter 1 (*LAT1*) and the inactivating type 3 deiodinase (*D3*) in the choroid plexus which forms the blood–cerebrospinal fluid barrier. This was confirmed by quantitative PCR which additionally indicated strongly increasing expression of *TTR* as well as detectable expression of the activating type 2 deiodinase (*D2*) and the (in)activating type 1 deiodinase (*D1*). In the brain capillaries forming the blood–brain barrier *in situ* hybridisation showed exclusive expression of *LAT1* and *D2*. The combined presence of LAT1 and D2 in brain capillaries suggests that the blood–brain barrier forms the main route for receptor-active T_3 uptake into the embryonic chicken brain. Expression of multiple transporters, deiodinases and TTR in the choroid plexus indicates that the blood–cerebrospinal fluid barrier is also important in regulating early TH availability. The impact of these barrier systems can be deduced from the clear difference in T_3 and T_4 levels as well as the T_3/T_4 ratio between the developing brain and the general circulation. We conclude that the tight regulation of TH exchange at the brain barriers from early embryonic stages is one of the factors needed to allow the brain to develop within a relative microenvironment.

1. Introduction

Thyroid hormones (THs) play an important role in the functioning and development of many tissues and organ systems. This is especially true for the brain where TH deficiency is linked to several neurodevelopmental impairments as well as depression and memory and concentration problems in adults (Bernal et al., 2003; Feldman et al., 2013). During neurodevelopment THs are key regulators in cell proliferation, differentiation and migration, neurite outgrowth, synaptogenesis and myelination (Williams, 2008). Control of TH access to the brain is therefore a prerequisite for normal brain development and functioning.

THs mainly act through binding of 3,5,3′-triiodothyronine (T_3) to TH receptors which control gene expression in the nucleus (Harvey and Williams, 2002). TH action in the brain thus depends on the intracellular availability of T_3 and its receptors. In the

Abbreviations: CSF, cerebrospinal fluid; D1, D2, D3, iodothyronine deiodinase type 1, 2, and 3; LAT1, LAT2, L-type amino acid transporter 1 and 2; MCT8, MCT10, monocarboxylate transporter 8 and 10; OATP1C1, organic anion transporting polypeptide 1C1; PTU, 6n-propylthiouracil; TH, thyroid hormone; TTR, transthyretin.

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circulation THs are bound to TH distributor proteins such as transthyretin (TTR). These TH distributor proteins prevent the lipophilic THs from partitioning into the cell membranes and could be involved in TH uptake into the brain (Richardson, 2007). The predominant TH in circulation is thyroxine (T_4) that can be deiodinated to the receptor-active T_3 by the type 2 deiodinase (D2) which is present in many cells. T_3 in turn can be inactivated to 3,3′-T_2 by the intracellular type 3 deiodinase (D3) (Gereben et al., 2008). To enter the cells THs require specific transporter proteins to cross the plasma membrane (Visser et al., 2008). TH transporter proteins include those belonging to the organic anion transporter family (OATPs), L-type amino acid transporters (LATs) and monocarboxylate transporters (MCTs), and have been found in the developing and adult brain of different vertebrates including mammals, birds, amphibians and fish (Arjona et al., 2011; Chan et al., 2011; Connors et al., 2010; Muzzio et al., 2014; Van Herck et al., 2013).

Local TH action results from the combined interaction of TH transporters, deiodinases and receptors which are expressed in a cell-specific pattern. In the brain an additional level of regulation exists at the brain barriers that separate the central nervous system from the rest of the body (Ek et al., 2012; Risau, 1994). The blood vessels (capillaries) in the brain form the blood–brain barrier, and the choroid plexus in the ventricles forms the blood–cerebrospinal fluid barrier (blood–CSF barrier). The cells of these barriers are linked by tight junctions (Liu et al., 2012; Møllgård and Saunders, 1975), inhibiting intercellular diffusion and imposing transmembrane transport for entrance into the brain. At the blood–brain barrier the tight junctions are situated at the level of the endothelial cells of the blood vessels. In contrast, the blood vessels within the choroid plexus are "leaky" while the outer epithelial cells of the choroid plexus contain the tight junctions and form the blood–CSF barrier. In this way these barriers are able to control the movement of compounds, such as hormones and nutrients, between the general circulation and the brain (Abbott et al., 2010; Saunders et al., 2013). Expression of TH transporters and deiodinases at the brain barriers can therefore strictly control the amount of (active) TH reaching the brain.

Some controversy remains on the exact timing of the onset of brain barrier function during development (Ek et al., 2012). While tight junctions are found in the blood vessels from the moment they start to invade the brain (Bauer et al., 1993; Møllgård and Saunders, 1986), decreased permeability appears to be a gradual process whereby macromolecules are excluded from passing the embryonic blood–brain barrier already at very early stages, but smaller molecules may pass more freely until a more dense network of endothelial junctions appears (Engelhardt, 2003; Stewart and Hayakawa, 1987). In the chicken embryo, the animal model used in this paper, tight junctions have been visualised in the endothelial cells of the blood–brain barrier at day 9 of the 21 day embryonic development (Nico et al., 1997). Injection experiments indicated a decreasing permeability of the brain blood vessels for Evan's blue and horseradish peroxidase from day 13 of development onwards (Ribatti et al., 1993; Wakai and Hirokawa, 1978).

The ontogenetic timing of the thyroid axis maturation and brain development in chickens largely correlates with the timing found in developing humans (Van Herck et al., 2013). In both species the functional maturation of the embryonic thyroid gland occurs around mid-embryonic development and also the major part of brain development occurs prenatally, in contrast to the situation in rodents (Howdeshell, 2002; Thommes, 1987). This, in combination with the fact that the chicken embryo can be easily reached through the egg and the possibility to synchronise the embryonic development of many animals in an incubator, makes it an appropriate and efficient model to investigate TH-regulated neurodevelopment, and development of the functional brain barriers.

In this paper we investigated the expression pattern of TH transporters, deiodinases and TTR at the developing brain barriers. Their expression was studied from day 8 of embryonic development, before the onset of embryonic thyroid gland functioning, until 11 days post-hatch, with the focus just prior to hatching as this is a period in which the brain undergoes rapid changes in TH metabolism (Reyns et al., 2003; Van der Geyten et al., 2002).

2. Materials and methods

2.1. Animal treatment and tissue sampling

Fertilised Ross (broiler) eggs were obtained from a commercial hatchery (Belgabroed, Merksplas Belgium), and incubated in a forced draft incubator at 37.5 °C and 50% relative humidity and automatically turned at a 45° angle every hour. The day on which incubation was started was called day 0 (E0). On days E8, E10, E11, E14, E17, E18, E19 and E20 some eggs were opened and embryonic tissues were sampled. At E20 the chicks hatch and make the transition to C0. Additional samples were collected on post-hatch stages C1 and C11.

Blood samples were taken by cardiac puncture (E14–E20) or carotid artery bleeding (post-hatch) in heparinised collection tubes. After centrifugation, plasma was stored at −20 °C until analysis. At each sampling stage the choroid plexus was collected from the lateral ventricles in the telencephalon and the remaining brain was divided into 5 parts: telencephalon (forebrain), diencephalon, mesencephalon (optic lobes) and rhombencephalon. The rhombencephalon was subdivided into cerebellum and brain stem. All tissue samples were snap frozen in liquid nitrogen and stored at −80 °C until analysis. Whole brain samples were collected for histological analysis. These samples were fixed overnight in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4, 4 °C) after which they were cryoprotected overnight in 20% sucrose in PBS (pH 7.4, 4 °C) and finally embedded in Tissue-Tek O.C.T. compound (Sakura finetek, Alphen aan den Rijn, The Netherlands). Samples were then frozen on a liquid nitrogen cooled metal stub and stored at −80 °C until cryosectioning. All the experimental protocols on animals were conducted in accordance with the European Communities Council Directive (2010/63/EU) and were approved by the Ethical Committee for animal experiments of the KU Leuven.

2.2. In situ hybridisation (ISH)

ISH procedures were performed using digoxigenin-UTP-labelled antisense riboprobes as previously described (Geysens et al., 2012), based on the protocol Hidalgo-Sanchez et al. (2005). Sense probes were used as negative controls for all genes and at different stages. Information on the specific probes is summarised in Table 1.

2.3. Reverse transcription quantitative PCR (qPCR)

RNA isolation, cDNA production and qPCR procedures were performed as previously described (Van Herck et al., 2012). Additional primers were designed using Primer3plus (Untergasser et al., 2012), and information on all primer pairs is summarised in Table 1. Relative expression values were calculated with the StepOne Software (Life Technologies). The expression data were normalised using the qBasePLUS Software (Biogazelle, Zwijnaarde, Belgium). Beta actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Cyclophillin A and Ubiquitin mRNAs were used for normalisation.