DOI:10.14753/SE.2017.2032

Investigation of regulatory factors underlying thyroid hormone mediated effects

Ph.D. Thesis

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Budapest
2017

DOI:10.14753/SE.2017.2032

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1. LIST OF ABBREVIATIONS

ANOVA	analysis of variance
AP-1	activator protein 1
bp	base pair(s)
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
cDNA	complementary deoxyribonucleic acid
cD2	chicken type 2 deiodinase
CpG	cytidine-phosphateguanosine
CREB	cAMP response element binding protein
cRNA	complementary ribonucleic acid
CSF	cerebrospinal fluid
D1	type 1 deiodinase
D2	type 2 deiodinase
D3	type 3 deiodinase
DBD	DNA binding domain
DIG	digoxigenin
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DRIP/TRAP	vitamin D receptor interacting protein/thyroid receptor associated protein
DTT	dithiothreitol
E	embryonic day, day of incubation
EDTA	ethylenediaminetetraacetic acid
EFsec	selenocysteine specific elongation factor
ER	estrogen receptor
FBS	fetal bovine serum
fmol	femtomole(s)
FSH	follicle stimulating hormone
GFAP	glial fibrillary acidic protein
HAT	histone acetyltransferase
HDAC	histone deacetylase

HPT	hypothalamo-pituitary-thyroid
HRE	hormone response element
kb	kilobase(s)
K_m	Michaelis-Menten constant
LBD	ligand binding domain
LH	luteinizing hormone
MCT	monocarboxylate anion transporter
µl	microliter
µM	micromole(s), micromolar
mRNA	messenger ribonucleic acid
mTRα	mouse thyroid receptor α
NCoR	nuclear receptor co-repressor
NGF	nerve growth factor
NLS	nuclear localization signal
nM	nanomole, nanomolar
nTRE	negative thyroid response element
OATP1C1	organic anion transporting polypeptides 1
PBS	phosphate buffered saline
PCAF	p300/CBP associated factor
PCR	polymerase chain reaction
PTU	propyl thiouracil
PVN	paraventricular nucleus
RNA	ribonucleic acid
rT_3	reverse T_3, 3,3’,5’-triiodothyronine
RTH	resistance to thyroid hormone
RT-PCR	reverse-transcription-polymerase chain reaction
RXR	retinoid X receptor
SBP	selenocysteine binding protein
SEAP	secretory alkaline phosphatase
SEM	standard error of the mean
SECIS	selenocysteine inserting sequence
SMRT	silencing mediator of retinoid and thyroid receptors
sORF	short open reading frame
SRC	steroid receptor co-activator

SSC	standard sodium citrate
SUN-CoR	small ubiquitous nuclear co-repressor
SV	simian virus
T_2	diiodothyronine
T_3	triiodothyronine, 3,5,3’-triiodothyronine
T_4	thyroxine, 3,5,3’,5-tetraiodothyronine
TBP	TATA-binding protein
TH	thyroid hormone
TK	thymidine kinase
TRE	thyroid hormone response element
TRH	thyrotropin releasing hormone
TRα	thyroid receptor α
TRβ	thyroid receptor β
tRNA	transfer ribonucleic acid
TSH	thyroid stimulating hormone, thyrotropin
UTR	untranslated region

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2. INTRODUCTION

Thyroid hormone (TH) plays a fundamental role in the development and function of various organ systems. TH is especially important for the development and function of the brain (Bernal et al., 2003). Thyroxine (T_4) is the most abundant (~80%) secretory product of the human thyroid gland. However, the presence of 3,5,3'-triiodothyronine (T_3) was also revealed in the human plasma by Gross and Pitt-Rivers (1952). T_3 is a deiodinated form of T_4 and it was established that T_3 is the compound responsible for most known TH-dependent biological effects despite its less than 20% presence in the thyroidal secretory output. Importantly, the existence of T_4 to T_3 conversion was proved by Sterling et al. (1976) indicating that not only the thyroid but also the prohormone T_4 can give rise to T_3.

The regulation of circulating TH levels is governed by the hypothalamo-pituitary-thyroid (HPT) axis (Fekete and Lechan 2014). The HPT axis is programmed to keep serum T_3 levels in the physiological range that is predominantly achieved by controlling the release of TH from the thyroid gland, especially via regulating the output of the long-lived T_4 prohormone. However, TH action occurs in tissue/cell compartments and its regulation requires quick and tissue-specific costumization (Gereben et al., 2015). Thus, the impact of the HPT axis on tissue TH levels is limited. According to the current consensus, a complex and tissue-specific regulatory system is responsible for the control of tissue TH action. The crucial players of this system are the members of the deiodinase enzyme family that catalyze TH activation and inactivation, the TH transporters, and the nuclear machinery of TH action (Gereben et al., 2008; Visser 2000).

2.1. Thyroid hormones are crucial factors of development

TH is essential for the development and differentiation of various cell types and exerts a striking impact on the developing central nervous system. A classical study established the correlation between iodine deficiency and cretinism (Ord 1888). It is well-known that congenital hypothyroidism is accompanied with detrimental effects. Therefore serum thyroid stimulating hormone (TSH) levels are subjected to routine screening in human neonates to ensure that TH supplementation will be performed in the critical

time window after birth to avoid the devastating and irreversible consequences (Fisher et al., 1979).

It took decades to accumulate data allowing a deeper understanding of the underlying mechanisms. TH was found to be crucial in the regulation of later neuron differentiation events: proper myelination (Balázs et al., 1969a and 1969b), dendrit and axon development and synaptogenesis. It was also demonstrated that suboptimal TH levels affect the expression of genes controlling myelin formation in rats, e.g. the ones encoding myelin basic protein, proteolipid protein and myelin associated glycoprotein (Farsetti et al., 1991; Rodriguez-Pena et al., 1993). Under prolonged hypothyroidism, the number of myelinated axons decreased and axons of lower diameter were found to be not myelinated in neonatal rats. These changes were also observed in cortical regions involved in visual, auditory and motoric activities. THs are inevitable for proper cell migration and formation of the layers of the cerebral cortex. Purkinje cells in the cerebellum and the pyramidal cells in the cortex were found highly sensitive to appropriate TH availability. Purkinje cells are unable to develop their dentritic tree in hypothyroidism (Legrand 1967a and 1967b) and this was underlain by affected cytoskeletal organization (Silva and Rudas, 1990) while the late migration of granular cells in the cerebellum was also severely affected. THs also affect glial cell differentiation (oligodendrocytes, astrocytes and microglia) (Gharami and Das 2000; Lima et al., 2001). The underlying mechanisms involve biochemical changes related to glucose-amino acid conversion, glutamine-dehidrogenase activity, decreased activity of oxidative enzymes (Balázs et al., 1971; Cocks et al., 1970) and also yet not fully revealed mechanisms governed by cell-cycle modulators such as cyclin D1, E2F-1 or p27 (Garcia-Silva et al., 2002). It was shown that the number of matured astrocytes and oligodendrocytes is reduced in the white matter tracts of hypothyroid rats (Schoonover et al., 2004; Martínez-Galán et al., 1997). Furthermore, THs also impact the development of the cytoskeleton via the upregulation of glial acidic fibrillary protein (GFAP) and F-actin, as demonstrated both in animal and cell culture models (Paul et al., 1996). THs enhance the secretion of different extracellular matrix proteins like laminin and fibronectin via growth factor secretion (Trentin et al., 1995 and 2001). Importantly, nerve growth factor (NGF) secreted by astrocytes has a crucial role in the control of neurit growth (Lindsay 1979; Charrasse et al., 1992). The abovementioned effects of

TH on glia cells also impact neurons. The role of TH played in neural development is summarized in **Figure 1.**

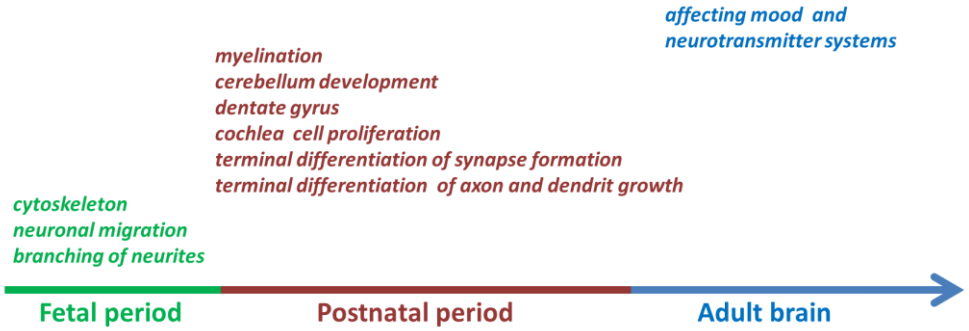


Figure 1. Role of thyroid hormones during different stages of brain development (based on Bernal 2015).

2.2. The structure and function of the HPT axis

The HPT axis is governed by hypophysiotropic thyrotropin releasing hormone (TRH) expressing neurons of the hypothalamic paraventricular nucleus (PVN). Axons of these neurons project to the external zone of the median eminence, located below the floor of the third ventricle where TRH is released from axon varicosities (Lechan and Fekete 2006). TRH reaches the anterior pituitary via the portal system and binds to the 7 transmembrane domain containing $G_{q/11}$ protein coupled TRH receptors of thyrotropes followed by the increase of thyrotropin (TSH) production and secretion. TSH is a glycoprotein that is formed by an α-subunit consisting of 92 amino acids (Salvatore et al., 2011). This subunit is very similar to those of the follicule stimulating hormone (FSH) and luteinizing hormone (LH) (Gray 1988). In contrast, the 110-amino acid-long β-subunit of TSH is specific for this molecule. TSH reaches the thyroid gland by the circulation and binds to the 7 transmembrane domain containing TSH receptors on thyrocytes. In contrast to the TRH receptor, the TSH receptor is coupled to G_s protein and acts via the activation of adenylate cyclase and consequently evokes cyclic adenosine monophosphate (cAMP) release. The capacity of a given amount of TSH to induce cAMP release is regulated by TSH bioactivity, a phenomenon dependent on TRH-promoted glycosylation of the TSH molecule (Salvatore et al., 2011). TSH promotes various steps of TH formation and release. These include the iodide uptake of the thyrocytes in the thyroid gland, synthesis and iodination of the thyroglobulin,

endocytosis of the colloid and activation of peroxidase. TSH is also able to increase the rate of cell division in the thyroid gland (Portulano et al., 2014).

TH regulates the HPT axis by negative feedback. Increased serum T_4 and T_3 levels decrease TSH and TRH secretion, while a decrease of circulating TH stimulates the hypothalamus and the anterior pituitary to release TRH and TSH, respectively. Tanycytes of the mediobasal hypothalamus play a very important role in the feedback mechanism. These specialized glial cells are located in the floor and the ventrolateral walls of the third ventricle (Bruni 1974, Krisch et al., 1978). Numerous tanycyte processes, especially those of β-tanycytes located in the floor of the third ventricle end in the median eminence and contact capillaries and axon terminals of neurosecretory neurons (Lechan and Fekete 2007). Importantly, the median eminence is located outside of the blood-brain barrier and its TH content originates both from local, tanycyte-generated sources and from the periphery (Kakucska et al., 1992). TRH expression in the PVN of hypothyroid rats could be normalized with exogenous T_3 only with a dose evoking hyperthyroidism in the periphery. This finding clearly underlines the functional importance of hypothalamic T_4 to T_3 activation in the regulation of TRH (Kakucska et al., 1992). Tanycytes are able to uptake T_4 either from the fenestrated capillaries of the median eminence, the intercellular space of the basal hypothalamus or from the cerebrospinal fluid (CSF). They actively convert T_4 to T_3 since these cells contain high amounts of deiodinase type 2 (D2) enzyme (for more details of D2 see Section 2.3). The generated T_3 is released either to the mediobasal hypothalamus, the CSF or the median eminence into the microenvironment located between tanycyte processes and axonal segments of parvocellular neurosecretory neurons. Hypophysiotropic TRH neurons contain monocarboxylate anion transporter 8 (MCT8) protein in the plasmamembrane of their axons that enables them to uptake T_3 in the median eminence and consequently this allows the regulation of TRH expression by T_3 of the median eminence (Kalló et al., 2012). This could work via an anticipated retrograde axonal transport of T_3 to reach the PVN but this remains to be proved. This is an important point to be revealed since TRH neurons in the PVN rely on external T_3 due to their inability to generate T_3 is underlain by the lack of D2 in these cells. Furthermore, a significant portion of TRH neurons do not contain the TH degrading deiodinase 3 enzyme (D3), thus their ability to actively regulate their intracellular T_3 content is limited, indicating that these cells are

programmed to accurately translate the hypothalamic T_3 levels into TRH expression (Kalló et al., 2012). Thus, in the mediobasal hypothalamus, D2-mediated local regulation of T_3 availablity functionally interacts with the regulation of the HPT axis. See **Figure 2** for summary.

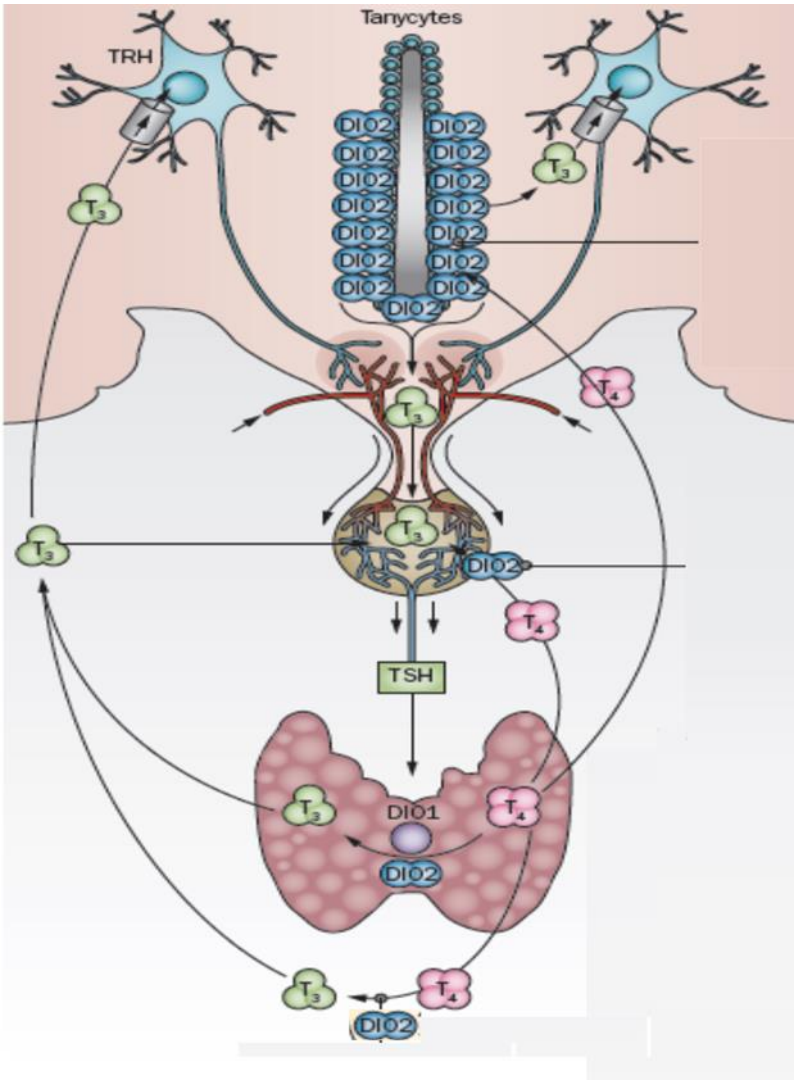


Figure 2. The hypothalamo-pituitary-thyroid axis and its interaction with local D2-mediated T_3 generation. TRH: thyrotropin releasing hormone, TSH: thyroid stimulating hormone, T_4: thyroxine, T_3: triiodothyronine, DIO1: deiodinase type 1, DIO2: deiodinase type 2 (modified from Gereben et al., 2015).

While TH is a major regulator of hypophysiotropic TRH neurons, these cells also receive afferents from different brain regions to allow proper response of the axis to the changing environment. Metabolic signals are transmitted from the arcuate nucleus,

circadian signals come from the suprachiasmatic nucleus while catecholaminergic afferents from the brainstem transmit information on changes of the external temperature (Fekete and Lechan 2014).

Set-point formation of negative feedback during ontogeny is a fundamental process for the regulation of the HPT axis and its consequences persist through the entire lifespan. However, only limited information is available on the onset of the negative feedback mechanism.

Both the development of the brain and the ontogeny of the HPT axis show marked species-specific differences. Rats are altricial animals, their brain is not well developed at birth and their hypothalamo-pituitary-thyroidal gland axis is not fully matured (Schwartz 1983, Legrand 1986). In contrast, the precocious sheep (Fisher 1991) and chickens have well developed HPT axes at the date of birth or hatching (Oppenheimer and Schwartz, 1997). Children at birth represent an intermediate state between these examples with an immature central nervous system but a fully developed axis. Thus rodents do not serve as appropriate model for the regulation of the development of the human HPT axis while the related mechanisms of hypothalamic feedback cannot be studied in humans due to ethical reasons. Therefore, chickens represent an invaluable model for these studies, since the developmental kinetics of the chicken HPT axis is rather similar to that of humans. In addition, the chicken embryo allows to study developmental phenomena in the absence of interfering maternal regulatory circuits. In contrast, data obtained from rats should be handled with care because of the significant differences exisiting between the development of the HPT axes of rodents and humans (Taylor et al., 1990).

In birds THs control piping, hatching, thermogenesis and growth, but also play a role in the neurulation of the chicken embryo (Flamant and Samarut 1998; Decuypere et al., 1990; Beckett and Arthur 1994). The onset of the thyroid function in chickens starts from embryonic day 9.5 (E9.5), T_4 and T_3 can be detected in the yolk and serve as maternal thyroid hormone supply (Prati et al., 1992). The thyroid gland starts to secrete its hormones in increasing amounts, but the TSHβ mRNA is increasing further until E19 as the negative feedback is not yet functional at this developmental stage (Gregory et al., 1998).

2.3. Regulation of tissue TH action by deiodination

Many organs can customize their TH action independently from circulating TH levels and deiodinase enzymes play a striking role in this event (Gereben et al., 2008). Thyroxine can be activated by 5' deiodination by removing iodine from the outer phenolic ring of thyroxine (activation pathway) while inner ring 5 deiodination results in the inactivation of T_4 or T_3 (**Figure 3**). Historically, the tissues were categorized based on their capability of utilizing TH. Tissues contributing to serum TH levels were considered as TH exporters while those tissues which do not, were considered as TH importers (Crantz et al., 1982). While this nomenclature properly recognized the importance of tissue-specific differences in TH economy, in light of recent data it falls short to address that i) tissue specific contribution to systemic TH levels is not constant but dependent on TH status and ii) in organs with complex cellular composition it also depends on cell-type specific events. For example, the rodent heart is not capable of producing T_3 locally and it is fully dependent on the circulating T_3 in the serum. The liver is TH importer in euthyroidism and TH exporter in hyperthyroidism. Importantly, the brain generates most of its T_3 locally, but does not contribute significantly to the T_3 level of the serum. Furthermore, striking compartmentalization exists inside the brain, as the glial compartment performs T_3 generation and the neuronal compartment consumes T_3. Thus, cell-type specific events ensure the cellular export and import of TH within the same tissue (see Section 2.4). In humans, T_3 originates predominantly from D2-mediated outer ring deiodination of the prohormone thyroxine in tissues (Maia et al., 2005). While biochemically both D2 and type 1 deiodinase (D1) can catalyse this process, due to its high K_m, D1 is not capable of generating T_3 under euthyroid conditions. Consequently, *in vivo*, D2 is the predominant activating deiodinase.

These enzymes belong to the selenodeiodinase enzyme family and contain the rare amino acid selenocysteine in their active center. In the deiodinase encoding mRNA selenocysteine is encoded by an in-frame UGA codon that serves otherwise as a stop codon in proteins that do not belong to the selenoprotein family. The translational readthrough of UGA is ensured by a specific mRNA secondary structure, the selenocysteine inserting sequence (SECIS) element, located in the 3' untranslated region (3'UTR) of the selenoprotein encoding mRNA (Berry et al., 1991a; Gereben et al., 1999). The SECIS binds the SECIS binding protein (SBP2) that interacts with

selenocysteine specific elongation factor (EFSec). The latter binds selenocysteine transfer RNA (tRNA) allowing cotranslational selenocysteine incorporation at the UGA on the ribosome (Bianco et al., 2002). Although this process is very complex and energy consuming, the resulting selenocysteine-containing deiodinase enzyme has much higher substrate affinity than the cysteine-containing version. This is because selenium can be ionized more easily at physiological pH than the sulphur of cysteine, turning the formed protein into a much more powerful oxido-reductase. On the other hand, this comes to the cost of the efficiency of translation that is ~50-100 times lower for the selenodeiodinase D2 than for its cysteine mutant version (Steinsapir et al.; 2000). Therefore, selenocysteine incorporation results in a low level of selenoenzyme that is highly efficient (Berry et al., 1991b).

D1 is capable of removing the iodine from both the outer and inner rings, while D2 is an exclusive outer ring deiodinase. An important difference between the two enzymes is that the *in vitro* K_m of D1 for T_4 deiodination is much higher (1-2 μM) compared to D2 (1-2 nM). D1 has low affinity and high capacity, at the same time D2 has high affinity and low capacity for T_4. While D1-mediated deiodination is strongly inhibited by 6*n*-propyl-thiouracil (PTU), D2 deiodination is not. This feature helped to discover the PTU-resistant 5' deiodination and as a consequence to identify D2 in the pituitary of hypothyroid rats (Silva and Larsen, 1977). D2 is the enzyme that can efficiently generate T_3 *in vivo*, while D1-mediated T_3 generation can only occur under hyperthyroid conditions. Although D1 predominantly inactivates reverse T_3 *in vivo*, the special kinetics of D2 enables this protein to activate thyroxine even at low substrate levels.

D1 was identified in several tissues. In rats liver, kidney, thyroid gland and the pituitary contain D1. Its 2.1 kb long cDNA was cloned by Berry et al. (1991a). D1 is anchored to the plasmamembrane (Baqui et al., 2000).

D2 is present in the glial compartment of various brain regions and in the pituitary (Silva and Larsen, 1977). It can also be found in brown adipose tissue, placenta, keratinocytes, myocardium, skeletal muscle and in the thyroid gland (Salvatore et al., 1996b; Croteau et al., 1996, Bianco et al., 2002). The first full length D2 cDNA of higher vertebrates was isolated from chicken (Gereben et al., 1999). The D2 mRNA

contains a SECIS element close to the end of 3'UTR. The coding region is of average size (840 bp) but the whole RNA is unusually long (6094 bp) due to the long untranslated regions and the distance between the SECIS and the UGA of the active center is almost 5 kb. D2 is localized in the endoplasmic reticulum (Gereben et al., 2008).

D3 represents the third known member of the family. This enzyme removes iodine from the inner ring of TH and its main role is to inactivate T_4 and T_3. D3 is expressed in the brain, palcenta, skin and fetal liver. D3-mediated T_3 inactivation is crucial in the regulation of TH availability in neurons and also prevents the fetus from high levels of maternal T_3 in specific phases of ontogeny (Tu et al., 1999; Gereben et al., 2008). D3 is localized in the plasmamembrane and in the dense core vesicles of neurosecretory neurons (Baqui et al., 2003; Kalló et al., 2012).

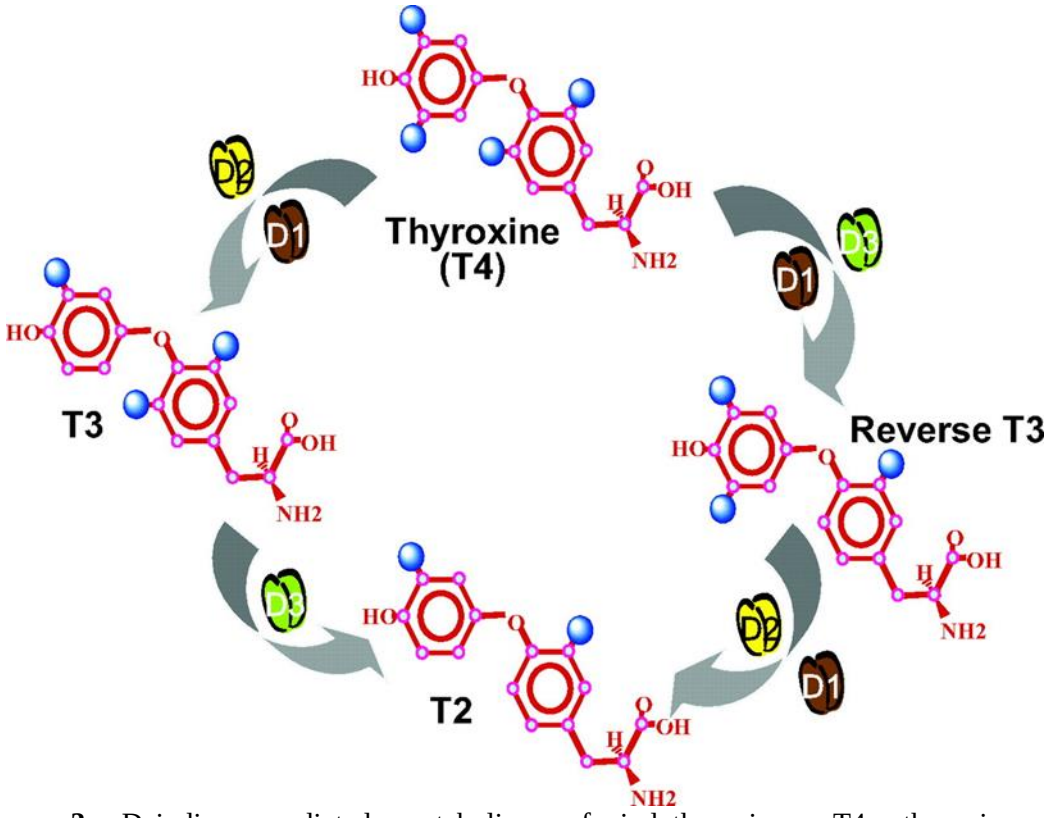


Figure 3. Deiodinase-mediated metabolism of iodothyronines. T4: thyroxine, T3: triiodothyronine, T2: diiodothyronine, reverse T3: reverse triiodothyronine (Gereben et al., 2008).

2.4. Regulation of thyroid hormone availability in the brain

T_4 and T_3 concentrations in the brain are strictly governed by complex, locally controlled mechanisms. This process is tightly regulated by deiodination-dependent TH

metabolism and neuro-glial TH transport (Freitas et al., 2010; Gereben et al., 2008). It was demonstrated that in the brain of rat fetuses T_3 is exclusively generated from local T_4 deiodination and the circulating T_3 virtually does not reach the brain and a high proportion of T_3 (>80% in the cerebral cortex) was shown to be locally generated in the adult rat brain (Crantz et al., 1982). As it was discussed in details in Section 2.2, the median eminence is a unique region of the brain which lies outside of the blood-brain barrier, so T_3 from the periphery can reach this area. In contrast, other tissues like the liver are readily accessed by serum T_3 (Calvo et al., 1990; Grijota-Martinez et al., 2011). Due to the presence of D3 in the axonal compartment of the external zone of the median eminence, T_3 availability is subjected to cell-type specific regulation in this crucial sensor region (Kalló et al., 2012).

In the brain, D2 and D3 are responsible for the regulation of TH availability. D2 is expressed in astrocytes in various brain regions and in tanycytes located in the floor and infralateral wall of the mediobasal hypothalamus (Tu et al., 1997). In contrast, D3 is expressed in neurons (Tu et al., 1999). Astrocytes generate T_3 from the T_4 by taking it up from the brain capillaries and provide activated TH to neurons (Freitas et al., 2010). This cell compartment regulates its intracellular TH levels via D3-mediated degradation of T_4 to rT_3 and T_3 to and T_2.

As it was discussed in details in Section 2.2, the highest D2 expression in the brain can be found in the tanycytes and this phenomenon has a crucial role in the feedback regulation of the hypophysiotropic TRH neurons. Cell-type specific ablation of D2 activity in astrocytes and in the pituitary of transgenic mice revealed that the astrocytic D2 pool exerts no impact on the regulation of the HPT axis. It also shed light on the complex functional interactions allowing the hypothalamus to keep the systemic TH economy in balance even in the absence of pituitary D2 (Werneck de Castro et al., 2015). Perivascular glial cells take up thyroxine from the blood vessels while a smaller fraction of TH is transported through the choroid plexus and the cerebrospinal fluid. TH passes the plasmamembrane via membrane transporters like the monocarboxylate anion transporter 8 and 10 and the natrium independent organic anion transporting polypeptides 1 (OATP1C1). MCT8 transporters are specific to iodothyronines and transport both T_3 and T_4. The transporter is expressed in the blood-brain barrier, choroid plexus, neurons, tanycytes and astrocytes (Heuer et al., 2005). OATP1C1 transports

mainly T_4 and it can be found in the endothelial cells of the blood-brain barrier and choroid plexus and in the astrocytes (Roberts et al., 2008). L-type amino acid transporters were also described in the astrocytes and neurons (Jansen et al., 2005).

Mutations in the MCT8 transporter protein are manifested in an X chromosome-linked psychomotor retardation, the Allan-Herndon-Dudley syndrome that is hallmarked in neonates by neurological symptoms, global developmental delay and mental deficiency due to the defects in the T_3 transport in critical development phases (Bernal 2005). However, MCT8-deficient mice show only negligible neurological impairments (Liao et al., 2011). This is underlined by a compensatory effect of OATP1C1 that was elegantly demonstrated by the severe phenotype of the double MCT8/OATP1C1 double knock-out (Mayerl et al., 2014). In parallel, it was demonstrated that the level of the OATP1C1 transporter is much lower in humans compared to rodents allowing less compensation for MCT8 deficiencies (Heuer et al., 2005).

2.5. Machinery of nuclear thyroid hormone action: thyroid hormone receptors and thyroid hormone response elements

In principle, hormones act in two different ways on their target cells: a group of hormones (polypeptides, monoamines, prostaglandins) do not enter the cells and bind to receptors on the cell surface, while another large group of hormones (small lipophilic molecules) enter the target cells and display their effects through intracellular receptors (Lazar 2011). TH receptors (TRs) belong to the nuclear hormone receptor superfamily and represent the cellular homologues of the v-erbA oncogen of the avian erythroblastosis retrovirus (Weinberger et al., 1986; Sap et al., 1986). Other members of this superfamily are receptors of

- other classical hormones (e.g.: glucocorticoid, mineralocorticoid, estrogen, androgen)
- vitamins (vitamin D, retinoic acid, retinoic X)
- metabolic intermediates and products (that ligand peroxisome proliferator-activated, liver X, bile acid receptor, Rev-Erb receptor)
- xenobiotics (that ligand pregnane X and constitutive androstane receptor) (Mangelsdorf et al., 1995)

DOI:10.14753/SE.2017.2032

Orphan receptors with unknown ligand were also described. A good example of this is the Rev-erb A receptor (Yen 2001).

The nuclear hormone receptors are hormone dependent transcription factors having a molecular weight in the range of ~100 kDa. For nuclear trafficking, a nuclear localization signal (NLS) is required to be attached to the receptor upon translation (Lazar 2011). Members of the nuclear receptor superfamily share a similar domain structure and contain separated domains for DNA binding and ligand binding (see **Figure 4)**. The *DNA binding domain* (DBD) and the *ligand binding domain* (LBD) are located at the amino and carboxy-terminal portion of the protein, respectively. Despite the structural similarity of this domain (with 12 α helical segment), slight molecular differences in the region account for the ligand specificity. The DNA binding domain is able to recognize specific DNA sequences (hormone response elements, HREs) in the promoter region of a responsive target gene. The DBD contains zinc fingers that contact the DNA, the region responsible for the recognition of the specific DNA hexamer is the P box that is basically a small stretch of amino acids (Lazar 2011). TR binds the AG**GT**CA hexamer, also called as half-sites (see **Figure 5**), while glucocorticoide receptors bind to AG**AA**CA. In some cases the receptor binds to extended half sites with C-terminal extension of the DNA-binding domain. TR predominantly binds the DNA heterodimerized with Retinoid X receptor (RXR). The dimers bind to the two half sites and the number of bases and the sequence between the half sites determine the target gene specificity. TRs usually bind to direct repeats separated by 4 bases between them (Umesono et al., 1991), but in some cases the orientation of the half-sites and their spacing is different (Williams and Brent 1995). RXR and TR bind to the 5’ and 3’ half site sequences, respectively. Upon ligand binding, TR undergoes a conformational change which alters its capacity to recruit coactivators or corepressors. This event is a prerequisite of the TR-dependent alteration of gene transcription. Whether the resulting effect will be manifested in activation or repression is primarily dependent on the TRE characteristics of a specific gene (see Section 2.6).

N terminal domain	DNA binding domain			Hinge region	Ligand binding domain		C terminal domain
A/B	C			D	E		F
Activation function-1 AF-1	P-box	Dimerization	NLS*			Activation function-2 (AF-2)	
			C term. extension				
Highly variable	Highly conserved				Moderately conserved		Variable

*nuclear localisation signal

Figure 4. General structure and functions of the nuclear receptors

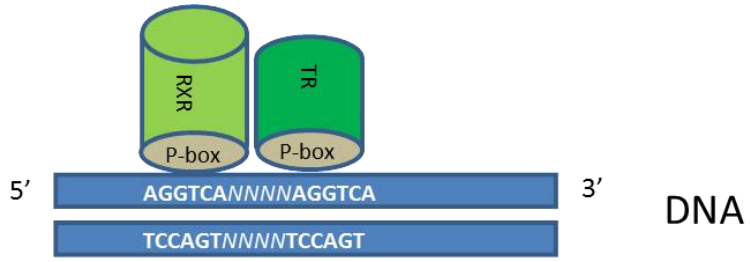


Figure 5. Binding of thyroid hormone receptors to DNA. Thyroid (TR) and retinoid X receptor (RXR) heterodimers bind to DNA half site sequences called TH response element (TRE).

2.5.1. Thyroid hormone receptor isoforms

Two TR isoforms have been identified. TRα was first isolated from chicken liver (Sap et al., 1986) and at the same time also from rat brain (Thompson et al., 1987). The TRβ was isolated from human placenta (Weinberger et al., 1986), chicken (Forrest et al., 1990 and 1991) and rat (Obregon et al., 1986). TRα and TRβ are encoded by two separate genes: TRα on the human chromosome 17, while TRβ on chromosome 3. TRα has two main subtypes, α1 and α2. TRα2 is an alternatively spliced variant, which is unable to bind thyroid hormones because some critical amino acids are replaced in the carboxy-terminal (Lazar et al., 1989b) and the dimerization properties of this receptor isoform are also changed. Some further protein products encoded by the TRα gene were also described such as TRα3 and the truncated ΔTRα1 and ΔTRα2 proteins. The function of the isoforms that do not bind the hormone is not clear, although these might have a role in attenuating the physiological effects of T_3 by competing with the T_3 binding variants (Lazar et al., 1989a).

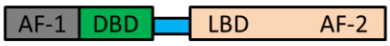
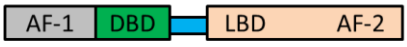
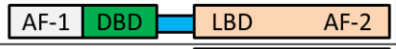
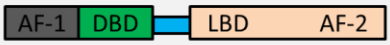
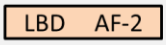
The TRβ gene encodes four different types of proteins: TRβ1, TRβ2, TRβ3 and ΔTRβ3. All of them are able to bind T_3 but the truncated ΔTRβ3variant cannot bind to the DNA.

DOI:10.14753/SE.2017.2032

The A and B domains of the TRβ receptors are different from each other but their DNA and ligand binding domains are very similar.

The tissue distribution of TR isoforms shows tissue-specific differences and their physiologic role depends on the given tissue in which they are expressed. It was e.g. demonstrated that TRα is important in the regulation of cardiac function, body temperature, intestinal and lymphocyte development. This receptor is also widely expressed in the brain (Morte et al., 2002). Some studies in mice confirmed the role of TRα in behaviour as it is responsible for the specification of the hippocampal neural circuits (Guadano-Ferraz et al., 2003). TRβ can be found in the pituitary, liver and cochlea. TRβ2 is the predominant TR isoform in the PVN (Bernal et al., 2003; Abel et al, 2001) and this subtype is responsible for the differentiation of the Purkinje cells in the cerebellum. For the different receptor types and their functions see **Table 1**.

Table 1. Thyroid hormone receptor isoforms, their tissue distribution and functions

Name of the protein	T3 binding	DNA binding	Figure of the structure	Tissue distribution
TRβ1	+	+		liver, kidney, cortex cerebellum (Purkinje cells)
TRβ2	+	+		anterior pituitary gland, hypothalamus (PVN), cochlea, retina
TRβ3	+	+		Kidney, liver, lung (only in rats!)
ΔTRβ3	+	-		?
TRα1	+	+		skeletal&cardiac muscle, cortex, cerebellum (granular cells), hippocampus, striatum, olfactory bulb, tract
TRα2	-	+		Several tissues incl. brain, testis
TRα3	-	+		?
ΔTRα1	-	-		?
ΔTRα2	-	-		?

(Based on Ortiga-Carvalho et al., 2014)

TRs are expressed in humans and in sheep well before the formation of the thyroid gland and the onset of thyroid hormone synthesis. In the human fetus the receptors can be detected from the 10th week of gestation while in sheep from the 50th day. TRα1 isoform is responsible for the vast majority of the total T_3 binding in the fetal brain (Schwartz et al., 1992; Strait et al., 1991). In the adult rat brain distribution of T_3

binding in the brain is approximately 60% α1, 30% β1 and 10% β2 (Schwartz et al., 1994).

Several types of thyroid hormone receptor mutations were described and TR knockout murine models provided novel insights into the physiological roles of the different receptor types (Ortiga-Carvalho et al., 2014). It was shown in mice that in the absence of TRβ neither behavioural nor neuroanatomical abnormalities were observed (Forrest et al., 1996b). The TRβ knockout mice are deaf, their colour vision is impaired indicating the role of this receptor type in the development of the cochlear cell and the retinal photoreceptor. Human patients without TRβ have normal mental development (Takeda et al., 1992), but the cochleo-vestibular development may be affected (Forrest et al., 1996a; Refetoff et al., 1967). Resistance to thyroid hormone syndrome (RTH) is typically caused by mutations in the TRβ gene (Jones et al., 2003). Patients with mutated TRβ have severe learning difficulties and reduced intelligence quotient. Their TSH and thyroid hormone levels are increased, goitre is developed accompanied with an impaired negative feedback of the HPT axis. The vision and hearing of these patients are also affected and heart defects, such as tachycardia, exacerbate the homeostatic functions (Refetoff and Dumitrescu 2007, Pazos-Moura et al., 2000). In general, mutations in TRβ manifest in more severe clinical signs than the complete absence of the receptor.

More recently, TRα1 mutant RTH patients have also been identified without presenting major disturbances in parameters of TH economy; in these cases TSH and circulating thyroid hormones are only mildly affected. On the other hand, the syndrome is represented by growth retardation, delayed bone development, cognitive deficits, severe constipation and impaired neuronal development (Van Mullem et al., 2012; Vennström et al., 2008).

It needs to be mentioned that the lack of TR function and hypothyroidism are manifested in different consequences since the deletion of TRs in knock-out mice do not lead to brain hypothyroidism. This is supported by the fact that the unliganded TRs can also act on their target gene, either in a positive or a negative manner, depending on the positive or negative regulation of the gene by T_3. This removal of TR function also abolishes the regulatory function of unliganded TRs (Chassande 2003).

2.6. Assessment of thyroid hormone action

Binding of TH to TR exerts either activating or repressory effect on TH responsive target genes (Glass and Rosenfeld 2000).

2.6.1. Positive regulation of gene expression by thyroid hormones

In contrast to the other nuclear homone receptors, e.g. the estrogen receptor (ER), the TR-RXR heterodimers are predominantly located in the nucleus even in the absence of ligand and even the unliganded TR can bind to TRE. Under uninduced conditions of a positively regulated TH sensitive gene the TR/RXR heterodimers bind co-repressor molecules. Binding of T_3 triggers a conformational change of TR that will release co-repressors and bind co-activators. This TH-mediated activation of a positively regulated gene consists of derepression followed by activation. See **Figure 6** for a schematic depiction of positively regulated genes (Lazar 2003).

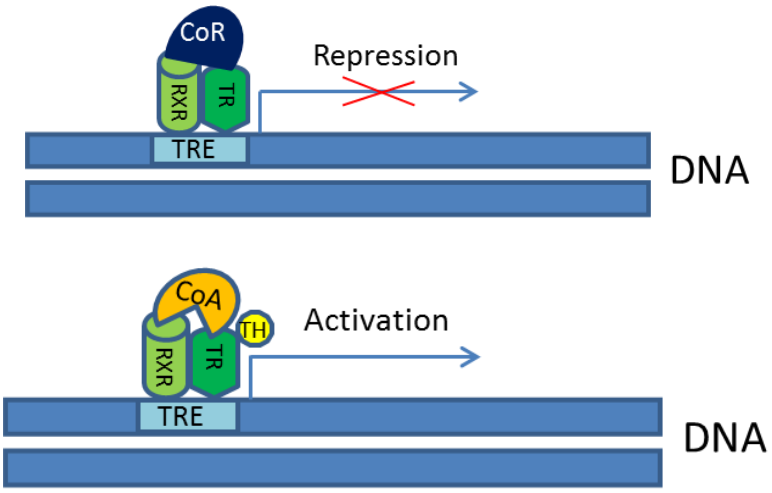


Figure 6. Activation and repression of positively regulated genes by thyroid hormone receptors. (Based on the model described by Lazar, 2003). (RXR: retinoid X receptor, TR: thyroid hormone receptor, CoR: co-repressor, CoA: co-activator, TRE: thyroid hormone response element, TH: thyroid hormone).

Several co-activators were identified that enhance the transcriptional activation of TH-responsive genes after TR binds to its ligand. Two important members of TH co-activators are the steroid receptor co-activator complex (SRC) and the vitamin D receptor interacting protein/thyroid receptor associated protein complex (DRIP/TRAP complex). SRCs have three subtypes: SRC-1, SRC-2 and SRC-3. Beside their capability to associate with TR they can also interact both with the CREB-binding protein (CBP) and p300. The CBP/p300 complex is able to interact with p300/CBP associated factor

(PCAF) that possesses histon acetylase (HAT) activity. Histon acetylases promote loosening the chromatin structure allowing transcription of the TH-responsive gene (McKenna et al., 1999). PCAF and CBP are able to interact with TATA-binding protein (TBP) associated factors and RNA polymerase II. Co-activators are able to bind to the helices 3, 5 and 6 of the ligand binding pocket of TR with their consensus LXXLL amino acid sequence. The components of the DRIP/TRAP pathway are able to associate with RNA polymerase II but they do not possess histone acetylase activity.

Major co-repressors of TH action are represented by the nuclear receptor co-repressor (N-CoR) and the silencing mediator of retinoid and thyroid receptors (SMRT). The latter is capable of anchoring further large multiprotein complexes that contain histon deacetylase (HDAC) activity. Histon deacetylases play a crucial role in maintaining the chromatin structure in a form that does not favour the basal transcription. DNA methylation may also attribute to basal repression since methyl-CpG-binding proteins associate with Sin3 containing co-repressor complex and HDAC (Nan X et al., 1998; Wade et al., 1999). Small ubiquitous nuclear co-repressor (SUN-CoR) was also described (Zamir et al., 1997). The nuclear receptor co-repressor (NCoR) and the SMRT are approximately 50% homologue based on their amino acids and have similar structural domains. In the interaction domain of NCoR and SMRT consensus sequences (I/L)xx(I/V)I were found, similarly to that of the co-activators described above (Lazar 2011).

2.6.2. Negative regulation of gene expression by TH

Mechanisms underlying TH-dependent negative regulation of gene expression are still incompletely resolved and different models have been worked out to explain the phenomenon. According to one of these models, TR binds to a negative TRE (nTRE). It means that in the case of hormone binding the receptor anchors to co-repressors (with HDAC activity), on the other hand in unliganded state the receptor binds to co-activators (with HAT activity). One of the *in vivo* examples for this model is represented by the negative effect of the TH bound receptor on TSH expression in the pituitary (Shibusawa et al., 2003) and on the regulation of TRH (see **Figure 7**).

DOI:10.14753/SE.2017.2032

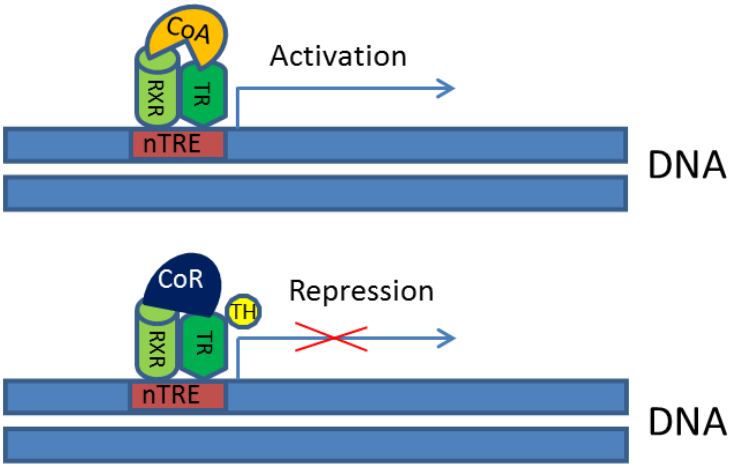


Figure 7. Activation and repression of negatively regulated genes by thyroid hormone receptors (Model 1). (Based on the model described by Shibusawa et al., 2003) (RXR: retinoid X receptor, TR: thyroid hormone receptor, CoR: co-repressor, CoA: co-activator, nTRE: negative thyroid hormone response element, TH: thyroid hormone).

It was also suggested that on negatively regulated target genes co-repressor complexes are recruited in the absence of TH. After T_3 binding co-activators are recruited, but at the same time the transcription is repressed (Astapova and Hollenberg 2013) (**Figure 8**).

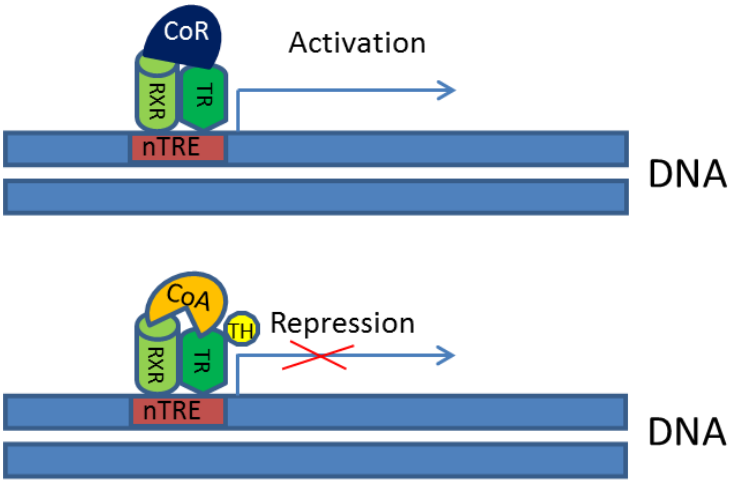


Figure 8. Activation and repression of negatively regulated genes by thyroid hormone receptors (Model 2). (Based on the model described by Astapova and Hollenberg 2013). (RXR: retinoid X receptor, TR: thyroid hormone receptor, CoR: co-repressor, CoA: co-activator, TRE: negative thyroid hormone response element, TH: thyroid hormone).

DOI:10.14753/SE.2017.2032

It was also suggested, that TR binding to DNA is not a prerequisite of the TH-mediated negative regulation (Zhang et al., 1991; Pfahl 1993). In this case, an activator protein 1 (AP-1) heterodimer (consisting of Jun and Fos) is involved in the process. Upon AP-1 binding TR recruits co-activators in the absence of the ligand and binds to co-repressor if T_3 is present (**Figure 9**).

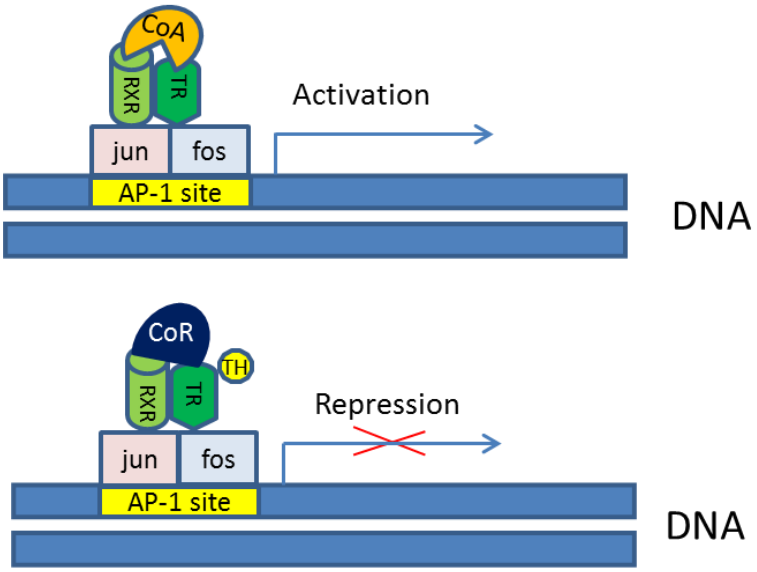


Figure 9. Activation and repression of negatively regulated genes by thyroid hormone receptors (Model 3). (Based on the model described by Zhang et al., 1991 and Pfahl, 1993). (RXR: retinoid X receptor, TR: thyroid hormone receptor, CoR: co-repressor, CoA: co-activator, AP-1 site: activator protein 1 binding site, TH: thyroid hormone).

This model can also involve regulation by non-T_3 dependent pathways, indicating that TR can compete with other nuclear receptors for co-activators or co-repressors (**Figure 10**). Without the ligand they bind ("steal") co-repressors, while in the presence of TH they bind to co-activators (Kamei et al., 1996 and Tagami et al., 1997).

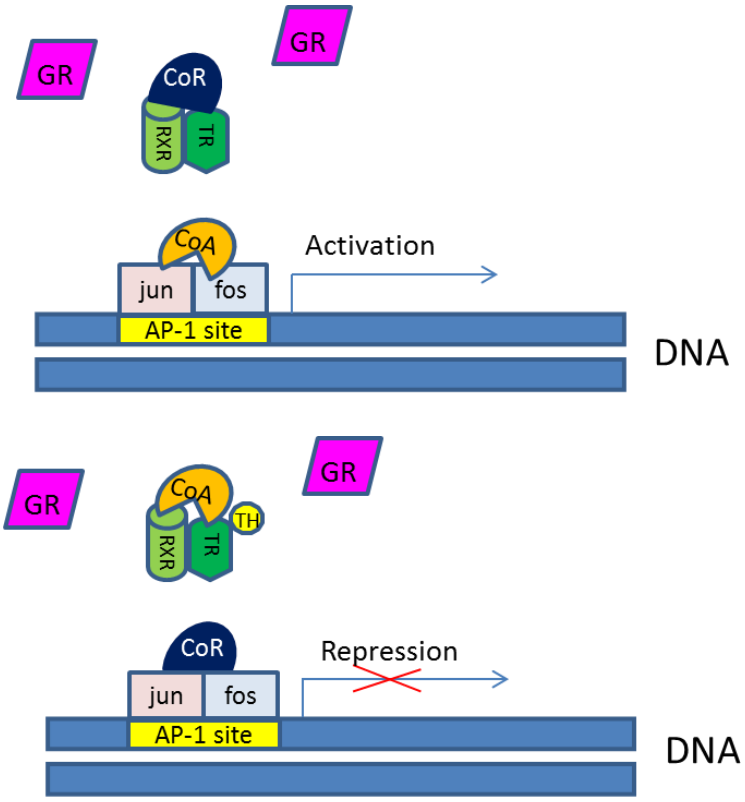


Figure 10. Activation and repression of negatively regulated genes by thyroid hormone receptors (Model 4) (Based on the model described by Kamei et al., 1996 and Tagami et al., 1997). (RXR: retinoid X receptor, TR: thyroid hormone receptor, CoR: co-repressor, CoA: co-activator, AP-1 site: activator protein 1 binding site, TH: thyroid hormone, GR: glucocorticoid receptor).

2.7. Posttranscriptional mechanisms regulating biological activity

2.7.1. Alternative splicing

Alternative splicing is a phenomenon in eukaryotes which increases the biodiversity of proteins encoded by the genome (Black 2003). Precursor messenger RNAs (or primary transcripts) can be modified by post-transcriptional processes. Alternative splicing can generate different mRNAs and as a consequence, different protein subtypes can be encoded by the same gene. There are two distinct mechanisms of alternative splicing, the exon skipping/switching and the intron slippage (Habener 2011). During exon skipping some exons are included while some of them may be excluded from the

primary transcript. Intron slippage is a process in which part of an intron is used in an exon, thus part of the intron becomes a coding region. Various examples are known when alternative splicing affects TH signaling. As it has been already highlighted in Section 2.5.1 the TRα2 is an alternatively spliced variant of the TRα1 isoform which is not able to bind T_3 (Lazar et al., 1989b).

2.7.2. The 5' untranslated region of the D2 mRNA

The 5' untranslated region (5'UTR) of a specific mRNA is located between the 5' proximity of the mRNA and the translational initiator ATG of the protein encoding region. The 5'UTR of the chicken D2 mRNA is unususally long (~600 bp). It contains short open reading frames (sORF) (Gereben et al., 1999). The sORFs are small alternative coding regions flanked by start and stop codons separated by an RNA segment consisting of bases in a number divisible by 3. Presence of the "Kozak consensus sequence" is the prerequisite of an efficient translational initiation of an eukaryotic open reading frame, represented by purine nucleotides (adenine or guanine) in the -3 position, where +1 is the first base of the ATG start codon (Kozak 1986). According to the scanning model of translation, the 40S ribosomal subunit moves 5' to 3' along the mRNA and upon reaching an ATG embedded into a Kozak consensus gets charged with methionine and by the help of additional factors recruits the 60S subunit in order to initiate translation (Alberts et al., 1994). Thus, sORFs in the 5'UTR of D2 may be subjected to translation.

2.8. Measurement of TH-dependent gene expression with promoter assays

2.8.1. Luciferase assays in general

Accurate measurement of gene transcription is a critically important tool to study genomic events underlying modulation of cell function. This is especially important for studies on TH-mediated events since most of the known effects of this hormone involve TH-induced transcriptional events. Taking advantage of bioluminescence is a state of the art approach to study transcriptional events. Bioluminescence, by definition, is the production and emission of visible light by a living organism as a result of a natural chemical reaction (McElroy et al., 1969). Several organisms are capable of producing light such as bacteria, insects, fungi and different marine organisms. During the

chemical reaction, the photon emitting luciferin gets oxidized by the luciferases. Luciferins are conserved but the luciferase enzymes are species-specific. The best established firefly luciferase originates from the North American firefly *Photinus pyralis* and is a 61 kDa protein. *Renilla* luciferase originating from *Renilla reniformis* represents another luciferase protein which was isolated from corals of coastal waters of North America (Lorenz et al., 1991). Recently, a novel luciferase enzyme Nano luciferase has been isolated from deep sea shrimp *Oplophorus gracilirostris*. It contains two smaller 19kDa and two larger 35 kDa subunits. This luciferase was used to develop the engineered luciferase NanoLuc in which the furimazine is used as a coelenterazine analogue. For cellular analytical assays this new luciferase has the advantage that it is more stable, showing brighter luminescence with sustained signal duration, has greater thermal, pH and urea stability, smaller size (19kDa) and monomeric structure. Its emission maximum is at 460 nm (Hall et al., 2012). Luciferase assays are rapid, sensitive and require non-radioactive substrates. In general, they are approximately 30-1000 times more sensitive than the classical chloramphenicol acetyltransferase reporter system (de Wet et al., 1987). It was also confirmed that luciferin is able to enter the cells enabling to conduct the luciferase expression studies in intact cells.

2.8.2. Investigation of thyroid hormone response elements

It is a crucial requirement of accurate gene expression studies that the reporter itself should not be affected by the factor used to modulate the promoter and its flanking region. However, in CV1 mammalian cells expression of the classical firefly luciferase was down-regulated by T_3 in a TR-dependent but promoter-independent manner (Tillman et al., 1993). Consequently, the CV1 cells cannot be reliably used for TRE structure or activity studies. This phenomenon was further evaluated with the analysis of unliganded thyroid hormone receptors on the Luc expression in HEK-293, COS-7 and JEG-3 cells (Maia et al., 1996). Among these cell lines the choriocarcinoma cell line JEG-3 was found to be the most sensitive for these studies. The authors suggested that an unidentified negative TRE should be present in the luciferase coding region and concluded that firefly luciferase cannot accurately measure the T_3-dependent gene expression.

Later, a cautionary note has been issued by Chan et al. (2008) regarding the use of the pBi-L (Clontech, Mountain View, CA, USA) dual expression plasmid for the generation

DOI:10.14753/SE.2017.2032

of transgenic mice. In these studies the expression of both TRα1 and luciferase were negatively regulated by T_3. This was independent of both the *cis* introduction of TR in the vector and the *trans* expression of TR from another vector. The negative regulation could be observed only in the presence of TRs and only in those vectors that contained the luciferase reporter. All these data above demonstrate that the use of firefly luciferase has its limitations in the T_3-dependent gene expression studies.

DOI:10.14753/SE.2017.2032

3. OBJECTIVES

Regulatory factors of thyroid hormone mediated effects were studied using molecular and cell biological approaches. We focused on the following issues.

I. Investigation of thyroid homone availability in the developing chicken hypothalamus

II. Understanding the RNA-dependent post-transcriptional regulation of the type 2 deiodinase (D2) encoding *dio*2 gene

III. Identification of authentic reporter proteins for studies on T_3-dependent gene transcription

We intended to address the following specific questions:

1. What is the distribution pattern of the D2 mRNA in the developing and adult chicken brain?
2. Do alternative splicing and the D2 5'UTR play a role in the post-transcriptional regulation of D2 activity?
3. Are novel luciferase reporters more accurate to assess T_3-mediated transcriptional changes than the classical firefly luciferase?

DOI:10.14753/SE.2017.2032

4. METHODS

4.1. Animals

Eight-week-old specific pathogen-free White Leghorn chickens and chicken embryos on the embryonic day (E)7, E8, E9, E10, E11, E13, E15 and E17 were obtained from the Central Veterinary Institute and Ceva-Phylaxia (Budapest, Hungary). The incubation was started at E0. Animal tissue samples were collected in accordance with the legal requirements of the Animal Care and Use Committee of the Institute of Experimental Medicine (Hungarian Academy of Sciences, Budapest).

4.2. Generation of expression constructs

4.2.1. Constructs prepared for the analysis of RNA-dependent post-transciptional regulation of D2

The backbone of the chicken D2 (cD2) reporter construct contained a cD2 coding region between *Ecor*I-*Hind*III and a rat D1 minimal SECIS element between *Hind*III-*Not*I (Gereben et al., 1999). Constructs were prepared with different UTR fragments cloned between the *Sac*II site of the D10 vector and *EcoRI*. To generate the cD2 5'UTR construct, *Sac*II-*EcoR*I fragment of the full-length cD2 cDNA was inserted between the corresponding sites of the cD2 reporter. The cORF(Wt)-cD2 construct was prepared by using oligonucleotides: sense tccccgcggG CCGAGAAACA ATGGGATAGC GCgaattcc and antisense, ggaattcGCG CTATCCCATT GTTTCTCGGC ccgcgggga. Oligonucleotides were annealed to generate double stranded DNA. For the cORF(Mut-ATG)-cD2 construct, the following oligonucleotides were used for annealing (sense, tccccgcggG CCGAGAAACA tTGGGATAGC Gcgaattcc; antisense, ggaattcGCG CTATCCCAaT GTTTCTCGGC ccgcgggga). The resulting inserts were cloned after *Sac*II-*EcoR*I digestion into the cD2 reporter. The cDNA encoding the Δ77cD2 protein was isolated as described in Section 4.4. The spliced cDNA was inserted between *Sac*II and *Not*I of the D10 mammalian expression vector using the same approach as described before for the wild-type cD2 mRNA (Gereben et al., 1999). The generated constructs were confirmed by automated sequencing.

4.2.2. Generation of expression constructs for the analysis of luciferase reporters

The thymidine kinase-luciferase (***TK-Luc)*** construct was generated by removing the TRE triplet of *pTRE-TK-Luc* by digestion with *BamH*I and *Bgl*II followed by religation

and confirmation of the final construct by sequencing. The original plasmid backbone, *pTRE-TK-Luc* contains the *thymidine kinase* minimal promoter of the *herpes simplex* virus and was kindly provided by Dr. AM Zavacki (Boston, MA, USA).

The ***TK-(dCpG)Luc*** was prepared using the *TK-Luc* plasmid backbone as follows. The *pMOD Luc-ShS v02* plasmid (InvivoGen, San Diego, CA, USA) was used as a template to amplify the *(dCpG)Luc* coding region with Vent PCR (oligonucleotides: sense, catgcc ATGGAGGATGCCAAGAATATTAAGAA; antisense, ggaattc TTATTTGCCACCCTTCTTGGCCTTGATCA). The amplicon was cut with *Nco*I and inserted into the *Nco*I - and the blunted *EcoN*I sites of *TK-Luc*. The construct was confirmed by sequencing.

The ***TK-NanoLuc*** was prepared by isolating the TRE lacking the minimal *TK* promoter from the *pTRE-TK-Luc* through digestion with *Bgl*II and *Hind*III and subsequent cloning of the released fragment into the corresponding sites of the *pNL1.1* vector (Promega, Madison, WI, USA) followed by confirmation of the final construct by sequencing.

The ***TK-Renilla-Luc*** was generated by truncating the 760 bp-long *TK* promoter of *pRL-TK* (Promega) using *Bgl*II and *Eco*RI digestion followed by blunting with Klenow polymerase and subsequent religation. This resulted in a minimal *TK* promoter between *Eco*RI and *Hind*III that is 31 bp shorter than the 128 bp-long minimal *TK* promoter of *TK-Luc*. 3' to the *TK* promoter, this construct also contains a 136 bp chimeric intron originating from *pRL-TK*. The construct was confirmed by restriction mapping.

The constructs are shown in **Figure 22**.

The ***mouse TRα*** *(mTRα)* expression construct was generated using the *TRαCDM* plasmid (Prost et al., 1998) (kindly provided by Dr. AM Zavacki, Boston, MA, USA) as a template to amplify *mTRα* coding region with Vent PCR (oligonucleotides sense: ggaattccat tATGGAACAG AAGCCAAGCA AGGT, antisense: ataagaatgc ggccgcTTAG ACTTCCTGAT CCTCAAAGA). The amplicon was cut with *Eco*RI and *Not*I and inserted into these sites of a *pCI-Neo* vector (Promega) and confirmed by sequencing.

The secreted embryonic alkaline phosphatase (SEAP) encoding *pSEAP2*-Promoter plasmid (Clontech) was used for transfection control.

4.3. DNA transfections

For studies on the *post-transcriptional regulation of D2* HEK-293 cells were transfected with calcium phosphate precipitation as described earlier (Brent et al., 1989). 10 micrograms of D10 based vector encoding the deiodinase was transiently transfected in the presence of 4 μg D15 helper vector required for the transcriptional activation of the promoter of D10 (Gossen and Bujard 1992). Results are given as the mean ± SEM of D2 activities of duplicate plates of at least three separate experiments as the percentage of the cD2 control.

For the investigation of the *luciferase reporters*, JEG-3 human choriocarcinoma cells (kindly provided by Dr. J. Szekeres, Pécs, Hungary) were cultured in 24-well plates in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). When ~70% confluency was reached, cells were transfected with 800 ng DNA/well (including 200 ng *Luciferase* reporter, 100 ng mouse *TRα*, 10 ng *pSEAP2* and 490 ng *pUC* as inert DNA) using Lipofectamine® 2000 (Life Technologies/Thermo, Carlsbad, CA, USA). After ~6 hours the transfection media was replaced with DMEM containing 10% hormone-free fetal bovine serum (FBS) and incubated for 40 hours (Egri and Gereben, 2014). Briefly, 100 mg charcoal (Sigma, St. Louis, MO, USA) and 50 mg dextran (Sigma) were preincubated overnight in 0.01 mol/l Tris buffer (pH=7.6). After centrifugation 40 ml FBS was added and incubated for 1 hour. The suspension was recentrifugated and the supernatant was used to supplement DMEM. The media was replaced with DMEM with 10% hormone-free FBS containing either 50 nM 3,5,3'-triiodothyronine (+T_3) or NaOH vehicle (-T_3). After 24 hours, the culture media was collected for SEAP measurement (see Section 4.7.2). The cells were washed with phosphate buffered saline (PBS) and harvested in 100 μl Passive lysis buffer (Promega).

4.4. RNA isolation and RT-PCR

For the investigation of the *ontogenic redistribution of D2*, brain samples of E7, E8, E9, E10, E11, E13, and E15 chicken embryos were dissected in duplicates, and total RNA was isolated with Trizol (Life Technologies, Inc.). E13 and E15 brains were separated for telencephalon+diencephalon (A) and brainstem+cerebellum (B) parts. RNA was subjected to first strand cDNA synthesis using an oligonucleotide-dT primer and

amplified with D2-specific primers, as described earlier (Gereben et al., 2002). The cD2 oligonucleotides were as follows (5'-3'): sense, CTG AAT TCA TCC GGC AGA AGA GAG; antisense, AGC TTC TCC TCC AAG TTT GA. The nonquantitative D2 amplification was performed using the following program: 94 °C for 2 min; 35 cycles of 94 °C for 30 sec, 58 °C for 30 sec, and 72 °C for 1 min; and then 72 °C for 4 min.

For the *studies on post-transcriptional regulation of D2* adult chicken telencephalons and livers were used. Total RNAs isolated from the telencephalon and liver were reverse transcribed using the antisense oligonucleotide CTCACCAGAA GGCCTGAAGA G and amplified by Taq polymerase (Sigma) by oligonucleotides, sense: CTGAATTCAT CCGGCAGAAG AGAG; antisense: AGCTTCTCCT CCAAGTTTGA. The amplicons generated on the brain cDNA were cloned into pGEM-T and subjected to automated sequencing. The amplifications were performed in two separate reactions.

4.5. Northern blots

The Northern blot *for the ontogenic D2 distribution study* was performed as previously described (Gereben et al., 1999). Briefly, total RNA was isolated with Trizol from the brains of E7, E8, E9, and E10 and hemispheres of E13, E15 and E17 chicken embryos as described in Section 4.4. A digoxigenin (DIG)-labelled single stranded cDNA probe complementer to 450 bp of the cD2 coding region was used to detect D2 in 30 μg of total RNA. The probe was labelled by linear PCR using the (5'-3') TGCACAATGCACACTCGCTC antisense oligonucleotide and DIG-deoxyuridine 5-triphosphate. As denominator for densitometry, the density of the 28S subunit of ethidium bromide stained gels was used.

4.6. *In situ* hybridization

The technique was used for the *analysis of D2 mRNA ontogenic redistribution* of the brains of E8 and E15 chicken embryos and 8-week-old chickens. The heads of three E8 embryos and the brains of three E15 embryos and three 8-week-old chickens were quickly frozen on dry ice and stored at –80 °C until used. Serial 12-μm-thick coronal sections were cut on cryostat, mounted on gelatine-coated slides, and dried at 42°C

overnight. The sections were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 30 minutes washed in 2-fold concentration of standard sodium citrate (2X SSC), acetylated with 0.25% acetic anhydride in 0.9 % triethanolamine for 20 min; and then treated in graded solutions of ethanol (70, 80, 96, 100%), chloroform and a descending series of ethanol (100, 96%) for 5 minutes each and hybridized with an approximately 840 bp single-stranded DIG-11-uridine 5-triphosphate (Roche Diagnostics GmbH, Mannheim, Germany)-labelled cRNA probe for the entire coding region of cD2. The hybridizations were performed under plastic coverslips in a buffer containing 50% formamide, 2-fold concentration of standard sodium citrate, 10% dextran sulfate, 0.5% sodium dodecyl sulfate, 250 μg/ml denatured salmon sperm DNA, and the DIG-labelled probe, diluted at 1:100 for 16 h at 56°C. The slides were washed in 1X SSC for 15 min and then treated with RNase (25 μg/ml) for 1 h, at 37°C. After additional washes in 0.1X SSC (2 X 30 min) at 65°C, sections were washed in PBS and treated with the mixture of 0.5% Triton X-100 and 0.5% H_2O_2 for 15 min and then with 2% bovine serum albumin (BSA) in PBS for 20 min to reduce the nonspecific antibody binding. The sections were incubated with a mixture of sheep anti-DIG-alkaline phosphatase Fab fragments (1:1000, Roche Diagnostics) overnight at 4°C. The alkaline phosphatase signal was detected using 5-bromo-4-chloro-3-indolyl-phosphate/4-nitroblue tetrazolium chromogen system (Roche Diagnostics) according to the manufacturer's instructions. The reaction was developed for 6 hours, and then, the sections were rinsed in Tris buffer (pH 7.6). The sections were coverslipped using Aquatex mounting medium (Merck, Darmstadt Germany), and the images were taken with an Axiophot microscope (Carl Zeiss Inc, Göttingen, Germany) equipped with real-time spot digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA). For semiquantitative analyses all samples were treated simultaneously. Three 20X field of the hypothalamus of each E15 and adult brain from three different anterior-posterior levels were analysed using ImageJ software (public domain from National Institutes of Health, USA). Background density points were removed by thresholding the image. The sum of integrated density values (density X area) was calculated for each animal. The specificity of hybridization was confirmed using a sense cD2 coding region probe, that resulted in the total absence of specific hybridization signal in the brain at all stages studied.

4.7. Assays

4.7.1. Deiodinase assay

For the investigation of the *ontogenic redistribution of D2*, brain samples of chicken embryos (E7, E8, E9, E10, E11, E13, and E15; n=5) were dissected and rapidly removed. The E13 and E15 brains were separated for telencephalon+diencephalon (A) and brainstem+cerebellum (B) parts. Samples were homogenized in ice-cold PE buffer [100 mM potassium phosphate, 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 6.9)] with 0.25M sucrose and 1 mM dithiothreitol (Gereben et al., 1999) and kept frozen at -80°C until used. The assays contained approximately 150-600 μg homogenate proteins in 300 μl PE buffer [100 mM potassium phosphate, 1 mM EDTA (pH 6.9)] supplemented with various amounts of cold T_4 (1 or 100 nM) and about 30,000 cpm of Sephadex LH-20 purified, labelled T_4 and 20 mM DTT (Larsen et al., 1979). Measurements using 100 nM T_4 were used to confirm the D2 nature of the measured 5' deiodinase activity (Salvatore et al., 1996a). Additional incubations were performed with 1 nM T_4 + 100 nM T_3 + 1 mM 6-n propylthiouracil (PTU) added to the reaction buffer to inhibit D1 and D3 activity. Incubation was carried out at 37°C for 2 hours.

The amount of protein used for assays was set to keep the percent of deiodination between 5 and 30%. The reactions were stopped by adding 200 μl horse serum (Invitrogen, Carlsbad, CA, USA) and 100 μl of 50% trichloroacetic acid for precipitation (Berry et al., 1990). A fraction of supernatant (400 of 600 μl) was applied on ion exchange chromatography through self-made Dowex 50WX (Amersham Pharmacia Biosciences, Uppsala, Sweden) columns to further separate iodine from other thyroid hormone metabolites. The fraction of supernatant containing the iodine but not thyroid hormones was eluted with 2 ml of 10 % acetic acid and counted in a γ-counter (Wizard-1470, PerkinElmer Life and Analytical Sciences, Inc., Boston, MA, USA). Assays were carried out in duplicates at least twice, and the activity level was expressed in femtomoles released (Larsen et al., 1979) per hour per milligram of protein. Total count and background were calculated from several blank tubes containing no homogenate. The Δ77cD2 protein and the cD2 reporter based 5'UTR constructs were expressed in HEK-293 cells and assayed for D2 activity in the presence of 2 nM T_4 according to the method described earlier (Gereben et al., 1999).

4.7.2. Luciferase assay

Luciferase activity was measured from 20 μl cell lysate with the Dual-luciferase Reporter Assay System (firefly luciferase for *pTRE-TK-Luc*, *TK-Luc, TK-(dCpG)Luc*; *Renilla* luciferase for *TK-Renilla-Luc*) as previously described (Zeold et al., 2006). Activity of the *TK-NanoLuc* was determined from 20 μl cell lysate with the Nano-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. Luciferase activity of each transfected well was assayed separately. Measurements with the Dual-luciferase Reporter Assay System were performed in duplicates that were averaged to express luciferase activity of each well. Duplicates were closely agreeing with an average difference of 7%±1.62 and 5.9%±1.2 (mean±SEM; n=14) for $-T_3$ and $+T_3$ groups of *pTRE-TK-Luc*, respectively. All measurements were performed with a Luminoskan Ascent Luminometer (Thermo, Waltham, MA, USA).

4.7.3. SEAP assay

SEAP activity was determined from 25 μl media with Nova Bright™ SEAP Enzyme Reporter Gene Chemiluminescent Detection system 2.0 (Invitrogen/Thermo) as previously described (Egri and Gereben 2014). SEAP was used for normalization by calculating Firefly luciferase (Luc)/SEAP light unit or *Renilla* luciferase (*Renilla*)/SEAP light unit ratios for each well. Experiments were performed at least eight times and presented as mean ± SEM.

4.8. Statistics

Statistical analysis on *deiodinase activity data* were performed using one-way analysis of variance (ANOVA) followed by Newman-Keuls test. The sums of integrated density values of *in situ* hybridization reactions were compared by unpaired *t* test.

Statistical analysis on *luciferase assay data* was performed with an unpaired two-sample *t*-test using a 95% level of confidence.

4.9. Sequences

The sequence of the Δ77cD2 coding region was deposited into the GenBank under accession no. AF401753.

DOI:10.14753/SE.2017.2032

5. RESULTS

5.1. Investigation of thyroid hormone availability in the developing chicken hypothalamus

Our aims were to determine:

- the expression of D2 mRNA in the brain of chicken embryos before and after the onset of the function of the thyroid gland
- thyroid hormone activating capacity of the developing chicken brain reflected by the activity of the D2 enzyme
- cell-type specific distribution of D2 mRNA expression in the brain of embryonic and adult chickens

5.1.1. Assessment of D2 mRNA expression in the developing chicken brain using RT-PCR and Northern blot

The D2 encoding mRNA transcript could be detected at all stages of the studied E7-E15 period with RT-PCR using intron spanning oligonucleotides amplifying the coding region of the mRNA. The telencephalon-diencephalon could be separated from the brainstem-cerebellum in samples of E13 and E15 allowing the isolated analysis of these regions. The D2 transcript could be detected in all studied brain regions (**Figure 11**). The size of the PCR amplicon matched exactly the deducted size calculated from the clones of the wild-type cD2 transcript (GenBank AF125575, Gereben et al., 1999) indicating that no D2 mRNA splice variant was expressed in a detectable amount using a sensitive PCR-based approach during this period of brain development.

DOI:10.14753/SE.2017.2032

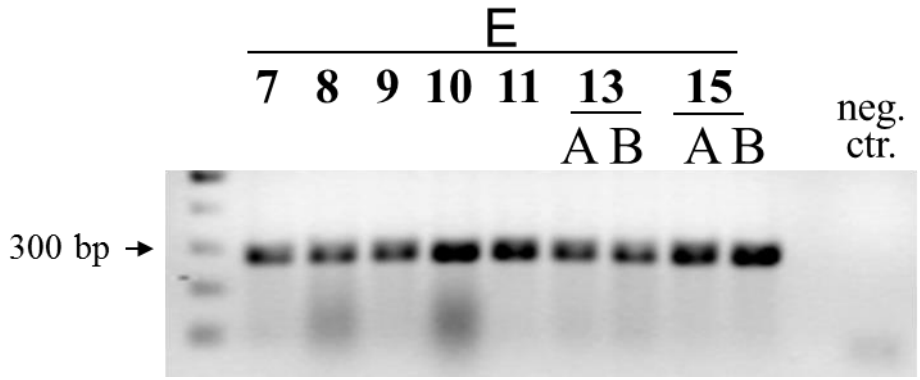


Figure 11. D2 mRNA expression in the developing chicken brain can be detected with RT-PCR. The D2 mRNA was detected with RT-PCR at all stages studied. Only the wild type but not the spliced Δ77cD2 variant transcript (see Section 5.2.1) could be detected. In the negative control (neg. ctr.) amplification was performed in the absence of template by replacing the cDNA with water. E: embryonic day; A: Telencephalon + diencephalon; B: Brainstem + cerebellum, bp: base pairs.

We then used Northern blot to quantify the amount of cD2 mRNA during chicken brain development from E7 to E17. A single transcript of expected size (~6 kb, GenBank AF125575) could be detected from E10 using a digoxigenin-labelled probe specific for the coding region of the cD2 mRNA (**Figure 12**). D2 expression underwent a robust increase during the studied period as represented by elevating D2/28 S density ratios (0.5, 2.1, 5.2, and 7.3 for E10, E13, E15 and E17, respectively) using density of ethidium bromide stained 28S ribosomal RNA fraction as denominator.

DOI:10.14753/SE.2017.2032

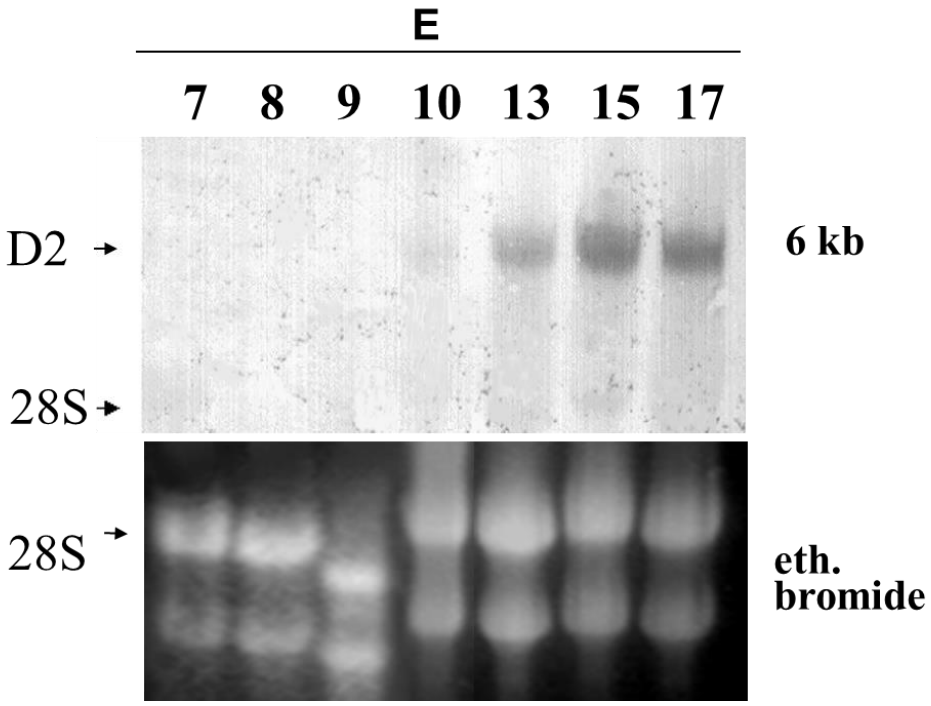


Figure 12. Ontogenic increase of D2 mRNA expression in the developing chicken brain. Northern blot using labelled cDNA probe was used to detect the D2 transcript. The lower panel demonstrates the ethidium bromide stained ribosomal RNA subunits as control for integrity and loading. The D2 mRNA was detected from E10 and increased robustly to stage E17. E: embryonic day, kb: kilobases

5.1.2. D2 activity in the developing chicken brain (E7-E15)

In order to gain a more direct insight into the TH activating capacity of the developing brain, we also measured the activity of the D2 enzyme. D2 activity could be detected from E7 (54 fmol/h/mg). From E13 a significant increase was found (p <0.001 by one way ANOVA followed by Newman Keuls posthoc-test) reaching a maximum of 148 fmol/h/mg at E15 (**Figure 12**). In the E13 and E15 samples (where the telencephalon+diencephalon and brainstem+cerebellum samples could be measured separately), no significant difference was found between D2 activities. In order to confirm the D2-dependent nature of the measured 5' deiodinase activity, we performed a fractional deiodination approach taking advantage from the highly different substrate sensitivity of types 1 and 2 deiodinases. Due to this highly different $K_m(T_4)$, D2 activity can be suppressed by 100 nM T_4 while this does not affect D1 enzyme activity. We found that only a very limited fraction of the measured 5' deiodinase activity could be attributed to D1, since in the T_4 saturation assay the deiodination of [125I]T_4 by the

brain homogenate was heavily suppressed at all investigated stages by the addition of 100 nM cold T_4 (**Table 2**). In addition, when 100 nM T_3 and 1 mM PTU were added to the assays (to exclude D3 activity) deiodination was only moderately affected. The inhibition of 1 nM T_4 outer ring deiodination was tested by adding 100 nM T_3 and 1 mM PTU and found to be slightly lower in E13-E15 samples compared to the E7-E8 ($p < 0.01$ by one way ANOVA followed by Newman-Keuls, **Table 2**). The activity was the highest in E13 and E15 samples (by one way ANOVA followed by Newman-Keuls, $p < 0.01$ when compared with E7 or E8). Thus the presented enzyme activity studies clearly confirmed the presence of authentic D2 activity in the brain of chicken embryos that increased during development.

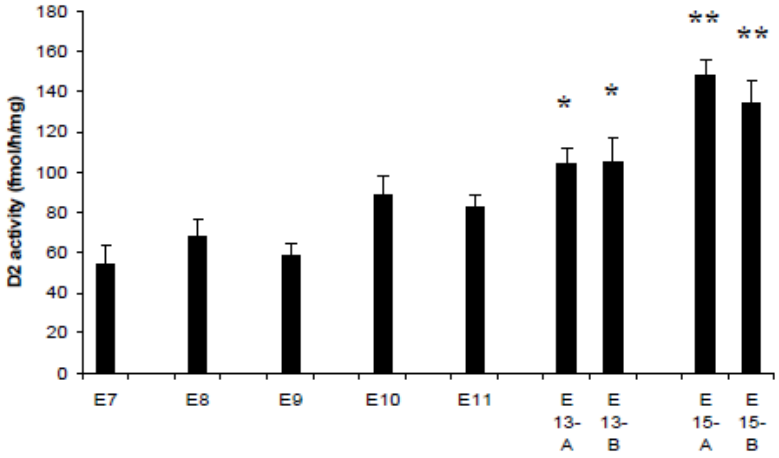


Figure 13. D2 activity in the brain of chicken embryos from E7 to E15. Specific low K_m D2 activity was present in the developing chicken brain from E7 to E15. Activity is expressed as femtomoles of iodine release /hour/mg protein. The increase of D2 activity was highly correlated with time during the whole investigated period (correlation coefficient 0.91, $p < 0.001$). From stage E13 D2 activity was significantly higher, compared with the earliest tested period (one way ANOVA followed by Newman-Keuls, $p < 0.001$). The whole brains of E7, E8, E9, E10, and E11 embryos were used, whereas at E13 and E15, the brains were separated for telencephalon + diencephalon (A) and brainstem + cerebellum (B) parts. *, $p < 0.001$ vs. E7; **, $p < 0.0001$ *vs*. E7 by one way ANOVA followed by Newman-Keuls (mean ± SEM, n = 5).

DOI:10.14753/SE.2017.2032

Table 2. Fractional deiodination in the chicken embryonic brain

	Fractional [^{125}I]T_4 deiodination relative to that at 1 nM concentration (%)	
Samples	**100 nM T_4**	**1 nM T_4 + 100 nM T_3 + 1 mM PTU**
E7	0	67.5 ± 6.7
E8	0.5 ± 0.8	60.9 ± 2.4
E9	1.8 ± 0.5	83.7 ± 6.8
E10	0.7 ± 0.5	67.9 ± 2.5
E11	3.4 ± 0.5	68.2 ± 1.4
E13A	0.7 ± 0.2	72.4 ± 2.8
E13B	3.1 ± 0.5	75.8 ± 3.4
E15A	5.2 ± 0.7	80.8 ± 1.8
E15B	5.1 ± 0.1	85.1 ± 1.5

Fractional inhibition of [^{125}I]T_4 deiodination by different assay conditions. For the given sample, T_4 deiodination at 1 nM concentration represents 100%. Assay conditions are shown *above the columns*. Data are given as mean ± SEM (n = 5). E, Day of incubation; A, telencephalon + diencephalon; B, brainstem + cerebellum.

5.1.3. Distribution of D2 mRNA in the brain of developing chicken

In order to study D2 expression at the cellular level, we used *in situ* hybridization to identify D2 mRNA in the developing brain. In the brain of E8 chicken embryos a rather weak D2 hybridization signal could be observed in scattered cell clusters (**Figure 14A**) using a digoxigenin-labelled probe specific for the D2 coding region. Signal intensity increased in perivascular like cell clusters throughout the brain of E15 embryos compared to the E8 stage (**Figure 14B-D**). No D2 hybridization signal was found in the ependymal cells lining the wall of the third ventricle (**Figure 14B**).

DOI:10.14753/SE.2017.2032

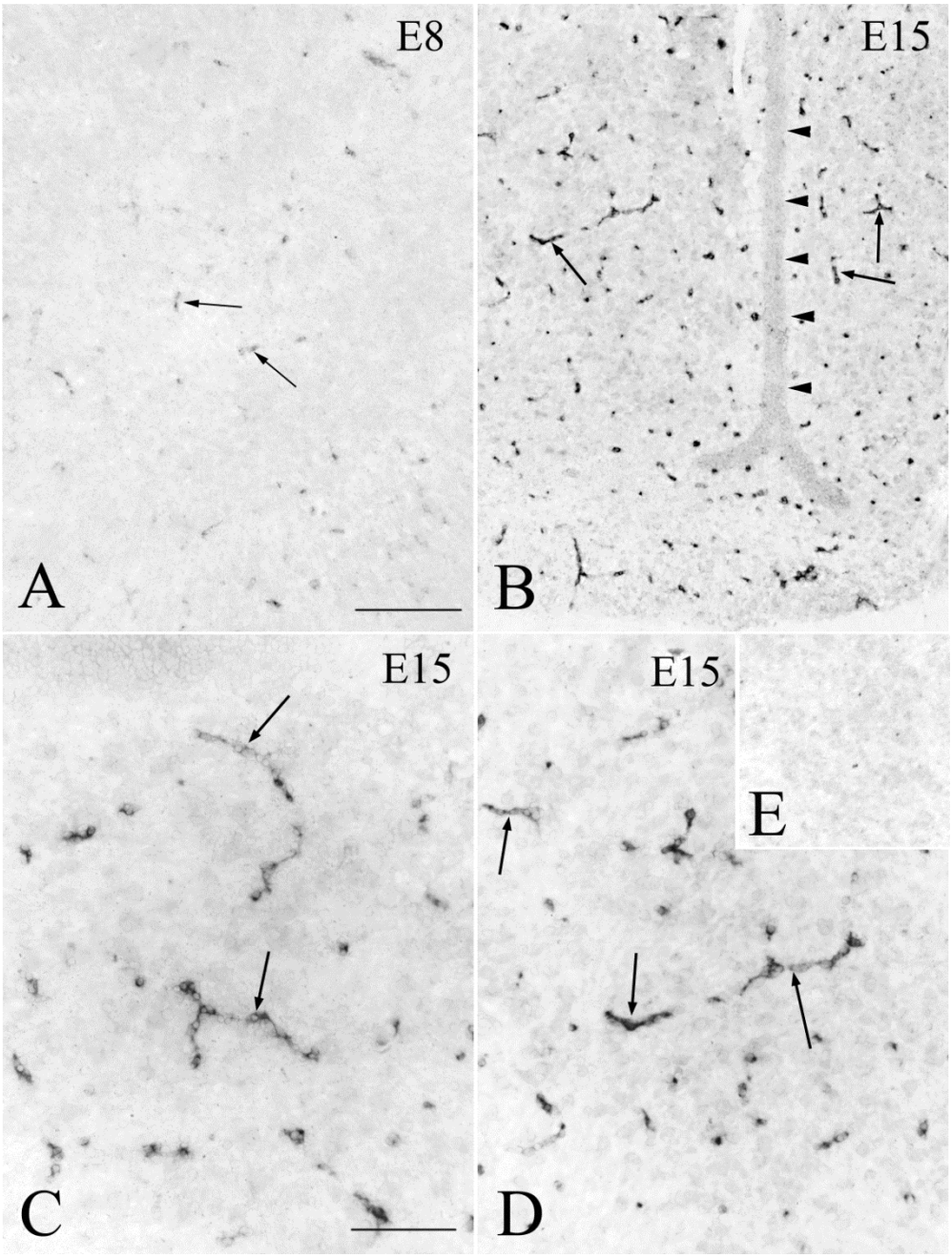


Figure 14. Distribution of D2 mRNA in the brain of E8 and E15 chicken embryos using *in situ* hybridization. The D2 hybridization signal is only very weak in the E8 brain sections (**A**). *Arrows* indicate modestly labelled cell clusters. The hybridization signal is markedly increased in the E15 brains (**B–D**). Low-magnification photomicrograph illustrates the D2 hybridization signal in the E15 hypothalamus (**B**). *Arrowheads* indicate the wall of the third ventricle. Note the lack of hybridization signal in the ependymal layer and the strong signal associated with elongated cell clusters *(arrows)*. Strong hybridization signal in elongated cell clusters *(arrows)* in the E15 neostriatum (**C**) and hypothalamus (**D**). No signal was detected using a sense D2 probe (**E**). *Scale bar*, 200 μm in A corresponds to A and B; scale bar, 100 μm in C corresponds to C–E. E8, E15: embryonic days 8 and 15.

5.1.4. Distribution of D2 mRNA in the brain of adult chicken

In the adult chicken no hybridization signal could be observed in the wall of the rostral part of the third ventricle (**Figure 15A**) and the lateral ventricles. A subset of the ependymal cells lining the floor of the third ventricle at the rostral pole of the median eminence were positive for D2 (**Figure 15B**). However, in the more posterior segment, the D2-expressing cells covered the ventral one half to two-thirds of the ventricular wall (**Figure 15C** and **D**). The distribution pattern of the labelled ependymal cells was reminiscent of that of tanycytes. The D2 hybridization signal in other parts of the brain was in similar cell clusters as in the E15 brains but the intensity of hybridization signal was markedly decreased [E15 *vs.* adult (integrated density units) 15.90 ± 0.23 vs. 3.34 ± 1.23, $p = 0.0043$] (compare **Figure 15A-D** and **G** with **Figure 14**). Strong D2 signal could be observed in isolated cells of the neostriatum (**Figure 15E**, **F**, and **H**).

DOI:10.14753/SE.2017.2032

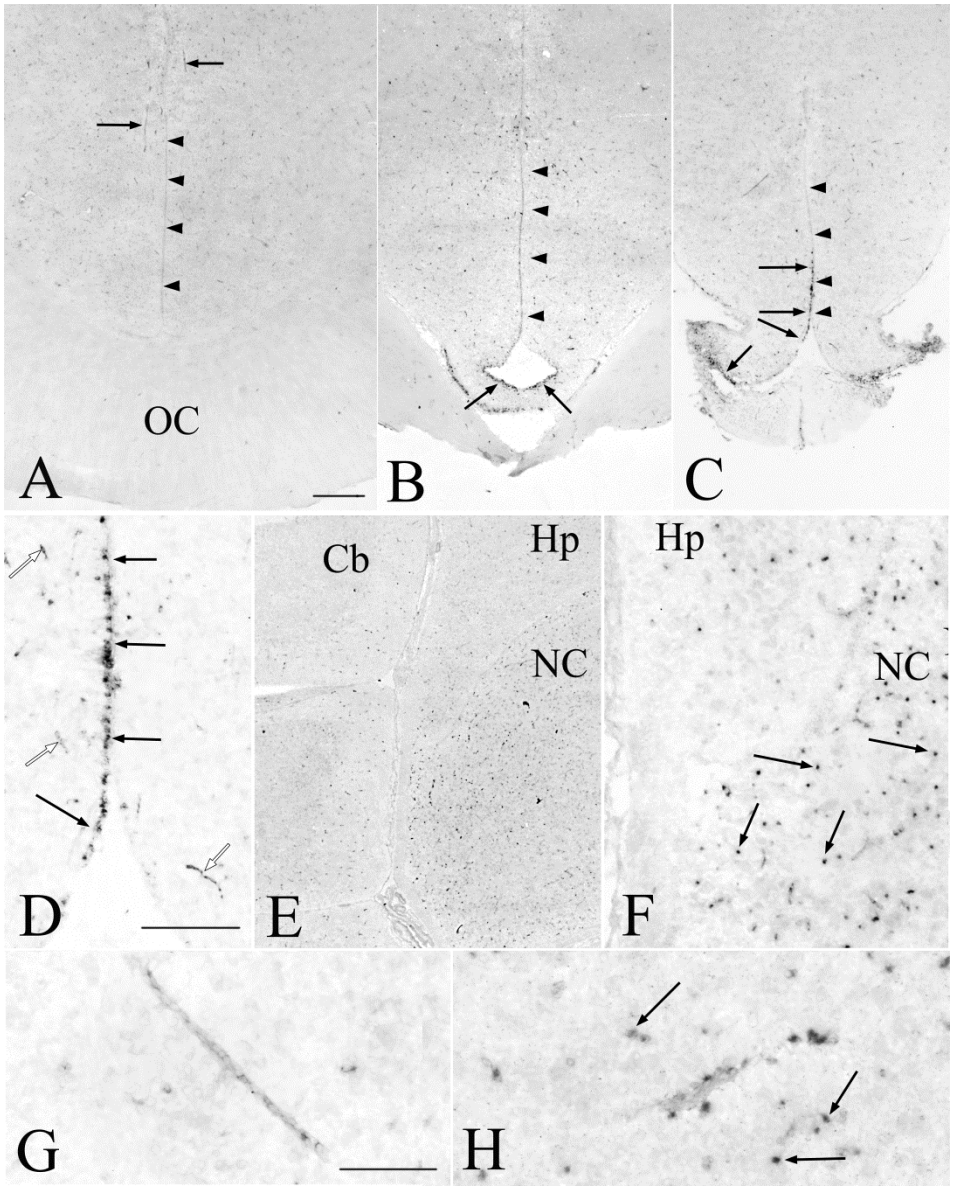


Figure 15. Distribution of D2 mRNA in the brain of adult chicken. The density of D2 hybridization signal associated with cell clusters is markedly decreased in the brain of adult chicken (A–H). **A**, No hybridization signal can be detected in the wall of the third ventricle rostral to the median eminence (*arrowheads* indicate the wall of the third ventricle, whereas *arrows* indicate modest signal in hypothalamic cell clusters). **B**, At the rostral pole of the median eminence, the hybridization signal was localized to the floor of the third ventricle (*arrows*). **C**, More caudally D2-expressing cells covered the ventral half of the ventricular wall (*arrows*). **D**, Higher-magnification micrograph illustrates the localization of D2 mRNA in the ependymal layer of the third ventricle (*arrows*). *Arrows* indicate examples for D2 hybridization signal in the adjacent hypothalamic tissue (*open arrows*). **E**, Low-magnification micrograph demonstrates the D2 hybridization signal in the cerebellum, hippocampus, and neostriatum caudale. **F**, Medium-power magnification of the same region is seen; *arrows* indicate D2 hybridization signal in isolated cells. **G**, High-power image of an elongated cell cluster in the hypothalamus. **H**, The D2 hybridization signal is also present in isolated cells (*arrows*) in the neostriatum. Cb, Cerebellum; Hp, hippocampus; NC, neostriatum caudale; OC, optic chiasm. *Scale bar*, 400 μm in A corresponds to A–C and E; *scale bar*, 200 μm in D corresponds to D and F; *scale bar* 100 μm in G corresponds to G and H.

5.2. Understanding the RNA-dependent post-transcriptional regulation of the type 2 deiodinase (D2) encoding *dio2* gene

Our aim was to analyse the role of mRNA structure in the post-transcriptional regulation of D2 gene in thyroid hormone activation. Specifically, we studied the role of i) the alternative splicing and ii) the 5'UTR of the D2 mRNA in the regulation of D2 activity.

5.2.1. Cloning and characterization of an alternatively spliced chicken D2 encoding transcript

D2 mRNA levels and enzyme activity are discrepant in specific tissues which is strikingly represented by the finding that the same amount of a ~6.1 kb D2 mRNA species results in a 2.6-fold higher D2 activity in the brain than in the liver of chicken (408 *vs.* 156 fmol T_4/h mg protein) (Gereben et al., 1999 and 2002). Therefore, we speculated whether post-transciptional events such as alternative splicing could play a role in the modulation of D2 activity. We hypothesized that a D2 mRNA species of slightly different size that would not appear on a routine screen could impact tissue-specific D2 activity by encoding a D2 protein of altered activity. Therefore, we isolated D2 encoding mRNAs from the telencephalon and liver of adult chickens using RT-PCR. The amplified fragments were cloned into plasmids and subjected to sequencing. This approach allowed us to identify a cD2 mRNA containing a 77-bp deletion in the coding region in the proximity of the exon/intron junction of the D2 encoding *dio*2 gene (**Figure 16A**). The sequence of the novel cD2 splice variant was deposited into the GenBank under accession #AF401753. We subjected the Δ77cD2 encoding mRNA to experimental testing to determine its activity by inserting the spliced coding region into a D10 expression vector 5' to SECIS element. The resulting construct was transiently transfected into HEK-293 cells. Δ77cD2 mRNA encoded an inactive D2 enzyme. The splicing-induced deletion resulted in the resetting of the reading frame of the cD2 coding region. The deduced amino acid sequence of the Δ77cD2 protein indicated a truncated D2 protein that is terminated N-terminal to the active center (**Figure 16B**).

DOI:10.14753/SE.2017.2032

A

```
                701                                                   750
cD2-AF125575    CACATGGTGC TGTTTCTGAG CCGCTCCAAG TCTGCGCGCG GCGAGTGGCG
cD2-clone#1     CACATGGTGC TGTTTCTGAG CCGCTCCAAG TCTGCGCGCG GTGAGTGGCG
cD2-clone#2     CACATGGTGC TGTTTCTGAG CCGCTCCAAG TCTGCGCGCG ..........
hD2 coding      CACGTGGTGC TGCTGTTGAG CCGCTCCAAG TCCACTCGCG GAGAGTGGCG

                751                                                   800
cD2-AF125575    GAGGATGCTG ACCTCGGAGG GGCTGCGCTG CGTCTGGAAC AGCTTCCTCC
cD2-clone#1     GAGGATGCTG ACCTCGGAGG GGCTGCGCTG CGTCTGGAAC AGCTTCCTCC
cD2-clone#2     .......... .......... .......... .......... ..........
hD2 coding      GCGCATGCTG ACCTCAGAGG GACTGCGCTG CGTCTGGAAG AGCTTCCTCC

                801
cD2-AF125575    TGGACGCCTA CAAGCAGGTC AAACTTGGAG GAGA
cD2-clone#1     TGGACGCCTA CAAGCAGGTC AAACTTGGAG GAGA
cD2-clone#2     .......... .......GTC AAACTTGGAG GAGA
hD2 coding      TCGATGCCTA CAAACAGGTG AAATTGGGTG AGGA
                                   ↑
                          ~8 kbp hdio2 intron
```

B

```
    ATGGGTCTGTTAAGTGCGGATTTGCTGATCACGCTTCAGATCTTGCCGGTCTTTTTCTCC
596 ----+---------+---------+---------+---------+---------+----- 655
    M  G  L  L  S  A  D  L  L  I  T  L  Q  I  L  P  V  F  F  S   -

    AATTGCCTCTTCCTTGCGCTGTATGACTCTGTGATCCTCCTGAAGCACATGGTGCTGTTT
656 ----+---------+---------+---------+---------+---------+----- 715
    N  C  L  F  L  A  L  Y  D  S  V  I  L  L  K  H  M  V  L  F   -

    CTGAGCCGCTCCAAGTCTGCGCGCGGTCAAACTTGGAGGAGAAGCTCCGAACTCCAGTGT
716 ----+---------+---------+---------+---------+---------+----- 775
    L  S  R  S  K  S  A  R  G  Q  T  W  R  R  S  S  E  L  Q  C   -

    AATCCACATAGCCAAGGGCAACGATGGCAGCAATAGCAGCTGGAAGAGTGTTGGT
776 ----+---------+---------+---------+---------+---------+ 830
    N  P  H  S  Q  G  Q  R  W  Q  Q  *                          -
```

Figure 16. Sequence analysis of the Δ77cD2 transcript. **A**, Alignment of the coding region of the chicken and human D2 coding regions (GenBank Acc. #AF125575 and U53506, respectively) to the sequence of the cD2. 77 bps (underlined) are missing from the cD2-clone#2. 5' and 3' nucleotides of the spliced region are indicated in bold. A polymorphism of cD2-clone#1 is shown in italics. The insertion site of the h*dio*2 intron is marked by an arrow, kbp: kilobase pair. **B**, Nucleic acid and deduced amino acid sequences of the alternatively spliced cD2 mRNA. The wild-type cD2 amino acid sequence is underlined and bold, the new junction is underlined and shown in bold italics. Numbers show positions in the cD2 mRNA GenBank Acc. #AF125575.

Then we aimed to determine the expression of the Δ77cD2 mRNA in the liver and brain compared to the wild-type cD2 mRNA. We set up a PCR-based detection system that specifically amplified the spliced region to allow simultaneous detection of the wild-type and spliced transcript in the same reaction. This approach can be used for semiquantitative detection of the wild-type and spliced amplicons due to their amplification by the same oligonucleotides in the same PCR (**Figure 17**). Using this system the coexpression of the wild-type and the Δ77cD2 mRNA could be confirmed in the telencephalon and liver of adult chicken. Compared to the wild-type, a higher amount of the splice variant could be detected in the liver, while this ratio was the opposite in the telencephalon (**Figure 18**).

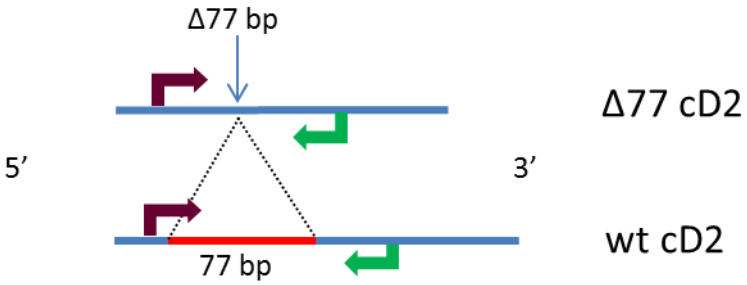


Figure 17. Schematic representation of the RT-PCR screening system allowing simultaneous detection of Δ77cD2 and wt cD2 mRNAs in chicken tissues.

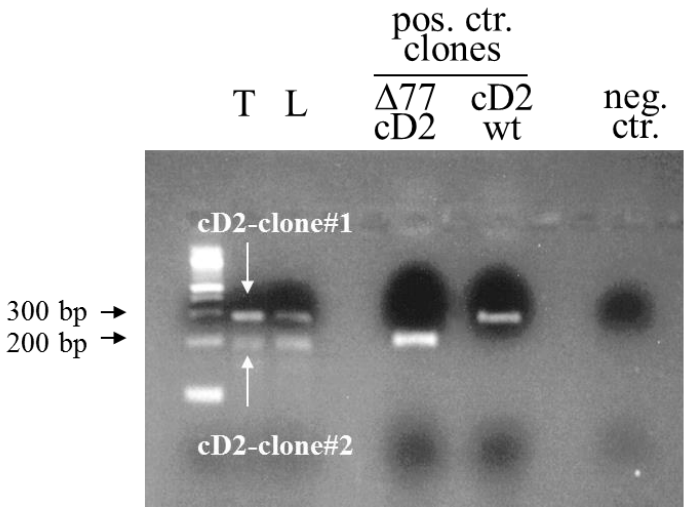


Figure 18. Expression of the Δ77cD2 transcript. Agarose gel electrophoresis of the PCR products from wild-type cD2 mRNA from telencephalon (T) and liver (L) from an adult chicken using oligos described in Section 4.2.1. The expected 277 bp band and a second ~200 bp product were generated. The products indicated by arrows were cloned and their sequences are shown in **Figure 16A** as cD2-clone#1 (wt) and cD2-clone#2 (Δ77cD2). The positive control (pos. ctr.) clones were plasmids containing the wild-type (cD2wt) and spliced (Δ77cD2) cD2 coding regions. As a negative control (neg. ctr.) cDNA was replaced by water in the PCR.

5.2.2. Investigation of the functional role of the 5'UTR of chicken D2 mRNA

The 5'UTR of the D2 mRNA is unusually long and we hypothesized that it could play a role in the regulation of D2 activity. We used a chicken D2-containing reporter to assess whether this mRNA region can modulate the activity of the D2 enzyme in HEK-293 cells. The chicken D2 5'UTR exerted a robust suppressory effect on the activity of the cD2 enzyme by decreasing its activity by 5-fold (**Figure 19**).

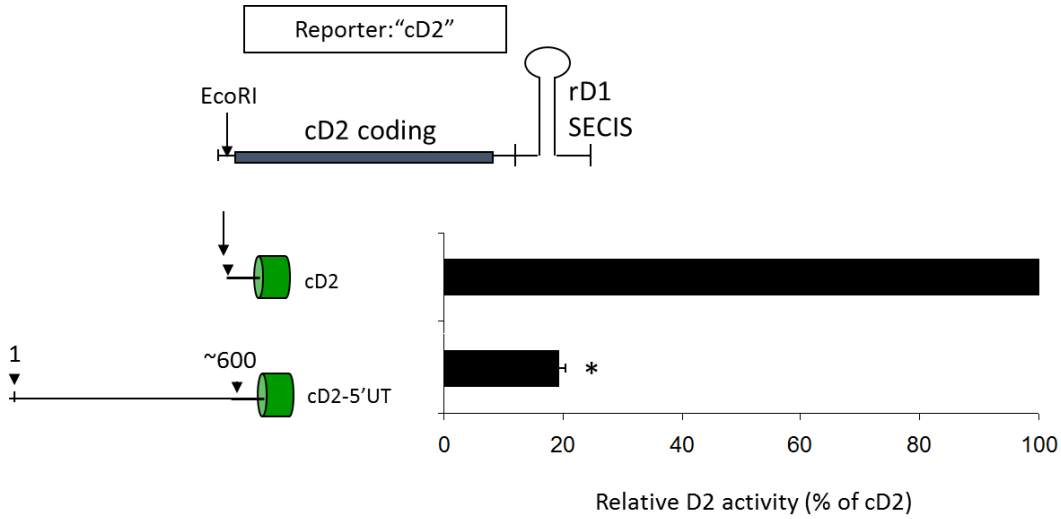


Figure 19. Effect of the chicken 5'UTR on D2 activity. The cD2 5'UTR was inserted 5' to the cD2 reporter containing the cD2 coding region followed by the rat D1 minimal SECIS element. Plasmids were transiently transfected into HEK-293 cells as described in Section 4.3. Chicken D2 5'UTR decreased the activity of the cD2 enzyme by 5-fold. Data are the mean ± SEM of relative D2 activities corrected for transfection efficiency of duplicate plates as a percentage of the activity of the cD2 reporter (n = 3; *, $p < 0.001$ *vs.* cD2 by t-test).

We aimed to understand the molecular mechanism underlying this inhibitory effect. The D2 5'UTR contains sORFs, a feature shared by the known D2 5'UTR of different species. First we performed sequence analysis to determine which of the sORFs contains a -3 purine (A or G) representing the Kozak consensus sequence, a prerequisite of efficient translational initiation in eukaryotes.

DOI:10.14753/SE.2017.2032

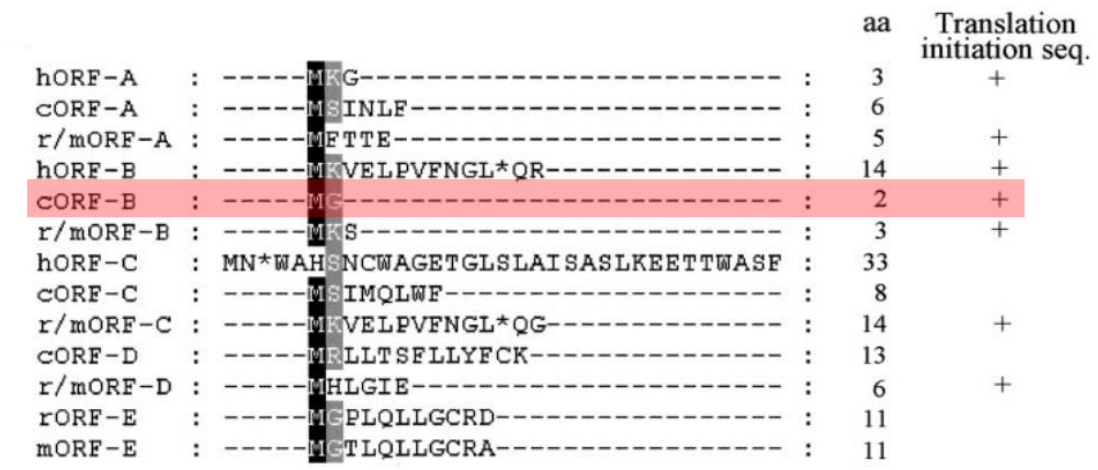


Figure 20. The cD2 5'UTR contain sORFs. The presence of a strong translational initiation sequence (-3 purine base where position 1 is the A of the ATG start codon) is indicated by + (Kozak 1986). Unambiguous stop codons (UAA, UAG) and in frame UGAs followed by purines were considered as translational terminators. In-frame UGAs in possible readthrough position (codon followed by a pyrimidine base) are indicated by an asterisk (McCaughan et al., 1995). Deduced amino acid sequences of the putative peptides are presented.

Sequence analysis revealed that among the four sORFs of the chicken 5' UTR only the second sORF from the direction of the transcriptional start site (cORF-B) met the set criterion (**Figure 20**). Therefore, we functionally tested the inhibitory potency of the isolated cORF-B on D2 activity using the abovementioned expression system in HEK-293 cells. The cORF-B caused a 2.5-fold suppression in D2 activity. Importantly, a point-mutation evoked deletion of the ATG initiation codon completely abolished the cORF-B-dependent inhibition of D2 activity (**Figure 21**). This finding proved that translational initiation occurs at the cORF-B and as a consequence this mechanism is involved in the 5'UTR-dependent decrease in D2 activity.

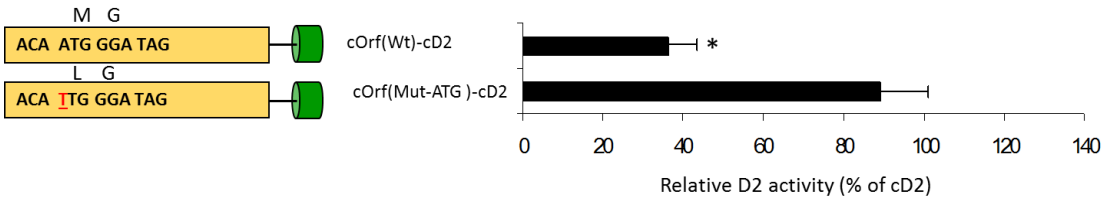


Figure 21. Role of cORF-B related translational initiation in the 5'UTR-dependent regulation of D2 activity. The cORF-B sequence was inserted into the cD2 reporter and transiently transfected into HEK-293 cells. The cORF-B caused a 2.5-fold suppression in D2 activity. ATG was mutated to TTG as shown in red. The point mutation completely abolished the cORF-B-dependent inhibition of D2 activity. Data are the mean ± SEM of relative D2 activities of duplicate plates as a percentage of the activity of the cD2 reporter (n = 3; * $p<0.05$ *vs.* cORF-B(Wt)-cD2 by unpaired t-test).

DOI:10.14753/SE.2017.2032

5.3. Identification of authentic reporter proteins for studies on T_3-dependent gene transcription

The T_3-evoked promoter independent downregulation of firefly luciferase is a phenomenon that undermines studies on TH-dependent transcriptional regulation. Our aim was to find different luciferase reporters that are less susceptible to promoter independent T_3-induced downregulation compared to the classical firefly luciferase. Mammalian expression constructs were generated containing the same minimal TK promoter as shown in **Figure 22**. This allowed to perform the experiments with the promoter type that proved to be unaffected by T_3 treatment (Tillmann et al., 1993). In addition, this harmonized promoter design helped to eliminate differences in promoter-dependent T_3-response of different constructs.

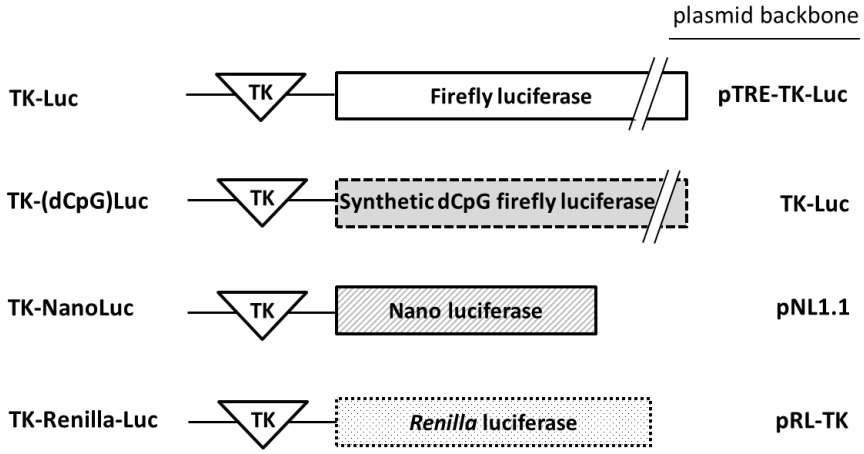


Figure 22. Schematic depiction of the generated mammalian expression constructs encoding different types of luciferase reporters driven by a minimal *thymidine kinase* (TK) promoter. The harboring plasmid backbone is indicated. The *firefly luciferase* cDNA of *TK-Luc* was replaced with the coding region of *dCpG firefly luciferase*. Thus, *TK-Luc* and *TK-(dCpG)Luc* are identical except for the regions encoding luciferase.

As controls, we used JEG-3 cells transfected either with *pTRE-TK-Luc* or with *TK-Luc firefly luciferase* in the presence of co-transfected mTRα. The luciferase activity of the *pTRE-TK-Luc* was induced approximately 3-fold with 50 nM T_3, while as expected, the same treatment suppressed luciferase activity of the no canonic TRE-containing *TK-Luc* reporter ~2.6-fold (**Figure 23**). We also tested the responsiveness of the SEAP construct used to monitor transfection efficiency. Importantly, T_3 treatment did not affect the level of the SEAP internal control (SEAP level in *pTRE-TK-Luc* wells -T_3: 0.97± 0.078 *vs.* +T_3: 0.79±0.084) /mean±SEM; n=14, p=0.13 with t-test/; SEAP level in *TK-Luc* wells -T_3: 0.86±0.105 *vs.* +T_3: 0.76±0.057 /mean±SEM; n=8, p=0.4 with t-test/).

DOI:10.14753/SE.2017.2032

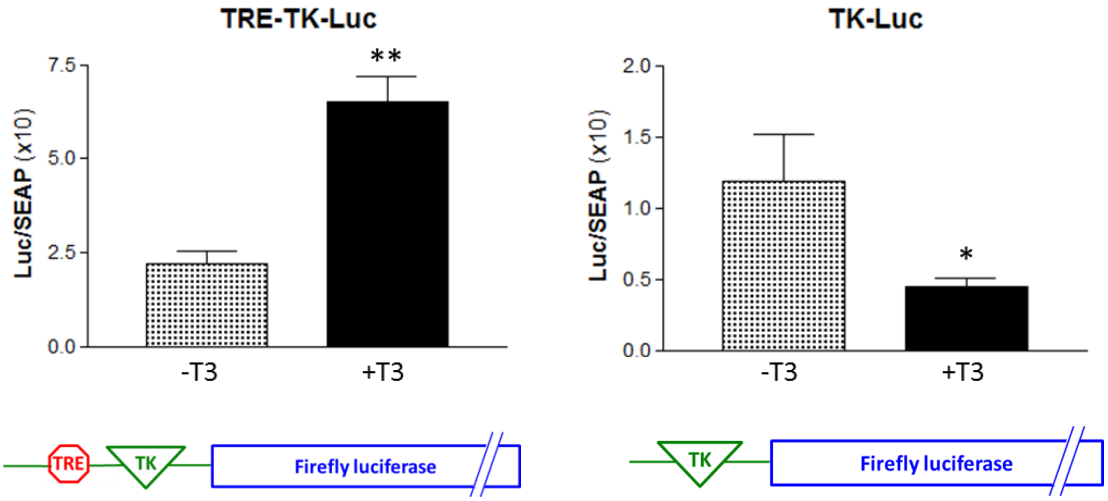


Figure 23. Response of *pTRE-TK-Luc* and *TK-Luc* control firefly luciferase reporters to 50 nM T_3 in JEG-3 cells. Reporters were co-transfected with mouse TRα. T_3 induced the luciferase activity of the *pTRE-TK-Luc* around 3-fold ($n = 14$; $p < 0.001$). The activity of *TK-Luc* was suppressed by 2.6-fold ($n = 8$; $p < 0.05$). Results are expressed as *firefly luciferase* (Luc)/SEAP light unit ratios as mean ± SEM.

Using this characterized system, we tested the responsiveness of various luciferase reporters to T_3 using the generated constructs that did not contain TRE in the canonical position 5' to the promoter sequence. Notably, the *TK-(dCpG)Luc* encoding a synthetic firefly luciferase was not affected by the addition of 50 nM T_3. The luciferase activity of the *TK-NanoLuc* showed a slight tendency of decreased activity, but it did not reach the level of statistical significance (**Figure 24**). On the other hand, T_3 treatment significantly decreased (by approximately 30%) the activity of cultures expressing the TK-*Renilla*-Luc (**Figure 24**).

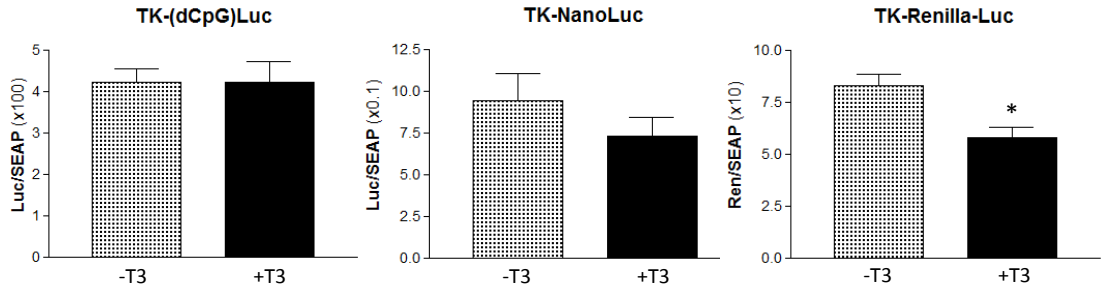


Figure 24. Response of *TK-(dCpG)Luc*, *TK-NanoLuc* and *TK-Renilla Luc* luciferase reporters to 50 nM T_3 in JEG-3 cells. Reporters were co-transfected with mouse TRα. T_3 did not significantly affect luciferase activity encoded either by the *TK-(dCpG)Luc* ($n = 28$; $p = 1$) or *TK-NanoLuc* ($n = 12$; $p = 0.27$). T_3 significantly reduced the activity of the *TK-Renilla-Luc* reporter ($n = 11$; $p < 0.005$). Results are expressed as firefly, Nano, or *Renilla* luciferase (Luc, NLuc, or Ren, respectively)/SEAP light unit ratios as mean ± SEM.

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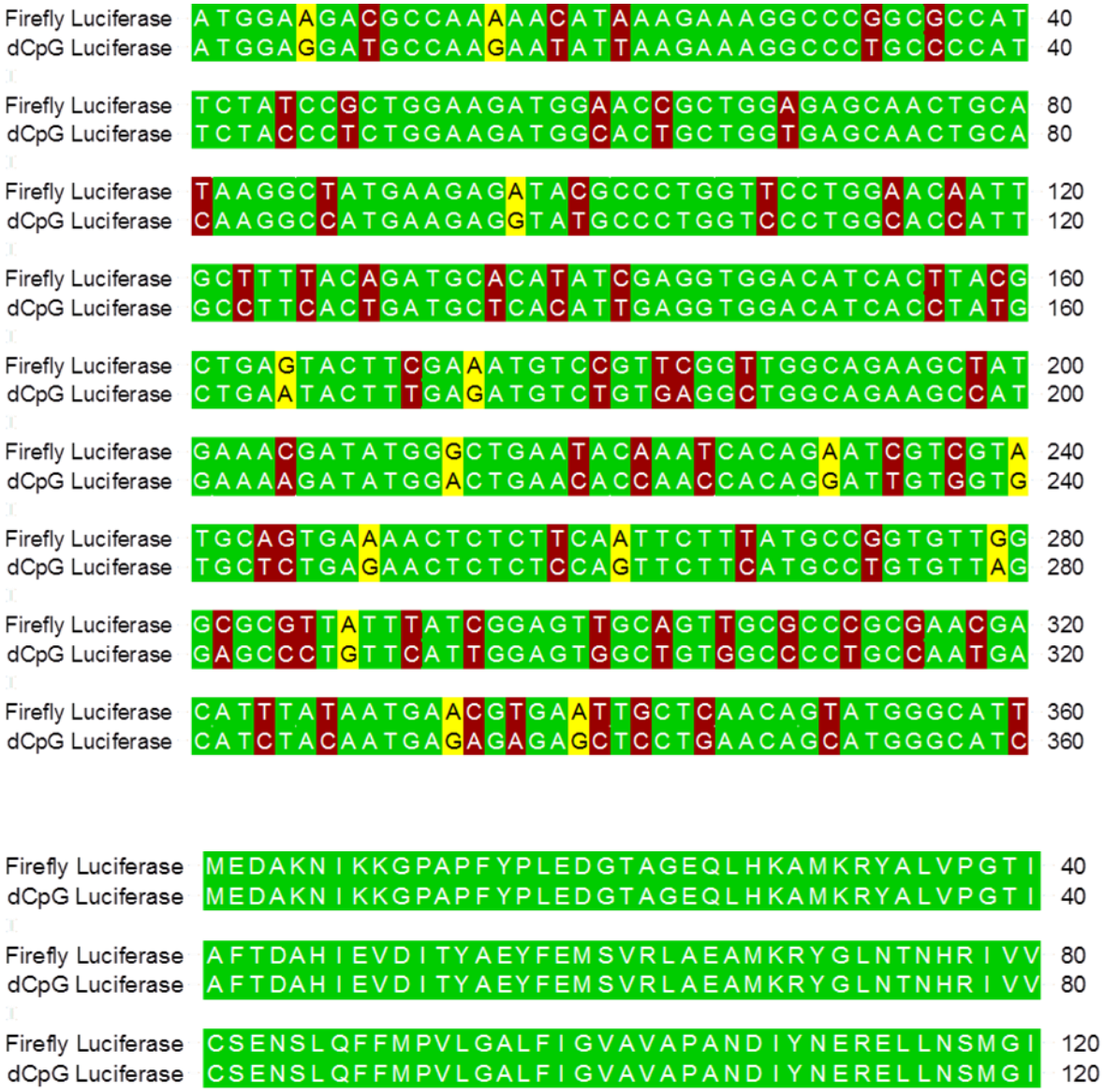

Figure 25. Alignment of the 5' 360 bp-long fragment of the coding region of *firefly luciferase* to the *(dCpG) Luciferase*. The DNA sequences of the full coding regions are only 77% identical (upper panel) but due to the silent nature of mutations they encode the same amino acid sequence (lower panel).

DOI:10.14753/SE.2017.2032

6. DISCUSSION

6.1. Investigation of thyroid hormone availability in the developing chicken hypothalamus

TH promotes cell differentiation during development by decreasing cyclin-D1 levels and inducing the expression of Nerve Growth Factor, thus consequently TH inhibits proliferation and promotes differentiation in the developing brain at the same time (Furumoto et al., 2005; Alvarez-Dolado et al., 1994). Therefore, the tight regulation of T_3 levels is one of the events essential to keep cell proliferation and differentiation under strict control. We aimed to study how D2, the enzyme responsible for TH activation is expressed in different brain regions during chicken development. In contrast to rodents, chickens have a relatively well developed HPT axis at hatching which makes the kinetics of their axis development similar to that of humans (Taylor et al., 1990). TH is already present in the egg yolk as maternal hormone supply thus the substrate of the D2 enzyme is available (Prati et al., 1992). Furthermore, TRα is already expressed in the neural plate and tube well before the blastula stage that would allow TH action taken that activated TH is present (Forrest et al., 1991).

Studies on the developmental regulation of TH availability in human embryos face ethical limitations. While no models are without shortcomings, still chickens provide a more useful approach with human relevance than rodents with respect to gain insight into the mechanisms regulating the TH economy in the developing brain. We addressed D2-mediated TH activation from different perspectives. RT-PCR was used to detect the presence of the D2 transcript in the developing chicken brain which allowed the detection of D2 expression from as early as E7. This was followed by Northern blot assisted quantification that revealed increasing D2 expession in the brain during ontogeny. The detected D2 mRNA encoded a functional enzyme as we demonstrated it by deiodinase assays. We also studied the fractional deiodination in the brain of chicken embryos to exclude the presence of D1 or D3 in the detected activity. The used 100 nM T_4 saturation assay took advantage of the low K_m nature of D2 activity. The finding that the overwhelming majority of 5' deiodinase activity of the developing chicken brain was suppressed by 100 nM T_4 demonstrated that the detected activity is authentic D2.

We also performed PTU inhibition, a drug that inhibits D1 but not D2, while T_3 excess was used to exclude D3 activity (Bianco et al., 2014). These results proved that D2 is

the predominant deiodinase enzyme in the developing chicken brain. The presence of D2 activity at early stages of chicken brain development is in accordance with findings that the cortex of the human fetus is able to perform D2-mediated TH activation already after 7-8 weeks of development (Chan et al., 2002). It was also demonstrated, that even in older chicken embryos (from E16 to hatching) D2 is the predominant activating deiodinase in the developing chicken brain (Van der Geyten et al., 2002).

Our data indicating a fractional decrease of 100 nM T_3 evoked suppression during the investigated period suggested that D3 activity decreased during development. This finding is in good agreement with previous data demonstrating lower D3 activity in the brain of hatched chickens compared to the second half of embryonic development (Reyns et al., 2003). More work need to be done to understand which factors can directly initiate D2 mRNA expression during brain development. It was recently suggested that Nkx2.1 could also play a role in this process (Mohácsik et al., 2016). Our Northern blot study indicated an increase in the D2 mRNA levels from E10. It should be noted that this approach is far less sensitive than PCR which clearly explains the difference between the appearance of D2 signals observed with the two distinct approaches. The appearance of the D2 signal in an amount detectable with Northern blot coincided with the onset of thyroidal secretion on E9.5 (Thommes et al., 1977) and it is in parallel with the period of intense glial cell proliferation (Rogers 1995). To better understand D2 expression at the cellular level during brain development, we used *in situ* hybridization in E8, E15 and adult chicken brains. D2 expression was found in elongated cell clusters with an appearance resembling perivascular cells. Since D2 is a glial enzyme (Mohácsik et al., 2011) we speculate that these D2 expressing cell clusters could be formed by perivascular astrocytes. D2 expression in this compartment increased from E8 and E15 that demonstrates with an independent approach that D2 expression increases during brain development. Importantly, we did not observe the presence of the Δ77cD2 alternatively spliced D2 splice variant in the developing brain indicating that the entire amount of the expressed D2 mRNA is capable of encoding a functional D2 enzyme at these developmental stages.

Remarkably, we observed D2 expression in the wall and the floor of the third ventricle of adult chickens in a distribution pattern reminiscent of tanycytes. Since D2 expression was earlier found in tanycytes of the rodent hypothalamus (Tu et al., 1997), our data

indicated that D2 expression, which is phylogenetically conserved in this cell type, plays a crucial role in the regulation of the HPT axis.

As already highlighted in Section 2, the onset of the negative feedback regulation of the HPT axis is poorly studied despite the fundamental impact of this phenomenon that persists throughout the entire lifespan. Interestingly, there is a gap between the onset of HPT feedback and the appearance of important factors of TH signalling. This suggests the need for a missing limiting factor. In chickens, the thyroid gland is functional from E9.5 while the anterior pituitary starts the stimulation of the secretion of the thyroid gland from E11.5 (Thommes et al., 1977). TRH is expressed well before the formation of the hypothalamus from E4.5 in the infundibulum and its level increases from E16 during the embryonic development (Thommes et al., 1983; Geris et al., 1998). In addition, TRβ2 receptors required for TH action in the PVN can already be detected from E14 in the chicken brain (Grommen et al., 2008). Recently, TH injection studies in chicken embryos demonstrated that the onset of negative feedback evolves from E19 until P2 in a D2-dependent manner (Mohácsik et al., 2016) suggesting that the intense, coordinated increase of D2 in the hypothalamus is the most important factor in the creation of the T_3 gradient. Our data demonstrated that the increase of D2 activity, which builds up the T_3 concentration required for hypothalamic feedback, starts already in the first half of embryonic development in chicken. On the other hand, D2 expression in tanycytes at embryonic stages did not exceed the detection level in our experiments that can be well explained by the lower sensitivity of a DIG-labelled cRNA probe compared to 35S-labelled probes. However, the question of the presence or absence of D2 expression in developing tanycytes between E8 and E15 is not a really relevant one in light of novel data demonstrating that not the tanycytic appearance of D2 but rather the increasing hypothalamic T_3 gradient is the key factor in the onset of TH-mediated negative feedback (Mohácsik et al., 2016).

In summary, we provided evidence that during brain development there is a profound ontogenically regulated increase in D2-mediated TH activation accompanied with ontogenic changes in the D2 mRNA expression. The developing chicken brain is able to generate T_3 locally via D2 well before the onset of the thyroidal secretion. This challenges the dogma of minimized TH action in the proliferative developmental stages.

6.2. Understanding the RNA-dependent post-transcriptional regulation of the type 2 deiodinase (D2) encoding *dio2* gene

Typically, the increase of an mRNA results in increased level or higher activity of the encoded protein. In the case of the D2 enzyme this correlation is far less tight. While D2 mRNA level and D2 activity increase in parallel by adrenergic mechanisms in the pineal gland or during its development (Tanaka et al., 1986; Kamiya et al., 1999; Campos-Barros et al., 2000), in other tissues a remarkable complexity of D2 regulation can be observed manifested in discrepancies between the amount of D2 mRNA and D2 enzyme activity. For example, the same amount of a ~6.1 kb D2 mRNA species results in 2.6-fold higher D2 activity in the brain than in the liver of chicken (408 *vs.* 156 fmol T_4/h mg protein) (Gereben et al., 1999). This phenomenon also occurs under pathological conditions. D2 activity ratios reflecting activity in hyperfunctioning human thyroid adenomas *vs*. normal tissues is ~5-fold in contrast to the 3-fold ratio of D2 mRNA expression of the two types of thyroid tissues (Murakami et al., 2001).

Therefore, we speculated whether post-transciptional events such as alternative splicing, a process known to interfere with protein encoding capacity of a given D2 mRNA could play a role in the modulation of D2 activity. The D2 mRNA is unusually long, it is ~6-7 kb in chicken and human, respectively (see Section 2.3). At least three types of mRNA transcripts differing by 500-700 nucleotides were detected in human thyroid, brain and other tissues (Croteau et al., 1996; Salvatore et al., 1996b; Bartha et al., 2000). However, the length of these mRNAs makes the identification of small changes quite difficult, despite their potential impact on the activity of the encoded protein. We speculated whether tissue-specific alteration in the ratio of D2 mRNA and activity could be underlain by the tissue-dependent presence of a D2 mRNA species which has a slightly different size and encodes an inactive D2 protein. We could identify a novel, alternatively spliced D2 transcript in the chicken brain and liver that encodes an inactive D2 protein. The mRNA encoding this inactive D2 protein lacks 77 nucleotides in the coding region. The deletion is adjacent to the conserved exon/intron junction of the D2 encoding *dio*2 gene indicated by the finding that the 3' end of the deleted region maps exactly to the exon/intron junction of the *dio*2 genes of human and mouse (**Figure 16**) (Bartha et al., 2000; Davey et al.; 1999; Song et al., 2000). Based on our PCR results, both the wild type and the Δ77cD2 transcripts are expressed in the chicken

telencephalon and liver. The different ratio of the two transcripts may account for the different D2 activity and mRNA ratios.

Alternative transcripts were also detected in human tissue samples, e.g. in thyroid tissue. However, in this case instead of deletion of nucleotides the insertion of a 108 bp long intronic region could be observed after codon 74 (Gereben et al; 2002). In contrast to Δ77cD2, the reading frame remained unchanged in the hD2+108 human D2 transcript. Interestingly, the derived hD2+108 protein is also inactive but this was achieved via a splicing mechanism different from the one generating the Δ77cD2 mRNA.

Other human D2 splice variants were also observed (GenBank Accession#AB041843) from a human cell line ECV304 (Ohba et al., 2001) and found to be present in the human brain, lung, kidney, heart and trachea. A truncated D2 coding region followed by the 3'UTR was also identified in a mouse cochlear cDNA library (GenBank AF177197) (Campos-Barros et al., 2000). In this case the divergence point was 35 bp 3' to the conserved exon/intron junction. No details are known about the translation of this transcript. In the human thyroid an alternatively spliced intron of the 5'UTR of human D2 was described (Bartha et al., 2000).

Our findings and the cited examples indicate that alternative splicing is an important mechanism to regulate the D2-mediated TH activation and tissue specific differences in the ratio of wild-type and spliced D2 transcript could affect D2 activity expressed by a specific amount of D2 mRNA. This phenomenon is phylogenetically conserved; it gives rise to differently spliced D2 mRNAs via different mechanisms. Further studies will be required to explore whether D2 alternative splicing can be regulated in an inducible manner or it is programmed to allow a tonic adjustment of D2 mRNA level in a given tissue.

In addition to the alternative splicing of the D2 mRNA we also tested how its unusually long 5'UTR impacts activity of the D2 enzyme and demonstrated that this region of the D2 mRNA massively suppress D2 activity. We aimed to understand the mechanism underyling this suppressory effect therefore studied the role of sORFs embedded in the 5'UTR region. 3-5 sORFs can be found in the 5'UTR of D2 mRNAs of different species including chicken, human, rat and mouse (**Figure 20**). In eukaryotes the presence of the Kozak consensus sequence is required to surround an ATG codon to

allow efficient translational initiation (see Section 2.7.2). We used this as a selection criterion to find sORF that can be potentially translated. Among chicken sORFs, only the second sORF (sORF-B) fulfilled this criterion, therefore, this was selected for functional studies. The sORF-B was able to cause a 2.5-fold decrease of D2 activity but only in the case when its initiator ATG was intact indicating that translational initiation occurs at the sORF-B. Thus the cORF-B in the 5'UTR serves as a translational roadblock to keep the translation of the D2-encoding reading frame low. This roadblock works via compromising ribosomal scanning of the D2 mRNA. Upon the small 40S ribosomal subunit reaches the initiator ATG of the sORF-B in the 5'UTR, it recruits the large 60S subunit to bind the complex and evoke the translational initiation. After translation of the sORF-B the ribosomal complex falls apart and only a limited number of 40S ribosomal subunits will be heading for further scanning of the more 3' located reading frame encoding the D2 enzyme. The gain of this mechanism is manifested in the facilitation of tight regulation of the catalytically highly active D2 enzyme. While the sequence and the appearance of the sORF encoded peptide seems to be unrelevant for the cells, the initiation process itself plays an important role in D2 regulation. We can conclude that the D2 mRNA behaves in a functionally polycistronic manner. It is presently unknown whether 5'UTR based suppression of D2 activity can be modulated by specific factors or conditions.

The 5'UTR and sORF based regulation is not unprecedented but uncommon and sORFs can be found only in 10% of the 5'UTR of vertebrate mRNAs (Kozak 1987). A few examples are represented by the Angiotensin II type IA receptor (Mori et al., 1996), the β2 adrenergic receptor (Parola and Kobilka 1994) and HER-2 receptor (Child et al., 1999). Among these the 5'UTR of the β2 adrenergic receptor is relatively well characterized and shows some similarity with D2 mRNA. Still, the sORF-based control is poorly understood in higher vertebrates. 5'UTR based translational control was also observed in yeasts and viruses (Hinnebusch 1994; Jackson and Kaminski 1995). It was also suggested that beyond sORFs other factors e.g. a secondary structure may also account for the translational inhibition (Kozak 1986).

In summary, our results confirmed that the alternative splicing of the D2 mRNA and the sORF based translation inhibition of the 5'UTR control the activity of D2 enzyme. It also needs to be kept in mind that beyond the studied posttranscriptional events

important post-translational mechanisms are also involved in the complex regulation of D2 protein level and activity. Importantly, the substrate-induced ubiquitination of the enzyme leads to rapid proteasomal degradation by reducing the half-life of the D2 protein (Gereben et al., 2000; Gereben et al., 2015). The multiplicity of regulatory mechanisms for D2 protein indicates that there is meticulous control of the deiodinative activation of T_4 to T_3. Therefore the correlation between D2 mRNA and activity is often weak and interpretation should be handled with care.

6.3. Identification of authentic reporter proteins for studies on T_3-dependent gene transcription

TH can impact gene transcription both in a positive and negative manner (see Section 2.6). While mechanism of T_3-mediated positive regulation of gene expression is well understood, the mechanism of T_3-mediated repression is more ambiguous and several molecular models exist to address this phenomenon. Studies on the effect of TH on a specific promoter are typically studied by luciferase reporters in transient expression systems. This is a fundamental approach to study the TH-mediated regulation of a specific gene. The assay is based on the fact that TH act predominantly via nuclear receptors and binding of T_3 to TR can either activate or repress the transcription of the target gene. In reporter assays it is crucial that the reporter itself should not be affected by the factor to be studied. Importantly, it has been demonstrated that the firefly luciferase reporter protein is downregulated by T_3 in a promoter and plasmid vector independent manner (Tillman et al., 1993; Maia et al., 1996). This phenomenon significantly undermines the accuracy of T_3 dependent luciferase assays therefore we aimed to develop a working alternative to overcome this problem.

It was revealed that T_3-mediated downregulation of luciferase remained constant even in different vectors if the same thymidine kinase promoter fragment was used. Applying mutant receptors revealed that the suppressive effect of T_3 was not observed if the DNA binding domain of the receptor was inactivated proving that the phenomenon is TR-dependent. In parallel, usage of mutant promoters with modified TR binding sites also justified that the suppression remained. Simian virus (SV) promoters were also involved in the investigation but the negative effect after T_3 addition remained unchanged. After further analysis of the effect of strong cis-linked positive TREs on the luciferase activity it was proven that only strong positive TREs are able to diminish the negative effect of

T_3 and TR on the luciferase activity (Tillman et al., 1993). In other studies, the effect of unliganded thyroid hormone receptors on luciferase activity was tested in HEK-293, COS-7 and JEG-3 cells and while the phenomenon was not cell-line dependent, JEG-3 cells proved to be the most sensitive to T_3-mediated downregulation (Maia et al., 1996). It was concluded that a negative TRE should exist in the luciferase coding region. Similarly to Tillman's group they also drew the conclusion that for studies on TH action the classical firefly luciferase does not provide an accurate reporter (Maia et al., 1996). The importance of this problem was also underlined by a cautionary note (Chan et al., 2008) regarding the use of pBi-L dual expression plasmid for the generation of transgenic mice (Clontech) and the vector was also suggested to contain an unwanted and not identified negative TRE. This negative regulation could be observed only in the presence of TR and only in vectors that contained luciferase marker. This phenomenon did not show cell line-dependent features. It was suggested that each individual vector should be evaluated on a case by case basis before applying them in TH-related studies. The suitability of the pGL-2 reporter series (Promega) for transient T_3-dependent expression studies was also questioned (Liu and Brent 2008) as the pGL-2 version contains numerous potential transcription sites.

Since the luciferase reporter based assay is a widely used, convenient and extremely sensitive non-radioactive approach, we attempted to identifiy alternative luciferase reporters lacking the susceptibility of T_3-mediated downregulation. Despite the well characterized positive TREs only very limited information is available regarding the negative TREs. Negative TRE was described in the TSHβ subunit (Shibusawa et al., 2003), in the CD44 (Kim et al., 2005) and CYP7A1 genes (Drover et al., 2002), but these vary in DNA sequence, position and configuration thus computer-assisted prediction of negative TREs is not feasible based on sequence data. Therefore, we performed the experimental testing of the T_3-responsiveness of various luciferase reporters.

Previously, the herpes simplex TK minimal promoter was proven to be unaffected by T_3 (Tillmann et al., 1993) therefore we designed the constructs with the same minimal TK promoter. We used JEG-3 human choriocarcinoma cell line, as it was shown in former transient expression studies to be the most sensitive cell line for T_3-mediated downregulation of the firefly luciferase gene (Maia et al., 1996). We set up controls by

using the pTRE-TK Luc that is upregulated after T_3 treatment and TK-Luc where we removed the classical TRE and observed the expected up and downregulation after T_3 treatment, respectively. We used this controlled system to test the response of various luciferase proteins to T_3. Testing of the dCpG luciferase revealed a complete resistance to T_3-mediated downregulation. The dCpG luciferase is a codon-optimized modified luciferase reporter containing silent mutations that remove 95 2'-deoxyribo-cytidine-phosphateguanosine (CpG) dinucleotides in a way that it is not reflected in its amino acid sequence. This way the dCpG luciferase coding region encodes the same amino acid sequence as the wild type firefly luciferase but the nucleotide sequence shows only 77% homology. The resistance of the dCpG construct to the T_3 treatment can be explained by the very likely disruption of the negative TRE(s) of the original sequence.

We also tested a novel luciferase NanoLuc. NanoLuc was isolated from deep-sea shrimp Oplophorus gracilirostris and a synthetic form of the small unit of the luciferase was created (Hall et al., 2012). This new luciferase system has several advantages compared to the firefly and *Renilla* based reporters (see Section 2), it is more stable to environmental stress (like changes in pH, temperature) and the duration of the signal is longer. In our studies we found that only a slight and not significant reduction was observed after T_3 treatment. Only a minimal decrease was observed, but it was within the range of the resolution limit of the assay. Our findings indicate that no potent negative TRE is present in the NanoLuc construct. To further widen the spectrum of the investigated constructs, *Renilla reniformis* luciferase was also tested (Lorenz et al., 1991) which is widely used as internal control in luciferase assays. The T_3 treatment caused a significant decrease in the luciferase expression compared to the control. However, the downregulation was less pronounced than in the case of the classical firefly but underlined that the popular *Renilla* luciferase reporter should be handled with care for T_3-mediated promoter studies.

In summary, we were able to identify alternative reporters, i.e the dCpG luciferase and the NanoLuc that can be more accurately used to assess TH responsiveness of a specific promoter than the classical firefly luciferase.

7. CONCLUSIONS

We focused on the better understanding of the D2-mediated TH activation in the embryonic and adult chicken brain. D2 mRNA could be detected as early as on embryonic day 7, and the presence of activity of the D2 enzyme could also be readily detected. We provided evidence that D2 message can be detected from E8 in scattered cell clusters. This became stronger and localized in elongated perivascular like cell clusters in the neostriatum and hypothalamus of E15 embryos. The rearrangement of the D2-labelled cells could be observed in the investigated adult chicken brain samples, namely labelled ependymal cells reminiscent of tanycytes were detected in the wall of the third ventricle, while the intensity of hybridization signal in other parts of the brain was markedly decreased. All these results confirmed that the developing chicken brain is able to activate the prohormone thyroxine with D2 at a very early stage, well before the onset of the thyroidal secretion. The distribution pattern of D2 is subjected to change during brain development via ontogenic regulation and contributes to the adjustment of appropriate T_3 levels in the central nervous system. Our findings challenge the dogma of minimized TH action during proliferative phases and calls to refine views on minimized T_3 availability in the developing central nervous system.

We confirmed the role of alternative splicing of the D2 mRNA in the post transcriptional regulation of the D2 encoding *dio*2 gene. We were able to identify and functionally test a novel alternatively spliced D2 mRNA in the brain and the liver of chickens. This transcript is 77 bp shorter than the wild-type D2 mRNA and encodes an inactive deiodinase enzyme. Our results indicate that alternative splicing is one of the several processing mechanisms that influence the level of D2 in a given tissue. Our results also confirm that D2 mRNA level quantification alone may be misleading in the interpretation of D2 enzyme activity levels of the different tissues. In addition, we demonstrated that the 5'UTR of the D2 mRNA down-regulates D2 activity and this effect is underlain by sORF based translation initiation. This mechanism helps to keep the translation of the D2 protein low which contributes to the tight regulation of this T_3 generating, highly active oxido-reductase.

According to the current consensus, T_3 exerts its biological effects predominantly via nuclear transcriptional events. Experimental assessment of this mechanism is a crucial approach to study thyroid hormone related signalling. T_3-mediated regulation of the

DOI:10.14753/SE.2017.2032

classical firefly luciferase reporter is a significant set back of these studies. Therefore, our findings on the resistance of the dCpG luciferase and the NanoLuc to T_3-mediated downregulation allow an accurate assessment of the potency of elements involved in the transcriptional regulation of TH-mediated gene expression in various reporter assays.

DOI:10.14753/SE.2017.2032

8. SUMMARY

Thyroid hormones play an important role in the development and function of various organ systems. The hypothalamo-pituitary-thyroid axis is programmed to keep serum thyroid hormone level relatively stable, but at the tissue level thyroid hormone evoked changes are regulated by a tightly controlled local machinery. Our aim was to investigate the regulatory factors underlying thyroid hormone mediated effects using molecular and cell biological approaches. We addressed the regulation of thyroid hormone availability in the developing chicken hypothalamus; performed studies to better understand the RNA-dependent post-transcriptional regulation of the type 2 deiodinase (D2) encoding *dio2* gene; and also aimed to identify authentic luciferase reporter proteins for transcriptional studies on T_3-dependent gene expression.

We provided evidence that the D2 mRNA expression and activity can be detected from embryonic day 7 and encodes an active enzyme in the developing chicken brain. We also observed redistribution of cellular elements responsible for D2-mediated thyroid hormone activation in the ontogeny of the chicken brain. Our results prove that the local T_3 generating machinery is functional already from early stages of development of the central nervous system that challenges the dogma of minimized TH action in the proliferative developmental stages.

We identified two RNA-dependent post-transcriptional molecular mechanisms regulating D2 activity in chicken. We isolated an alternatively spliced D2 mRNA that encodes an inactive enzyme that could impact tissue specific D2 activity in the brain and liver of chicken. We also demonstrated that the 5' untranslated region of D2 mRNA downregulates the activity of the D2 enzyme and the underlying molecular mechanism is based on short open reading frame associated translational initiation.

We demonstrated that two novel luciferase reporters, the dCpG luciferase and the NanoLuc accurately measure the activity of T_3-dependent gene expression in contrast to the classical firefly. The established approach allows the authentic assessment of transcriptional effects of thyroid hormone.

Our studies contribute to the better understanding of cellular and molecular mechanisms underlying the effect of thyroid homone on the brain and other tissues.

DOI:10.14753/SE.2017.2032

9. ÖSSZEFOGLALÁS

A pajzsmirigyhormonok fontos szerepet játszanak a különböző szervrendszerek fejlődésében és működésében. A hipotalamusz-hipofízis-pajzsmirigy tengely a szérum pajzsmirigyhormon szint viszonylagos stabilitásának fenntartására van programozva, azonban a szövetekben a pajzsmirigyhormonok által kiváltott változásokat szigorú szabályozás alatt álló helyi mechanizmusok irányítják. Célunk a pajzsmirigyhormon hatás szabályozó tényezőinek tanulmányozása volt, ehhez molekuláris és sejtbiológiai módszereket használtunk. Vizsgáltuk a fejlődő csirke hipotalamuszban a pajzsmirigyhormon elérhetőség szabályozását; kísérletet tettünk a kettes típusú dejodázt (D2) kódoló *dio*2 gén RNS-függő poszttranszkripcionális szabályozásának jobb megértésére; továbbá a T_3-függő génexpresszió transzkripciós tanulmányozására alkalmas luciferáz riporter fehérjék azonosítását tűztük ki célul.

Bizonyítottuk, hogy a D2 mRNS expressziója és enzimaktivitása már a 7. embrionális naptól kimutatható a fejlődő csirkeagyban. Megfigyeltük a D2 által kiváltott pajzsmirigyhormon aktiváció sejtes elemeinek újrarendeződését a csirke agyfejlődése folyamán. Eredményeink azt igazolják, hogy a T_3 előállító rendszer már a központi idegrendszer korai fejlődési stádiumában működőképes, ami megkérdőjelezi azt az elképzelést, miszerint a pajzsmirigyhormon hatás minimalizálva van proliferatív fejlődési szakaszokban.

Azonosítottunk két olyan RNS-függő, poszttranszkripcionális molekuláris mechanizmust, ami szerepet játszik a D2 aktivitás szabályozásában csirkében. Izoláltunk egy alternatívan hasított, inaktív enzimet kódoló D2 mRNS-t, ami szerepet játszhat a szövetspecifikus D2 aktivitás kialakulásában csirke agyban és májban. Kimutattuk továbbá, hogy a D2 mRNS 5’ nemtranszlálódó régiója csökkenti a D2 enzim aktivitását és e folyamat molekuláris hátterében a rövid nyitott olvasási keret transzlációs iniciációja áll.

Igazoltuk, hogy két új riporter, a dCpG és a NanoLuciferáz, a klasszikus “firefly” luciferázokkal ellentétben, pontosan méri a T_3-függő génexpresszió aktivitását. A kidolgozott módszer lehetővé teszi a pajzsmirigyhormonok által kiváltott transzkripciós változások pontosabb megítélését.

Vizsgálataink hozzájárulnak a pajzsmirigyhormonok agy- és egyéb szövetekben kifejtett hatásának hátterében álló sejtes és molekuláris folyamatok jobb megértéséhez.

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DOI:10.14753/SE.2017.2032

11. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

11.1. List of publications the thesis based on

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Gallus gallus type 2 iodothyronine 5'-deiodinase splice variant mRNA, complete cds, alternatively spliced. Deposited to GenBank under Accession #AF401753.

11.2. Other publications

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DOI:10.14753/SE.2017.2032

12. ACKNOWLEDGEMENTS

I would like to express my honest gratitude to my promoter Dr Balázs Gereben for his intensive support in supervising my scientific work as early as from my undergraduate years.

I would like to thank to Dr Csaba Fekete, Petra Mohácsik, Andrea Juhász, Dr Péter Egri and Zsuzsanna Kvárta-Papp for their generous help in the laboratory work.

I thank to Dr Zsolt Liposits for his support of my work performed as an external PhD student.

I am grateful to Dr Zoltán Pénzes at my office at Ceva-Phylaxia Co. Ltd. for his support in giving me the chance to finish my PhD studies in parallel with my work.

I would like to thank my husband Csaba, my sons Bertalan and Barnabás and my parents for their tolerance and patience during all these years.