Investigation of regulatory factors underlying thyroid hormone mediated effects

Ph.D. Thesis

Anna Kollár D.V.M.

Semmelweis University
János Szentágothai Doctoral School of Neurosciences

Institute of Experimental Medicine
Hungarian Academy of Sciences

Consultant: Balázs Gereben, D.V.M., D.Sc.

Official reviewers: Péter Gálfi, D.V.M., D.Sc.
Zsuzsanna Tóth, Ph.D.

Head of the final examination committee:
Alán Alpár, M.D., D.Sc.

Members of the final examination committee:
Attila Patócs, M.D., Ph.D.
Emília Madarász, D.Sc.

Budapest
2017

1. INTRODUCTION

Thyroid hormone (TH) plays an essential role in the development and function of various organ systems. TH impacts fundamental events of cellular function, including the regulation of cell proliferation/differentiation and modulation of cellular energy metabolism. TH is especially important for the development and function of the brain. Disturbed TH economy is associated with severe symptoms in humans; untreated congenital hypothyroidism is manifested in the detrimental signs of cretinism while mutations causing improper TH signalling evoke cognitive and peripherial dysfunction.

The regulation of circulating TH levels is governed by the hypothalamo-pituitary-thyroid (HPT) axis. This centrally governed circuit is programmed to keep serum T_3 levels in the physiological range. This is predominantly achieved by controlling the release of TH, especially the long-lived T_4 prohormone from the thyroid gland. However, TH action occurs in tissue/cell compartments and its regulation requires quick and tissue-specific costumization. Thus, the impact of the HPT axis on tissue TH levels is limited.

Intense efforts of the past two decades revealed numerous mechanisms forming the basis of the molecular network that establishes customized TH action and regulates its activity. The cloning of the members of the selenodeiodinase family was a hallmark of this process. Type 2 deiodinase enzyme (D2) is the crucial factor to activate TH in the brain by converting the inactive prohormone thyroxine (T_4) to triiodothyronine (T_3) by removing iodine from the outer phenolic ring of T_4 by 5' deiodination. This occurs in a spatially separated manner in the brain tissue; D2-mediated T_3 production takes place in glial cells while neurons cannot generate T_3 for themselves and need to take up the activated hormone. The essential role of this event played in the regulation of TH availability of the brain is underlined by findings demonstrating that not T_3 but the T_4 prohormone is the compound that can efficiently gain access to the brain parenchyma. Thus T_3 needs to be generated *in situ* in the brain tissue.

Tight regulation of TH availability is a prerequisite of TH-related biological effects. It is well known that the HPT axis is regulated by TH-mediated negative feedback. This is achieved by mechanisms that functionally interlink the central regulation of the HPT axis with local D2-mediated regulation of TH availability in tanycytes of the mediobasal hypothalamus (MBH) located in the floor and the ventrolateral walls of the third ventricle. The hypophysiotropic TRH neurons of the hypothalamic paraventricular nucleus (PVN) cannot generate T_3 due to

their lack of D2 expression and therefore their TH uptake is dependent on the T_3 level of the MBH.

The consensus supports the idea of minimized TH action in proliferative developmental stages, but data are limited on the T_3 generating capacity of the brain at early stages of brain development. Chickens represent an invaluable model for studies on the TH availability in the developing brain since in contrast to rats, 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.

D2 is a highly active oxido-reductase and its activity is tightly controlled by complex mechanisms at multiple regulatory levels. These mechanisms are criticial to achieve to allow TH to exert tissue-specific and timely controlled effects on cellular function. The discrepancies between the level of D2 mRNA expression and activity suggest the active role of post-transcriptional events in the regulation of D2-mediated TH activation, possibly alternative splicing of the D2 encoding mRNA. The D2 mRNA itself is approximately four times longer than a usual eukaryotic mRNA and contains long untranslated regions. Interestingly, its long 5' untranslated region (5' UTR) contains several short open reading frames (sORF) that are small alternative coding regions flanked by start and stop codons separated by an RNA segment consisted of bases in a number divisible by 3 that may be subjected to translation.

Having born, T_3 exerts its biological effects predominantly by liganding the nuclear hormone receptor TR that binds DNA as a heterodimerization partner of Retinoid X receptor (RXR). The TR-RXR heterodimers are located in the nucleus even in the absence of ligand and even the unliganded TR can bind to a TH response element(s) (TRE) of a target gene. 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. Mechanisms underlying TH-dependent negative regulation of gene expression are still incompletely resolved and different models have been worked out to explain the phenomenon.

Bioluminescence-based assays represent the state of the art approach of transcriptional studies. 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, it was demonstrated that the classical firefly luciferase is down-regulated by T_3 in a TR-dependent but promoter-independent manner and it was suggested that negative TRE should

exist in the luciferase gene. Several studies demonstrated limitations that firefly luciferase faces in T_3-dependent gene expression studies. These observations were calling for an unbiased approach to study T_3-mediated gene expression, the mechanism allowing T_3 to impact cellular function.

Thus, we aimed to investigate cellular and molecular regulatory factors underlying thyroid hormone mediated effects in the brain and other tissues.

2. 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?

3. METHODS

3.1. 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).

3.2. Constructs

Analysis of RNA-dependent post-transcriptional 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. Constructs were prepared with different UTR fragments cloned between the *Sac*II site of the D10 vector and *EcoR*I. 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 and the spliced cDNA was inserted between *Sac*II and *Not*I of the D10 mammalian expression vector. The generated constructs were confirmed by automated sequencing.

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 direct sequencing.

The *TK-(dCpG)Luc* was prepared using the *TK-Luc* plasmid backbone as follows. The *pMOD Luc-ShS v02* plasmid was used as a template to amplify the *(dCpG)Luc* coding region with Vent PCR. 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 *mouse TRα (mTRα)* expression construct was generated using the *TRαCDM* as a template to amplify *mTRα* coding region with Vent PCR. 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 was used for transfection control.

3.3. DNA transfections

HEK-293 cells were transfected with calcium phosphate precipitation. 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. 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. JEG-3 human choriocarcinoma cells 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. After ~6 hours the transfection media was replaced with DMEM containing 10% hormone-free fetal bovine serum and incubated for 40 hours. 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. The cells were washed with phosphate buffered saline (PBS) and harvested in 100 μl Passive lysis buffer.

3.4. RNA isolation and RT-PCR

Brain samples of E7, E8, E9, E10, E11, E13, and E15 chicken embryos were dissected in duplicates, and total RNA was isolated with Trizol. RNA was subjected to first strand cDNA synthesis using an oligonucleotide-dT primer and amplified with D2-specific primers. Total RNA isolated from the telencephalon and liver was reverse transcribed using the antisense oligonucleotide CTCACCAGAA GGCCTGAAGA G and amplified by Taq polymerase with

D2-specific primers. The amplicons were cloned into pGEM-T and subjected to automated sequencing. The amplifications were performed in two separate reactions.

3.5. Northern blots

The Northern blot *for the ontogenic D2 distribution study* was performed as previously described. 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. 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.

3.6. *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 fixed with 4% paraformaldehyde in PBS and hybridized with an approximately 840 bp single-stranded DIG-11-uridine 5-triphosphate labelled cRNA probe for the entire coding region of cD2 followed by washing and incubation with a mixture of sheep anti-DIG-alkaline phosphatase Fab fragments (1:1000, Roche Diagnostics GmbH, Mannheim, Germany) 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 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.

3.7. Assays

3.7.1. Deiodinase assay

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 and kept frozen at -80°C until used. The assays contained approximately 150-600 μg homogenate proteins in 300 μl PE buffer supplemented with various amount 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. 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. The iodine containing supernatant was counted with a γ-counter. The activity level was expressed in femtomols released 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.

3.7.2. 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*). 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. All measurements were performed with a Luminoskan Ascent Luminometer (Thermo, Waltham, MA, USA).

3.7.3. SEAP assay

SEAP activity was determined from 25 μl media with Nova Bright™ SEAP Enzyme Reporter Gene Chemiluminescent Detection system 2.0 (Invitrogen/Thermo). 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.

3.8. 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 and *luciferase assay data* were compared with an unpaired two-sample *t*-test using a 95% level of confidence.

3.9. Sequences

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

4. RESULTS

4.1. 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

4.1.1. 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. Size of the PCR amplicon matched exactly the size calculated from the sequence of the wild-type cD2 transcript (GenBank AF125575) indicating that no D2 mRNA splice variant is expressed in a detectable amount using a sensitive PCR-based approach during this period of brain development.

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. 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.

4.1.2. 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 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. 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. Using this approach 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. 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 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.

4.1.3. 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 using a digoxigenin-labelled probe specific for the D2 coding region. Signal intensity increased in perivascular like cell clusters throughout the brain compared to the E8 stage. No D2 hybridization signal was found in the ependymal cells lining the wall of the third ventricle.

4.1.4. 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 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 was positive for D2. However, in the more posterior segment, the D2-expressing cells covered the ventral one half to two-thirds of the ventricular wall. 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, $p = 0.0043$]. Strong D2 signal could be observed in isolated cells of the neostriatum.

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

Our aim was to understand the role of mRNA structure in the post-transcriptional regulation of the *dio2* 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.

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

D2 mRNA levels and enzyme activity is 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 2.6-fold higher D2 activity in the brain than in the liver of chicken (408 *vs.* 156 fmol T_4/h mg protein). 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. 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. The spliced coding region was inserted into a D10 expression vector and it 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. 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 D2 transcripts 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. 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.

4.2.2. 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. 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 initiaton in eukaryotes.

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. 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. 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.

4.3. 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. This allowed to perform the experiments with the promoter type that proved to be unaffected by T_3 treatment, this harmonized promoter design helped to eliminate differences in promoter-dependent T_3-response of different constructs. 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. 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/). 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. On the other hand, T_3 treatment significantly decreased (by approximately 30%) the activity of cultures expressing the *TK-Renilla-Luc*.

5. 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 the 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 *dio2* 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 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 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 classical firefly luciferase reporter is a significant set back of these studies. Therefore our findings on the resistance of the dCpG luciferase and the NanoLuciferase 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.

6. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

6.1. List of publications the thesis based on

Kollár A, Kvárta-Papp Zs, Egri P, Gereben B (2016) Different types of luciferase reporters show distinct susceptibility to T3-evoked downregulation. Thyroid, 26: 179-182.

Gereben B, Pachucki J, **Kollár A**, Liposits Z, Fekete C (2004) Ontogenic redistribution of type 2 deiodinase messenger ribonucleic acid in the brain of chicken. Endocrinology, 145: 3619-3625.

Gereben B, **Kollár A**, Harney JW, Larsen PR (2002) The mRNA structure has potent regulatory effects on type 2 iodothyronine deiodinase expression. Mol Endocrinol, 16: 1667-1679;
Gallus gallus type 2 iodothyronine 5'-deiodinase splice variant mRNA, complete cds, alternatively spliced. Deposited to GenBank under Accession #AF401753.

6.2. Other publications

Gereben B, **Kollár A**, Bartha T, Buys N, Decuypere E, Rudas P (1998) 3,3',5-Triiodothyronine (T_3) uptake and expression of thyroid hormone receptors during the adapation to hypothyroidism of the brain of chicken. Acta Vet Hung, 46: 473-485.