Final report

(K125247)

The aim of our project was to address novel aspects of tissue-specific thyroid hormone (TH) action under physiological and pathological conditions. The project also involved a year of no-cost extension due to a delay caused by both the pandemics and by an animal-facility infection that slowed down animal experiments.

For our studies, The model is based on a TH-responsive luciferase reporter system while all members of the local TH signaling machinery remain intact. Preliminary data of the present grant was partially based on data obtained on THAI mouse. During the course of this project, we obtained patents for THAI mouse of the United States Patent and Trademark Office (#10,694,724 B2), the European Patent Office (#EP3223606) and the Hungarian Intellectual Property Office (#HU230964).

Using this model, we studied the pathogenesis of Nonthyroidal Illness Syndrome (NTIS). NTIS is a frequently observed phenomenon of the endocrine praxis and present in virtually all ICU patients. It is hallmarked by the uncoupling of the hypothalamo-pituitary-thyroid (HPT) axis from peripheral TH levels. It is still controversial, whether this condition requires therapy or is beneficial; highly probably an acute existence of the phenomenon is beneficial adaptation to reduced availability of energy equivalents, while its long-term existence would require correction. We found that serum TH levels were falling in THAI mice after bacterial lipopolysaccharide (LPS) induction of NTIS. However, local hyperthyroidism existed (measured by the expression of the luciferase THAI reporter) both in the hypothalamic median eminence/arcuate nucleus region, where the D2 expressing (and this way T3 generating) tanycytes are located. Similarly, local increase of TH action existed in the hypothalamic paraventricular nucleus (PVN) containing the cell bodies of the hypophysiotropic TRH neurons, that at the same time decreased their TRH expression. Consequently, TSH also decreased in the pituitary but interestingly, local TH action was decreased in the pituitary. Since TSH is known to be negatively regulated by TH, these findings demonstrate, that in NTIS not the local TH economy of the pituitary, but rather the decreased TRH of the hypothalamus generates the decreased TSH expression. Thus, we found that localized hypothalamic hyperthyroidism supressed the HPT axis in NTIS. At the periphery, hepatic TH action remained

unchanged while that in the GI tract was found to be decreased. Thus, we found coexistence of eu-, hypo and hyperthyroidism in different tissues of the same animal and our data demonstrate that local increase of hypothalamic TH action uncouples the HPT axis from peripheral TH levels. These data also prove how inadequate the "TSH only" measurement can be to assess to TH status of an individual.

We also found that aging interferes with the effects of NTIS since 1-year-old animals showed a blunted TSH response; the lack of downregulation of *Tshβ* and free T4 (fT4) in LPS-treated aged THAI mice suggests an aging-evoked decrease of the responsiveness of the HPT axis. TRH is known to be the main hypothalamic regulator of TSH and TRH expression is known to decrease in NTIS. Our data demonstrated that this central signal is superior to the local TH feedback in the pituitary during TSH regulation in NTIS. Since TRH expression decreased both in aged and young animals while TSH response was blunted in old mice, we wanted to understand whether TRH basal levels could be increased in aged animals, *e.g.* due to reduced negative feedback. Therefore, we performed TRH *in situ* hybridization in the PVN and demonstrated that TRH integrated density was not significantly changed by age. This indicates that an age-dependent elevation of TRH levels in the PVN should not be considered as a causative role in the observed age-dependent difference in HPT axis responsiveness.

Based on our studies to uncover the mechanisms of NTIS with our THAI mouse model, we expanded our investigation to another form of NTIS, caused by fasting. We subjected THAI mice to 24 and 48h fasting and found that fasting induced NTIS was not associated with altered TH signaling in the hypothalamus. Thus, although the HPT axis is inhibited both in LPS and fasting-induced NTIS, LPS achieves this by centrally inducing local hyperthyroidism in the ARC-ME region, while fasting acts without affecting hypothalamic TH signaling. The LPS-induced tissue-specific hypo-, eu- and hyperthyroidism in different tissues of the same animal indicate that under certain conditions TH levels alone could be a poor marker of tissue TH signaling. In conclusion, decreased circulating TH levels in these two forms of NTIS are associated with different patterns of hypothalamic TH signaling. Our findings have implications for the efforts that aim to improve the condition of ICU patients using TH supplementation *(Sinkó et al. Thyroid 2022).*

We also studied the impact of environmental pollutants on tissue TH economy. We used THAI mice to assess the endocrine disruptor activity of two compounds, tetrabromobisphenol A (TBBPA) and diclazuril. TBBPA is the most common flame retardant used for the

production of printed electronic circuit boards that was also detected in aquatic food samples, further increasing human exposure. Consequently, TBBPA was also found in human tissues, milk and serum. In addition, TBBPA showed neurotoxic, nephrotoxic and hepatotoxic effects and also impacted reproductive health in various animal models. Diclazuril is widely used as an antiprotozoal agent and acts by targeting the chlorophyll a-D1 complex. It is used to prevent and treat coccidiosis in multiple species and is also applied against equine protozoal myeloencephalitis, and to a lesser extent, toxoplasmosis and neosporosis. As chickens are often treated with medicated food containing diclazuril, human exposure is a realistic scenario. Thus, the effect of diclazuril on the human TH economy needs to be further investigated. Diclazuril is considered to be safe against toxoplasmosis during pregnancy in a mouse model. However, continuous exposure leads to stable plasma levels, which raises human concerns and calls for further studies. Furthermore, its potential to disrupt hormonal signaling is poorly documented, but its ability to bind androgen receptors was shown, along with data of its potential to antagonize TH receptors in a high-throughput cell-based reporter gene assay.

Since TH signaling is a prerequisite of normal tissue function, therefore environmental pollutants with the potential to disrupt endocrine functions represent an emerging threat to human health and agricultural production. THAI mouse was exposed to Tetrabromobisphenol A (TBBPA; 150 mg/bwkg/day orally for 6 days) and diclazuril (10.0 mg/bwkg/day orally for 5 days). Tissue-specific changes of TH action were assessed in 90-day-old THAI mice by measuring the expression of a TH-responsive luciferase reporter in tissue samples and by in vivo imaging (14-day-long treatment accompanied with imaging on day 7, 14 and 21 from the first day of treatment) in live THAI mice. This was followed by promoter assays to elucidate the mechanism of the observed effects. TBBPA and diclazuril impacted TH action differently and tissue-specifically. TBBPA disrupted TH signaling in the bone and small intestine and impaired the global TH economy by decreasing the circulating free T4 levels. In the promoter assays, TBBPA showed a direct stimulatory effect on the *hdio3* promoter, indicating a potential mechanism for silencing TH action. In contrast, diclazuril acted as a stimulator of TH action in the liver, skeletal muscle and brown adipose tissue without affecting the HPT axis.

Our data obtained in the THAI mouse demonstrate that TBBPA and diclazuril exert a distinct tissue-specific impact on mammalian TH action detectable in living animals and in isolated tissue samples. The obtained data demonstrated the tissue-specific effects of TBBPA and diclazuril on local and global TH economy. They also proved that the THAI mouse provides a powerful and novel *in vivo* mammalian model to selectively screen the potential of

compounds to disrupt TH signaling and identify TH disruptors and their tissue-specific effects *(Sinkó et al. IJMS 2022).*

We developed a biomarker system that allows the assessment of the human tissue TH economy. To achieve this, first we used Next Generation Sequencing (NGS) to generate the TH-dependent expression profile of human hair follicles originating from patients with different TH status (hypo-, hyper- and euthyroid). This approach identified a human gene set that we used as biomarker candidates of tissue TH action. We then performed an independent confirmation of the identified marker gene candidates by qPCR on 150 human hair follicle samples collected in total (based on our original and a renewed ethical permission) that include samples from patients with different TH status and with or without amiodarone treatment. Amiodarone (Cordarone) is an antiarrhythmic drug that represents the only effective therapeutic choice for specific patients bit it knows to interfere with serum TH parameters. This allowed to narrow down our list of candidate marker genes using an independent approach. We selected four marker genes showing the best performance on TH dependence and basal expression level. One of these markers is also expressed in leukocytes that raises the possibility of parallelly detect of tissue TH action and in cells facing the blood stream, a situation that is functionally represents an intersection of tissue TH economy and blood TH levels.

Clinically applicable TH-dependent human markers became of great interest since it is now widely accepted that serum TH levels and especially TSH alone are often inadequate to validly report on TH signalling in tissues (*e.g.* in NTIS or amiodarone treatment), although TH action occurs in the tissue pools. Our above summarized results are publishable. However, we aim to come forward with our data in a more complex translational setting. Based on our generated marker expression database we finished building a logistic regression model using non-amiodarone treated samples as calibrators for a prediction model. In order to determine whether the correlation of TSH-based prediction and tissue-based prediction is similar between amiodarone treated patients and non-amiodarone treated patients, the performance of our new prediction model is compared between these two patient groups, with special respect to amiodarone treatment. The clinical consequence of this diagnosis is very significant since in order to avoid thyrotoxicosis and thyroid storm, it results in the discontinuation of treatment and there is no affective antiarrhythmic alternative treatment available for this patient group. Therefore, it is critically impotent that discontinuation should only occur if tissue TH economy is really impacted but this cannot be validly judged only by serum TH parameters. Our

preliminary analysis suggest that hyperthyroidism based on serum TH levels of amiodarone-treated patients is not associated with hyperthyroidism at the tissue level. Since the availability of independent samples of amiodarone treated patients is limited, we are still extending the collection of those by the help of an extended number of clinical collaborators and as it is finished, we will submit the manuscript.

We also developed a parallel collaboration to better understand the alterations of deiodination-regulated cardiac TH action in cardiology patients.

We also put intense efforts to study the impact of amiodarone in THAI mouse model. We had to develop this model since it is not available in the literature. Animals showed a massive decrease (more than 80%) in pituitary D2 activity and expression of the luciferase reporter showed a decreasing trend. This was accompanied with unaltered *dio3* expression, encoding the enzyme responsible for TH inactivation. *Tshβ* expression remained unchanged, and this was reflected in unchanged serum fT3 and fT4 levels. Cortical D2 activity remained unchanged as well, while cortical luciferase expression showed a ~50% decrease. This could not be related to cortical *dio3* or TH transporter expression levels. In both treatment groups we observed macroscopic signs of liver steatosis. Hepatic luciferase showed a decreasing trend in the five-day-long treatment, but remained unchanged after 22 days. In summary the present data indicate that a dose of Cordarone, that is comparable to the one used to treat humans, does not generate tissue hyperthyroidism in the THAI mouse model. We have still ongoing studies using amiodarone containing diet, in order to also test this treatment regime.

According to the plan, we also developed a mouse model of mild hypo- and hyperthyroidism in mice, that we use in our ongoing studies. This was monitored by the measurement of serum fT4, fT3 and pituitary *Tshβ* expression (this was detailed in interim reports). To generate borderline hyperthyroidism, mice are placed on iodine free diet. Drinking water is supplemented both with 0.1% sodium perchlorate plus 0.5% methimazole and with TH. To achieve the appropriate concentration of TH in the drinking water, body weight and the volume of consumed drinking water is measured 2-3 times a week and TH concentration of the drinking water is adjusted accordingly. Volume of daily consumed drinking water should contain 2µg/100g BW/day TH with a 5:1 ratio of T4 and T3, respectively. For borderline hypothyroid, mice are placed on iodine free diet. Drinking water is supplemented both with 0.1% sodium perchlorate plus 0.5% methimazole and with TH. To achieve the appropriate

concentration of TH in the drinking water, body weight and the volume of consumed drinking water is measured 2-3 times a week and TH concentration of the drinking water is adjusted accordingly. Volume of daily consumed drinking water should contain 1µg/100g BW/day TH with a 5:1 ratio of T4 and T3, respectively.

The studies addressing the impact of exogenous T4 excess on D2-mediated T3 generation, a situation tissues face at T4 monotherapy will go beyond this grant Our data suggest marked brain region specific differences in the response of TH action to exogenous T4. These studies are still under way and reach beyond the period of the grant.

Based on our expertise and our developed THAI model of tissue specific TH action, we were invited to contribute to a study on BAT TH economy. THAI mouse provided a unique model to generate well-controlled and localized BAT hyperthyroidism. This allowed to elucidate the mechanisms underlying TH action in adipocyte progenitors residing in BAT and provided a framework for better understanding of the TH effects on hyperplastic growth and adaptive thermogenesis in BAT depot at a single-cell level *(Liu et al, Nature Comm 2022)*. We also contributed to studies that revealed that endocannabinoid signaling in hypothalamic tanycytes regulate the HPT axis and impacts glucose homeostasis and fat deposition *(Kővári et al. Neuroendocrinology 2022)* while on another study we contributed to address the role of the prenatal peak of type 2 deiodinase-catalysed TH activation in the mammalian liver. Our data also revealed the role of this mechanism in alcoholic liber steatosis *(Fonseca et al. Alcohol Clin and Exp Research. 2019).*

Our current views on tissue specific thyroid hormone economy were summarized for the most cited endocrine journal, the *Endocrine Reviews*. The PI gave invited symposium talks on leading international endocrinology meetings (*The American Endocrine Society's Annual Meeting, The European Congress of Endocrinology*, three symposium talks at the *Annual Meeting of the European Thyroid Association*) and among other events delivered an invited plenary talk on the *Meeting of the Hungarian Society for Endocrinology and Metabolism*.

Dissemination of our results for the public was done in radio interviews (Kossuth Radio, Info Radio, Klub Radio) and numerous Web news portals covered our work. The project also contributed to a PhD thesis that will be defended next year.