Central regulation of the HPT axis

PhD thesis

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1. Introduction

Thyroid hormones (TH) produced by the thyroid gland are involved in the regulation of most of our organs and physiological processes and responsible for the maintenance of normal function and development of most tissues, including the bone, liver, cardiovascular system and brain. Furthermore, THs play critical role in the regulation of a number of physiological functions, including food intake and energy expenditure.

The two major forms of TH are the thyroxine (T4) and triiodothyronine (T3). T4, the major product of thyroid gland is considered a prohormone. T4 has to be converted to T3 by removal of one iodine residue from the outer ring. This activation is catalyzed by deiodinase enzymes. The activation of TH is catalyzed primarily by type 2 deiodinase (D2) under euthyroid conditions. D2 is the only deiodinase enzyme that can catalyze the TH activation in the central nervous system. The local TH activation or inactivation catalyzed by the deiodinases ensure that TH signaling in different tissues can change independently from the serum TH levels. Tissue specific regulation of deiodinase enzymes can highly increase the local TH action in certain tissues to meet the demand of tissues even if the circulating TH level is unchanged or decreased.

The relatively steady circulating levels of TH that is necessary for the preservation of the normal function of brain and peripheral organs is maintained by the hypothalamic-pituitary-thyroid (HPT) axis. The central regulator of this neuroendocrine system is a group of thyrotropin-releasing hormone (TRH)-synthetizing neurons in the hypothalamic paraventricular nucleus (PVN). These hypophysiotropic TRH neurons release TRH into the portal circulation of the pituitary in the external zone of median eminence (ME) where their axons terminate. The released TRH then reach the thyrotrophs of the anterior pituitary and stimulates the thyroid-stimulating hormone (TSH) synthesis, glycosylation and secretion and thus stimulates the TH production of the thyroid gland. In the PVN, not all TRH neurons are involved in the negative feedback regulation of HPT axis. Only the hypophysiotropic TRH neurons that project to the external zone of ME are involved in the control of thyroid gland. The negative feedback regulation of the HPT axis is responsible for the stable circulating TH level. The negative feedback effect of TH is exerted directly on the hypophysiotropic TRH neurons.

Tanycytes are special glial cells of the hypothalamus lining the ventrolateral walls and the floor of the third ventricle behind the optic chiasm. The tanycytes play critical role in the negative

feedback regulation of HPT axis. Only tanycytes produce D2 within the mediobasal hypothalamus (MBH), and thus only these cells can convert T4 to T3 in this brain region. Tanycytes synthetize the TRH degrading enzyme, the pyroglutyamyl-peptidase II (PPII). TRH neurons are surrounded by tanycyte processes, it is likely that PPII can degrade the released TRH before it could enter into the portal capillaries.

Endocannabinoids are retrograde neurotransmitters widely spread in the brain, released from postsynaptic neurons and act on presynaptic axon terminals. One major type of endocannabinoids is the 2-arachydonoylglicerol (2-AG) which is synthesized by the postsynaptically located diacylglycerol lipase α (DAGLα). Recently, our group has described a novel microcircuit between the hypophysiotropic TRH axons and tanycytes which regulates the TRH release via the endocannabinoid system. Tanycytes express DAGLα and inhibition of endocannabinoid synthesis markedly increases the TRH release of ME explants, suggesting that tanycytes tonically inhibit the TRH release of hypophysiotropic axons. Glutamate regulates the endocannabinoid synthesis and release of postsynaptic neurons by stimulating DAGLα. Glutamate release of the hypophysiotropic TRH neurons may stimulate the endocannabinoid synthesis of tanycytes.

Under most conditions, the HPT axis maintains stable circulating TH levels by the negative feedback regulation. During development, a set point of this feedback regulation develops that is characteristic for the HPT axis of the individual and this set point is stable for the whole life. The set point is the interrelationship of TRH and free thyroxine (fT4) levels that is optimal for the individual. Any deviations from this TRH-fT4 relationship results in counterregulatory action. This set point develops around birth. The mechanism of this set point development is poorly understood. The timing of the development of the set point of HPT axis is different among species. Beside the maternal thyroid status, environmental factors also influence the development of the offspring's HPT axis in the sensitive period. The long-term effects of environmental factors or perturbations of internal milieu during the late gestational or perinatal periods can be mediated by epigenetic modifications of genes. These changes of gene expression by methylation ensure the adaptation to the environmental changes. Changes in TH status at the early postnatal period when the set point of the HPT axis develops can cause epigenetic modification of TH dependent genes, but this mechanism is poorly understood yet.

2. Aims

The aim of our study was to better understand the underlying mechanisms of the development of the HPT axis set point and the central regulation of the negative feedback of HPT axis in mice, therefore our specific aims were:

1. To investigate how tanycytes regulate the TRH release of hypophysiotropic TRH neurons and the activity of the HPT axis via the endocannabinoid system.

2. To elucidate, how early, perinatal disturbance of the thyroid status influences the HPT axis and metabolism of mice at adulthood.

3. Methods

3.1. Experimental animals and treatments

The experiments were carried out using newborn and adult mice housed under standard environmental conditions (12h light/dark cycle, temperature 22±1 °C, chow and water *ad libitum*). The mouse strains used for the experiments and the experimental design are found in **Table 1**. All experimental protocols were reviewed and approved by the Animal Welfare Committee at the Institute of Experimental Medicine and the Animal Health and Food Control Station, Budapest (PE/EA/1102-7/2020).

Table 1: Summary of experimental design and strains used in the experiments.

	Strain	**Treatment**	**Duration of treatment**	**Age during treatment**	**Sacrifice**
Experiment 1	CD1	-	-	-	55-65
	Rax-CreERT2 Rax-CreERT2//Dagla$^{fl/fl}$	175µg/bwg Tamoxifen	4 days	45-55	55-65
Experiment 2	TRH-IRES-tdTomato	200 ng/bwg T4 or vehicle	1 day	1,2,3,4,5,6,7,10	1,2,3,4,5,6,7,10
	FVB/Ant	200 ng/bwg T4 or vehicle	1 day	1,3,4,7	1,3,4,7
Experiment 3	TRH-IRES-tdTomato	1 µg/bwg T4 or vehicle	5 days	2-6	55-70
	THAI	1 µg/bwg T4 or vehicle	5 days	2-6	55-70

3.2. Tissue preparation

3.2.1. Preparation of tissues for gene expression analysis

Adult Rax-CreERT2 and Rax-CreERT2//Dagla$^{fl/fl}$ mice (Experiment 1) were decapitated 1 week after the last tamoxifen treatment for collection of blood to measure free TH levels, pituitary samples for qPCR analysis and whole brain samples for TRH *in situ* hybridization (ISH). Serial, 12 µm coronal sections through the PVN were cut on a cryostat.

FVB/Ant and TRH-IRES-tdTomato mouse pups (Experiment 2) were decapitated for collecting whole brain samples for TRH ISH, and liver and pituitary samples for qPCR analysis. 12 μm coronal sections through the PVN were cut on a cryostat.

Postnatally treated, adult thyroid hormone action indicator (THAI) mice (Experiment 3) were decapitated and liver, brown adipose tissue (BAT), small intestine, hippocampus and hypothalamus samples were collected for qPCR measurements.

Postnatally treated, adult TRH-IRES-tdTomato mice (Experiment 3) were anaesthetized with a mixture of ketamine and xylazine (ketamine 50 mg/kg, xylazine 10 mg/kg body weight, i.p.). Liver samples for qPCR analysis were collected and blood was drawn for free TH level measurements. For ISH, anaesthetized mice were perfused transcardially with 10 ml RNAse-free 0.01 M phosphate-buffered saline (PBS) pH 7.4, followed by 50 ml 4% paraformaldehyde (PFA) in RNAse-free PBS. The brains were removed and transferred into 4% PFA for 2 hours, then the brains were incubated in 20% sucrose in RNAse-free 0.01 M PBS overnight at 4 ℃ and frozen in powdered dry ice. Serial 18 μm thick coronal sections through the PVN were cut on a cryostat.

3.2.2. Tissue preparation for immunohistochemistry

One week after the last tamoxifen treatment, Rax-CreERT2 and Rax-CreERT2//Dagla $^{fl/fl}$ mice (Experiment 1) were deeply anesthetized with ketamine and xylazine (ketamine 50 mg/kg, xylazine 10 mg/kg body weight, i.p.). The animals were transcardially perfused with 10 ml 0.01 M pH 7.4 PBS, followed by 50 ml 4% PFA in 0.1 M phosphate buffer pH 7.4. The brains were removed and transferred into 4% PFA for 2 hours, then were incubated in 30% sucrose in 0.01 M PBS overnight at 4 ℃ and frozen in powdered dry ice. Series of 25 μm coronal sections were cut using freezing microtome.

3.2.3. Tissue preparation for laser capture microdissection

Postnatally treated, adult TRH-IRES-tdTomato (Experiment 3), CD1, Rax-CreERT2 and Rax-CreERT2//Dagla$^{fl/fl}$ mice (Experiment 1) were deeply anaesthetized as described above. For isolation of tanycytes with Laser Capture Microdissection (LCM) system, mice were transcardially perfused with 30 ml ice cold 10% RNAlater dissolved in 0.01M RNase-free PBS, then the brains were removed and frozen in -40°C isopentane. 12 μm thick coronal sections were cut on a cryostat through the rostrocaudal extent of the ME. The sections were stained with 0.6% cresyl violet.

Table 2: Summary of tissue preparation and sectioning.

Experiment	Strain	Fixative	Sectioning
DAGLα immunohistochemistry (Exp. 1)	Rax-CreERT2 Rax-CreERT2//Dagla fl/fl	4% PFA 30% sucrose	freezing microtome ME 25 µm
In situ hybridization (Exp. 1,2,3)	postnatally treated, adult TRH-IRES-tdTomato	4% RNase-free PFA 20% RNase-free sucrose	cryostat PVN 18 µm Superfrost Plus slide
	Rax-CreERT2 Rax-CreERT2//Dagla fl/fl FVB/Ant pups	-	cryostat PVN 12 µm gelatine-coated slide
Laser capture microdissection (Exp. 1,3)	CD1 Rax-CreERT2 Rax-CreERT2//Dagla fl/fl postnatally treated, adult TRH-IRES-tdTomato	10% RNAlater	cryostat ME 12 µm PenSlide

3.3. Immunohistochemistry for DAGL*α* in the ME

Sections of the ME of tamoxifen treated Rax-CreERT2 and Rax-CreERT2//Dagla$^{fl/fl}$ mice (Experiment 1) were treated in 0.5% Triton X-100 and 0.5% H_2O_2 in 0.01 M PBS for 20 min, then the sections were placed in 2% normal horse serum in PBS for 20 min. The sections were incubated in guinea-pig anti-DAGLα antibody at 1:4000 dilution, then in biotinylated donkey anti-guinea pig IgG and treated with avidin-biotin-peroxidase complex. The DAGLα immunoreactivity was visualized with Ni-DAB developer.

3.4. Measurement of blood glucose and serum free TH levels

Blood glucose levels of Rax-CreERT2 and Rax-CreERT2//Dagla$^{fl/fl}$ mice (Experiment 1) were determined from one drop of blood collected from a small tail incision.

Blood drawn from caudal vena cava of anaesthetized TRH-IRES-tdTomato mice before the perfusion and trunk blood of Rax-CreERT2 and Rax-CreERT2//Dagla$^{fl/fl}$ mice was collected.

The free TH levels were measured from tamoxifen treated Rax-CreERT2 and Rax-CreERT2//Dagla$^{fl/fl}$ mice (Experiment 1) by AccuBind ELISA fT3 and fT4 kits using Microplate Absorbance Reader.

FT4 and fT3 levels of postnatally treated, adult TRH-IRES-tdTomato mice (Experiment 3) were measured by AccuLite CLIA fT3 and fT4 kits using a luminometer.

3.5. Radioactive ISH for determination of proTRH mRNA levels in the PVN

Every third section of the PVN of T4 treated and control 1, 3, 4 and 7 day old FVB/Ant mouse pups (Experiment 2) and postnatally treated, adult TRH-IRES-tdTomato animals (Experiment 3) or every fourth section of the PVN of Rax-CreERT2//Dagla$^{fl/fl}$ and their control Rax-CreERT2 mice (Experiment 1) were processed for ISH with single stranded [35S]UTP-labeled cRNA probe for mTRH. Following stringency washes, sections were dehydrated in ascending series of ethanol, air-dried, and dipped into Kodak NTB autoradiography emulsion. The concentration of radiolabeled probe used for the different experiments and expositions times are described in **Table 3**.

Table 3: Concentration of mTRH probe and exposition times used for the ISH experiments.

Experiment	Strain	probe (cpm/µl)	Exposition time
Experiment 1	Rax-CreERT2 Rax-CreERT2//Dagla fl/fl	30000	7 days
Experiment 3	Adult TRH-IRES-tdTomato	60000	20 days
Experiment 2	P1 FVB/Ant	50000	18 days
	P3 FVB/Ant		15 days
	P4 FVB/Ant		15 days
	P7 FVB/Ant		11 days

3.6. Examination of DAGLα, Dio2, PPII, glutamate transporter and receptor gene expression in tanycytes isolated with LCM

3.6.1. Isolation of tanycytes using LCM

The cell bodies of β2-tanycytes from CD1, Rax-CreERT2 and Rax-CreERT2//Dagla$^{fl/fl}$ mice (Experiment 1) and α- and β-tanycytes from adult TRH-IRES-tdTomato mice (Experiment 3)

were microdissected separately with LCM System and pressure-catapulted into 0.5 ml Adhesive cap, opaque tubes using a 20X objective lens.

3.6.2. RNA isolation and determination of RNA quality

RNA isolation was performed using the Arcturus PicoPure RNA Isolation Kit and DNase treatment was carried out using RNase-Free DNase Set. The RNA integrity number (RIN) and the concentration of the isolated RNA samples were measured with Agilent 2100 Bioanalyzer using Agilent RNA 6000 Pico Kit. Samples with RIN below 4 were excluded from further studies.

3.6.3. Reverse transcription and amplification

The RNA samples were transcribed using ViLO Superscript III cDNA Reverse Transcription Kit. Concentration of single-stranded complementary DNA (cDNA) was determined with Qubit Fluorometer by using Qubit ssDNA Assay Kit. The cDNA was preamplified for every studied and housekeeping genes by using Preamp Master Mix Kit.

3.6.4. Custom TaqMan Gene Expression Array Card

384-well Custom TaqMan Gene Expression Array Cards were used to examine the presence of glutamate transporters and glutamate receptor subunits in β2-tanycytes of CD1 mice (Experiment 1). The microfluidic card was preloaded by the manufacturer with selected gene expression assays for our target receptors, transporters and housekeeping genes. ViiA 7 real-time PCR platform with Array Card Block and comparative CT method was used for thermal cycles of the qPCR. The diacylglycerol lipase alpha (Dagla) and type 2 deiodinase (Dio2) served as positive controls, while the metabotropic glutamate receptor 1 (Grm1) and the metabotropic glutamate receptor 5 (Grm5) were used as negative controls.

3.6.5. TaqMan quantitative PCR analysis

Equal amount (10 ng) cDNA was applied in each TaqMan reaction using Taqman Fast Universal PCR Mastermix. For the qPCR analysis, ViiA 7 real-time PCR platform was used for thermal cycles with Fast-96 well block and comparative CT method.

3.7. Determine the expression of TH sensitive genes in different tissues

The RNA was isolated from pituitary samples by Arcturus PicoPure RNA Isolation Kit and DNase treatement was performed by RNase-Free DNase Set.

From MBH samples, the RNA isolation was performed by RNeasy lipid tissue mini kit.

NucleoSpin RNA kit with DNase treatment was used to isolate total RNA from liver, BAT, small intestine, hypothalamus and hippocampus samples. RNA concentration was determined with Qubit Fluorometer using a Qubit hs-RNA assay kit or with Smart Spec Plus Spectrophotometer. Reverse transcription was carried out with High-capacity cDNA reverse transcription kit, then cDNA concentration was measured with Qubit Fluorometer using Qubit ss-DNA assay kit. MBH, liver, BAT, hypothalamus and hippocampus samples of THAI mice were premaplified for luciferase (luc) gene expression. The TaqMan qPCR analysis was performed as described in **3.6.5**.

3.8. Body composition analysis and indirect calorimetry

Male TRH-IRES-tdTomato mouse pups (Eyperiment 3) were injected with T4 or vehicle and two months later, body composition analysis and indirect calorimetry measurements were performed. One week after the last tamoxifen treatment, the same measurements were performed on Rax-CreERT2 and Rax-CreERT2//Dagla$^{fl/fl}$ mice (Experiment 1). The body composition of the animals was determined by EchoMRI whole body magnetic resonance analysis. The food intake, the locomotor activity and the calorimetric parameters of the animals were continuously monitored for 72 hours by TSE PhenoMaster System. The metabolic data was analyzed with the TSE PhenoMaster software.

4. Results

4.1. Determine the importance of the endocannabinoid production of tanycytes in the regulation of the HPT axis

4.1.1. β2-tanycytes express glutamate receptors and glutamate transporters in mice

Gene expression assay of β2-tanycytes from CD1 mice was performed to determine whether the tanycytes might be sensitive for the glutamate and TRH released from the hypophysiotopic TRH neurons. The β2-tanycytes were isolated by LCM and gene expression of DAGLα, Dio2, glutamate receptor subunits, glutamate transporters and TRH receptors was measured by using TaqMan gene expression array cards. High level of glutamate transporter SLC1A3 expression was detected in β2-tanycytes. Mainly AMPA and kainite receptor subunits also expressed in β2-tanycytes, whereas the NMDA receptor subunits and metabotropic glutamate receptors present in less abundance. Both TRH receptor, TRHR1 and TRHR2, were absent in β2-tanycytes. These results indicating that glutamate can act primarily on the β2-tanycytes via kainite and AMPA receptors and glutamate transporter SLC1A3.

4.1.2. Validation of the tanycytes specific ablation of DAGLα in the Rax-CreERT2//Dagla$^{fl/fl}$

We determined whether the genetic manipulation decreased the DAGLα expression in the β2-tanycytes. While immunocytochemistry demonstrated DAGLα-immunoreactivity in the cell bodies and basal processes of β2-tanycytes in the ME of Rax-CreERT2 mice, markedly decreased DAGLα-immunoreactivity was observed in the tanycytes of the ME in the tamoxifen treated Rax-CreERT2//Dagla$^{fl/fl}$ (T-DAGLα KO) mice. The genetic manipulation did not influence the DAGLα-immunoreactivity in other brain regions. The expression of DAGLα mRNA in β2-tanycytes isolated by LCM was significantly decreased in β2-tanycytes of T-DAGLα KO mice compared to the controls, demonstrating that the Rax-CreERT2//Dagla$^{fl/fl}$ mice can be used to study the role of the DAGLα expression of tanycytes.

4.1.3. Tanycyte specific ablation of DAGLα influences the HPT axis

We examined how tanycyte specific ablation of DAGLα influences the HPT axis. ISH revealed that expression of proTRH mRNA did not change in the T-DAGLα KO mice compared to the control group. Densitometric analysis of autoradiograms revealed no significant differences between the integrated density unit of proTRH mRNA of T-DAGLα KO mice compared to the control group. However, the TSHβ mRNA level showed a two-fold increase in the T-DAGLα

KO mice compared to the controls. In accordance with the increased TSH production of the pituitary, the circulating fT4 level showed ~20% increase in the T-DAGLα KO mice compared to the control animals. However, the fT3 level was not influenced by the genotype.

Gene expression of Dio2 from isolated β2-tanycytes showed significant decrease in T-DAGLα KO mice, indicating that the tanycytes reduce the local T3 production as a result of elevated TSHβ and fT4 levels. However, the expression of TRH degrading enzyme PPII was not changed significantly.

4.1.4. Effect of tanycyte specific ablation of DAGLα on body composition and metabolism

THs play critical role in the regulation of development and energy homeostasis, therefore the effect of genetic manipulation on the body composition and metabolism was studied by indirect calorimetry measurements. During this 3-day-long period before and after the 72-hour-long indirect calorimetry measurements, the T-DAGLα KO animals gained significantly more weight than the controls. Furthermore, the fat mass normalized by lean body mass (LBM) significantly increased in the T-DAGLα KO mice, however, the LBM normalized by the total body weight (TBW) and the hydration ratio of the animals did not show significant changes. Interestingly, the blood glucose of the T-DAGLα KO group was significantly lower compared to the controls. In accordance with that the higher blood glucose level inhibits food intake, the T-DAGLα KO showed tendency for increased LBM normalized food intake compared to the control animals. The XY activity of the T-DAGLα KO mice was significantly increased in their home cages. The energy expenditure, the resting energy expenditure and respiratory exchange ratio (RER) of the animals did not show significant changes.

4.2. Development of the HPT axis negative feedback regulation in mice

4.2.1. THs already regulate the TRH expression in the PVN and the TSHβ expression in the pituitary in newborn mice

The setpoint of the HPT axis develops around birth, but the exact timing is unknown in mice, therefore, we examined the development of the feedback regulation of the HPT axis in newborn mouse pups. The TSHβ mRNA expression of the pituitary in the T4-treated TRH-IRES-TdTomato mouse pups decreased significantly in all age groups compared to the respective controls, indicating that the feedback regulation at the pituitary level is already developed after birth in mice. We repeated the treatments in a new cohort of 1, 3, 4 and 7 day old FVB/Ant

pups and proTRH mRNA level was determined by ISH in the mid-level of PVN. Representative images of all age groups showed that proTRH mRNA level was decreased in the T4-treated pups compared to the control animals. Densitometric analysis of the selected images revealed significant decrease of the proTRH mRNA level of postnatal day (P) 3, P4 and P7 T4-treated mouse pups. However, P1 age group showed only a strong tendency for decrease of proTRH expression in T4 group compared to the control.

4.2.2. Effect of early postnatal hyperthyroidism on the expression of Dio1 in the liver

Liver expression of the highly TH sensitive type 1 deiodinase (Dio1) was examined as a marker of circulating TH levels in 1-7 and 10 day old TRH-IRES-TdTomato mouse pups. Dio1 mRNA expression was significantly increased in the liver in all T4-treated animals compared to the respective controls, indicating the hyperthyroid state of the treated animals.

4.3. Characterize the effects of early postnatal hyperthyroidism on the HPT axis, metabolism and tissue specific TH action of adult mice

4.3.1. Early postnatal hyperthyroidism induced lifelong changes of the HPT axis

To determine the effect of early postnatal hyperthyroidism in adult mice, male TRH-IRES-TdTomato and THAI mouse pups were injected subcutaneously daily with 1μg/bwg T4 between P2-P6 and sacrificed 2 months later. Early postnatal T4 administration resulted in a marked decrease of the proTRH mRNA hybridization signal in the PVN of adult TRH-IRES-TdTomato mice compared to the control group. By image analysis, postnatal T4 treatment induced an approximately 30% reduction in the density values of proTRH mRNA hybridization signal in the PVN of adult mice. However, the TSHβ mRNA expression of the pituitary did not change significantly, but the circulating fT4 level decreased significantly in the group treated with T4 in the postnatal period compared to the control group. The presence of decreased TRH expression and decreased fT4 level supports the existence of central hypothyroidism in this mouse model. The fT3 level, however, was not altered by the treatment, suggesting that changes of peripheral TH metabolism can normalize the fT3 level despite of the lower fT4 level.

4.3.2. Tanycytes are not involved in the regulation of the HPT axis set point by postnatal hyperthyroidism

As the TH activating capability of tanycytes can influence the feedback regulation of the TRH neurons and the Dio2 in the MBH is only expressed by the tanycytes, we isolated α- and β-

tanycyte subgroups separately by LCM of postnatally treated TRH-IRES-TdTomato adult mice. The Dio2 mRNA expression did not change significantly neither in α-, nor in β-tanycytes. Tanycytes can also influence the activity of the HPT axis by degrading TRH in the ME by PPII enzyme. Neither α-, nor β-tanycytes showed significant change in the expression of PPII between the control and postnatally T4-treated, adult TRH-IRES-tdTomato mice. As D2 activity is highly regulated posttranslationally, we also studied whether the TH action is influenced by the postnatal T4 treatment in the MBH of adult mice. For this experiment, we used THAI mice in which the expression of luc is highly regulated by THs. In the MBH of postnatally T4-treated adult THAI mice, luc expression - the indicator of TH action - was not influenced by the early postnatal treatment. These results demonstrate that the tanycytes are not involved in the regulation of the HPT axis set point by postnatal hyperthyroidism.

4.3.3. Early postnatal hyperthyroidism influences the body composition of adult mice

THs play critical role in the regulation of development and energy homeostasis, therefore the effect of early postnatal hyperthyroidism was studied on the body composition and energy homeostasis of adult TRH-IRES-tdTomato mice. The adult, postnatally T4-treated animals showed significantly decreased body weight compared to the controls. Furthermore, the body length of the T4 group was significantly decreased compared to the control animals. The body composition of mice was mesaured by EchoMRI whole body magnetic resonance analyser. Neither the fat mass / LBM, nor the LBM / TBW ratio were different between the control and T4 treated groups. The LBM normalized food intake was not different between the groups. Thus, the marked decrease of body weight is not due to altered body composition, but rather gross retardation, since the thyroid status influences the bone growing.

4.3.4. Early postnatal hyperthyroidism influences the activity and energy homeostasis of adult mice

The locomotor activity and energy homeostasis of adult TRH-IRES-TdTomato mice was determined by indirect calorimetry measurements. Despite the decreased fT4 levels, the postnatally T4-treated mice showed significantly increased locomotor activity in the metabolic cages, including XY activity, travelled distance and speed. The higher activity was accompanied by significantly higher energy expenditure. Furthermore, the resting energy expenditure of the postnatally T4 treated mice was also significantly higher, suggesting that the central hypothyroidism is compensated at the level of tissues. The RER was not different between the two groups.

4.3.5. Effects of early postnatal hyperthyroidism on the TH action in tissues of adult mice

THAI mice were treated with 1μg/bwg thyroxin or vehicle between P2-6 and were sacrificed at adulthood. To determine the tissue specific TH action, luc expression (marker of TH action in the THAI mice) was studied in different tissues. Neither the hypothalamus, nor the hippocampus showed significant changes in the luc expression in T4-treated mice compared to the controls. We also examined the luc expression in TH sensitive peripheral tissues. The luc expression decreased only in the small intestine in the postnatally T4-treated adult mice, but in BAT, the luc mRNA level was unchanged.

4.3.6. Early postnatal hyperthyroidism induced changes in TH-releated genes of adult mouse liver

Since the liver is one of the main target tissues of THs, expression of TH-related genes were examined by TaqMan qPCR analysis in the liver of postnatally T4-treated adult TRH-IRES-TdTomato mice. Early postnatal T4 treatment resulted in marked, approximately 90% decrease of Dio1 expression in the liver. To determine the tissue specific TH action, luc expression was also studied in the liver of postnatally T4-treated THAI mice. However, the luc expression as a marker of thyroid hormone action did not change significantly in the liver indicating euthyroid status in this tissue. The TH receptor α and β (THRA, THRB) expression did not change significantly in this tissue. The TH responsive (Spot14) gene is known to respond rapidly to TH and is responsible for the tissue-specific regulation of lipid metabolism as a lipogenic gene in the liver. Interestingly, the lower fT4 level of the animals did not change the gene expression of Spot14 in the postnatally T4-treated mice. The expression of other genes involved in the fatty acid metabolism and regulated positively by TH were also studied. Both the malic enzyme 1 (ME1) and fatty acid synthase (FASN) showed significant decrease in the T4-treated group compared to the control animals. The expression of the catalytic subunit of glucose-6-phosphatase (G6PC) - which is essential for endogenous glucose production - did not change significantly.

4.3.7. Effect of early postnatal hyperthyroidism on the expression of DNA methyltransferase enzymes in the liver

To determine whether the changes in the expression profile of certain TH-dependent genes in the liver could be due to epigenetic regulation, the expression of genes involved in DNA methylation were measured in liver of T4-treated and control 2 and 6 day old mouse pups. DNA-methyltransferase 1 (Dnmt1) level showed a tendency to increase in the T4 group, but

the change was not significant, while DNA-methyltransferase 3A (Dnmt3a) and DNA-methyltransferase 3B (Dnmt3b) expression was significantly increased in the T4 groups compared to the controls at P2. The level of these genes did not show significant changes at P6, indicating that the P2 but not the P6 mice are in the sensitive period, when the thyroid status may influence the set point of TH dependent genes in the liver and induce epigenetic changes by DNA methylation.

5. Conclusions

The present study provides a better understanding of the central regulation of the HPT axis by endocannabinoids and how disturbances in thyroid status induce lifelong changes in this sensitive system.

It was known that tanycytes can influence the HPT axis via several mechanisms. Recently, our group revealed a novel regulatory microcircuit, which involves the tanycytic endocannabinoid production. In the current study, we showed that the tanycytes specific genetic ablation of DAGLα results in increased TSH synthesis and elevated fT4 level, supporting the view that the TRH release is tonically inhibited by the endocannabinoid production of tanycytes. Despite of the elevated fT4 level, the energy expenditure of the T-DAGLα KO mice was not increased suggesting that the increased TH level is compensated by TH metabolism in tissues. Interestingly, T- DAGLα KO mice had decreased blood glucose level indicating that the 2-AG production of tanycytes is also involved in the regulation of glucose homeostasis, but the mechanism of this effect needs further examination.

Our experiments revealed that the thyroid status has critical effect on the development of the feedback regulation of the HPT axis in the early postnatal period. Hyperthyroidism in this critical period induces lifelong changes of the set point of this regulation, suggesting that epigenetic modifications occur in certain TH dependent genes of the HPT axis. This hypothesis is supported by the TH regulation of DNMT enzymes in P2 mouse pups.

Despite of the hypothyroidism of adult mice induced by the early postnatal hyperthyroidism, most tissues of these animals are euthyroid and they have normal energy expenditure suggesting that the peripheral tissues can compensate the lower fT4 level of this model. However, tanycytes play critical role in the regulation of the HPT axis, our data indicate that these cells are not involved in the mediation of the effects of early postnatal hyperthyroidism on the HPT axis.

These results support that not only hypothyroidism, but also hyperthyroidism in the developmental period has major life-long effect.

6. List of publications

6.1. List of publication the thesis based on

Kővári D, Penksza V, Szilvásy-Szabó A, Sinkó R, Gereben B, Mackie K, Fekete C. (2021) Tanycyte specific ablation of diacylglycerol lipase alpha stimulates the hypothalamic-pituitary-thyroid axis by decreasing the endocannabinoid mediated inhibition of TRH release. Journal of Neuroendocrinology, 2022;34:e13079. IF:3,27

Farkas E, Varga E, Kovács B, Szilvásy-Szabó A, Cote-Vélez A, Péterfi Z, Matziari M, Tóth M, Zelena D, Mezriczky Z, Kádár A, Kővári D, Watanabe M, Kano M, Mackie K, Rózsa B, Yvette R, Tóth B, Máté Z, Erdélyi F, Szabó G, Gereben B, Lechan RM, Charli JL, Joseph-Bravo P, Fekete C. (2020) A glial-neuronal circuit in the median eminence regulates thyrotropin-releasing hormone-release via the endocannabinoid system. iScience, 27;23(3):100921 IF:5,458

6.2. Other publications

Varga E, Farkas E, Zséli G, Kádár A, Venczel A, Kővári D, Németh D, Máté Z, Erdélyi F, Horváth A, Szenci O, Watanabe M, Lechan RM, Gereben B, Fekete C. (2019) Thyrotropin-releasing-hormone-synthesizing neurons of the hypothalamic paraventricular nucleus are inhibited by glycinergic inputs. Thyroid, 29:12 pp. 1858-1868 IF: 5,227

Ruska Y, Szilvásy-Szabó A, Kővári D, Kádár A, Mácsai L, Sinkó R, Hrabovszky E, Gereben B, Fekete C. (2021) Expression of glucagon-like peptide 1 receptor in neuropeptide Y neurons of the arcuate nucleus in mice. Brain Structure & Function, 227, pages 77-87(2022) IF: 3,27

Cumulative IF: 17,225