Final report

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Thyroid hormone (TH) is fundamental regulator of cellular energetics and cell cycle and represents an indispensable factor for proper brain development and function. Our aim was to reveal novel cellular and molecular regulators of hypothalamic TH signalling in order to better understand the regulation of the hypothalamo-pituitary-thyroid (HPT) axis and adult hypothalamic neurogenesis.

TH activation is performed by type 2 deiodinase (D2) catalysed deiodination that results in conversion of thyroxine/T4 to T3 to allow its binding to TH receptor (TR). TH economy is regulated by two major regulatory systems; the HPT axis and the local/tissue-specific regulatory machinery of TH action: the two systems are functionally interconnected in the hypothalamus, representing the center of the endocrine brain. In the hypothalamus, D2 is expressed in tanycytes in the wall and floor of the third ventricle. We demonstrated earlier that D2 undergoes substrate induced ubiquitination and proteasomal degradation along the endoplasmic-reticulum-associated-protein-degradation pathway. Tanycytes coexpress the WSB1 and Teb4/MARCH6 D2 ubiquitin ligases, that is not the case in astrocytes, representing the major D2-expressing cell-type outside of the hypothalamus. This allowed to hypothesize, that ubiquitination mediated fast post-translational D2 regulation could be more complex in tanycytes than in astrocytes. Using a coexpression approach we mimicked this situation n HEK293 cells and studied structural and subcellular aspects that could impact D2-mediated T3 generation. We clarified the topology of the D2-MARCH6 interaction by demonstrating direct protein-protein interaction between the D2 globular domain and the N-terminal RING-domain of MARCH6 in living cells by Fluorescence resonance energy transfer. We also proved that the interaction is increased upon T4 exposure as an underlying mechanism of substrate induced downregulation of the D2 protein. The expression of MARCH6 was not affected by TH. We also generated chimeric recombinant deiodinase proteins and identified the minimal combination of structural elements required and sufficient for the instability and targeting of deiodinase proteins for ubiquitination. By the construction of chimeric deiodinases, we identified the minimal structural elements required for the instability and ubiquitination of a deiodinase protein, that involve the combinates presence on of ubiquitinated lysines and a D2 specific loop sequence and ER localization. Our data demonstrates that WSB1 and MARCH6-

D2 coexpression allows efficient ubiquitin ligation to the D2 molecule *(Egri and Gereben J. Mol. Endocrinol 2014).*

To better understand pathways affecting intracellular TH signalling as the regulator of adult hypothalamic neurogenesis, we studied novel regulators of D2 mediated T3 generation of tanycytes. The pituitary adenylate cyclase-activating polypeptide (PACAP) is regulated by cAMP, an intracellular messenger representing at the same time an impotent regulator of the D2 expressing dio2 gene. We used luciferase promoter assays to demonstrate that PACAP can induce the dio2 promoter and this effect can be abolished by the mutagenesis of the cAMP response element of the dio2 gene. We also demonstrated the presence of the PACAP specific PAC1R receptor on rat tanycytes. Using stereotaxic assisted *icv.* hypothalamic PACAP administration we could detect increased D2 activity in the mediobasal hypothalamus. These findings proved that PACAP is a regulator of T3 generation in tanycytes. The PACAP-D2-T3 signalling pathway also had physiological consequences since it could decrease TRH expression in the paraventricular nucleus (PVN) of mice indicating that the generated T3 excess in the hypothalamus, beyond its potential effect on hypothalamic neurogenesis can also impact the HPT axis *(Egri et al. Endocrinology 2016).*

Proopiomelanocortin (POMC) is known to be expressed in neurons. In a collaborative study we demonstrated that POMC is also expressed in the ventral wall of the hypothalamic third ventricle were tanycytes are located. These data suggested that tanycyte-derived POMC, acting in a paracrine/autocrine manner, may be involved in hypothalamic cell proliferation including an effect on the proliferative/neurogenic functions of tanycytes themselves *(Wittmann et al. J. of Comp. Neurology 2017).* We also demonstrated that Musashi, a cell cycle regulator protein is expressed in tanycytes and studied its role in D2 regulation. We generated D2 3'UTR recombinant DNA constructs and demonstrated the presence of a Musashi responsive region in the 3' UTR of the D2 mRNA in HEK293 cells. Our data demonstrate that Musashi is a potential regulator of hypothalamic T3 generation.

To better understand factors regulating adult hypothalamic neurogenesis we also performed studies on TH transporters known to affect intracellular TH levels. We and others showed before that MCT8 and OATP1c1 TH transporters are expressed in the hypothalamus. Therefore, we investigated cell-type specific aspects and adaptational changes of hypothalamic and extrahypothalamic TH transport in a collaboration study. We showed that, the initial phase

of bacterial lipopolysaccharide (LPS) induced nonthyroidal illness transporter expression is not changed in astrocytes and neurones, in contrast to the blood brain barrier *(Wittmann et al. Endocrinology 2015; Wittmann et al. Fluids Barriers CNS 2015).*

We set up various approaches to manipulate gene expression in tanycytes. We generated a Nestin-Cre-ERT2 X tdTomato(Flx) mouse line, that following tamoxifen induction expressed the reporter in tanycytes. However, since reporter expression was not restricted to tanycytes we moved to Rax2-Cre, a marker more restrictively expressed in tanycytes in the hypothalamus. The Rax-Cre-ERT2 X tdTomato animals showed reporter expression only in tanycytes 2 weeks after Tam induction. We used this model to study adult hypothalamic neurogenesis where i) tdTomato indicates Rax2 expression and this way labels cells of tanycyte origin; ii) HuC/D positivity demonstrates the neuronal phenotype; iii) while BrdU (delivered by an osmotic minipump for 2 weeks) captures newborn cells. In each animal cell were counted in three consecutive sections. We observed numerous Rax2(Tomato) and BrdU positive cells but these two groups only minimally overlapped. In the arcuate nucleus we also detected HuC/D-BrdU positive cells, i.e. newborn neurons of non-tanycytic origin. On contrast, another cell group coexpressed Rax2(Tomato) and HuC/D but these were typically BrdU negative. It can be hypothesized that the HuC/D/BrdU cells could represent precursors in which Rax2(Tomato) expression could start at a later stage and these cells potentially could give rise to either neurons or tanycytes. The presence of Rax2(Tomato)/Hu without BrdU could represent a neuronal cell group that express Rax2 independently of the tanycyte cell lineage. We conclude that in the used experimental paradigm the newborn BrdU positive hypothalamic neurons of tanycyte origin (indicated by Rax2(Tomato) positivity) represent a minority of newborn neurones in the adult hypothalamic arcuate nucleus - median eminence region.

We also generated Rax-Cre-ERT2 X D2(Flx) animals to suppress D2 in tanycytes using various Tam injection protocols but the achieved 50% decrease in D2 mRNA expression was still not enough to achieve significant decrease in the activity of the D2 enzyme. This problem could not be overcome with the addition of tamoxifen containing diet in addition to the treatment regime. D2 expression in tanycytes is very abundant and tanycytes are self-renewing cells that could interfere with the ablation. We also tried to overcome this problem by hypothesizing the insufficient tanycytic Cre action in tanycytes is the consequence of dense chromosomal gene packaging and this could be resolved by transcriptionally induce the D2 encoding dio2 gene before Cre recombination in order to allow better access to the recombinase protein to

less densely packaged DNA strands. This approach was proved to be efficient for others to knock down the expression of the leptin receptor in these cells that similarly to D2, failed before using the induction approach. We have attempted to knock down D2 in tanycytes of the Rax-Cre-ERT2 X D2(Flx) mice using histone decomposition. We performed transcriptional induction of the D2 encoding dio2 gene before Cre recombination by LPS application to mice using our established protocol in order to allow better access to the recombinase protein to less densely packaged DNA strands. However, this approach could not achieve D2 knock-down indicating that insufficient Cre recombinase action in tanycytes could be explained by dense chromosomal gene packaging.

Therefore, we have set up an *in vitro* CRISPR/Cas9 system in HEK293 to facilitate the test guide RNAs aimed to knock-down D2. We generated fusion vectors containing a D2 encoding fragment in frame of the 5' end of a luciferase coding region. This was cotransfected by corresponding D2 targeting guide RNA encoding vectors along with a CAS9 expressing vector followed by the assessment of luciferase activity. This represents the amount of the D2-target-luciferase fusion DNA. We did not find this approach effective *in vitro* therefore we decided to move directly to an *in vivo* approach bases on AAV-mediated expression of D2 specific guide CRISPR RNAs in CAS9 mice to suppress D2 in tanycytes. Studies are still in progress, but we already performed a technical optimization step using injections of a floxed EYFP expressing virus (rAAV-DIO-ChR2-EYFP) into Tam induced Rax2-Cre-ERT2 mice that demonstrated that multilocular injections (injections using minimized injection volume on both sides into the arcuate nucleus) can achieve expression in both ventral alpha-, and beta tanycytes. As an *in vitro* contingency approach, we have also set up primary tanycyte cultures during the extension period that will allow to work on tanycytes originating from D2 knock-out mice.

Hypothalamic T3, generated in tanycytes, also play a fundamental role in regulating the HPT axis by negative feedback. The underlying mechanisms of his process are largely unknown despite their fundamental impact on TH economy on tissue function that remarkably persist throughout the entire lifespan. Thus, we aimed to determine the onset of feedback on hypophysiotropic TRH neurons of the PVN and assesses the role of D2-mediated T4 activation in this process, and also attempted to identify the underlying cellular and molecular events. Since rodents provide a suboptimal model for these studies due to their strikingly different kinetics of HPT axis development compared the humans, we used chicken embryos, a well-established developmental model for our studies that also allowed *in ovo* embryonic

manipulation in the absence of modifying maternal effects. We studied the effect of T4 and T3 challenges on the developing chicken hypothalamus and determined TRH expression in the PVN by *in situ* hybridization along with TSHβ assessment in the pituitary with TaqMan PCR. We demonstrated that negative TH feedback on TRH neurons forms between embryonic day 19 and two days after hatching during chicken ontogenesis. Our studies on the underlying mechanisms revealed that feedback formation is accompanied by intense and coordinated hypothalamic increase of D2 activity required to build the sufficient T3 gradient for set point formation. Using promoter assay in glioma cells and *in situ* hybridisation at different developmental stages we also provided evidence that Nkx2.1-mediated transcriptional events could play a role in this process. Since human maternal TH status impacts foetal TH availability and altered TH levels during development could result in shifted HPT set point with persisting effects, our data could provide novel arguments to the ongoing debate whether maternal TH levels should be routinely monitored during human pregnancy, especially in the sensitive period when set point of the HPT axis is formed. Studies on primates would be useful to confirm the importance of the presented mechanism in higher vertebrates *(Mohácsik et al. Endocrinology 2016).*

We have also generated a transgenic mouse model (THAI, thyroid hormone indicator mouse) that allows the tissue specific measurement of TH action in tissue samples and live animals including the brown adipose tissue that is a locus of TH and norepinephrine regulated nonshivering thermogenesis and represents a target of obesity research (*Mohácsik et al. Endocrinology 2018*). The model has been patented (see details in the utilization section) since it also allows *in vivo* testing of TR isoform-specific TH analogue candidate molecules. We performed studies to pick the most suitable Luciferase reporter since the classical firefly luciferase is subjected to T3 evoked but promoter independent downregulation. To overcome this problem, we characterized the TH responsiveness of various luciferase reporters. We demonstrated that the dCpGLuciferase, representing a luciferase with unchanged amino acid sequence with numerous silent mutations can accurately assess TH action *(Kollár et al. Thyroid 2016*). We used the THAI model to study hypothalamic TH signalling during nonthyroidal illness and revealed the region-specific changes of TH action in the rodent hypothalamus. The studies were also extended to assess the responsiveness of the HPT axis during aging. This model will be also useful to monitor TH signalling in hypothalamic regions of adult neurogenesis.

We summarized our results on hypothalamic TH signalling in two review articles *(Gereben et al. Nature Rev Endocrinology 2015; Bianco et al. Endo Reviews accepted 2019*).

The PI also gave numerous invited symposia talks at leading scientific meetings and workshops on the subject of the project, e.g.: 38th Annual Meeting of the European Thyroid Association, Santiago de Compostella Spain 2014; International Thyroid Congress Lake Buena Vista (FL) USA 2015; Joint Meeting of the Hungarian Pharmacological, Anatomical, Microcirculation and Physiological Societies, Pécs Hungary 2016; Selected Topics in Clinical Endocrinology, Quo vadis glandula thyreoidea? Balatonfüred Hungary 2017; European Society of Endocrinology Winter School, Budapest 2017; European Society of Endocrinology Meeting, Barcelona Spain 2018; the 41st European Thyroid Association Annual Meeting, Newcastle UK 2018.

As dissemination, we gave interviews to the "Novum Magazin" of the TV channel MTVA M5 (09/07/2017) and for the Journal „Innotéka Magazin" (30-33 2017 December, http://www.innoteka.hu/cikk/felfedezo_kutatastol_a_szabadalomig.1610.html) to introduce research addressing TH signalling in the brain to the public.

In summary, we developed cellular and transgenic mouse models to study TH signalling and identified cellular and molecular pathways regulating hypothalamic TH economy. While knocking-down gene expression in hypothalamic tanycytes *in vivo* turned out to be more challenging than expected due to the self-renewing nature of these cells, we attempted to develop alternative approaches to overcome this problem and utilization of these approaches is ongoing.