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. 200ng/bwg thyroxin was administered to 1-7 and 10 day old pups and the animals were sacrificed 8h later.

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

The TSHβ mRNA expression of the pituitary in the T4-treated TRH-IRES-TdTomato mouse pups decreased significantly in all age groups (**Figure 1**, p<0.0001) compared to the respective controls, indicating that the feedback regulation at the pituitary level is already developed after birth in mice. Therefore, we examined if the feedback regulation is also developed at the hypothalamic level after birth. 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 **(Figure 2)**. Densitometric analysis of the selected images revealed significant decrease of the proTRH mRNA level of P3 (**Figure 2D**, Control *vs.* T4 (Int. dens. sum): 86.68±7.96 *vs.* 63.98±2.98, p=0.02, t=2.839, df=11, N=6-7), P4 (**Figure 2F**, Control *vs.* T4 (Int. dens. sum): 183.20±39.58 *vs.* 63.96±8.95, p=0.03, t=2.680, df= 9, N=5-6) and P7 (**Figure 2H**, Control *vs.* T4 (Int. dens. sum): 222.40±24.67 *vs.* 150.10±13.41, p=0.03, t=2.573, df=10, N=6) T4-treated mouse pups. However, P1 age group **(Figure 2B)** showed only a strong tendency (p=0.06) for decrease of proTRH expression in T4 group compared to the control (Control *vs.* T4 (Int. dens. sum): 183.00±51.41 *vs.* 74.41±23.58, t=2.142, df=8, N=4-6) which can be due to the low sample size.

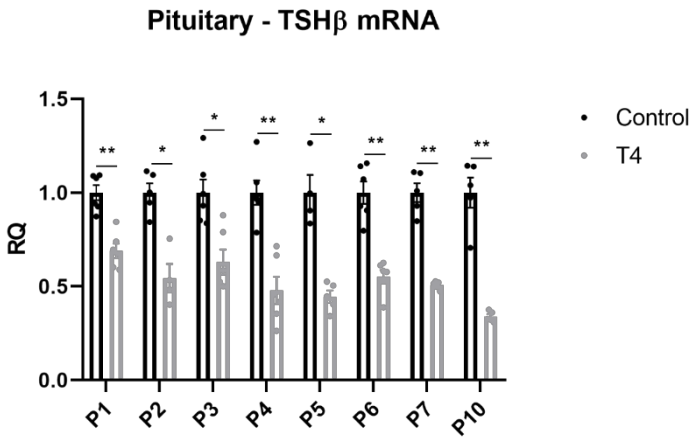

Figure 1: Changes of TSHβ expression after T4 treatment in P1-10 mouse pups.

*TaqMan qPCR analysis of TSHβ mRNA expression in pituitary 8 hours after injection of 200 ng/bwg T4 or vehicle in P1-7 and 10 day old TRH-IRES-TdTomato mouse pups. Pituitary TSHβ mRNA levels were significantly decreased in all age groups compared to the controls. Data are compared with Two-way ANOVA followed by Bonferroni post hoc test. Data are shown as mean ± SEM. N=4-6, *p < 0.05; **p < 0.01. Abbreviations: TSHβ - thyroid stimulating hormone beta, P - postnatal day, T4 – thyroxine.*

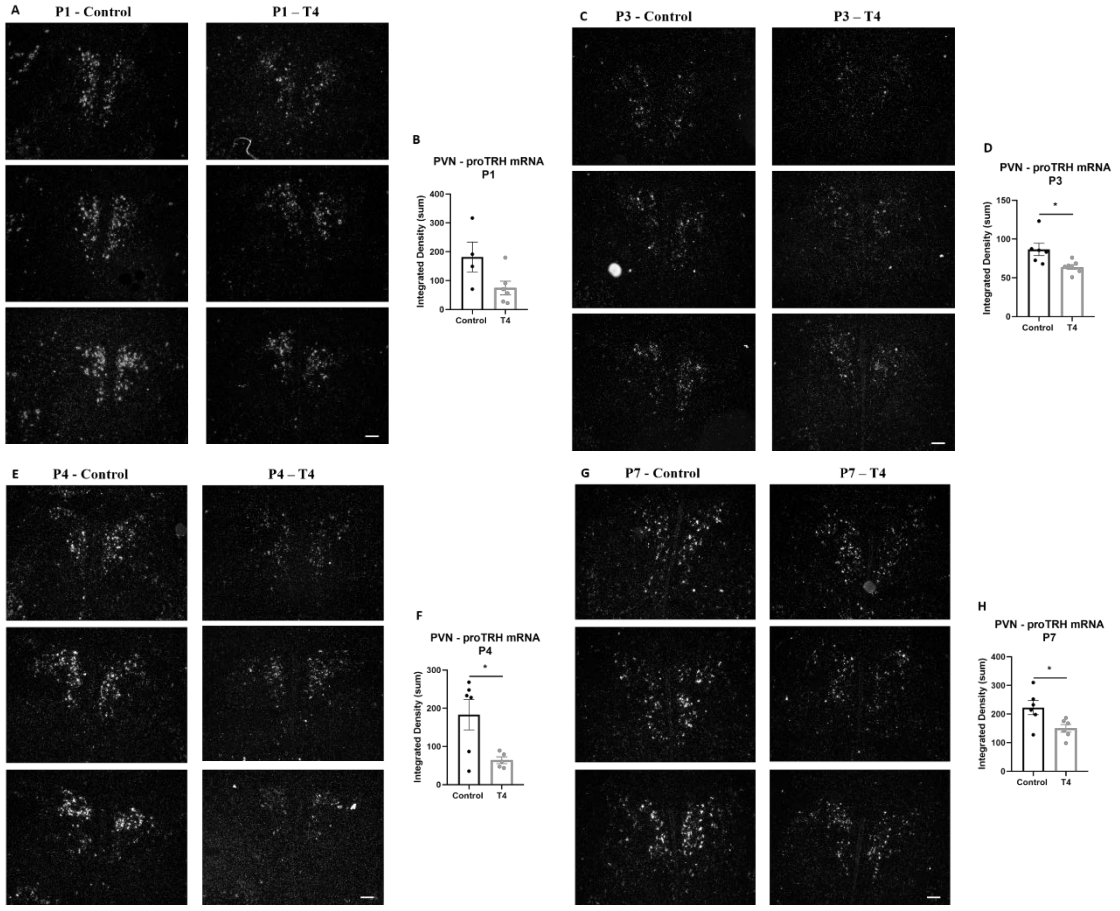

Figure 2: Response of proTRH expression in the PVN to peripheral T4 treatment in mouse pups.

*1, 3, 4 and 7 day old FVB/Ant mouse pups were treated with 200 ng/bwg T4 or vehicle and sacrificed 8 hours later to determine TRH expression in PVN. Representative images of proTRH mRNA ISH autoradiograms in 1 (A), 3 (C), 4 (E), 7 (G) day old FVB/Ant mouse pups. Densitometric analysis of the selected images of proTRH mRNA hybridization signal in the PVN showed significant decrease in 3 (D), 4 (F) and 7 (H) day old (N=5-7) T4 treated groups compared to the controls and tendency for decrease in P1 (B, N=4-6) age group. Data are compared with Student's t test. Data are shown as mean ± SEM. *p < 0.05. Abbreviations: P - postnatal day, T4 – thyroxine, ISH – in situ hybridization, PVN - hypothalamic paraventricular nucleus, proTRH – pro-thyrotropin releasing hormone.*

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

Liver expression of the highly TH sensitive 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 **(Figure 3)** was significantly increased in the liver ($p<0.0001$) in all T4-treated animals compared to the respective controls, indicating the hyperthyroid state of the treated animals.

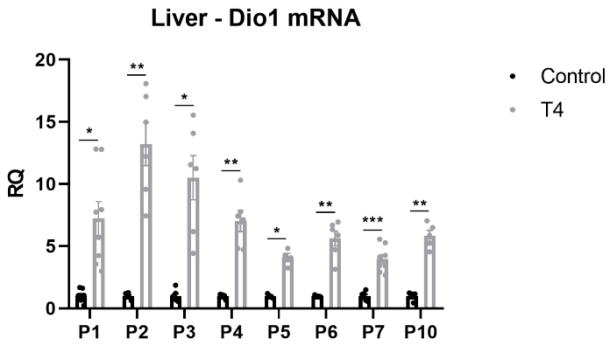

Figure 3: Expression of Dio1 in liver of P1-7, 10 mouse pups.

*TaqMan qPCR analysis of Dio1 mRNA expression (N=4-8) in liver 8 hours after injection of 200 ng/bwg T4 or vehicle in P1-7 and P10 mouse pups. Liver Dio1 mRNA levels were significantly increased in all age groups compared to the controls by two-way ANOVA followed by Bonferroni post hoc test and shown as mean ± SEM. *p < 0.05; **p < 0.01, ***p < 0.001. Abbreviations: Dio1 – type 1 deiodinase, P - postnatal day, T4 – thyroxine.*

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

To determine the effect of early postnatal hyperthyroidism in adult mice, male TRH-IRES-TdTomato and Thyroid Hormone Action Indicator (THAI) mouse pups were injected subcutaneously daily with 1µg/bwg T4 between P2-P6 and sacrificed 2 months later. The THAI mice express luciferase (luc) protein under the control of a minimal TK promoter that was made TH sensitive by three copies of the TH response element of the human *dio1* gene. Therefore, the expression of luc can be used as the marker of tissue specific TH action [133].

Early postnatal hyperthyroidism induced lifelong changes of the HPT axis

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 **(Figure 4A-F)**. By image analysis **(Figure 4G)**, postnatal T4 treatment induced an approximately 30% reduction in the density values of proTRH mRNA hybridization signal in the PVN of adult mice (Control *vs.* T4 (Int. dens.): 296.00±23.91 *vs.* 212.80±20.27, p=0.02, t=2.654, df=16, N=9).

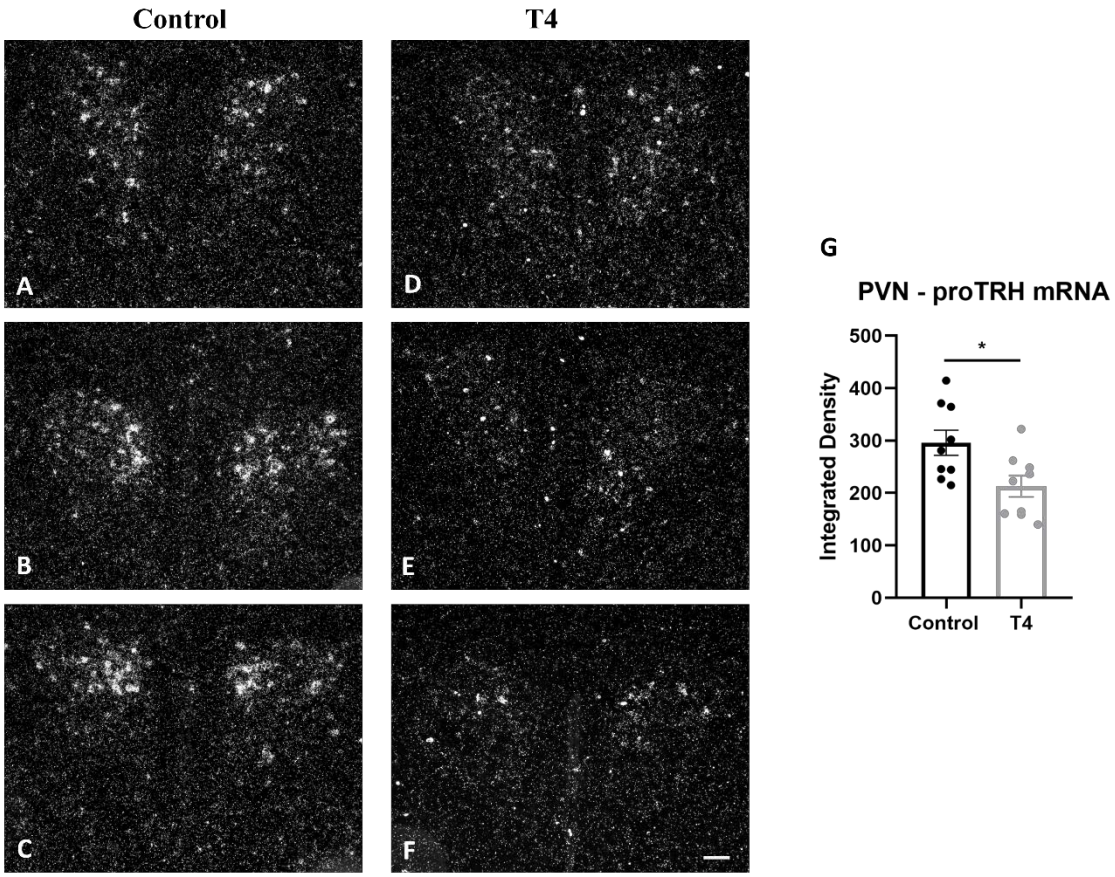

Figure 4: Effect of early postnatal hyperthyroidism on the TRH expression in the PVN of adult mice. *TRH-IRES-TdTomato male mouse pups were treated with 1µg/bwg T4 or vehicle between P2-6 days and sacrificed at adulthood to determine the TRH expression in PVN by ISH. Darkfield images (A-F) and densitometric analysis (G, N=9) of proTRH mRNA expression of the PVN showed that the early postnatal T4 treatment resulted in marked decrease of TRH synthesis in adult mice. Data are compared with Student's t test and shown as mean ± SEM. *, p < 0.05. Abbreviations: T4: thyroxine, PVN – hypothalamic paravnetricular nucleus, proTRH – pro-thyrotropin releasing hormone.*

However, the TSHβ mRNA expression of the pituitary **(Figure 5A)** did not change significantly despite of the reduced hypothalamic TRH expression (Control *vs.* T4 (RQ): 1.00±0.07 *vs.* 0.92±0.09, p=0.46, t=0.759, df=19, N=10-11), but the circulating fT4 level **(Figure 5B)** decreased significantly in the group treated with T4 in the postnatal period compared to the control group (Control *vs.* T4 (ng/dl): (0.82±0.03 *vs.* 0.68±0.04, p=0.02, t=2.527, df=26, N=14). The presence of decreased TRH expression and decreased fT4 level supports the existence of central hypothyroidism in this mouse model. The fT3 level, however, **(Figure 5C)** was not altered by the treatment (Control *vs.* T4 (ng/dl): 3.10±0.39 *vs.* 2.78±0.30; p=0.52, t=0.656, df=25, N=13-14), suggesting that changes of peripheral TH metabolism can normalize the fT3 level despite of the lower fT4 level.

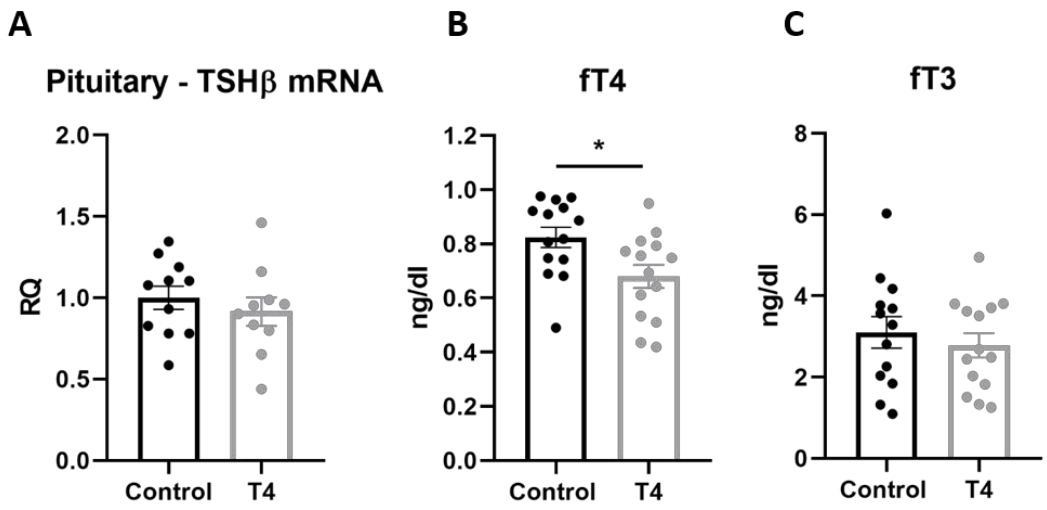

Figure 5: Effect of early postnatal hyperthyroidism on the TSHβ expression and TH levels in adult mice. *TRH-IRES-tdTomato male mouse pups were treated with 1µg/bwg T4 or vehicle between P2-6 days and sacrificed at adulthood to measure TSHβ expression in the pituitary by TaqMan qPCR analysis and TH levels from sera. The pituitary TSHβ mRNA (A, N=10-11) level was not change significantly in the groups. Serum fT4 (B, N=14) level was significantly lower in the adult, postnatally T4 treated mice. However, the serum fT3 (C, N=13-14) was not influenced by the treatment. Data are compared with Student's t test and shown as mean ± SEM. *, p < 0.05. Abbreviations: T4: thyroxine, TSHβ - thyroid stimulating hormone beta subunit, fT4 – free thyroxin, fT3 – free triiodothyronine.*

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

The TH activating capability of tanycytes can influence the feedback regulation of the TRH neurons [78]. Therefore, the expression of dio2 gene coding the TH activating enzyme D2 was studied in the MBH of adult, postnatally T4 treated THAI mice to investigate whether the central hypothyroidism resulted by the postnatal T4 treatment is due to altered TH activating capacity of tanycytes. Since 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- (**Figure 6A,** Control *vs.* T4 (RQ): 1.00±0.10 *vs.* 0.96±0.20, p=0.88, t=0.159, df=10, N=6), nor in β-tanycytes (**Figure 6B,** Control *vs.* T4 (RQ): 1.00±0.29 *vs.* 0.85±0.26, p=0.71, t=0.385, df=7, N=4-5).

Tanycytes can also influence the activity of the HPT axis by degrading TRH in the ME by PPII enzyme. Neither α- (**Figure 6C,** Control *vs.* T4 (RQ): 1.00±0.06 *vs.* 1.01±0.10, p=0.93, t=0.932, df=10, N=6), nor β-tanycytes (**Figure 6D,** Control *vs.* T4 (RQ): 1.00±0.23 *vs.* 0.50±0.13, p=0.09, t=1.965, df=7, N=4-5) 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. 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 (**Figure 6E,** Control *vs.* T4 (RQ): 1.00±0.13 *vs.* 0.81±0.11, p=0.31, t=1.056, df=11, N=6-7) excluding the possibility that in this model, the effect of postnatal T4 treatment on the HPT axis of adult mice is mediated by tanycyte induced alteration of the feedback regulation of TRH neurons.
These results demonstrate that the tanycytes are not involved in the regulation of the HPT axis set point by postnatal hyperthyroidism.

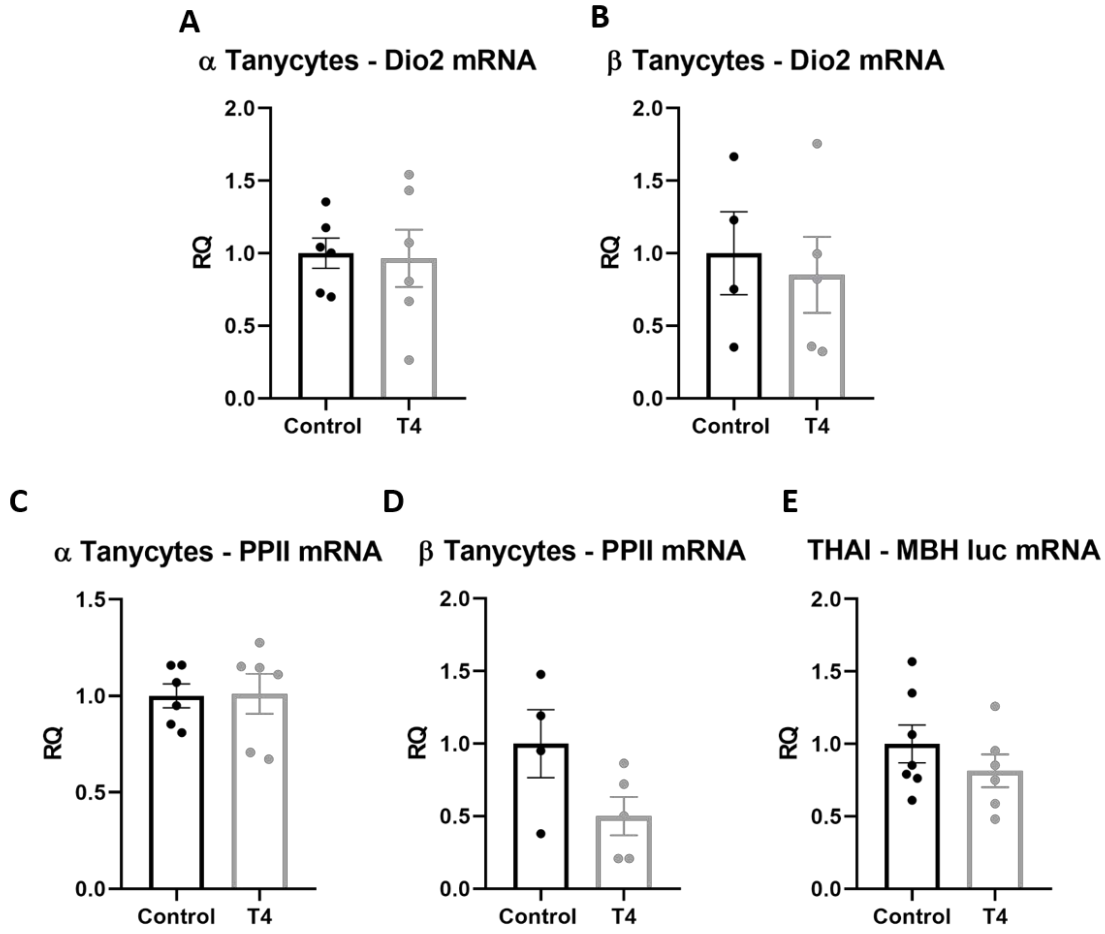

Figure 6: Early postnatal hyperthyroidism did not influence the TH activating and TRH degrading capacity of tanycytes.

Male THAI and TRH-IRES-TdTomato mouse pups were treated with 1µg/bwg T4 or vehicle between P2-6 days and sacrificed at adulthood. The Dio2 expression in α- (A) and ß-tanycytes (B) did not change significantly between control and T4-treated mice. The treatment also did not influence the PPII mRNA level in α- (C) or ß-tanycytes (D) and luc expression of MBH of THAI mice (E). Data are compared with Student's t test and shown as mean ± SEM. Abbreviations: THAI – thyroid hormone action indicator mouse, MBH – mediobasal hypothalamus, Dio2 – type 2 deioidinase, luc – luciferase, PPII – pyroglutamyl-peptidase II.

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 (**Figure 7A,** Control *vs.* T4 (g): 22.84±0.63 *vs.* 19.18±0.50, p=0.001, t=4.637, df=22, N=11-13). Furthermore, the body length of the T4 group was significantly decreased compared to the control animals (**Figure 7B,** Control *vs.* T4 (cm): 9.54±0.19 *vs.* 8.79±0.16, p<0.0001, t=9.657, df=19, N=10-11). The body composition of mice was mesaured by EchoMRI whole body magnetic resonance analyser. Neither the fat mass / LBM ratio (**Figure 7C**, Control *vs.* T4 (%): 14.09±1.08 *vs.* 13.63±0.74, p=0.72, t=0.357, df=22, N=11-13), nor the LBM / body weight ratio (**Figure 7D**, Control *vs.* T4 (%): 84.05±0.79 *vs.* 84.44±0.62, p=0.7, t=0.397, df=22, N=11-13) were different between the control and T4 treated groups. The LBM normalized food intake was not different between the groups (**Figure 7E,** Control *vs.* T4 (g/h/kg lbw): 7.68±0.72 *vs.* 8.13±0.35, p=0.55, t=0.616, df=10, N=5-7). 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.

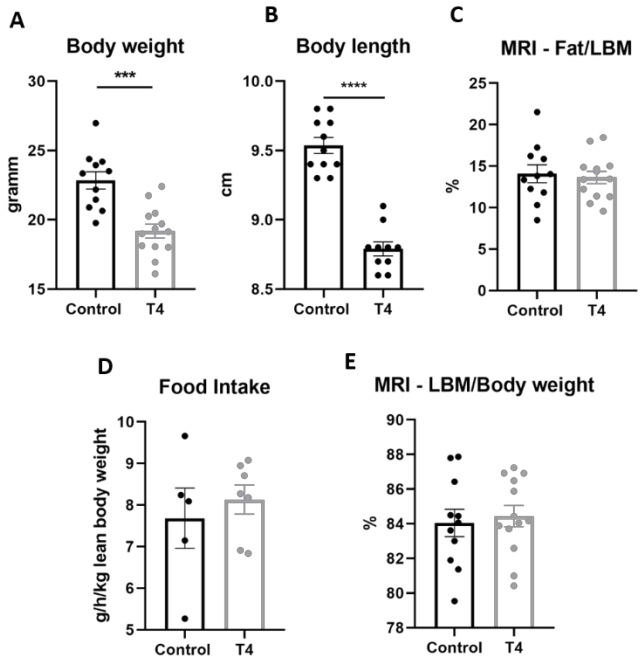

Figure 7: The effect of early postnatal T4 treatment on body weight and body composition.
*Early postnatal T4 treatment resulted in significantly decreased body weight (A, N=11-13) and body length (B, N=10-11) at adulthood. Body composition analysis did not show significant differences in fat and LBM (C, N=11-13) and in LBM and body weight (D, N=11-13) ratios between the groups. The LBM normalized food intake was not different significantly between the groups (E, N=5-7). Data are compared with Student's t test and shown as mean ± SEM. ***p < 0.001. Abbreviation: LBM – lean body mass.*

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 using TSE PhenoMaster System. Despite the decreased fT4 levels, the postnataly T4-treated mice showed significantly increased locomotor activity in the metabolic cages, including XY activity (**Figure 8A**, Control *vs.* T4 (cnts/h): 3433±392.60 *vs.* 4691±349.40, p=0.04, t=2.371, df=10, N=5-7), travelled distance (**Figure 8B**, Control *vs.* T4 (cm/h): 11383±554.10 *vs.* 22240±3648.00, p=0.03, t=2.466, df=10, N=5-7) and speed (**Figure 8C**, Control *vs.* T4 (cm/sec): 3.14±0.16 *vs.* 6.17±1.02, p=0.03, t=2.471, df=10, N=5-7). The higher activity was accompanied by significantly higher energy expenditure (**Figure 8D**, Control *vs.* T4 (Kcal/h/kg lbw): 24.64±0.73 *vs.* 27.39±0.54, p=0.01, t=3.098, df=10, N=5-7). Furthermore, the resting energy expenditure of the postnatally T4 treated mice was also significantly higher (**Figure 8E**, Control *vs.* T4 (Kcal/h/kg lbw): 22.61±0.67 *vs.* 24.91±0.60, p=0.03, t=2.516, df=10, N=5-7), suggesting that the central hypothyroidism is compensated at the level of tissues. The RER was not different between the two groups (**Figure 8F**, Control *vs.* T4 (RER/h): 0.84±0.02 *vs.* 0.84±0.01, p=0.98, t=0.026, df=10, N=5-7).

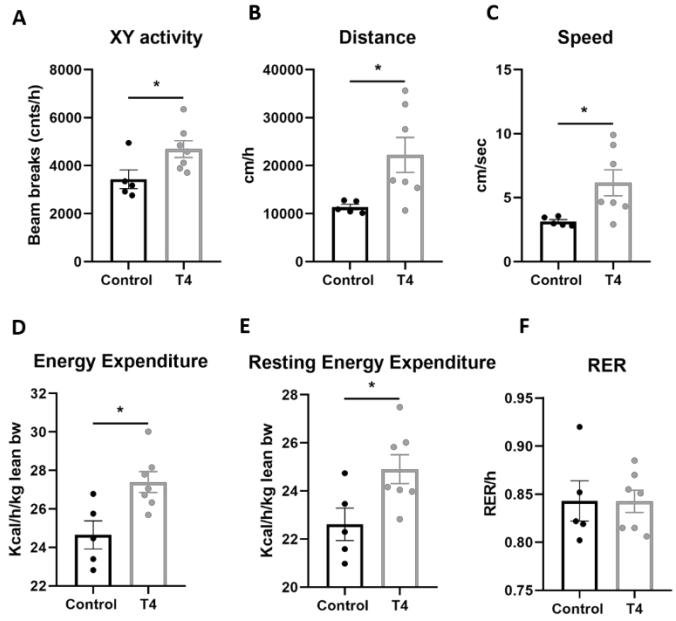

Figure 8: The effect of early postnatal hyperthyroidism on the activity and metabolism of adult TRH-IRES-TdTomato mice.

*The postnatally T4-treated group showed significant increase in the XY activity (A), travelled distance (B) and speed (C) parameters compared to the control group. The LBM normalized energy expenditure (D) and resting energy expenditure was significantly increased in T4-treated mice compared to the controls. The RER (F) was not significantly different between the groups. Data are compared with Student's t test and shown as mean ± SEM. N=5-7; *p < 0.05. Abbreviation: RER – Respiratory Exchange Ratio.*

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, luciferase expression (marker of TH action in the THAI mice) was studied in different tissues. Neither the hypothalamus **(Figure 9A)**, nor the hippocampus **(Figure 9B)** showed significant changes in the luc expression in T4 treated mice compared to the controls (Control *vs.* T4 (RQ): hypothalamus: 1.00±0.21 *vs.* 0.85±0.19, p=0.6, t=0.533, df=13, N=7-8; hippocampus: 1.00±0.13 *vs.* 0.76±0.28, p=0.42, t=0.853, df=8, N4-6). We also examined the luc expression in TH sensitive peripheral tissues. The luc expression decreased only in the small intestine **(Figure 9C)** in the postnatally T4 treated adult mice, but in BAT **(Figure 9D)**, the luc mRNA level was unchanged (Control *vs.* T4 (RQ): small intestine: 1.00±0.12 *vs.* 0.72±0.05, p=0.04, t=2.202, df=14, N=8; BAT: 1.00±0.31 *vs.* 0.62±0.16, p=0.28, t=1.129, df=13, N=7-8).

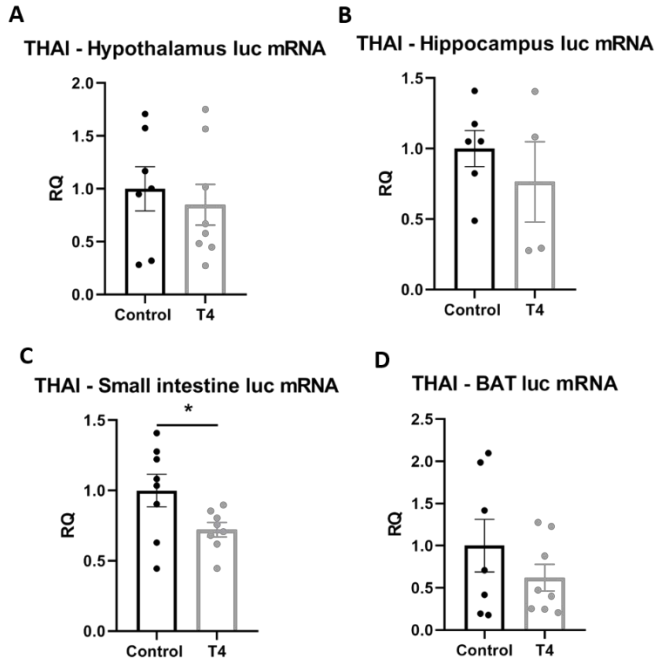

Figure 9: Tissue specific TH action.

*THAI mice were treated with 1µg/bwg thyroxin or vehicle between P2-6 and were sacrificed at adulthood and tissues were collected to determine tissue specific TH action. TaqMan qPCR analysis revealed no significant differences in luc expression of hypothalamus (A), hippocampus (B) and BAT (D), but in the small intestine (C), the early postnatal T4 treatment resulted in decreased luc expression. Data are compared with Student's t test and shown as mean ± SEM. N=6-8; *p < 0.05.. Abbreviation: THAI – thyroid hormone action indicator mouse, luc – luciferase, BAT – brown adipose tissue.*

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. First, we determined the Dio1 expression of liver that is considered to be the most sensitive marker of TH action in this tissue. Early postnatal T4 treatment resulted in marked, approximately 90% decrease of Dio1 expression in the liver (**Figure 10A**, Control *vs.* T4 (RQ): 1.00±0.16 *vs.* 0.09±0.02, p=0.0007, t=4.340, df=14, N=6-10). 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 (**Figure 10B**, 1.00±0.16 *vs.* 1.14±0.15, p=0.55, t=0.619, df=11, N=6-7) indicating euthyroid status in this tissue. To better understand the thyroid status of liver in this animal model, the expression of other TH sensitive genes was also determined. The TH receptor α and β (THRA, THRB) expression did not change significantly in this tissue (**Figure 10C,D**, Control *vs.* T4 (RQ): THRA: 1.00±0.10 *vs.* 0.98±0.09, p=0.90, t=0.131, df=18, N=10; THRB: 1.00±0.13 *vs.* 0.84±0.06, p=0.27, t=1.136, df=18, N=10). 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 [136]. Interestingly, the lower fT4 level of the animals did not change the gene expression of Spot14 in the postnatally T4-treated mice (**Figure 10E**, Control *vs.* T4 (RQ): 1.00±0.13 *vs.* 0.85±0.23, p=0.59, t=0.555, df=17, N=9-10). The expression of other genes involved in the fatty acid metabolism and regulated positively by TH [137, 138] were also studied. Both the malic enzyme 1 (ME1, **Figure 10F**) and fatty acid synthase (FASN, **Figure 10G**) showed significant decrease in the T4-treated group compared to the control animals (Control *vs.* T4: ME1 (RQ): 1.00±0.10 *vs.* 0.58±0.07, p=0.003, t=3.441, df=16, N=9; FASN: 1.00±0.15 *vs.* 0.54±0.03, p=0.01, t=2.898, df=15, N=8-9). The expression of the catalytic subunit of glucose-6-phosphatase (G6PC) - which is essential for endogenous glucose production [139] - did not change significantly (**Figure 10H**, Control *vs.* T4 (RQ): 1.00±0.11 *vs.* 0.92±0.10, p=0.62, t=0.511, df=18, N=10).

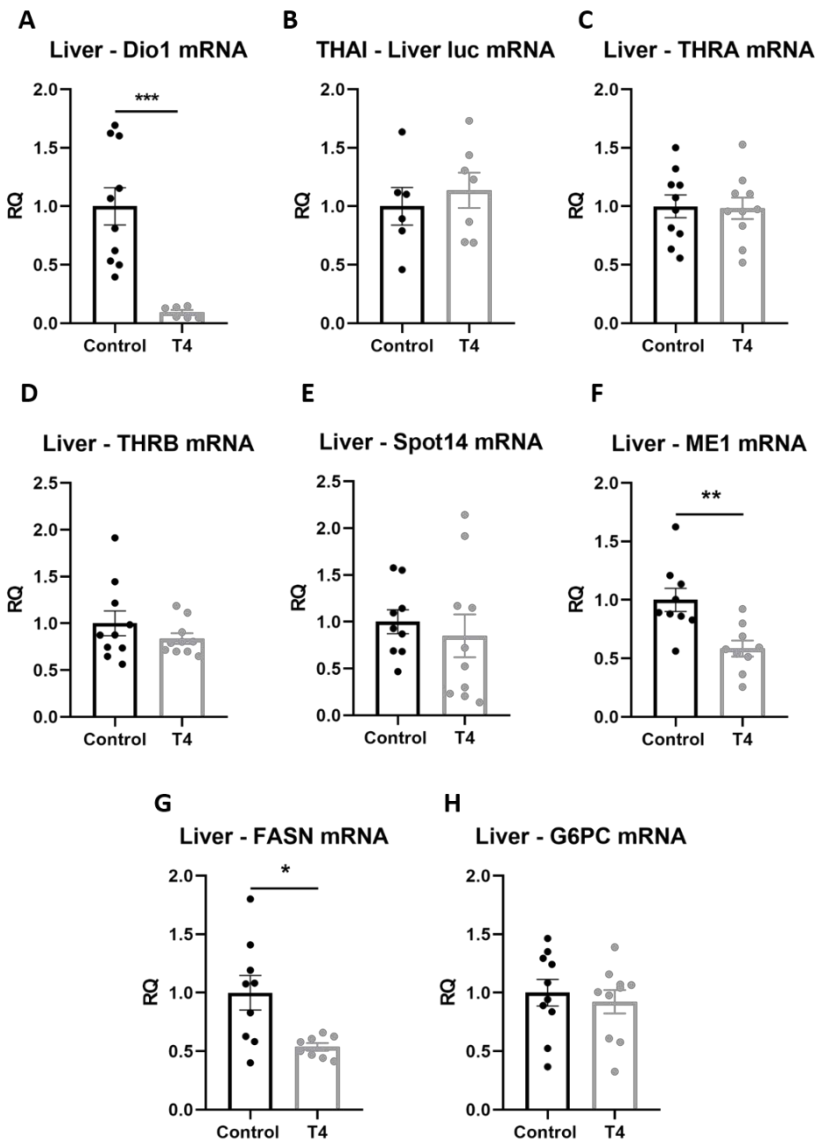

Figure 10: Effect of early postnatal hyperthyroidism on TH-releated genes of adult mouse liver

*TaqMan qPCR analysis in liver of adult TRH-IRES-TdTomato male mice treated with 1µg/bwg T4 or vehicle between P2-6 days. Liver Dio1 expression (A, N=6-10) decreased significantly in T4-treated group. The liver luc expression of THAI mice did not change significantly (B, N=6-8). The gene expression of TH receptors THRA (C), THRB (D) and Spot14 (E) did not change significantly in this group. The postnatal T4 treatment resulted in decreased mRNA level of ME1 (F) and FASN (G) compared to the control. The expression of G6PC (H) did not change significantly. C-H: N=8-10. Data are compared with Student's t test and shown as mean ± SEM. *p < 0.05; **p < 0.01. Abbreviations: Dio1 – type 1 deiodinase, THAI – thyroid hormone action indicator mouse, luc – luciferase, THRA - TH receptor α, THRB - TH receptor β, Spot14 - TH responsive, ME1 – malic enzyme 1, FASN - fatty acid synthase, G6PC - glucose-6-phosphatase.*

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. Dnmt1 level (**Figure 11A,** Control *vs.* T4 (RQ): 1.00±0.06 *vs.* 1.28±0.14, p=0.13, t=1.667, df=9, N=5-6) showed a tendency to increase in the T4 group, but the change was not significant, while Dnmt3a (**Figure 11B,** Control *vs.* T4 (RQ): 1.00±0.05 *vs.* 1.37±0.10, p=0.009, t=3.337, df=9, N=5-6) and Dnmt3b (**Figure 11C,** Control *vs.* T4 (RQ): 1.00±0.03 *vs.* 1.37±0.08, p=0.004, t=3.855, df=9, N=5-6) 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 (**Figure 11D-F,** Control *vs.* T4 (RQ): Dnmt1: 1.00±0.05 *vs.* 1.04±0.10, p=0.76, t=0.316, df=10, N=6); Dnmt3a: 1.00±0.05 *vs.* 1.10±0.08, p=0.35, t=0.984, df=10, N=6; Dnmt3b: 1.00±0.04 *vs.* 1.02±0.14, p=0.1, t=0.207, df=10, N=6), 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.

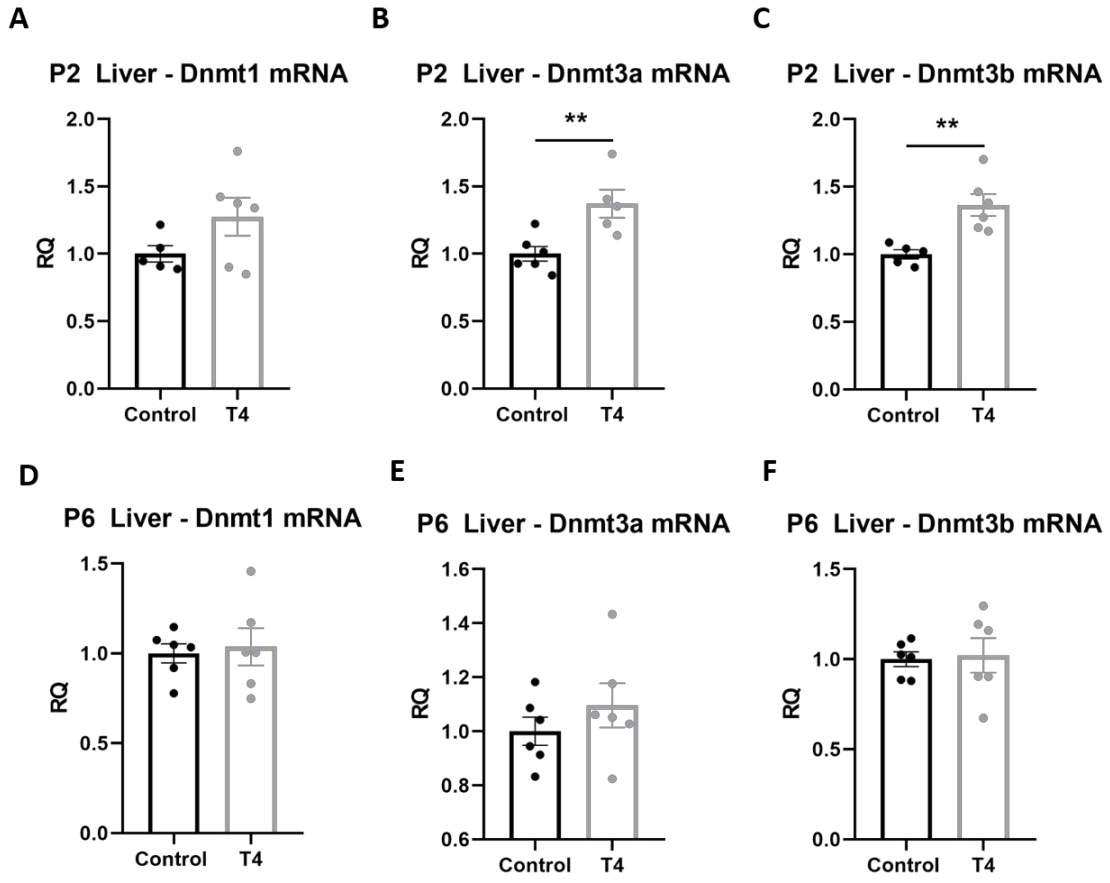

Figure 11: Expression of DNA methylation-related genes in the liver of T4 treated P2 and P6 mouse pups.

*TaqMan qPCR analysis of Dnmt1, Dnmt3a and Dnmt3b mRNA expression in liver 8 hours after injection of 200 ng/bwg T4 or vehicle in P2 (A-C, N=5-6) and P6 (D-F, N=6) pups. Liver Dnmt1 (A) expression was not changed significantly in P2, while Dnmt3a (B) and Dnmt3b (C) expression was significantly increased in P2 animals. The Dnmt1 (D), Dnmt3a (E) and Dnmt3b (F) levels of P6 animals did not change signifcantly. Data are compared with Student's t test and shown as mean ± SEM. *p < 0.05; **p < 0.01, ***p < 0.001. Abbreviations: P - postnatal day, T4 – thyroxin, dnmt1 - DNA (cytosine-5)-methyltransferase 1, dnmt3a - DNA (cytosine-5)-methyltransferase 3a, dnmt3b - DNA (cytosine-5)-methyltransferase 3b.*

Our results indicate that the early postnatal hyperthyroidism induces lifelong central suppression of the HPT axis. The tanycytes do not seem to be involved in this regulation. We have isolated RNA from LCM isolated TRH neurons of control and postnatally TH T4 treated adult mice and performed TAQMAN PCR analysis of the expression of the genes might be involved in the regulation of TH signalling and TRH synthesis. We currently analyse the data. We also isolated TRH neurons for analysis of the methylation status of genes that are regulated by postnatal T4 treatment in the TRH neurons. We plan to finish these experiments within six months. This delay is caused by the fact that the tdTomato reporter of our TRH-IRES-tdTomato mice completely loses its fluorescence after drying and therefore, it is not suitable for laser capture microdissection. To solve this problem, we had to generate a new mouse line, the TRH-IRES-CREERT2 mouse line what we could cross with ZSGreen reporter mouse line. This new mouse line solved the problem, but the time of generation and testing took considerable time.

We also found that the T4 treatment increases the expression of DNA methyltransferase enzymes in the liver on P2, but not on P6. These data indicate that there is a short time window in the early postnatal life when thyroid hormones can induce epigenetic regulation of liver genes. As the expression of Luc and several TH sensitive genes suggests that the liver is euthyroid in this animal model, we hypothesized that the 90% decrease of dio1 expression in the liver of postnatally T4 treated adult mice is due to epigenetic regulation of this gene. Therefore, we performed bisulfite sequencing of the dio1 promoter from the liver of control and postnatally T4 treated adult mice.

As a result of our studies, we hypothesized that epigenetic mechanisms may underlie the change of the expression of the Dio1 gene. We thought that an alteration of the DNA methylation pattern could accompany or result in a decrease of the gene expression. DNA methylation, which affects CG dinucleotides, inhibits gene expression by various means, e.g. preventing transcription factor binding. Based on bioinformatics and literature data, we selected four regions in the Dio1 gene whose methylation patterns were compared between treated and control animals. We chose an important promoter region, which is located close to the HNF4a binding site, which plays an important role in regulation. In addition, we also selected an intronic sequence that has a T4R binding site, which has been shown to be active. In addition, we selected two regions surrounding the T4R binding sites, which were shown to have open chromatin and active epigenetic marks when the gene is expressed in hepatocytes.
We decided to use the targeted bisulfite sequencing method. Bisulfite treatment is a chemical treatment, which converts unmodified cytosines to uracils then sequenced as thymines, while methyl cytosines are resistant to the treatment and are sequenced as cytosines. We investigated the four genomic regions in 6 treated and 6 control animals. We developed a new method used for the first time

in the present study for bisulfite amplicon sequencing by NGS. The amplifions were amplified after the bisulfite treatment. These primers contained an adapter, the same for all amplifions. Then, a second round of amplification was performed with primers containing the adapter and the same individual mouse-specific sequence tag on both ends of the primers. This minimized the necessary primers for the sequencing. We sequenced by Novogene 2x250bp paired-end sequencing.
The quality of the reads were conform to the expected, >95% of the nucleotides were good quality for sequence analysis. The individual samples gave similar amounts of reads. The analysis of the obtained sequences showed the early postnatal T4 treatment caused an approximately 20% increase of the methylation of the dio1 gene around a putative T4 response element suggesting that the TH regulation of the dio1 gene is lost due to the postnatal hyperthyroidism.

We plan to publish these data within a year.

As in addition to the HPT axis, the GLP-1 also plays critical role in the regulation of the energy homeostasis, we have studied the presence of the GLP-1 receptor (GLP-1R) in the feeding related neuronal circuits.

We have shown that GLP-1R-immunoreactivity was associated with the cell membrane of POMC neurons and with axon terminals forming synapses on these cells. The GLP-1 analog exendin 4 (Ex4) markedly increased the firing rate of all examined POMC neurons and depolarized these cells. These effects of Ex4 were prevented by intracellular administration of the G-protein blocker guanosine 5'-[β-thio]diphosphate trilithium salt (GDP-β-S). Ex4 also influenced the miniature postsynaptic currents (mPSCs) and evoked PSCs of POMC neurons. Ex4 increased the frequency of miniature excitatory PSCs (EPSCs) and the amplitude of the evoked EPSCs in half of the POMC neurons. Ex4 increased the frequency of miniature inhibitory PSCs (IPSCs) and the amplitudes of the evoked IPSCs in one-third of neurons. These effects of Ex4 were not influenced by intracellular GDP-β-S, indicating that GLP-1 signaling directly stimulates a population of axon terminals innervating the POMC neurons. The different Ex4 responsiveness of their mPSCs indicates the heterogeneity of the POMC neurons of the ARC. In summary, our data demonstrate that in addition to its direct excitatory effect on the POMC neurons, GLP-1 signaling also facilitates the presynaptic input of these cells by acting on presynaptically localized GLP-1R.

We also demonstrated that GLP-1R-immunoreactivity was observed in NPY neurons and in axons ensheathing the majority of NPY neurons. Ultrastructural studies confirmed that GLP-1R-immunoreactivity is associated with the outer membrane of NPY perikarya as well as with axons forming symmetric type, inhibitory synapses on NPY-containing neurons. Double-labeling in situ hybridization experiments demonstrated the expression of GLP-1R mRNA in approximately 20% of NPY mRNA-containing neurons of the ARC. In summary, our data demonstrate the presence of GLP-1R protein and mRNA in NPY neurons of ARC and also reveal the innervation of NPY neurons by GLP-1R-containing inhibitory neurons. These observations suggest that GLP-1 signaling can influence NPY neurons both directly and indirectly. Furthermore, GLP-1 signaling on energy homeostasis appears to involve both direct and indirect effects of GLP-1 on the orexigenic NPY neurons, in addition to the previously known effects via the anorexigenic POMC neuronal system.

These two studies are already published.