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PROGRAMMING OF CARDIOVASCULAR AND METABOLIC FUNCTIONS BY THYROID HORMONE SIGNALING

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Karolinska Institutet

Stockholm 2014

Published by Karolinska Institutet.
Printed by Åtta.45

ISBN 978-91-7549-682-5

Programming of Cardiovascular and Metabolic Functions by Thyroid Hormone Signaling
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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AKADEMISK AVHANDLING
som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i CMB auditorium, Berzelius väg 21, fredagen den 17 oktober, 2014, klockan 09.30

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To my wonderful parents

ABSTRACT

Thyroid Hormones (TH) regulate myriad processes, such as development, metabolism and cardiovascular functions, by controlling gene expression through binding to nuclear thyroid hormone receptors, TRα and TRβ. While patients with mutations in the TRβ gene are well known, patients with TRα mutations have only recently been identified. To determine potential phenotypes of such patients, our lab previously created a mutant mouse line (TRα 1+/m), in which the receptor affinity to TH is reduced. TRα1+/m mice display a wide range of abnormalities including hypermetabolism and mild bradycardia, contrary to what was predicted from receptor-mediated hypothyroidism. The aim of this thesis is to analyze the unexpected metabolic and cardiac phenotype in greater detail. Furthermore, it aims to elucidate the physiological phenotype of offspring from TRα1 mutant mothers.

Hepatic glycogen content serves as a readout of the metabolic status of mammals. In **paper I** we show that hypermetabolism leads to completely depleted glycogen storage in adult TRα1 mutants. Furthermore we show that the livers of these mice compensate for this loss by up-regulating glucose production over glucose degradation, in contrast to what is observed in T3-treated animals, thus raising the possibility that additional TR-mediated regulatory mechanisms are at play. Remarkably, exposure to high maternal thyroid hormone fully restored glycogen levels in the TRα1+/m adult mice, demonstrating that genetic and maternal factors orchestrate the glycogen set point of the embryo.

Studies from our lab showed that cardiomyocytes isolated from the TRα1+/m mice exhibit signs of severe hypothyroidism. Surprisingly, TRα1+/m animals are only slightly bradycardic despite a striking reduction in expression of the pacemaker gene, Hcn2, which in itself can induce a marked reduction of heart rate, **paper II**. We show that the autonomic regulation of heart rate in TRα1+/m mice fails to switch from sympathetic to parasympathetic tone in response to environmental stimuli such as temperature. **In paper III**, we find that impaired thyroid hormone signaling during development leads to an irreversible reduction in parvalbumin cell number in the hypothalamus, and that these cells regulate blood pressure and temperature-dependent changes in the heart rate. These data reveal a novel link between thyroid hormones and central regulation of the cardiovascular system.

Recent studies suggest that maternal factors during pregnancy, such as hormones and nutrients, epigenetically affect the development of the embryo, with long-lasting effects on offspring metabolism and behavior. **In paper IV** we show that TRα1+/m mutant dams produce male wild type offspring that have a metabolically favorable outcome characterized by their decreased body fat mass, increased lean mass and elevated glucose utilization. However, these mice display an increased voluntary wheel-running behavior, reminiscent of animal models of Attention Deficit Hyperactivity Disorder and addictive behavior. We provide evidence that a likely cause of this phenotype is decreased expression of genes involved in regulation of reward circuits and that at least one of these genes is epigenetically regulated. Moreover, we show that the phenotype is transmitted to the second generation, through the paternal line.

In conclusion, we show that proper thyroid hormone signaling is important during development to i) program the glycogen set point of the embryo; ii) regulate development of a novel hypothalamic cell population that controls cardiovascular functions; and iii) epigenetically regulate gene expression in the wild type embryo with long-lasting consequence for metabolism and behavior.

LIST OF PUBLICATIONS

I. **Vujovic, M**., Nordström, K., Gauthier, K., Flamant, F., Visser, T. J., Vennström, B. and Mittag, J. (2009). "Interference of a mutant thyroid hormone receptor alpha1 with hepatic glucose metabolism." Endocrinology 150(6): 2940-2947.

II. Mittag, J., Davis, B., **Vujovic, M**., Arner, A. and Vennström, B. (2010). "Adaptations of the autonomous nervous system controlling heart rate are impaired by a mutant thyroid hormone receptor-alpha1." Endocrinology 151(5): 2388-2395.

III. Mittag, J., Lyons, D. J., Sällstrom, J., **Vujovic, M**., Dudazy-Gralla, S., Warner, A., Wallis, K., Alkemade, A., Nordström, K., Monyer, H., Broberger, C., Arner, A. and Vennström, B. (2013). "Thyroid hormone is required for hypothalamic neurons regulating cardiovascular functions." J Clin Invest 123(1): 509-516.

IV. **Vujovic, M**., Warner, A., Höfig, C., Björnholm, M., Rozell, B., Vennström, B. and Mittag, J. (2014). “A dominant negative maternal mutation in thyroid hormone receptor alpha 1 affects the metabolism and behavior of wild type offspring”.
Manuscript

PUBLICATIONS NOT INCLUDED IN THE THESIS

I. **Vujovic, M.,** Dudazy, S., Hård, J., Solsjö, P., Warner, A., Vennström, B. and Mittag, J. (2014). "Regulation of Carbonic Anhydrase 4 in diverse tissues by Thyroid Hormones".
Manuscript

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LIST OF ABBREVIATIONS

αKG	Alpha Ketoglutarate
AHA	Anterior Hypothalamic area
ANS	Autonomic Nervous System
AV	Atrioventricular
BAT	Brown Adipose Tissue
CBR	Cannabinoid Receptor
DNMT	DNA methyltransferase
GC	Glucocrticoid
GFP	Green Fluorescent Protein
GR	Glucocorticoid Receptor
HCN	Hyperpolarization Cyclic Nucleotide
HPT axis	Hypothalamic-Pituitary-Thyroid axis
IAP	Intracistenal A Particle
IGF	Insulin Growth Factor
Mgll	Monoacylglycerol lipase
MHC	Myosin Heavy Chain
PEPCK	Phosphoenolpyruvate carboxykinase
PV	Parvalbumin
Pyrk	Pyruvate Kinase
RTH	Resistance to Thyroid Hormone
RXR	Retinoid X Receptor
SA	Sinoatrial
SERCa2	Sarcoplasmic Reticulum Ca(2+) ATPase
TET	Ten-eleven translocation methylcytosine dioxygenase
TH	Thyroid Hormones
TR	Thyroid Hormone Receptors
TRE	Thyroid Hormone Response Elements
TRH	Thyrotropin-Releasing Hormone
TSH	Thyroid-Stimulating Hormone
WAT	White Adipose Tissue

1 GENERAL INTRODUCTION

Thyroid Hormones (TH) are required for development and proper functioning of a variety of organs and tissues in the body, beginning in the embryo, in which they play critical roles in metabolism and brain development. As will be discussed below, minor disturbances in TH signaling during this period can lead to altered metabolic tone, increasing the risk of developing metabolic and cardiovascular diseases as adults. In cases of severe TH deficiencies, even more deleterious and irreversible defects such as mental retardation and growth abnormalities can arise. This thesis addresses the effects of maternal receptor-mediated hypothyroidism on the developing embryo.

1.1 Thyroid Hormone production and release

THs are produced by follicular cells of the thyroid gland situated in front of the trachea, and exist in two forms: 3, 3,`5, -triiodothyronine and 3, 3`,5, 5`- tetraiodothyronine, or T3 and T4, respectively. The production and secretion of THs is regulated by a negative-feedback mechanism that involves the hypothalamic-pituitary-thyroid (HPT) axis. Thyrotropin-releasing hormone (TRH) released from the hypothalamus stimulates the pituitary to produce and release the thyroid-stimulating hormone (TSH), which in turn stimulates the thyroid gland to release T4 and T3 into the circulation. THs exert negative feedback control on both TSH and TRH (Figure 1).

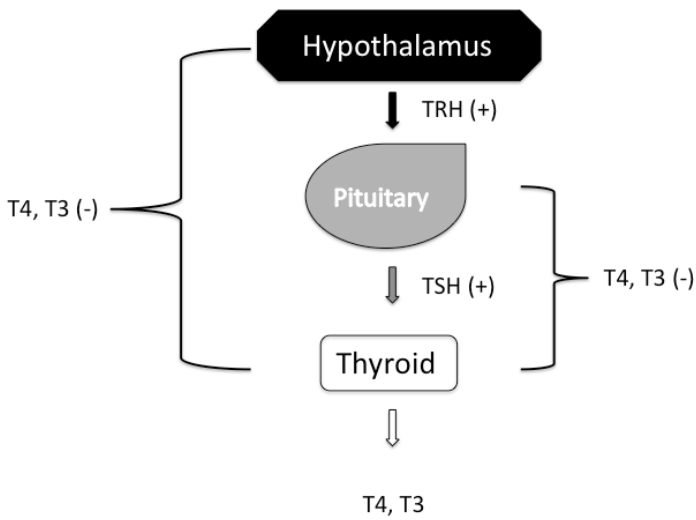

Figure 1. The HPT axis. TRH and TSH stimulate T3 and T4 release, while their own release is inhibited by these hormones. (+), stimulation; (-), inhibition

Although T4 is the most abundant form released by the thyroid, it binds to thyroid hormone receptors with weaker affinity than T3 and is therefore considered to be mainly a prohormone (Sandler et al., 2004). Most circulating TH is bound to plasma proteins such as thyroxin-binding globulin (TBG), thyroxin binding prealbumin and human serum albumins.

Previously, it was thought that THs passively diffuses over the cell membrane. However, converging lines of evidence have culminated in a new paradigm, namely that THs are transported by cellular transmembrane transport mechanisms involving specific transporter proteins, e.g. organic anion transporting polypeptide OATP and monocarboxylate transporters such as MCT8. Once inside the cell, T4 is converted by enzymes called deiodinases that remove an iodine from the outer ring of the T4, thus generating the active form T3. This function is performed by type 1 and type 2 deiodinases (D1 and D2). When D1 and D3 remove iodine from the inner ring, the hormone is inactivated (Figure 2) (Bianco et al., 2002).

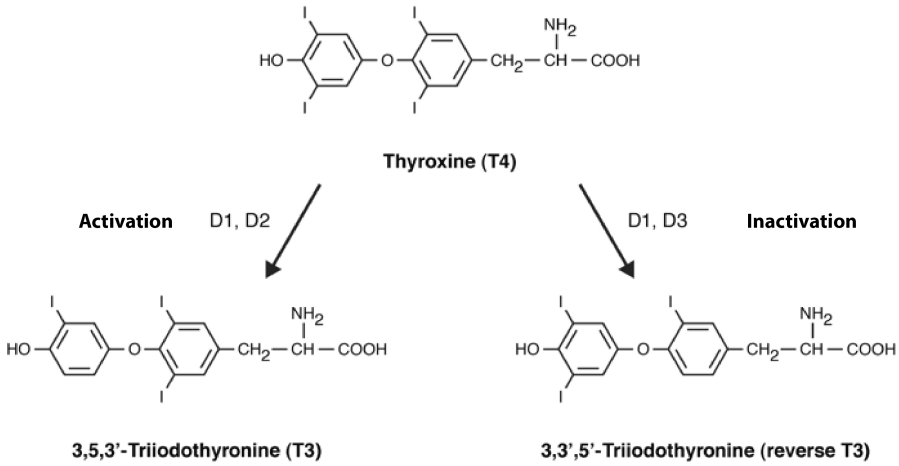

Figure 2. Different deiodinases regulate activation or inactivation of THs.

1.2 Thyroid Hormone Receptors

T3 exerts its action by binding Thyroid Hormone Receptors (TRs). These are transcription factors in the nuclear receptor superfamily and are encoded by two separate genes, THRA and THRB. Through alternative splicing, these give rise to mainly 4 different isoforms, TRα1/α2 and TRß1/ß2 (Figure 3). TRs contain a DNA-binding domain, which binds to Thyroid Hormone Response Elements (TREs) in the regulatory regions of target genes. TRs have been described to bind either as monomers or heterodimers, often together with the Retinoid X Receptor. TRα1 and both TRß isoforms bind T3 in their carboxy-terminal ligand-binding domains. By contrast, although TRα2 binds weakly to TREs, it has a truncated ligand-binding domain and different carboxy terminal domain, and consequently cannot associate with the ligand; its function remains enigmatic (O'Shea and Williams, 2002).

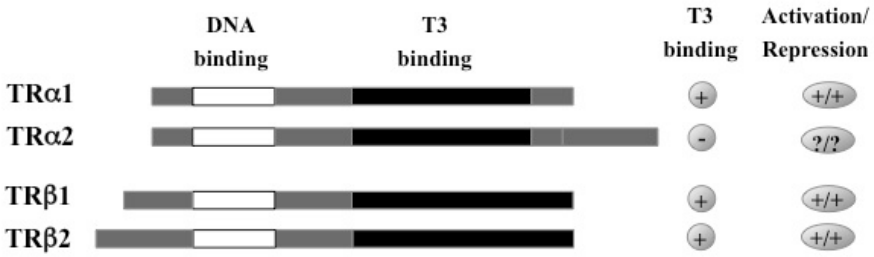

Figure 3. Thyroid Hormone Receptor isoforms

TRs are active also as aporeceptors, i.e. in the absence of ligand binding. Thus, target genes that are activated by ligand are repressed in its absence, and vice-versa, via recruitment of co-repressors such as nuclear receptor co-repressor 1 (NCoR) and co-activators such as steroid receptor co-activator (SRC) respectively. Because of this, aporeceptor activity leads to more detrimental outcomes for the organism following loss of thyroid hormone than loss of its receptor (Flamant et al., 2002; Gothe et al., 1999; O'Shea and Williams, 2002). However, the mechanisms of gene repression are to date not fully understood. In general, ligand binding leads to a transcriptional switch in which co-repressor binding is destabilized and co-activators are recruited in their place (Figure 4).

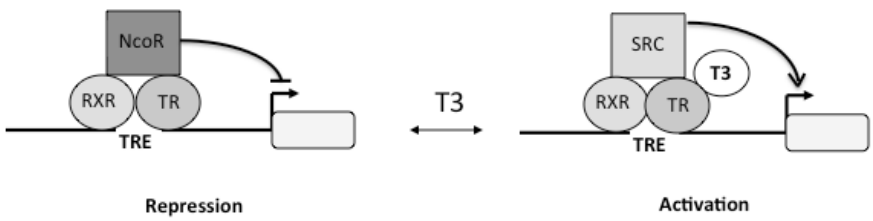

Figure 4. Schematic representation of ligand induced positive regulation of target genes by TR.

TR isoforms are expressed in almost all tissues with TRα1 being the most abundant isoform in the heart, brain and bone while TRß2 is found in hypothalamus, pituitary and auditory system. TRß1, meanwhile, is the predominantly expressed in liver and kidney (Brent, 1994).

1.3 Disorders associated with Thyroid Hormone Signaling

Given the wide distribution of TRs and TH-responsive genes, it is not surprising that disturbances in TH signaling can lead to deleterious effects. Hyperthyroidism, i.e. excess of TH, is often caused by the autoimmune condition Graves disease, in which autoantibodies over-stimulate the thyroid gland to produce T4 and T3, leading to a range of symptoms such as hyperactivity, tachycardia, depression, weight loss and heat intolerance. Conversely, low levels of TH cause hypothyroidism. This common endocrine disorder is associated with symptoms such as depression, weight gain, cold

sensitivity and dry skin and can be caused by inflammation of the thyroid gland, iodine deficiency or the autoimmune disorder Hashimoto's Thyroiditis, in which follicular cells of the thyroid are lost (Roberts and Ladenson, 2004). The causes of these autoimmune disorders are not clear but are believed to include genetic factors, age, gender, lifestyle choices and presence of other immune disorders.

Congenital hypothyroidism (CH) is the most common endocrine disorder after diabetes, affecting 1 in 3500 infants (Kopp, 2002) and results in hypothyroidism from birth onwards. Today, all newborns are screened for this condition. If not treated within the first days after birth, CH leads to severe mental retardation and growth abnormalities. After iodine (essential component of TH synthesis) deficiency, the most common cause of CH is defective thyroid gland development, which is at least in some cases due to mutations in genes regulating thyroid gland development (Mansouri et al., 1998).

Resistance to thyroid hormone ß (RTHß) is caused by mutations in the TRß gene that lead to reduced binding to T3 (Refetoff et al., 1993). Symptoms of RTHß include elevated serum levels of TH and TSH, goiter and tachycardia (Refetoff and Dumitrescu, 2007). Symptoms resulting from low or high TH action differ in different tissues, depending on which TR isoform is predominantly expressed.

1.3.1 Mice with a mutation in the TRα1 gene (R438C)

RTHß was first described in 1967 and since then around 3000 cases have been identified. However, until 2012, no naturally occurring mutations in the TRα1 gene had been found. This was explained by the possibility that such a mutation could be lethal or alternatively would not be characterized by the usual symptoms associated with disrupted thyroid hormone signaling. It was reasoned that an understanding of the role of TRα1 in mice might uncover previously unassociated symptoms of resistance to thyroid hormone, i.e. an RTHα phenotype. One such mutant mouse line, TRα1R438C, is the subject of this thesis.

The amino acid substitution introduced into the ligand-binding domain of the TRα1 gene was originally identified in TRß of a RTH patient family. This mutation leads to a 10-fold reduced affinity for T3, allowing the mutant receptor to be reactivated by supraphysiological doses of T3. *In utero*, the receptor can be reactivated through exposure to high TH, either by treating the mother with T3 or by using TRß-/- mothers in which the negative feedback on TSH is disrupted, exposing the embryo to endogenously high TH throughout gestation. TRα1R438C mice, hereafter referred to as TRα1+/m mice, are euthyroid, i.e. have normal TH levels and normal to low TSH levels. The mice exhibit a plethora of abnormalities, including postnatal retardation in growth and development, high anxiety and cardiac abnormalities (the latter aspect is further discussed in Papers II and III). Furthermore, these animals display a striking metabolic phenotype such as hyperphagia, resistance to diet induced obesity and an increased basal metabolic rate, which consequently leads to a lean phenotype (Tinnikov et al., 2002; Venero et al., 2005; Wallis et al., 2008). Surprisingly, this resembles hyperthyroidism, as opposed to hypothyroidism, which would be predicted from a dominant mutation that leads

to a reduced affinity to T3. Further studies identified impairment in heat conservation and dissipation through the tail vein (Warner et al., 2013) which caused these animals to activate brown adipose tissue (BAT) via sympathetic stimulation, increasing the turnover of lipids and carbohydrates in several tissues, in particular BAT, in order to maintain heat. Moreover, hepatic glycogen was completely depleted to fuel this function of BAT in these animals, yet they remained euglycemic (further discussed in Paper I).

1.3.2 Additional animal models with a mutation in the TRα1 gene

Three additional mouse models harboring mutations in the TRα1 gene have been created (Kaneshige et al., 2001; Liu et al., 2003; Quignodon et al., 2007). The chosen mutations either reduced binding to T3 or abolished the binding completely. As expected, given the high distribution of TRα1 in neurons, bone and cardiac tissue, most of these strains display abnormalities in fetal and postnatal growth and development and exhibit a cardiac phenotype. None of the strains display high fluctuations in TH levels, however most of them show different levels of TSH that vary with age. A notable difference between these models is their metabolic phenotype. For example, the TRα1P398H mice are obese, arguing that the site of the mutation has high impact on the phenotype (Liu et al., 2003). Indeed, it was shown that this particular mutation interferes with Peroxisome proliferator-activated receptor alpha (PPARα) signaling and, consequently leading to abnormal gene regulation and obesity.

1.3.3 RTHα in patients harboring mutations in the TRα gene

In 2012, the first description of a patient with a mutation in the TRα1 gene was published, followed by identification of additional 10 patients, all diagnosed with the newly named RTHα (Bochukova et al., 2012; Moran et al., 2014; Moran et al., 2013; van Mullem et al., 2012) (unpublished data). In all cases, the HPT axis was not as clearly affected as in patients with previously characterized conditions associated with TH disturbances such as RTHβ. However, all patients displayed abnormalities in T3/T4 ratio, which is still the best biochemical marker. As expected from the animal models, these patients showed delayed bone development, impaired neuronal development and bradycardia. However, none of the patients displayed a lean phenotype as seen in our mice. One possible explanation for this discrepancy would be that the site of the mutation results in differential affinity for T3 and/or other transcription factors.

1.4 Thyroid Hormone signaling during pregnancy

Subclinical hypothyroidism (SCH), also referred to as mild thyroid failure, has an incidence of 2-3% among pregnant women (Lazarus et al., 2014). However, this number varies in different countries and can reach up to 13.7% as seen in Northern Spain (Aguayo et al., 2013). TSH levels higher than the upper limit for pregnancy- related reference range, together with normal T4 levels, characterize SCH. Disturbances in TH signaling during pregnancy have detrimental effects on the developing embryo, especially on the developing brain, in which TH promotes synaptogenesis, myelination and neuronal migration.

Importantly, fetal TH synthesis is only initiated around the beginning of the second trimester, so the offspring is completely dependent on the maternal supply prior to this time (de Escobar et al., 2007). However, the mother's body faces its own challenges in maintaining euthyroidism, as the pregnancy itself sets a high demand on TH regulation: Firstly, chorionic gonadotropin hormone (hCG) secreted from placenta can mimic the TSH signal and thus lead to higher production of TH. Secondly, the pregnancy-associated increase in estrogen levels leads to higher TBG levels in the blood and hence reduced free T4 levels. The decreased negative feedback stimulates TSH secretion, which leads to increased secretion of TH to maintain normal TH levels. Thirdly, demand for iodine is increased during pregnancy, in response to (i) the body's own demand for TH synthesis, (ii) increased iodine clearance by the kidney, and (iii) the demands of the fetus (Nathan and Sullivan, 2014). In developing countries, lack of iodine is the biggest cause of maternal hypothyroxinemia, which results in endemic cretinism in the offspring (de Escobar et al., 2007). This is a relatively common condition, characterized by severe mental retardation and growth defects. The World Health Organization (WHO) has estimated that 50 million people suffer from some degree of iodine deficiency-related damage, which can lead to lower performance on IQ tests and difficulties in everyday life. In 2011, the WHO estimated that 1.88 billion people worldwide have insufficient dietary iodine intake (Andersson et al., 2012). Considering the ease with which this type of maternal and fetal hypothyroidism can be reversed, simply through iodine supplementation, the WHO has issued an effort to address iodine deficiency in the 54 most affected countries. Screening for TH disturbances during pregnancy is still not routinely done anywhere in the world. In Sweden for example, pregnant women must specifically ask for a TSH/TH test at their first check-up, which is as late as around week 10-12 of pregnancy (http://www.1177.se/Stockholm/Tema/Gravid/). This could lead to severe damage to the embryo due to its absolute dependence on maternal TH prior to this time.

Besides iodine intake, adequate nutritional status of the mother is also important for maintaining sufficient levels of selenium, which has an essential role for TH metabolism, firstly for deiodinase function and secondly for function of glutathione peroxidase, which metabolizes the H_2O_2 produced when iodine and tyrosine are coupled to form TH (Arthur, 1991). Other adverse effects of low nutritional intake during pregnancy are reduced thyroid gland activity, TH synthesis, metabolism, and expression of TRs. In addition to causing neurological defects, this leads to impaired gene expression in peripheral tissues and consequently reduced tissue growth and metabolism (Dauncey et al., 2001).

Maternal hyperthyroidism occurs in 0.4-1.7% of pregnant women and is mostly caused by gestational transient thyrotoxicosis, which in turn is triggered by elevated hCG levels (Nathan and Sullivan 2014). Another common cause is Graves disease. Both conditions can lead to fetal thyrotoxicosis, which is characterized by fetal tachycardia, accelerated bone maturation and intrauterine growth retardation. The latter has adverse consequence for the offspring metabolism and behavior (Polak et al., 2004). Maternal hyperthyroidism can also lead to reduced thyroid gland and abnormalities in thyroid morphological characteristics in children, which can cause congenital hypothyroidism in those children (Dussault, 1993).

2 AIMS

The subject of this thesis is to study effects of mutated TRα1 on liver metabolism (paper I), cardiac function (paper II and III) and offspring physiology (paper IV). TRα1+/m mice have been instrumental in the identification of patients harboring TRα1 mutations; as such mutations in mice are characterized by overlapping but distinct phenotypes, further analysis of the TRα1+/m model should help the search for new patients. Numerous studies have determined that hormones and nutrients from the pregnant mother affect the future metabolism and behavior of the unborn offspring. An analysis of the phenotype of offspring born to mothers with disruptive thyroid hormone signaling could therefore help identify any potential conditions these individuals might have, despite not having inherited the mutant allele. Specifically, the work comprising this thesis aims to:

I) determine how the liver of the TRα1+/m mice is affected by hypermetabolism, and investigate a potential role of TRα1 signaling in glucose metabolism;

II) understand how TRα1 signaling interacts with the autonomic control of heart rate;

III) determine central effects of thyroid hormone on cardiovascular function;

IV) understand how the wild type offspring of TRα1+/m females are affected.

3 EFFECTS OF THYROID HORMONE ON THE LIVER

Previously it was shown that TRα1+/m mice display hypermetabolism due to sympathetic stimulation of BAT. In this study, we wanted to investigate i) how liver tissue responds to hypermetabolism and ii) a potential genetic interaction between mutant TRα1 and genes involved in glucose metabolism.

3.1 The murine liver and Thyroid Hormones

One of the many functions of the liver is to maintain glucose homeostasis in the body. This is accomplished by i) producing glucose to be transported in the blood to other organs such as BAT or ii) by degradation of glucose.

Thyroid Hormones play important roles in regulating hepatic cholesterol and triglyceride metabolism, glycolysis and gluconeogenesis (Yen, 2001). Hypothyroid patients often develop non-alcoholic fatty liver disease, which is associated with obesity and/or type 2 diabetes. Many of the TH target genes in liver are down-regulated in hypothyroidism, leading to increased cholesterol and triglycerides. Conversely, in hyperthyroidism, excessive lipid oxidation can lead to tissue damage due to generation of reactive oxygen species, substantially increasing the risk of liver failure. TH also induces glucose-producing genes, such as phosphoenol-pyruvate kinase (PEPCK), the rate-limiting enzyme involved in gluconeogenesis, and represses pyruvate kinase (Pyrk), the rate-limiting enzyme in glycolysis. Consequently, hyperthyroidism increases and hypothyroidism decreases glucose production and utilization by peripheral tissues (Yen et al., 2003). TH function in the liver is mostly mediated by TRß, which regulates ~85% of TH-responsive genes, with TRα1 believed to regulate the balance (Flores-Morales et al., 2002).

3.2 Consequence of mutant TRα1 on the liver (paper I)

In **paper I** we found that TRα1+/m mice respond to hypermetabolism by increasing gluconeogenesis, which requires PEPCK, and decreasing glycolysis, which requires Pyrk. As T3 positively regulates PEPCK and suppresses Pyrk, this finding was unexpected, and the inverse of what was expected to occur in receptor-mediated hypothyroidism. We also found that the TRα1+/m mice have completely depleted glycogen storage, suggesting that the increased gluconeogenesis serves to replenish the depleted glycogen storage. Upon exposure of TRα1+/m embryos to high maternal thyroid hormone, in order to reactivate the mutant receptor *in utero*, the glycogen phenotype was reversed. The data suggest that the liver phenotype of the TRα1+/m mice is caused by a combination of defective TRα1 signaling in the liver, affecting the PEPCK and Pyrk expression, and hypermetabolism. Importantly, these results show that genetic and maternal factors interact to determine the metabolic set point in the offspring.

In order to disassociate effects of T3 and metabolism, a desirable experiment would be to induce fetal hyperthyroidism as opposed to exposing the mother to T3, as this already impacts offspring metabolism. Nevertheless, a requirement for proper maternal TH during

fetal development for glycogen content has since been observed in sheep (Forhead et al., 2009). In this study T3 levels were manipulated during mid to late gestation by a combination of T3 infusion in utero and fetal thyroidectomy. Induced hypothyroidism led to decreased glycogen content in adult sheep, while T3 infusion to embryos with intact thyroids caused an increase in hepatic glycogen.

The precise mechanism of mutant TRα1 activity in glucose metabolism has yet to be determined. However, this would require the generation of a liver-specific TRα1+/m mutant. Lastly, it would be interesting to see whether the expression levels of the PEPCK and Pyrk are restored when TRα1+/m receptor is activated in utero, as this would demonstrate that the increased gluconeogenesis and decreased glycolysis serves as a compensatory mechanism to replenish the depleted glycogen stores and that expression of genes involved in these processes are dependent on proper TRα1+/m signaling *in utero*.

4 EFFECTS OF THYROID HORMONE ON CARDIAC MUSCLE

TRα1+/m mice only display mild bradycardia despite the severe decrease in contractility of isolated cardiomyocytes. In the following studies we aimed to identify tissue-autonomous defects and to unravel mechanisms that mask the mild cardiac phenotype.

4.1 The contractive cardiac muscle

The function of the heart is to distribute blood to the body, which it does by the coordinated contraction of its cardiomyocytes (Rapila et al., 2008). Although cardiomyocytes, in contrast to skeletal and smooth muscle, are characterized by rhythmic contractility, the coordinated contraction of these cells is regulated by specialized cardiomyocytes, the cardiac pacemaker cells, that are only weakly contractile but produce electrical impulses that are propagated along the length of the myocardium. The heart consists of four chambers: the left atrium and left ventricle, which transport oxygenated blood to the body, and the right atrium and right ventricle, which transport de-oxygenated blood to the lungs. Each contraction cycle (heart beat) includes two major actions: (1) contraction of the atria causes blood that has moved into these chambers to pass into the ventricles, which (2) is then transported from the right ventricle to the pulmonary circulation, via the pulmonary artery and from the left ventricle to the systematic circulation, via the aorta (Young, 2010).

Under normal resting conditions, the heart is not dependent on control from the nervous system or hormonal stimulation to contract, because of the pacemaker cells, which are located in the sinoatrial (SA) node on the right side of the heart, the atrioventricular (AV) node in the septum, and Purkinje fibers that branch throughout the ventricular wall (Young, 2010). The SA node, also known as the heart's pacemaker, is located in the upper wall of the right atrium. Through spontaneous depolarization, i.e. a positive change in membrane potential, it initiates an action potential, leading to atrial contraction, or systole. The action potential reaches the AV node, situated in the lower right atrium, and leads to the ventricular systole. Following the atrial systole, the membrane potential of atrial myocytes returns to the resting state, or diastole, by hyperpolarization of the membrane potential. The coordination of these steps is essential to allow the chambers time to refill following their respective contractions. This regulation of contractility is mediated by different ion channels expressed on the cell and sarcoplasmic membranes of myocytes (Young, 2010).

The velocity of the heart rate is also regulated by changes in the periphery, which are mediated by the autonomic nervous system (ANS) and hemodynamic changes. The autonomic nervous system controls visceral functions and is divided into 1) the parasympathetic nervous system, which controls bodily activities that occur at rest, e.g. respiratory rate and digestion, and 2) the sympathetic nervous system, which controls the "fight-or-flight" response (Hildreth et al., 2009).

The systemic circulatory system carries oxygenated blood from the heart to the rest of the body and returns deoxygenated blood back to the heart. The velocity of vascular circulation is dependent on the pressure exerted by blood on the wall of blood vessels. This in turn is dependent on the pressure generated by the heart. During each heart beat, arterial pressure is at its highest during the systole (contraction of the cardiac muscle) and at its lowest during the diastole (cardiac relaxation). Changes in arterial pressure and resistance lead to altered blood volume and consequently an altered cardiac response in order to maintain homeostasis.

The heart is a major target organ for thyroid hormone (TH) action, and marked changes in cardiac function occur in patients with hyper-or hyporthyroidism (Kahaly and Dillmann, 2005). The link between the thyroid gland and the heart was discovered in 1825 by C. Parry, who noticed the correlation between an enlarged thyroid area and palpitation, a typical symptom of hyperthyroidism (Kahaly and Dillmann, 2005). However, effects of hypothyroidism on cardiac performance remained unrecognized until 1918 when H. Zondek noted classical clinical features of hypothyroidism and found that these changes could be ameliorated by treating patients with thyroid extract (Kahaly and Dillmann, 2005).

Today, we know that TH increases peripheral oxygen consumption, causing a secondary increase in cardiac contractility, as well as directly regulating cardiac contractility. THs exerts its action on the myocardium by at least three different routes: 1) through direct gene regulation (further discussed in Paper II); 2) through the sympathetic nervous system (further discussed in paper II and III); and 3) through hemodynamic changes (further discussed in paper III) (Dillmann, 2002).

4. 2 Thyroid Hormone action on the heart through direct gene regulation

As described in section 1.2, TH exerts its effects by binding to Thyroid Hormone Receptors (TRs). TRs bind the TREs on target genes as holo- and aporeceptors and regulate the expression of these genes (Sap et al., 1986; Weinberger et al., 1986).

T3 has been shown to shorten diastolic relaxation and hypothyroidism to prolong it in all mammalian species (Mintz et al., 1991). Decreasing the concentration of free calcium ions in the cytosol is the primary mechanism for myocyte relaxation, inhibiting the incorporation of calcium ions in troponin C, the thin filament of myofibrils (muscle fibers). Sarcoplasmic Reticulum Ca(2+) ATPase (SERCa2), the key calcium pump involved in regulating cytosolic calcium, is localized on the sarcoplasmic reticulum (SR), a membranous organelle surrounding the myofibrils. SERCa2 is strongly upregulated in response to T3, and three TREs have been found in the regulatory region of this gene (Hartong et al., 1994; Rohrer and Dillmann, 1988). T3 negatively regulates the expression and function of the SERCa2 inhibitor phospholamban (Plb), thereby increasing the activity of SERCa2 protein as well (Kiss et al., 1994; Ojamaa et al., 2000). The expression of ryanodine channels that contribute to free cytosolic calcium in the SR is also increased by TH (Arai et al., 1991). The increased expression and activity of these channels underlies the increased heart rate that accompanies hyperthyroidism. At the end of the systole, the membrane potential of the myocytes returns to

the resting state by repolarization. This is achieved through opening of potassium channels and the resulting flow of K+ ions out of the cell. Several voltage-gated potassium channels are regulated by TH, e.g. KCNA5, KCND2, and KCND3, thus coordinating the electrochemical responses of the myocardium (Ojamaa et al., 1999). TH also regulates proteins directly related to mechanical contraction of the heart, such as Myosin heavy chain (MHC) isoforms. MHCa, which has high ATPase activity and contractile properties, is positively regulated by T3 and accelerates contraction of the hyperthyroid heart, whereas MHCb, which has low ATPase activity and contractile properties, is negatively regulated by TH, resulting in decreased velocity of contraction in hypothyroid heart (Morkin, 1993). Additionally, other components of the contractive machinery such as actin and troponin I are also positively regulated by TH (Dieckman and Solaro, 1990). TH also positively regulates the ion channel in the SA node, hyperpolarization cyclic nucleotide 2 (HCN2) that plays a pivotal role in cardiac pacemaker activity (Pachucki et al., 1999).

4.3 Animal models of Thyroid Hormone Receptors and the cardiovascular phenotype

Given the importance of direct regulation of cardiac genes by TH and its receptor, TR, several TR-deficient animal models have been generated. Wikström et al (1998) was the first to show that TRα1 plays a central role in the heart, as mice deficient of this receptor displayed bradycardia, accompanied with prolonged repolarisation. Further studies confirmed that TRα1 is the predominantly expressed isoform in the murine heart, accounting for 70% of total cardiac TR transcripts, with TRß1 contributing the remaining 30% (Gloss et al., 2001; Johansson et al., 1999).

Mouse mutants lacking TRα are collectively referred to as TRαKO and include TRα1-/- and TRα0/0 (Gloss et al., 2001; Wikstrom et al., 1998). These mutants recapitulate the effects of hypothyroidism in the heart, displaying bradycardia that is accompanied by decreased levels of the Hcn2 and Hcn4 pacemaker genes. Expression of K^+ channels involved in action potential repolarization is also decreased in TRαKO mice. Moreover, whereas the contractile machinery components such as SERCa2 and MHC-a are also markedly decreased in TRαKO mice, the negative regulator of contractility, MHC-b, is increased. By contrast, TRßKO animals recapitulate the effects of hyperthyroidism in the heart due to the derepression of Tsh that leads to higher TH levels acting on TRα1, and these mutants accordingly display tachycardia. Consistent with this, when TRßKO animals are made euthyroid, the heart tissue of these animals no longer shows significant electrophysiological or contractile changes, nor changes in the expression of corresponding genes such as HCN2/4 and SERCa2, supporting the evidence that these genes are targets of TRα (Gloss et al., 2001).

T3 treatment of TRα1-/- mice leads to a partial rescue of the decreased heart rate, suggesting that TRß may compensate the effects of TH in the heart to a certain extent, given that TRα2 cannot bind T3 (Wikstrom et al., 1998). However, in wild type mice, the TRß-selective agonist GC-1 does not enhance cardiac output to the same level as T3, underscoring that TRα1 is the predominant receptor through which TH exerts its effects on the heart (Trost et al., 2000).

4.3.1 The consequences of aporeceptor activity on gene expression and cardiac function

As described in section 1.2, TRs act in the absence of ligands as aporeceptors to repress their target genes. For example, it has been shown that unliganded TRα1 represses Hcn2: Pax8-/- mice, which fail to produce TH, display normal expression of Hcn2 when crossed with TRα0/0 mutants, whereas in Pax8-/-;TRß-/- double mutants, expression remains low due to repression by the unliganded TRα1 (Flamant et al., 2002). To analyze the TRα1 aporeceptor activity, four animal models have been created in which TRα1 affinity to T3 is either reduced or abolished, resulting in a dominant negative and receptor-mediated hypothyroidism. The three with reduced affinity to T3 or the co-activators exhibit bradycardia, whereas the fourth, in which affinity to T3 is abolished, shows no alteration (Kaneshige et al., 2001; Liu et al., 2003; Quignodon et al., 2007; Tinnikov et al., 2002).

4.3.2 Heart phenotype of the TRα1+/m mice

Mice with a dominant-negative mutation of TRα1R384C (TRα1+/m mice) were generated in our lab (Tinnikov et al., 2002). As described in section 1.3.1, this mutation leads to a 10-fold reduced affinity to T3: consequently the receptor can be reactivated by supraphysiological levels of T3. Isolated cardiomyocytes from these animals fully recapitulated the hypothyroid phenotype with severely slowed contractions, a prolonged diastole and impairment of Ca^{2+} handling (Tavi et al., 2005). ß-adrenergic stimulation, which has previously been shown to result in phosphorylation of PLB and thereby increased SERCa2-dependent Ca^{2+} release and uptake (Koss and Kranias, 1996) resulted in a greater relative increase of contractility in the isolated mutant cardiomyocytes than in their wild type counterparts. However, even with ß-adrenergic stimulation, the rates of Ca^{2+} decline and relaxation were not normalized. Moreover, despite the higher responsiveness to ß-adrenergic stimulation, injection of the mice with catecholamine isoprenalin failed to increase the heart rate of TRα1+/m mice to the same level as wild type mice (Tavi et al., 2005). In **paper I**, we measured Hcn2 expression by qPCR of mutant and wild type total heart cDNA and found a 50% reduction in its expression compared to wild type animals. As even minor reductions of expression of this gene result in severe bradycardia under normal conditions (Gloss et al., 2001), this was surprising, given that the TRα1+/m mice displayed only mild bradycardia (Tinnikov et al., 2002). Nevertheless, these results raise the possibility that the sympathetic drive in the TRα1+/m mutants is increased, masking the intrinsic defects in cardiomyocytes, discussed further in paper II and III.

4.4 Interaction between Thyroid Hormones and the sympathetic system in regulation of the heart rate

The interactions between thyroid hormone and sympathetic signaling have long been studied, as both hyperthyroidism and an excess of the sympathetic catecholamine ß-adrenaline induce tachycardia, forming the basis for treating hyperthyroid patients with sympatholytic agents/beta blockers to ameliorate changes in heart rate velocity. Surprisingly, however, neither plasma nor urine levels of catecholamines are increased in hyperthyroidism

(Coulombe et al., 1976), leading to the hypothesis that increased thyroid hormone levels result in sensitization to the sympathetic drive. However, analyses of human patients have shown that increased activity of the sympathetic system is also observed in long-term hypothyroidism (Cacciatori et al., 2000). Moreover, animal studies have reported that ß-adrenergic receptors are up-regulated by TH (Hammond et al., 1987) and T3-bound TR has been shown to bind a TRE on the ß1-adrenergic receptor gene (Bahouth et al., 1997) causing a 4-fold increase of expression (Bahouth, 1991). Taken together, these data raise the possibility that the increased number of ß-adrenergic receptors could enhance the sympathetic sensitivity of the hyperthyroid heart. On the other hand, a recent study suggest that the effects of TH on the heart may be independent of ß-adrenergic signaling as effects of hyperthyroidism on the heart rate were similar between ß-adrenergic receptor KO and wild type mice (Bachman et al., 2004).

4.5 Consequence of mutant TRα1 on the autonomic regulation of the heart (paper II)

To better understand the interaction between these pathways, TRα1+/m mice were analyzed in **paper II**: we observed no difference in the mRNA levels of either ß-adrenergic receptor or parasympathetic muscarinic receptor levels as compared to wild type controls. Recent studies have demonstrated that TH centrally affects the autonomic control of peripheral tissues such as liver and brown adipose tissue (Klieverik et al., 2009; Lopez et al., 2010). However, upon injection, of freely moving TRα1+/m mice reared at room temperature, with either scopolamine, an inhibitor of parasympathetic signaling, or timolol, which blocks sympathetic signaling, no changes in the heart rate were observed relative to wild type controls, suggesting that the mutant TRα1 receptor does not affect the regulation of the heart rate by the ANS at room temperature. However, TRα1+/m mice fail to switch between the two branches of the ANS as indicated by the fact that the differences in heart rate became more pronounced following a stress stimulus or normal activity at night (**paper II**). Both of these conditions require a change of autonomic control for homeostasis (Dampney et al., 2008; Ikeda et al., 2007), raising the possibility that intact TRα1 signaling is required for ANS-mediated central adjustment of heart rate to environmental changes. The murine heart is predominantly controlled by the sympathetic nervous system at room temperature (Walsh, 1969), whereas at 30ºC it switches to parasympathetic control (Swoap et al., 2008). When the mice were reared at thermoneutrality, both genotypes displayed slower heart rate compared to those reared at room temperature. When injected with scopolamine, the heart rate in wild type mice remained increased after the initial stress response while the TRα1+/m heart rate was unaffected by the treatment. When injected with timolol, the heart rate of the TRα1+/m mice decreased dramatically, whereas the basal heart rate of the wild type mice remained unaffected, implicating that TRα1+/m mice failed to switch the autonomic control of the heart rate as they stayed on the sympathetic control even at thermoneutrality. As the ANS is regulated centrally (Saper, 2002), our results demonstrate that the mutation in TRα1 impairs the central adaptation of the cardiac control.

4.5.1 Consequence of mutant TRα1 on central regulation of cardiovascular functions (paper III)

While recent studies have unraveled some of the molecular mechanisms of TH-mediated central regulation of peripheral tissues, little is known about the molecular mechanisms governing the central regulation of the heart rate. **Paper III** has helped to clarify this issue. In TRα1+/m mutants, we found a 70% reduction of parvalbumin positive (pv+) neurons in the anterior hypothalamic area (AHA) region of hypothalamus, the master regulator of the ANS (Saper, 2002). Reactivation of the mutant receptor failed to normalize the number of pv+ cells, indicating that intact TRα1 signaling is necessary for the development of these cells. This was further confirmed by the reduced number of pv+ neurons at P10 and P14 in the TRα1+/m mutants relative to wild type animals. Patch-clamp recordings of the AHA pv+ neurons revealed that all pv+ neurons tested responded to changes in temperature ranging from 25ºC to 40ºC. To determine their physiological role, pv+ cells were selectively ablated in wildtype mice expressing Cre recombinase in these cells, using diphtheria toxin, yielding 40% reduction in cell number. Heart rate measurements were taken from freely moving animals using radio telemetry transmitters, revealing slight tachycardia at room temperature in pv+ neuron-ablated animals, which became more pronounced at night as well as upon exposure to cold as compared to control animals. As the autonomic innervation of the heart shifts in rodents with changes in temperature and environment (Paper II), (Dampney et al., 2008; Ikeda et al., 2007) we next tested whether the ablation of AHA pv+ neurons changes the autonomic control of the heart rate. Upon denervation of the heart with both scopolamine and timolol, reduced sympathetic and parasympathetic input to the heart was observed in mice with ablated AHA pv+ cells but not control animals, demonstrating the important role of these neurons in the autonomic control of cardiovascular function.

4.5.2 Interaction between Thyroid Hormones and the systemic vascular system in regulation of the heart rate (paper III)

Patients with hyperthyroidism have previously been shown to display increased systolic pressure and decreased systemic vascular resistance, which activates the renin-angiotensin-aldosterone axis. Increased aldosterone promotes renal sodium resorption, increasing blood volume and hence cardiac output (Klein and Ojamaa, 2001). Hypothyroidism is characterized by an increase in both diastolic pressure and systemic vascular resistance, resulting in decreased blood volume (Kahaly and Dillmann, 2005). However, both conditions lead to hypertension but the mechanism underlying the changes in blood pressure and vascular resistance are unclear.

Cardiovascular functions are regulated centrally through a complex interplay between hypothalamus, pituitary gland, adrenal gland and kidney. As lesions of the AHA cause a dramatic increase in blood pressure (Folkow et al., 1959), we assessed these parameters in the pv+ neuron-ablated animals and found hemodynamic changes, including increased systolic, diastolic and mean arterial pressure relative to the non-ablated controls, suggesting a role for these cells in the central regulation of blood pressure that could contribute to the

environment-dependent tachycardia observed in pv+ neuron-ablated animals (**paper III**). Ablation of these cells did not lead to changes in body weight, respiratory quotient or activation of brown adipose tissue as seen in the TRα1+/m mutants (Sjogren et al., 2007; Tinnikov et al., 2002) or changes in renin angiotensin system, suggesting that the AHA pv+ cells control cardiovascular function via the direct regulation of the autonomic nervous system, rather than through endocrine alterations.

To test whether the hemodynamic changes were another aspect of the TRα1+/m animals, recapitulated by the ablation model, we next measured blood pressure and associated parameters in TRα1+/m mutants. Although we found that the renin-angiotensin-aldosterone axis components pulmonary Ace and serum angiotensin II were decreased in these mutants, blood pressure was surprisingly unchanged. As we believed that this defect was caused by peripheral actions of the mutant TRα1 on gene expression, we reactivated the receptor with supraphysiological doses of T3. Indeed, subsequent reactivation of the mutant receptor normalized Ace expression and angiotensin II levels, which was now accompanied by an increase in blood pressure parameters (systolic, diastolic and mean arterial pressure) as compared to T3-treated wild type controls, suggesting that the acute reactivation of the mutant receptor unmasked the underlying hypertension.

4.5.3 Thyroid Hormone signaling and the central regulation of cardiovascular functions (paper II and III)

TH has long been known to regulate cardiac function by direct gene regulation, hemodynamic changes, and via the ANS, but the mechanisms by which it regulated the latter two processes have remained enigmatic. In **papers II and III**, our research shows that TH signaling is required to integrate these processes and respond to environmental changes centrally, through the output of the previously unidentified pv+ neurons of the AHA (Figure 5). We have identified at least 4 different subtypes of these cells, based on their response to temperature and TRH. The viral vector carrying pv promoter-driven diphtheria toxin targets all of these cells in the AHA, which makes it difficult to fully recapitulate the loss of these cells in TRα1+/m mice. It therefore remains unclear whether the full range of functions of these cells has been determined by these studies. Future experiments to better understand the development and identity of the individual subtypes, including the role of TRα1, should suggest routes to elucidate their functional roles. This could be done by e.g. Fluorescence-activated cell sorting of mechanically dissociated anterior hypothalamic area from the TRα1+/m mutant and control Green Fluorescent Protein (GFP) mice (available in our lab) (Wallis et al., 2010), followed by RNA sequencing from single cells. Subsequent gene expression from major subpopulation could be analyzed in hypothalamic area from these mice.

The mild but opposing heart rate defects seen at room temperature in the TRα1+/m mutants and pv+ neuron-ablated animals indicate that the tissue autonomous defect in TRα1+/m mutants i.e. the reduced HCN2 expression, has an overriding bradycardic effect on the altered autonomic cardiovascular regulation. Therefore, the central regulation of the cardiovascular

system and direct regulation of cardiac gene expression constitute a further integration of extrinsic and intrinsic regulatory mechanisms, counterbalancing the autonomic output of pv+ cells. Thus, TH/TRα1 signaling plays a key role in fine-tuning cardiovascular output, which is critical for robustness in response to environmental alterations. Whether the altered autonomic tone is due to an increase in the sympathetic tone remains to be determined. Further aspects of this role for TRα1 may include higher metabolic demand in these animals, which also would to lead to changes in the cardiac output.

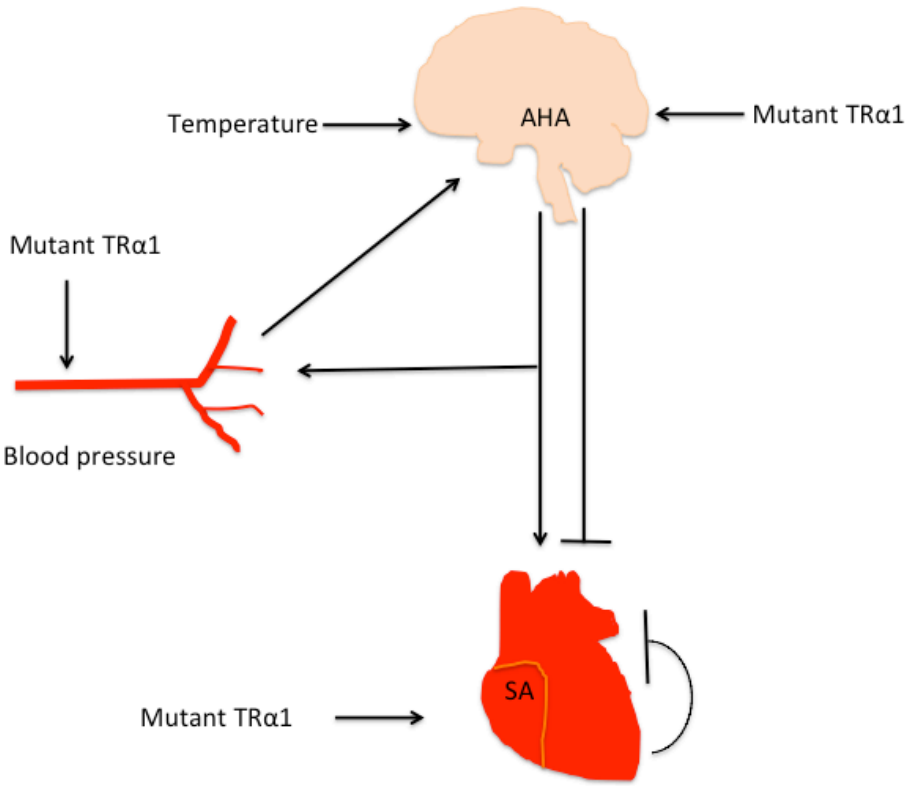

Figure 5. The heart receives several inputs, which lead to a changed heart rate. Firstly, the mutant TRα represses the Hcn2 gene expression in the SA node of the heart, leading to severe bradycardia. Secondly, pv+ neurons in the AHA integrate signals from the periphery, including temperature changes and blood pressure parameters, which modulates the central autonomic output and hence the heart rate.

In summary, we have identified developmental hypothyroidism as a cause of hypertension due to permanent defects in brain development. Whether TH exerts a central control of cardiovascular functions in humans remains to be elucidated. Analysis of the angiotensin system and the cardiovascular responses to temperature in the recently identified patients with a mutation in TRα1 (Bochukova et al., 2012; Moran et al., 2013) should shed light on this issue.

5 EFFECTS OF A MUTANT THYROID HORMONE RECEPTOR ALPHA 1 ON OFFSPRING METABOLISM

Disturbances in maternal TH signaling can affect the metabolic tone of the offspring and persists into adulthood. In **paper IV**, we investigate how maternal receptor-mediated hypothyroidism with consequent hypermetabolism and anxiety affects the metabolism and behavior of the offspring.

5.1 In utero programming of offspring metabolism

Research over recent decades has culminated in the birth of the field of developmental origins of health and disease (DOHaD) that strives to explain the epidemics of obesity, type 2 diabetes and cardiovascular diseases. It has become clear that, in addition to the genetic predisposition of the embryo, maternal behavior during pregnancy contributes to the metabolic health outcome and adult phenotype of the embryo. Indeed, maternal nutrition and state of mind are critical regulators of fetal development, conveying information about the metabolic status of the mother, thereby allowing the embryo to adjust its metabolism to the world outside of the womb. Astonishingly, it has been estimated that as much as 62% of the variation in human birth weight results from the intrauterine environment, compared with 20% and 18% from maternal and paternal genes respectively (Holt, 2002).

An association between environmental factors, e.g. nutrition, and cardiovascular disease later in life was first observed 80 years ago (Kermack et al., 1934). Records of children conceived during the 6 month long Dutch famine of 1944-45 indicate that babies born to nutrient-restricted mothers had low birth weight and were more likely to develop obesity, type 2 diabetes and cardiovascular disease later in life (Ravelli et al., 1976). These human observations prompted several animal studies that mimic such conditions, which showed that nutrient restriction, both in terms of caloric restriction or protein reduction, lead to different outcomes in adulthood, depending on which developmental stage the event occurred, as well as the nutrient supply postnatally. For example, caloric restriction exclusively during the prenatal period leads to obesity and insulin resistance in adult animals, whereas normal postnatal weight follows when it occurs during both the prenatal and lactation periods (McMillen and Robinson, 2005). Likewise, children conceived to nutrient-restricted mothers during the 1941-1944 Siege of Leningrad exhibited reduced birth weight that was not correlated to metabolic phenotypes such as obesity, glucose intolerance or cardiovascular disease in adulthood (Stanner et al., 1997). The negative outcomes observed in the Dutch cohort are due to rapid catch up growth during the childhood, made possible because of an abundant nutrient supply immediately after the war (McMillen and Robinson, 2005) as will be discussed in detail below.

Many research groups have hypothesized about the mechanisms by which early life events shape the adult phenotype. In 1962, Neel suggested that “thrifty” genes were evolutionarily selected during periods when food was scarce, leading to improved capacity to store fat when food was plentiful and consequently to enhanced fitness of the individual. With food

abundant in the West today, this would place a substantial proportion of the population at high risk of developing obesity and insulin resistance (Neel, 1962). However, due to a lack of genes correlated with metabolic disorders, it became clear that a thrifty genotype would not be sufficient to explain the alarming epidemic of these disorders today (Gluckman et al., 2007). In 1992, Barker and Hales proposed the thrifty *phenotype* hypothesis, in which factors in the womb determine the development of the embryo. Thus, poor intrauterine conditions stimulate the developing embryo to optimize the use of existing nutrients in order to ensure growth and survival (Hales and Barker, 1992). This could be achieved through blood flow and nutrient redistribution to favor the brain at the expense of e.g. liver, pancreas and muscle and/or through changes in the production of fetal and placental hormones that act as fetal growth programming factors (Barker, 2002). Others proposed "fetal programming" (also known as metabolic plasticity) to define an adaptive response of the embryo to ensure survival and postnatal growth, in which a given genotype is likely to give rise to different phenotypes depending on the environmental factors. Thus, if there is a mismatch between fetal and postnatal environment, the individual would be placed at risk for disease later in life as seen in those conceived during the Dutch famine (Gluckman et al., 2007).

While the precise mechanisms remain unclear, the current view is that fetal programming is an adaptive process that occurs (1) through epigenetic regulation of genes during critical developmental windows, namely when tissues are in their proliferative and/or differentiating phases; and (2) by the coordinated action of glucocorticoids (GC), IGF-1, IGF-2, insulin, thyroid hormones (TH), and leptin (Fowden, 1995) which directly regulate either gene expression and/or the production of growth factors and other hormones by feto-placental tissues (Fowden and Forhead, 2004).

Several reports support the thrifty phenotype and fetal programming hypotheses. For example, severe intrauterine conditions in the mother and consequent decreased energy supply across the placenta can lead to loss of structural units such as nephrons, hepatocytes, cardiomyocytes and pancreatic beta cells in the offspring (McMillen and Robinson, 2005), leading to life-long changes in function and structure of these tissues. For example, in rats, maternal starvation during four last days of the pregnancy led to 15% reduction in liver weight. The authors found that restricted nutrient availability in utero signals to hepatocytes to regulate the cell cycle, reducing proliferation and hence liver mass in the offspring (Gruppuso et al., 2005). Another study found that low-protein diet in mice leads to epigenetic silencing of hepatocyte nuclear factor 4a (Hnf4a), a regulator of islet beta cell transcription, and this leads to impaired beta cell differentiation and consequently altered glucose homeostasis (Sandovici et al., 2011).

5.2 Programming by in utero factors

The main roles of the placenta are to supply nutrients and oxygen to the developing embryo and to produce hormones and growth factors to support the embryo. Several factors, such as glucose, thyroid hormones, leptin and glucocorticoids, can cross the placenta, whereas others, e.g. insulin, are expressed by the placenta itself and reflect the metabolic status of the mother

(Hiden et al., 2009). Malnutrition leads to reduced oxygen delivery capacity of the placenta. In addition, the size of the placenta is dependent on the nutrient supply, and its size is a determining factor in the amount of glucose that can be transferred to the embryo, with obvious implications for fetal programming (Coan et al., 2008).

Glucocorticoids (GC) are the best example of an in utero factor that can easily pass the placenta and program the embryo. Studies have shown that the HPA axis can be prenatally programmed by malnutrition in the form of caloric restriction or low protein diet, as well as maternal stress, which leads to elevated GC concentration in the offspring (McMillen and Robinson, 2005; Welberg and Seckl, 2001). Administration of GC during the critical developmental window results in impaired renal development, hypertension, glucose intolerance and insulin resistance (Gicquel et al., 2008). For example, in rats, daily treatment with synthetic GC during the final week of gestation results in elevated basal plasma corticosterone levels in those offspring as adults (Welberg and Seckl, 2001) leading to increased blood pressure and obesity (Rosmond and Bjorntorp, 2000). Prenatal exposure to GC also alters glucocorticoid receptor (GR) gene expression in adult tissues such as liver, hypothalamus and hippocampus (Fowden and Forhead, 2004; Weaver et al., 2004), causing further alterations to the HPA axis in adulthood. Moreover, it induces changes in placental GLUT gene expression, which may alter transplacental glucose transport to the fetus and with it fetal metabolism and growth (Hahn et al., 1999). Taken together, GCs exert their functions through the altered gene expression of receptors, enzymes, growth factors, transcription factors, ion channels, and transporters in the embryo, altering hormone levels of the fetal programming factors such as IGF1/2 and T3, respectively. Ultimately, these perturbations lead to altered proliferation, differentiation, and tissue growth (de Moura and Passos, 2005).

5.3 Programming by epigenetic mechanisms

In utero factors can directly act on genes through epigenetic mechanisms, which result in changes in gene expression without altering the actual DNA sequence. Such modifications are essential for proper development, as they regulate gene expression in a tissue-specific manner. There are three major classes of epigenetic modifications: DNA methylation, histone modifications and non-coding RNAs. Genes that are required later in development are typically silenced by histone modifications that can be easily reversed once the genes need to be turned on again. Long-term silencing, on the other hand, is largely based on DNA methylation and is enriched at centromeres and transposable elements that have been incorporated into the DNA over the course of evolution. Moreover, this process regulates female X chromosome inactivation and imprinted genes. However, besides these events, it has become clear that certain genes can be subjected to altered DNA methylation by environmental factors, such as stress or nutrition, leading to an altered phenotype. Sometimes these traits can even be inherited across several generations (Anway et al., 2005). As **paper IV** of the thesis concerns a change in DNA methylation, I will mostly focus on this class of modification.

Epigenetic modifications are erased across the genome twice in an animal's life cycle: firstly at the time of gametogenesis and secondly during the pre-implantation period of embryonic development. Primordial germ cells (PGCs) give rise to gametes, and their genomes undergo DNA de-methylation, including at imprinted genes at E10.5-E11.5, followed by de novo methylation and re-acquisition of imprints. This latter process continues up to E18.5 in males, while in female maturing oocytes, it ends immediately before ovulation in females. The second round of epigenetic reprogramming is initiated by fertilization and occurs during the pre-implantation period at which time all modifications with the exception of those at imprinted genes are removed (Reik, 2007).

DNA methylation is associated with gene silencing, as it often occurs in the promoter or enhancer regions of genes. In mammals, methylation occurs in most cases at cytosine nucleotides that are followed by guanine nucleotides: so-called CpG islands. De novo methylation, in which a methyl group is added to the 5-carbon of cytosine by enzymes called DNA methyltransferases (DNMTs), is introduced during gametogenesis by DNMT3A and is maintained between the mitotic division by DNMT1s in embryonic and adult tissues. De-methylation occurs by both passive and active mechanisms: in the former, when a new DNA strand is synthesized during replication, the methylation pattern is reestablished by DNMT1, whereas the latter occurs during the pre-implantation period by removal of all methyl groups. Recent studies have identified the TET family of enzymes as capable of disrupting such highly stable carbon-carbon bonds. These studies have shown that TET enzymes oxidize 5-methylcytosine to form 5-hydroxymethylcytosine, which is then actively reverted to cytosine through iterative oxidation and thymine DNA- mediated base excision repair (He et al., 2011; Ito et al., 2011).

Methylation silences genes by either changing transcription factor binding of DNA and/or by attracting methyl-CpG-binding domain proteins (MBDs) that in turn recruit chromatin-remodeling proteins, resulting in heterochromatin formation, i.e. chromatin compaction. Histones, the main proteins that make up chromatin, are positively charged and associate with the negatively charged DNA to form nucleosomes. Each nucleosome contains DNA wrapped around four pairs of histone proteins called H2A, H2B, H3 and H4. The tails of nucleosomes are subjected to covalent modifications that will either lead to stronger DNA binding (compaction) and hence transcriptional repression, or to looser DNA binding and increased accessibility to the transcriptional machinery. These modifications include lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, and lysine ubiquitination and sumoylation (Vaquero et al., 2003). Histones are regulated by a set of enzymes that will add or remove these modifications, including histone acetyltransferases (HATs), hitone deacetylases (HDACs), histone methyltransferases (HMTs) and histone demethylases (HDMs). Epigenetic regulation can also be influenced by RNA interference, that is the presence of non-protein coding RNAs that can interfere with gene expression through interaction with RNA, DNA or chromatin.

5.3.1 Crosstalk between metabolism and epigenetics

The activity of many DNA- and histone-modifying enzymes is dependent on a cell's metabolic status. For example, DNMTs and HMTs both transfer a methyl group from S-adenosyl methionine (SAM) to the substrate. As SAM is derived from the essential amino acid methionine, it is possible to alter its levels through the diet (Poirier et al., 2001). Dietary intake of methyl donors such as folate is linked lo levels of DNA methylation. In the Agouti mouse model (Wolff et al., 1998) it has been shown that maternal methyl group providing supplements increased DNA methylation, and this was correlated with a reversion to brown coat color and decreased future development of obesity and insulin resistance. Moreover, it has been shown that glutathione depletion leads to genome-wide DNA hypomethylation due to depletion of methionine pools (Lertratanangkoon et al., 1997) and it has been suggested that a poor intrauterine environment leads to oxidative stress, possibly due to reduced oxygen transfer through the placenta, which is known to reduce glutathione levels (Pham et al., 2003). A third example is the protein-restricted diet rat model. Adult offspring of these rats develop high blood pressure and cardiovascular disease (Watkins et al., 2008), and exhibit increased expression of GR and PPARα in the liver. It was shown that this increase correlates with decreased promoter methylation, probably due to lack of essential amino acids. TET activity is also thought to depend on the metabolic status of the cell. These enzymes depend on α-ketoglutarate (αKG) to oxidize 5-methylcytosine to 5-hydroxymethylcytosine. αKG is the key metabolite of the TCA cycle and can be derived either from glucose or glutamine (Lu and Thompson, 2012). Other examples that connect metabolism to epigenetics are summarized in Figure 6.

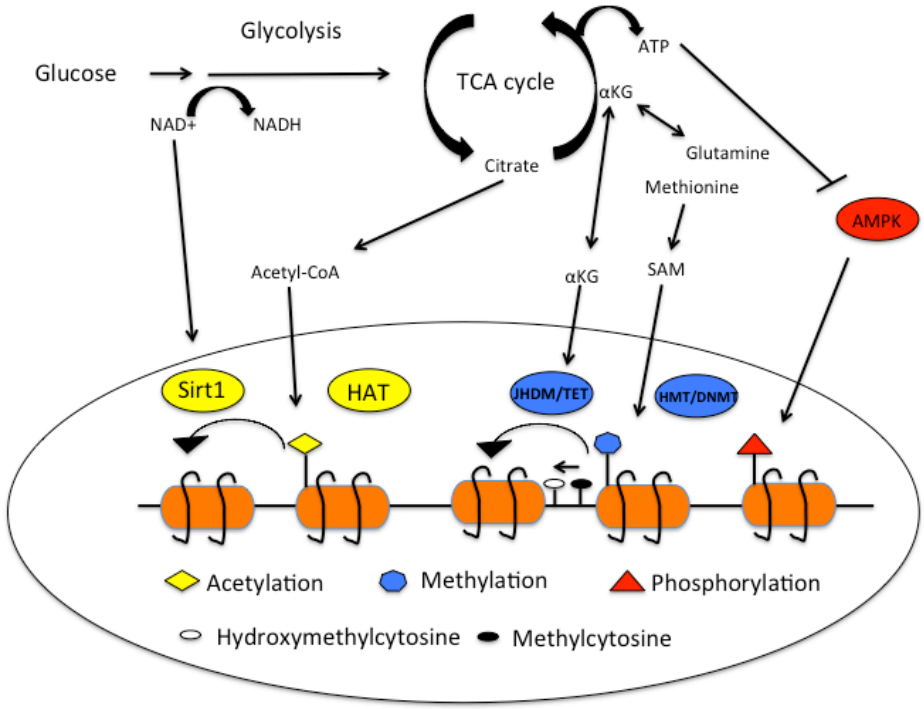

Figure 6. Glucose entry through the glycolysis determines the NAD^+/NADH ratio, which regulates Sirt1 activity. TCA cycle intermediates, citrate and aKG, are exported out of mitochondria. Citrate is converted to acetyl-CoA which is used as a donor for HAT-mediated histone acetylation. TET and JHDM use aKG as a cofactor for DNA demethylation reactions. The essential amino acid Methionine synthesize SAM, which is then used as substrate for HMT and DNMT. Lastly, a low ATP/AMP ratio can activate AMPK, which can phosphorylate histones. NAD, Nicotinamide adenine dinucleotide; Sirt, sirtuin histone deacetylase; TCA, citric acid cycle; αKG, α-ketoglutarate; Acetyl-CoA, acetyl coenzyme A; HAT, histone acetyltransferase; TET, Ten-eleven translocation methylcytosine dioxygenase; JHDM, Jumonji Domain-containing Histone Demethylase; SAM, S-Adenosyl methionine; HMT, histone methyltransferase; DNMT, DNA methyltransferase; ATP, adenosine triphosphate; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase A.

5.3.2 Epigenetic inheritance

Importantly, epigenetic marks that are established throughout the postnatal lifespan of the organism will not be transmitted to the next generation unless they are established in the gametes. In order for an epigenetic mark to be transferred to the next generation, it must arise during gonadal development and resist epigenetic reprogramming. Though there is some evidence that this occurs in mice, little is known about epigenetic inheritance in humans. Lumley and colleagues reported that children born to mothers during the Dutch famine were smaller than average and that this effect could last two generations (Lumey, 1992); however, same authors failed to find the same connections to these findings (Stein and Lumey, 2000). Using a mouse model to mimic the Dutch famine, reduced birth weight, glucose intolerance

and obesity were observed in both first and the second generations (Jimenez-Chillaron et al., 2009). However, only embryo transfer experiments to non-exposed dams would exclude effects of the in utero environment and show that the epigenetic information is inherited via the gametes.

There are several studies using animal models that report trans-generational inheritance, in some cases up to the fourth generation (F4). For example, pregnant rats that were exposed to the endocrine disruptor vinclozolin during the period of gonadal sex determination induced a phenotype in the adult F1 males, which included decreased viability and cell number in the sperm and higher infertility. This was correlated to altered DNA methylation persisting to F4 by paternal transmission (Anway et al., 2005).

The most frequently cited example of epigenetic inheritance through the gametes is the Agouty viable yellow A^{vy} allele that arose as a result of intracistenal A particle (IAP) insertion upstream of the Agouty locus. Expression of agouti protein is directed by promoter elements in retro-transposons and leads to yellow fur, obesity, diabetes and increased susceptibility to tumors. The DNA methylation status of the promoter is correlated to transcriptional activity and the mice carrying the allele display coat colors raging from yellow to pseudoagouti (brown). Trans-generational inheritance was only seen through the female line as yellow dams produce a high percentage of yellow offspring, which was not observed when A^{vy} was transmitted through the paternal line. Embryo-transfer showed that the phenotype was transferred through gametes, as surrogates still produced a higher percentage of yellow dams (Morgan et al., 1999). Another well-known example of gametal trans-generational inheritance is the Axin fused allele ($Axin^{Fu}$), which also contains an IAP element that, depending on the methylation status within the transposon, influences expression of linked genes, and results in a kinked tail phenotype. It was shown that the epigenetic state at $Axin^{Fu}$ was inherited trans-generationally by both maternal and paternal transmission (Rakyan et al., 2003).

However, none of the aforementioned studies proposed a mechanism by which such epigenetic marks could be inherited, one study even arguing that DNA methylation was not the inherited mark responsible for the phenotype in the Agouty mice (Blewitt et al., 2006), despite reports showing that IAPs are resistant to reprogramming (Lane et al., 2003). It has been hypothesized that chromosome modifications promoting DNA methylation, RNA-based mechanisms in the sperm, or factors in the semen or some diffusible factor in the egg cytoplasm damns may be responsible (Daxinger and Whitelaw, 2012).

Caution should be exercised when interpreting data to determine whether trans-generational inheritance through gametes occurs. For example, rats nurtured by stressed mothers are more likely to become stressed, and this phenotype is transmitted across generations. Stressed rodent mothers fail to adequately care for their offspring, changing the serotonin drive to the hippocampus of the offspring. This in turn leads to altered release of a transcription factor that binds the promoter of GR, stimulating DNA methylation and histone acetylation, and consequently leading to altered GR expression and HHPA axis tone that persists into the

adult life of the offspring (Francis et al., 1999). It is therefore not surprising that the offspring exhibit the same behavior to which they were exposed, thus perpetuating the phenotype across generations. This is en example of trans-generational inheritance that does not occur through the gametes but through an adaptive response linked to an epigenetic change that theoretically better equips the offspring to meet the stressful life outside the womb. In order to consider epigenetic inheritance through the gametes, it is not sufficient to only analyze the phenotype in F2 generation: if the pregnant mother (F0) is exposed during the gonadal development of the offspring (F1), then an epigenetic phenotype will also be observed in F2 without further exposure; however, if F3 shows the trait, gametal transmission should be considered.

5.4 Sexual dimorphism in metabolism and behavior

Metabolic differences in lipid profiles have been observed between men and women conceived during the Dutch famine. Middle-aged daughters showed elevated cholesterol concentrations and triglyceride content, which was not observed in middle-aged sons (Lumey et al., 2009). By contrast, studies on intrauterine growth restriction in rats showed that males were more prone to hypertension as compared to female offspring (Grigore et al., 2008). Moreover, maternal exposure to stress puts males at greater risk of neurodevelopmental disorders such as autism and schizophrenia (Gabory et al., 2013). This could be explained by the finding that stress in early pregnancy leads to sex-dependent effects on placental gene expression, modifying the fetal transport of key growth factors and nutrients. For example, glucocorticoids indirectly increase expression of insulin-like growth factor binding protein 1 (IGFBP-1) (Degenhardt et al., 2006) which is known to down-regulate genes involved in fetal growth (Kajimura et al., 2005). As reductions in growth factors during critical developmental periods have been linked to affective and neurodevelopmental disorders, the decrease in available growth factors may play a role in male fetal programming. However, while this example outlines how altered placental gene expression could affect a fetus, it does not explain how only male placentas are affected in the first place, which has yet to be elucidated. Converging lines of evidence suggest that sex differences in metabolic and behavioral phenotypes originate early in development, in particular in the placenta. Many placental genes display different epigenetic marks that reflect the gender of the embryo and respond differently to the nutrient supply. For example, embryonic female placenta of pregnant mice fed either high fat, low fat or intermediate diet showed drastic differences in gene expression as compared to male placenta in response to each maternal diet (Mao et al., 2010). This is consistent with the finding that high- fat diet induces hypomethylation only in female placentas at E15.5 (Gabory et al., 2013), clearly indicating sexual dimorphism of the placental tissue, suggesting that female placentas are more sensitive to nutritional fluctuations than the male counterparts.

5.5 Consequence of a mutant TRα1 on offspring metabolism (paper IV)

As described in section 1.3.1, TRα1+/m mutants represent a novel model of receptor mediated maternal hypothyroidism, and are very relevant for the study of such effects on offspring metabolism and behavior due to the recent finding of a human female patient with a similar mutation (Moran et al., 2013). As serum corticosterone levels are unchanged in these animals (Paper IV), we wanted to investigate how these events would program the developing embryo on the molecular level.

5.5.1 Mh mice unexpectedly exhibit a favorable metabolic outcome

In **paper IV**, we show that male but not female wild type mice (mh mice) born to mutant TRα1+/m mothers weigh less, display reduced body fat mass and increased lean mass. Their glycogen levels are decreased by 60%, accompanied by accelerated glucose clearance and increased oxygen consumption, while food intake remained normal. On the molecular level, we found decreased liver expression of Mgll, the enzyme responsible for metabolizing the cannabinoid receptor ligand arachidonyl glycerol (2-AG), which was associated with hypermethylation of a specific CpG site in the Mgll promoter region. Mgll expression was also decreased in gastrocnemius muscle. Interestingly, we observed a modest decrease in cannabinoid 1 receptor (CB1R) expression in the hypothalamus of the mh mice. As cannabinoids have been shown to be involved in mechanisms of behavioral reward (Keeney et al., 2008), we placed running wheels in the cages and observed an increase in running wheel behavior in male mh mice but not females. Thus, although these animals have an apparently favorable metabolic phenotype, the desire to run more intensively might reflect a mental disorder that is reminiscent of addiction or ADHD.

5.5.2 Mh mice as a possible model for mental disorders?

It has been shown that running wheel behavior is highly rewarding and hence addictive, even to such extent that mice are willing to cross an aversive water barrier to receive wheel-running reward (Keeney et al., 2008). High runner (HR) mice, which are selectively bred for this behavior, resemble the mh mice in terms of both metabolic and wheel running phenotypes. When HR mice are denied running, they show differential neuronal activity, as indicated by cFos, in several brain regions involved in arousal (lateral hypothalamus), natural reward (medial frontal cortex) and initiation of locomotion (caudate putamen) (Rhodes et al., 2003). Interestingly, CB1R is expressed in all these regions (Cota et al., 2003; Lisboa et al., 2010; Mailleux and Vanderhaeghen, 1993) and administration of rimonabant, a CB1R receptor antagonist, decreased wheel running in HR mice (Keeney et al., 2008). Therefore, it would be interesting to perform these experiments in mh mice to assess the role for CB1R in the mh phenotype, thus linking the maternal in utero environment to addiction.

Addiction is neurologically similar to ADHD, and the two conditions often coincide (Kaye et al., 2014), raising the possibility that an ADHD like disorder causes the running wheel phenotype in mh mice. Indeed, administration of Ritalin, a pharmacological agent for ADHD

treatment, has also been shown to decrease wheel running in HR mice (Rhodes et al., 2005). Ritalin inhibits the dopamine transporter, and the dopamine system is a critical component of reward behavior. ADHD is a complex disorder, with few genes directly implicated; however, fetal programming can play a role, as obesity among pregnant women has been associated with 2.8-fold increase in prevalence of ADHD in children (Buss et al., 2012). Moreover, in another similarity with the mh phenotype, ADHD is sexually dimorphic, with men more susceptible than women (Biederman et al., 2008). Whether defective dopamine or endocannabinoid signaling however underlies the phenotype of mh male mice remains yet to be established.

As proper brain development depends on an interplay between the individual´s genotype and conditions during early development (McMillen and Robinson, 2005) one might hypothesize that the in utero environment of TRα1+/m dams leads to altered susceptibility to psychological disorders such as addiction and/or ADHD, and it will be interesting to test this possibility. A complex interplay between peripheral organs and the central nervous system, particularly the hypothalamus, has been shown to regulate numerous physiological processes (paper III), (Yadav et al., 2009). The extent to which such mechanisms underlie the phenotype of the mh mice is an open question, but it would be interesting to explore the potential for a link between the decreased expression of liver and muscle Mgll and the decreased CB1R expression in the hypothalamus.

5.5.3 Potential factors that could cause the phenotype

Since the available blood glucose in the circulation of hypermetabolic TRα1+/m dams to a large extent serves to replenish BAT energy stores (Sjogren et al., 2007), it is reasonable to speculate that reduced glucose could be transferred to the embryo. As summarized in figure X, lower glucose transfer can lead to decreased levels of NAD+/NADH and aKG that could potentially change the methylation patterns of the genes in somatic tissues or developing gametes, resulting in hypermethylation of the Mgll promoter. Although this should be tested by treating mutant dams with glucose or T3 throughout the pregnancy and lactation period, as mutant dams are euglycemic under normal conditions (Sjogren et al., 2007), other factors are more likely to contribute to the phenotype.

Leptin has been proposed to act as a programming factor (de Moura and Passos, 2005). As reduced fat depots in mutant dams lead to decreased circulating leptin levels (Sjogren et al., 2007), one could hypothesize that decreased leptin availability through the placenta could contribute to the mh phenotype. However, low leptin levels during gestation or lactation leads to obesity in the offspring, which is not observed in the mh mice. However, as mh mice are exposed to similar conditions during lactation as in utero, cross-fostering to wild-type mothers would provide new clues.

Although TRα1+/m mice display high levels of anxiety (Venero et al., 2005), corticosterone levels in TRα1+/m dams were normal under standard conditions, hence it is not known which factor produced by this physiological condition could lead to the phenotype observed in the

mh mice. Maternal depression is one of the most common prenatal complications in women (Sandman et al., 2014), and the pregnancy itself could have induced several additional anxiety-related symptoms that would alter glucocorticoid levels in the TRα1+/m mutant dams and consequently affect the embryo. Thus it would be interesting to test corticosterone levels and anxiety phenotype in pregnant TRα1+/m dams.

Hypothalamus is the master regulator of metabolism that integrates CNS and peripheral tissues and could act as a source for fetal programming factors. Gene expression profiling of hypothalamus from mutant females and possibly pregnant mutant females could lead to novel insights. Given the importance of fetal programming by the placenta, gene expression profiling of the placentas from mh male and female embryos versus wild-type male and female embryos should be analyzed in order to provide new clues to different responses to the in utero environment. Furthermore, to test whether the phenotype is transferred through the gametes, F3 male generation should also be analyzed.

It seems unlikely that the phenotype is caused by fluctuation in maternal nutrient supply, as female offspring do not display a metabolic phenotype and evidence suggest that females are also highly responsive to any metabolic disturbances that happen during the fetal development. Mechanisms directing sexual dimorphism are at work during organogenesis, and it has been shown that chromatin structures and epigenetic marks differ between males and females in the brain and liver, two tissues, which show a striking phenotype in mh mice. Besides epigenetic differences between the sexes, anatomical differences have been observed between male and female brains, notably in the hypothalamus and cortex (Ngun et al., 2011), and sexual dimorphism has also been observed in the behavioral and neurochemical responses to cannabinoid signaling (Rubino and Parolaro, 2011). These findings raise the possibility that the reason for differences between mh male and female mice might be due to distinct responses to an adaptation that was made during fetal life.

Genome-wide association studies have been inadequate in finding genetic contributions to several current health problems such as obesity, diabetes, or cardiovascular disorders. Therefore the current view is that other processes, such as *in utero* factors and epigenetic mechanisms are involved. However, little is known how these work on a molecular level. Endocrine disorders play a big role in this and can largely be caused by endocrine disruptors that we are exposed to daily as they can be found in our food, food containers, clothes, medical equipment or toys. Some of the well-known compounds that are still in use or have just been banned include bisphenol A, phthalates and parabens. Therefore it is paramount to study the consequences of maternal endocrine problems on the offspring. In this study we have focused on effects of hypothyroidism and more specifically on TRα1 mediated hypothyroidism, which is specially important today as recently identified TRα1 mutations in humans were found to be compatible with life and even reproduction. Here we provide insights to how the children of such mothers could be affected, even if they don't inherit the maternal TRα1 mutation. Moreover, the study could help finding molecular links for

addiction and ADHD like phenotype, which are other unsolved problems likely caused by epigenetic variables.

6 SUMMARY

The research conducted in this thesis has provided novel insights into roles of THs during development in programming of cardiovascular and metabolic functions as well as behavior. We show that THs program hepatic glycogen stores, which is of high relevance throughout postnatal life, as hepatic glycogen serves to maintain normal glucose levels. We also show that TH signaling affects peripheral and central mechanisms controlling cardiovascular functions, for instance through the developmental regulation of recently identified cell type in the hypothalamus, which alters autonomic output in response to environmental changes. Lastly, we show that maternal TH signaling modulates central and peripheral metabolic and behavioral pathways in the offspring through epigenetic regulation of gene expression. Our findings contribute to a better understanding of thyroid hormone action in development, and are of immediate clinical relevance, as they support the diagnosis and the treatment of patients with a mutation in TRα1, which were recently identified.

7 ACKNOWLEDGEMENTS

My supervisors

Björn Vennström: Thank you for taking me on as a student and for all the fantastic scientific input over the years. You have always allowed me to speculate and to think outside the box. It has been really fun to "talk science" with you. Even outside of work, you contribute with fun and interesting facts and views on diverse subjects ranging from the Nobel Assembly to birds, politics as well as parenting. It has been a great experience to work closely with you.

Jens Mittag: Where to start…Thank you so much for choosing me for the undergraduate project and for always being there for me as if you were my main supervisor. You have really taught me to be efficient in planning, executing the right experiments, and writing. I think you are an amazing scientist and you make everything look so easy. I hope that I one day will be able to write as nicely as you and know how to make that fantastic pitch that summarizes everything in a beautiful and clear way. It has been so much fun to see you go from a post-doc in the lab, head of the pub-crew to a professor without ever losing sense of who you are. You are impressive in many ways. Wish you and your lovely family all the best!

To both Björn and Jens: Thank you for giving me the opportunity to explore a new diverging field within our field, which has really helped me to find my path in science.

Juleen Zierath: Thank you for letting me join your journal clubs!

Collaborators

Marie Björnholm and **Björn Rozell:** thank you for all the help with paper IV.

Anders Arner: Thank you for your enthusiasm and fantastic input into papers II and III and a special thanks for reading my thesis!

Former and present members of the lab

Kristina: For your magic hands in the lab, that I have seen serve you very well in your new hobby. **Maria:** When I came to the lab, your thesis really helped me to understand the field. Still remember how impressive it was to hear your defense! **Karin**: It was so much fun hanging out in the lab. Remember the crazy nights at the pub and all the fun discussions we had. Good luck with everything and big hugs to your lovely family. **Susi:** You are as perfect a PhD mate as one could ever wish for. It has been great to have a true friend in the lab and outside to talk to about everything and nothing. Your attitude to life, work and people is great and I know you will be successful with whatever you decide to do! **Amy**: thanks for teaching me words like burlesque ☺ and for inspiring me to communicate science to undergraduates, it was a lot of fun! Good luck at Astra. **Caro:** Your dedication, hard work and the ease with which you grasp new ideas has been inspiring. You will do great! **Anneke, Saida, Max, Joanna, Melanie, Krissi, Peter, Dina, Ann-Sophie** and **Simon**: thanks for all the fun times in the lab and at conferences.

CMB backbone

Matti: Thank you for your genuine care and great advice. You make everything sound possible. We are so lucky to have you at CMB! **Linda Lindell:** For your efficiency, shiny smiles and pleasant ways. **Linda Thörn:** For helping us organize such a great conference in Turkey. **Hannele:** For all the help with administrative issues. **Micke:** For the technical help and good advice over the years. **Zdravko**: for your great jokes, smiley face and help with the nitrogen tank. **Elisabeth and Rosa Amanda:** for shiny smiles and sharing stories about your children. **Irene, Lina** and **Margaret:** for all the help and your positive ways.

Members of Johan Ericson's lab

Lizzy: for really nice chats over the years. It´s a lot of fun to be around someone so energetic as you are. **Sanja:** For all your crazy stories and honest way of being. **Jose:** For nice lunches and chats. And a special thanks for the EndNote lesson ☺**. Zhanna, Masha, Ashwini** and **Johan**: thanks for nice chats and for sharing our big moments with us.

Members of Lorenz Poellinger's lab

Former and present members, especially thanks to **Micha:** you have a great way with people and you can easily see their needs. You will do great. And…you are also very funny ;) **Nino**: fun to share the corridor with you after sharing classroom in Södertörn, **Catta**: always as nice to talk to you, **Sandra**: for fun chats at the coffee machine, good luck with everything!

Former and present members of the pub crew

- Keep up the great work. It was loads of fun being part of it for some years.

Other friends from KI

Jonathan: Thanks so much for your input into paper IV! Good luck with everything! **Ersen:** Good luck with your company and hope to see you soon in Sweden. **Tove and Daniel:** see you around in the neighborhood. Looking forward to taking the little ones to öppna förskolan ☺ **Henriette:** For your graceful manner and fun chats over the years. Good luck with your new little family ☺. **Misia and Samer:** Welcome to parenthood! Can't wait to see the little cutie ☺ **Indira:** Always nice to talk to you. Good luck with the final sprint and hope to see you and your beautiful family more often. **Christian and Ulrika:** Looking forward to many more moments with you guys and our little ones ☺ **Sonal and Sameer:** Guys, I really miss seeing you. Hope you will eventually move back to Stockholm. **Emma and Nico:** You are a wonderful couple with great stories up your sleeves. Looking forward seeing kids playing together and I am glad Sophie will finally have another girl in the group ☺ **Blanchi:** Always a full house when you are around and definitely never boring. I am so glad my dear friend has such a good guy. **Helena:** The queen of fashion ☺ We have had so much fun over the years. Good luck with the last year/s? ☺ Stay in touch! **Nasim:** You made the last years of university so much fun! I remember all the cool scientific discussions we had and all the dreams we shared. Many of them have come true. Good luck with everything!

Pegah: Thanks for your friendship over the years, wise thoughts, caring ways, and for always being there for me. You really inspire me, both in science and privately.

Friends outside of academia

Karro: Vart du än är i världen så är du bara ett telefonsamtal bort. Varje gång vi pratar är det som att vi precis har setts. Du kommer alltid att förbli en av mina närmaste vänner som jag kan anförtro allt! **Fernanda:** Min fina vän som jag har känt längst av alla mina nära vänner. Vi har verkligen gått igenom mycket ihop och du känner mig ut och in. Är så oerhört tacksam att jag har dig i mitt liv! **Bodil:** Min kloka, starka, modiga vän! Jag tror på dig och vet att du kommer att lyckas med alla dina planer. **Anna-Klara:** Min allt i allo…Min dagbok, spegel, inspiration, min andra soul-mate ☺ Du är som familj till mig!

In-Laws

Philip and Becky: Can't wait to see you guys get married. Looking forward to Sophie and the little one having more cousins to play with. **Jim and Martha:** Having wonderful parents in law is a true blessing but to be as lucky as to feel so close to the grandparents in law is granted to few. I have so enjoyed all our trips together and am looking very much forward to Christmas in Guatemala. **Kathy and Fred:** You have really enriched my life and I feel so lucky to be part of your wonderful family. I feel at home with you and couldn't wish for better extra parents.

Extended family

Neno: Moving to the US wouldn´t be as hard, knowing we are closer to you. I love hanging out with you and your family and wish we could see you guys more often! **Nila:** Moja lipa Nile, svaki put kad cujem tvoj glas sijetim se divnih lijeta i momenata sa vama uz more, divnu hranu i dobra vina. Jedva cekam da vas vidim sve! **Dunja:** My dear sweet cousin who is always following her dreams and not scared of taking new directions. You are really awesome! **Vesna:** Din styrka är imponerande! Önskar vi kunde ses oftare. **Goga:** Min modiga coola kusin som vågar bryta normer och är ärlig mot sig själv. Du har alltid inspirerat mig med ditt hårda arbete och förmågan att komma över svåra situationer, både privat och under studietiden. Är så glad att du har tackat ja till Gudmor rollen ☺ **Nela:** full of energy, wonderful, crazy, inspiring, clever, brave, absolutely gorgeous, so much fun to be around…attributes are endless to describe you…one just has to know you. You never cease to surprise and your life is a real adventure. I totally dig you! **Milan:** Moj striko i madionicar! Da nije bilo tebe u pravo vrijeme ko zna da li bi ikad uzela ovaj put. Hvala ti sto si uvijek bio uz mene! Ti si jedna velika inspiracija jer znas kako da ostvaris svoje snove. Veselim se budicih vecera i fika sa tobom i dragom **Beom**. **Ana:** Moja draga seka koja je uvijek tako pazljiva i misli na sve nas. Volim te puno!

My parents: Ne znam ni gdje da pocnem.. Ljubav i divljenje koje osjecam prema vama je ne opisivo. Kroz toliko toga smo zajedno prosli i cesto u teskim situacijama pomislim na vas i kako ste se borili i kako ste fantasticno jaki ljudi. Vi ste moja najveca inspiracija u zivotu i ne mogu zamisliti bolje roditelje. U svakom trenutku ste bili uz mene i znali mi pokazat pravi put, cak i da mi pomognete izabrat fakultet. Taso moj, dao si mi puno tvojih dobrih strana i vrlo sam ponosna sa tim. Mamo moja, ostajes mi najbolja prijateljica i nadam se da Sophie i ja mozemo imat vezu kao sto je nasa.

My family

Till min ofödda son: Jag längtar så mycket efter dig!

Sophie: Du har gett mig så mycket glädje och lycka. Mitt liv har fått en djupare mening.

Chris: I can´t believe I was so lucky in life to find you. You are everything to me!

8 REFERENCES

Aguayo, A., Grau, G., Vela, A., Aniel-Quiroga, A., Espada, M., Martul, P., Castano, L., and Rica, I. (2013). Urinary iodine and thyroid function in a population of healthy pregnant women in the North of Spain. Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements *27*, 302-306.

Andersson, M., Karumbunathan, V., and Zimmermann, M.B. (2012). Global iodine status in 2011 and trends over the past decade. The Journal of nutrition *142*, 744-750.

Anway, M.D., Cupp, A.S., Uzumcu, M., and Skinner, M.K. (2005). Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science *308*, 1466-1469.

Arai, M., Otsu, K., MacLennan, D.H., Alpert, N.R., and Periasamy, M. (1991). Effect of thyroid hormone on the expression of mRNA encoding sarcoplasmic reticulum proteins. Circulation research *69*, 266-276.

Arthur, J.R. (1991). The role of selenium in thyroid hormone metabolism. Canadian journal of physiology and pharmacology *69*, 1648-1652.

Bachman, E.S., Hampton, T.G., Dhillon, H., Amende, I., Wang, J., Morgan, J.P., and Hollenberg, A.N. (2004). The metabolic and cardiovascular effects of hyperthyroidism are largely independent of beta-adrenergic stimulation. Endocrinology *145*, 2767-2774.

Bahouth, S.W. (1991). Thyroid hormones transcriptionally regulate the beta 1-adrenergic receptor gene in cultured ventricular myocytes. The Journal of biological chemistry *266*, 15863-15869.

Bahouth, S.W., Cui, X., Beauchamp, M.J., and Park, E.A. (1997). Thyroid hormone induces beta1-adrenergic receptor gene transcription through a direct repeat separated by five nucleotides. Journal of molecular and cellular cardiology *29*, 3223-3237.

Barker, D.J. (2002). Fetal programming of coronary heart disease. Trends in endocrinology and metabolism: TEM *13*, 364-368.

Bianco, A.C., Salvatore, D., Gereben, B., Berry, M.J., and Larsen, P.R. (2002). Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. Endocrine reviews *23*, 38-89.

Biederman, J., Kim, J.W., Doyle, A.E., Mick, E., Fagerness, J., Smoller, J.W., and Faraone, S.V. (2008). Sexually dimorphic effects of four genes (COMT, SLC6A2, MAOA, SLC6A4) in genetic associations of ADHD: a preliminary study. American journal of medical genetics Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics *147B*, 1511-1518.

Blewitt, M.E., Vickaryous, N.K., Paldi, A., Koseki, H., and Whitelaw, E. (2006). Dynamic reprogramming of DNA methylation at an epigenetically sensitive allele in mice. PLoS genetics *2*, e49.

Bochukova, E., Schoenmakers, N., Agostini, M., Schoenmakers, E., Rajanayagam, O., Keogh, J.M., Henning, E., Reinemund, J., Gevers, E., Sarri, M., *et al.* (2012). A mutation in the thyroid hormone receptor alpha gene. The New England journal of medicine *366*, 243-249.

Brent, G.A. (1994). The molecular basis of thyroid hormone action. The New England journal of medicine *331*, 847-853.

Buss, C., Entringer, S., Davis, E.P., Hobel, C.J., Swanson, J.M., Wadhwa, P.D., and Sandman, C.A. (2012). Impaired executive function mediates the association between maternal pre-pregnancy body mass index and child ADHD symptoms. PloS one *7*, e37758.
Cacciatori, V., Gemma, M.L., Bellavere, F., Castello, R., De Gregori, M.E., Zoppini, G., Thomaseth, K., Moghetti, P., and Muggeo, M. (2000). Power spectral analysis of heart rate in hypothyroidism. European journal of endocrinology / European Federation of Endocrine Societies *143*, 327-333.

Coan, P.M., Angiolini, E., Sandovici, I., Burton, G.J., Constancia, M., and Fowden, A.L. (2008). Adaptations in placental nutrient transfer capacity to meet fetal growth demands depend on placental size in mice. The Journal of physiology *586*, 4567-4576.
Cota, D., Marsicano, G., Lutz, B., Vicennati, V., Stalla, G.K., Pasquali, R., and Pagotto, U. (2003). Endogenous cannabinoid system as a modulator of food intake. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity *27*, 289-301.

Coulombe, P., Dussault, J.H., and Walker, P. (1976). Plasma catecholamine concentrations in hyperthyroidism and hypothyroidism. Metabolism: clinical and experimental *25*, 973-979.

Dampney, R.A., Horiuchi, J., and McDowall, L.M. (2008). Hypothalamic mechanisms coordinating cardiorespiratory function during exercise and defensive behaviour. Autonomic neuroscience : basic & clinical *142*, 3-10.

Dauncey, M.J., White, P., Burton, K.A., and Katsumata, M. (2001). Nutrition-hormone receptor-gene interactions: implications for development and disease. The Proceedings of the Nutrition Society *60*, 63-72.

Daxinger, L., and Whitelaw, E. (2012). Understanding transgenerational epigenetic inheritance via the gametes in mammals. Nature reviews Genetics *13*, 153-162.

de Escobar, G.M., Obregon, M.J., and del Rey, F.E. (2007). Iodine deficiency and brain development in the first half of pregnancy. Public health nutrition *10*, 1554-1570.

de Moura, E.G., and Passos, M.C. (2005). Neonatal programming of body weight regulation and energetic metabolism. Bioscience reports *25*, 251-269.
Degenhardt, T., Matilainen, M., Herzig, K.H., Dunlop, T.W., and Carlberg, C. (2006). The insulin-like growth factor-binding protein 1 gene is a primary target of peroxisome proliferator-activated receptors. The Journal of biological chemistry *281*, 39607-39619.
Dieckman, L.J., and Solaro, R.J. (1990). Effect of thyroid status on thin-filament Ca2+ regulation and expression of troponin I in perinatal and adult rat hearts. Circulation research *67*, 344-351.

Dillmann, W.H. (2002). Cellular action of thyroid hormone on the heart. Thyroid : official journal of the American Thyroid Association *12*, 447-452.

Dussault, J.H. (1993). Neonatal screening for congenital hypothyroidism. Clinics in laboratory medicine *13*, 645-652.

Flamant, F., Poguet, A.L., Plateroti, M., Chassande, O., Gauthier, K., Streichenberger, N., Mansouri, A., and Samarut, J. (2002). Congenital hypothyroid Pax8(-/-) mutant mice can be rescued by inactivating the TRαlpha gene. Molecular endocrinology *16*, 24-32.

Flores-Morales, A., Gullberg, H., Fernandez, L., Stahlberg, N., Lee, N.H., Vennstrom, B., and Norstedt, G. (2002). Patterns of liver gene expression governed by TRßeta. Molecular endocrinology *16*, 1257-1268.

Folkow, B., Johansson, B., and Oberg, B. (1959). A hypothalamic structure with a marked inhibitory effect on tonic sympathetic activity. Acta physiologica Scandinavica *47*, 262-270.

Forhead, A.J., Cutts, S., Matthews, P.A., and Fowden, A.L. (2009). Role of thyroid hormones in the developmental control of tissue glycogen in fetal sheep near term. Experimental physiology *94*, 1079-1087.

Fowden, A.L. (1995). Endocrine regulation of fetal growth. Reproduction, fertility, and development *7*, 351-363.

Fowden, A.L., and Forhead, A.J. (2004). Endocrine mechanisms of intrauterine programming. Reproduction *127*, 515-526.

Francis, D., Diorio, J., Liu, D., and Meaney, M.J. (1999). Nongenomic transmission across generations of maternal behavior and stress responses in the rat. Science *286*, 1155-1158.

Gabory, A., Roseboom, T.J., Moore, T., Moore, L.G., and Junien, C. (2013). Placental contribution to the origins of sexual dimorphism in health and diseases: sex chromosomes and epigenetics. Biology of sex differences *4*, 5.

Gicquel, C., El-Osta, A., and Le Bouc, Y. (2008). Epigenetic regulation and fetal programming. Best practice & research Clinical endocrinology & metabolism *22*, 1-16.

Gloss, B., Trost, S., Bluhm, W., Swanson, E., Clark, R., Winkfein, R., Janzen, K., Giles, W., Chassande, O., Samarut, J*., et al.* (2001). Cardiac ion channel expression and contractile function in mice with deletion of thyroid hormone receptor alpha or beta. Endocrinology *142*, 544-550.

Gluckman, P.D., Hanson, M.A., and Beedle, A.S. (2007). Early life events and their consequences for later disease: a life history and evolutionary perspective. American journal of human biology : the official journal of the Human Biology Council *19*, 1-19.

Gothe, S., Wang, Z., Ng, L., Kindblom, J.M., Barros, A.C., Ohlsson, C., Vennstrom, B., and Forrest, D. (1999). Mice devoid of all known thyroid hormone receptors are viable but exhibit disorders of the pituitary-thyroid axis, growth, and bone maturation. Genes & development *13*, 1329-1341.

Grigore, D., Ojeda, N.B., and Alexander, B.T. (2008). Sex differences in the fetal programming of hypertension. Gender medicine *5 Suppl A*, S121-132.

Gruppuso, P.A., Boylan, J.M., Anand, P., and Bienieki, T.C. (2005). Effects of maternal starvation on hepatocyte proliferation in the late gestation fetal rat. Pediatric research *57*, 185-191.

Hahn, T., Barth, S., Graf, R., Engelmann, M., Beslagic, D., Reul, J.M., Holsboer, F., Dohr, G., and Desoye, G. (1999). Placental glucose transporter expression is regulated by glucocorticoids. The Journal of clinical endocrinology and metabolism *84*, 1445-1452.
Hales, C.N., and Barker, D.J. (1992). Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. Diabetologia *35*, 595-601.

Hammond, H.K., White, F.C., Buxton, I.L., Saltzstein, P., Brunton, L.L., and Longhurst, J.C. (1987). Increased myocardial beta-receptors and adrenergic responses in hyperthyroid pigs. The American journal of physiology *252*, H283-290.

Hartong, R., Wang, N., Kurokawa, R., Lazar, M.A., Glass, C.K., Apriletti, J.W., and Dillmann, W.H. (1994). Delineation of three different thyroid hormone-response elements in promoter of rat sarcoplasmic reticulum Ca2+ATPase gene. Demonstration that retinoid X receptor binds 5' to thyroid hormone receptor in response element 1. The Journal of biological chemistry *269*, 13021-13029.

He, Y.F., Li, B.Z., Li, Z., Liu, P., Wang, Y., Tang, Q., Ding, J., Jia, Y., Chen, Z., Li, L.*, et al.* (2011). Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. Science *333*, 1303-1307.

Hiden, U., Glitzner, E., Hartmann, M., and Desoye, G. (2009). Insulin and the IGF system in the human placenta of normal and diabetic pregnancies. Journal of anatomy *215*, 60-68.
Hildreth, V., Anderson, R.H., and Henderson, D.J. (2009). Autonomic innervation of the developing heart: origins and function. Clinical anatomy *22*, 36-46.
Holt, R.I. (2002). Fetal programming of the growth hormone-insulin-like growth factor axis. Trends in endocrinology and metabolism: TEM *13*, 392-397.

Ikeda, H., Yong, Q., Kurose, T., Todo, T., Mizunoya, W., Fushiki, T., Seino, Y., and Yamada, Y. (2007). Clock gene defect disrupts light-dependency of autonomic nerve activity. Biochemical and biophysical research communications *364*, 457-463.

Ito, S., Shen, L., Dai, Q., Wu, S.C., Collins, L.B., Swenberg, J.A., He, C., and Zhang, Y. (2011). Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science *333*, 1300-1303.

Jimenez-Chillaron, J.C., Isganaitis, E., Charalambous, M., Gesta, S., Pentinat-Pelegrin, T., Faucette, R.R., Otis, J.P., Chow, A., Diaz, R., Ferguson-Smith, A.*, et al.* (2009). Intergenerational transmission of glucose intolerance and obesity by in utero undernutrition in mice. Diabetes *58*, 460-468.

Johansson, C., Gothe, S., Forrest, D., Vennstrom, B., and Thoren, P. (1999). Cardiovascular phenotype and temperature control in mice lacking thyroid hormone receptor-beta or both alpha1 and beta. The American journal of physiology *276*, H2006-2012.

Kahaly, G.J., and Dillmann, W.H. (2005). Thyroid hormone action in the heart. Endocrine reviews *26*, 704-728.

Kajimura, S., Aida, K., and Duan, C. (2005). Insulin-like growth factor-binding protein-1 (IGFBP-1) mediates hypoxia-induced embryonic growth and developmental retardation. Proceedings of the National Academy of Sciences of the United States of America *102*, 1240-1245.

Kaneshige, M., Suzuki, H., Kaneshige, K., Cheng, J., Wimbrow, H., Barlow, C., Willingham, M.C., and Cheng, S. (2001). A targeted dominant negative mutation of the thyroid hormone alpha 1 receptor causes increased mortality, infertility, and dwarfism in mice. Proceedings of the National Academy of Sciences of the United States of America *98*, 15095-15100.

Kaye, S., Gilsenan, J., Young, J.T., Carruthers, S., Allsop, S., Degenhardt, L., van de Glind, G., and van den Brink, W. (2014). Risk behaviours among substance use disorder treatment seekers with and without adult ADHD symptoms. Drug and alcohol dependence.

Keeney, B.K., Raichlen, D.A., Meek, T.H., Wijeratne, R.S., Middleton, K.M., Gerdeman, G.L., and Garland, T., Jr. (2008). Differential response to a selective cannabinoid receptor antagonist (SR141716: rimonabant) in female mice from lines selectively bred for high voluntary wheel-running behaviour. Behavioural pharmacology *19*, 812-820.

Kermack, W.O., McKendrick, A.G., and McKinlay, P.L. (1934). Death-rates in Great Britain and Sweden: Expression of Specific Mortality Rates as Products of Two Factors, and some Consequences thereof. The Journal of hygiene *34*, 433-457.
Kiss, E., Jakab, G., Kranias, E.G., and Edes, I. (1994). Thyroid hormone-induced alterations in phospholamban protein expression. Regulatory effects on sarcoplasmic reticulum Ca2+ transport and myocardial relaxation. Circulation research *75*, 245-251.

Klein, I., and Ojamaa, K. (2001). Thyroid hormone and the cardiovascular system. The New England journal of medicine *344*, 501-509.

Klieverik, L.P., Janssen, S.F., van Riel, A., Foppen, E., Bisschop, P.H., Serlie, M.J., Boelen, A., Ackermans, M.T., Sauerwein, H.P., Fliers, E.*, et al.* (2009). Thyroid hormone modulates glucose production via a sympathetic pathway from the hypothalamic paraventricular nucleus to the liver. Proceedings of the National Academy of Sciences of the United States of America *106*, 5966-5971.

Kopp, P. (2002). Perspective: genetic defects in the etiology of congenital hypothyroidism. Endocrinology *143*, 2019-2024.

Koss, K.L., and Kranias, E.G. (1996). Phospholamban: a prominent regulator of myocardial contractility. Circulation research *79*, 1059-1063.

Lane, N., Dean, W., Erhardt, S., Hajkova, P., Surani, A., Walter, J., and Reik, W. (2003). Resistance of IAPs to methylation reprogramming may provide a mechanism for epigenetic inheritance in the mouse. Genesis *35*, 88-93.

Lazarus, J., Brown, R.S., Daumerie, C., Hubalewska-Dydejczyk, A., Negro, R., and Vaidya, B. (2014). 2014 European thyroid association guidelines for the management of subclinical hypothyroidism in pregnancy and in children. European thyroid journal *3*, 76-94.

Lertratanangkoon, K., Wu, C.J., Savaraj, N., and Thomas, M.L. (1997). Alterations of DNA methylation by glutathione depletion. Cancer letters *120*, 149-156.

Lisboa, S.F., Reis, D.G., da Silva, A.L., Correa, F.M., Guimaraes, F.S., and Resstel, L.B. (2010). Cannabinoid CB1 receptors in the medial prefrontal cortex modulate the expression

of contextual fear conditioning. The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum *13*, 1163-1173.

Liu, Y.Y., Schultz, J.J., and Brent, G.A. (2003). A thyroid hormone receptor alpha gene mutation (P398H) is associated with visceral adiposity and impaired catecholamine-stimulated lipolysis in mice. The Journal of biological chemistry *278*, 38913-38920.

Lopez, M., Varela, L., Vazquez, M.J., Rodriguez-Cuenca, S., Gonzalez, C.R., Velagapudi, V.R., Morgan, D.A., Schoenmakers, E., Agassandian, K., Lage, R.*, et al.* (2010). Hypothalamic AMPK and fatty acid metabolism mediate thyroid regulation of energy balance. Nature medicine *16*, 1001-1008.
Lu, C., and Thompson, C.B. (2012). Metabolic regulation of epigenetics. Cell metabolism *16*, 9-17.

Lumey, L.H. (1992). Decreased birthweights in infants after maternal in utero exposure to the Dutch famine of 1944-1945. Paediatric and perinatal epidemiology *6*, 240-253.

Lumey, L.H., Stein, A.D., Kahn, H.S., and Romijn, J.A. (2009). Lipid profiles in middle-aged men and women after famine exposure during gestation: the Dutch Hunger Winter Families Study. The American journal of clinical nutrition *89*, 1737-1743.

Mailleux, P., and Vanderhaeghen, J.J. (1993). Dopaminergic regulation of cannabinoid receptor mRNA levels in the rat caudate-putamen: an in situ hybridization study. Journal of neurochemistry *61*, 1705-1712.

Mansouri, A., Chowdhury, K., and Gruss, P. (1998). Follicular cells of the thyroid gland require Pax8 gene function. Nature genetics *19*, 87-90.

Mao, J., Zhang, X., Sieli, P.T., Falduto, M.T., Torres, K.E., and Rosenfeld, C.S. (2010). Contrasting effects of different maternal diets on sexually dimorphic gene expression in the murine placenta. Proceedings of the National Academy of Sciences of the United States of America *107*, 5557-5562.

McMillen, I.C., and Robinson, J.S. (2005). Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. Physiological reviews *85*, 571-633.

Mintz, G., Pizzarello, R., and Klein, I. (1991). Enhanced left ventricular diastolic function in hyperthyroidism: noninvasive assessment and response to treatment. The Journal of clinical endocrinology and metabolism *73*, 146-150.

Moran, C., Agostini, M., Visser, W.E., Schoenmakers, E., Schoenmakers, N., Offiah, A.C., Poole, K., Rajanayagam, O., Lyons, G., Halsall, D.*, et al.* (2014). Resistance to thyroid hormone caused by a mutation in thyroid hormone receptor (TR)alpha1 and TRαlpha2: clinical, biochemical, and genetic analyses of three related patients. The lancet Diabetes & endocrinology *2*, 619-626.

Moran, C., Schoenmakers, N., Agostini, M., Schoenmakers, E., Offiah, A., Kydd, A., Kahaly, G., Mohr-Kahaly, S., Rajanayagam, O., Lyons, G.*, et al.* (2013). An adult female with resistance to thyroid hormone mediated by defective thyroid hormone receptor alpha. The Journal of clinical endocrinology and metabolism *98*, 4254-4261.

Morgan, H.D., Sutherland, H.G., Martin, D.I., and Whitelaw, E. (1999). Epigenetic inheritance at the agouti locus in the mouse. Nature genetics *23*, 314-318.

Morkin, E. (1993). Regulation of myosin heavy chain genes in the heart. Circulation *87*, 1451-1460.
Nathan, N., and Sullivan, S.D. (2014). Thyroid disorders during pregnancy. Endocrinology and metabolism clinics of North America *43*, 573-597.

Neel, J.V. (1962). Diabetes mellitus: a "thrifty" genotype rendered detrimental by "progress"? American journal of human genetics *14*, 353-362.

Ngun, T.C., Ghahramani, N., Sanchez, F.J., Bocklandt, S., and Vilain, E. (2011). The genetics of sex differences in brain and behavior. Frontiers in neuroendocrinology *32*, 227-246.

O'Shea, P.J., and Williams, G.R. (2002). Insight into the physiological actions of thyroid hormone receptors from genetically modified mice. The Journal of endocrinology *175*, 553-570.

Ojamaa, K., Kenessey, A., and Klein, I. (2000). Thyroid hormone regulation of phospholamban phosphorylation in the rat heart. Endocrinology *141*, 2139-2144.

Ojamaa, K., Sabet, A., Kenessey, A., Shenoy, R., and Klein, I. (1999). Regulation of rat cardiac Kv1.5 gene expression by thyroid hormone is rapid and chamber specific. Endocrinology *140*, 3170-3176.

Pachucki, J., Burmeister, L.A., and Larsen, P.R. (1999). Thyroid hormone regulates hyperpolarization-activated cyclic nucleotide-gated channel (HCN2) mRNA in the rat heart. Circulation research *85*, 498-503.

Pham, T.D., MacLennan, N.K., Chiu, C.T., Laksana, G.S., Hsu, J.L., and Lane, R.H. (2003). Uteroplacental insufficiency increases apoptosis and alters p53 gene methylation in the full-term IUGR rat kidney. American journal of physiology Regulatory, integrative and comparative physiology *285*, R962-970.

Poirier, L.A., Wise, C.K., Delongchamp, R.R., and Sinha, R. (2001). Blood determinations of S-adenosylmethionine, S-adenosylhomocysteine, and homocysteine: correlations with diet. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology *10*, 649-655.

Polak, M., Le Gac, I., Vuillard, E., Guibourdenche, J., Leger, J., Toubert, M.E., Madec, A.M., Oury, J.F., Czernichow, P., and Luton, D. (2004). Fetal and neonatal thyroid function in relation to maternal Graves' disease. Best practice & research Clinical endocrinology & metabolism *18*, 289-302.

Quignodon, L., Vincent, S., Winter, H., Samarut, J., and Flamant, F. (2007). A point mutation in the activation function 2 domain of thyroid hormone receptor alpha1 expressed after CRE-mediated recombination partially recapitulates hypothyroidism. Molecular endocrinology *21*, 2350-2360.
Rakyan, V.K., Chong, S., Champ, M.E., Cuthbert, P.C., Morgan, H.D., Luu, K.V., and Whitelaw, E. (2003). Transgenerational inheritance of epigenetic states at the murine

Axin(Fu) allele occurs after maternal and paternal transmission. Proceedings of the National Academy of Sciences of the United States of America *100*, 2538-2543.

Rapila, R., Korhonen, T., and Tavi, P. (2008). Excitation-contraction coupling of the mouse embryonic cardiomyocyte. The Journal of general physiology *132*, 397-405.

Ravelli, G.P., Stein, Z.A., and Susser, M.W. (1976). Obesity in young men after famine exposure in utero and early infancy. The New England journal of medicine *295*, 349-353.

Refetoff, S., and Dumitrescu, A.M. (2007). Syndromes of reduced sensitivity to thyroid hormone: genetic defects in hormone receptors, cell transporters and deiodination. Best practice & research Clinical endocrinology & metabolism *21*, 277-305.

Refetoff, S., Weiss, R.E., and Usala, S.J. (1993). The syndromes of resistance to thyroid hormone. Endocrine reviews *14*, 348-399.

Reik, W. (2007). Stability and flexibility of epigenetic gene regulation in mammalian development. Nature *447*, 425-432.

Rhodes, J.S., Gammie, S.C., and Garland, T., Jr. (2005). Neurobiology of Mice Selected for High Voluntary Wheel-running Activity. Integrative and comparative biology *45*, 438-455.
Rhodes, J.S., Garland, T., Jr., and Gammie, S.C. (2003). Patterns of brain activity associated with variation in voluntary wheel-running behavior. Behavioral neuroscience *117*, 1243-1256.

Roberts, C.G., and Ladenson, P.W. (2004). Hypothyroidism. Lancet *363*, 793-803.
Rohrer, D., and Dillmann, W.H. (1988). Thyroid hormone markedly increases the mRNA coding for sarcoplasmic reticulum Ca2+-ATPase in the rat heart. The Journal of biological chemistry *263*, 6941-6944.

Rosmond, R., and Bjorntorp, P. (2000). Occupational status, cortisol secretory pattern, and visceral obesity in middle-aged men. Obesity research *8*, 445-450.

Rubino, T., and Parolaro, D. (2011). Sexually dimorphic effects of cannabinoid compounds on emotion and cognition. Frontiers in behavioral neuroscience *5*, 64.

Sandler, B., Webb, P., Apriletti, J.W., Huber, B.R., Togashi, M., Cunha Lima, S.T., Juric, S., Nilsson, S., Wagner, R., Fletterick, R.J*., et al.* (2004). Thyroxine-thyroid hormone receptor interactions. The Journal of biological chemistry *279*, 55801-55808.

Sandman, C.A., Buss, C., Head, K., and Davis, E.P. (2014). Fetal Exposure to Maternal Depressive Symptoms Is Associated With Cortical Thickness in Late Childhood. Biological psychiatry.
Sandovici, I., Smith, N.H., Nitert, M.D., Ackers-Johnson, M., Uribe-Lewis, S., Ito, Y., Jones, R.H., Marquez, V.E., Cairns, W., Tadayyon, M*., et al.* (2011). Maternal diet and aging alter the epigenetic control of a promoter-enhancer interaction at the Hnf4a gene in rat pancreatic islets. Proceedings of the National Academy of Sciences of the United States of America *108*, 5449-5454.

Sap, J., Munoz, A., Damm, K., Goldberg, Y., Ghysdael, J., Leutz, A., Beug, H., and Vennstrom, B. (1986). The c-erb-A protein is a high-affinity receptor for thyroid hormone. Nature *324*, 635-640.

Saper, C.B. (2002). The central autonomic nervous system: conscious visceral perception and autonomic pattern generation. Annual review of neuroscience *25*, 433-469.
Sjogren, M., Alkemade, A., Mittag, J., Nordstrom, K., Katz, A., Rozell, B., Westerblad, H., Arner, A., and Vennstrom, B. (2007). Hypermetabolism in mice caused by the central action of an unliganded thyroid hormone receptor alpha1. The EMBO journal *26*, 4535-4545.

Stanner, S.A., Bulmer, K., Andres, C., Lantseva, O.E., Borodina, V., Poteen, V.V., and Yudkin, J.S. (1997). Does malnutrition in utero determine diabetes and coronary heart disease in adulthood? Results from the Leningrad siege study, a cross sectional study. Bmj *315*, 1342-1348.

Stein, A.D., and Lumey, L.H. (2000). The relationship between maternal and offspring birth weights after maternal prenatal famine exposure: the Dutch Famine Birth Cohort Study. Human biology *72*, 641-654.

Swoap, S.J., Li, C., Wess, J., Parsons, A.D., Williams, T.D., and Overton, J.M. (2008). Vagal tone dominates autonomic control of mouse heart rate at thermoneutrality. American journal of physiology Heart and circulatory physiology *294*, H1581-1588.

Tavi, P., Sjogren, M., Lunde, P.K., Zhang, S.J., Abbate, F., Vennstrom, B., and Westerblad, H. (2005). Impaired Ca2+ handling and contraction in cardiomyocytes from mice with a dominant negative thyroid hormone receptor alpha1. Journal of molecular and cellular cardiology *38*, 655-663.

Tinnikov, A., Nordstrom, K., Thoren, P., Kindblom, J.M., Malin, S., Rozell, B., Adams, M., Rajanayagam, O., Pettersson, S., Ohlsson, C*., et al.* (2002). Retardation of post-natal development caused by a negatively acting thyroid hormone receptor alpha1. The EMBO journal *21*, 5079-5087.

Trost, S.U., Swanson, E., Gloss, B., Wang-Iverson, D.B., Zhang, H., Volodarsky, T., Grover, G.J., Baxter, J.D., Chiellini, G., Scanlan, T.S*., et al.* (2000). The thyroid hormone receptor-beta-selective agonist GC-1 differentially affects plasma lipids and cardiac activity. Endocrinology *141*, 3057-3064.
van Mullem, A., van Heerebeek, R., Chrysis, D., Visser, E., Medici, M., Andrikoula, M., Tsatsoulis, A., Peeters, R., and Visser, T.J. (2012). Clinical phenotype and mutant TRαlpha1. The New England journal of medicine *366*, 1451-1453.

Vaquero, A., Loyola, A., and Reinberg, D. (2003). The constantly changing face of chromatin. Science of aging knowledge environment : SAGE KE *2003*, RE4.

Venero, C., Guadano-Ferraz, A., Herrero, A.I., Nordstrom, K., Manzano, J., de Escobar, G.M., Bernal, J., and Vennstrom, B. (2005). Anxiety, memory impairment, and locomotor dysfunction caused by a mutant thyroid hormone receptor alpha1 can be ameliorated by T3 treatment. Genes & development *19*, 2152-2163.

Wallis, K., Dudazy, S., van Hogerlinden, M., Nordstrom, K., Mittag, J., and Vennstrom, B. (2010). The thyroid hormone receptor alpha1 protein is expressed in embryonic postmitotic neurons and persists in most adult neurons. Molecular endocrinology *24*, 1904-1916.

Wallis, K., Sjogren, M., van Hogerlinden, M., Silberberg, G., Fisahn, A., Nordstrom, K., Larsson, L., Westerblad, H., Morreale de Escobar, G., Shupliakov, O., *et al.* (2008). Locomotor deficiencies and aberrant development of subtype-specific GABAergic interneurons caused by an unliganded thyroid hormone receptor alpha1. The Journal of neuroscience : the official journal of the Society for Neuroscience *28*, 1904-1915.

Walsh, R.R. (1969). Heart rate and its neural regulation with rising body temperature in anesthetized rats. The American journal of physiology *217*, 1139-1143.

Warner, A., Rahman, A., Solsjo, P., Gottschling, K., Davis, B., Vennstrom, B., Arner, A., and Mittag, J. (2013). Inappropriate heat dissipation ignites brown fat thermogenesis in mice with a mutant thyroid hormone receptor alpha1. Proceedings of the National Academy of Sciences of the United States of America *110*, 16241-16246.

Watkins, A.J., Ursell, E., Panton, R., Papenbrock, T., Hollis, L., Cunningham, C., Wilkins, A., Perry, V.H., Sheth, B., Kwong, W.Y., *et al.* (2008). Adaptive responses by mouse early embryos to maternal diet protect fetal growth but predispose to adult onset disease. Biology of reproduction *78*, 299-306.

Weaver, I.C., Cervoni, N., Champagne, F.A., D'Alessio, A.C., Sharma, S., Seckl, J.R., Dymov, S., Szyf, M., and Meaney, M.J. (2004). Epigenetic programming by maternal behavior. Nature neuroscience *7*, 847-854.

Weinberger, C., Thompson, C.C., Ong, E.S., Lebo, R., Gruol, D.J., and Evans, R.M. (1986). The c-erb-A gene encodes a thyroid hormone receptor. Nature *324*, 641-646.
Welberg, L.A., and Seckl, J.R. (2001). Prenatal stress, glucocorticoids and the programming of the brain. Journal of neuroendocrinology *13*, 113-128.

Wikstrom, L., Johansson, C., Salto, C., Barlow, C., Campos Barros, A., Baas, F., Forrest, D., Thoren, P., and Vennstrom, B. (1998). Abnormal heart rate and body temperature in mice lacking thyroid hormone receptor alpha 1. The EMBO journal *17*, 455-461.

Wolff, G.L., Kodell, R.L., Moore, S.R., and Cooney, C.A. (1998). Maternal epigenetics and methyl supplements affect agouti gene expression in Avy/a mice. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *12*, 949-957.

Yadav, V.K., Oury, F., Suda, N., Liu, Z.W., Gao, X.B., Confavreux, C., Klemenhagen, K.C., Tanaka, K.F., Gingrich, J.A., Guo, X.E., *et al.* (2009). A serotonin-dependent mechanism explains the leptin regulation of bone mass, appetite, and energy expenditure. Cell *138*, 976-989.

Yen, P.M. (2001). Physiological and molecular basis of thyroid hormone action. Physiological reviews *81*, 1097-1142.

Yen, P.M., Feng, X., Flamant, F., Chen, Y., Walker, R.L., Weiss, R.E., Chassande, O., Samarut, J., Refetoff, S., and Meltzer, P.S. (2003). Effects of ligand and thyroid hormone receptor isoforms on hepatic gene expression profiles of thyroid hormone receptor knockout mice. EMBO reports *4*, 581-587.

Young, D.B. (2010). In Control of Cardiac Output (San Rafael (CA)).