From the Department of Cell and Molecular Biology
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FROM MOLECULE TO NEURON: THYROID HORMONE ACTION IN THE BRAIN

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From molecule to neuron:
Thyroid hormone action in the brain

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By

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To my family
with all my love

Hold fast to dreams, for if dreams die,
life is a broken-winged bird that cannot fly.
- Langston Hughes -

ABSTRACT

The importance of thyroid hormone (TH) for mental health has been known for decades. With the identification of the thyroid hormone receptors α and β (TRα, TRβ), TR dependent mechanisms involved in brain development were investigated, identifying TRα1 as the main TR isoform in the brain. However, the lack of reliable antibodies has hampered the identification of TRα1 expressing cell types and TRα1 target genes. Here, we used two mouse lines to unravel TRα1 action in the developing brain. Firstly, using the TRα1-GFP mice circumvented the need for specific TRα1 antibodies in the study of TRα1 expression and target genes. Secondly, the TRα1+m mice which express a dominant negative TRα1 allowed the study of a receptor mediated hypothyroidism that furthermore can be ameliorated by supraphysiological doses of TH. These mice exhibit a delayed differentiation of parvalbumin expressing (PV+) GABAergic interneurons in the neocortex.

In **paper I** we describe the expression of TRα1 in the developing and mature brain of TRα1-GFP mice. TRα1 expression occurred in postmitotic neurons, which persisted in most mature neurons and in the glial tanycytes in the hypothalamus; the postnatal cerebellum however showed a transient expression in Purkinje cells. This mouse model was further utilized in **paper II** to identify TRα1 target genes in the TRα1-GFP mice by establishing a ChIP assay. This enabled us to enrich specifically for TRα1-bound DNA with GFP antibodies and to use wildtype chromatin as perfect background control. In **paper III** and **IV** this method led to identification of further target genes. **Paper III** we revealed a tissue specific regulation of carbonic anhydrase 4 (Car4) expression by TH. In brain and liver, Car4 was downregulated in TRα1+m mice, which could not be normalized by TH treatment, indicating the importance of proper TRα1 signaling during development for establishing the Car4 expression level in the adult. In contrast, renal Car4 expression was unaltered in the mutants but downregulated in response to TH treatment, revealing a suppressive function of renal TH. In **paper IV**, we investigated the role of TRα1 in developing PV+ cells and showed that TRα1 does not interfere with proliferation or migration of these cells but instead postpones their final step of differentiation. This was accompanied by a reduced expression of the neurotrophin NT-3. In summary, we described the temporal and spatial expression of TRα1 in the brain and established a method to reliable identify TRα1 target genes which is crucial to further understand the mechanism involved in TH dependent brain development.

POPULAR SCIENCE - GERMAN

Müdigkeit, Abgeschlagenheit und Konzentrationsschwäche werden leicht mit unzureichendem Schlaf, falschem Essen oder zu wenig Bewegung assoziiert, aber oft verbirgt sich dahinter jedoch eine Fehlfunktion der Schilddrüse. Eine Schilddrüsenunterfunktion, die dazu führt, dass der Körper unzureichend mit Hormonen versorgt wird, kann jedoch leicht mit Schilddrüsenhormon-Supplementen behandelt werden. Tritt diese Unterversorgung allerdings während der Schwangerschaft auf, sind irreversible neurologische Schädigungen im Neugeboren häufig. Darüberhinaus gibt es eine angeborene Schilddrüsenunterfunktion, den sogenannten kongenitalen Hypothyroidismus, die zu neurologische Schädigungen wie zum Beispiel einem reduzieren IQ führen kann, wenn sie nicht rechtzeitig erkannt und behandelt wird. Bislang ist allerdings noch unzureichend verstanden, welche Regionen und Zellen im Gehirn genau davon betroffen sind. Mit einem Mausmodell konnten wir nun zeigen, dass sich spezielle inhibierende Zellen, die sogenannten Parvalbumin-Neurone, im Gehirn nicht richtig entwickeln wenn nur unzureichend Schilddrüsenhormon zur Verfügung steht. Ein Verlust dieser Zellart ist assoziiert mit Epilepsie und Autismus.

Der genau Wirkungsmechanismus der Schilddrüsenhormone auf zellulärer Ebene ist ebenfalls nicht geklärt. Gründe dafür sind zum einen die fehlenden Daten über die zellspezifische Verteilung der Schilddrüsenhormon – Bindungsstellen (Rezeptoren) im Gehirn und zum anderen das lückenhafte Wissen über die involvierten Gene, die von den Hormonen in ihrer Aktivität reguliert werden. Meine Doktorarbeit fokussierte sich daher auf die Aufklärung des Wirkmechanismus der Schilddrüsenhormonen in der Entwicklung der inhibierenden Zellen. Wir konnten zeigen, dass die im Gehirn vorherrschende Form des Schilddrüsenhormonrezeptors Alpha 1 in allen Nervenzellen zu finden ist. Außerdem konnten wir eine neue Methode etablieren, mit deren Anwendung wir neue Zielgene der Schilddrüsenhormone in Gehirn identifizieren konnten. Aufbauend auf diesen Erkenntnissen konnten wir letztendlich einen Wirkungsmechanismus vorzuschlagen, wie Schilddrüsenhormone die Reifung der inhibierenden Zellen auf molekularer Ebene kontrollieren. Mit den erhaltenen Ergebnissen könnte eine neue Behandlungsstrategie für Patienten mit schilddrüsenhormonbedingten neurologischen Defekten entwickelt werden. Darüberhinaus trägt die Arbeit dazu bei, die immense Bedeutung der Schilddrüsenhormone in der Gehirnentwicklung zu unterstreichen und somit die irreversiblen Schädigungen durch unerkannte Schilddrüsenprobleme in der Schwangerschaft und in Neugeborenen zu vermeiden.

LIST OF SCIENTIFIC PAPERS

I. Wallis K, **Dudazy S**, van Hogerlinden M, Nordström K, Mittag J, Vennström B (2010). The thyroid hormone receptor alpha1 protein is expressed in embryonic postmitotic neurons and persists in most adult neurons. *Molecular Endocrinology* 24 (10): 1904-1916.

II. **Dudazy-Gralla S**, Nordström K, Hofmann PJ, Meseh DA, Schomburg L, Vennström B, Mittag J (2013). Identification of thyroid hormone response elements in vivo using mice expressing a tagged thyroid hormone receptor α1. *Bioscience Reports* 33(2): e00027. doi: 10.1042/BSR20120124.

III. Vujovic M*, **Dudazy-Gralla S***, Hård J, Solsjö P, Warner A, Vennström B, Mittag J (2015). Thyroid hormone drives the expression of mouse carbonic anhydrase Car4 in kidney, lung and brain. *Mol Cell Endocrinol*. Aug 28. pii: S0303-7207(15)30057-5. doi: 10.1016/j.mce.2015.08.017.

IV. **Dudazy-Gralla S**, Kloppsteck AS, Mittag J and Vennström B (2015). A dominant negative thyroid hormone receptor alpha 1 suppresses cortical expression of neurotrophin-3 and delays the maturation of parvalbumin positive interneurons. Manuscript.

* These authors contributed equally.

PUBLICATIONS NOT INCLUDED IN THE THESIS

Hadjab-Lallemend S*, Wallis K*, van Hogerlinden M, **Dudazy S**, Nordström K, Vennström B, Fisahn A (2010). A mutant thyroid hormone receptor alpha1 alters hippocampal circuitry and reduces seizure susceptibility in mice. *Neuropharmacology* 58(7): 1130-9.

Mittag J, Lyons DJ, Sällström J, Vujovic M, **Dudazy-Gralla S**, Warner A, Wallis K, Alkemade A, Nordström K, Monyer H, Broberger C, Arner A, Vennström B (2013). Thyroid hormone is required for hypothalamic neurons regulating cardiovascular functions. *The Journal of Clinical Investigation* 123(1): 509-16.

CONTENTS

LIST OF ABBREVIATIONS

3'UTR	Untranslated region
AKT	Protein kinase B
BDNF	Brain-derived neurotrophic factor
CA1	Cornu ammonis area 1 of the hippocampus
Car	Carbonic anhydrase
ChIP	Chromatin immunoprecipitation
CNS	Central nervous system
CR	Calretinin
DIO	Deiodinases
DR [number]	Direct repeat
E [number]	Embryonic day
GABA	Gamma aminobutyric acid
GAD	Glutamic acid decarboxylase
GFP	Green fluorescent protein
HCN	Hyperpolarization-activated cyclic nucleotide-gated channel
HPT axis	Hypothalamus-pituitary-thyroid axis
Lhx6	LIM homeobox 6
MCT [number]	Monocarboxylate transporter
MGE	Medial ganglionic eminence
Ncor	Nuclear receptor co-repressor
NeuN	Neuronal nuclei
NGF	Nerve growth factor
NMDAR	N-methyl-D-aspartate receptor
NT [number]	Neurotrophin
P [number]	Postnatal day
POa	Preoptic area
PTZ	Pentylenetetrazol
PV	Parvalbumin
RNF	Ring finger protein
RTH	Resistance to thyroid hormone

RXR	Retinoid-X receptor
Sema	Semaphorin
SMRT	Silencing mediator of retinoic acid/ thyroid hormone receptor
SST	Somatostatin
SVZ	Subventricular zone
T3	Triiodothyronine
T4	Thyroxine
TH	Thyroid hormone
TRE	Thyroid response element
TRH	Thyrotropin releasing hormone
Trk	Tyrosine receptor kinase
TSH	Thyroid stimulating hormone
Wt	Wildtype

1 INTRODUCTION

Fatigue, concentration deficits, weight gain and mood disorders are often correlated with a lack of sleep, unhealthy food and work overload. However, these are also typical symptoms for a shortage of thyroid hormone, a condition called hypothyroidism. Depending on degree and temporal onset, hypothyroidism causes a plethora of symptoms ranging from metabolic to behavioral phenotypes, growth and mental retardation, locomotor disorders, anxiety, mood disorders and heart failure. Most effects of TH are mediated by the nuclear thyroid hormone receptors α and β (TRα, TRβ), with TRα1 being the predominant TR isoform in the brain. Even though the importance of TH for brain development and function has been described decades ago, a lack of reliable TR antibodies has hampered the identification of many TH target genes and the unraveling of molecular mechanism causing the described symptoms. My thesis therefore focused on understanding the role of TRα1 in brain development by detecting TRα1 on the cellular level, identifying novel TRα1 target genes and unraveling the role of TRα1 in the maturation of GABAergic interneurons.

1.1 THYROID HORMONES

In humans, three to four weeks after gestation the thyroglossal duct develops at the base of the tongue and atrophies into thyroid tissue to create the two lobes of the thyroid gland. Follicular cells build the endocrine unite of the thyroid gland and are responsible for the production of thyroid hormone (TH).

1.1.1 Production and release of thyroid hormones

In the follicles tyrosine residues on thyroglobulin are iodinated to synthesize two kinds of thyroid hormones, T4 (3,3',5,5'– tetraiodothyronine) and T3 (3,3',5- triiodothyronine) negative feedback system regulated by the hypothalamus-pituitary-thyroid (HPT) axis controls the production and secretion of TH. The hypothalamus secretes thyrotropin-releasing hormone (TRH), which stimulates the pituitary to release thyrotropin-stimulating hormone (TSH). TSH in turn activates the thyroid gland to produce and secrete TH into the circulation (Fig. 1). Both T3 and T4 serve as negative feedback regulator on TSH and TRH (Yen, 2001; Bauer *et al.*, 2008), e.g. decreased TH levels increase TRH and TSH secretion leading to higher production of TH. The majority of TH secreted by the thyroid gland is T4, which is regarded as a pro-hormone. This pro-hormone is peripherally converted into the biological

active form T3 by tissue specific deiodinases; this extrathyroidal synthesis of T3 accounts for approximately 80% (Kohrle, 1999; Sandler *et al.*, 2004).

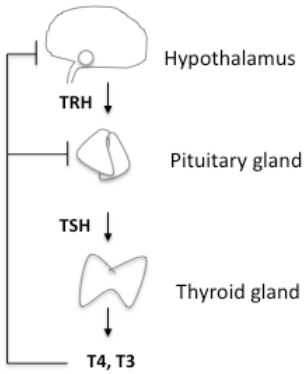

Figure 1: The HPT axis. TRH and TSH stimulate T4 and T3 release, whereas T4 and T3 send a negative feedback to inhibit further TRH and TSH expression.

1.1.2 Deiodinases

Specific proteins transport TH across the cell membrane. The two major families are the monocarboxylate transporters (e.g. MCT8) and organic-anion transporter polypeptides (e.g. OATP1c1) (Friesema *et al.*, 2003; Friesema *et al.*, 2008; Schweizer & Kohrle, 2013). In the cell, deiodinases regulate T3 availability in response to TH levels in a tissue and spatial dependent manner. They either activate T4 by removing iodine from the outer ring to produce T3 (DIO1, DIO2) or inactivate T4 by facilitating the inner ring deiodination (DIO1, DIO3) to generate reverse T3 (rT3) (Bianco & Kim, 2006). DIO2 converts T4 to T3 in the central nervous system (CNS), including hypothalamus, pituitary and cortex, where it is responsible for 75% of the nuclear T3 (Crantz *et al.*, 1982). The inactivating DIO3 is mostly expressed in the placenta, uterus and CNS (Kohrle, 1999; St Germain *et al.*, 2005). Lastly, DIO1 with affinity to both rings is expressed in liver, kidney, thyroid and pituitary (Bates *et al.*, 1999; Bianco *et al.*, 2002) (Fig. 2). However, studies on mice lacking the DIO2 gene and endogenously low levels of DIO1 demonstrated normal T3 and increased serum T4 and TSH levels, indicating the presence of an compensating mechanism most likely due to an adaption of thyroidal TH production (Bianco & Kim, 2006).

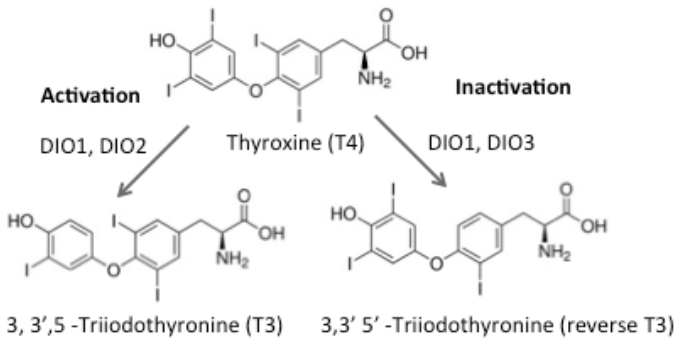

Figure 2: Structures of T4, T3 and reverse T3 with deiodinases catalyzing activation or inactivation of the hormone.

1.2 THYROID HORMONE RECEPTORS

In the 19th century hypothyroid patients were treated with sheep thyroid extract to ameliorate their symptoms, but the active components in the extract was not known until 1915 when thyroid hormone was isolated and crystalized by Kendall. However, it took another 40 years to identify T3, the active form (Gross & Pitt-Rivers, 1952). Its vital function was well acknowledged but it was not apparent how the hormone exerted its function (Hamdy, 2002). Finally, in 1986 the first thyroid hormone receptor isoforms were identified, which allowed research on their mechanism of action in the cell (Sap *et al.*, 1986; Weinberger *et al.*, 1986).

1.2.1 Thyroid hormone receptors and gene expression

T3 exerts its function through binding to the thyroid hormone receptors (TRs) α and β**.** TRs are encoded by the *Thra* and *Thrb genes* (Sap *et al.*, 1986; Weinberger *et al.*, 1986; Koenig *et al.*, 1988), alternative splicing give rise to four isoforms: the ligand binding TRα1, TRβ1, TRβ2 and the non-ligand binding TRα2 (Fig. 3A) (O'Shea & Williams, 2002).

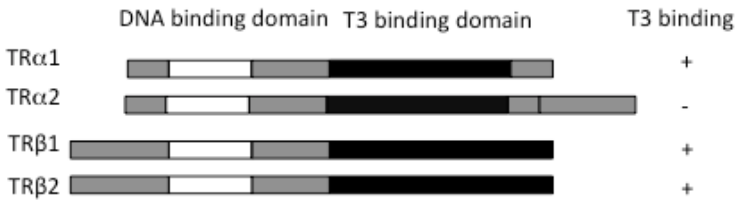

Figure 3A: Thyroid hormone receptors. Schematic representation of TR isoforms with functional domains including DNA binding and T3 binding domain as well as T3 binding ability.

TRs show similar structural organization with an amino-terminal A/B domain, a ligand binding C-domain and a DNA binding N-domain (Yen, 2001). However, the TRβ isoforms differ in their amino-terminal regions, whereas the divergent carboxy-terminal region of TRα2 prevents ligand binding and its function is poorly understood (Moran & Chatterjee, 2015). TRs belong to the family of nuclear receptors and are ligand modulated transcription factors. They usually bind as heterodimers in association with retinoid X receptor (RXR) to thyroid response elements (TRE) of target genes and generally activate their transcription upon ligand binding (Fig. 3B).

Non-liganded receptors recruit co-repressors such as Ncor, SMRT and Alien and bind histone deacetylases to silence positively regulated target genes. Ligand binding then leads to conformational changes resulting in the release of co-repressors and the recruitment of co-activators, such as SRC-1, TIF-2, CBP, with subsequent histone acetyltransferase binding and gene activation (Fig. 3B) (Koenig, 1998; Moran & Chatterjee, 2015). It can be said that in the euthyroid organism, the circulating level of TH balances transcriptional repression and activation to achieve an optimal target gene expression. Negatively regulated genes, e.g. TRH and TSH, are repressed upon ligand binding and activated by the apo-receptor; however not many negatively regulated genes have been identified so far and the molecular mechanisms remain obscure (Chin *et al.*, 1998; Costa-e-Sousa & Hollenberg, 2012).

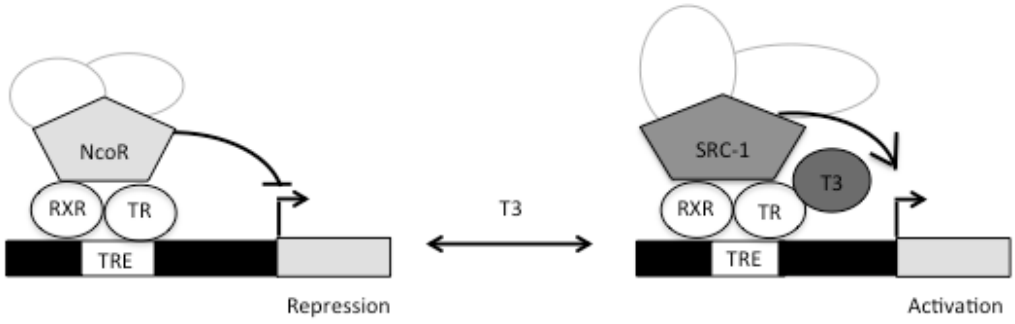

Figure 3B: Repression and activation of a positively regulated gene. TR and RXR bind as a heterodimer to TRE. Binding of Ncor and other co-factors lead to target gene repression. T3 binding initiates recruitment of co-activators such as SRC-1 and activation of gene expression.

TRs are ubiquitously expressed, with TRα1 being the most abundant isoform in the brain, bone, heart and gastrointestinal tract. In contrast, TRβ1 is the predominant isoform in kidney and liver, while TRβ2 expression is limited to the hypothalamus, pituitary, eye and inner ear (Brent, 1994; Forrest *et al.*, 1996a).

1.2.2 Characteristics of thyroid hormone response elements

In order to elucidate the molecular mechanism causing the syndromes related to TH dysregulation, target genes need to be identified. However, the high variety of sequences and the lack of reliable TR antibodies have hampered the detection of thyroid hormone response elements (TRE) and consequently the identification of thyroid hormone target genes. In previous studies, Thompson et al. used gel shift assays to narrow down the putative TRE sequence for hairless, a protein known to be regulated by TH (Thompson, 1996). Gel shift assays were performed with radioactive labeled DNA, RXR and TR. Fragments that were found to bind the labeled complex were subcloned and reloaded to a gel shift assay. This was performed multiple times until a TRE sequence was identified (Thompson & Bottcher, 1997). Another TRE was described using co-immunoprecipitation with anti-TR antibodies and footprint analysis. This technique also helped to determine the TRE in RC3, a gene implicated among others in synaptic plasticity and regulated by TH (Martinez de Arrieta *et al.*, 1999). Although neither method allowed to conclude which TR isoform binds to the TREs, they helped together with other studies to characterize TREs. However, genome wide analyses in neural cells revealed partly overlapping target genes but also TR isoform specific gene transcription (Chatonnet *et al.*, 2013), which was in line with previous observation in TR overexpressing HepG2 cells (Chan & Privalsky, 2009). Tissues expressing both isoforms, revealed distinct phenotypes when one or the other isoform was blocked. This finding suggested different gene repertoires for TRα1 and TRβ in one tissue (Winter *et al.*, 2006), which was later supported when a receptor specific preference of T3 target genes was identified (Chatonnet *et al.*, 2013).

However, from previous studies it can be summarized that, TREs are generally located upstream of the promoter (Chatterjee *et al.*, 1989; Carr *et al.*, 1992; Bigler & Eisenman, 1995; Yen, 2001) and that the consensus hexamer half-site for TREs is encoded by (G/A)GGTC(C/G)A (Cheng *et al.*, 1987). Most target genes possess more than one half-site which is organized as direct, inverted or everted repeats with an optimal spacing of zero, four or six nucleotides respectively (Fig. 4). The majority of described TREs are direct repeats with a spacing of four nucleotides (DR4), such as in the promotor of kruppel like factor (klf9) (Denver & Williamson, 2009; Paquette *et al.*, 2014).

Consensus half-site	(G/A)GGTC(C/G)A
Direct Repeat	AGGTCANNNNAGGTCA
Everted Repeat	TGACCTNNNNNNAGGTCA
Inverted Repeat	AGGTCATGACCT

Figure 4: Sequences and organization of thyroid hormone response elements.

1.3 THYROID HORMONE DISORDERS

In a healthy individual serum TH levels are stable and fine balanced via the HPT axis. However, iodine deficiency, autoimmune disorders or pregnancy can cause dysregulation of TH levels. Given the ubiquitous expression of TRs it is not surprising that alterations in TH levels lead to adverse affect in the body.

1.3.1 Hypothyroidism

A lack of TH caused by e.g. insufficient iodine intake or the autoimmune disorder Hashimoto's disease (loss of follicular cells) leads to weight gain, cold sensitivity, tiredness and mood disorders, symptoms that easily can be associated with other dysfunctions. However, low TH levels can be stabilized with hormone supplementation, when identified. During pregnancy the embryo depends largely on TH supply from the mother as TRs can already be detected in the fetal brain at 9-12 weeks of gestation, i. e. before endogenous TH production is established at around 17-19 weeks of gestation (de Escobar *et al.*, 2004; Chan *et al.*, 2009). Even though it was shown in numerous studies that already a mild reduction of TH levels during pregnancy leads to neuronal alterations in the brain, mental retardation and growth defects in the offspring (Haddow *et al.*, 1999; Pop *et al.*, 1999; Utiger, 1999; Auso *et al.*, 2004; de Escobar *et al.*, 2004; Lazarus *et al.*, 2012) a general screening for subclinical hypothyroidism is currently not advised, as there are only limited data on the outcome of the treatment of mild hypothyroidism during pregnancy (Lazarus *et al.*, 2014). This however is controversially discussed and about 40% of endocrinologists in Europe test TH levels during pregnancy routinely (Vaidya *et al.*, 2012), but the number might be different for gynecologists.

Congenital hypothyroidism

Also, hypothyroidism can occur in the newborn child, a syndrome called congenital hypothyroidism. This disease is mostly caused by deficits in thyroid gland development or thyroid hormone production, and leaves the child mentally retarded with motor dysfunctions, short statue, obesity and delayed overall development, if the condition remains untreated in the first weeks of live (Bernal, 2007; Rastogi & LaFranchi, 2010; Krude *et al.*, 2015). Fortunately, thyroid TSH levels in newborns are routinely tested in most developed countries (Horn & Heuer, 2010), and timely treatment prevents mental retardation entirely (Gruters & Krude, 2012). However, also mutations in the T3 transporter MCT8 cause severe mental and motor retardations including inability to speak or hear and poor head control (Schwartz & Stevenson, 2007). As the T3 transport is inhibited, the T3 serum levels are increased. Consequently, children suffer additionally of hyperthyroidism leading to hypermetabolism, tachycardia or anorexia; unfortunately, a treatment of this disease in not yet established (Krude *et al.*, 2015).

1.3.2 Hyperthyroidism

Not only reduced levels of TH have consequences for health and wellbeing, also the increased production of TH leads to disease. Goiter and adenomas can cause an excessive TH production, although the quite common autoimmune Grave's disease causes the majority of the hyperthyroidism related diseases (Nathan & Sullivan, 2014). This disorder involves autoimmune antibodies that stimulate the TSH receptor, thereby increasing the production and secretion of TH. Elevated TH levels lead to symptoms such as anxiety, restlessness, depressive disorders, impaired concentration and decreased appetite (Bauer *et al.*, 2008). During pregnancy hyperthyroidism increases the risk for miscarriages, maternal heart failure and hypertension as well as growth retardation and increased risk for congenital hypothyroidism in the child (Nathan & Sullivan, 2014). The latter is due to an impaired development of the HPT axis in a hyperthyroid environment (Kempers *et al.*, 2003). Like hypothyroidism, hyperthyroidism is not always immediately identified by physicians, as the symptoms are quite vague and blood test are needed. Once identified, TH level can be corrected by anti-thyroid drugs that reduce TH production.

1.4 MOUSE MODELS WITH MUTATION IN THYROID HORMONE RECEPTOR ISOFORMS

For decades it has been an enigma how thyroid hormones exerted their functions. With the identification of thyroid hormone receptors (Sap *et al.*, 1986; Weinberger *et al.*, 1986; Koenig *et al.*, 1988) researchers became eager to elucidate the function of those receptors. Subsequent creation of several mouse models with mutations in different thyroid hormone receptor alleles revealed a variety of phenotypes.

1.4.1 Phenotypes of various TR knock out mice

Studies on various TR knockout animals revealed TR specific roles in development and physiology of target tissues. In general, TRα1 was found to be crucial for postnatal development and cardiac function, whereas TRβ is mainly involved in inner ear and retina development, liver metabolism and regulation of thyroid hormone (Flamant & Samarut, 2003).

The phenotype of mice lacking both of TRβ1 and TRβ2 expression is characterized by goiter, deafness as well as high levels of TSH and TH, indicating an involvement of TRβ in the HPT axis (Forrest *et al.*, 1996b). However, TRβ2 knockout mice exerted normal hearing and only slightly increased T3 and TSH levels, indicating a predominant function of TRβ1 in hearing development and pituitary control, with the latter being modulated by TRβ2 (Ng *et al.*, 2001). Additional, TRβ2 contributes to vision development by regulating late stage differentiation of M-cones, consequently TRβ2 knockout led to M-cones loss (Ng *et al.*, 2001; Roberts *et al.*, 2006).

TRα1 knockout mice with preserved TRα2 function showed low heart rate and reduced body temperature (Wikstrom *et al.*, 1998). Mice lacking only TRα2 were viable with a mixed hyper- and hypothyroid phenotype including increased fat mass, growth retardation but reduced body weight, elevated heart beat and higher body temperature. As the TRα2 knockout lead to overexpression of TRα1, the phenotype was ascribed to the increased TRα1 action (Salto *et al.*, 2001).

Finally, TRα1/TRβ knockout mice exhibit a hyperactive HPT axis, poor female fertility, retarded growth and bone development, i.e. characteristics not seen so prominently in the single knockout mice indicating the existence of common and distinct pathways for the TR isoform. Interestingly, due to the lack of the receptors, the endogenous excess of TH in the

double knockouts does not lead to a hyperthyroid phenotype. Moreover, induced hypothyroidism in those mice had no affect on their phenotype either, demonstrating that TRα1 and TRβ are the only T3 responsive receptors, and that TRα2 may only have a modulating function (Gothe *et al.*, 1999).

1.4.2 Dominant negative TRα1

Since a lack of TRs caused a milder phenotype than the lack of TH as seen for example in cretinism, it was postulated that the non-ligand receptor suppresses gene expression (Gothe *et al.*, 1999; Morte *et al.*, 2002). As abolished TH binding to TRβ leads to the condition of resistance to thyroid hormone (RTH), it became of great interest to study a similar mutation in TRα1. Since no patients with mutations in TRα1 had been found before 2012, it was speculated that the phenotype of RTHα patients is either too mild for diagnosis, lethal leading to abortions, or difficult to associate with a thyroid hormone receptor disorder. To study the outcome of a TRα1 aporeceptor, Björn Vennström's group introduced a mutation into the TRα1 locus originally identified in the TRβ gene (Adams *et al.*, 1994). Tinnikov and colleagues changed arginine to cysteine in the ligand-binding domain of TRα1 leading to a 10-fold reduced affinity to T3, leaving the receptor in a non-ligand state (TRα1R384C). The mutant receptor acts in a dominant negative manner and can be reactivated by supraphysiological doses of TH (Tinnikov *et al.*, 2002). Most homozygous mice die shortly after birth for unknown reasons, whereas heterozygotes (TRα1+m) generally survive to adulthood. The postnatal phenotype of these TRα1+m mice is low birth weight and a retarded development including ossification, eye opening, tooth eruption, maturation and puberty. TH administration throughout pre- and postnatal development relieved those symptoms (Tinnikov *et al.*, 2002). Adult mice show slightly reduced heart rate and reduced blood pressure (Mittag *et al.*, 2010b). They are hyperphagic, hypermetabolic and have a 10-15% reduced body weight due to decreased white adipose tissue (Sjogren *et al.*, 2007). Endocrine markers in adults such as TSH were found to be normal to elevated in adults, T4 was low to normal, whereas T3 showed no alteration (Tinnikov *et al.*, 2002). Neurologically, TRα1+m mice exhibit anxiety and reduced cognition, which can be normalized with T3 treatment in adulthood (Venero *et al.*, 2005). They are resistant to chemical induced seizures, but susceptible to audiogenic seizures (Hadjab-Lallemend *et al.*, 2010). Moreover, they show reduced grip strength, abnormal gait, everted hind paws and poor limp coordination similar to

what is seen in cretinism. Reduced grip strength was ameliorated by the combined fetal and postnatal TH treatment (Wallis *et al.*, 2008).

Interestingly, other mouse models with similar mutations in the ligand-binding domain show somewhat different phenotypes, ranging from dwarfism to near normal growth and from lean to obese mice. The differences might be explained by the location of the mutation, which may alter cofactor binding (Liu *et al.*, 2007; Mittag *et al.*, 2010b). Consequently, Liu et al. demonstrated a mutation dependent interaction with peroxisome proliferator activated receptor α (PPARα), a regulator of lipid metabolism. Mice harboring a slightly different point mutation in TRα1 (TRα1P398H) show visceral obesity, whereas TRα1R384C mice are lean. Interestingly, TRα1P398H reduced the binding of PPARα to its response elements compared to wildtype TRα1 whereas TRα1R384C did not affect PPARα binding and showed similar transactivation properties as the wt TRα1 (Liu *et al.*, 2007). These findings explain at least partly the diverse metabolic phenotypes and demonstrate the mutation dependent transactivation of target genes.

1.5 PATIENTS WITH RESISTANCE TO THYROID HORMONE

Mutations in the ligand-binding domain of TRs lead to tissue-specific hyposensitivity towards TH. For decades only patients with mutations in TRβ were found, as guidelines to identify TRα1 patients were not available, until recently the first TRα1 patients were characterized (Bochukova *et al.*, 2012; Moran *et al.*, 2013; van Mullem *et al.*, 2013; Moran & Chatterjee, 2015). Now we distinguish between two disorders: resistance to thyroid hormone α (RTHα) or resistance to thyroid hormone β (RTHβ). In both syndromes the mutated receptors exert a dominant negative function over the wildtype receptor, hence, inhibiting its function and repressing the transcription of most genes that are normally activated by ligand binding. This is most likely caused by failure of corepressor dissociation by the dominant negative receptor (Yoh *et al.*, 1997; Moran & Chatterjee, 2015).

1.5.1 Resistance to thyroid hormone β

3000 individuals with mutation in TRβ have been identified to date (Dumitrescu & Refetoff, 2013). The autosomal inherited mutation concerns the ligand binding domain of TRβ and leads to impaired or abolished binding of T3 to TRβ, leaving TRβ in the aporeceptor stage

and, hence, suppressing the transactivation of positively regulated genes. However, the negatively regulated gene TSH is increased in RTHβ patients regardless of their elevated TH levels. Since transactivation of TSH is initiated by the aporeceptor, the inability of the mutant receptor to bind TH leads to an overexpression of TSH, which demonstrates the failure of the mutant receptor to regulate gene expression (Mittag *et al.*, 2010b; Dumitrescu & Refetoff, 2013).

RTHβ patients show heterogeneous symptoms, but goiter, tachycardia, attention deficit hyperactivity disorder (ADHD), learning disability and developmental delay are the common clinical signs (Refetoff *et al.*, 1993).

1.5.2 Resistance to thyroid hormone α

The first case of RTHα was described in 2012, to date there are 14 reported patients from 10 families (Moran & Chatterjee, 2015), exhibiting e.g. *de novo* mutations (Bochukova *et al.*, 2012; Moran *et al.*, 2013), or inherited RTHα (van Mullem *et al.*, 2013). Depending on the mutation T3 binding can either be diminished or abolished, but both cases lead to a dominant negative apo- TRα1. Hallmarks of the syndrome are hypothyroid features such as short statue, delayed bone age, impaired ossification, bradycardia, severe constipation, macrocephaly, reduced muscle strength, developmental delay and cognitive impairment, with normal TH and TSH levels, low rT3 and subnormal T3/T4 ratio. In some cases T4 treatment improved constipation, metabolic rate, general activity, but showed no effect on developmental delay, growth rate or bradycardia (Bochukova *et al.*, 2012; Moran *et al.*, 2013; van Mullem *et al.*, 2013). These observations integrate well with our findings in TRα1+m mice: developmental delay was rescued by pre- and postnatal treatment with high doses of TH, whereas postnatal treatment was not sufficient to ameliorate growth retardation or reduced heart rate (Tinnikov *et al.*, 2002). Interestingly, whole genome sequencing of patients with autism spectrum disorders identified a patient with a R384C mutation in TRα1, identical to the mutation in our TRα1+m mouse strain (Yuen *et al.*, 2015). Unfortunately, no further data about that patient are available to date. Finally, in January 2015, the sequence of 60 000 anonymous exomes were released by the Exac database (http://exac.broadinstitute.org/) revealing the existence of 68 *THRA* mutations, so the incidence seems to be around 1:10.000! Unfortunately, the Exac database does not give access to personal data.

1.6 THYROID HORMONE ACTION IN THE BRAIN

The behavior and locomotor phenotype of TRα1+m mice is accompanied by impaired development of GABAergic interneurons in the brain (Venero *et al.*, 2005; Gilbert *et al.*, 2007; Wallis *et al.*, 2008). In the following my focus will be on the development of the brain and in particular on GABAergic cells and the influence of TH in response to the neurological phenotype of TRα1+m mice.

1.6.1 Brain development

Before closure, the anterior end of the neuronal tube forms the three primary brain vesicles: prosencephalon, mesencephalon (later midbrain) and rhombencephalon (later hindbrain). The prosencephalon consists of the diencephalon (later thalamus) and the telencephalon. The latter is responsible for higher brain function and gives rise to the neocortex, hippocampus, basal ganglia and olfactory bulb (Stiles & Jernigan, 2010). The neocortex is the largest and most crucial part of the telencephalon. It is involved in cognition, sensory perception and locomotor processes. Distinct well-organized areas of the neocortex are responsible for certain tasks, and differ by neuronal constellation, connectivity and density, and are generally divided in six layers (lamina). Neurons of the distinct layers target different areas of the brain, e.g. pyramidal cells in layer II and III project to other areas of the neocortex, whereas pyramidal cells of layer IV and V send their axons outside the cortical layers (e.g. thalamus). Pyramidal cells belong together with spiny stellate cells to the group of glutamatergic excitatory neurons which account for 80% of all neocortical neurons and exist in fine balance with the remaining 20% of inhibitory interneurons in the neocortex (Sultan *et al.*, 2013).

1.6.2 GABAergic interneurons

Interneurons using the neurotransmitter gamma aminobutyric-acid (GABA) control the information distribution in the cortex; they time firing of excitatory pyramidal cell, synchronize network activity, fine tune neuronal firing to oscillation and keep a well-adjusted excitatory and inhibitory balance. Defects in GABA transmission are involved in neurological and psychiatric diseases, such as epilepsy, anxiety or autism (Cobos *et al.*, 2006; Rudy *et al.*, 2011). The different tasks require highly specialized cells. I

Generally, GABAergic cells are spiny and partially spiny non-pyramidal cells that express the glutamic acid decarboxylase (GAD) required for GABA synthesis from glutamate. They are

connected with excitatory and inhibitory cells in their vicinity and can extend horizontal or vertical axons, with their cell body mostly in layer II-IV in the cortex. Their inhibitory synapses target soma, dendrites, axons or presynaptic boutons on particular neurons to establish inhibitory circuits (Fig. 5). Consistently, interneurons are a heterogenous group of cells that can be distinguished by their morphology, electrophysiology and neurochemical properties (Brandao & Romcy-Pereira, 2015). One possibility is to subdivide interneurons neurochemically into three major groups. Members of those groups express either the calcium binding protein parvalbumin (PV), the neuropeptide somatostatin (STT) or the ionotropic serotonin receptor (5Ht3aR). Each group consists of several types of interneurons with a different morphological and electrophysiological profile but the same origin. PV identifies fast spiking basket and chandelier cells and accounts for 40% of GABAergic cells in the cortex. 30% of GABAergic interneurons express SST, which serves as markers for burst-spiking Martinotti cells and cells that specifically target layer IV, whereas 5Ht3aR stains the remaining 30% of GABAergic interneurons including vasointestinal protein (VIP) non/expressing cells (Lee *et al.*, 2010; Rudy *et al.*, 2011). It is of note that other calcium binding proteins such as calretinin or calbindin are often used to distinguish interneurons, but they overlap with PV, SST or VIP expression.

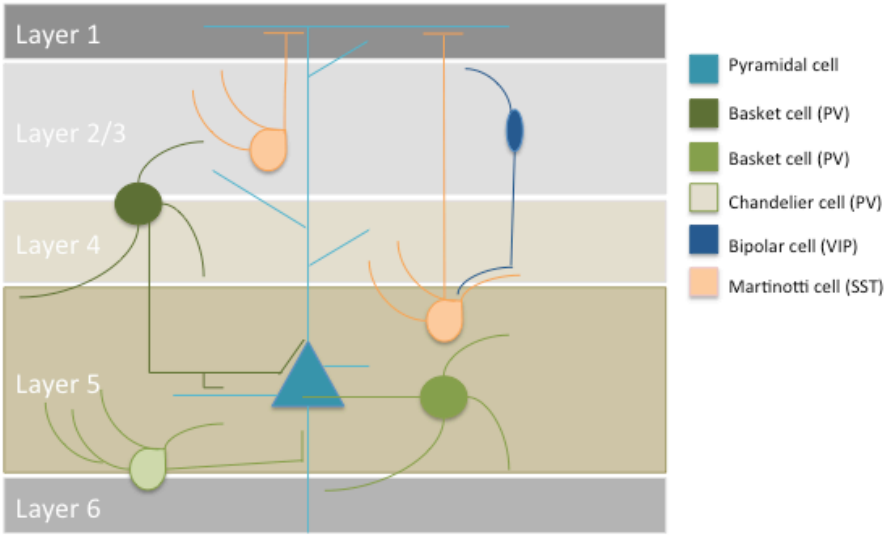

Figure 5: Schematically representation of interneuron distribution and connectivity in the laminar structure of the cerebral cortex. Modified from (Marin, 2012).

1.6.2.1 Origin and migration of interneurons

Interneurons subgroups originate of distinct areas of the developing brain. 5Ht3aR cells, CR and/or VIP expressing cells originate in the caudal ganglionic areas. In contrast, PV+ and

SST+ cells arise from subpallial progenitors in the medial ganglionic eminence (MGE). Specifically, PV+ interneurons are born in the ventral part of MGE (vMGE), whereas the dorsal MGE (dMGE) generates SST+ cells (Cobos *et al.*, 2006; Flames *et al.*, 2007). After proliferation in the ventricular zone, MGE derived interneurons migrate tangentially to their destination in the cortex by generating a leading process with multiple branches to detect chemical cues on their journey to their final destination. Once the cell decided for a moving direction, the leading process is stabilized, the organelles form a presomal swelling into the process followed by a nucleus translocation towards the swelling (Guo & Anton, 2014).

At E11.5 an early stream of interneurons migrate from the MGE on a superficial path through the marginal zone (MZ), circumventing the striatum to form the preplate at the surface of the cortex. At E12.5- E.14.5 a second stream of interneurons migrate through a deep route along the intermediate zone (IZ) and the superficial MZ to avoid the striatal mantel and cortical plate (Fig. 6) (Marin & Rubenstein, 2001). In late corticogenesis cells mainly migrate through the IZ/SVZ to invade the cortical plate; however, migration streams can also be observed in the MZ and subplate. The latter arises from the preplate. Once interneurons reach the cortical plate they switch to radial migration to find their destination (Sultan *et al.*, 2013). Early born MGE cells are located in the deep layers of the cortex, whereas younger cells migrate past the older cell and invade upper layers of the cortex.

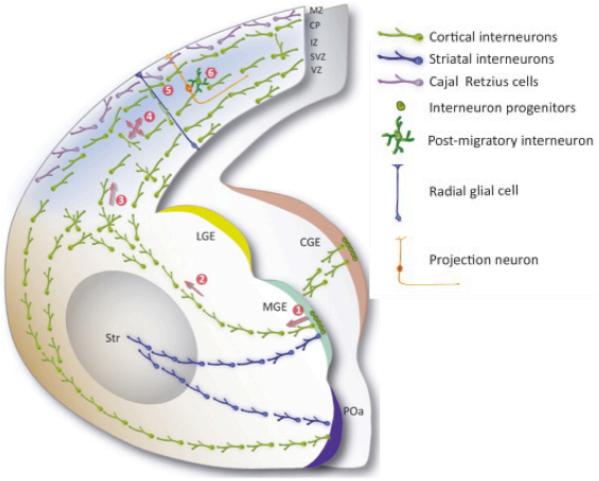

Figure 6: Migration streams in the developing forebrain: 1 Exit of proliferation site, 2-4 selection of migration stream and orientation, 5 identification of final destination, 6 termination of migration. CGE: caudal ganglionic eminence, CP: cortical plate, LGE: lateral ganglionic eminence, MGE: medial ganglionic eminence, MZ: marginal zone, IZ: intermediate zone, POa: preoptic area, SVZ: subventricular zone, VZ: ventricular zone. Modified from (Guo & Anton, 2014).

Migration onset is initiated by the chemorepellent ephrin-5, which is expressed in the ventricular zone (VZ) of the ganglionic eminence (GE) and signals through its receptor EphA4 expressed in newborn cells, consequently downregulation of EphA4 leads to an infiltration of the VZ (Zimmer *et al.*, 2008). Once released from the VZ mitogenic factors hepatocyte growth factor (HGF) and the glial cell derived neurotrophic factor (GDNF) were found to be required for migration, as a lack of these genes lead to migration deficits (Powell *et al.*, 2001; Pozas & Ibanez, 2005). Interestingly, MGE derived cells express the neurotrophin receptor TrkB that binds to brain derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4). Disruption of BDNF signaling reduced the number of interneurons, indicating at least a modulating function of neurotrophins on interneuron motility (Polleux *et al.*, 2002; Guo & Anton, 2014) as it was later shown that neurotrophoic factors are dispensable or may only affect specific interneuron subpopulations (Marin, 2013).

During migration chemoattractant and chemorepellent factors provide interneurons with direction cues. MGE derived interneurons avoid entering the preoptic area (POa) and striatum, due to region specific expression of chemorepellents. Ephrin B-3 expressed in the POa interacts with its EphA4 receptor in MGE cells, repelling them and keeping them on the right path to the cortex. This is accompanied by secretion of the chemorepulsive factors class III semaphorins (Sema3A, Sema3F) in the striatum, with its receptor neuropilin expressed in MGE cells (Rudolph *et al.*, 2010; Hernandez-Miranda *et al.*, 2011). Consistently, cells targeting the striatum do not express those receptors. Additionally, the transcription factor Nkx2.1 is downregulated to enhance the repellent function of Sema3A/ Sema3F, consistently, high levels of Nkx2.1 reduce the repellent function of Sem3a, and cells infiltrate the striatum (Nobrega-Pereira *et al.*, 2008; Guo & Anton, 2014).

Neuregulin-1 acts together with its receptor ErbB4 as the only identified chemoattractant, which attracts MGE derived cells to the neocortex (Flames *et al.*, 2004). Moreover, migrating interneurons become responsive for neurotransmitters, with increased sensitivity for GABA and glutamate when leaving the subpallium to the neocortex, with a dopamine 1 receptor activating migration and with glycine controlling nucleokinesis (Guo & Anton, 2014). Interstingly, GABA and glutamate depolarize cells in the post-mitotic cortex through their receptors, leading to a Ca^{2+} transient which keeps cells migrating (Brandao & Romcy-Pereira, 2015). Once in the neocortex the chemoattractant CXCL12 - expressed in the MZ and SVZ - keeps the MGE interneurons in the migration stream, but only if they express both CXCL12 receptors (CXCR4/CXCR7). The responsiveness to CXCL12 is downregulated when interneurons perform the final step of migration – a switch from tangential to radial

migration. As the radial migration is perturbed in the absence of the adhesion protein Connexin 43, it is believed that radial glia interneuron interaction is required additionally for the transition (Guo & Anton, 2014). The final placement may depend on interaction with pyramidal cells and neuonal activity. With the expression of the potassium chloride channel KCC2, only few hours after the cells reach the cortex, GABA becomes hyperpolarizing which in turn inhibits the motility of cells (Brandao & Romcy-Pereira, 2015). During maturation and migration interneurons express different combinations of transcription factors believed to be involved in interneuron specification.

1.6.2.2 Interneuron specification

Two mechanisms are important for cell fate specification, the intrinsic molecular cues, which are the expression of distinct sets of transcription factors in distinct groups of interneuron precursors, responsible for the early commitment to a specific neuronal fate, and the extrinsic cues provoked by the cell environment such as neural activity (Cobos *et al.*, 2006). The origin of SST+ cells, the dMGE, is enriched in Nkx6.2 and Gli, whereas the major source of PV+ cells the vMGE showed an enrichment in Dlx5/6 and Lhx6. Furthermore, in the early MGE, Nkx2.1 promotes specification of SST+ and PV+ cells. During that stage sonic hedgehog (Shh) signaling is stronger in the dMGE than in the vMGE favoring SST+ cell development (Fogarty *et al.*, 2007). Consistently, high levels of Shh signaling were found to support SST fate acquisition (Xu *et al.*, 2010). Loss of Nkx2.1 on the other hand leads to a fate shift from MGE cells to LGE cells, reducing the number of cortical interneurons by more than 50% (Sussel *et al.*, 1999).

As a direct target of Nkx2.1, Lhx6 expression is induced and coordinates intracellular processes required for the specification of both cell types. Deletion of Lhx6 leads to a loss of almost all PV+ and SST+ cells in the cortex besides a small number of scattered cells in the deep layers, which were later identified as potentially POa derived PV+ cells (Liodis *et al.*, 2007; Brandao & Romcy-Pereira, 2015). Lhx6 expression is maintained in mature cells, and is exclusively found in PV+ and SST+ cells (Fogarty *et al.*, 2007; Liodis *et al.*, 2007). Sox 6 (and Satb1) acts downstream of Lhx6 and is required to generate the accurate number of PV+ and SST+ cells and involved in their placement and maturation (Batista-Brito *et al.*, 2009). Moreover, the Dlx homeobox genes are widely expressed in postmitotic and mature cells. Dlx5 or Dlx5/6 knockout mice exhibit specific loss of PV+ cells in the cortex, whereas the deletion of Dlx1 reduces SST but not PV expressing cells.

Most characteristics of the interneuron subtypes are only present in a later postnatal brain. Additionally, immature PV and SST cells express similar transcription factors, which hinder the identification and distinction of immature PV and SST cells (Fig. 7). They only become neurochemically distinguishable when the cells undergo the final step of differentiation and start to express PV around P7 or SST from P0 on (Sanchez *et al.*, 1992; Lee *et al.*, 1998; Hof *et al.*, 1999). Given the similar origin, migration path and expression of transcription factors it is astonishing that only PV+ cells are affected in the TRα1+m mice.

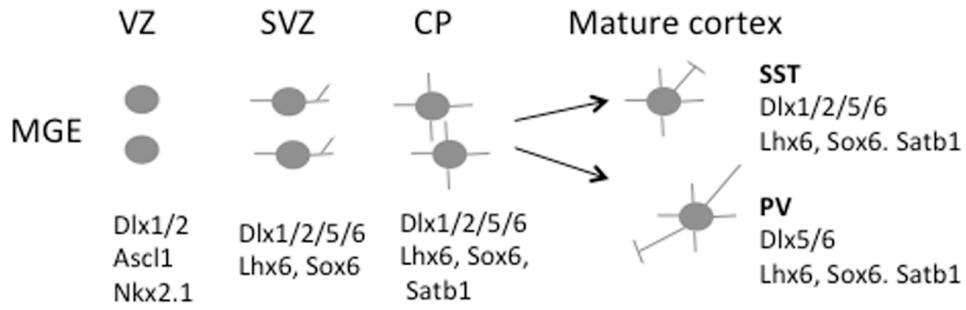

Figure 7: Expression of transcription factors through out maturation of MGE derived SST+ and PV+ cells. Note the identical set of transcription factors expressed by both subpopulations. VZ: ventricular zone, SVZ: subventricular zone, CP: cortical plate. Modified from (Cauli *et al.*, 2014).

1.6.2.3 Neuronal activity

The maturation of GABAergic cells is modulated by the activity of their postsynaptic partners and by the expression of activity dependent growth factors (Patz *et al.*, 2004). Consequently dark reared animals exert reduces PV expression and reduced GABAergic inhibition in the visual cortex, with the latter improving drastically by visual input (Morales *et al.*, 2002; Tropea *et al.*, 2006).

In the cortex neuronal activity depends on synaptic transmission, neuronal connectivity and proper pH levels. Excitation leads to the release of glutamate and dopamine from the presynaptic cell. These neurotransmitters bind in case of inhibition to its receptors on inhibitory interneurons, which in turn release GABA. GABA than binds to its postsynaptic receptors where it initiates hyperpolarization by opening Cl^- and K^+ channels, leading to a negatively charged postsynaptic cell (hyperpolarization). The change in the electrochemical gradient is accompanied by an extracellular alkaline shift caused by an net influx of CO_2 and HCO_3^- efflux through the $GABA_A$ receptor gated Cl^- channel leading to postsynaptic acidosis

(Kaila & Voipio, 1987; Chesler, 2003; Sinning & Hubner, 2013). Interestingly, breathing of 5% CO_2 inhibits seizures, most likely due to an enhanced inhibition caused by increased intracellular acidosis (Pavlov *et al.*, 2013). Like inhibition, excitation leads to an extracellular alkaline shift which is HCO_3^- independent, but arises due to an extracellular H^+ sink (Chesler & Kaila, 1992). This is accompanied by a Na^{2+} influx that depolarizes the postsynaptic cell.

The alkaline shift in both cases is buffered by carbonic anhydrases, which hydrate CO_2 and dehydrate HCO_3^- ($CO_2 + H_20 \leftrightarrow HCO_3^- + H^+$). The membrane bound carbonic anhydrase 4 and 9 (Car4, Car9) buffer the extracellular alkaline shifts. Knockout studies demonstrated the ability of both to substitute for each other, whereas a lack of both isoform led to a stronger extracellular alkalization (Huang *et al.*, 1995; Shah *et al.*, 2005). Additionally, Cars also influences synaptic transmission by buffering H^+, which was shown to modulate the glutamate NMDA receptor activity; consequently a lack of Cars increases intracellular H^+ and reduced NMDA receptor response (Taira *et al.*, 1993).

1.7 TR DISTRIBUTION IN THE BRAIN

The importance of TRs in brain development was described decades ago. Unfortunately, a lack of reliable antibodies against the TR moiety made it impossible to dissect the cell specific expression of TRs in the brain. Alternative methods were used to create a rather vague picture of spatial and temporal TR expression. Briefly, TRα1 is the predominant TR isoform in the brain where it is ubiquitously expressed and accounts 70-80% of total T3 binding (Schwartz *et al.*, 1992; Ercan-Fang *et al.*, 1996). In particular TRα1 was present in areas such as cortex, hippocampus, cerebellum and amygdala in postnatal to adult rats, with a peak in the first three weeks of life and an expression onset at E5 in chicken and E14 in rat (Forrest et al, 1996a; Mellstrom et al, 1991). In contrast, TRβ expression was restricted to selected rather mature neuronal subpopulations. Consistently only low levels were detected prenatally but increased during postnatal development, with expression in hippocampal granule and pyramidal cells, cerebellar Purkinje cells and paraventricular hypothalamic neurons (Horn & Heuer, 2010).

1.7.1 Effects of TRα1 alteration on brain structure and function

In the following years the role of TRα1 in brain development and function was studied in several brain regions. The focus was on the cerebellum with an import role in motor control,

the hippocampus involved in memory consolidation and space as well as the motor cortex, which plans, controls and executes voluntary movements.

1.7.1.1 Cortex

The neocortex regulates higher function such as motor commands, conscious thoughts and language. In hypothyroidism the number of PV+ terminals in the neocortex is strongly reduced (Berbel *et al.*, 1996). Wallis et al. demonstrated that the dominant negative mutation in TRα1 (TRα1+m) affects the development of GABAergic interneurons. In particular, the number of PV expressing interneurons in the motor cortex of TRα1+m mice were significantly reduced in P14 mice but normalized in adulthood, whereas calretinin expressing cells were increased in juvenile and adult mice. Consistently, staining for GAD67, an enzyme expressed in GABAergic cells, revealed no alteration in cell number. However, PV+ cell development was rescued by high doses of TH from around birth on, whereas high TH levels during embryogenesis did not improve PV+ cell development.

Behavior test revealed locomotor disabilities and poor performance in the hanging wire test, but normal muscle development. These defects improved by both embryonic (E10.5 – E13.5) and postnatal TH treatment (Wallis *et al.*, 2008).

1.7.1.2 Hippocampus

The functionality of the rodent hippocampus can be tested by several behavior tests targeting memory formation. Contextual fear experiments engage the hippocampus and amygdala, whereas the cued fear test depends on the integrity of the amygdala and lastly, the novel object recognition test, which measures hippocampus dependent visual memory (Guadano-Ferraz *et al.*, 2003).

As the deletion of TRα1 lead to less exploratory behavior in open field experiments and a higher freezing response in contextual fear experiments - but not in the cued fear test - it was suggested that TRα1 plays a crucial in hippocampal function. On a neuronal level, fewer GABAergic terminals on pyramidal cells in the CA1 region of the hippocampus accompany the described behavior phenotype. These findings indicate an involvement of TRα1 in the development of inhibitory circuits in the hippocampus and, hence, a modulating function on creating emotions and hippocampal memory (Guadano-Ferraz *et al.*, 2003). In 2005, Venero

and colleagues used the TRα1+m mouse strain to understand the role of the aporeceptor in brain development and function. They discovered anxiety and reduced hippocampus-dependent visual recognition memory in adult mutant mice, which they connected to a reduced number of PV+ perisomatic terminals in the CA1 region of the hippocampus. All defects were ameliorated with TH treatment in adulthood (Venero *et al.*, 2005). Additionally, TRα1+m mice showed reduced seizure susceptibility to chemically induced seizures, accompanied by hyperpolarization and reduced cell activation upon seizure induction. These findings were reversed by TH treatment during both embryonic and postnatal development. Histological analysis of brain sections revealed increased numbers of inhibitory calretinin cells in the hippocampal hilus, suggesting a stronger inhibition or reduced excitability of the hilus leading to reduced seizure susceptibility (Hadjab-Lallemend *et al.*, 2010).

1.7.1.3 Cerebellum and hypothalamus

Congenital hypothyroidism is associated with cerebellar misdevelopment and locomotor disabilities. Hypothyroidism in wildtype but not in TRα1 knockout mice caused a delayed postnatal migration of external granule cells to the internal granular layer of the cerebellum and arrested development of Purkinje cells, suggesting an involvement of the apo-receptor in inhibiting cell migration and partly Purkinje cell maturation (Morte *et al.*, 2002). This hypothesis was confirmed by using TRα1+m mice, which showed a delayed migration of external granule cells and a minor delay in Purkinje cell maturation accompanied by locomotor impairment on the Rotarod. Thyroid hormone treatment in adulthood improved the neurological phenotype, whereas postnatal treatment was necessary to ameliorate the locomotor alterations (Venero *et al.*, 2005). Additional immunohistological studies identified an overall delay of PV, calbindin and calretinin expressing interneurons in TRα1+m, which normalized at around P9 (Wallis *et al.*, 2008). To elucidate whether T3 acts directly or indirectly on cell maturation a mouse model with a floxed mutant TRα1 was used to express the dominant negative receptor selectively in different cell types (Fauquier *et al.*, 2014). This study identified a cell autonomous affect of TRα1 on Bergman glia and Purkinje cell development, which in turn modulates granule cell migration. The expression of a dominant negative TRα1 in Purkinje cells, Bergman glia and interneurons increased the amount of immature GABAergic cells, and reduced the number of mature PV+ cells and GABAergic synapses in juvenile mice. Further studies revealed the influence of Purkinje cells on interneurons during early postnatal development whereas Bergman glia affected interneuron

maturation in a later stage. However, as no interneuron specific mouse line was studied, a cell autonomous affect of TRα1 on interneuron development could not be excluded (Fauquier *et al.*, 2014).

Additionally, in the hypothalamus a reduced expression of PV+ cells was identified in TRα1+m mice, which could not be rescued by TH treatment in adulthood or postnatally. The additional deletion of TRβ decreased their number even further, suggesting the importance of a proper TRα and TRβ signaling during embryogenesis (Mittag *et al.*, 2013).

Taken together, all examined brain regions of TRα1+m mice revealed disturbed PV+ cell development, with the majority being rescued by TH treatment.

2 AIMS

The overall purpose of my PhD thesis was to identify the role of TRα1 in interneuron development by determining TRα1 expression in the tissue, identifying novel target genes and unraveling a possible mechanism by which TRα1 affects parvalbumin+ cell development. The specific aims of my thesis were to:

1. identify the spatial and temporal expression of TRα1 in the developing and adult brain by analyzing a mouse strain expressing a chimeric TRα1-GFP protein;
2. establish a method to reliably detect TRα1 target genes using the TRα1-GFP mouse strain;
3. characterize Carbonic anhydrase 4 (Car4) as novel TRα1 target gene in the brain; and
4. unravel the molecular mechanism underlying the delayed appearance of parvalbumin expressing interneurons in TRα1+m mice.

3 RESULTS

3.1 TRα1 EXPRESSION IN THE DEVELOPING AND MATURE BRAIN - PAPER I

Several studies have described the importance of proper TRα1 activity in the brain and identified cell types depending on TRα1. Wallis et al. describe a delayed appearance of cortical GABAergic PV+ cells in mice with a dominant negative TRα1 (Wallis *et al.*, 2008); a similar delay was described in the cerebellum (Venero *et al.*, 2005; Fauquier *et al.*, 2014). However, the lack of reliable TRα1 antibodies prevented the proper identification of TRα1 expressing cell types and hence, hampered the understanding of molecular mechanisms causing the interneuron phenotype.

Generation of mice and phenotype analysis

In order to determine the temporal and spatial expression of TRα1 in the brain, we generated a transgenic mouse line carrying a GFP sequence in the reading frame of *Thra*. The sequence was inserted in frame 3' to exon 9 of *Thra*, resulting in the expression of a chimeric TRα1-GFP protein from the endogenous *Thra* locus. Since the expression is regulated by the endogenous promotor, the chimeric protein is generally expressed where and when TRα1 is normally activated. The functionality of the chimeric protein was first tested in transfection assays, where it showed a slightly reduced ability to suppress target gene expression. This was later confirmed in heart tissue of homozygote TRα1-GFP ($TR\alpha1^{gfp/gfp}$) mice that revealed increased expression of the negatively regulated target gene MyHCβ (cardiac muscle myosin heavy chain beta), whereas the positively regulated MyHCα (Dillmann, 1990) was unaltered. These findings indicated a somewhat reduced suppression of transcription in the homozygote mice but normal gene activation.

We further analyzed the *Thra* transcripts after GFP introduction into the mouse genome. Northern blots revealed the expected increase of *Thra* transcript size, but it also showed a reduction of TRα2 expression in the $TR\alpha1^{+/gfp}$ mice and a loss of the TRα2 transcript in the homozygote mice, accompanied by an increased expression of the TRα1-GFP. These results were confirmed with qPCRs. As the knock-in led to a decreased transcriptional suppression and abolished TRα2 levels we tested physiological and behavioral parameters. We found small variations in weight development of heterozygote male mice, which normalized in adulthood. Eye opening as parameter for development was unaltered. Heart and brain size

were unchanged whereas the liver size of TRα1$^{gfp/gfp}$ mice was decreased. To exclude a functional deficit in the liver caused by the TRα1-GFP we examined liver deiodinase 1, which is regulated by TH. No difference was observed, indicating no TH mediated functional deficit in the liver.

Since our study focused on TRα1 in the brain we examined the T3 regulated genes RC3 and hairless (Thompson & Bottcher, 1997; Martinez de Arrieta *et al.*, 1999) in the developing brain and could not find any alteration. Also the expression of the corepressors Ncor and Alien were unchanged in the juvenile brain. Adult mice behavior was than tested in SHIRPA where no alteration was observed.

In summary, no differences could be detected in the brains of TRα1-GFP mice compared to wildtypes. TH target gene expression was not effected and the behavior of TRα1-GFP mice did not show any abnormalities. Since we observed a decreased repression of genes in the homozygotes we used heterozygote mice for TRα1 expression studies.

TRα1 expression in the mature brain

In order to characterize TRα1 expression in the brain we used anti-GFP antibodies to detect TRα1-GFP in coronal brain sections. Immunohistochemistry revealed exclusively nuclear TRα1 expression in the neocortex, striatum, hippocampus, hypothalamus and cerebellum, supporting previous findings (Mellstrom *et al.*, 1991; Bradley *et al.*, 1992). It was not detected in white matter, indicating a major expression in neurons.Colocalization for TRα1-GFP and the neuronal marker NeuN confirmed expression of TRα1-GFP in virtual all neurons of the adult brain, with the exception of Purkinje cells in the cerebellum (see below). Additionally, TRα1-GFP colocalized with parvalbumin and calretinin expressing interneurons, cell populations diversely affected in mice harboring a mutation in TRα1 (Wallis *et al.*, 2008). In glial cells TRα1 was detected in tanycytes lining the third ventricle of the hypothalamus, which constitute an important barrier between cerebrospinal fluid and hypothalamus, and in mature oligodendrocytes of the hypothalamus. Earlier observations proposed a TH dependent maturation of oligodendrocytes through a sequential expression of TRβ and TRα (Billon *et al.*, 2001; Billon *et al.*, 2002), which could be supported by our finding.

TRα1 in the cerebellum

In the cerebellum TRα1 was absent in mature Purkinje cells, but showed transient expression in immature Purkinje cells at P7, which is in line with previous observations (Heuer & Mason, 2003). Adjacent to Purkinje cells we identified parvalbumin expressing stellate/ basket cells that were positive for TRα1.

The influence of TH on granule cell proliferation and migration was described previously. The apo-TRα1 was associated with a delayed migration onset of the granule cells of the external granular layer (Morte *et al.*, 2002; Venero *et al.*, 2005). In our study, TRα1 was absent in proliferating cells of the external granular layer but appeared in migrating cells in the molecular layer. Moreover, GFAP (Glial fibrillary acidic protein) positive glial cells of the granular layer expressed TRα1.

TRα1 in the developing cortex

We further investigated the expression onset of TRα1 during neuronal development. At E9.5 TRα1 was not detected in the developing brain but appeared at E13.5 in the cortical plate and marginal zone, whereas proliferation sites were negative for TRα1 expression. TRα1 cells were identified with β-tubIII as immature postmitotic neurons. This is in line with our observations in the cerebellum, where TRα1 was found in migrating cells of the molecular layer, but was absent in the proliferation site (external granular layer). TRα1 was detected in the cortical plate throughout development, with a stronger expression in the deeper layer where cells have already matured. After finalized cortical lamination, TRα1 showed even expression throughout the layers.

Taken together, the TRα1-GFP mouse enabled us to detect TRα1 protein expression in the brain for the first time: from E13.5 TRα1 is expressed in postmitotic cells and continues to be expressed in virtually all neurons of the adult brain except for Purkinje cells, indicating a role in cell maturation and maintenance. Moreover, TRα1 is expressed in tanycytes and mature oligodentrocytes of the hypothalamus.

3.2 IDENTIFICATION OF TRα1 TARGET GENES - PAPER II

The TRα1-GFP mouse strain was a valuable tool to detect TRα1 expression on a cell level in the brain. We hypothesized that this mouse line could also be used to identify TRα1 target genes with chromatin immunoprecipitation (ChiP), as our mouse line enabled us to circumvent the need for a TRα1 antibody by using GFP antibodies. We then successfully establish a protocol to use the TRα1-GFP mouse strain in ChIP assays in order to identify TRα1 target genes.

ChIP assay and method validation

Protein-DNA interaction can be detected with chromatin immunoprecipitation. Briefly, tissue is incubated in PFA to cross-link DNA and attached protein, followed by formaldehyde quenching and homogenization of tissue. Cells are lysed, sonicated to break DNA in smaller pieces (500-1000kb) and the ChIP DNA is incubated in an antibody solution. The DNA-protein-antibody complex is then immunoprecipitated (pull-down) with protein G-coated magnetic beads. After heat induced reverse crosslinking and protein digestion, the enrichment of the specific DNA sequence in ChIP DNA compared to untreated input DNA is verified with qPCR and normalized to background noise in wt ChIP and the corresponding input DNA.

ChIP assays were performed on TRα1$^{gfp/gfp}$ brain and heart tissue using a GFP antibody and wildtype chromatin as control. We investigated previously described TREs in hairless (Thompson & Bottcher, 1997) and RC3 (Martinez de Arrieta *et al.*, 1999) to validate our system and included genomic DNA without TREs as negative control. We observed TRα1-GFP binding to RC3 and hairless TREs whereas the negative control did not produce any pull-down.

Next we investigated the heart as an additional TRα1 target organ. We identified TREs in the promotor regions of the TH regulated genes Sema3a (Semaphorin 3a), HCN2 and HCN4 (hyperpolarization-activated cyclic nucleotide-gated ion channel 2 and 4) (Mittag *et al.*, 2010a), which bound to TRα1 in the assay. Electrophoretic mobility shift assay (EMSA) confirmed the binding ability of the previous described and the majority of novel identified TREs as heterodimer with RXR to TRα1 and TH. We further analyzed the functionality of TREs in luciferase assays after cotransfection of cells with TRα1 and a TRE containing

plasmid (Hofmann *et al.*, 2009). All TREs increased luciferase expression upon TH treatment, independent of their localization in the promotor.

Identification of novel TH target genes

We aimed to identify new target genes by cloning the sheared chromatin precipitate. Sequencing of random clones revealed RNF166 (ring finger protein 166) as possible candidate. A putative TRE was identified in the 3'UTR (untranslated region) downstream of the promotor region and ChIP assay revealed binding of TRα1 to the TRE. EMSA confirmed the binding ability, as the TRE bound monomeric or as heterodimer with RXR to TR. The TRE was than cloned into a promotor vector for luciferase assay where it induced luciferase expression upon TH treatment. Lastly, qPCR revealed a reduced expression of RNF166 mRNA upon TH treatment.

Protein-DNA interaction in hypo and hyperthyroid mice

It is hypothesized that TRs are bound to DNA in absence and presence of ligands. We addressed this question and treated animals either with an anti-thyroid drug (methiamazole) to reduce their TH levels to a hypothyroid state or injected TH to increase TH levels. Subsequent ChIP assay revealed no effect of ligand availability on TRα1-DNA binding when compared to the euthyroid mice, supporting the hypothesis of a steady binding of TRα1 to DNA.

Taken together animal models expressing a GFP tagged transcription factor are an excellent tool to analyze DNA-protein interactions *in vivo* and can be used to identify new target genes.

3.3 CAR4: A NOVEL TRα1 TARGET GENE - PAPER III

Equipped with the established ChIP assay we were eager to identify additional TRα1 target genes. Carbonic anhydrases 4 (Car4) regulates together with other Car isoforms the activity dependent pH shift in GABAergic cells and is involved in synaptic transmission (Urbanics *et al.*, 1978; Tong *et al.*, 2000). Moreover, Car4 is expressed in the kidney and lung, which functions are closely related to proper TH signaling. These made Car4 an interesting candidate for further investigation.

Car4 expression in brain, kidney and lung

In situ hybridization studies for Car4 showed a reduced expression in the cortex and thalamus of the TRα1+m mice, which slightly increased upon TH treatment, indicating an early established defect on Car4 expression. Similar to what was found in the brain, Car4 mRNA expression was also reduced in the lung of TRα1+m mice and did not recover upon TH treatment. The TH treatment efficiency was verified by additional qPCRs for the TH regulated genes surfactant protein A and serpina 3G, which showed the expected regulation (Ramadurai *et al.*, 1998; Barca-Mayo *et al.*, 2011). Since Car4 is involved in the regulation of pulmonary CO_2 (Fleming *et al.*, 1993), we measured respiratory rate and oxygen saturation as readout for *in vivo* lung function. None of them were affected in the mutant mice; however, TH treatment increased the respiratory rate in both genotypes. Analysis of mRNA levels of other Cars did not show a difference between genotypes, but TH treatment led to increased expression of Car2 and Car14 in the mutant mice.

In the kidney Car4 is involved in renal regulation of pH levels and bicarbonate reabsorption (Purkerson & Schwartz, 2007). Here, its expression was not affected by the apo-TRα1 in the mutant mice, but was down-regulated on the RNA and protein levels when treated with TH in both genotypes, indicating a TH dependent repressive regulation of Car4 transcription probably mediated by TRβ. Deiodinase1 served as treatment control and showed the expected increase induced by TH treatment. However, the reduced Car4 levels did not influence pH levels in urine, which prompted us to investigate other Cars. Indeed, Car7 and Car15 were induced upon TH treatment, probably to compensate for the suppressed Car4 or as result of transcriptional activation by TH.

Car4 a new target gene for TRα1

We identified four putative TREs in the promotor region of Car4. Three of them showed enriched TRα1 binding in the ChIP assay and one of them demonstrated the expected transcriptional repression upon TH treatment in luciferase assay. In the same setting a plasmid containing the specific TRE with mutated bases to alter TRα1 binding, failed to regulate luciferase activity, whereas a plasmid upgraded to contain a perfect TRE upregulated luciferase activity. With this experiment, we identified Car4 as a novel negatively regulated TH target gene by a TRE in position -2385 relative to the translation start site.

Taken together, we identified tissue specific Car4 regulation by TH. In tissues predominately expressing TRα1 (brain and lung), we discovered a permanent repression of Car4, whereas the kidney predominately expressing TRβ showed acute TH regulation leading to suppressed Car4 expression upon TH treatment. The latter is of great interest, as Car4 can be used as an additional novel marker for TH status in the kidney.

3.4 TRα1 ACTION IN INTERNEURON DEVELOPMENT - PAPER IV

A previous publication from our lab described a delayed appearance of a GABAergic cell subpopulation in the cortex of TRα1+m mice. In particular, the number of parvalbumin (PV) expressing interneurons was decreased at P14 but their number normalized in adulthood (Wallis *et al.*, 2008). Somatostatin (SST) cells, which undergo a similar migration and developmental path and express the same transcription factor as PV+ cells (Marin & Rubenstein, 2003), were not affected by the dominant negative receptor. In **paper I** we showed that TRα1 is expressed in PV+ interneurons as well as in immature neurons. In this project, we investigated whether the mutation affects migration, survival or differentiation of PV interneurons and used ChIP assays to characterize possible target genes. The animals were studies at P7, when immature PV cells start to undergo their final step of maturation and at P14 when the delayed appearance of PV+ cells becomes apparent.

Identification of immature PV cells in the cortex

First, we analyzed mRNA levels with qPCR of markers expressed in PV+ and SST+ cells. BMPR1a and 1b showed no alteration between the genotypes at both time points. The same result was found for NMDA receptor 1 as well as for ERbB4, which expression is restricted to 85% of PV+ cells and 15% of SST+ cells. We next examined the expression of the PV+ cell exclusive marker Kv3.1b, which revealed no difference between the genotypes. From that we concluded that the immature PV+ cells reach the cortex but fail to undergo the final step of differentiation, which is associated with the onset of PV expression.

Analysis of transcription factors

In a next step we investigated the transcription factors Dlx5 and Lhx6, both needed for PV+ cell differentiation. QPCR revealed upregulation of both genes in the mutant mice at P7,

which normalized at P14. Subsequent ISH for the PV and SST specific transcription factor Lhx6 at P7 and P14 showed no alteration between the genotypes and quantification of Lhx6 expressing cells revealed a similar number of Lhx6 positive cells in the cortex of both genotypes. ISH for Dlx5 at P7 confirmed the increases expression. Lastly, we determined PGC1α expression, a modulator for PV expression, which did not show alteration between the genotypes.

These results supported the idea that the immature PV cells reach the cortex but fail to differentiate. Additionally, the increased mRNA levels of Dlx5 and Lhx6 at P7 might indicate a compensatory effect for the delayed differentiation.

Neuronal activity

The expression of PV is modulated by neuronal activation (Patz *et al.*, 2004). Neuronal activity depends among others on the availability of neurotransmitters. Consequently we analyzed the protein levels of the inhibitory neurotransmitter GABA and the excitatory neurotransmitters dopamine and glutamate. ELISAs revealed no difference between the genotypes. In the following we wanted to increase the cortical activity by injecting the convulsant drug PTZ (pentylenetetrazol). PTZ binds to the $GABA_A$ receptor and initiates depolarization by increasing calcium and sodium influx and hence increases excitation. This in turn leads to release of glutamate, which acts on inhibitory interneurons (Huang *et al.*, 2001). Immunohistochemistry for PV revealed a loss of PV+ cells in the mutant mice compared to untreated mutants, whereas wt mice were unaffected. From that we concluded that the excitatory/ inhibitory system is not fully developed and that the increased excitation might have induced cell death.

Dominant negative TRα1 affects the expression of neurotrophin-3

Neurotrophins are crucial players in interneuron development and especially BDNF was shown to regulate PV+ cell differentiation in an activity dependent manner (Patz *et al.*, 2004). We analyzed mRNA levels of neurotrophin receptors TrkB, TrkC and p75 and their ligands BFND, NGF, NT-3 and NT-4/5. TrkC, p75, BDNF, NGF and NT-4/5 were unaffected in the mutants at both time points, whereas TrkB expression was significantly elevated at P14 in the mutant cortex, whereas NT-3 levels were significantly reduced in this animals. Western blot analysis revealed reduced NT-3 protein levels also at P7. We then investigated whether the

NT-3 genomic sequence contained TRE sequences. We identified two putative TREs in the promotor region and subsequent ChIP assay revealed TRα1 binding to a TRE located -606 relative to the translation start site. Next we analyzed the expression of downstream genes of NT-3 and identified a reduced phosphorylation of AKT (phosphoAkt) and a slightly but not significant reduction of MEK1/2 phosphorylation, both indicating altered intracellular signaling caused by the mutant TRα1.

In summary, marker genes for PV+ and SST+ cells were similarly expressed in juvenile wt and TRα1+m mice. Together with the unaltered number of Lhx6 cells in mutant mice we concluded, that immature PV cells reach the cortex, but do not undergo the final step of maturation. We further observed a reduced expression of NT-3 and phospoAKT in the mutant animals and identified a TRα1-TRE interaction in the NT-3 promotor region, establishing NT-3 as a novel TRα1 target gene.

4 DISCUSSION

The delayed appearance of PV+ cells in the cortex of TRα1+m mice was the center of my PhD thesis. In the following I discuss my results regarding neuronal maturation including proliferation, migration, signal transductions regulated by neurotrophins and cortical activity. Lastly, I discuss our findings regarding target gene regulation and TREs.

4.1 PROLIFERATION

TRα1+m mice exert a reduced number of PV expressing cells in the juvenile cortex and a prolonged proliferation in the external granular layer of the cerebellum (Venero *et al.*, 2005). Hence, we investigated whether TRα1 modulates proliferation. However, TRα1 was absent in proliferation sites of the developing brain including the medial ganglionic eminence where interneurons are born and proliferate (Marin & Rubenstein, 2003). This was in line with our observation in the cerebellum, where TRα1 was not detectable in the external granular layer were cells undergo proliferation. However, an involvement of TRα1 in proliferation was described earlier in intestinal epithelia cells and optic nerve oligodendrocyte precursor (Billon *et al.*, 2002; Plateroti *et al.*, 2006). These diverse findings might be explained by the tissue specific functionality of TRs, which was described earlier for hairless and RC3. In the brain hairless is responsive to TH whereas in the skin hairless is not inducible by TH (Engelhard & Christiano, 2004). Additionally, TH regulates the expression of the TRα1 target gene RC3 in a temporal and spatial dependent manner, which was associated to region specific trans-acting elements (Guadano-Ferraz *et al.*, 1997; Chatonnet *et al.*, 2015).

In summary, form out data it seems that TRα1 is not involved in cortical interneuron and external granular layer proliferation, whereas the proliferation of other cell types is affected by TRα1. This can be explained by a cell specific functionality of TRα1.

4.2 INTERNEURON MIGRATION IN THE DEVELOPING CORTEX AND CEREBELLUM

As TRα1 was detected in migrating cells of the developing cortex as well as in migrating granule cells of the cerebellum (**paper I**), we investigated whether TRα1 influences interneuron or granule cell migration.

In the developing brain TRα1 is expressed in tangentially migrating cells (**paper I**), which is the preferred migration route of immature PV and SST interneurons (Marin & Rubenstein, 2003). Interestingly, we did not detect an interneuron specific migration failure in TRα1+m mice, as the number of cells expressing the PV+ and SST+ cell exclusive marker Lhx6 was unaltered in the juvenile brain (**paper IV**). This is supported by the unaffected PV+ cell migration into the somatosensory cortex of hypothyroid rats (Morte *et al.*, 2002; Auso *et al.*, 2004). These observations raise the possibility that the unliganded TRα1 is necessary for proper migration. In line with this hypothesis, only recently it was postulated that during embryogenesis before the onset of endogenous TH production the transcriptional repression of the unliganded TRα1 is of greater importance than during postnatal development (Yen, 2015). This would explain the normal migration of interneuron in TRα1+m mice and during hypothyroidism. Moreover, it would be in line with rescuing the delayed appearance of PV+ cells by TH treatment from birth on, when the liganded receptor gains more importance (Wallis *et al.*, 2008).

In the postnatal cerebellum cells proliferate in the external granular layer (EGL) before they start to migrate postmitotically through the molecular layer into the internal granular layer (in adults referred to granular layer). As mentioned above this event is delayed in TRα1+m mice (Venero *et al.*, 2005). However proliferation seems not affected by TRα1, hence, we were wondering if TRα1 was involved in EGL cell migration. TRα1 was expressed in migrating cells as well as in maturing Purkinje cells. As granule cells are the presynaptic partners of Purkinje cells it can be speculated that the disturbed granule cell migration in TRα1+m is due to failures in Purkinje cell development caused by the mutant TRα1. Although Purkinje cells showed no obvious alterations in TRα1+m mice, synapses have not been tested, leaving room for speculations (Venero *et al.*, 2005). Recent studies supported the importance of proper TRα1 activity for Bergman glia and Purkinje cell development, that in turn affects granule cell migration (Fauquier *et al.*, 2014).

Taken together, in the cortex the unliganded TRα1 does not interfere with proper migration, as interneurons reach the cortex in hypothyroid animals as well as in TRα1+m mice. From these data we concluded that immature PV cells reach the cortex but are prevented to perform the final step of maturation. Further investigations are needed to quantify the number of immature PV cells in the mutant and wildtype animals. That can be achieved by combined in situ hybridization and immunohistochemistry for Lhx6, SST and PV on wt and TRα1+m section.

In the contrary, in the cerebellum it is suggested that TRα1 acts via Purkinje cells in early development to regulate granule cell migration.

4.3 NEUROTROPHINS

We could show that immature PV cells reach the cortex in TRα1+m mice but their differentiation was blocked by the mutant TRα1. Interstingly, when different factors involved in PV+ cell differentiation were investigated, only neurotrophin-3 (NT-3) was found dysregulated in the mutants (**paper IV**). This is line with previous observations, showing that NT-3 expression is regulated by TH *in vivo* and *in vitro* (Lauterborn *et al.*, 1994; Neveu & Arenas, 1996). However, to date it is not clear if the reduced expression of NT-3 is involved in the delayed PV+ cell maturation.

In general, neurotrophins regulate via receptor activation the development of interneurons. Neuronal growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) are the members of the neurotrophin family and activate receptor tyrosine kinases (TrkA, TrkB, NT-4/5 and TrkC) and the neurotrophin receptor p75 (Fig. 8).

Figure 8: Neurotrophins and their receptors. Black arrows: high affinity receptors, grey arrows: lower affinity.

Ligand binding leads to transphosphorylation of the Trk receptors and to the activation of one of the signaling pathways involved in e.g. cell differentiation (PI3K- MAP kinase pathway), cell survival and growth (PI3K - Akt pathway) or synaptic plasticity (plcɣ1 pathway) (Vaillant *et al.*, 1999; Reichardt, 2006).

Interestingly, several studies have shown that BDNF and NT-4/5 promote PV+ cell development via TrkB activation (Patz *et al.*, 2004; Itami *et al.*, 2007), whereas NT-3 did not rescue PV+ cell development in organotypical cultures (Patz *et al.*, 2004). Nevertheless, mice carrying NT-3 alleles in place of the BDNF alleles could rescue the PV+ cell number in BDNF knockout mice (Berghuis *et al.*, 2006). Together with the observed severe phenotype of NT-3 knockout mice compared to the rather mild phenotype of TrkC knockouts (NT-3 receptor), the data indicate that NT-3 is may bind to other receptors and hence, compensate for BDNF loss (Coppola *et al.*, 2001). However, in TRα1+m mice a compensatory mechanism seems to arise from BDNF-TrkB signaling, as mRNA levels of TrkB were elevated at P14 (**paper IV**).

Nevertheless, several studies showed an elevated expression of NT-3 in the first postnatal week, suggesting a function in cortical maturation (Ernfors *et al.*, 1990; Lauterborn *et al.*, 1994). Consequently, the reduced NT-3 levels in the mutant mice might indicate a delayed developmental increase of NT-3 compared to wt mice. Measurement of NT-3 throughout development might help to identify whether the peak of NT-3 expression is delayed or not detectable.

The differentiation of cells expressing both p75 and TrkC were shown to depend on NT-3 (Simmons *et al.*, 2013). TrkC is only expressed in 12% of adult cortical PV+ cells (Gorba & Wahle, 1999). This number does not coincide with the reduction of PV+ cells in our mutant mice (Wallis *et al.*, 2008). However, like NT-3, TrkC showed a transiently elevated expression during early postnatal development (Ringstedt *et al.*, 1993). This finding is of particular interest, since it may indicate that NT-3 –TrkC signaling is involved in early cell differentiation. Moreover, the number of TrkC expressing cells during development might be higher than in adults, consequently a lack of NT-3 in early development would affect more cells than reported in the adults. From that it can be hypothesized that NT-3 regulates cell development in a greater extent than suggested in previous reports.

Not only the cortical signaling is important for PV+ cell development; removal of cortico-thalamic connections in cortical cultures led to a delayed PV expression onset but not a loss of PV+ cells (Patz *et al.*, 2004), similar to what we have seen in our animal. Interestingly, an involvement of NT-3 and its receptor TrkC in afferent innervation has been postulated, as the transiently elevated expression of TrkC in the neocortex is accompanied by an increased expression of NT-3 in thalamic nuclei. These findings suggested that thalamic NT-3 and neocortical TrkC communicate to stabilize cortico-thalamic innervations (Ringstedt *et al.*,

1993; Lauterborn *et al.*, 1994). As TRα1 is also expressed in the thalamus (**paper I**), and ChIP assay identified NT-3 as target gene (**paper IV**), NT-3 might be down regulated in the thalamus as well, leading to reduced innervation and hence to a decelerated expression of PV. Cortico-thalamic connections were not tested in this study, but need to be followed up to further understand the function of NT-3 and TRα1 in innervation.

However, reduced NT-3 level led to inhibited signal transduction and hence, to reduced activation of neurotrophin induced pathways. We identified a reduced phosphorylation of AKT at P7, which is involved in cell survival. As discussed in **paper IV**, the reduced AKT phosphorylation might be a mechanism to reduce the increased number of calretinin cells in TRα1+m mice. Additionally, we identified a slightly reduced phosphorylation of MEK1/2, which is suggested to affect PV+ cell differentiation. Consequently, blocking of MEK2 was shown to reduce PV+ cell differentiation in culture (Patz *et al.*, 2004). From that we can speculate that the mutant TRα1 affects neurotrophin induced signaling pathways. On one hand the mutant receptor inhibits the phosphorylation of AKT leading to reduced cell survival and on the other hand it reduces MEK phosphorylation, which inhibits PV+ cell differentiation.

Taking together, even though NT-3 is not the typical neurotrophin involved in PV+ cell differentiation, there are several NT-3 dependent mechanisms that modulate PV+ cell maturation. In the cortex, the reduced NT-3 levels leads to inhibited signal transduction necessary for cell differentiation. In the thalamus, NT-3 is required for proper cortico-thalamic innervations, which is essential for proper PV+ cell development. Lastly, elevated TrkB levels might indicate a compensation mechanism for the delayed PV+ cell differentiation.

4.4 CORTICAL ACTIVITY

Previous studies have revealed the requirement of neuronal activity for neurotrophin and PV expression *in vitro* (Thoenen, 1995; Patz *et al.*, 2004). As carbonic anhydrases (Car) are involved in neuronal excitation via buffering the alkaline pH shifts (Chesler, 2003; Shah *et al.*, 2005), we speculated that the excitation rate is reduced in TRα1+m mice due to lower Car4 levels observed in **paper III**. This idea is supported by Tang et al. who showed that low Car expression reduces seizure susceptibility through the suppression of glutamate receptors by an excess of extracellular H^+ (Tang *et al.*, 1990).

The capability of Car14 to functionally compensate for Car4 absence in the brain was shown previously (Shah *et al.*, 2005). Interestingly, Car14 levels are not altered in the mutants (observation by Dudazy-Gralla) indicating a normal pH buffering capacity in the cortex. However, TRα1+m mice show a reduced seizure susceptibility accompanied by an increased expression of the calcium binding protein calretinin in the hippocampus (Hadjab-Lallemend *et al.*, 2010). Interestingly, hippocampal GABAergic cells depolarized pyramidal cells upon PTZ treatment, indicating an enhanced amount of extracellular HCO_3^-, which was shown to shift the typical GABA induced inhibition to excitation (Kaila & Voipio, 1987; Chesler & Kaila, 1992). As Car4 is responsible for dehydrating HCO_3^-, a lack of Car4 can easily cause enrichment. Nevertheless, neurotransmitter and activity-dependent BDNF expression are normal in the cortex of mutant mice arguing against a reduced cortical activity (**paper IV**). However more studies are needed to clarify whether cortical activity is disturbed in the mutant cortex. Measurement of the inhibitory and excitatory postsynaptical potential may contribute to a better understanding of neuronal connectivity. In order to increase excitation pharmacologically *in vivo*, injections with the glutamate analog kainate acid can be considered, as it leads to increased excitation resulting in a strong inhibitory response (Ben-Ari & Cossart, 2000). This treatment should be adequate to provide the putative missing excitation when used in lower dosage, as hyperactivation can lead to cell death, similar to what we have seen after PTZ injections (**paper IV**).

4.5 IDENTIFICATION OF TARGET GENES

Several attempts have been made to find additional TR target genes either by microarray after TH treatment or by transfecting tagged-TRs into cell lines followed by TH treatment and transcriptom analysis. These experiments revealed over 4000 putative TR target genes, with an overlap of only 734 genes between the experiments, depicturing the difficulties to reliable identify target genes (Chatonnet *et al.*, 2015). Explanation for the high variety between the studies is on the one hand the technical aspects such as TH treatment or statistical analysis. On the other hand, target gene regulation differ between cell types, developmental stage and is influenced by the altered chromatin structure in cell cultures (Engelhard & Christiano, 2004; Bernal & Morte, 2013). With our mouse line we can pull down TRα1 specific TREs in mouse tissue. In our admittedly exploratory experiments, we identified three novel TRα1 target genes in the brain and characterized novel TRα1 responsive TREs in heart tissue. However, the localization and sequence of the TRE did not allow any conclusions about their regulation of transcriptional control, that needed to be tested *in vitro* and *in vivo*,

With the established ChiP assay we identified two negatively regulated TH target genes: Car4 and RNF166. Interestingly, the TREs were located in different regions of the respective genes, with the TRE of RNF166 in the 3'UTR (untranslated region) and the TRE of Car4 in the proximity to the promotor. This is a peculiar finding as it suggests that the direction of transregulation is not predictable by the genomic context. Indeed, cistrome analysis revealed 70% of positively regulated genes contain no TRE in the promotor proximity and binding sited were identified upstream and downstream of the promotor (Dong *et al.*, 2009; Chatonnet *et al.*, 2013).

However, the mechanism causing transrepression might depend on TRE localization. Consistently, the TRα1 responsive TRE of Car4 was located in the promotor region. Cloning of Car4 into a promotor vector led to a reduced luciferase induction upon TH treatment, which was in line with *in vivo* result. On the contrary cloning of the RNF166 TRE into the promotor led to transactivation *in vitro*. From that it can be hypothesized that TREs localized in 3'UTR require additional factors to exhibit transrepression (Bigler & Eisenman, 1995), which are not available in cell cultures. However, the long distance to the promotor can be overcome by chromatin looping, which enables transactivation at long distances. This process was shown for other nuclear receptors and was postulated for TRs by Bigler and colleagues (Bigler & Eisenman, 1995; Flamant & Gauthier, 2013)

Taken together, TRE proximity to the promotor does not allow conclusion about target gene activation and requires additional *in vivo* or *in vitro* experiments after TH level manipulation. Long distance between TRE and transcriptional start site can be overcome by chromatin looping.

The advantage of our system when combined with ChiP sequencing compare to the available transcriptom analysis is the specific enrichment for TRα1 *in vivo*, circumventing the need for specific TR antibodies and cell culture experiments.

5 SUMMARY AND FUTURE PERSPECTIVES

In my thesis, I describe for the first time the expression pattern of the thyroid hormone receptor alpha 1 protein in the developing and mature brain on a cell level. Importantly, our discovery that virtually all postmitotic neurons express the receptor clarifies the previously long standing question: "in which neurons is TRα1 expressed, and when?". With this knowledge, subsequent understanding of, and research into TRα1 action in the brain will be significantly simplified.

In the search for TRα1 target genes we established ChIP assays using TRα1$^{gfp/gfp}$ mice, circumventing the need for specific - and not available - TRα1 antibodies. Here, we identified RNF166 as novel target genes and discovered TRα1 responsive TREs in the genomic sequence of the TH regulated genes Sema3a, HCN2 and HCN4 in heart tissue. The method was further utilized to identify carbonic anhydrase 4 (Car4) as novel target gene. We describe a tissue specific regulated by TH, with Car4 in lung and brain being dependent on proper signaling during early development, whereas renal TH was not affected by the mutant TRα1 but decreases its expression upon TH treatment. Finally, we discovered that immature PV cells are arrested at a late stage of differentiation in the cortex of TRα1 mutant mice. This finding was accompanied by a reduced expression of NT-3 and an inhibited phosphorylation of its down stream genes, indicating a disturbed intracellular neurotrophin signaling.

In the future the TRα1-GFP mouse strain may be used to identify further target genes in the brain or other tissues, either via individual ChIP assays or untargeted approaches like ChIP sequencing. However, the regulation of putative target genes needs to be tested *in vivo* by examination of mRNA levels of the gene of interest, by microarrays or RNA sequencing in hypo-, eu- and hyperthyroid animals.

To identify the role of Car4 in brain maturation and seizure susceptibility in the mutant mice the suggested extracellular enrichment of HCO_3^- needs to be measured in a cell culture setting. As the seizure susceptibility was normalized upon TRα1 activation during embryogenesis and postnatal live, the same treatment might be sufficient to ameliorate the Car4 phenotype. Additionally, overexpression of Car4 might normalize GABA response - that was shown to be depolarizing in the hippocampus of TRα1+m mice - and hence alleviate the seizure phenotype. Although this proposed role of the downregulation of Car4 may seem far reaching, the hypothesis fits with known functions of the involved genes and should therefore be tested experimentally.

The mechanisms causing the delayed differentiation of PV+ cells needs further investigations. To determine whether NT-3 is directly involved in PV+ cell differentiation, cortical injecting of an adenoassociated virus (AAV) expressing NT-3 can be considered (Boyce *et al.*, 2012). This virus infects neurons at the injection site that will express NT-3. In turn, the increased NT-3 levels may induce PV+ cell differentiation. However, how this ultimately, through a direct or indirect mechanism, normalizes PV expression requires additional experiments, as NT-3 was found to be expressed in pyramidal cells and to a smaller extend in interneurons (Pascual *et al.*, 1998; Boukhaddaoui *et al.*, 2001). To address the question, I have already cloned the dominant negative TRα1 sequence between lox P sites and the vector was linked to an adenoassociated virus construct, intended for studies on interneuron specific Cre mouse lines. Using an Lhx6 Cre line, this approach may initiate the expression of a dominant negative TRα1 in all maturing SST+ and PV+ interneurons, and in case of a cell autonomous should decrease the number of PV+ interneurons. Lastly, in case of PV+ cell rescue, assessment of behavior and locomotor might answer the question whether the defect in PV+ cell maturation causes the phenotype.

Further investigation of both mice strains will help to understand the mechanisms involved in developmental and adult hypothyroidism. Ultimately, the findings may contribute to identify a treatment regime to alleviate the brain retardation and other symptoms described in e.g. congenital hypothyroidism or RTHα.

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Only from the alliance of the one,
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are great things born.
- Antoine de Sainte-Exupéry -

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