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EXPRESSION AND FUNCTION OF THYROID HORMONE RECEPTOR ALPHA 1 IN THE BRAIN

Karin Wallis

Karolinska Institutet

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Cover picture shows neurons expressing TRα1-GFP (green) and parvalbumin (red) in the CA3 subfield of the hippocampus in the adult mouse brain.

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Till minne av min mormor
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ABSTRACT

Thyroid hormone is fundamentally important for development and maintenance of adult brain functions and maternal hypothyroxinemia during pregnancy can lead to a severe mental retardation known as endemic cretinism. The first realization that endemic cretinism is caused by iodine deficiency and its association with thyroid hormone was made a century ago; this eventually resulted in dietary supplementation programme, e.g. iodination of table salt. Moreover, routine screening of newborns and subsequent treatment prevents the irreversible psychomotor defects caused by congenital hypothyroidism. However, the understanding of the mechanism for how thyroid hormone exerts its effects during brain development is limited. In addition, the fetal consequences of maternal hypothyroxinemia in the absence of iodine deficiency are not generally accepted.

The aim of the work in this thesis was to elucidate functions of thyroid hormone in the developing nervous system. For this we studied the cellular mediators of thyroid hormone action, i.e. nuclear thyroid hormone receptors (TRs). Recent publications suggested that many of the consequences of hypothyroidism in the brain are caused by the repressor activity of the unliganded isoform TRα1. We therefore generated mice in which a mutant TRα1, with lower affinity to ligand, confers a "receptor-mediated hypothyroidism". In **paper I** we show that these mice have locomotor aberrancies that bear a striking resemblance to that seen in endemic cretinism. Indeed, we could show that the defects were founded during pregnancy and that the offspring was dependent on maternal thyroid hormone for proper motor functions in the adult. Furthermore, we identified that specifically the parvalbumin subtype of GABAergic interneurons in the cortex showed a delayed development, correlating with the locomotor phenotype. This was accompanied by an impaired neuronal network activity and a lowered number of fast-spiking interneurons. In **paper II** we investigated if the reduced inhibition resulted in lowered seizure susceptibility. Surprisingly, the mutant mice were partially resistant to seizures induced by the $GABA_A$ receptor antagonist pentylenetetrazol, a result that was mirrored in hippocampal slice preparations *in vitro*. Moreover, patch clamp recordings revealed that the pyramidal cells of the mutant mice were hypoexcitable.

Although the TRs were cloned over 20 years ago their expression in specific cell types in the brain was still unknown due to a lack of reliable antibodies against them. We therefore decided to generate mice that express a chimeric TRα1-GFP protein from the *Thra* locus. The results in **papers III** and **IV** showed that TRα1-GFP was first expressed in postmitotic neurons of the embryonic telencephalon, the postnatal cerebellum and in the adult hippocampal neurogenic niche. In the adult, essentially all mature neurons expressed TRα1, the exception being Purkinje cells in the adult. Expression in glia was limited to tanycytes lining the third ventricle and to the cerebellum. The effect of the unliganded TRα1 on adult neurogenesis was explored in **paper IV.** Here we could demonstrate that the aporeceptor activity of TRα1 caused a reduction in survival of postmitotic neuroblasts during adult-onset hypothyroidism.

We have made significant advancement towards understanding the damage resulting from endemic cretinism by identification of cells that develop improperly as a result of insufficient supply of fetal thyroid hormone and establishing that TRα1 expression is first turned on during later stages of neuronal maturation.

LIST OF PUBLICATIONS

I. **Wallis K***, Sjögren M*, van Hogerlinden M, Silberberg G, Fisahn A, Nordström K, Larsson L, Westerblad H, Morreale de Escobar G, Shupliakov O, Vennström B (2008) Locomotor deficiencies and aberrant development of subtype-specific GABAergic interneurons caused by an unliganded thyroid hormone receptor alpha1. *The Journal of Neuroscience* 28 (8): 1904-1915

II. Hadjab-Lallemend S*, **Wallis K***, van Hogerlinden M, Dudazy S, Nordström K, Vennström B, Fisahn A (2010) A mutant thyroid hormone receptor alpha 1 alters hippocampal circuitry and reduces seizure susceptibility in mice. *Neuropharmacology* 58 (7): 1130-1139

III. **Wallis K**, Dudazy S, van Hogerlinden M, Nordström K, Mittag J, Vennström B (2010) The thyroid hormone receptor α1 protein is expressed in embryonic postmitotic neurons and persists in most adult neurons. *Molecular Endocrinology* 24 (19): 1904-1916

IV. Kapoor R*, van Hogerlinden M*, **Wallis K**, Ghosh H, Nordström K, Vennström B, Vaidya VA (2010) Unliganded thyroid hormone receptor α1 impairs adult hippocampal neurogenesis. *FASEB Journal* 24 (12): 4793-4805

*These authors contributed equally

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

BrdU	5-bromo-2'-deoxyuridine
CA1, CA2, CA3	*Cornu Ammonis* area 1, 2 and 3 of the hippocampus
CB	Calbindin
CR	Calretinin
D1, D2, D3	Iodothyronine deiodinases type I, II and III
E [number]	Embryonic day
EGL	External granular layer
IGL	Internal granular layer
GABA	γ-aminobutyric acid
GCL	Granular cell layer
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
HPT axis	Hypothalamus-pituitary-thyroid axis
MGE	Medial ganglionic eminence
NeuN	Neuronal nuclei
OL	Oligodendrocyte
OPC	Oligodendrocyte precursor cell
P [number]	Postnatal day
PTZ	Pentylenetetrazol
PV	Parvalbumin
RTH	Resistance to thyroid hormone syndrome
T3	Triiodothyronine
T4	Thyroxine
TR	Thyroid hormone receptor
TRE	Thyroid hormone response element
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone
SGZ	Subgranular zone
SOM	Somatostatin
SVZ	Subventricular zone
Wt	Wildtype

INTRODUCTION

Thyroid hormone affects most of our bodies' organs. Especially, it is a key regulator of metabolism and absolutely essential for normal development and function of the brain. Goiter (enlargement of the thyroid gland) is the sign of a diseased thyroid and is associated with both hypo- and hyperthyroidism. The syndrome was described already in the ancient Greek culture and we know that cretinism (thyroid hormone deficiency causing mental and physical retardation) was observed in the Alpes in the thirteenth century. It was during the Renaissance that the thyroid gland was discovered but not until 1820 that Swiss physician Coindet successfully treated goiter with iodine. In the 1880s goiters were removed surgically and it was noted that the patients nervousness disappeared. However, the disease was soon replaced with a new one: without a thyroid the patients became slow of mind and socially non-functional. In 1891 Murray, a British physician, treated hypothyroid patients with injections of sheep thyroid extracts, still unknowing of the active substance. This was not solved until 1915 when thyroid hormone (thyroxine, T4) was the first hormone to be isolated and crystallized (Kendall, 1983). The biologically more active form, triiodothyronine (T3), was discovered in 1952 (Gross and Pitt-Rivers, 1952). It was however still a mystery how this hormone could be so vital to our physiology. How do the cells know how to behave in presence or absence of thyroid hormone? Clues came in 1963 when it was shown that the increase in body weight that followed administration of thyroid hormones to thyroidectomized rats was accompanied by altered levels of mRNA expression (Tata et al., 1963). In 1986 it was shown that the sensing and response to thyroid hormone was conducted by the thyroid hormone receptors working as transcription factors (Sap et al., 1986; Weinberger et al., 1986).

The World Health Organization has proclaimed iodine deficiency as the world's greatest single cause of preventable mental retardation and has together with UNICEF implemented worldwide programmes for the elimination of iodine deficiency through supplementation of salt. In addition, all newborns in developed countries are screened for hypothyroidism. Many of the functions of thyroid hormone are well characterized, but we still do not understand the severe consequences on brain development caused by thyroid hormone deficiency during the fetal and postnatal period. Moreover, even though it is over 20 years since the thyroid hormone receptors where identified, we do not know which specific cells in the brain express these receptors and during what stages of their development.

THYROID HORMONES

Thyroid hormone homeostasis, metabolism and transport

Production of thyroid hormone

To synthesize thyroid hormone the body needs iodine, which is supplied via food intake. Iodine is transported in the blood from the intestine to the thyroid gland where the thyroid hormones are synthesized by iodination of tyrosine residues on thyroglobulin. Release of thyroid hormones is regulated by thyroid stimulating

hormone (TSH), produced by the pituitary (Fig. 1). TSH secretion is in turn stimulated by thyrotropin releasing hormone (TRH) produced in the hypothalamus. The hypothalamic-pituitary-thyroid (HPT) axis is under negative feedback control by thyroid hormone, i.e. increased serum levels of thyroid hormone suppress both TRH and TSH production, thus leading to decreased production of thyroid hormone and vice versa. The two thyroid hormones secreted into the serum by the thyroid gland are T4 and T3. The latter is the biologically active form, but the majority of thyroid hormone released from the thyroid is T4, which must therefore be converted to T3 peripherally. This process, as well as thyroid hormone deactivation, is performed by iodothyronine deiodinases. About 70-80% of T3 in the rat brain has been estimated to derive from local intracellular production (Crantz et al., 1982).

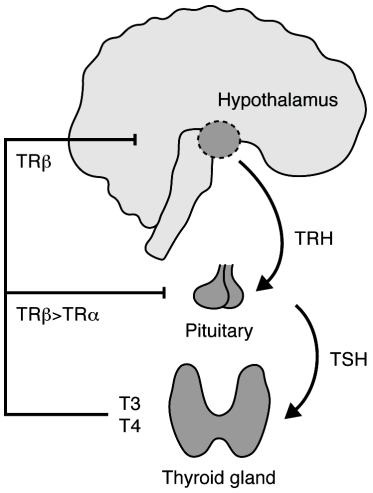

Figure 1. The HPT axis. Thyroid hormone negatively regulates its own synthesis and release by feedback inhibition of TRH and TSH in the hypothalamus and pituitary gland, respectively.

The deiodinases: local control of thyroid hormone levels

Type I and type II deiodinases (D1 and D2) are the activating enzymes (Fig. 2). But whereas D1 can deiodinate both rings of all iodothyronines, D2 can only deiodinate the outer ring (or 5'-deiodination). D3 deiodinates the inner ring (5-deiodination) of T3 and T4 and is the major inactivating enzyme. The deiodinases also differ in their expression pattern: the highest levels of D1 is found in the thyroid, liver and the kidney and D2 in the central nervous system, pituitary, brown adipose tissue and placenta (Bianco and Kim, 2006; St Germain et al., 2005). D3 activity is high in the placenta and uterus during pregnancy and in the central nervous system (Galton et al., 1999; Huang et al., 2003; Wasco et al., 2003).

From studying mice devoid of one or several deiodinases it has become apparent that D2 is critical for determining the local thyroid hormone content within tissues (Galton et al., 2009; Schneider et al., 2001; Schneider et al., 2006). D1 in the liver and kidney generates T3 to be exported to the blood and has also been suggested to work as a scavenger enzyme important for iodine recycling especially during iodine deficiency (Galton et al., 2009). Through regulating expression of these enzymes in different

tissues an individual can have euthyroid serum levels but be hypo or hyperthyroid on the tissue level (Bianco and Kim, 2006). This becomes particularly evident in the tadpole, where the organs have different requirements for thyroid hormone during distinct stages of metamorphosis, which is matched by local regulation of D2 and D3 activity (Becker et al., 1997). A role for D3 in protection from high levels of thyroid hormone was also demonstrated in cochlear development, where mice devoid of D3 showed a premature differentiation with deafness as a result (Ng et al., 2009).

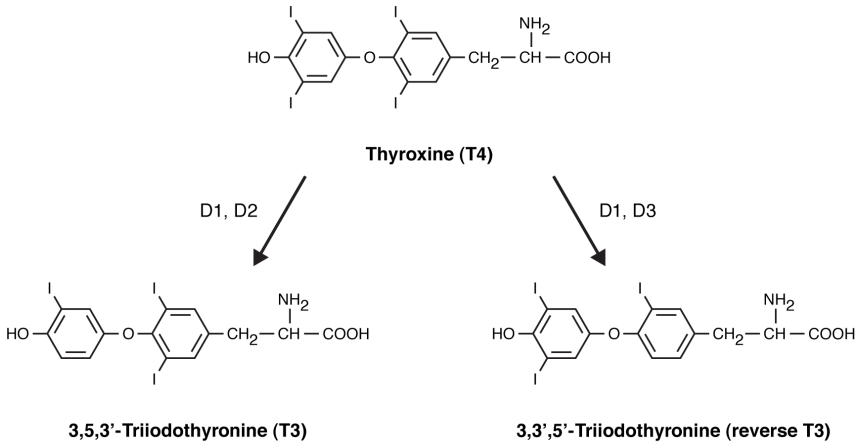

Figure 2. Deiodination. Structures of T4, T3 and reverse T3 and the reactions catalyzed by the deiodinases D1, D2 and D3.

Thyroid hormone transport

Another level for regulating intracellular thyroid hormone is the transport across the plasma membrane. After the identification of the first thyroid hormone transporting molecule (Abe et al., 1998), many transporting proteins have been reported, but to date there are only three that have been shown to have high affinities for the thyroid hormones: the monocarboxylate transporters 8 and 10 (MCT8 and MCT10) and organic anion transporting polypeptide 1C1 (OATP1C1) (Friesema et al., 2003; Friesema et al., 2008; Pizzagalli et al., 2002; Sugiyama et al., 2003; Tohyama et al., 2004).

Thyroid hormone receptors and gene regulation

Thyroid hormone mediates its effects through the thyroid hormone receptors (TRs) α and β, transcribed from the genes *Thra* and *Thrb* respectively (Sap et al., 1986; Weinberger et al., 1986). The TRs belong to the family of nuclear hormone receptors and are transcription factors that in response to hormone binding regulate gene expression (Mangelsdorf et al., 1995). By alternative splicing *Thra* and *Thrb* encode four major isoforms: the ligand binding TRα1, TRβ1, TRβ2 and the non-ligand binding TRα2, the function of which is unclear (Benbrook and Pfahl, 1987; Flamant et al.,

2002; Hodin et al., 1989; Koenig et al., 1988; Lazar et al., 1988, 1989; Mitsuhashi et al., 1988; Mittag et al., 2005; Salto et al., 2001; Thompson et al., 1987). Expressed from the opposite strand of the TRα locus is rev-erbA, which is implicated in circadian rhythm regulation (Forman et al., 1994; Ueda et al., 2002; Yin et al., 2007).

Regulation of gene expression occurs in the nucleus where TRs bind to thyroid hormone response elements (TREs) of target genes (Yen, 2001; Zhang and Lazar, 2000). Through interaction with T3 and transcriptional modulators, i.e. coactivators or corepressors, they either activate or repress target genes (Koenig, 1998). The TR isoforms have common structural features, with a DNA binding domain at the N-terminus and a ligand binding domain at the C-terminus, the latter also mediating receptor dimerization and coactivator binding (Fig. 3A). TRs generally bind to TREs as heterodimers with retinoid X receptor, but monomers and homodimers also occur. Genes that harbour a positive response element are repressed by the non-ligand bound aporeceptor (Fig. 3B). Binding of T3 induces a conformational change in the receptor and replacement of corpressors such as NCoR, SMRT and Alien with coactivators, e.g. SRC-1, TIF-2 and CBP. The latters recruit factors of the basal transcriptional machinery, resulting in gene activation. Genes harbouring negative TREs basically function in the opposite manner: they are activated by the aporeceptor and repressed in the presence of T3. The molecular details of negative regulation by TRs have not been fully elucidated.

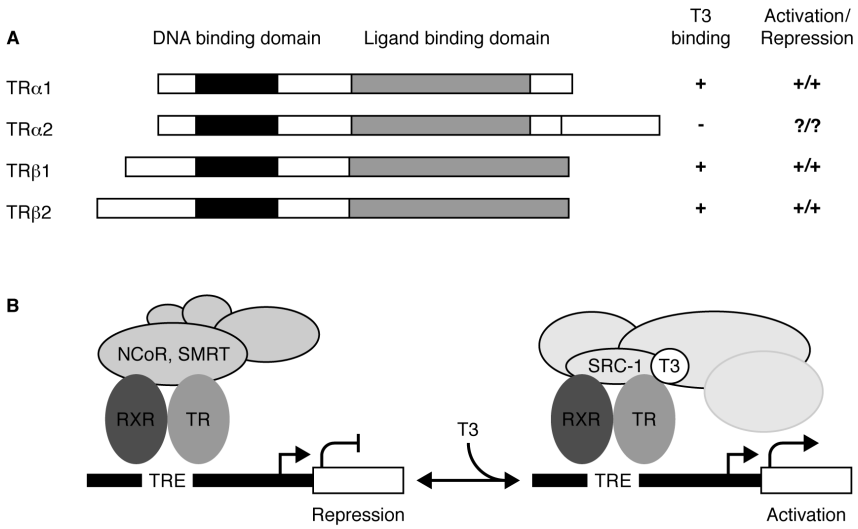

Figure 3. The thyroid hormone receptors. A: schematic representation of the TR isoforms, indicating their functional domains, ability to bind ligand and their function as transcriptional regulators. B: example of transcriptional repression and activation of a positively regulated target gene. Thyroid hormone binding leads to displacement of corepressors (e.g. NCoR, SMRT) and the recruitment of coactivators (e.g. SRC-1) and factors of the basal transcriptional machinery. NCoR, nuclear receptor corepressor, RXR, retinoid X receptor, SMRT, silencing mediator of retinoic and thyroid receptor, SRC-1, steroid receptor coactivator 1.

Thyroid hormone receptor knockout mice

When generating mice devoid of TR isoforms it was the general belief that the phenotype of these mice would resemble a strong hypothyroidism (Flamant and Samarut, 2003; Forrest and Vennstrom, 2000). Indeed, these mice have been important for the understanding of TR function in a variety of tissues. From such studies (combined with expression analyses) we have learnt that TRα is the predominant isoform in the brain, bone and heart, whereas TRβ is critical for color vision, auditory and liver function and for HPT axis regulation. However, congenital hypothyroidism is more deleterious than absence of all TR isoforms (Gauthier et al., 1999; Göthe et al., 1999; Mansouri et al., 1998). It is now well recognized that many of the effects of hypothyroidism, especially in the brain, are caused by the aporeceptor activity of the unliganded receptor (Flamant et al., 2002; Morte et al., 2002; Venero et al., 2005).

Thyroid hormone disorders

Hypothyroidism

In humans, hypothyroidism is associated with fatigue, decreased appetite, weight gain, depression, mental impairment, cold intolerance, decreased sweating, bradycardia and menstrual disturbances (Braverman and Utiger, 2000a). When occurring in the adult, most symptoms of hypothyroidism are reversed by treatment with thyroid hormone. However, if not discovered within weeks after birth in the newborn, it will cause irreversible mental retardation and motor dysfunctions. The most common reason for adult hypothyroidism worldwide is iodine deficiency. Other causes are diseases of the thyroid gland, such as autoimmune thyroiditis (Hashimoto's disease), irradiation-induced hypothyroidism (following treatment of hyperthyroidism or tumor) (Braverman and Utiger, 2000a). Less frequent is central hypothyroidism, which is caused by deficiency in stimulation of the thyroid by the hypothalamus or pituitary.

Hyperthyroidism

The symptoms of hyperthyroidism (or thyrotoxicosis, which is the clinical term for hypermetabolism caused by high concentrations of serum T4 and T3) are reverse of those of hypothyroidism: increased metabolism with weight loss, anxiety, hyperactivity, increased sweating and tachycardia. Thyrotoxicosis is in 60-90% of all cases caused by Grave's disease, which is an inherited autoimmune disease where the body produces antibodies that stimulate the TSH receptor (Braverman and Utiger, 2000b; Davies, 2000). Other causes for thyrotoxicosis are toxic multinodular goiter and toxic adenomas. The principal treatments are radioiodine therapy, thyroidectomy or in the case of Grave's disease antithyroid drugs that inhibit T4 and T3 synthesis.

Resistance to thyroid hormone

More than 300 patient families have been identified to date that suffer from resistance to thyroid hormone (RTH), which is an inherited syndrome of reduced tissue responsiveness to thyroid hormone (Refetoff et al., 1967; Weiss and Refetoff, 2000). A majority of affected individuals have a mutation in TRβ that diminishes binding of thyroid hormone. Because TRβ is the main regulator of TSH release from the pituitary (Fig. 1), these patients have elevated levels of serum thyroid hormones, but at the tissue level they often display a mixed hypo and hyperthyroid phenotype. Affected

individuals usually present a goiter and may suffer from learning disabilities, attention deficit hyperactivity disorder (ADHD), low IQ and hearing defects.

THYROID HORMONE IN THE DEVELOPING BRAIN

The brain is a major target for thyroid hormone and hypothyroidism during gestation and the postnatal period results in irreversible mental retardation. Hypothyroidism in the neonate is referred to as congenital hypothyroidism, whereas thyroid hormone deficiency in the pregnant mother in iodine deficient areas results in endemic cretinism in the child.

Congenital hypothyroidism

The incidence of congenital hypothyroidism is 1 in 3770 births in Europe (Klein and Mitcell, 2000) and today most industrialized countries screen all newborns for hypothyroidism. Introduction of the screening programme, and the immediate treatment of affected induviduals with T4, in the United States in 1974 raised the mean IQ in affected children from 80 to normal values in addition to a normal distribution of individual IQs (Klein and Mitcell, 2000). It has been shown that hypothyroid neonates lose 3-5 points in IQ score for each month that treatment is delayed (Foley, 2000). After iodine deficiency, the most common cause of congenital hypothyroidism is improper development of the thyroid, but it can also be the result of defects in hormone synthesis or more rarely mutations in the TSH receptor or TSHβ (Foley, 2000).

Endemic cretinism and maternal thyroid hormone deficiency

In iodine deficient areas up to 5-15% of the population may suffer from the severe neurological and physical underdevelopment caused by endemic cretinism (Delange, 2000). The symptoms cannot be reversed after birth, but population-wide iodine supplementation programmes have efficiently prevented endemic cretinism in many areas. There are two forms of endemic cretinism, neurological and myxedematous cretinsism, which differ both in their underlying cause and in their symptoms. Neurological cretinism manifests in mental retardation, abnormal stance and gait and in the most severe cases, deaf-mutism. In spite of this, the patients are euthyroid and the prevalence of goiter is not higher than in the rest of the population. Myxedematous cretins on the other hand suffer from long-term hypothyroidism, which has resulted in a less severe mental disability, dwarfism, and retardation in growth of body proportions, nasoorbital features and sexual development.

With the successful treatment of congenital hypothyroidism after birth it was for many years a consensus that the fetal brain does not require thyroid hormone. We now know that this is not true. In fact, thyroid hormone receptors are expressed in the human brain already at 8-10 weeks of gestation (Bernal and Pekonen, 1984; Iskaros et al., 2000). In contrast, the human fetal thyroid gland is not functional until midgestation. Before this time the developing brain is thus dependent on maternal T4 for local conversion to T3 and the requirement continues throughout pregnancy (Bernal and Pekonen, 1984;

Kester et al., 2004; Morreale de Escobar et al., 2004). During iodine deficiency, the body will maintain euthyroidism by prioritizing T3 synthesis over T4, which is the optimal strategy for most maternal organs, but detrimental for the fetal brain. It is now recognized that the harmful effects of neurological cretinism on brain development are not caused by maternal hypothyroidism but by low levels of T4 (Morreale de Escobar et al., 2004). This symptomless state of low T4 levels but normal T3 and TSH is referred to as hypothyroxinemia. In contrast, children with congenital hypothyroidism are born by mothers with normal T4 and T3 levels and the fetal brain therefore develops in the presence of thyroid hormone making treatment after birth effective.

Even a mild transient hypothyroxinemia of the mother affects brain development in the euthyroid offspring. In iodine deficient areas psychomotor development of the whole population is affected, not only in children suffering from cretinism (Morreale de Escobar et al., 2000). Furthermore, it has been reported that low maternal T4 concentrations at 12 weeks of gestation, but not at 32 weeks, give an increased risk of impaired neurodevelopment in apparently healthy pregnancies in an iodine sufficient population (Pop et al., 1999). Recently, a Dutch cohort study comprising 3659 children demonstrated maternal hypothyroxinemia as a risk factor for delayed development of cognitive skills (Henrichs et al., 2010). Another more immediate threat in our part of the world is untreated hypothyroidism such as Hashimotos disease during pregnancy. The requirement for thyroid hormone will increase in these patients during pregnancy and thyroid function should be assessed already a few weeks after implantation (DeGroot et al., 1996).

Structural alterations caused by developmental hypothyroidism and hypothyroxinemia

To understand the effects that thyroid hormone exerts on the brain it is necessary to know the basic mechanisms of normal brain development. The mammalian forebrain develops from the anterior neural tube and comprises the telencephalon (cerebral cortex and basal ganglia) and the diencephalon (thalamus and hypothalamus). Higher function such as motor commands and sensory perception is regulated from the layered structure of the cerebral cortex called the neocortex. The excitatory projection neurons of the neocortex are born in the ventricular zone (Fig. 4). After cell cycle exit neurons migrate to form the preplate at the surface of the cortex. This layer is split into the marginal zone and subplate as a new group of neurons arrives to form the cortical plate. The neocortex grows in an inside-out pattern, i.e. later born cells will migrate past older neurons to form a new layer as they differentiate. The interneurons of the neocortex are born in an area of the ventral telencephalon called the medial ganglionic eminence (MGE) and the postmitotic neurons migrate tangentially to reach the neocortex (Fig. 4A). It is of importance to know that once in the neocortex, interneurons will migrate radially before reaching their laminar position and integrate within the neuronal network. Cell-cell interactions are important both for this latter process of dendrite growth and synapse formation and for migration. Migration along radial glia is the most studied process (Fig. 4B) but neurons will also migrate along axons. Neuronal survival and differentiation are promoted by neurotrophins that bind to receptors on the responsive cell. Some neurotrophins, i.e. brain derived neurotrophic factor (BDNF) and

neurotrophin-4 (NT4), have in addition been shown to stimulate migration of neurons that express the receptor TrkB. In the opposite manner, chemorepellants, e.g. Slit1, will prevent migrating neurons to enter certain areas as for example the ventricular zone (Marin and Rubenstein, 2003; Nadarajah and Parnavelas, 2002).

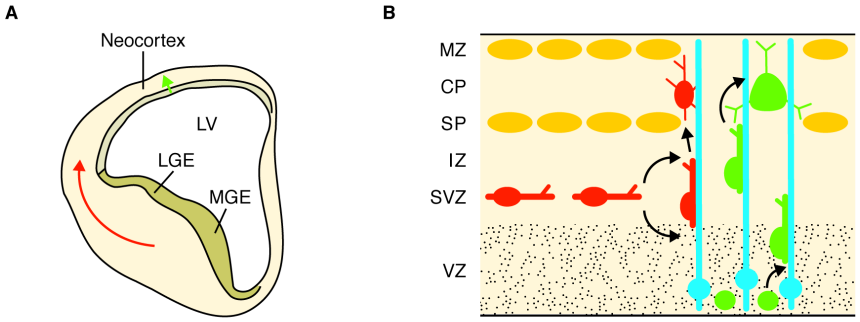

Figure 4. Migration of neurons in the embryonic telencephalon. A: source of radially migrating pyramidal neurons (green) and tangentially migrating interneurons (red), illustrated in a coronal section. B: Interneurons arriving to the neocortex at the SVZ migrate radially to reach the cortical plate. Pyramidal neurons leave the ventricular zone after cell cycle exit and migrate along radial glia scaffolds to the cortical plate for terminal differentiation. CP, cortical plate, IZ, intermediate zone, LGE, lateral ganglionic eminence, LV, lateral ventricle, MGE, medial ganglionic eminence, MZ, marginal zone, SP, subplate, SVZ, subventricular zone, VZ, ventricular zone.

Most studies on thyroid hormone action in the brain were conducted in rats or genetically modified mice. When translating findings from rodents to humans certain differences need to be considered; the newborn rat is comparable to the human fetus at midgestation and the newborn human to the rat ten days after birth (Bernal, 2007). This means that some of the processes that take place postnatally in the rodent, such as development of the cerebellum, mainly occur before birth in the human (Fig. 5). There are also differences between mice and rats: the gestation period is 21 days in the mouse and 23 days in the rat.

Thyroid hormone in the developing forebrain

Early work on rats by Legrand and colleagues in the 1970-80's established the effects of hypothyroidism on cell maturation, migration, synaptogenesis and myelin formation and thyroid hormone has later been shown to regulate specific genes involved in these processes (Thompson and Potter, 2000). These results were specified and extended when it was demonstrated that early maternal and perinatal hypothyroidism delays radial distribution of neurons in the neocortex, barrel field formation and affect arborization and bouton number (Auso et al., 2001; Berbel et al., 2001; Berbel et al., 1996; Calikoglu et al., 1996). Furthermore, maternal hypothyroidism in rats causes a delay in the development of radial glia scaffolds and reduced thickness of the visual cortex (Martinez-Galan et al., 2004). If this is an effect of differential expression of *reelin* mRNA, a gene believed to be of importance for the laminar distribution of neurons and known to be low during perinatal hypothyroidism remains to be

established (Alvarez-Dolado et al., 1999). Other potential mechanisms could include the reduction in BDNF, NT3 and the neurotrophin receptors TrkA and TrkB during hypothyroidism (Thompson and Potter, 2000).

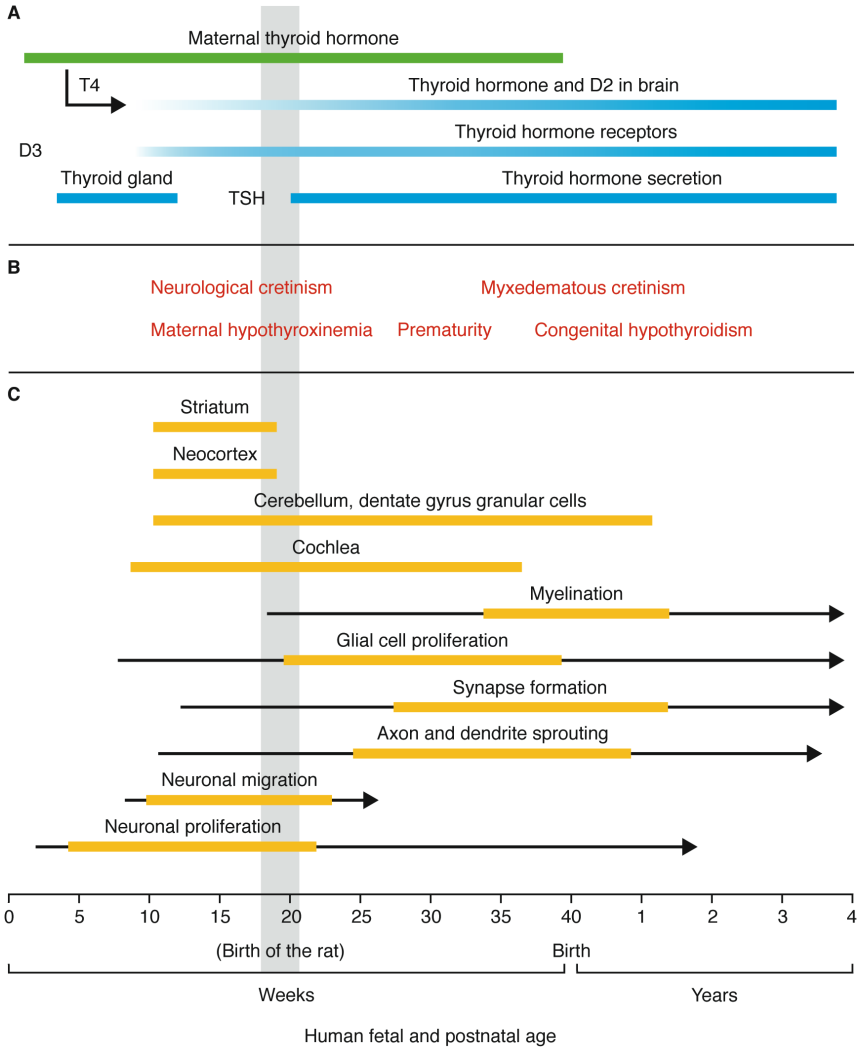

Figure 5. Overview of rat and human brain development in relation to thyroid hormone availability. A: development of the thyroid gland, thyroid hormone secretion and thyroid hormone receptor expression in the fetal brain. Maternal thyroid hormone is available during most of pregnancy, and is the source of T4, which is converted to T3 by D2 in the fetal brain. D3 is present from early gestation. B: temporal representation of insults caused by thyroid hormone deficiency. C: timing for different neurodevelopmental processes. Modified from (Bernal, 2007).

In the experiments described above, hypothyroidism or iodine deficiency was induced in pregnant rats and in several cases continued after birth. Because of the difficulties in distinguishing between early and late events, but also maternal thyroid hormone deficiency from fetal defects in hormone synthesis, strategies for inducing early maternal hypothyroxinemia have been developed. This revealed that maternal hypothyroxinemia in rats alters the neuronal cytoarchitecture of the cortex in the offspring (Lavado-Autric et al., 2003). Specifically, a time window was been defined, during which the fetus is dependent on maternal thyroid hormone. During this period, embryonic day 10-13 (E10-13) in mice and E12-15 in rats (corresponding to the end of first trimester to mid gestation in humans), even a mild hypothyroxinemia can affect the layering of the rat neocortex (Auso et al., 2004) and guidance of migrating neurons in mice (Cuevas et al., 2005). Importantly, rats with a transient hypothyroxinemia during this critical time also show an increased susceptibility to audiogenic seizures (Auso et al., 2004) and impairments in spatial learning and long-term potentiation response, probably correlated with an absence in *c-fos* activation and increase in the postsynaptic proteins PSD-95, NR1 and TrkB (Opazo et al., 2008). These experiments argue for the necessity of appropriate thyroid hormone levels during pregnancy, but do not prove that the consequences of maternal hypothyroxinemia in humans arise from defects in cortical layering and migration.

Thyroid hormone in the developing cerebellum

A substantial amount of work on thyroid hormone in rodent brain development has focused on the cerebellum. Hypothyroidism during the postnatal period causes structural defects in the cerebellum, most strikingly in granule cell migration and maturation as well as on Purkinje cell differentiation. Granule cells are born and proliferate in the external granular layer (EGL) and then migrate to the internal granular layer (IGL) where differentiation into mature granule cells takes place. Hypothyroidism delays this process, and it has been shown that the ligand-bound TRα1 has a permissive effect, as TRα1-/- mice are protected from the migration deficits caused by hypothyroidism (Morte et al., 2002). This was further evidenced in mice with a point mutation in TRα1 (TRα1R438C), resulting in a receptor with a 10-fold lower affinity to thyroid hormone: the heterozygotous animals (TRα1+/m mice), which are euthyroid but suffer from a receptor-mediated hypothyroidism, show the same delay in granule cell migration as wildtype (wt) mice made hypothyroid (Tinnikov et al., 2002). It however remained unknown if this is a cell autonomous effect or if TRα1 is expressed in substrate cells providing migratory signals. A second prominent effect of hypothyroidism is on Purkinje cell terminal differentiation, which has been shown to be dependent on TRα1 (Heuer and Mason, 2003; Morte et al., 2002). The involvement of TRβ is disputed: whereas Morte and colleagues partially rescued the defects on Purkinje cell development caused by hypothyroidism when treating the mice with a TRβ selective T3 analog, Heuer and Mason could demonstrate an insensitivity to T3 of primary Purkinje cells *in vitro* derived from TRα1-/- mice but not of those derived from TRβ-/- mice (Heuer and Mason, 2003; Morte et al., 2002). In addition, the TRs have been implicated in the development of cerebellar γ-aminobutyric acid (GABA)ergic interneurons from Pax-2 expressing precursor cells (Manzano et al., 2007).

Finally, the role of thyroid hormone on development of glia cells in the cerebellum has been investigated, firstly establishing that it is needed for Bergmann glia maturation

(Clos et al., 1980). For maturation of cerebellar astrocytes and Golgi epithelial cells, interplay between TRα1 and TRβ has been suggested as hypothyroidism normalizes the altered expression pattern of astrocyte markers observed in TRα1-/- mice and the aberrant expression is further enhanced by a TRβ specific ligand in the same mice (Morte et al., 2004).

Adult neurogenesis

Neurogenesis in the adult brain is confined to two areas; the dentate gyrus of the hippocampal formation and the subventricular zone (SVZ) adjacent to the lateral ventricles. In recent years it has been demonstrated that adult-onset hypothyroidism affects the generation of new neurons in both these regions (Ambrogini et al., 2005; Desouza et al., 2005; Lemkine et al., 2005). Proliferating granular cell progenitors of the dentate gyrus are located in the subgranular zone (SGZ). After cell cycle exit the neuroblasts migrate to the granular cell layer while forming dendritic arbors and sequentially expressing stage-dependent markers. Only a subset of progenitors will differentiate into new neurons or glia and many will instead undergo apoptosis. In two studies adult-onset hypothyroidism did not affect the proliferative state of progenitors, but significantly reduced their survival (Ambrogini et al., 2005; Desouza et al., 2005). In a later report, proliferation in the SGZ was also shown to be affected by hypothyroidism (Montero-Pedrazuela et al., 2006). In addition Montero-Pedrazuela and colleges found a reduction in both number of doublecortin positive (DCX+) immature neurons and altered growth of their dendritic tree, but no difference in cell survival. In the second adult neurogenic area, the SVZ, hypothyroidism decreased proliferation and the number of cells that progressed and differentiated into neurons, effects that were ascribed to the liganded TRα1 (Lemkine et al., 2005). The reasons for the different outcomes have not been clarified, but may result from different BrdU incorporation protocols or means of inducing hypothyroidism (goitrogens or thyroidectomy). Apart from the indication obtained by Lemkine and colleges *in vitro*, the TR isoform responsible for the thyroid hormonal effects on adult neurogenesis have remained unknown.

THYROID HORMONE ON MAINTENANCE OF BRAIN FUNCTION

Thyroid hormone and behavioural alterations

The consequences of thyroid hormone deficiency on behaviour have been studied extensively in the rodent. From this work it is clear that lack of thyroid hormone is associated with anxiety, increased fear, impaired cognition and reduced explorative behaviour. These problems have been linked to structural alterations in the brain, but the molecular actions are still to be unravelled. Mice lacking TRα1 (TRα1-/- and TRα0/0 mice) do not display a severe neurological phenotype, although behavioural analyses have revealed a moderately increased anxiety and fear, decreased explorative behaviour and impairments in learning (Guadano-Ferraz et al., 2003; Wilcoxon et al., 2007). Because TRα0/0 mice lack the whole TRα locus including TRα2 it is however not clear which isoform causes the phenotype. Indeed, locomotor activity is decreased

in TRα0/0 mice but not affected when only TRα1 is ablated (Wikstrom et al., 1998). It should also be noted that TRα0/0 mice are hypothyroxinemic and it is therefore possible that some of the behavioural defects are caused by developmental hypothyroidism rather than lack of TRα. TRα1+/m mice with a receptor-mediated hypothyroidism show a striking behavioural phenotype, with high anxiety and reduced cognition (Venero et al., 2005). Because the mutation does not completely abolish binding of ligand, it can be reactivated by administration with supraphysiological levels of thyroid hormone. Reactivation of the mutant receptor in adult life normalizes the behaviour of the mutant mice, suggesting that activity of the unliganded TRα1 hampers adult brain function.

The functional effects of altered cellular and synaptic organization caused by thyroid hormone deficiency have only started to become elucidated. This is critical because without such data there is no proof that the abnormalities found using immunohistochemistry actually cause the behavioural alterations described above. Electrophysiological recordings have revealed that developmental hypothyroidism reduces excitatory synaptic transmission, impairs long-term potentiation, reduces inhibition and affects mechanisms of neurotransmitter release (Dong et al., 2005; Gilbert and Sui, 2006; Gilbert et al., 2007; Niemi et al., 1996; Vara et al., 2002). Also, iodine deficiency or hypothyroidism throughout gestation and lactation result in impaired induction of long term potentiation and reduced expression of the immediate early genes *c-fos* and *c-jun* (Dong et al., 2005). A correlation between a behavioural phenotype and defects in network properties remains to be established.

Excitability and seizure generation

Feedback inhibition of excitatory neurons projecting their axons between cortical areas is of outmost importance, as continuous firing would lead to seizure propagation and eventually cell death. Excitatory neurons utilizing the neurotransmitter glutamate are therefore under constant control by local circuit inhibitory GABAergic interneurons that modulate the output from the cortex. A variety of interactions between thyroid hormone and components of the GABA system have been reported such as regulation of GABA receptor function, GABA synthesis, release and reuptake, as reviewed earlier (Wiens and Trudeau, 2006). Hypothyroidism is known to increase seizure susceptibility and a mild transient hypothyroxinemia results in increased audiogenic seizure susceptibility in the offspring (Auso et al., 2004). Also, TRβ deficiencient mice are susceptible to auditory seizures (Ng et al., 2001). The GABAergic interneurons can be divided into subtypes based on their morphology, electrophysiological properties and neurochemical phenotype, such as their differential expression of calcium binding proteins (Markram et al., 2004). Hypothyroid rats show a decreased expression of the calcium binding protein parvalbumin (PV) in the somatosensory cortex and hippocampus (Berbel et al., 1996; Gilbert et al., 2007). In addition, deficiency in TRα1 results in a reduced density of inhibitory perisomatic terminals expressing PV in the hippocampus (Guadano-Ferraz et al., 2003; Venero et al., 2005). In TRα1+/m mice, T3 administration during adulthood normalizes the number of PV+ terminals (Venero et al., 2005). Other means of reduced inhibition that could explain increased excitability and seizure susceptibility have also been reported, such as high mu opioid receptor and

reduced benzodiazepine receptor binding during adult-onset hypothyroidism (Ortiz-Butron et al., 2003).

EXPRESSON OF THYROID HORMONE RECEPTORS IN THE BRAIN

T3 binding activity has been demonstrated as early as at E14 in the rat (Perez-Castillo et al., 1985) and TRα and β are differently expressed during brain development (Bradley et al., 1989; Forrest et al., 1990; Strait et al., 1990). Later, it was demonstrated that TRα1 contributes to 70-80% of all TR expression in the brain (Schwartz et al., 1992). However, these and other attempts to define the distribution of TR isoforms in the brain used *in situ* hybridization techniques on tissue sections and immunoprecipitation or quantitative PCR performed on homogenized tissue or primary cell culture explants. Unfortunately, the lack of readily available antibodies against the TRs has prevented double labelling of cells for identification of TR isoform expression in specific cell types on brain sections, and results from *in vitro* experiments are of limited use. Below follows a summary of previous results on TR expression in the three main cell types of the brain: neurons, astrocytes and oligodendrocytes, including their precursors in the developing brain. The differences observed between *in vitro* and *in vivo* data are likely to be caused by the absence of diffusible factors such as neurotrophins, or contacts with astrocytes, neurons and their axons in tissue culture preparations.

Distribution of TR expression in the brain

In the developing rat brain, Mellström and colleagues studied TRα1 and TRβ1 mRNA expression from E14 until adulthood (Mellstrom et al., 1991). From E19 until birth TRα1 was found to be expressed in the outermost part of the cerebral cortex, CA1 in the hippocampus, hypothalamus and thalamus. The authors also noted a distinct peak in expression between the first and third week after birth in areas such as the cortex, amygdala, hippocampus and cerebellum. This was followed by a lowered expression that persisted until adulthood. A probe that recognized both TRα1 and TRα2 transcripts showed a similar expression pattern. TRβ was very low or absent in the embryo but increased drastically after birth. Expression was especially high in the caudate putamen, the CA1 field of the hippocampus and certain layers of the olfactory bulb but notably not detected or very low in the cerebellum, thalamus and brain stem. In parallel studies, Bradley and colleges found a wide distribution of TRα1 and α2 mRNA throughout gestation into adulthood (Bradley et al., 1992; Bradley et al., 1989). The earliest expression was found at E11.5 in the neural tube. TRβ1 expression was first detected at E11.5 and was only present in a subset of TRα expressing areas.

During the same time*, in situ* hybridization on chicken brains showed TRα to first be expressed at E5 and continued to be present throughout development in many fore-, mid, and hindbrain areas, including various layers of the telencephalon, the hippocampus and the hypothalamus (Forrest et al., 1991). TRα was also strongly expressed in the developing cerebellum and was present both in the EGL and later in the IGL after inwards migration of the developing granule cells. In contrast to the

continuous expression of TRα, TRβ was sharply induced after E19. At this time its expression pattern was similar to that of TRα with the important exception of the cerebellum where TRβ was absent in the EGL but highly expressed in the white matter. In the Purkinje cells TRα was strongly expressed whereas TRβ expression was only slightly above background.

TR expression in glia

Several research groups have demonstrated that TRα1 is expressed in both oligodendrocyte precursor cells (OPCs) and mature oligodendrocytes (OLs), whereas TRβ is only expressed in mature OLs (Baas et al., 1994; Billon et al., 2002; Billon et al., 2001; Carlson et al., 1994; Fierro-Renoy et al., 1995). However, there has been some controversy because of other reports of expression of TRβ1 and TRβ2 in rat OPCs (Barres et al., 1994; Gao et al., 1998; Kondo and Raff, 2000). These differences are likely to arise from the types of techniques and preparations used, in addition to origin of the examined cells.

Mature OLs have been shown to express all TR protein isoforms, but a shift from TRα1 to TRβ expression has also been reported (Carlson et al., 1994; Carre et al., 1998). These *ex vivo* data from rat brain showed that TRα decreases dramatically after postnatal day 20 (P20), and that very few mature OLs express TRα after P40 (Carre et al., 1998) whereas most express TRβ (Carlson et al., 1994). In addition, TRβ1 and TRβ2 mRNA was not expressed in cultures of purified optic nerve OPC from rat(Billon et al., 2001). Work on mice deficient of TR isoforms has enhanced and confirmed previous data: TRβ-/- mice have normal numbers of optic nerve oligodendrocytes and their OPCs show a normal response to thyroid hormone in timing of differentiation (Billon et al., 2001). In contrast, TRα1-/- mice have a decreased number of oligodendrocytes in the optic nerve and these fail to stop dividing and differentiate in response to thyroid hormone (Billon et al., 2002). Moreover, overexpression of TRα1 accelerates the differentiation into oligodendrocytes, suggesting that the amount of TRα1 available to the cell is limiting its capacity to respond to thyroid hormone and differentiate.

Dussault and colleagues have performed a number of immunohistological studies on TR expression in cultured neurons and astrocytes in addition to one report on TR expression in the adult rat brain (Garza et al., 1990; Luo et al., 1989; Puymirat et al., 1991). These experiments were however performed using an antibody that binds to all TR isoforms and it is not clear whether it cross-reacts with other nuclear hormone receptors such as the retinoic acid receptor. Later, the presence of TRα1, TRα2 and TRβ1 mRNA in rat primary cultures of both from neurons and astrocytes were reported (Lebel et al., 1993; Leonard et al., 1994; Puymirat et al., 1992).

AIMS

The research presented in this thesis aimed towards elucidating the role of the TRα1 in brain development and function. In doing this I wanted to identify specific deficits in the brain that are associated with the symptoms of endemic cretinism and bring further evidence for the importance of maternal thyroid hormone during pregnancy. Specific questions addressed in the papers are:

(I) How does a mutation in TRα1, causing a receptor-mediated hypothyroidism, affect the development of motor skills? If such defects are found, when during brain development are they established, and what specific alterations on the tissue level are they caused by?

(II) Does the mutation in TRα1 and the hampered neuronal network it results in lead to altered seizures susceptibility? Here we also further investigated the neuronal network impairments described in paper I.

(III) What is the temporal and spatial expression of TRα1in the brain? The aim of this study was to generate a mouse line that could be used to determine what specific cells in the brain express TRα1, and its role for these cells to develop and/or function properly.

(IV) Adult hippocampal neurogenesis is of importance for memory, learning and mood regulation and is affected by adult-onset hypothyroidism. In this paper we wanted to know if TRα1 is expressed in the hippocampal neurogenic niche and determine if it regulates neuronal survival and differentiation in the adult brain.

RESULTS AND DISCUSSION

LOCOMOTOR DEFICIENCIES AND DEVELOPMENT OF GABAERGIC INTERNEURONS IN MICE WITH A RECEPTOR-MEDIATED HYPOTHYROIDISM (PAPER I)

Locomotor dysfunctions of TRα1+/m mice

Recent research has demonstrated that many of the defects in the brain during hypothyroidism are caused by the unliganded TRα1 and we therefore generated mice with a mutation in this receptor (TRα1R384C), which reduces the affinity to ligand. Under physiological levels of thyroid hormone the receptor will remain largely unliganded, resulting in a receptor-mediated hypothyroidism in the mice, as the mutant receptor is dominant over the wt counterpart. The mice display a severe neurological phenotype with high anxiety and memory impairments (Venero et al., 2005). Locomotor deficiencies of cerebellar origin (i.e. rotarod performance) had also been demonstrated.

The severe consequences of iodine deficiency during pregnancy on psychomotor development in the offspring are well established, but the primary cause at the tissue level has remained unknown. Previously, thyroid hormone related locomotor deficiencies have been associated with cerebellar defects, but because the cerebellum develops relatively late (postnatally in the rat), aberrancies in brain regions that mature earlier are also to be expected. In addition, it was not known how thyroid hormone exerts its effect in this context, i.e. through what receptor isoform and in which cell type. We therefore decided to investigate the locomotor aberrancies of the TRα1+/m mice further, primarily using the robust hanging wire test. This revealed that the mutant mice were impaired in clinging to a wire grid, whereas wt mice were able to hang upside down from the wire for at least one minute. This performance of the mutant mice was accompanied by reduced grip strength.

The anxiety and memory impairments of the TRα1+/m mice had successfully been treated with T3 in the drinking water during adulthood, whereas the performance on the rotarod could only be ameliorated by treating juvenile mice (Venero et al., 2005). To determine when the motor defects were founded, we administrated thyroid hormone during P10-35 or as adults; however this did not result in improved performance in the hanging wire test. To test if the lesion causing the inability was founded during an earlier time period we used mice that are, in addition to the mutation in TRα1, deficient in TRβ (TRα1+/mTRβ-/- mice). Because TRβ is needed to maintain thyroid hormone homeostasis through the HPT axis, these mice have 10-fold elevated thyroid hormone levels, sufficient to reactivate the mutant receptor (Forrest et al., 1996; Tinnikov et al., 2002). To reactivate the mutant receptor during pregnancy when the fetus is dependent on maternal thyroid hormone, we used TRβ deficient dams. We found that the ability in the hanging wire test was only restored when the mutant mice received elevated levels of thyroid hormone during early fetal development in combination with high levels throughout subsequent development, as hyperthyroid TRα1+/mTRβ-/- mice born by hyperthyroid TRα1+/+TRβ-/- dams performed as good as wt mice, whereas their

euthyroid TRα1+/mTRβ+/- littermates were not enhanced in their performance. To pinpoint the time when the fetus is most dependent on thyroid hormone for performance in the hanging wire test we injected euthyroid pregnant dams carrying TRα1+/mTRβ-/- pups with thyroid hormone during E10.5-13.5, before fetal secretion of thyroid hormone began. The treatment resulted in an improved performance, demonstrating that the fetus requires maternal thyroid hormone during this time of development. In addition to the hanging wire test we identified aberrancies in locomotor performance and gait using the beam walk test and foot print analysis. These disabilities were of mixed perinatal origin as some could be restored by postnatal reactivation of the mutant receptor whereas others required prenatal reactivation.

Delayed development of parvalbumin cells and an increased number of calretinin neurons

To identify the cause of the locomotor dysfunctions we performed a systematic analysis of tissues involving motor function. We excluded a muscular deficiency, abnormal innervation and gross myelination defects of nerves descending from the spinal cord. As the TRα1+/m mice have a reduced number of PV+ perisomatic terminals in the hippocampus (Venero et al., 2005) and the locomotor deficiencies were of prenatal origin, we decided to investigate the development of PV+ interneurons in the motorcortices of mutant mice. Interneurons are born in the MGE of the ventral telencephalon and then migrate tangentially to the neocortex where they differentiate. The first neurons of the PV subtype are not differentiated until P10 whereafter the number of PV+ cells increases. We found a remarkable reduction in PV+ interneuron number at P14 persisting until adulthood. In addition, the mutant mice had an increased number of calretinin positive (CR+) cells, whereas the numbers of other subclasses of interneurons were not affected. We concluded that there was not a direct regulation of PV expression by TRα1, because treating the mice with thyroid hormone for three days (P10-13) did not increase PV levels. However, TRα1+/mTRβ-/- mice had an increased number of PV+ cells, correlating PV interneuron development with the locomotor phenotype. In contrast, the numbers of other subtypes of GABAergic interneurons, i.e. calbindin (CB)+ and somatostatin (SOM)+ neurons were equal to that found in wt mice, while CR+ cells were increased. We next explored if the delayed development of PV cells resulted in functional defects such as impaired network properties of TRα1+/m brains. This was done using an *in vitro* slice setup, demonstrating that the mutant mice had a reduced number of fast spiking cells and lower frequency of rhythmic network oscillations in the gamma frequency range. As the PV interneurons are fast spiking cells important for setting the frequency of gamma oscillations, our results suggest that the mistimed development of GABAergic interneurons results in aberrancies of the neuronal network that could explain the locomotor deficiencies of the mutant mice.

SEIZURE SUSCEPTIBILITY AND HIPPOCAMPAL CIRCUITRY FUNCTION IN MICE WITH A MUTANT TRα1 (PAPER II)

Reduced seizure susceptibility *in vivo*

In paper I we showed that mice with the TRα1R384C mutation, in addition to their reduced number of PV+ terminals in the CA1 field of the hippocampus, showed a mistimed development of GABAergic interneurons and alterations in cortical network activity. Reduction in the number of GABAergic interneurons is expected to lead to unbalance between excitation and inhibition and perhaps development of seizures. TRα1+/m mice do not display spontaneous seizures and we therefore decided to induce seizures chemically using the convulsant drug pentylenetetrazol (PTZ). To our surprise, the mutant mice were less susceptible to seizures than the wt controls: none of the mutant mice developed tonic-clonic convulsions in contrast to wt mice, which all displayed generalized clonus shortly after PTZ injection. The development of seizures in the wt mice was associated with *c-fos* induction in the hippocampus, identifying this region as the main area for seizure propagation. Quantitative PCR of $GABA_A$ receptor subunits excluded an increased number, or altered composition, of the receptor complex as a cause for the resistance to the $GABA_A$ receptor antagonist PTZ. Administration of thyroid hormone during different time periods during development and in adult life demonstrated that thyroid hormone is required both during early gestation, when the embryo is dependent on maternal thyroid hormone, and at later developmental events after birth for a normalized response to PTZ stimulation.

Altered hippocampal circuitry and hypoexcitability of pyramidal neurons

To test the properties of the hippocampal network we performed electrophysiological recordings in the CA3 region of hippocampal slices. In paper I we demonstrated that the mutant mice have a reduced frequency of gamma oscillations induced with kainic acid, an agonist at the kainate class of ionotropic glutamate receptor. These physiological activities can be converted into pathological epileptic-like interictal waves and we found that a lower percentage of mutant mice displayed such pathological activities than wt controls. Moreover, when decreasing inhibitory neurotransmission with PTZ only 31% of mutant slices developed interictal waves as compared to 73% of wt slices. These *in vitro* recording therefore mirrored the *in vivo* finding that the mutant mice are resistant to PTZ-induced seizures. To elucidate the cellular mechanism behind the reduced seizure susceptibility we performed patch clamp recordings of pyramidal cells. This revealed that both mutant and wt mice responded to superinfusion with kainic acid by depolarization, but while the subsequent addition of PTZ did not change the membrane voltage in the wt mice, it resulted in hyperpolarization of pyramidal neurons of mutant mice. In conclusion, our *in vitro* findings are consistent with the reduced susceptibility to seizures observed *in vivo*.

Lack of dorsoventral gradient of calretinin-positive hilar cells

We next hypothesized that the hyperpolarizing effect of PTZ on pyramidal neurons is caused by depolarization by GABA. A possible reason for depolarization could be an altered chloride ion distribution across the plasma membrane, as is normally found early in development when GABA has an excitatory effect on neurotransmission. Indeed, quantitative PCR revealed that TRα1+/m mice had a decreased expression of the chloride importer Na/K/Cl cotransporter 1 channel (NKCC1), indicating that TRα1+/m mice may have a lower concentration of intracellular Cl^-. However, if GABA would have a depolarizing effect in the mutant mice they should display spontaneous seizures unless there are other abnormalities that counteract seizure development. Such defects could lie anywhere from the perforant path to the dentate gyrus to the CA1 and CA3 regions. Deletion of mossy cells in the hilus of the hippocampus has been proposed to decrease the excitability of granule cells (that subsequently relay information to the CA3 region). Mossy cells express CR in a dorsoventral gradient, with more CR+ cells in the ventral hippocampus. We found that this gradient is absent in the mutant mice, which express CR in a greater number of cells in particularly the dorsal region. We accordingly proposed the increased CR expression to be compensating for the decreased inhibition in the mutant mice. However, there are other explanations of our observations that should also be considered and alternative synaptic changes in the TRα1+/m mice therefore need to be elucidated. For example, the effect of PTZ on GABA release and alterations in glutamate signalling should be explored.

TEMPORAL AND SPATIAL EXPRESSION OF TRα1 IN THE BRAIN (PAPER III)

Generation of TRα1-GFP knock-in mice

As confirmed in paper I and II lack of thyroid hormone has profound effects on brain development. In recent years we and others have identified specific cell types and brain areas that are particularly vulnerable to thyroid hormone deficiency because of the repressor activity of the unliganded TRα1. However, the lack of reliable antibodies against the TRs has been a great limitation to a detailed understanding of how thyroid hormone exerts these effects. Especially studies on thyroid hormone function in the brain have suffered because of its regionalization and great diversity in cell types. We decided to address this problem by generating transgenic mice that express TRα1 and green fluorescent protein (GFP) as a chimeric protein from the endogenous *Thra* locus. This was done through gene targeting by inserting the sequence of GFP *in frame* 3' to exon 9 of *Thra*, resulting in knock-in mice that express TRα1-GFP under the native promotor, i.e. where and when the gene is normally active.

The mouse line was validated by determination of mRNA expression from the *Thra* locus and analyses of endocrine and physiological parameters. This showed that the gene targeting event resulted in an increased amount of TRα1-GFP as compared to wt TRα1 and, unexpectedly, an abolished expression of TRα2. However, analyses of organ weights and parameters known to be influenced by thyroid hormone only revealed

minor aberrancies in the transgenic mice. Specifically, the body, brain and heart weights of the TRα1-GFP mice were normal. However, liver weights were slightly reduced in homozygote male mice. Furthermore, the serum levels of T3 and T4 were the same as those of wt mice as were the mRNAs for TSHβ and liver Dio1, thus indicating normal thyroid hormone metabolism. We also found that mice homozygote for TRα1-GFP failed to suppress fully the cardiac target gene MyHCβ. This is likely to reflect a diminished efficacy of the TRα1-GFP aporeceptor to repress target genes, which had also been indicated in transfection experiments. The primary application of the TRα1-GFP mice was to determine the expression of TRα1 in the brain and we therefore tested for a potential effect of the chimeric protein on the brains of the mice. We found that in the neonatal brain there was no significant difference in the gene expression of thyroid hormone regulated genes RC3 or hairless or for the corepressors alien and NCoR. Furthermore, studies of the behaviour of TRα1-GFP mice using the SHIRPA protocol revealed no abnormalities. In summary, the data suggest that there are no major deficiencies in brain function of TRα1-GFP mice as compared to wt littermates. Nevertheless, homozygous TRα1-GFP mice may have minor impairments in the regulation of TRα1 target genes and we therefore decided to conduct all forthcoming studies on heterozygote mice.

TRα1 is expressed in essentially all mature neurons in the adult brain

Double immunohistochemistry against GFP and neuronal nuclei (NeuN) revealed that essentially all mature neurons in the adult brain expressed TRα1. This included the neocortex, the caudate putamen, the CA1 and CA3 regions, the granular and hilar cells of the dentate gyrus, thalamus and hypothalamus. The only exception was mature Purkinje cells of the cerebellum. TRα1 was also found in glial fibrillary acidic protein (GFAP) expressing tanycytes lining the third ventricle in the hypothalamus. In contrast, TRα1 was not expressed in GFAP+ astrocytes in any other part of the forebrain or midbrain. TRα1 was also absent in SRY related HMG box 10 (Sox10) expressing mature oligodendrocytes other than in the hypothalamus.

TRα1 expression in the cerebellum

In the cerebellum of adult mice, we found TRα1 to be expressed in stellate/basket cells, non-interneuronal NeuN+ cells of the molecular layer, granule cells and glia. Thyroid hormone is needed for the differentiation of Purkinje cells but we found no TRα1 expression in these cells of adult mice. However, at P7 the Purkinje cells indeed expressed TRα1, in line with previous results (Heuer and Mason, 2003). It is known that lack of thyroid hormone leads to delayed migration of cerebellar granule cells and that TRα1 mediates this effect (Morte et al., 2002). When we examined TRα1 expression in the developing cerebellum we found that TRα1 was expressed in cells of the molecular layer with increased intensity as the cells reach the IGL. In contrast, TRα1 was absent in the EGL, indicating that TRα1 is not needed to maintain granule cell precursors in a proliferative state. The identity of the TRα1+ cells in the molecular layer at this time is not known, but their morphology and location suggest that they may be migrating granule cells.

TRα1 is expressed in postmitotic immature neurons

We next decided to determine when during neuronal development TRα1 expression is turned on. At the earliest time point investigated, E9.5, we found no TRα1 expression in any part of the brain. At E13.5 TRα1 was expressed in the cortical plate and marginal zone. In proliferating cells, i.e. in the ventricular zone, TRα1 was absent. This is in accordance with what we found in the cerebellum where TRα1 was expressed in the IGL but not in the EGL. TRα1+ cells at both E13.5 and E17.5 coexpressed β-tubulin III (β-tubIII), demonstrating that they were immature postmitotic neurons. TRα1 continued to be expressed in the cortical plate throughout development, but was at earlier time points markedly higher in neurons that had already found their laminar position within the neocortex than in subsequent neurons of the cortical plate.

ROLE OF TRα1 IN ADULT HIPPOCAMPAL NEUROGENESIS (PAPER IV)

Expression of TRα1 in an adult neurogenic niche

Adult-onset hypothyroidism reduces hippocampal neurogenesis, but the role of different TR isoforms in this process is not known. We used the TRα1-GFP mice to determine the expression of TRα1 in proliferating and postmitotic neuronal progenitors. Using BrdU incorporation to label mitotic cells we could show that TRα1 was not expressed in proliferating cells of the SGZ. After cell cycle exit TRα1 was turned on in the SGZ and granular cell layer (GCL) as demonstrated with immunohistochemistry against DCX and the basic helix-loop-helix transcription factor NeuroD. This established TRα1 as a possible mediator of thyroid hormone actions during hippocampal neurogenesis and we therefore decided to elucidate this role further.

Effect of the unliganded TRα1 on proliferation and survival of adult hippocampal progenitors

The potential function of TRα1 in hippocampal neurogenesis was investigated in three different mouse lines: TRα1-/-, TRα2-/- and TRα1+/m mice. Proliferation was assessed with BrdU injections two hours before the mice were sacrificed and with the endogenous mitotic marker PCNA. The result showed no difference in cell cycling in any of the mouse lines as compared to wt controls. However, when examining the number of surviving cells 30 days after BrdU incorporation, opposing effects on survival were obtained in TRα1-/- mice as compared to TRα2-/- and TRα1+/m mice. The finding that TRα1-/- mice had an elevated number of cells expressing stage-specific markers for postmitotic neuroblasts, e.g. DCX, stathmin and PSA-NCAM, corroborated the result. In contrast, TRα2-/- and TRα1+/m mice showed a reduced number of postmitotic progenitors. TRα2-/- mice overexpress TRα1 and abnormalities previously defined in these mice were explained by this increased expression while no phenotype could be assigned to TRα2 (Salto et al., 2001). We therefore hypothesized that the TRα2-/- mice have an imbalance in the ligand/receptor ratio leading to TRα1 aporeceptor activity under physiological levels of thyroid hormone (see paper IV, fig.

10). Treating the mice with thyroid hormone tested this hypothesis. Indeed, thyroid hormone administration rescued the decline in DCX cell number of TRα2-/- mice, suggesting that a limited availability of ligand for TRα1 was causing the decline in neuronal progenitors. A similar result was obtained with the TRα1+/m mice in which thyroid hormone administration normalized the number of surviving cells 30 days after BrdU incorporation. In addition, NeuroD+ cell number was reduced in both TRα2-/- and TRα1+/m mice, but thyroid hormone treatment only increased the expression significantly in TRα1+/m mice. In all these experiments thyroid hormone administration also increased the number of BrdU, DCX and NeuroD+ cells in wt animals. In conclusion, the results suggest that the survival of adult hippocampal progenitors is regulated by the availability of ligand for TRα1.

Differentiation of newborn progenitors

To assess if TRα1 influences the number of progenitors that differentiate into mature neurons, cells were stained for BrdU and NeuN 30 days after BrdU injection. In TRα1-/- and TRα2-/- mice there was no difference in the number of cells that were positive for these markers. Similarly, there was no change in differentiation of cells into glia, as shown with GFAP immunohistochemistry. In TRα1+/m mice however, there was a decreased number of cells positive for BrdU and NeuN, which was normalized with thyroid hormone treatment. These mice also showed a decline in the number of CR+ cells in the SGZ, representing a fraction of postmitotic progenitors that are destined for neuronal differentiation. We therefore found that the reduction in progenitor survival in the TRα1+/m mice resulted in a decreased number of differentiated neurons whereas differentiation was not altered in the TRα1-/- or TRα2-/- mice.

Differences between overexpression and a mutation in TRα1

Recent reports have implicated adult-onset hypothyroidism with reduced neurogenesis. In this paper we found a decline in survival and differentiation of adult hippocampal progenitors in mice that overexpress TRα1 or that have a TRα1-mediated hypothyroidism. Our results show a resemblance to what is observed during adult-onset hypothyroidism, suggesting that unliganded TRα1 causes a reduction in neurogenesis. Lack of TRα1 had the opposite effect, indicating that it is the repressing activity of the aporeceptor, which is absent in the TRα1-/- mice, that reduces progenitor survival. In addition, there was a reduction in differentiation of progenitors into mature neurons in the TRα1+/m mice but no change in TRα1-/- and TRα2-/- mice. The reason for these differences may be caused by the strong aporeceptor activity in the TRα1+/m mice as compared to overexpression of TRα1 in the TRα2-/- mice. In addition, differences in TR stoichiometry may underlie the variations.

GENERAL DISCUSSION

Effects of the TRα1 aporeceptor on brain development and function

In papers I and II we demonstrated that thyroid hormone deficiency during fetal and postnatal development leads to impaired development of GABAergic interneurons and defects in cortical network activity. Thus, functions for thyroid hormone during maturation of the cortex are expected. Our result in paper IV that TRα1 is expressed in postmitotic neurons and subsequently in all NeuN+ neurons of the adult brain establishes that TRα1 has the potential to modulate differentiation of all neuronal types. The reason for the increased vulnerability of the CR and PV subclasses of interneurons to the mutation in TRα1 is currently not known. Our results indicate that similar dysfunctions may exist for other classes of neurons, perhaps at other time periods during brain development. In addition, we could demonstrate in paper II that the TRα1+/m mice are resistant to PTZ induced seizures. Even though we speculated that an increased expression of CR in the hilus contributed to the seizure phenotype, our recordings of pyramidal cells indicated that thyroid hormone also affect neurotransmission at the synaptic level. The full extent of this function is still to be explored.

Cellular mechanism of TRα1 action in neuronal development

In papers III and IV we showed that TRα1 expression was switched on in neuronal progenitors at a stage during their development that was the same in three distinct brain regions that mature at different times relative to each other. Although the expression pattern largely agreed with available *in situ* hybridization data, it was unexpected to find that TRα1 was expressed in essentially all developing and adult neurons.

The results give rise to the question of how TRα1 exerts its effects on progenitor survival and/or neuronal maturation. In the embryo we found colocalization of TRα1 with β-tubIII, identifying the cells as postmitotic neurons. In agreement with this, TRα1 expression preceded the mature neuronal marker NeuN. In the adult brain TRα1 was expressed in DCX+ and NeuroD+ progenitors. In neither the developing brain or in the adult was TRα1 associated with regulation of progenitor proliferation. Despite of these novel findings, the exact stage of TRα1 upregulation during neuronal development is still to be determined. This data could be obtained with BrdU injections at different time points and with additional stage-specific markers. Identification of TRα1 interaction with target genes such as *NeuroD* or cofactors and coregulators would shed further light on the exact role of TRα1 in progenitor differentiation and survival (Chantoux and Francon, 2002).

Possible cell-autonomous effects and secondary effects through for example neurotrophins also need to be distinguished. Previous attempts to determine the contribution of neurotrophins such as BDNF and NT3 to thyroid hormone mediated cerebellar maturation have been inconclusive. Briefly, both a cell autonomous effect of TRα1 in the Purkinje cells and promotion from granule cells have been suggested

(Heuer and Mason, 2003; Neveu and Arenas, 1996). Our results from paper III that TRα1 is expressed in migrating granule cells outside the EGL and in early postnatal Purkinje cells corroborate both studies.

How does TRα1 regulate PV cell development?

In paper I we presented evidence that the unliganded TRα1 causes two impairments affecting GABAergic cells: a retardation of PV+ cell development and an increased number of CR+ cells seen as early as P14. That a three-day thyroid hormone administration failed to normalize the number of PV+ cells argues that TRα1 does not directly regulate PV expression. The delayed development of the PV cells may therefore be due to defects in differentiation or an impaired migration of the maturing cells from the MGE to the neocortex. Tangential migration is defective on hypothyroid cortical mounts (Cuevas et al., 2005) and the delayed appearance of PV+ cells in the TRα1+/m mice could therefore be caused by defective expression of migratory cues such as guiding cell surface proteins or secreted factors. However, a preliminary study using CB as a marker for tangentially migrating precursors at E13.5-17.5 revealed no gross changes in the mutant embryos (data not shown).

The subclass identity of PV+ and CR+ cells is predicted by their temporal and spatial origins (Butt et al., 2005). The mutant TRα1 could thus cause imbalance between the PV and CR cells by skewing the temporal choice of a precursor cell to enter the respective differentiation programmes. The transcription factor code that specifies interneuron development and migration has only recently begun to be unravelled. In particular, the LIM homeodomain transcription factor Lhx6 is expressed in specifically the PV+ and SOM+ subtypes and is required for both their tangential migration and specification (Liodis et al., 2007). We have used Lhx6 *in situ* hybridization at P14 to visualize PV precursors before they express PV. The preliminary result indicated that there was no obvious difference in Lhx6 expression between mutant and wt mice (Dudazy, Wallis and Vennström, data not shown). However, quantification of Lhx6+ cells in distinct cortical layers might reveal defects in radial migration. Such defects, which would ultimately result in delayed final differentiation and hence expression of PV, could be caused by the previously reported delay in postnatal development of radial glia during hypothyroidism (Martinez-Galan et al., 2004). Collectively, the results in paper I, together with our unpublished data suggest that the migration of interneurons from the MGE is not perturbed in the mutant mice, but that the TRα1 aporeceptor regulate the final maturation of PV cells. That TRα1 is first expressed in the cortical plate and marginal zone at later stages of neuronal maturation further support this idea.

Implications for human brain development

Surprisingly little has been known about the brain alterations underlying the severe mental retardation and motor dysfunctions that are the symptoms of endemic cretinism. In paper I we demonstrated that locomotor dysfunctions caused by the aporeceptor activity of TRα1 correlate with delayed development of GABAergic interneurons in the

motor cortex. The resemblance of the locomotor phenotype of the mice to that seen in endemic cretinism suggest that aberrant development of interneurons could occur also when thyroid hormone is absent during human brain development. This may be a risk also in iodine-sufficient countries, as even a mild transient hypothyroidism of the mother can affect brain development in the euthyroid offspring (Henrichs et al., 2010; Pop et al., 1999). This is emphasized by the suggestion to perform mass screening for maternal hypothyroidism and thyroid autoimmunity during pregnancy to prevent irreversible mental retardation (Morreale de Escobar et al., 2004). Our finding that a receptor-mediated hypothyroidism leads to functional deficits in the mice gives further credence to this view. The demonstration that TRα1 is expressed in all developing and mature neurons furthermore suggests a broad role for thyroid hormone during brain development and implies that maternal hypothyroidism or hypothyroxinemia may be associated with additional neurological disabilities.

Hypothyroidism in the adult human is associated with depression, attention and cognitive disorders (Davis and Tremont, 2007). These hippocampal-dependent defects have been correlated with reduced hippocampal neurogenesis (Deng et al., 2010). In addition, adult neurogenesis can influence the efficacy of treatment with antidepressants (Deng et al., 2010). Recently, it was demonstrated that adult-onset hypothyroidism results in deficient neurogenesis (Ambrogini et al., 2005; Desouza et al., 2005; Lemkine et al., 2005). One study correlated the reduction in neurogenesis with depressive-like behaviour and showed that thyroid hormone treatment both reversed the behaviour and increased the number of neuroblasts in the dentate gyrus (Montero-Pedrazuela et al., 2006). Our results in paper IV strongly suggest that the aporeceptor activity of TRα1 causes the defects in adult neurogenesis during hypothyroidism. In this context it is interesting to note that the TRα1+/m mice also exhibit impairments in hippocampal-associated behaviour (Pilhatsch et al., 2010). This merits further study to determine the implication of impaired adult neurogenesis on behavioural defects during adult-onset hypothyroidism.

SUMMARY AND FUTURE PERSPECTIVES

In the projects of my thesis work we have demonstrated that a receptor-mediated hypothyroidism through TRα1 results in locomotor dysfunctions during development that manifest in the adult mouse. We found that for development of these functions, the embryo is dependent on maternal thyroid hormone during E10.5-13.5 in addition to its own production throughout life. This is the first study that correlates maternal thyroid hormone deficiency through a distinct TR isoform with functional deficits in addition to histological evidence of aberrant brain development. In these mice we also made important and novel observations on GABAergic neurotransmission and hypoexcitability resulting from maternal hypothyroidism. To determine when and in what type of brain cells TRα1 is expressed, we generated mice that express TRα1 and GFP as a chimeric protein. With this new tool we established that TRα1 expression is first turned on during postmitotic stages of neuronal development and persists in essentially all mature neurons.

I have focused my studied on the TRs, which bind thyroid hormone and mediate its effects through regulation of gene expression. The results recognized the necessity of binding of thyroid hormone to TRα1 for progression of neuronal differentiation. Moreover, I found that TRα1 has the potential to affect all neuronal subtypes. This highlights the importance of thyroid hormone availability at the right time for proper brain development and emphasizes that for the full comprehension of thyroid hormone action during neuronal development, knowledge of expression of factors that regulate cellular thyroid hormone availability, such as deiodinases and transporters, are needed. Transcriptional coregulators further determine the response of target genes to thyroid hormone. Despite many efforts only a limited number of genes harbouring a TRE are known today. The TRα1-GFP mice will aid the identification of both novel target genes and coregulators through applications that previously have been challenging because of the lack of TRα1 specific antibodies, such as immunohistochemical techniques and immunoprecipitation. Finally, the exact stage of progenitor maturation during which TRα1 is first expressed needs to be decided. This would aid elucidation of the cellular mechanism of thyroid hormone induced progression in neuronal development and substantially enhance our understanding of thyroid hormone action in the developing nervous system.

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