In vitro validation of peptide-T3 conjugates as a treatment option for MCT8-deficiency via a “Trojan Horse”-like mechanism

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Abstract

Cells use transmembrane transporters to take up substances that are important for functionality and survival of the organism. One example of these substances are thyroid hormones (THs), tyrosine-based hormones containing iodine that are released by the thyroid gland. The most important THs, thyroxine (T4) and triiodothyronine (T3) are transported in and out of cells by several transporters including the very specific TH transporter monocarboxylate transporter 8 (MCT8). This transporter is expressed in the central nervous system (CNS) among other tissues. Unfortunately, several inactivation mutations of the gene SLC16A2 (coding for MCT8 on the X-chromosome) have been found in mostly male patients. They suffer from severe psychomotor retardation, known as Allan-Herndon-Dudley syndrome (AHDS), due to lack of THs during fetal development. Since MCT8 is the crucial transporter for the influx of THs into the brain, the patients are rendered hypothyroid in the CNS, but hyperthyroid in the periphery. Treatment options with TH derivates are currently limited, so development of a new therapy is urgently needed.

Here, we investigate a novel therapeutic mechanism, circumventing transporters altogether by using the internalization mechanism of G protein-coupled receptors (GPCRs). This concept, dubbed the “Trojan Horse”-like mechanism, includes the conjugation of T3 with a peptide ligand. The peptide part of the conjugate can specifically activate GPCRs, leading to an internalization of the receptor-ligand complex. Once the conjugate enters the cell, T3 is liberated from a special linker and can start gene regulation as if it were transported over a transmembrane transporter. Another advantage of this concept is the target-specific delivery of the conjugated hormone. The choice of the peptide-receptor pair directs the hormone to the target tissues, but spares tissues that do not express the specific GPCR.

For this project, two peptide-T3 conjugates are characterized and validated *in vitro*. The first, Glucagon-like peptide 1 (GLP1)-T3 is derived from the idea of using the “Trojan Horse”-like approach for the treatment of metabolic diseases and is used as a proof-of-principle compound to establish an *in vitro* toolbox. It could be shown that GLP1-T3 activates signaling and internalization of the target receptor GLP1 receptor (GLP1R) as well as canonical and non-canonical TH signaling in the presence, but not in the absence of the GLP1R. In conclusion, GLP1-T3 acts over the “Trojan Horse”-like mechanism and does not show any adverse effects *in vitro*.

The second conjugate, oxytocin (OT)-T3 was designed to target the hypothyroid brain areas of AHDS patients. It was characterized with the methods established with GLP1-T3, but showed biased signaling effects on its two target GPCRs, OT receptor (OTR) and Vasopressin arginine receptor 1A (V1AR). Unfortunately, the results show that OT-T3 is not stable in culture conditions and was not able to use the “Trojan Horse”-like mechanism to enter the cell.

This project gave an insight into the “Trojan Horse”-like mechanism *in vitro*. The investigations showed how the efficiency of the conjugate is dependent on the size of the peptide as well as the type of target receptor. Specifically, the structure of the binding pocket of the GPCR determines whether the signaling was changed in comparison to the unconjugated peptide. Additionally, the conjugates stability is crucial for a successful internalization of the conjugated TH. The investigations with the here established *in vitro* toolbox were able to identify adverse effects to avoid unnecessary *in vivo* assays.

Zusammenfassung

Zellen verwenden Transmembrantransporter, um Substanzen aufzunehmen, die wichtig sind für ihre Funktion und das Überleben des Organismus. Ein Beispiel für diese Substanzen sind Schilddrüsenhormone (SDH), Tyrosin-basierende, Iod-enthaltene Hormone, die über die Schilddrüse abgegeben werden. Die wichtigsten SDH, L-Thyroxin (T4) und Triiodthyronin (T3) werden über verschiedene Transporter in und aus Zellen transportiert. Einer dieser Transporter ist der für SDH sehr spezifische Monocarboxylat-Transporter 8 (MCT8), der unter anderen im zentralen Nervensystem (ZNS) exprimiert wird. Unglücklicherweise wurden mehrere inaktivierende Mutationen im Gen SLC16A2 (kodierende für MCT8 auf dem X-Chromosom) gefunden. Die hauptsächlich männlichen Patienten leiden an einer schweren psychomotorischen Retardierung, dem Allan-Herndon-Dudley-Syndrom (AHDS) genannt. Bei diesem Patienten prägt sich der Phänotyp aufgrund fehlenden SDH schon während der fetalen Entwicklung aus. Da MCT8 ein essentieller Transporter für den Einstrom von SDH ins Gehirn ist, haben die Patienten eine Hypothyreose im ZNS, während ihre Peripherie hyperthyreot ist. Behandlungsoptionen mit SDH-Derivaten ist derzeit limitiert, weshalb die Entwicklung von neuen Therapien dringend notwendig ist.

In dieser Arbeit wird eine neue Behandlungsidee untersucht, bei der Transporter umgangen wird durch die Nutzung des Internalisierungs-Mechanismus von G-Protein gekoppelten Rezeptoren (GPCRs). Dieses Konzept, „Trojan Horse“-ähnlicher Mechanismus genannt (zu Deutsch: „trojanisches Pferd“) beinhaltet die Konjugation von T3 mit einem Peptidliganden. Der Peptidanteil des Konjugates kann einen GPCRs spezifisch aktivieren und so die Internalisierung des gesamten Rezeptors-Liganden-Komplexes herbeiführen. Ist das Konjugat einmal in der Zelle, kann T3, was über einen speziellen Linker an das Peptid gebunden ist, freigesetzt werden und Genregulation ähnlich wie T3 über einen SDH-Transporter starten. Ein weiterer Vorteil dieses Konzepts ist die zielgerichtete Lieferung des konjugierten Hormons. Die Wahl des Peptid-Rezeptor-Paars definiert die Gewebe, die das Konjugat anzielt, während Gewebe, die den spezifischen GPCR nicht exprimieren, unberührt werden.

In diesem Projekt wurden zwei Peptid-T3-Konjugate *in vitro* charakterisiert und validiert. Das erste, Glucagon-like peptide 1 (GLP1)-T3 entstand aus der Idee, den „Trojan Horse“-ähnlichen Mechanismus für die Behandlung von metabolischen Krankheiten zu verwenden und wurde hier als Grundsatzbeweis sowie für die Entwicklung von *in vitro*-Methoden verwendet. Es konnte gezeigt werden, dass GLP1-T3 Signalisierung und Internalisierung am GLP1-Rezeptor (GLP1R) aktiviert. Zusätzlich konnte die kanonische und nicht-kanonische SDH-Signalisierung in der Anwesenheit aber nicht in der Abwesenheit vom GLP1R gemessen werden. Zusammenfassend konnte gezeigt werden, dass GLP1-T3 über den „Trojan Horse“-ähnlichen Mechanismus transportiert wird und keine Veränderungen in der Signalisierung zwischen GLP1 und GLP1-T3 *in vitro* festgestellt werden konnten. Das zweite Konjugate, Oxytocin (OT)-T3 wurde anhand der hypothyreoten Gehirnareale der AHDS-Patienten entwickelt. Es wurde mit den durch GLP1-T3 etablierten Methoden charakterisiert, zeigte aber funktionelle Selektivität an seinen zwei Zielrezeptoren, OT-Rezeptor (OTR) und Vasopressin-Arginin-Rezeptor 1A (V1AR). Unglücklicherweise wurde zusätzlich festgestellt, dass OT-T3 in Kulturbedingungen

nicht stabil und damit nicht in der Lage war, die Zelle über den „Trojan Horse“-ähnlichen Mechanismus in die Zelle eindringen kann.
Diese Arbeit gibt einen Einblick in den „Trojan Horse“-ähnlichen Mechanismus *in vitro*. Die Untersuchungen zeigten, dass die Effizienz des Konjugates von der Größe des Peptids als auch von der Art des Zielrezeptors abhängig ist. Besonders die Beschaffenheit der Bindungstasche des GPCRs bestimmt, ob sich die Signalisierung im Vergleich zum unkonjugierten Peptid verändert. Zusätzlich ist die Stabilität des Konjugates maßgeblich für die erfolgreiche Internalisierung des konjugierten SDH. Die Untersuchungen mit der hier etablierten *in vitro* Toolbox waren in der Lage, unerwünschte Effekte zu identifizieren, um unnötige *in vivo*-Analysen zu vermieden.

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1 Introduction

The discovery of thyroid hormones (THs) and their numerous functions was over a century ago and a large amount of information has been accumulated since then, with new verdicts about TH action being constantly revealed. Nevertheless, many questions, especially about the pathophysiology of disturbed TH action, are still unsolved. One example is the correlation between a TH transporter mutation and the severe Allan-Herndon-Dudley syndrome, a psychomotor retardation known since 1944. It took another 60 years after the definition of the syndrome to find the link between the disease and the molecular cause, first published in 2004. To the present day, therapeutic options for this complex disease and its devastating effect on development are limited. In this work we investigate a new treatment approach *in vitro* to reveal possible adverse effects.

1.1 Thyroid hormones

In 1915, Edward Kendall isolated a crystalline form of an iodine containing substance from the thyroid gland. He found that the compound exerts physiological effects such as increased pulse rate and metabolism, and named it thyroxine (Kendall 1915). In the last 100 years, numerous investigations have shown that the thyroid gland produces and releases several kinds of iodinated hormones that are important metabolic and developmental regulators (Boelaert and Franklyn 2005).
Thyroid hormones are chemically based on a tyrosine-backbone and are bound to three or more iodine atoms. The most important are thyroxine (3,3',5,5'-Tetraiod-L-thyronin, T4), triiodothyronine (T3) and reverse triiodothyronine (rT3).

1.1.1 Biosynthesis of thyroid hormones

The thyroid gland is a butterfly shaped organ close to the trachea in the neck. It consists of two lobes, which are connected by the *isthmus glandularis*. It produces THs and releases them into the blood stream. The thyroid gland is connected to the arterial blood stream by the superior and inferior artery (Nobori et al. 1994).
THs are produced by a special cell type, the thyrocytes, which are arranged in follicles surrounded by epithelia cells, called C cells, that produce the hormone calcitonin (Mundy and Guise 1999). These small spherical structures vary in size and consist of one-layered thyrocytes and a lumen, called the colloid, and are surrounded by blood vessels to ensure iodine and TH exchange (Johnson 1955). The colloid contains thyroglobulin, a larger protein that contains tyrosine residues and is a precursor for THs. The iodine is actively transported from the blood stream by the cells using a sodium-iodine-symporter (NIS), a basolateral located ion channel (Dohan et al. 2003) and diffuses through the apical side by a second ion channel called pendrin (Yoshida et al. 2002).

By oxidizing the iodine, thyroperoxidase (TPO) catalyzes the attachment of the iodine ions to the thyroglobulin, mediated by hydrogen peroxide (H_2O_2), produced by thyroid oxidases (THOX1 and THOX2), two enzymes closely associated with the TPO at the apical membrane of the thyrocytes (De Deken et al. 2000). The attachment of iodine to thyroglobulin results in monoiodotyrosine (MIT) and diiodothyrosine (DIT), that are then connected by TPO to form T4 (DIT coupled with DIT) or T3 (MIT coupled with DIT) (Taurog, Dorris, and Doerge 1996).
To release THs into the blood stream, hormones containing thyroglobulin are taken up by endocytosis and convey to lysosomes for final processing inside the thyrocytes (Bernier-Valentin et al. 1990).

1.1.2 Regulation of TH synthesis via the hypothalamus-pituitary-thyroid axis (HPT)

Since THs are important regulators in metabolism and development, their biosynthesis is in tight control to ensure balanced TH concentrations. It underlies a neuroendocrine system that depends on the hypothalamus, the anterior pituitary and the thyroid gland (**Figure 1**). The hypothalamus, as part of the brain, includes TH sensing neurons. At low concentration of circulating T4 and T3, the hypothalamus releases thyrotropin-releasing hormone (TRH), which can bind to the TRH receptor (TRHR), a G protein-coupled receptor (GPCR) in the pituitary. The pituitary responds to TRH by producing thyroid-stimulating hormone (TSH), which then stimulates the thyroid to produce and secrete THs into the blood stream by binding to the TSH receptor (TSHR), another GPCR located in the basolateral membrane of thyrocytes. Higher concentrations of THs exert a negative feedback loop to the pituitary as well as the hypothalamus, to suppress TRH and TSH expression and therefore stop TH release (Kopp 2001).

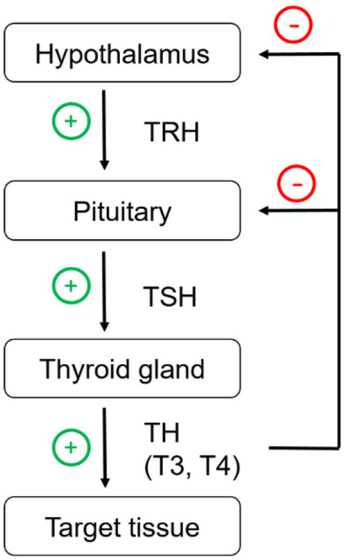

Figure 1: The HPT axis is the control system of TH synthesis. Sensing neurons in the hypothalamus respond to low TH concentrations by releasing TRH. Receptors in the pituitary are activated by TRH and the pituitary secretes TSH. In the thyroid gland TSH, bind to the TSH receptor. The thyroid starts the biosynthesis and release of THs, mainly T4 and T3. To protect from overproduction, high TH concentration in the blood stream inhibits the release of TSH by the pituitary and TRH by the hypothalamus as a negative feedback loop. THs in the blood stream activate responses in different target tissues. TH: thyroid hormone; TRH: thyrotropin-releasing hormone; TSH: thyroid-stimulating hormone; T3: triiodothyronine; T4: 3,3',5,5'-Tetraiod-L-thyronin

1.1.3 Metabolism of TH

Inside their target cells, the majority of THs need to be metabolized in order to administrate an effect. Since only a fraction of free THs is the biologically active T3 (20%), the inactive form T4 needs to be metabolized to T3. Specialized enzymes, the deiodinases catalyze the activation and deactivation of THs by reductive elimination of iodine. There are three isoforms coded by the DIO genes. Those deiodinases contain a non-canonical amino acid, a selenocysteine, which is important for enzyme function. Type II deiodinase (DIO2) is mainly expressed in the brain, here mostly in glia cells, in brown adipose tissue (BAT), thyme and placenta and only catalyzes the reaction from T4 to T3 by deiodination of the phenyl ring (5'-deiodination on the outer ring). Type III (DIO3) only catalyzes by eliminating the iodine from the phenyl ring (5-deiodination, inner ring) to turn T4 into rT3. It is expressed in the brain, placenta and fetus during gestation. Type I deiodinase (DIO1) catalyzes both reaction from T4 to T3 as well as from T4 to rT3 and is mostly expressed in liver, kidney, pituitary and thyroid gland. Hepatic DIO1 activity controls the serum T3 concentrations (Kohrle 2002). THs can be broken down into further partly biological active metabolites by other enzymes like decarboxylases and monoaminoxidases. One example for those is 3,5-Diiodothyronine, (3,5-T2), which is known to have metabolic effects such as reduction of body weight and temperature (Pietzner et al. 2015).

1.1.4 Diseases of disturbed TH production: Hyper- and hypothyroidism

Any kind of deregulations of the HPT axis can lead to constantly too high or constantly too low concentrations of TH in the blood stream. Hyperthyroidism, an excessive production of THs (thyrotoxicosis) can be the result of an autoimmune inflammation, e.g. in the case of Grave's disease (Weetman 2000). On the other hand, hypothyroidism, low circulating TH concentrations might be the result of a defective thyroid anlage or dyshormonogenesis (Grüters and Krude 2012).
Several studies showed that passage of maternal THs during pregnancy are important for brain development of the fetus. An infant exposed to reduced TH concentrations during gestation from a hypothyroid mother is likely to show decreased cognitive functions and increased susceptibility to behavior problems later in life (de Escobar, Obregón, and del Rey 2004). In untreated congenital hypothyroidism (CH), patients show a short stature, mental retardation as well as obesity. Treatment with L-T4 should start within the first three month of life to stabilize TH levels and therefore normalize growth and development (Krude, Kühnen, and Biebermann 2015).
The cases of CH can be classified into different types. Thyroid dysgenesis includes missing or hypoplastic thyroid gland as well as defects in hormone biosynthesis, whereas central hypothyroidism is mostly based on defects of the hypothalamus or pituitary (Gruters, Krude, and Biebermann 2004). Several causes for CH have been found including mutations in: the transcription factors PAX8 (Macchia et al. 1998), NKX2.1 (Devriendt et al. 1998), FOXE1 (Clifton-Bligh et al. 1998) as well as in genes of TSHR (Duprez et al. 1994), TPO (Abramowicz et al. 1992), thyroglobulin (Ieiri et al. 1991), NIS (Fujiwara et al. 1997), the two thyroid oxidases THOX1 and THOX2 (Moreno et al. 2002), the β-subunit of TSH (TSHβ) (Hayashizaki et al. 1989) and the

guanine nucleotide binding protein α-subunit (GNAS) (Patten, Smallwood, and Eil 1989). Another TH deficiency due to a transporter defect will be introduced in 1.2.

1.1.5 Target tissues of THs

After release of THs by the thyroid gland, the hormones reach target tissues throughout the body. Within the blood stream, THs are bound to various proteins and only a fraction is unbound. Proteins binding THs are thyroxine-binding globulin (TBG), albumin, transthyretin and lipoproteins. In a bound state, THs are inactive, but stabilized and are hindered from unspecific binding to lipid membranes. It also ensures a fast exchange between bound (inactive) and free (active) T4/T3 in an unbalanced situation acting as a TH reserve that reacts quicker than the HPT axis (Schussler 2000; Pappa, Ferrara, and Refetoff 2015). Single cells take up T3 and T4 by TH transporters (introduced in detail in 1.2).
THs target a broad number of tissues including brain, heart, bone, liver and fat tissue. The effects of THs on target tissues are summarized in **Table 1**.

Table 1: Summarizes effects of THs on their target tissues

Target tissue	THs promote	Hypothyroid state	Hyperthyroid state	References
Brain	Cognition and motor function	Decreased neuronal function and development, impaired myelination (during gestation)	Early neuronal differentiation and termination of neuronal proliferation	(Wirth and Meyer 2017; Pappa, Ferrara, and Refetoff 2015; Nicholson and Altman 1972)
Bone	Growth and bone turnover	Growth arrest, malformations	Accelerated growth, increased bone turnover	(Bassett and Williams 2003; Williams, Robson, and Shalet 1998; Kamegai et al. 2001)
Heart	Heart rate, blood pressure	Decreased heart rate, low blood pressure	Increased heart rate, high blood pressure	(Moolman 2002; Vargas-Uricoechea, Bonelo-Perdomo, and Sierra-Torres 2014)
Liver	Energy expenditure, weight regulation, thermogenesis	Decreased metabolism, weight gain, decreased thermogenesis	Increased metabolism, weight loss, increased thermogenesis	(Tam, Lecoultre, and Ravussin 2012; Mullur, Liu, and Brent 2014)

1.1.5.1 Brain

In the brain, the neuronal development is especially dependent on optimal TH supply of the fetus as well as the infant after birth. Since the brain is segregated from the rest

of the blood circulation by the blood-brain barrier (BBB), neurons are arranged in special functional units, the tripartite synapse together with astrocytes. These cells are star-shaped glia cells, that provide the neurons with energy substrates, neurotransmitters and also hormones like THs from the endothelia cells of the BBB (Wirth and Meyer 2017; Pappa, Ferrara, and Refetoff 2015). Maternal TH concentrations influence brain development during gestation, especially before the onset of fetal thyroid function. Maternal hypothyroidism can impact neuronal proliferation, migration, differentiation and myelination as well as synaptic plasticity and neurochemistry resulting in impaired motor function and cognition (Moog et al. 2017). In rats, hyperthyroidism has been shown to promote early neuronal differentiation as well as early termination of proliferation of granular layer cells in the cerebellum (Nicholson and Altman 1972).

1.1.5.2 Bone

Next to developmental regulations in the brain, THs are especially important in growth. In bone, they influence linear growth and skeletal development in infants and maintain adult bone mass. Patients with untreated congenital hypothyroidism with thyroid dysgenesis due to athyrosis or hypoplasia, resulting in TH deficiency, develop severe skeletal defects and growth arrest. An excess of THs can lead to accelerated growth and bone formation in infants and increased bone remodeling in adults (Bassett and Williams 2003).
The mechanism for TH action on bone is the direct effect of T3 on the bone resorption. Both, osteoclasts and osteoblasts have been shown to respond to THs with bone turnover (Williams, Robson, and Shalet 1998). In addition to the influence on the pituitary as a negative feedback signal for their own synthesis, THs regulate growth hormone (GH) synthesis and secretion by influencing the pituitary as well as hypothalamic functions, shown by hypothyroidism leading to decreased GH secretion (Kamegai et al. 2001).

1.1.5.3 Heart

For the cardiovascular system, THs regulate the expression of myocardial genes that are important for calcium administration. These can affect both systolic and diastolic myocardial function. The hormones have also indirect and direct effects on peripheral vascular smooth muscle tone and can alter the connection between the left ventricle and the artery system (Moolman 2002). Hyperthyroidism can lead to an increase in resting heart rate, blood volume, stroke volume, myocardial contractility, and ejection fraction, whereas hypothyroidism can cause lower heart rate, weakening of myocardial contraction and relaxation, with prolonged systolic and early diastolic times (Vargas-Uricoechea, Bonelo-Perdomo, and Sierra-Torres 2014).

1.1.5.4 Liver and fat

The liver is the main actor in metabolism. Together with fat tissue, it regulates the lipid homeostasis. THs are known to influence body weight and energy expenditure. In BAT, containing numerous small lipid droplets and being the main source of non-shivering thermogenesis (Tam, Lecoultre, and Ravussin 2012), THs induce expression of uncoupling protein 1 (UCP1) which increases thermogenesis and promotes weight

loss. In liver, THs influence cholesterol and fatty acid metabolism directly and indirectly (Mullur, Liu, and Brent 2014). Patients with hyperthyroidism show increased resting energy expenditure, weight loss and decreased cholesterol levels. Reduced TH concentrations in hypothyroidism show the opposite effect on the metabolism. These effects are due to T3 action in white adipose tissue (WAT) and BAT. In WAT, a tissue composing of a single large lipid droplet as storage, T3 induces lipolysis and promotes weight loss (Mullur, Liu, and Brent 2014).

1.1.6 Molecular effects of TH in the cell

Similar to the depot generation with TH binding proteins in the blood circulation, a protein inside the cell keeps THs within the cytoplasm. This protein, the μ-crystallin (CRYM) was identified as a NADPH-dependent cytosolic T3-binding protein in the kangaroo eye lens (Kim, Gasser, and Wistow 1992; Vie et al. 1997) and has been shown to be abundant in the human cochlea and vestibular tissue of the inner ear (Abe et al. 2003).
As the biologically most active TH, T3 is known to have canonical as well as non-canonical effects within its target cells.

1.1.6.1 Canonical effects of T3

Once transported into the cell and converted into T3, the canonical action of THs is mediated through TH receptors (TR) belonging to the class of nuclear receptors. TR are ligand-induced transcription factors, that are activated by binding to T3 and form homo- or heterodimers, together with other nuclear receptors, especially the retinoid X receptor (RXR) (Lazar, Berrodin, and Harding 1991), translocate to nucleus and bind to specific thyroid hormone response elements (TRE) to start gene expression. They are encoded by the genes THRA on chromosome 17 and THRB on chromosome 3 with either of them having three splicing variants. Most variants, TRα1, TRβ1, TRβ2 and TRβ3, are known to bind to THs (Williams 2000). The splicing variant TRα2 and 3 are not able to bind hormones (Harvey and Williams 2002). The TRα isoform is important for early development, whereas expression of the TRβ isoform takes place in later development, being mainly expressed in liver, pituitary, kidney, thyroid, heart, retina, and several brain areas (Cheng, Leonard, and Davis 2010).
The transcription factors all consist of a single polypeptide chain with a very variable regulatory A/B domain at the N-terminus as a nuclear localization signal, a DNA-binding domain in the center and a C-terminal T3-binding domain. The latter two domains are separated by a hinge region, containing additional localization signals (Zhang, Roggero, and Allison 2018). The TRβ variants differ in the N-terminal A/B domain and are very homologous throughout the other two domains (Cheng, Leonard, and Davis 2010), whereas the TRα variants differ in the length of the C-terminal domain (Plateroti et al. 2001). Additional to binding hormones, the C-terminal domain is also involved in interactions with co-regulators, like corepressors or coactivators (Feng et al. 1998) and heterodimerization with the RXR (Cheng, Leonard, and Davis 2010).
TRE contain two hexameric half-sites with the consensus sequence G/AGGTC/GA and are arranged in palindromic, inverted palindrome, or direct repeat separated by four nucleotides (DR4). TRE are located in the promotor region of T3-regulated genes

involved in metabolic, cell proliferation and apoptosis and others (Harvey and Williams 2002). Liganded and free TR compete for these TRE, unliganded TR have even been shown to act as transcriptional repressors in many studies (Hashimoto et al. 2001; Chassande 2003; Venero et al. 2005; Wallis et al. 2008; Araki et al. 2009; Kapoor et al. 2010).

1.1.6.2 TR deficiencies

For both TRα and TRβ, mutations have been known in humans. For TRβ, all known mutations are located in the T3 binding domain (Sakurai et al. 1989). Patients show a mostly hyperthyroid state of heart and several brain areas, but can also suffer from hypothyroid symptoms in the periphery, due to variable tissue expression. The manifestations of the mutation vary from case to case with the majority having a mild phenotype (Krude, Kühnen, and Biebermann 2015). Refetoff et al was the first to describe this TH resistance and the disease was therefore named Refetoff syndrome. The patient suffered from deaf-mutism and goiter among other symptoms (Refetoff, DeWind, and DeGroot 1967). Later the group could link these symptoms to an impairment in the TRβ gene (Refetoff and Dumitrescu 2007).
TRα mutations on the other hand show a more severe outcome for patients. The first mutation was found very recently in 2012 (Bochukova et al. 2012) and 28 cases have been identified to date (van Gucht et al. 2017). Like for TRβ, most of the known mutations are located in the ligand-binding domain. The patients suffer from mild hypothyroidism with impaired growth, mild to moderate mental retardation, mild skeletal dysplasia with specific facial features and severe constipations (Tylki-Szymanska et al. 2015). **Table 2** summarizes the symptoms reported for TRα and TRβ deficiencies.

Table 2: Overview of symptoms of TR deficiencies

Affected thyroid hormone receptor	Reported symptoms	References
TRα	Comprise growth retardation, mild-to-moderate mental retardation, mild skeletal dysplasia, severe constipation, hypothyroidism-like	(Bochukova et al. 2012), (Schoenmakers et al. 2013), (Moran et al. 2013), (Moran et al. 2014), (Tylki-Szymanska et al. 2015)
TRβ	Highly variable phenotype, in some cases IQ less than 85, delayed speech development, attention-deficit hyperactivity disorder (ADHS), deaf-mutism, thyroid gland enhancement or goiter and short stature	(Refetoff, DeWind, and DeGroot 1967), (Sakurai et al. 1989), (Brucker-Davis et al. 1995), (Refetoff and Dumitrescu 2007)

1.1.6.3 Mice studies for TR deficiencies

Several mice studies revealed the function and contributions of the different isoforms of TRs. Knock-outs (KOs) for the Thra gene displayed behavioral abnormalities due to reduced GABAergic synaptic density (Guadano-Ferraz et al. 2003) with lower heart rate and decreased body temperature (Wikström et al. 1998). Mice carrying mutations found in human patients exhibit impaired growth, heart rate and motor functions, as well as variable metabolic phenotypes with extremely obese or extremely lean mice dependent on the mutation (Vennström, Mittag, and Wallis 2008). The KO of the Thrb gene showed altered maturation of the retina and the inner ear as well as dysfunction in the HPT axis (Jones et al. 2003), whereas mutations in the gene caused severe cognitive developmental defects and deafness (Flamant et al. 2006). A double KO (DKO) of both TR revealed severe developmental defects such as hyperactive HPT-axis, reduced growth and bone maturation (Göthe et al. 1999). These findings indicate that the isoforms can substitute each other in some functions, but also retain tissue-specific action.

1.1.6.4 Non-canonical effects of T3

In addition to the well-known canonical effects, T3 can activate non-canonical pathways, both dependent and independent of TR. This has been speculated, since the canonical pathway could not explain the whole phenotype of KO mice models (Shibusawa et al. 2003) and indicated an admission site close to the plasma membrane or in the cytoplasm. It has been shown that T3 rapidly regulate membrane potential and cellular depolarization by modulating plasma membrane ion channels and GPCRs (Sakaguchi, Cui, and Sen 1996; Sun et al. 2000; Sen, Sakaguchi, and Cui 2002) as well as mediating glucose uptake (Segal and Ingbar 1990). After that, an involvement of T3 in the phosphatidylinositol 3-kinase (PI3K)/protein kinase Akt pathway was discovered, which takes place in the cytoplasm after TH transport into the cell (Davis, Goglia, and Leonard 2016). In addition, TR can also phosphorylate the Mitogen-activated protein kinase (MAPK, also called ERK1/2, extracellular signal-regulated kinase 1/2) and activate the respective pathway (Lin et al. 2003). It is unclear whether TRα, TRβ or both are capable of this type of non-canonical signaling. There have been several studies describing the direct inhibition of PI3K by TRβ and even excluded an involvement of TRα (Storey et al. 2006; Martin et al. 2014). Others claim the opposite with data that show the activation of the PI3K/Akt pathway by a direct interaction with TRα, but not TRβ (Hiroi et al. 2006) or the involvement of a membrane-bound translational variant of TRα (p30), that starts a cascade leading to MAPK and PI3K activation (Kalyanaraman et al. 2014).
Another TR-independent signaling pathway was revealed when a TH binding domain was found in a protein of the plasma membrane, the integrin αVβ3 (Bergh et al. 2005). Binding of the hormones to αVβ3 initiates complex cellular events such as angiogenesis and cell proliferation transduced by phospholipase C (PLC) and protein kinase C (PKC) activating MAPK. This kinase then phosphorylates further targets such as signal transducer and activator of transcription (STAT) 1α, estrogen receptor (ER)-α and TRβ1 (Davis et al. 2000), starting shedding of coactivators or repressors and protein trafficking to the cell nucleus (Hammes and Davis 2015). **Figure 2** depicts the possible pathways for TH action within the cell. A recent article proposed a more

precise nomenclature for TH signaling pathways. First, it was suggested that the terminology is changed from former genomic vs. non-genomic to canonical vs. non-canonical, due to the word “non-genomic” being misleading. Furthermore, four possible models of action were considered. Type 1 summarizes the effects known from the canonical pathway as a TR-dependent signaling with direct binding to DNA, type 2 includes the recent discovery of indirect interaction of THs activated TR with DNA inducing chromatin remodeling (Grøntved et al. 2015). It is suggested that TR is interacting with other transcription factors, though further investigation is needed. Type 3 sums up the TR-dependent signaling of THs without DNA binding, such as PI3K and ERK1/2 activation, and type 4 is suggested to classify TR-independent TR signaling such as integrin activation and CRYM interactions (Flamant et al. 2017).

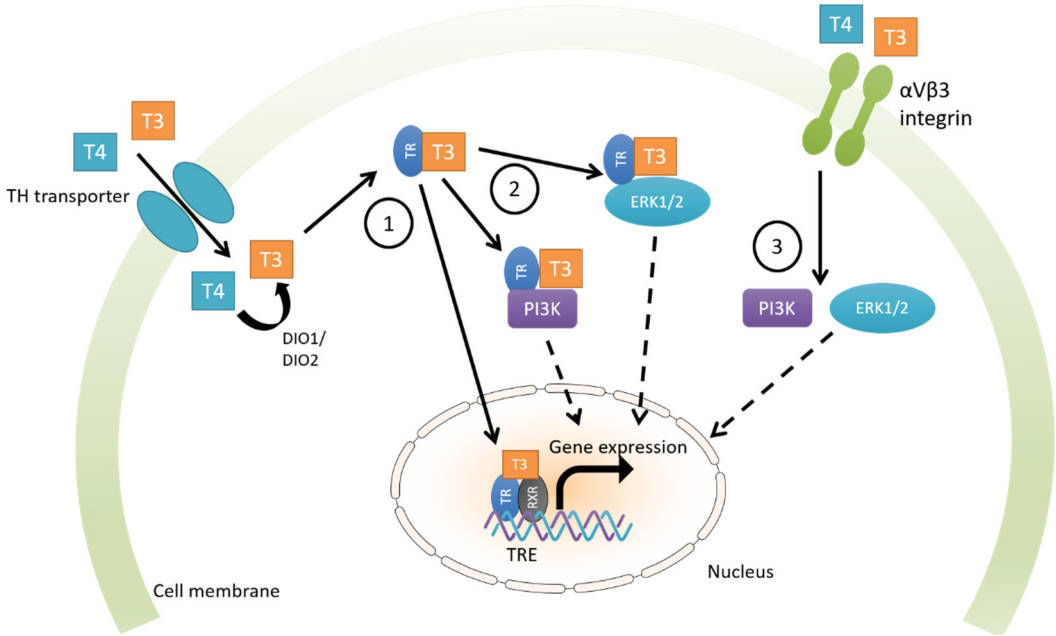

Figure 2: **Canonical and non-canonical pathways of TH. 1: The canonical pathway of TH action includes the transport of T3 and T4 via TH transporter. Intracellular deiodinases catalyze the reaction from T4 into T3 (DIO1 and DIO2). The bioactive T3 binds to TR in the nucleus (here TRβ is exemplified. With its co-receptor RXR, the TR-T3 complex binds TRE in promotor regions of target genes to activate gene expression. 2: The TR-T3 complex can also bind to PI3K and the MAPK ERK1/2 to activate the respective signaling pathway and target gene expression. 3: Additionally, THs are also known to bind to the integrin αVβ3 and activate PI3K and ERK1/2, which lead to gene expression via the respective signaling cascades.**

1.2 Thyroid hormone transporters

For a long time, it was thought that THs pass the plasma membrane by simple diffusion due to their small size and lipophilic properties (Hennemann et al. 2001). In the 1970s however, several studies suggested that membrane transporters facilitate the TH uptake by amino acid transporter or similar proteins (Stitzer and Jacquez 1975).
Since the first discovery, a number of TH transporters have been identified, cloned and functionally characterized. They are categorized into different families, the monocarboxylate transporters (MCTs), the organic anion transporter polypeptides (OATPs), and the L-type amino acid transporters (LATs). They all differ in their

substrate specificities and tissue distribution. All of them belong to the major facilitator superfamily (MFS) (Kinne, Schülein, and Krause 2011).
Many OATPs are known to transport different iodothyronines and their derivates, but also transport several other organic compounds like bile salts and steroid hormones in a sodium-dependent manner. Some of the members of this superfamily are expressed only in a specific tissue whereas others are expressed more broadly. The genes coding for these transporters are grouped into the solute carrier family (SLC) (Hagenbuch and Meier 2004). The most prominent members concerning TH transport are MCT8 and OATP1C1; both are known to be expressed in the brain, among other tissues. Notably, the contribution of the latter in the human brain differs from the murine brain (Mayerl et al. 2014). In the human brain, OATP1C1 is mainly expressed in astrocytes. Here it is thought to facilitate the uptake of the inactive T4 to be converted into the active T3 by the deiodinase 3 (DIO3). In the murine brain, it is highly enriched in the choroid plexus and cerebral micro vessels, therefore on the blood-brain-barrier (Roberts et al. 2008), as well as in cortical neurons (Wirth, Roth, Blechschmidt, Hölter, Becker, Racz, Zimmer, Klopstock, Gailus-Durner, and Fuchs 2009).
Some of the amino acid transporters have been shown to also transport THs due to tyrosine backbone of iodothyronines. For example, the L-type amino acid transporters facilitate the uptake of several TH derivates over the cell membrane, such as LAT-1 and LAT-2 (Friesema et al. 2001). They mainly facilitate the exchange of neutral amino acid between the blood stream and several tissues, such as the small intestine, skeletal muscle, liver, kidney and brain. They consist of a light and a heavy chain, that are connected via disulfide bond (Wagner, Lang, and Bröer 2001). The heavy subunit functions as an escort protein that is required for trafficking to the cell surface, whereas the light chain facilitates actual substrate transport (Palacín, Errasti-Murugarren, and Rosell 2016).
The family of MCTs consists of 14 homologous proteins, which are known to transport monocarboxylates such as pyruvate and lactate. The genes encoding for these transporters consists of the SLC16 family (Halestrap 2012). MCT8 (SLC16A2) as well as MCT10 (SLC16A10) have been identified as TH transporters with MCT8 being the only transporter specifically for TH uptake (Friesema et al. 2003; Friesema et al. 2008). Despite the structural similarity of both transporters, MCT10 was shown to have a broader substrate specificity and additionally transports aromatic amino acids (Halestrap 2012). Therefore, MCT8 is considered a primary TH transporter, whereas MCT0, some OATPs and the above mentioned LAT1 and LAT2 with a wider substrate spectrum belong to the secondary TH transporters (Kinne, Schülein, and Krause 2011). **Table 3** summarizes the substrate specificity of the mentioned transporters.

Table 3: Substrate specificity of TH transporters, numbers of + are indicating the degree of affinity

Protein	3,3'-T2	T3	T4	rT3	References
MCT8	+	+++	++	+	(Friesema et al. 2003; Friesema et al. 2006)
MCT10	++	+++	++	+++	(Friesema et al. 2008)
OATP1C1		+	+++	+++	(Kinne, Schülein, and Krause 2011; Zevenbergen 2015)
LAT1/LAT2	+++	++	+/-	+/-	(Friesema et al. 2001; Kinne et al. 2015)

1.2.1 Monocarboxylate transporter 8

The gene encoding for MCT8 (SLC16A2) is located on the X chromosome position q13.2 and consist of six exons. When it was first cloned, it was annotated after its N-terminal domain consisting mainly of proline (P), glutamate (E), serine (S) and threonine (T) repeats, XPCT for X-linked PEST-containing transporter (Lafrenière, Carrel, and Willard 1994). After the identification as a specific TH transporter, further investigations tried to elucidate the gene and protein structure as well as the transport mechanism.

In contrast to other investigated species with only one defined translational start, the human MCT8 gene reveals two possible translational start sites, giving two hMCT8 isoforms. The long hMCT8L codes for 613 amino acids, whereas the short isoform hMCT8S is 74 amino acids shorter (539 amino acids). Since human liver transcripts have shown the presence of both mRNA species (Friesema, Visser, and Visser 2010), the purpose of the additional N-terminal domain has been investigated. It has been shown to enhance proteasomal degradation and is a target of ubiquitin conjugation (Zwanziger et al. 2016). General MCT8 expression have been shown in several tissues, such as brain, liver, kidney, heart, adrenal and thyroid gland (Visser, Friesema, and Visser 2011). The transporter protein consists of 12 transmembrane helices with both, N- and C-terminus positioned intracellularly and connecting intra- and extracellular loops (Kinne et al. 2010). MCT8 is known to form homodimers or even higher order oligomers (Biebermann et al. 2005; Visser, Philp, et al. 2009), which have been found crucial for the function as substrate transporter (Fischer et al. 2013). Proteasomal degradation dependent on the expressed isoform can influence the amount of transporter and subsequently affect the capacity for oligomerization (Zwanziger et al. 2016).

The same study that showed high affinity of MCT8 to THs (K_M = 2-5 µM) could also prove specific transport over the cell membrane. In contrast to the other known members of the MCT family, MCT8 does not facilitate the uptake of any other aromatic amino acid (Friesema et al. 2003). The transport seemed to be sodium- and proton-

independent and is assumed to follow the “rocker-switch” model developed for MFS transporters (Kleinau et al. 2011). This model theorizes that substrate binding facilitates conformational changes within the protein. The transporter changes from a “open to outside” into a “open to inside” conformation and therefore transports the substrate over the plasma membrane (Schweizer et al. 2014; Sun et al. 2000). The distinctive recognition of THs by the transporter has been investigated by mutation studies and comparison to the highly homologous MCT10 based on the homology models. The following amino acid residues have been shown to change substrate specificity and are compatible with the homology models: H192 (Braun et al. 2013), R445 and N498 (Groeneweg et al. 2013), A224 in the substrate channel, S310 in the substrate pocket and F287 (Johannes, Braun, et al. 2016). A study combining functional and structural information about MCT8 pointed out two amino residues, R301 and H415 worth future experimental investigation (Kleinau et al. 2011).
Besides structural investigations, studies have been performed due to the identification of MCT8 as a major TH transporter in the human brain and several loss-of-function mutations linked to a severe psychomotor retardation, the Allan-Herndon-Dudley syndrome (Dumitrescu et al. 2004; Schwartz et al. 2005).

1.2.2 Allan-Herndon-Dudley syndrome

The group of Theo Visser was the first to identify a specific transporter for thyroid hormones, especially for T3 (Friesema et al. 2003). Later, it was speculated that in patients with elevated T3 concentration this transporter might be disrupted (Friesema et al. 2004). The Allan-Herndon-Dudley syndrome (AHDS) has been described as early as 1944 as an X-linked mental retardation with early onset hypotonia, athetoid limb movements, motor speech impairments and muscle hypoplasia. Allan, Herndon and Dudley examined a large family of 7 generations, with 29 affected males all showing the same symptoms of the disorder (Allan, Herndon, and Dudley 1944). In 1990, Bialer et al found a second family with ADHS, with similar facial features and severe mental retardation in the United States (Bialer et al. 1992). In the following years, unrelated families have been identified in Brazil (Passos-Bueno et al. 1993; Zorick et al. 2004), Germany (Dumitrescu et al. 2004; Friesema et al. 2004), the Netherlands (Friesema et al. 2004) and Israel (Gika et al. 2010).
The symptoms could now be linked to single-nucleotide point mutations in the MCT8 gene for investigated subjects. Later other groups identified additional variants in the MCT8 gene (Dumitrescu et al. 2004). Shortly after, more AHDS patients showed similar TH concentrations and additional MCT8 mutations could be identified (Papadimitriou et al. 2008). Since then, several different mutations have been found in the MCT8 gene, leading to functional characterization *in vitro*. These gene mutations include 11 gross deletions, 10 small deletions, 13 small insertions, two splice-site mutations, 39 missense or nonsense mutations and one complex rearrangement (Schwartz et al. 2005; Jansen et al. 2008; Visser, Jansen, et al. 2009; Visser et al. 2013) with differences in the phenotype dependent on the cell type (Kinne et al. 2009) and localization of the mutated protein. Milder phenotypes can be due to an only slightly lower expression pattern compared to wild type and therefore residual activity of the transporter (Jansen et al. 2008).

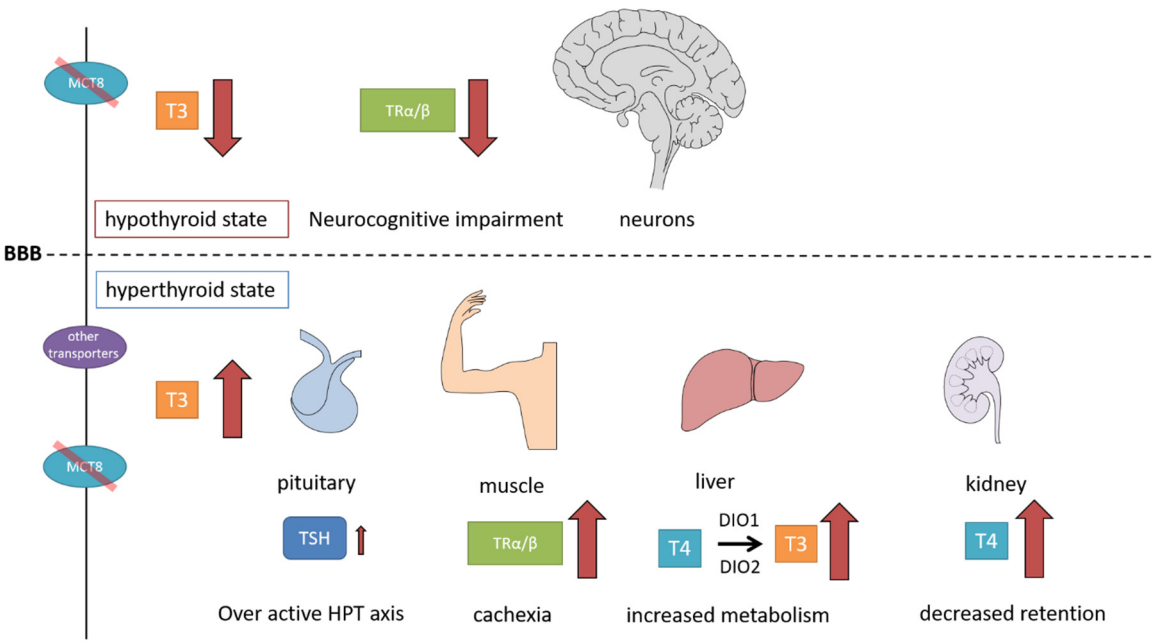

Figure 3: Overview of altered thyroid state of different tissues in AHDS patients. Inactivating mutations of the MCT8 lead to a hypothyroid state of brain tissue and a hyperthyroid state in the periphery. An insufficient amount of THs are transported into neurons, so TR signaling is decreased and neuronal development is impaired. In peripheral tissues, other TH transporters support the influx of THs leading to slightly elevated TSH generation of the pituitary and therefore an overactive HPT axis, increased TR signaling in muscle cells inducing cachexia, increased metabolism in liver cells and decreased retention of T4 in the kidney.

Since a MCT8 deficiency has a devastating effect on global development, especially neuronal development, the transporter is likely to be important for TH supply in the brain and only in a lesser extent for other organs, probably due to the presence of additional transporters that are able to compensate for the loss. Neuronal development starts within the third gestational week (Stiles and Jernigan 2010) and for the first trimester the embryo is provided with maternal TH (de Escobar et al. 2008). In comparison to congenital hypothyroidism, where the fetus had maternal TH supply until birth, MCT8 deficient patients are lacking TH from the beginning of gestation due to inefficient uptake by the compromised transporter. In 2014, López-Espíndola et al. published the first post-mortem brain histopathology of a MCT8-deficient male fetus (week 30 of gestation) and an 11-year old male patient. Both brains showed histological signs of immaturity, deficient myelination, and altered expression of TH-dependent neuronal proteins like the light neurofilament subunit (NEFL) and the Ca^{2+}-binding proteins parvalbumin (PVALB) and calbindin-D28k (CALB) in the frontal cortex and cerebellum. They could also show increased DIO2 and decreased DIO3 activities (López-Espíndola et al. 2014).

Due to the inefficient uptake into the brain, T3 exceeds in the periphery. Other transporters than MCT8 on the other hand can facilitate the transport of T4 over the BBB, so serum T4 concentrations are low. This results in a peripheral hyperthyroid state with elevated T3 concentrations that leads to an overactive HPT axis, meaning reduced TH secretion, as well as an increase in deiodination. The liver is in a hyperthyroid state, as well as skeletal muscle cells resulting in raised energy expenditure. Therefore, patients exhibit a hypermetabolic state with low body weight, increased perspiration, tachycardia, muscle wasting which can lead to a life-

threatening cachexia (Groeneweg et al. 2016). TH retention through the kidney is reduced due to MCT8 deficiency leading to a T4 accumulation within the kidney tissue (Bernal, Guadaño-Ferraz, and Morte 2015). **Figure 3** visualizes the altered thyroid state and the resulting symptoms of AHDS patients.

1.2.3 Transgenic models for MCT8 deficiency

To investigate MCT8 and its functions further, a MCT8 KO mouse model was generated. Though it replicated the thyroid state and serum TH concentrations of the affected patients (Dumitrescu et al. 2006), the mice lacked most of the behavioral impairments (Wirth, Roth, Blechschmidt, Hölter, Becker, Racz, Zimmer, Klopstock, Gailus-Durner, and Fuchs 2009), though the uptake of T3 into the brain has been shown to be compromised (Trajkovic et al. 2007). Although the KO model delivered an insight in the mechanism of MCT8 deficiency for the thyroid state (Di Cosmo et al. 2010), the neuronal development is thought to be rescued by another TH transporter, OATP1C1. It has been shown that a Mct8/Oatp1c1 DKO reflect a bit more the state of human MCT8 deficiency such as the cerebellar development, the reduced myelination, and locomotor abnormalities of MCT8-deficient patients (Mayerl et al. 2014).
Due to the limitations of mice models for MCT8, other organisms have been investigated, such as zebrafish with the *slc16a2* gene sharing 56-57% identity with the human SLC16A2 (Arjona et al. 2011). This KO model showed a neurological phenotype with impaired embryonic development (Vatine et al. 2013), altered behavioral performance and hypomyelination (Zada et al. 2014). With a phenotype closer to the human state of MCT8 deficiency than mice and the transparency of zebrafish larvae, life-imaging studies can be performed easily.
Since the transgenic KO of a whole gene does usually not mimic the pathogenic condition in the patients, a new approach was described in 2017. Here, fibroblasts from two affected AHDS patients and several control samples were taken and reprogrammed into induced pluripotent stem cells (iPSCs). Additionally, the gene-editing tool CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats) was used to either induce or correct the MCT8 mutation. The iPSCs were then used to generate two different models: a neural culture to investigate TH transport, neural differentiation and maturation, as well as induced brain microvascular endothelia cells (iBMECs) to establish a new BBB-model when seeded into a transwell system and examine TH transport over this cell layer. From the study it was concluded that although MCT8-deficient neurons had a significantly lower TH transport, T3-dependent gene expression was not impaired. It has to be noted that T3 concentration exposed on cells did not emulate *in vivo* conditions. On the other hand, the BBB-model has been shown to impair transport and efflux rates for MCT8-deficient cells. Therefore, the authors concluded, that the BBB is the critical barrier for TH transport and an affected MCT8 can lead to under-supply of the brain (Vatine et al. 2017).

1.2.4 Treatment options for MCT8 deficiency

Due to the early onset of TH deprivation during gestation, therapeutic options for MCT8 deficiency are currently limited and the developmental brain impairments are almost certainly irreversible. The most promising treatments so far are based on T3 analogs that could enter the cell independently of MCT8. A potential treatment approach is using 3,5-diiodothyropropionic acid (DITPA), a T3 analog, that is able to activate TRs (Pennock et al. 1992), but maintains normal metabolic activity (Moreno et al. 2008). A treatment of Mct8 KO mice resulted in an improvement of the peripheral hyperthyroid state, but was not able to act on the hypothyroid brain (Ferrara et al. 2015).
Another TH analog, 3,5,3'-triiodothyroacetic acid (Triac), a metabolite present in the normal organism in small concentrations, has also been shown to be transported MCT8-independently, though the mechanism is unknown. In contrast to DITPA, Triac was able to restore neural differentiation in Mct8/oatp1c1 KO mice (Kersseboom et al. 2014), though a recent study used Mct8 KO only and observed normalized TH serum levels, but no measurable Triac concentration in the brain as well as almost no effect on brain development. The authors deductively warned that the decreased concentration of free T4 would limit the amount of THs delivered to the brain and would aggravate the hypothyroid state even further (Bárez-López et al. 2016). It should be noted that the two mentioned studies above used different mice models. As stated in the last section, the Mct8 KO mouse model show only a mildly hypothyroid brain and therefore do not exhibit the abnormalities in neuronal development (Mayerl et al. 2014). For those reasons, the results from the latter study should be handled with caution.

1.3 Peptide-hormone conjugates

Hormonal treatment as an instrument in medical intervention, such as hormone replacement therapy with steroids (Scharbo-Dehaan 1996) or TH treatment of CH (Krude, Kühnen, and Biebermann 2015) have been done for a long time. Though a central administration can be beneficial in some cases, for several hormones negative side effects have been observed in certain tissues. The search for a possibility to exploit positive effects of a treatment, and omit negative, gave rise to the idea of target specific delivery of hormones. A new approach to deliver hormones specifically into target tissues is the hybridization with a peptide ligand for a G protein-coupled receptor. Here, binding of the ligand to receptor and the following internalization of this complex is utilized to bypass the influx over a transporter. The peptide is acting as guidance to specific target tissues that express the respective receptor. The conjugate is then thought to be processed intracellularly and the hormone is released from the peptide. For example, a peptide-hormone conjugate has been published very recently utilizing the metabolic effects of T3 under the guidance of glucagon. Here the therapeutic benefits of both, glucagon and T3, and their synergistic effects have been shown to correct symptoms of metabolic disease by targeting the liver, a glucagon receptor-expressing organ. The treatment with the conjugate did not affect the cardiovascular system and therefore reduced the adverse effects of a systemic T3 application (Finan et al. 2016).

Before that, in 2012, the first peptide-hormone conjugate was published: Glucagon-like peptide 1 (GLP1) as guidance for estrogen to ensure target specific delivery only into GLP1 receptor (GLP1R) expressing tissue (Finan et al. 2012). The steroid estrogen is known to modulate energy expenditure and feeding behavior (Mauvais-Jarvis 2011; Xu et al. 2011), but also exhibit undesired gynecological and tumor-promoting effects (Paruthiyil et al. 2004). Therefore, a central estrogen treatment for obesity is not a therapeutic option. The synthesized GLP1-estrogen conjugates have been pharmacologically tested to show the beneficial effects on weight loss in mice without the unwanted side effects on the uterus (Finan et al. 2012). The efficacy of this conjugate has been demonstrated in several *in vivo* and *in vitro* studies (Tiano et al. 2015; Schwenk et al. 2015; Vogel et al. 2016).
The proposed mechanism behind these findings has been based on the internalization mechanism of GPCRs. To gain full understanding of this mechanism, the properties of receptors will be introduced in the following chapters.

1.4 G protein-coupled receptors (GPCRs)

Communication between cells and their neighboring cells or the extracellular space is important for any biological process. The key players for the transmission of signals from the outside to the cytoplasm are receptors. The largest family of receptors is the family of transmembrane GPCRs that are abundant in all living organisms (Schöneberg et al. 2007). They can act on various types of outside signals such as light, mechanical impulses, and chemical compounds and conduct these signals to proteins in the intracellular space (Rosenbaum, Rasmussen, and Kobilka 2009). There are five classes of GPCRs based on sequence homologies, functional similarities and a phylogenetic analysis: Class A, the rhodopsin-like GPCRs, class B, the secretin receptor family, class C, the metabotropic glutamate GPCRs, adhesion and frizzled/taste GPCRs (Fredriksson et al. 2003).

1.4.1 Structure of GPCRs

All GPCRs have a basic structure in common and consist of seven transmembrane α-helices (transmembrane helices, TMH1-7) with an extracellular N-terminus and an intracellular C-terminus, three intracellular (ICLs) and three extracellular loops (ECLs). The largest class of GPCRs, class A contains 19 subclasses and includes a variety of receptor types such as neurotransmitter, hormone or light receptors, which are usually interact with a binding pocket inside of the THMs and ECLs (Rosenbaum, Rasmussen, and Kobilka 2009). They all share several highly conserved amino acids in their TMHs (Chelikani et al. 2007). Contrastingly, class B consists of a small group of GPCRs (around 25 members) with little homology to the class A receptors. They mainly bind smaller endogenous peptide ligands such as glucagon or secretin with their comparatively large N-terminal extracellular domain of ~100 to 160 amino acids (Hoare 2005)

1.4.2 Signaling of GPCRs

The eponymous property of GPCRs is the signaling via G proteins within the cytoplasm. Usually binding to an agonist causes changes in the receptor conformation, which leads to the coupling of G proteins. They are heterotrimeric proteins consisting of an α, β and γ subunit, that interact with the intracellular part of the receptor (Dohlman et al. 1991). The α-subunit exhibits a GTPase activity, hydrolyzing guanine triphosphate (GTP) to form guanine diphosphate (GDP) upon receptor activation. Therefore, the GPCR serves as guanine triphosphate exchange factor (GEF), which leads to the dissociation of the βγ-subunit from the α-subunit (Hurowitz et al. 2000). There are four distinguished G protein classes, referred to by their α-subunits: $G_{\alpha s}$ stimulating the adenylate cyclase (AC), $G_{\alpha i}$ inhibiting the AC and $G_{\alpha q/11}$ activating phospholipase C (PLC) and $G_{12/13}$ activating Rho guanine-nucleotide exchange factors (RhoGEFs). Those downstream factors are characteristic for the respective G protein and start signaling cascades (Pierce, Premont, and Lefkowitz 2002).
The AC, activated by $G_{\alpha s}$ and inhibited by $G_{\alpha i}$, catalyzes the reaction from adenosine triphosphate (ATP) from cyclic adenosine monophosphate (cAMP) by dephosphorylation. cAMP acts as a second messenger by binding to the regulatory chain of protein kinase A (PKA) and therefore activation the catalytic subunit. The targets of the PKA are diverse and are dependent on the cell type. Its general function is the regulation of gene expression. In a feedback loop, an enzyme called the phosphodiesterase deactivates the PKA by converting cAMP to AMP.
The activation of PLC-β by $G_{\alpha q/11}$ leads to the hydrolysis of phosphatidylinositol-4,5 bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol triphosphate (IP_3). The latter acts as a second messenger and binds to IP_3 receptors located in the membrane of the endoplasmic reticulum (ER). This triggers the release calcium ions (Ca^{2+}) into the cytoplasm. The calcium immobilization can have various effects such as muscle contraction, glycogen release or vesicle transport. Ca^{2+} together with DAG also binds to protein kinase C (PKC), which has several target proteins such as ERK1/2 (Jeremy M Berg 2002; New and Wong 2003). The βγ-subunits of G proteins are also capable of interaction with second messengers, leading to the activation of MAPK pathway (ERK1/2) (Faure, Voyno-Yasenetskaya, and Bourne 1994).
Since not every GPCR couples to every known G protein, the signaling properties for every receptor have to be investigated. These properties can differ depending on the type of ligand activating the receptor as well as the interaction of the GPCR with other transmembrane or cytosolic proteins. This functional selectivity is also known as “biased signaling” (Shukla 2014). One example for biased agonism would be the activation of a receptor by a newly discovered or synthetic agonist that shifts the signaling preference of the GPCR to different cascades than it is known for its endogenous ligand. Biased receptors are two distinct GPCRs that prefer divergent signaling pathways despite being activated by the identical ligand due to structural differences within the two, for example. The activated signaling cascades can also be pleiotropic, meaning dependent on the cell environment a GPCR-ligand pair can differ in different cell system due to differential expression of cofactors (Smith, Lefkowitz, and Rajagopal 2018). **Figure 4** depicts the four most prominent G proteins and their pathway on the left side.

1.4.3 Internalization of GPCRs

Upon agonist-dependent receptor activation, it has to be ceased at some point in order to inhibit continuous signaling. This desensitization mechanism is regulated by G protein-independent signaling involving arrestins. Their family is divided into visual and non-visual, ubiquitously expressed subtypes and they bind to GPCRs in order to initiate receptor internalization. For this step, the C-terminal part of the GPCR is phosphorylated by GPCR kinases (GRK) to initiate the binding of the arrestin molecule. Clathrin-coated pits mediate the internalization process causing the complex of receptor and bound ligand to undergo endocytosis. The internalized GPCR is then sorted to degradation or recycling back to the cell surface (Moore, Milano, and Benovic 2007). As they move from early to late endosomes, the pH decreases, which cause the receptor to release the bound ligand. Studies on the LDL (low-density lipoprotein) receptor and the liver-specific asialoglycoprotein receptor suggest that the ligand is then degraded in the lysosome (Lodish 2000). In addition to the desensitization and internalization mechanism, arrestins act as signaling scaffold proteins for several pathways, in particular the MAPK pathway. This signaling cascade controls many cellular functions, such as cell cycle progression, transcriptional regulation, and apoptosis (DeWire et al. 2007). As a scaffolding protein, arrestins facilitate kinase-interaction, ensure signaling specificity, and maintain subcellular distribution of the proteins (Pierce and Lefkowitz 2001). Some publications suggested that β-arrestins were actual initiators of these signaling cascades and referred to G protein-independent, β-arrestin-dependent signaling activation, mainly the ERK1/2 phosphorylation pathway (Eichel, Jullié, and von Zastrow 2016; Shenoy et al. 2006). Recently, the involvement in actual activation of this pathway by β-arrestins has been questioned, though the scaffolding role was not dismissed. New gene editing techniques such as CRISPR/Cas9 made complete KO of G proteins or β-arrestins possible, rather than a knock down using siRNA. A recent paper was able to show that β-arrestins at “zero functional G” failed to initiate ERK phosphorylation and morphological changes, whereas β-arrestin1/2 KO cells had an increase in ERK phosphorylation after agonist stimulation, hinting towards solely desensitization as the main role of arrestins and maybe a less pronounced role as a scaffold protein (Grundmann et al. 2018). The internalization process is depicted in **Figure 4** on the right side.

Recent findings have suggested that the internalization process initiated by β-arrestins does not necessarily result in an uncoupling of the α-subunit of the G protein and the rapid termination of signaling (Pavlos and Friedman 2017). Sustained intracellular (also called endosomal) cAMP signaling has been described for the TSHR (Calebiro et al. 2009), the luteinizing hormone receptor (LHR) (Lyga et al. 2016), the parathyroid receptor (PTHR) (Vilardaga et al. 2012), the GLP1R (Kuna et al. 2013), the vasopressin 2 receptor (V2R) (Feinstein et al. 2013), the β_2 adrenergic receptor (β_2AR) (Irannejad et al. 2013) and the dopamine receptors 1, 2 and 3 (D1R, D2R, D3R) (Kotowski et al. 2011) in physiological related cells or cell lines for most cases. This process seems to be dependent on the cell type, the receptor family, as well as the agonist activating the GPCR (Vilardaga, Jean-Alphonse, and Gardella 2014).

The termination of endosomal signaling and the final step to dissociate receptor and ligand is facilitated the recruitment of the multiprotein endosomal sorting actin-sorting nexin 27 (SNX)-retromer tubule (ASRT) complex, which replaces β-arrestin. From there, the receptor is either degraded or recycled in a rapid or slow fashion (Pavlos and Friedman 2017).

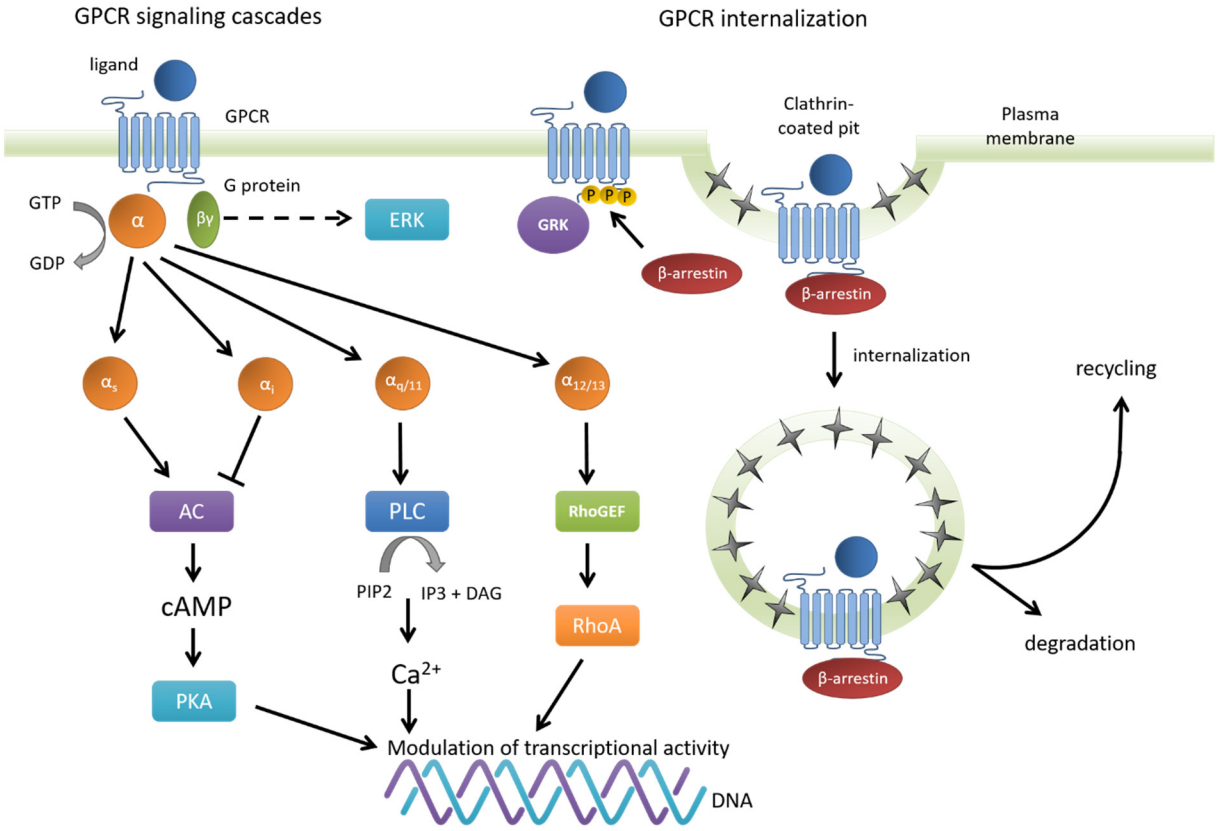

Figure 4: Graphical overview of GPCR signaling cascades and internalization mechanism. On the left side, pathways of the four most prominent G proteins are depicted. Upon ligand binding and receptor activation, a conformational change in the membrane protein leads to the exchange of a GDP to a GTP in the α-subunit of a coupled G protein. This subunit hydrolyses GTP to GDP and dissociates from its βγ-subunits. There are four prominent α-subunits: $G_{\alpha s}$ increases intracellular cAMP concentration by activation of AC, $G_{\alpha i}$ decreases cAMP by inhibition of AC, $G_{\alpha q/11}$ activates PLC, which leads to an increase in intracellular Ca^{2+} and $G_{\alpha 12/13}$ activating the RhoA pathway. All of these signaling cascades result in different gene regulations. On the right side, GPCR internalization is depicted. Upon activation, receptor signaling needs to be ceased to prevent overstimulation of the cell. GRKs phosphorylate the C-terminal part of the GPCR, which results in the recruitment of β-arrestins. The receptor-ligand complex gets internalized into clathrin-coated pits. After this endocytosis, receptors are either degraded or recycled back to the plasma membrane. AC: adenylate cyclase; PKA: protein kinase A; ERK: extracellular-signal regulated kinase; PLC: phospholipase C; GRK: GPCR kinase; Rho homology gene family, member A; RhoGEF: Rho Guanine exchange factor

1.4.4 Glucagon-like peptide 1 receptor (GLP1R)

The Glucagon-like peptide 1 receptor (GLP1R) is a class B receptor that is activated by incretins in pancreatic β-cells (Hoare 2005). The incretins GLP1 and glucose-dependent insulinotropic polypeptide (GIP) are produced by cells of the intestine and released into the blood stream after meal ingestion. A third natural agonist for GLP1R

is glucagon, which is released by the pancreatic α-cells when insulin concentration in the bloodstream is low to control glycolysis (Voet and Voet 2011). Agonist-dependent activation of the GLP1R leads to an increase in intracellular cAMP, which results in an accumulation of intracellular calcium and thus mediates insulin secretion, proliferation and inhibition of apoptosis. Additionally, GLP1R is expressed in neurons of the hypothalamus, where agonists promote reduced food intake and satiety. In the pancreas, GLP1R signaling controls insulin secretion and biosynthesis as well as β-cell proliferation and neogenesis (Baggio and Drucker 2007).
As mentioned above, the main signaling cascades activated by GLP1R is the G_S-pathway, accumulating intracellular cAMP (Skoglund, Hussain, and Holz 2000). Additionally, GLP1R in *rattus norvegicus* is reported to induce calcium response as well as ERK1/2 phosphorylation, suggesting coupling to the $G_{q/11}$ protein and $G_{i1,2}$ (Wheeler et al. 1993; Montrose-Rafizadeh et al. 1999). Co-expression and consequently interaction with GIP receptor (GIPR) was shown to significantly decrease cAMP and calcium production as well as ERK1/2 phosphorylation, whereas the interaction with glucagon receptor (GCGR) impaired calcium response and to a lesser extend ERK accumulation, but not cAMP production (Roed et al. 2015). As mentioned in the last chapter, GLP1R was reported to exhibit endosomal cAMP signaling in a pancreatic β-cell line suggesting implications for receptor-mediated regulation of insulin secretion (Kuna et al. 2013).
The peptide GLP1 is the result of a posttranscriptional cleavage of proglucagon, coded by the glucagon gene and is only processed in L cells, an endocrine cell type located in the ileum and the small intestine (Doyle and Egan 2007).

1.4.5 Oxytocin receptor and vasopressin arginine 1 A receptor

1.4.5.1 Oxytocin receptor (OTR)

The oxytocin receptor (OTR) belongs to the class A GPCRs and is known to couple to $G_{q/11}$ as well as G_i proteins resulting in increased Calcium mobilization and ERK1/2 phosphorylation, and decreased intracellular cAMP concentrations (Strakova et al. 1998). Its endogenous ligands oxytocin (OT) and the structural closely related arginine vasopressin (AVP), as well as synthetic drugs can activate the receptor. The two closely-related natural agonists are small neuropeptides, consisting of nine amino acids and differ only in two amino acids: the third position, an isoleucine for OT and a phenylalanine for AVP, and the eighth position, a leucine for OT and an arginine for AVP (Ganten et al. 1986). They originate from the same chromosomal locus and arose from an early gene duplication event in evolution (Gwee et al. 2009). Their structural similarity leads to the overlap in receptor targets, including the OTR and the three vasopressin receptors (V1AR, V1BR V2R). Both peptides are synthesized in magnocellular neurons in the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) of the hypothalamus. From there, their producing neurons project into the posterior lobe of the pituitary, where the neuropeptides are stored in vesicles and can be released into the peripheral circulation to reach OTR expressing tissues (Meyer-Lindenberg et al. 2011).
The expression of OTR is most prominent in uterus, placenta, kidney, heart and the CNS (Kimura et al. 1992). Its biological functions include mediation of uterine

contractions, lactation, cardiovascular and kidney functions, bone and muscle formation as well as sexual and social behaviors, such as maternal behavior and interpersonal bonding (Gimpl and Fahrenholz 2001; Zingg, Bourque, and Bichet 2012).

1.4.5.2 Vasopressin arginine 1 A receptor (V1AR)

The vasopressin arginine 1 A receptor (V1AR) also belongs to the Class A GPCRs and shares 45% sequence with the OTR. The receptor has a higher affinity to AVP in comparison to OT (Åkerlund et al. 1999; Tahara et al. 1997; Tahara et al. 1998). The main pathway is the activation of $G_{q/11}$/PLC- pathway and consequently ERK phosphorylation (Tahara et al. 1999; Schöneberg et al. 1998; Thibonnier et al. 1994)
In the periphery, V1AR is expressed in liver, kidney and vasculature, whereas the behavioral functions are mediated by the expression throughout the CNS, in particular hippocampus, hypothalamus, olfactory bulb, ventral tegmental area, substantia nigra, superior colliculus, dorsal raphe, nucleus of the solitary tract and inferior olive (Johnson et al. 1993; Caldwell et al. 2008). It has been under intense investigation concerning pair bonding, anxiety, circadian rhythm and renal function in rodents (Bielsky et al. 2003; Bales et al. 2007; Li et al. 2009). In humans, compared to rodents, V1AR is not expressed in the cortex and is investigated in the context of personality traits and autism (Loup et al. 1991; Meyer-Lindenberg et al. 2009).

1.5 The “Trojan Horse”-like mechanism

Although the efficacy of peptide-hormone conjugates has been shown in *in vivo* models, the underlying mechanism behind it is not completely elucidated. It is proposed that the GPCR-conjugate-complex is internalized upon receptor activation. Inside the cell, the pH-sensitive linker between peptide and hormone releases the hormone, which can then activate its respective signaling responses. Since this approach uses an unconventional way to deliver hormones into target cell by linking it to another compound, it has been dubbed the “Trojan Horse” approach or the “Trojan Horse”-like mechanism. Here the hormone conjugated to a peptide is only taken up into cells where the peptide receptor is expressed and thereby directed the hormone to specific target cells. In case of MCT8 deficiency, it could be a therapeutic option by choosing one or more target receptors that are expressed in hypothyroid areas of the brain (**Figure 5**).
Here, the choice of guidance peptide and target receptor needs careful consideration. The peptide should be easy to administer and have no or few adverse effects. Optimally, it should add to the overall objective of the treatment. The expression of its target receptors should align with the affected areas of the transporter deficiency. Expression in additional areas and the effects on these tissues need to be kept in mind for adverse side effects. Binding of the conjugate to the GPCR should start a signaling cascade, which could be G protein- or β-arrestin–dependent. Most importantly, the internalization process needs to be initiated by the conjugate. Negative effects of the linker position on the binding capacity of the peptide to the receptor need to be avoided or at least biased signaling should be noted as it could have diverse effect from the

administration of the peptide alone. Lastly, upon internalization the hormone should start its respective signaling cascades similar to a hormone transported via a transmembrane transporter. To find the perfect peptide-receptor pair for the targeted application, *in vitro* studies are necessary, especially to minimize animal testing.
The first created conjugate that would target the central nervous system was GLP1-T3. It has been developed for metabolic interventions and should primarily target pancreatic β-cells, but was the perfect conjugate for proof-of-principle studies. The second conjugate was designed derived from the symptoms of MCT8-deficient patients and their probably most affected brain areas: Oxytocin-T3 (OT-T3). T3 was attached to oxytocin at the position 8 (Leu) with a pH-sensitive linker. Here, the two important target receptors in mind were V1AR and OTR, both highly expressed in several brain areas.

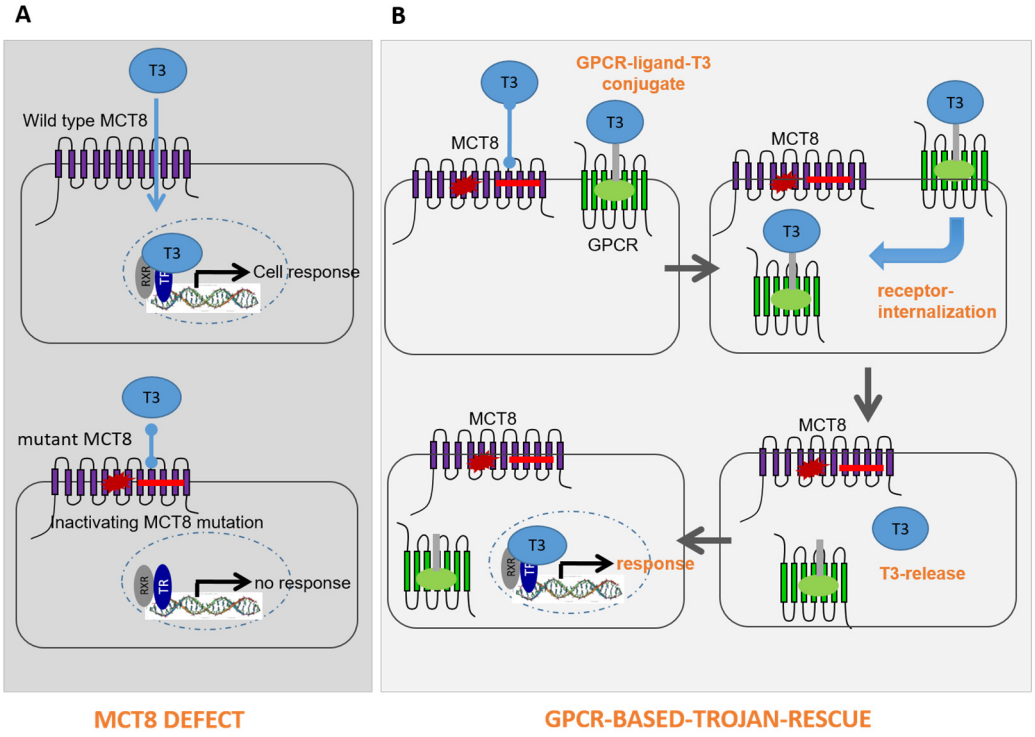

Figure 5: MCT8-deficiency and the "Trojan Horse"-like mechanism (A) The monocarboxylate transporter 8 (MCT8) is able to control efflux and influx of thyroid hormones (TH) T3 and T4. The biological active T3 binds to nuclear TH receptors (TR). The complex translocates into the nucleus, where a cell response is started. An inactivation mutation of MCT8 leads to a decreased uptake in the central nervous system and therefore to a severe psychomotor retardation.
(B) A therapeutic option for MCT8-deficiency could be the "Trojan Horse"-approach with the administration of peptide-T3 conjugates. The proposed mechanism is based on the internalization of G protein-coupled receptor (GPCR. The conjugate activates the receptor, the GPCR-ligand complex is internalized and T3 is released into the cytoplasm. Here it can bind to the TRs and start a cell response.
Picture credit: Heiko Krude

2 Aim of the study

From *in vivo* studies of already designed conjugates, it is known that the “Trojan Horse”- mechanism is generally working. So far, this mechanism was used to increase energy expenditure in order to treat obesity and related complications such as diabetes (Finan et al. 2012; Finan et al. 2016). Yet, this approach was never used to treat a rare disorder such as MCT8 deficiency. Therefore, the intention of this study was the elucidation of *in vitro* aspects that are prerequisites to use the “Trojan Horse”-like mechanisms in MCT8 deficiency. Ideally, a perfect ligand-receptor pair would be found that can be used as treatment for this devastating disease.
New peptide-T3 conjugates have been synthesized by our cooperation partners at the chemistry department of the Indiana University (Bloomington, USA), tested *in vivo* in Munich (Helmholtz Zentrum, Institute for Diabetes and Obesity), and need to be characterized in addition to *in vivo* studies. The investigation will be covering the following questions for every tested conjugate:

a) Does the conjugate activate the receptor and its signaling cascades? Here, all known signaling pathways for the respective receptor will be analyzed in comparison to the unconjugated peptide.
b) Does the conjugate initiate the receptor internalization? This is the crucial step for the “Trojan Horse”-like mechanism and will be investigated using internalization assays as well as fluorescence-based imaging methods.
c) Does the conjugate activate both canonical and non-canonical TH-dependent signaling pathways? Cells over-expressing the specific receptor are compared with cells that do not express the receptor. Only if T3-dependent signaling increases in the presence of the peptide receptor, it shows that the conjugate enter the cell through the “Trojan Horse”-like mechanism. If possible, the endogenously expressed TH transporter should be inhibited using known TH transporter blocker. If T3-dependent signaling is still present after inhibition, the T3 entered the cell through the “Trojan Horse”-like mechanism.

The first available conjugate used to establish the assays was GLP1-T3. It was developed to treat metabolic diseases, but was the ideal compound for proof-of-principle experiments. The conjugate that was developed with MCT8-deficiency in mind was oxytocin-T3. It has two target receptors that are expressed in the brain: The oxytocin receptor (OTR) and the Vasopressin arginine receptor 1 A (V1AR). It will be investigated using the established methods.

3 Material and methods

3.1 Materials

3.1.1 Technical equipment

Table 4: Machines used and their corresponding supplier company

Machine	Company
Anthos Plate reader 2001	Anthos Mikrosysteme GmbH, Krefeld, DE
Cell culture hood Lamin Air HBB 2448	Heraeus Instruments, Hanau, DE
Centrifuge Sorvall RC 6 Plus	Thermo Fisher, Waltham, MA, USA
Deep freezer Forma -80C ULT-Freezer	Thermo Fisher, Waltham, MA, USA
DNA sequencer 3130xL Genetic Analyzer	Applied Biosystem, Thermo Fisher
Gel chambers Whatman compact XS/S	Biometra, Jena, DE
Gel documentation system GeneFlash	Syngene, Cambridge, UK
Heating block Thermomixer Compact	Eppendorf, Hamburg, DE
Incubator certomat® BS-1	B. Braun, Melsungen, DE
Incubator kelvitron® t	Heraeus Instruments
Lab balance CPA 223S-OCE	Satorius, Göttingen, DE
LUNA™ Automated Cell Counter	Logos biosystems, Villeneuve d'Ascq, FR
Microscope Axiovert10	Zeiss, Oberkochen, DE
MilliQ-system Millipore Water Purification Systems	Merck Millipore, Darmstadt, DE
Mini centrifuge Galaxy Mini	VWR, Darmstadt, DE
pH-meter Seven easy	Mettler Toledo, Gießen, DE
Photometer BioPhotometer	Eppendorf
Plate reader Mithras LB 940	Berthold Technologies, Bad Wildbad, DE
Shaker IKA-vibrax-VXR	Janke&Kunkel, Staufen, DE
Shaker Vari-Shaker	Dynatech Laboratories Ltd., Sussex, UK
Table centrifuge Centrifuge 5417R/C	Eppendorf
Thermocycler Mastercycler® Gradient	Eppendorf

3.1.2 Chemicals and consumables

The companies that supplied consumables (Table 5A) and chemicals (Table 5B) are listed below.

Table 5: Supplier companies for consumables (A) and chemicals (B)

A	B
Becton Dickinson Biosciences	Invitrogen, Darmstadt, DE
Berthold Technologies	Merck, Darmstadt, DE
Biozym Scientific GmbH, Hessisch Oldendorf, DE	Promega, Mannheim, DE
BRAND GmbH + CO KG, Wertheim, DE	Roth, Karlsruhe, DE
Eppendorf	Sigma-Aldrich, Taufkirchen, DE
Greiner bio-one, Frickenhausen, DE	
PerkinElmer, Rodgau, DE	
Sarstedt, Nürnbrecht, DE	
Thermo Scientific	
TPP Techno Plastic Products, Trasadingen, CH	

3.1.3 Kits

Table 6: Overview of the commercially available kits that were used in the course of this project

Kit	Supplier
ABI Prism® BigDye® Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems
AlphaScreen™ cAMP Assay Kit	Perkin Elmer
CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS)	Promega
Luciferase Assay System, Reporter Lysis 5x Buffer	Promega
NanoBRET™ Nano-Glo® Detection System	Promega
Nano-Glo® HiBiT Extracellular Detetction System	Promega
Pure Yield™ Plasmid Midiprep System	Promega
Pure Yield™ Plasmid Miniprep System	Promega
SsoFast™ Evagreen Supermix®	Bio-Rad, München, DE
Wizard® SV Gel and PCR Clean Up System	Promega

3.1.4 Buffers, reagents

All buffers have been prepared using HPLC or Milli-Q water.

Table 7: Buffers and reagents commercially purchased or prepared in the lab

Buffer/solution	Components/Supplier/Comments
Ampicillin stock solution	50 mg/ml Ampicillin
cAMP assay medium	138 mM NaCl, 6 mM KCl, 1mM $MgCl_2$ * 6 H_2O, 5.5 mM Glucose, 20 mM Hepes, 1mM CaCl2 * 2 H_2O, 0.1% BSA, adjusted to pH 7.4
4′,6-diamidino-2-phenylindole (DAPI) stock solution	5 mg/ml dissolved in HPLC H_2O, Roche Applied Science, Mannheim, DE)
dNTP mix stock	50 mM, Invitrogen
dNTP mix	10 mM dNTP-Mix diluted from dNTP mix stock
70 % Ethanol	70 % (v/v) (96 % Ethanol diluted in HPLC water)
HPLC water	HPLC Gradient Grade, purchased from Fisher Chemicals, Schwerte, DE
3-Isobutyl-1-methylaxanthine (IBMX) stock solution	500 mM IBMX (Sigma-Aldrich), solved in DMSO (Dimethylsulfoxide)
Kanamycin stock solution	50 mg/ml Kanamycin
Loading dye	0.05 % (w/v) Bromphenolblue sodium salt, 0.05 % (w/v) Xylencyanol, 50 % (v/v) 1 x TBE, 50 % (v/v) Glycerol
LO buffer (basic solution for LI buffer)	0.1 % BSA (Bovine Serum Albumin), 0.3 % Tween® 20, 5 nM Hepes, adjusted to pH 7.4
LI buffer (lysis buffer for AlphaScreen™)	1 mM IBMX to LO buffer
Paraformaldehyde (PFA) for cell fixation	4% PFA in PBS
PBS Dulbecco	w/o Ca^{2+}, w/o Mg^{2+}, instamed 9.55 g/l, 1x: diluted in 5 l Milli-Q water, purchased from Biochrom AG, Berlin, DE
PBS-T	0.05 % (w/v) Tween® 20, in 1 x PBS
5 x TBE stock solution (Running buffer for gel electrophoresis)	0.5 M Boracic acid, 10 mM EDTA, 0.5 M Tris, adjusted to pH 8.0

3.1.5 Stimulation agents/Blockers

Table 8: Stimulation agents used in this project

Stimulant/Blocker	Stock solution	Supplier
2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH)	10 mM in H_2O	Sigma-Aldrich
3,3′,5-Triiodo-L-thyronine, T3	10 mM in DMSO	Sigma-Aldrich
3-iodothyronamin (3-T_1AM)	1 mM in DMSO	Santa Cruz
Bromosulphtalein (BSP)	4 mM in H_2O	Sigma-Aldrich
Desipramine hydrochloride (DMI)	10 mM in H_2O	Sigma-Aldrich
Forskolin	10 mM in DMSO	BioChemica - AppliChem, Darmstadt, DE
Glucagon-like-peptide 1 (GLP1)	1 mM in PBS + 0.1% BSA	Indiana University, Bloomington, USA
GLP1-T3 conjugate	1 mM in PBS + 0,1% BSA	Indiana University, Bloomington, USA
Oxytocin	1 mM in DMSO	Indiana University
Oxytocin-T3	1 mM in DMSO	Indiana University
Pertussis toxin (PTX)	50 µg/ml	Sigma-Aldrich
Probenecid	10.3 mM in NaOH, adjusted to pH 7.4	Sigma-Aldrich
Silychristin	20 mM in EtOH	Sigma-Aldrich
Thyrotropin-stimulating hormone from bovine pituitary (bTSH)	10 IU/ml in PBS + 0,1% BSA	Sigma-Aldrich

BSA: Bovine Serum Albumin; PBS: Phosphate buffered saline; DMSO: Dimethylsulfoxide; NaOH: Sodium hydroxide; EtOH: Ethanol

3.1.6 Enzymes

All reactions using the following commercially available enzymes were prepared with the supplied appropriate buffers.

Table 9: Commercially available enzymes used for this project.

Enzyme	Supplier
Carboxy-Flexi® Enzyme Blend (*Sgf*I*, EcoICR*I)	Promega
Flexi® Enzyme Blend (*Sgf*I*, Pme*I)	Promega
Mango-Taq-DNA Polymerase	Bioline
Pfu-Turbo DNA Polymerase	Agilent Technologies
Restriction endonucleases (*Dpn*I*, EcoR*I*, Hind*III*, Nhe*I*, Pac*I*, Xba*I*, Xho*I)	New England Biolabs
T4 DNA Ligase	New England Biolabs
Trypsin/EDTA-solution (0,5/0,2% in PBS)	Biochrom

3.1.7 Expression vectors

Table 10: Expression vectors utilized in the course of this project; * marks

Name	Supplier
pBiT3.1-secN	Promega
pcDNA3	IEPE (Charité)
pcDps	IEPE (Charité)
p(DR4)2-SV40-luc+ (pGL3)	IEE (Charité)
pFC14A	Promega
pFC32K	Promega
pGL4.3-[luc2P/NFAT-RE/Hygro]	Promega
pGL4.3-[luc2P/SRE/Hygro]	Promega
pRS-rTRα1	IEE (Charité)
$pSNAP_f$	New England Biolabs

3.1.8 cDNA of proteins of interest

Table 11: Sources for cDNA of proteins of interest for this project

Gene	Protein	Supplier
rARRB2	β-arrestin2 (*rattus norvegicus*)	Dr. Vera Knäuper College of Biomedical and Life Sciences, Cardiff University, UK
GLP1R	Glucagon-like peptide 1 receptor	Source Bioscience, Nottingham, UK
OTR	Oxytocin receptor	DNASU plasmid repository
V1AR	Vasopressin Arginine receptor 1 A	DNASU plasmid repository

3.1.9 Fluorescent dyes, gel electrophoresis ladder

Table 12: Fluorescent dyes and DNA markers implemented for this project

Agent	Supplier
SNAP Surface® A488	New England Biolabs
1 kb DNA Ladder (0.5 µg/lane)	Invitrogen, BIOLINE
Easy Ladder I	Invitrogen, BIOLINE

3.1.10 Cell culture reagents and media

Table 13: Overview of cell cultured related reagents and media for this project

Agent	Supplier
Advanced MEM (w Non-essential amino acids, w 110 mg/l Na- pyruvate, w/o L-glutamine)	Gibco
Charcoal-treated Fetal Bovine Serum (FBS)	Biochrom
Dulbecco´s MEM (w 3.7 g/l NaHCO3, w 4.5 g/l D-Glucose, w/o L-Glutamine, w/o Na- Pyruvate, low endotoxin)	Biochrom
Fetal Bovine Serum (FBS)	Biochrom
FuGene HD®	Promega
FBS (charcoal-stripped, sterile-filtered)	Sigma-Aldrich
MEM Earle´s (w 2.2 g/l $NaHCO_3$, w stable glutamine, low endotoxin)	Biochrom
Metafectene®	Biontex Laboratories GmbH, Martinsried, DE
Mounting medium with DAPI	Vectastain
Non-essential amino acids (NEA)	Biochrom
Opti-MEM I Reduced Serum Medium (1x)	Gibco
Opti-MEM I Reduced Serum Medium (1x), w/o phenol red	Gibco
Penicillin/streptomycin	Biochrom
Phenol/water/chloroform	Applied Bioscience, Beverly Hills, CA, USA
Phosphate buffered saline (PBS), 1x, Dulbecco (w/o Ca^{2+}, w/o Mg^{2+}, low endotoxin)	Gibco
Poly-L-lysine (0.1 mg/ml)	Biochrom

Cells were cultivated in complete media with additive specified as:

Table 14: Composition for complete media for the different cell lines used in the course of this project

Complete medium for	Basis medium	additives
HEK 293	MEM	5% (v/v) FBS, 0.5% (v/v) NEA
COS7	DMEM	10% (v/v) FBS, 0.5% (v/v) Pen/Strep
Freezing medium	FBS	10% (v/v) DMSO

3.1.11 Bacteria culture reagents and media

All media are prepared using MilliQ water.

Table 15: Composition for bacterial culture reagents and media implemented for this project

Medium	Composition	Antibiotics
LB medium	0.5% (w/v) yeast extract, 1% (w/v) tryptone/peptone from casein, 1% (w/v) sodium chloride, adjusted to pH 7.4	100 µg/ml ampicillin or kanamycin
LB agar plates	1,5% (w/v) agar-agar in LB medium	100 µg/ml ampicillin or kanamycin
SOB medium	0.5% (w/v) yeast extract, 2% (w/v) tryptone/peptone from casein, 0.05% (w/v) sodium chloride, 25 mM potassium chloride, adjusted to pH 7.4	
SOC medium	20 mM magnesium chloride, 20 mM glucose in SOB medium	

3.1.12 Bacterial strains and eukaryotic cell lines

Table 16: Bacterial strains and eukaryotic cell lines used in the course of this project

Strain/cell line	Properties
E. coli Max Efficiency® DH5α™	Chemically competent bacterial strain, 10^9 transformants/µg plasmid DNA, purchased from Invitrogen
HEK 293	Human embryonic kidney cell line, immortalized by transformation with adenoviral fragments, adherent
COS7	*Cercopithecus aethiops*, African green monkey kidney fibroblast-like cell line, immortalized by transformation with SV40 T antigen, adherent

3.1.13 Computer software

Table 17: Computer software used for this project

Program	Supplier
GraphPad Prism 6	GraphPad Software, La Jolla, CA, USA
Microsoft Office	Microsoft, Unterschließheim, DE
MikroWin 2000	Mikrotek Laborsysteme, Overath, DE
ImageJ	National institute of health, MD, USA

3.2 Methods

3.2.1 Molecular biology methods

In this project, peptide-T3 conjugates were validated using numerous *in vitro* assays. Therefore, the target receptors were cloned into the expression vector specific for the various *in vitro* assays. Afterwards, characterization of the conjugates was performed focusing on two main topics: 1) GPCR activation and internalization by the compound and 2) the TH signaling as proof of the “Trojan Horse”-like mechanism. **Figure 6** depicts the workflow for the project:

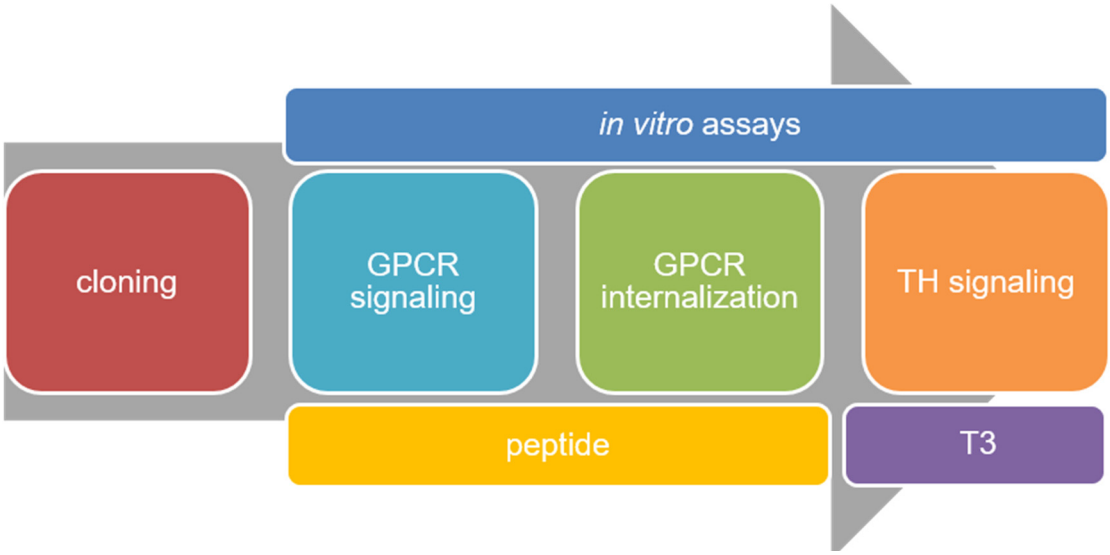

Figure 6: Schematical depiction of the workflow for this project. The cDNA of the target receptors was cloned into various expression vectors that are specific for the *in vitro* assays. Afterwards, GPCR signaling and internalization as well as TH signaling was investigated using various *in vitro* assays.

3.2.1.1 Cloning strategy

For the validation of the peptide-hormone conjugates, several *in vitro* assays were employed. For these, cDNA of the target receptors was cloned into different expression vectors following the scheme in **Figure 7:**

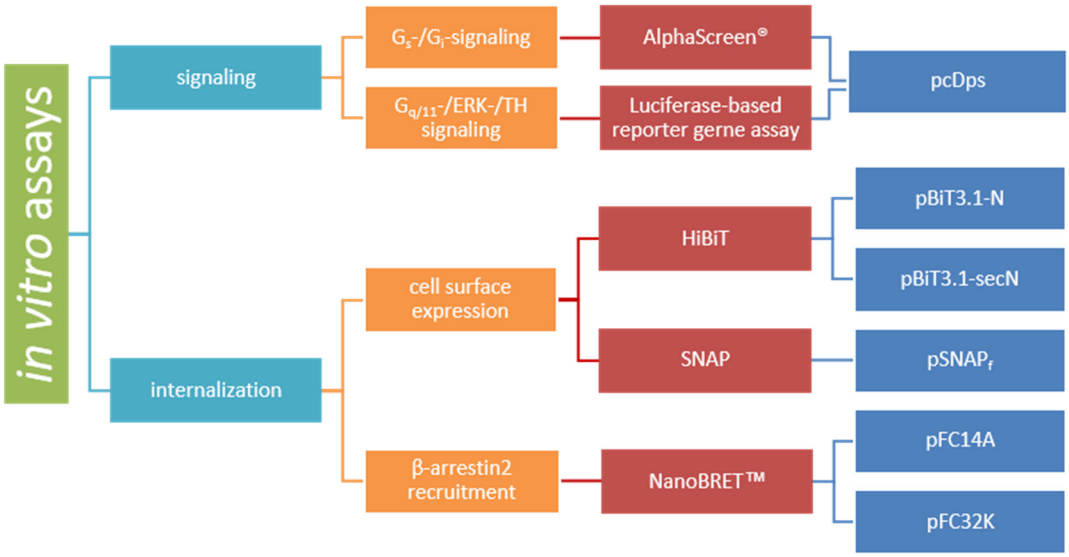

Figure 7: Overview of *in vitro* assay and the expression vectors used for cloning for the specific assays. The *in vitro* assays were used to monitor signaling and GPCR internalization. Signaling assays were either luciferase-based reporter gene assays or a competitive-based AlphaScreen™ assay. For all of them, GPCRs were cloned into the expression vector pcDps. For the internalization assays, cell surface expression was measured using either the HiBiT system, which uses the expression vectors pBiT3.1-N or pBiT-3.1secN, or the SNAP-tag system that requires cloning into the expression vector pSNAP$_f$. Part of the internalization mechanism is the β-arrestin2 recruitment of GPCRs, which was measured using a protein interaction assay, the NanoBRET™, a system that uses pFC14A as the acceptor fusion vector and pFC32K as the donor fusion vector.

The untagged versions of the GPCRs have been cloned into the expression vector pcDps, which utilizes a SV40 promotor for expression. The optical visualization to investigate the behavior of GPCRs towards the peptide-hormone conjugates used several fusion proteins generated by expression vectors. These needed to be cloned into different expression vectors in the course of this project. The first generated N-terminally SNAP-tagged constructs of GPCRs with the pSNAP$_f$ expression vector. Additionally, fusion proteins for a new proximity assay (NanoBRET™) were generated, adding either the luciferase NanoLuc or the protein tag HaloTag®, both C-terminally, using the expression vector pFC32K or pFC14A respectively. Lastly, a small protein tag called HiBiT has been added to the N-terminus of the GPCRs by employing the expression vectors pBiT3.1-N or pBiT3.1-secN. The GPCRs used for tagging were GLP1R, V1AR, and OTR. All of these plasmids express the insert under the control of the CMV promotor. Furthermore, all cDNA sequences were checked for cutting sites beforehand, to make sure that the enzymes do not cut inside of the amplicon.

3.2.1.1.1 Cloning of SNAP-tagged GPCRs

To investigate the internalization of GPCRs, N-terminally SNAP-tagged receptors have been generated. The advantage of the pSNAP$_f$ expression vector was the fact that it has two multiple cloning sites (MCS) to be able to generate both, N- and C-terminally tagged fusion proteins. Since the GLP1R includes a signal peptide, a two-step cloning strategy was used to position the signal peptide N-terminally of the SNAP-tag, whereas the rest of the GPCR is fused to the C-terminus of the tag. The receptors V1AR and OTR do not need signal peptides to be delivered to the plasma membrane, so no additional cloning step was needed.

For DNA amplification of all GPCRs, primers were designed to include the restriction enzymes *Xho*I for the forward primer and *Pac*I for the reverse primer. The GLP1R signal peptide cDNA was amplified using primers containing *Nhe*I for the forward primer and *EcoR*I for the reverse primer. All restriction enzymes are contained either in the first MCS of the expression vector, located N-terminally from the SNAP-tag (*Nhe*I*, EcoR*I) or in the second MCS on the C-terminal site of the tag (*Xho*I*, Pac*I).

3.2.1.1.2 Cloning fusion proteins for NanoBRET™

To generate the GPCRs fused with the BRET partners, the expression vectors from the Flexi cloning system (Promega) were utilized. These vectors enclose a protein-encoding region flanked by the restriction enzymes *Sgf*I and *EcoICR*I as cloning sites, endonucleases that do not affect most human open reading frames. The PCR amplicons were generated using primers that add a *Sgf*I restriction site to the 5' end and a *Pme*I restriction site on the 3' end. After digestion, these proceed into a sticky and a blunt end site respectively. Cloning primers were designed omitting the stop codon and append a GTT codon (encoding a valine). Ligation to the blunt end produced by *Eco*I*CR*I in the plasmid results in the elimination of the stop codon in the *Pme*I restriction site, allowing the fusion with one of the two BRET partners. All GPCRs were cloned into both expression vectors for NanoBRET™, resulting in fusion proteins with NanoLuc and HaloTag® on the C-termini of the receptors.

3.2.1.1.3 Cloning HiBiT-tagged GPCRs

A new surface expression assay employs a small protein tag (eleven amino acids) called HiBiT. To generate HiBiT-tagged GPCRs, the HiBiT MCS-cloning system (Promega) was used. Here, choosing different restriction sites in the vector can employ a variable Gly/Ser linker. For the GLP1R a very long spacer of 24 amino acids was chosen and the cDNA was cloned into the pBiT3.1-secN expression vector, containing an N-terminal IL6 signal peptide to replace the signal peptide of the GLP1R. The used restriction enzymes were *Xba*I and *Hind*III and were added via PCR to the amplified sequence.

3.2.1.2 Polymerase chain reaction (PCR)

Polymerase chain reaction is an enzyme-dependent method to amplify specific double-stranded DNA sequences exponentially (Schorderet 1994). This method was used to amplify target sequences from expression vectors with the polymerase PfuTurbo. The reaction was prepared according to **Table 18**:

Table 18: PCR preparation and cycler program

PCR preparation		Cycler program		
ingredient	amount	time	Temperature	cycles
template DNA	50 – 300 ng	2 min	95°C	1
PfuTurbo-Buffer	1x	30 s	95°C	30
dNTP mix	0.4 mM	30 s	55°C	
Forward primer	125 ng	5 min	72°C	
Reverse primer	125 ng	10 min	72°C	1
PfuTurbo	2.5 U	30 min	4°C	1
HPLC water	Add to 25 µl	∞	20°C	1

After the reaction, the amplicon was purified using the column-based "wizard SV gel and PCR clean-up system" (Promega) according to manufacturer's protocol to remove proteins, salts and unused nucleotides. For further cloning, the DNA underwent digestion with the appropriate restriction enzymes, another purification and ligation into the respective expression vector. Afterwards the ligated vectors were propagated in chemically competent *E.coli* (DH5α) by transformation.

3.2.1.2.1 Site-directed mutagenesis

In order to introduce insertion, deletion or substitutions within the target DNA, site-directed mutagenesis can be used. This method utilizes a customized PCR with primers, complementary to the template, containing the desired modification, preferably in the middle of the sequence. After the reaction, the amplicon is not methylated in contrast to the template sequence, which does not contain the desired modifications. To remove the template, a digestion with *Dpn*I is used. This restriction enzyme recognizes and cleaves methylated DNA, which evolve by DNA-methylases in *E.coli*, and can be used to remove the methylated template DNA, leaving the amplicon intact (Kunkel 1985). In the course of this project, the PCR was performed with PfuTurbo. The reaction was prepared according to **Table 19**:

Table 19: Site-directed mutagenesis PCR preparation and cycler program

PCR preparation		Cycler program		
ingredient	amount	time	Temperature	cycles
template DNA	10 – 200 ng	30 s	95°C	1
PfuTurbo-Buffer	1x	1 min	95°C	18
dNTP mix	0.4 mM	1 min	55°C	
Forward primer	125 ng	5 min	68°C	
Reverse primer	125 ng	10 min	68°C	1
PfuTurbo	2.5 U			
HPLC water	Add to 50 µl	∞	4°C	1

After the reaction, digestion with *Dpn*I (20 units) and transformation into chemically competent *E.coli* (DH5α) was performed.

3.2.1.2.2 DNA sequencing

DNA sequencing is a standard procedure to verify the correctness of distinct sequences. In the course of this project, sanger-sequencing was performed (Sanger, Nicklen, and Coulson 1977). This technique relies on the principle of PCR with additional differently fluorescently labeled dideoxynucleotides (ddNTPs). Here, only one primer is used to amplify single-stranded DNA with randomly introduced ddNTPs, which results in the termination of the elongation due to the lack of the 3'-hydroxy group. This produces a mix of DNA fragments of various lengths. To analyze the sequence, these fragments are then sorted by length in automated sequencing machines that read out the fluorescent labels on the 3'-end (Shendure et al. 2011). In the course of this project, DNA sequencing was conducted using the Big Dye polymerase. The reaction was prepared according to **Table 20**:

Table 20: Sequencing PCR preparation and PCR program

PCR preparation		Cycler program		
ingredient	amount	time	Temperature	cycles
template DNA	100 – 300 ng	2 min	95°C	1
Big Dye sequencing buffer	0.75x	30 s	95°C	30
		30 s	55°C	
primer	0.5 µM	5 min	60°C	
Big Dye Mix V3.1	0.75 µl	7 min	60°C	1
HPLC water	Add to 10 µl	∞	4°C	1

After the reaction, the DNA was precipitated using 0.1 M sodium acetate and 20 µl 96 % ethanol. After a centrifugation step at 15°C for at least 30 min, the supernatant was removed by suction and the pellet was washed with 70% ethanol. After a second centrifugation step for 15 min the supernatant was removed again and pellets were dried for 5 min at 37°C. The dry pellet was stored in -20°C until it was suspended in sequencing buffer and analyzed using the sequencing machine (ABI PRISM 3130xl)

3.2.1.3 Restriction digestion

For the insertion of double-stranded DNA into an expression vector, digestion with restriction enzymes is used to create compatible ends to join. Depending on the enzyme used for the digestion, 3'- or 5'-sticky ends or blunt ends can be created by a break of the phosphodiester link of the DNAs backbone. The specific cutting sites are palindromic, so that sticky ends can only be joined with the complementary end respectively, whereas blunt end can be joined with any other blunt end. To inhibit spontaneous re-ligation of the expression vector after restriction, the 5'-phosphate group needs to be removed by additional incubation with calf intestinal alkaline phosphatase (CIP) (Arber and Linn 1969). The reaction was prepared according to **Table 21**:

Table 21: Restriction digestion preparation for expression vector and insert

Digestion preparation for expression vector		Digestion preparation for DNA insert	
ingredient	amount	ingredient	amount
Digestion buffer	1x	Digestion buffer	1x
Expression vector	4 µg	Expression vector	1 µg
Restriction enzyme I	1,5 µl	Restriction enzyme I	2,5 µl
Restriction enzyme II	1,5 µl	Restriction enzyme II	2,5 µl
HPLC water	Add to 30 µl	HPLC water	Add to 50 µl
Incubation at 37°C for 45 min		Incubation at 37°C for 1h	
CIP	1,5 µl		
Incubation at 37°C for 15 min			

To identify and purify the desired fragments, agarose gel electrophoresis was implemented. Respective bands were cut out, identified by their length (in bp) and purified using the column-based “wizard SV gel and PCR clean-up system” (Promega) according to manufacturer’s protocol. The next step is the joining of expression vector and insert using ligation.

3.2.1.4 Ligation

The actual joining reaction of two double-stranded DNA molecules is executed by the T4 ligase enzyme and is called ligation. The ligase joins a 5’-phosphate with a 3’-hydroxy group to create a phosphodiester bond between two nucleotides and uses ATP (Lehnman 1974). The reaction was prepared with a volume ratio between vector and insert ranging from 1:1 to 1:5, depending on the length of insert. The reaction was prepared and modified according to **Table 22**:

Table 22: Ligation preparation

ingredient	amount
Ligation buffer	1x
Digested expression vector	1 µl
Digested insert DNA	1 µl /3 µl /5 µl
T4 ligase	200 units
HPLC water	Add to 20 µl
Incubation at room temperature for 1-2h or 16°C overnight	

For propagation of correctly joined expression vector and insert, the ligation reaction was transformed into chemically competent *E.coli* (DH5α).

3.2.1.5 Transformation

Bacterial transformation of plasmid DNA is a technique to amplify generated expression vectors that include the desired insert sequence. An *E.coli* strain DH5α, made chemically competent by the rubidium chloride method (Hanahan, Jessee, and Bloom 1991) is forced to take up and incorporate exogenous DNA by the heat shock method. The DNA is then amplified through bacterial replication and selected using

resistance encoded by the expression vector. The desired plasmid DNA can be stored by freezing glycerol stocks of transformed bacteria stored at -80°C.
For this project, 50 µl of competent DH5α cells were placed on ice for 10 to 15 min, then incubated with either 20 µl of ligation preparation or 50 µl of *Dpn*I digested mutagenesis preparation for 20 to 30 min on ice and afterwards the heat shock was applied for 90 sec at 42°C. In the following, the cells chilled for 2 min on ice and 250 µl pre-warmed SOC-medium was added. The mixture was incubated while shaking for 30 to 60 min at 37°C. Afterwards cells were plated on agar plates supplemented selective antibiotics (ampicillin or kanamycin) and incubated at 37°C overnight. A successful transformation yielded colonies of clones, which were picked to inoculate mini preparations of 5 ml LB-medium supplemented with selective antibiotics overnight. The plasmid DNA was isolated using "Pure Yield™ Plasmid Miniprep System" (Promega) according to manufacturer's protocol. The correctness of inserted DNA sequences was checked using sequencing PCR. Upon conformation, midi preparations (200 ml) were inoculated overnight and the 500 µl culture was used to generate glycerol stocks. DNA isolation was performed using "Nucleobond Xtra Midi Prep" (Macherey & Nagel).

3.2.2 Cell culture methods

3.2.2.1 Cultivation of cell lines

The cultivation of mammalian cell lines took place in tissue culture treated polystyrene flask (75 cm^2) at standard cell culture conditions of 37°C and 5% CO_2 in appropriate cell culture media.
Passaging was accomplished by washing the cells with 10 ml Dulbecco's PBS and trypsinizing with 1x Trypsin/EDTA for 3 to 6 min at 37°C until cells are detached. Light tapping was performed to support detachment and cells were then harvested by re-suspended with complete cell culture media added to 10 ml volume. As an ingredient of the media, serum oversaturates trypsin and stops the enzymatic digestion of cell matrix. The re-suspended cells were then split and seeded.
For performance of a functional assay, cells were counted using a cell counting device. The appropriate cell concentration was set and the cells were seeded in the respective dish format. For TH-signaling assays, cells were seeded with MEM, containing charcoal-treated and therefore TH-free FBS (5%).
For storage of cell aliquots, the cell suspension was centrifuged at 200 g for 10 min, the pellet was re-suspended using freeze media containing FBS with 10% DMSO and placed into a cryo tube. The freezing process was conducted using a "Mr. Frosty" cooling device for gradually freezing and placed at -80°C. For re-cultivation, cells were thawed in a water bath at 37°C shortly and seeded in pre-warmed complete cell culture media in a 75 cm^2 culture flask. The next day, the media was exchanged to remove DMSO and cell debris.

3.2.2.2 Transfection

Transfection is a method to incorporate expression vectors into mammalian cells in order to create cells overexpressing a desired protein. Depending upon whether a selective pressure is applied (e.g. using a selective antibiotic) or not, the transfection

will be permanent (stable transfection) or only for a few days (transient transfection). For cell lines that are easy to transfect, transfection reagents can be used that form a complex with the DNA to mask its negative charge. This results in the DNA entering the cell and an expression in a high rate.
For all studies conducted in this project, only transient transfections were performed.

3.2.2.2.1 Metafectene™

Functional studies were performed in HEK293 or COS7 cells cultured in MEM (Earl's minimal essential medium) supplemented with 5% FBS (fetal bovine serum) and 1% non-essential amino acids or DMEM (Dulbecco's MEM) containing 10% FBS and 100 U/ml penicillin, 100 µg/ml streptomycin respectively. For that, cells were seeded into 96 well plates at a cell density of $1.5x10^4$ cells/well and 5% CO_2. For HEK 293 cells, plates were pretreated with poly-L-lysine (1:2 in PBS) for 10 min and washed twice with PBS. Transfection was performed with 45 ng plasmid DNA/well and 0.45 µl Metafectene™/well 24h after seeding. The transfection reagent was premixed in half of the needed volume of serum-free medium and incubated for 5 min at room temperature. The DNA was added to the other half of the volume and the prepared Metafectene™ mix was added to the DNA preparation. After incubation for 15-20 min at room temperature, the complete medium was sucked off the cells and replaced with the transfection mix. Cultivation was continued at 37°C and 5% CO_2. For cAMP assays, cells were transfected with the appropriate GPCR expression vector or empty vector. For reporter gene assays, equal amounts of the appropriate reporter construct containing the firefly luciferase gene and the respective GPCR were co-transfected. For T3 response reporter gene assays, equal amounts of constructs containing rTRα1 have also been co-transfected to have an abundance of TR in the cell.

3.2.2.2.2 FuGene® HD

In comparison to Metafectene™, FuGene® HD has a lower autofluorescence and can therefore be used for imaging studies that involve fluorescent dyes. This transfection reagent was used to transfect HEK293 cells on cover slips in a 6 well plate ($1.7x10^5$ cells/well) 24h after seeding. Transfection of SNAP-tagged GPCRs was performed using FuGene® HD in a reagent-DNA-ratio of 2.5:1 and according to manufacturer's protocol. In details, 3.3 µg/well DNA was added to Opti-MEM, mixed with 8.3 µl FuGene® HD and incubated for 5-10 min at room temperature. For transfection, 150 µl of the transfection preparation was added dropwise to each well and cultivation was continued at 37°C and 5% CO_2.
Additionally, FuGene® HD was used for the NanoBRET™ assay. Here, HEK293 cells in 6 well plates ($8.0x10^5$ cells/well) were transfected 4-6 h after seeding in a reagent-DNA-ratio of 3:1 and according to manufacturer's protocol. The BRET partners were kept in a donor-acceptor-ratio of 1:10, diluted in carrier DNA (pGEM3Z, Promega). In details, a transfection mixture was prepared with 2 µg of HaloTag® plasmid and 0.2 µg of NanoLuc plasmid and 0.2 µg carrier DNA in 100 µl Opt-MEM for each well. To each mixture, 8 µl FuGene® HD was added and incubated for 10 min. The mixture was then added dropwise to each well with attached cells and incubated for approximately 20h at 37°C, 5% CO_2 to allow protein expression.

3.2.3 Biochemical methods

3.2.3.1 Characterization of intracellular signaling transduction

GPCR signaling can be influenced by many factors, including fusion with other proteins or modified ligands. In order to make sure that T3 conjugated peptide ligands are able to activate a signaling cascade in the same manner as the unmodified ligand, a functional characterization of the conjugates took place. Additionally, modifications of any kind can also lead to deviating behavior of GPCRs, known as biased signaling (Shukla, Singh, and Ghosh 2014). Depending on the type of signaling pathway the respective receptor was involved, different approaches were used to measure the accumulation of second messengers.
Next to the pathway activations of GPCRs, intracellular signaling can be activated by TH. The T3 conjugated ligands are supposed to release the T3 in the intracellular space, where several signaling cascades can be activated. Therefore, reporter gene assays were used to analyze different signaling pathways.

3.2.3.1.1 Intracellular cAMP accumulation assay for measurement of G_S- and G_i-activation

Cyclic AMP is a downstream product of the interaction between the GPCR and the G_S protein. It is enzymatically produced by the adenylate cyclase, which is activated by the α-subunit of the G_S protein. For a direct conclusion of receptor activation to intracellular cAMP concentration, the degrading enzyme phosphodiesterase needs to be inhibited by adding 3-Isobutyl-1-methylxanthine (IBMX) while receptor stimulation is in progress. Intracellular cAMP concentration was determined using the AlphaScreen™ (Amplified Luminescent Proximity Homogeneous Assay, PerkinElmer) method. This competitive assay is based on the reaction of two different kinds of beads. The acceptor beads are conjugated to a cAMP antibody and bind to accumulated cAMP in the sample. The streptavidin-coated donor beads are added together with biotinylated cAMP, which is then bound to both donor and acceptor beads. Excitation of the donor beads at 680 nm leads to a conversion of ambient oxygen to a more excited singlet state. In close proximity, this molecule diffuses across to react with the acceptor bead, which generates a chemiluminescence emitting light in the range of 520 to 620 nm. Since this reaction only occurs, when donor and acceptor beads are bound to the same molecule, the biotinylated cAMP is creating signals, being in competition to the intracellular cAMP. Therefore, this method is an indirect determination of cAMP concentration (Eglen et al. 2008). The principle of the AlphaScreen™ is depicted in **Figure 8**:

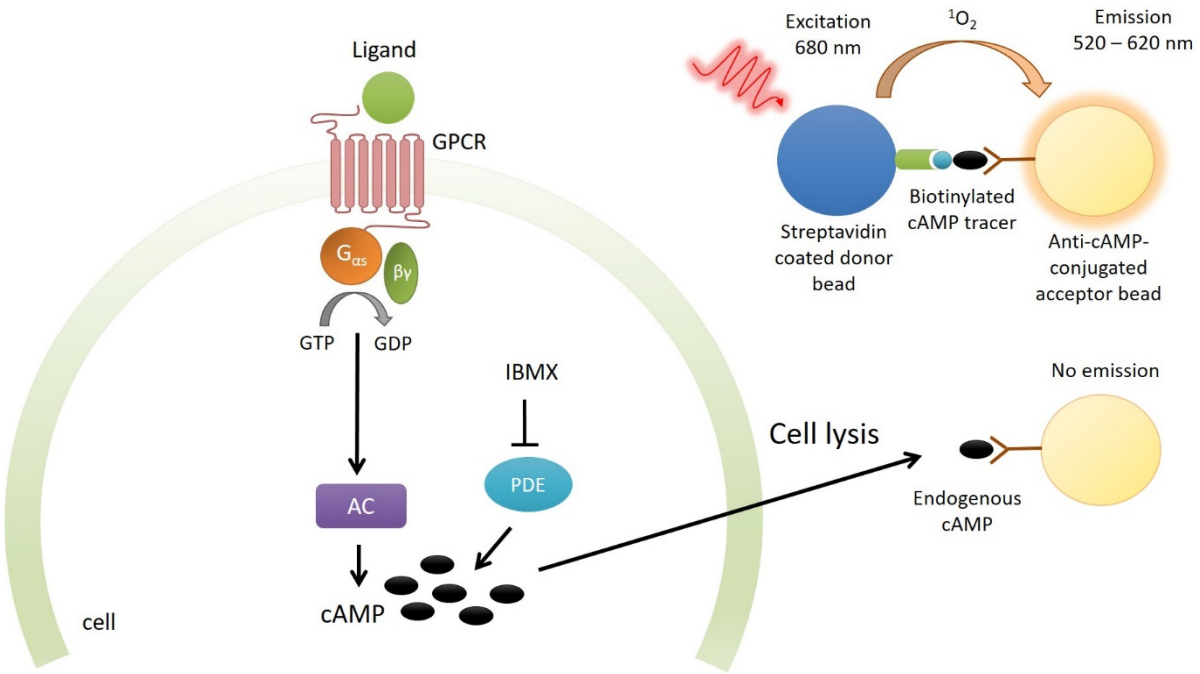

Figure 8: Schematical depiction of the AlphaScreen™ assay used for determination of intracellular cAMP concentration. Upon stimulation of cells expressing G_s-coupled GPCRs with their specific ligands, intracellular cAMP increases. IBMX, a PDE inhibitor blocks the degradation of cAMP in the cell. The determination is then performed with cell lysate after 45 min of stimulation. The competitive-based AlphaScreen™ assay utilizes a biotinylated cAMP tracer that competes with intracellular cAMP for the anti-cAMP-conjugated acceptor beads. Only the tracer can be bound by the streptavidin-coated donor beads, which brings donor and acceptor in close proximity. Excitation of the donor bead leads to the conversion of ambient oxygen to a more excited singlet state, which reacts with the acceptor beads nearby. This leads to the generation of chemiluminescent light. Binding of endogenous cAMP does not lead to light emission. Therefore, the cAMP assay is a competitive measurement. GPCR: G protein-coupled receptor; AC: adenylate cyclase; cAMP: cyclic AMP; PDE: phosphodiesterase; IBMX: 3-Isobutyl-1-methylxanthin

After seeding ($1.5x10^4$ cell/well) and transfection with vectors containing the respective GPCR or empty vector using Metafectene™, HEK293 or COS7 cells were stimulated with various ligands (50 µl/well) in stimulation buffer containing IBMX (500 µM) for 40 min at 37°C. For G_i –activity measurements, cells were stimulated with forskolin in parallel, which penetrates the cell membrane to activate the adenylate cyclase unspecifically. Afterwards, cells were lysed using lysis buffer containing IBMX at 4°C on a horizontal shaker. Cell lysates were stored at -20°C. In order to conclude from raw emission data to concentration, a cAMP standard is prepared according to manufacturer's protocol using lysis buffer. 5 µl of standard and samples are transferred to a 384 well plate and 10 µl of diluted acceptor beads (1:100 in lysis buffer) are added in minimal lighting. After incubation for 30 min at room temperature in the dark, 10 µl of diluted donor beads containing biotinylated cAMP (1:100 for beads, 1:16 for cAMP in lysis buffer) is added in minimal lighting. After incubation for an additional hour in the dark, the emission of the acceptor beads are measured using a plate reader (Berthold Mithras LB 940).

3.2.3.1.2 Reporter gene assays to determine PLC activation (read out used for $G_{q/11}$), MAPK-signaling and canonical T3-dependent signaling

Luciferase-based reporter gene assays are used to identify the activation of different signaling pathways such as $G_{q/11}$ and MAPK-signaling. The promotor region of the luciferase gene contains a response element, which starts the expression of luciferase when bound by the respective second messenger. For $G_{q/11}$/PLC activation, the response element NFAT RE (nuclear factor of activated T-cell response element) was used, whereas the ERK1/2 phosphorylation (MAPK pathway), which can be activated by both GPCR activity and intracellular T3, was determined using the SRE (serum response element) (Singleton 2010). To analyze the canonical nuclear T3-pathway, a DR4 (Thyroid hormone receptor response element) was used (Hofmann, Schomburg, and Kohrle 2009). An overview of response elements used in luciferase-based reporter gene assays in the course of this project give **Table 23**:

Table 23: Overview of the pathways investigated with luciferase-based reporter gene assays

Pathway	Response element	Vector	References
$G_{q/11}$/PLC activation	NFAT	pGL4.3-luc2	(Boss, Talpade, and Murphy 1996)
ERK 1/2 phosphorylation	SRE	pGL4.3-luc2	(Hawes et al. 1995)
Canonical TH-dependent signaling	DR4	pGL3-luc2	(Hofmann, Schomburg, and Kohrle 2009)

After seeding ($1.5x10^4$ cells/well) and co-transfection with the reporter gene, the appropriate GPCR or empty vector and thyroid hormone receptor α (only for investigation of T3-dependent signaling) using Metafectene™, HEK293 cells were stimulated with the respective ligands (50 µl/well) in MEM without supplements at 37°C. Depending on the assay, stimulation time varied. For GPCR functional characterization studies, cells were stimulated for 6h. If PTX pretreatment was necessary, cells medium was changed 16-18h before stimulation to complete media containing 50 ng/ml PTX. Non-pretreated cells were also changed to complete media without PTX. For T3-dependent signaling studies, stimulation was either performed in a time-dependent or concentration-dependent manner. For time course experiments, cells were incubated with 1 µM T3 for 10, 30, 60, 180 and 360 min. Afterwards, the cells were washed with 50 µl PBS and incubation continued with 50 µl with T3-depleted media until 24h were completed to allow sufficient luciferase expression. For concentration-response experiments, cells were incubated with different concentrations of T3 for 1h and again washed and incubated as stated above. For experiments using TH transport blockers, the cells were incubated with the compounds with or without 1 µM T3 for 1h, followed again by a washing step and incubation for another 23h in T3-depleted medium. Subsequently, cells were lysed using passive lysis buffer (Promega) at room temperature according to manufacturer's protocol. Cell lysates were stored at -20°C. The measurement of luciferase activity was performed

by transfer of 10 µl cell lysate to black 96 well plates, injection of luciferase substrate (40 µl/well) and measurement of emission at 560 nm using a plate reader (Berthold Mithras LB 940).

3.2.3.1.3 NanoBRET™ assay to determine the interaction between GPCR and β-arrestin2

The interaction with β-arrestins is the initiation of internalization and subsequently desensitization of GPCRs and their signal transduction. Arrestins can also act as scaffolding proteins to activate several signaling pathways, which they share with G protein-dependent signaling, such as MAPK pathways. Therefore, it is difficult to characterize the activation of β-arrestins by determining downstream messengers. To study this important step for GPCR internalization, a Bioluminescence resonance energy transfer (BRET) assay was employed. It is a method to detect protein-protein interaction in live cells. **Figure 9** depicts the concept of a BRET assay:
NanoBRET™, a very recent version of this assay, uses a smaller and brighter bio-engineered version of the *Renilla* luciferase, called the NanoLuciferase (NanoLuc) as an energy donor. The second interaction partner is tagged with a protein tag called HaloTag®, which can bind covalently to an energy acceptor, the NanoBRET™ ligand 618. In comparison to the usual used BRET methods, this version allows a convenient determination of background by omitting the addition of the energy acceptor. The combination of these BRET partners achieved a good spectral separation to avoid

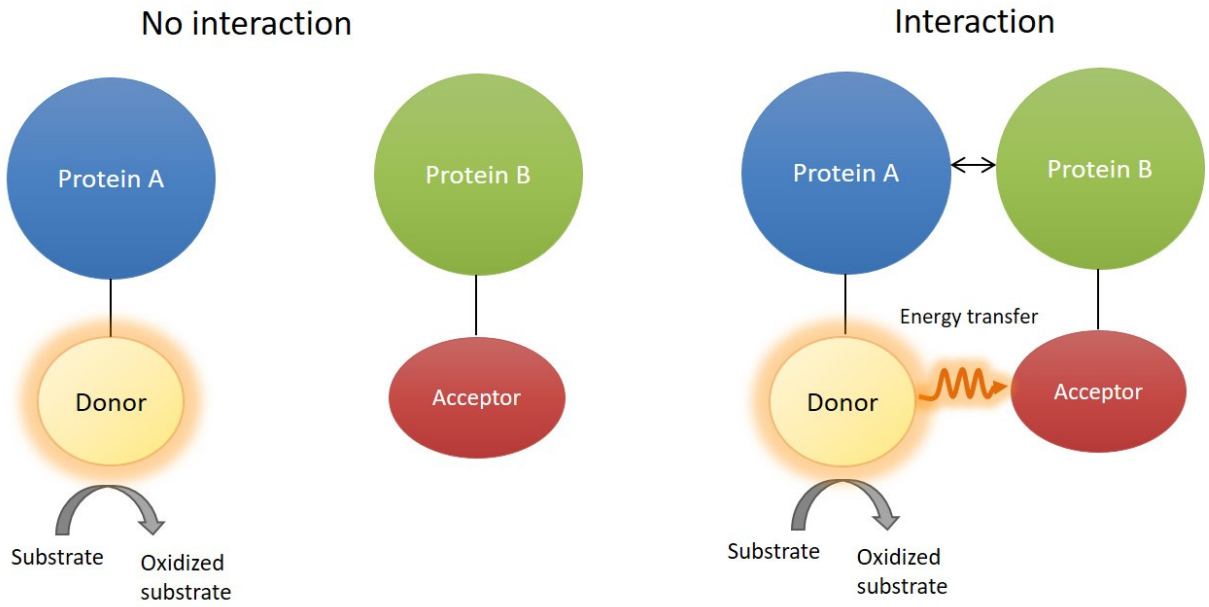

Figure 9: Concept of a bioluminescence resonance energy transfer (BRET) assay. This method is used to detect protein-protein interactions in lives cells. Putative interaction partners are fused with either an energy donor, a luciferase or an energy acceptor, a fluorophore. The luciferase can oxidize its substrate and emits light. In case of an interaction between the two BRET partners, the energy acceptor is in close proximity to the donor and radiation-free energy transfer takes place. This leads to the emission of fluorescence from the acceptor fluorophore. Both the luminescent signal of the donor and the fluorescent light of the acceptor are measured and the ratio between these signals.

bleed-through of the donor emission into the acceptor channel, increasing the detection sensitivity of the assay (Machleidt et al. 2015).
After seeding the cells in 6 well plates ($8.0x10^5$ cells/well) and transfection using FuGene® HD, cells were detached approximately 20h after transfection. After a centrifugation step (130 g, 5 min, RT), the medium containing phenol red was removed, and cells were re-suspended in Opt-MEM without phenol red containing 4% FBS. The cell suspension was diluted to $2.2x10^5$/ml and separated into two pools adding either NanoBRET™ Ligand 618 (1 µl/ml cells of 0.1 mM solution) or the same amount of DMSO to the cells. Afterwards, cells were reseeded into opaque white 96 well plates (90 µl/well) in triplicates for each condition tested and let cells attach about 4-6h at 37°C and 5% CO_2.
Due to spatial configurations of the putative interaction partners, not every combination of tagged donor/acceptor pair is optimal for BRET measurements. Therefore, the ideal combination needed to be established for every investigated GPCR, before concentration-response assay could be performed. Here, all available tag variants of receptor and arrestin was combined and stimulated with 1 µM of agonist for 5 min. Since the interaction between receptor and β-arrestin2 is located in the intracellular part, the receptors were tagged at the C-terminus only, limiting the combination of donor/acceptor pairs. The BRET pair with the highest ligand-promoted NET BRET was chosen for further assays. For concentration-dependent experiments, cells were incubated with a serial dilution of compound at 37°C for 5 min.
For measurement, 25 µl/well of Nano-Glo substrate (50 µM) were injected using a plate reader (Berthold Mithras LB 940). The donor and acceptor emission were measured at 460 nm and 610 nm respectively. The BRET ratio was calculated using the formula:

$$\text{BRET ratio} = \frac{\text{emission}_{\text{acceptor}}}{\text{emission}_{\text{donor}}}$$

Afterwards, the ratio was corrected by subtraction of the background (no acceptor control) and conversion to milliBRET Units (mBU):

$$\text{corrected BRET ratio [mBU]} = (\text{BRET ratio}_{\text{sample}} - \text{BRET ratio}_{\text{no acceptor control}}) \times 1000$$

To calculate the difference between stimulated and basal interaction, the NET BRET was acquired using the formula:

$$\text{NET BRET} = \text{corrected BRET ratio}_{\text{stim}} - \text{corrected BRET ratio}_{\text{basal}}$$

3.2.3.2 HiBiT Internalization assay

The HiBiT Extracellular Detection Assay can be utilized to monitor internalization of GPCRs. The assay is based on a split luciferase, the NanoLuc, divided into a very small (eleven amino acids, SmBiT) and a very large part of the protein (LgBiT). The SmBiT is used as an N-terminal tag at the GPCR and should be expressed very low in the cell. The transfection was performed with 0.45 ng DNA/well of HiBiT and Carrier-DNA (pGEM-3Zf(+)) was used to add to 45 ng/Well. Measurement took place after 30 minutes of stimulation with varying concentration of stimulant by mixing LgBiT protein (1:100) and luciferase substrate (1:50) in substrate buffer. This mixture (50 µl/well) was injected using a plate reader (Berthold Mithras LB 940) with a low speed at room temperature and the plate was not shaken to mix. The reagent is slightly denser than culture media for rapid equilibration of HiBiT-tagged proteins with LgBiT Protein and

the substrate. After four minutes of incubation, luminescence was measured at 460 nm. For a background control, data from cells transfected with pcDNA3 were recorded and subtracted from the sample data. **Figure 10** depicts the principle of this assay.

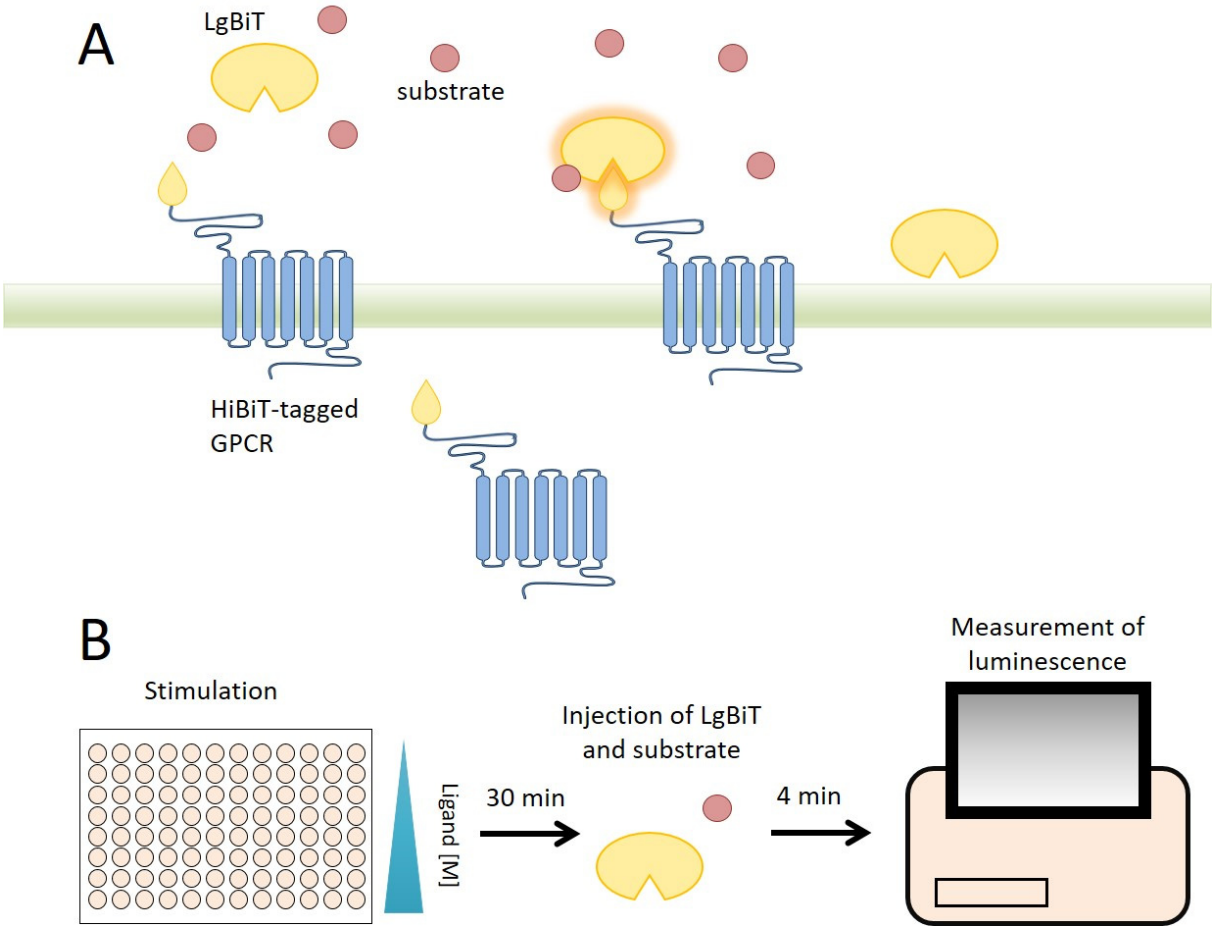

Figure 10: Graphical depiction of the HiBiT Cell surface expression assay. A: When the receptor is located on the cell surface, the N-terminal HiBiT-tag is situated in the extracellular space. It is then available for the LgBiT-protein that cannot overcome the cell membrane. Once HiBiT and LgBiT are complemented, they resemble a functional luciferase that is able to oxidize its substrate and produces luminescence. B: The target receptor was stimulated with a serial dilution of compounds for 30 min. Afterwards, the LgBiT and substrate were injected into the wells and luminescence was measured using a plate reader after 4 min of incubation.

3.2.4 Imaging methods

3.2.4.1 Imaging of SNAP-tagged GPCRs

Fusion proteins between a GPCR and the SNAP-tag can be imaged using a fluorescent dye, which covalently binds to the tag. It is a highly engineered version of AGT (alkylguanine DNA alkyltransferase) and forms a covalent thioester bond with the dye (Gautier et al. 2008). In the course of this project, GPCRs were tagged N-terminally with the SNAP-tag. Thus, tagged GPCRs in the plasma membrane had the tag localized in the extracellular space. Addition of a lipophobic SNAP-dye, that is not able to overcome the plasma membrane, could only bind to the extracellular tags and

therefore only receptors localized on the cell membrane were labeled. After the addition of an agonist, internalization of the GPCR is triggered. This process could be detected by microscopy in a time-dependent manner.
48 h after transfection, cells were incubated with SNAP surface A488 (1:500 in HEK complete media) for 30 min at 37°C. Afterwards, cells were washed briefly with DMEM three times and stimulated with appropriate ligand or conjugate (1 µM) for various time periods at 37°C. To stop the stimulation, cells were washed with PBS three times for 5 min and fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature. After an additional two washing steps with PBS for 5 min, cells were mounted with mounting media including DAPI (Vectastain) and dried at 4°C overnight. In order to display the internalization process, microscopy was applied (Zeiss Axiovert). Images were edited using ImageJ software.

3.2.5 Cell viability assay

Treatment with various compounds can lead to poor cell viability due to cytotoxicity. To measure survival of cells during an assay, cell viability assays can be utilized. Here, the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) from Promega was used. Cells were treated as described for luciferase-based reporter gene assays with additional non-transfected cells as well as just medium for background controls. After stimulation, 10 µl solution per well was added and incubated at 37°C for an hour. Calorimetric measurements were carried out with a plate reader (Anthos reader 2000) at 492 nm with a reference wavelength of 620 nm. Background values were subtracted from the sample values and data was normalized to mean of background.

3.2.6 Statistical analysis

The statistical analysis of data collected from experiments is necessary to evaluate the results and be able to discuss and conclude a possible explanation. In the course of this project, statistical analysis was performed using the GraphPad Prism 6 software. For Concentration-response experiments, X-values were transformed into $\log_{10}(x)$ and non-linear regression was applied using the equation “log(agonist) vs. response (three parameters)” for stimulants to calculate EC_{50} or IC_{50} and span values or “log(inhibitor) vs. response (three parameters)” for blockers.
To evaluate the significant difference between transfections, treatments or time points, a two-way ANOVA (analysis of variance) with multiple comparison was implemented with an $\alpha = 0.05$. Statistical significance assessed by the p-value was indicated with * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** or ### for $p \leq 0.001$.

4 Results

The result part of this project is organized in two main subchapters. First, the findings of the functional characterization are depicted for both investigated conjugates, GLP1-T1 and OT-T3. Here, the focus will be on activation of signaling cascades as well as internalization processes. These actions are initiated by the peptide part of the conjugates. The second part will then present the results obtained from TH signaling assays, initiated by the T3 part of the conjugate. The general structure of the result part is depicted in **Figure 11:**

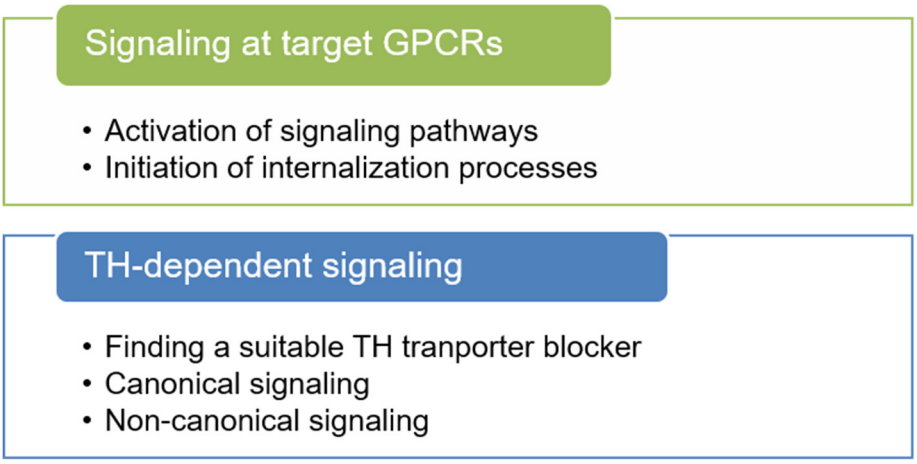

Figure 11: The general structure of the results part. First, findings of the functional characterization will be shown, afterwards results for TH signaling assays are presented.

4.1 Functional characterization of the conjugates at their respective GPCR

4.1.1 Activation of GLP1R signaling

The prerequisite of the “Trojan horse”-paradigm is internalization of a GPCR for which the ligand was used to couple to T3. The process of GPCR internalization and subsequently desensitization for extracellular stimuli is preceded by receptor activation. Therefore, the activation of the GLP1R by GLP1-T3 is crucial for the channeling of T3 into the cell. Here, we performed functional characterization studies in order to determine whether GLP1-T3 activates the signaling cascades of GLP1R, and subsequently internalization, in the same manner as the endogenous ligand GLP1. GLP1R is part of the GPCR family B. Its main pathway is reported to be G_S coupling, leading to an increase in intracellular cAMP concentration (Skoglund, Hussain, and Holz 2000). HEK293 cells were transfected with either a mock control or GLP1R, stimulated with T3, GLP1 or GLP1-T3 (each 1 µM) and intracellular accumulation of cAMP was determined using the AlphaScreen™ assay. GLP1-T3 was able to activate the GLP1R in the same manner as GLP1, leading to an accumulation for both of around 12 fold over the basal control (GLP1: 12.24 ± 2.87, GLP1-T3: 12.79 ± 2.20) (**Figure 12A**). Additionally, GLP1R of *rattus norvegicus* is reported to induce calcium

response as well as ERK1/2 phosphorylation, suggesting coupling to $G_{q/11}$ (Wheeler et al. 1993; Montrose-Rafizadeh et al. 1999). Therefore, we measured NFAT as read-out for $G_{q/11}$ phospholipase C (PLC) activation, and SRE for activation of the MAPK pathway. In these experiments, GLP1R exhibited a mean basal activity of 14.36 ± 0.84 fold over basal control in PLC activation that could not be increased further with stimulation with either T3, GLP1 nor GLP1-T3 (**Figure 12B**). In HEK293 cells, we were not able to determine significant ERK phosphorylation at the human GLP1R (**Figure 12C**).

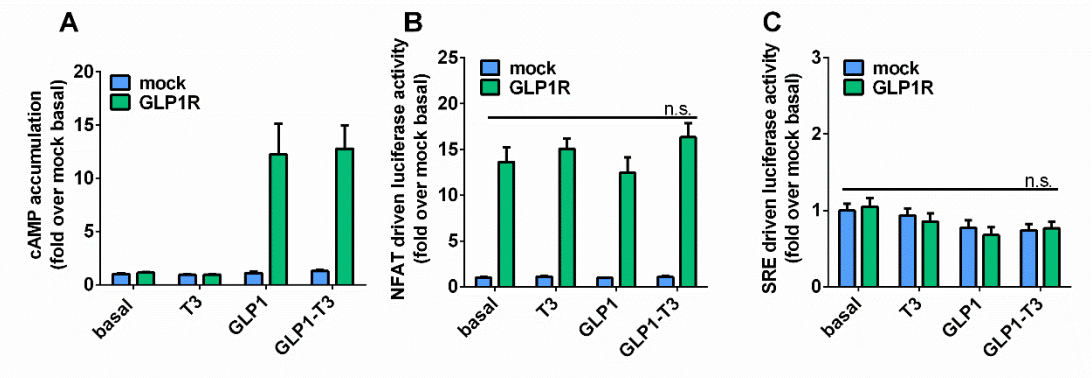

Figure 12: GLP1-T3 is able to activate GLP1R in the same manner as GLP1. Functional studies of the GLP1R (in green) to induce cAMP accumulation (A), Calcium mobilization (B) and ERK phosphorylation (C) were performed in comparison to mock transfected cells (in blue). Cells were stimulated with T3, GLP1 or GLP1-T3 (each 1 µM). Data represent four independent experiments, each performed in triplicates. For statistical analysis, a two-way ANOVA with multiple comparison was performed comparing different transfections. Values represent mean ± SEM

4.1.2 Initiation of GLP1R internalization

In order to monitor receptor internalization, GLP1R tagged with a SNAP-tag was transfected was into HEK293 cells. The SNAP-tag is a recombinant derivate of the O6-guanine nucleotide alkyl transferase, which covalently binds to benzyl guanine substrates conjugated to fluorescent dyes.
Living cells were stained with SNAP Surface®-A488, a dye that cannot overcome the plasma membrane, before stimulation with GLP1 or GLP1-T3 for 30 min. In the basal state, receptors are distributed evenly throughout the cell. Pre-stained receptors on the cell surface accumulated within the cell plasma upon activation (**Figure 13A**).
Additionally, we investigated β-arrestin2 recruitment using BRET. The interaction with arrestins is known to mediate receptor internalization by clathrin-coated pits and also scaffolding G protein independent signaling, such as ERK-phosphorylation (Moore, Milano, and Benovic 2007). Class B receptors, such as GLP1R, are reported to bind to both β-arrestin1 and β-arrestin2 with high affinity (Tobin, Butcher, and Kong 2008). Conjugate-dependent recruitment of β-arrestin2 would therefore indicate receptor internalization. Before a concentration-dependent interaction was investigated, the optimal BRET partner combination needed to be established. Therefore, all available combination of BRET pairings were tested to find the combination with the highest ligand-promoted NET BRET. The results are depicted in the supplements (**Figure S 2A and B**). Afterwards, a concentration-response assay was performed with the best BRET pair, which was ARRB2-NL and GLP1R-HT with a NET BRET of 14.18 ± 0.39

mBU. Both unconjugated GLP1 and GLP1-T3 were able to induce β-arrestin2 interaction in a similar manner with a higher EC_{50} for GLP1-T3 (188.0 ± 0.15 nM) compared to the EC_{50} of GLP1 (50.06 ± 0.18 nM) (**Figure 13B**).

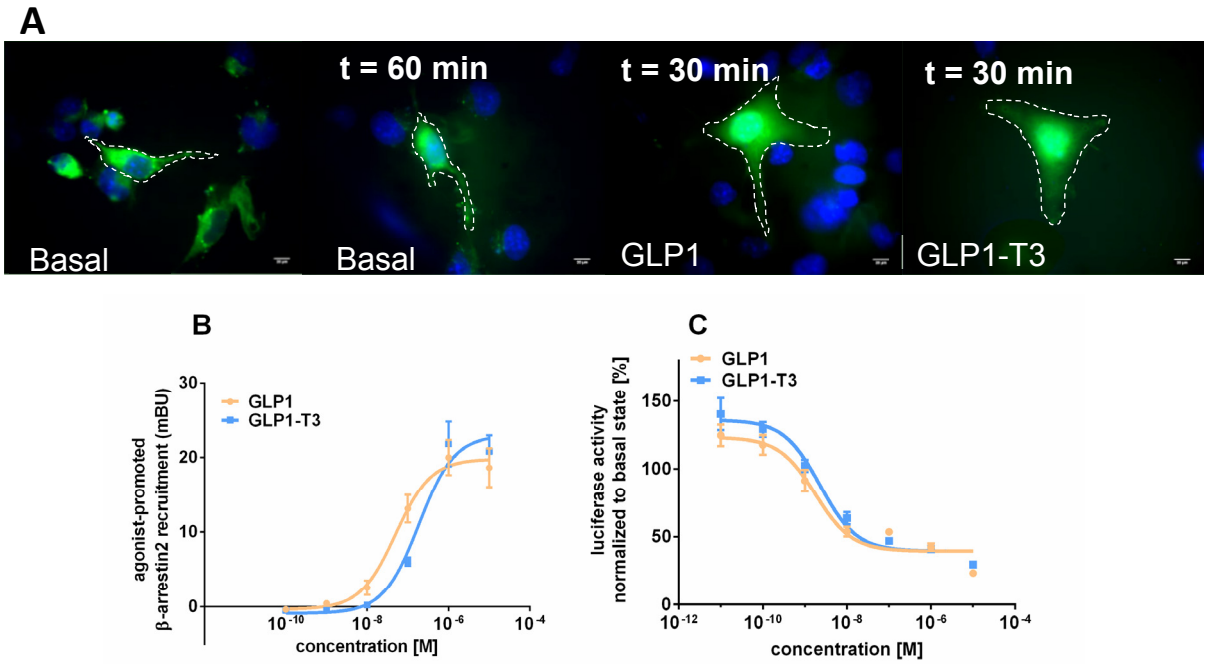

Figure 13: GLP1-T3 is able to initiate GLP1R internalization in a similar fashion to GLP1.
Imaging studies in (A) to monitor receptor internalization in HEK293 cells pre-stained with SNAP Surface-A488 (green) stimulated with GLP1 (1 µM) or GLP1-T3 (1 µM), and subsequently fixated. Nuclei were stained with DAPI (blue) and cell outline was added digitally (white dashed line). In the basal state, SNAP-tagged GLP1R are evenly distributed over the cell surface immediately after live staining (t = 0 min) and after an hour of no stimulation (t = 60 min). Following stimulation with either GLP1 or GLP1-T3 for 30 min, pre-stained receptors accumulated in the cytosol due to internalization processes.
β-arrestin2 recruitment in (B) was measured using NanoBRET™ in HEK293 cells after stimulation with either GLP1 (1 µM) or GLP1-T3 (1 µM) for 5 min and determined as the difference between basal and stimulated BRET ratios. Studies reveal a similar interaction profile after the stimulation of GLP1 (blue) and GLP1-T3 (yellow). The recruitment of β-arrestin2 can hint to the initiation of internalization processes after stimulation with specific agonists. Data represent three independent experiments, each performed in triplicates. Values represent mean ± SEM.
GLP1R shows a concentration-dependent decrease of cell surface expression after challenge with either GLP1 (blue) or GLP-T3 (yellow) (each 1 µM) in (C) Cells surface expression as measured using a small N-terminal protein tag that is part of a split luciferase. N-terminally tagged receptors were stimulated with GLP1 (1 µM) or GLP1-T3 (1 µM) for 30 min. Data represent three independent experiments, each performed in triplicates. Values represent mean ± SEM.

Furthermore, internalization would reduce the amount of receptors present on the plasma membrane that can be monitored using a cell surface expression assay. GLP1, as well as the conjugate were able to reduce the amount of GLP1R expressed on the cell surface in a concentration-dependent manner with a surface expression decrease of 83.93 ± 5.44% for GLP1 and 96.63 ± 5.23% for GLP1-T3. The compounds had a very similar IC_{50} with 1.78 ± 0.15 nM for GLP1 and 2.31 ± 0.13 nM for GLP1-T3 (**Figure 13C**).

4.1.3 Activation of OTR and V1AR

Functional characterization of OT-T3 was performed on two target receptors, the OTR and the V1AR. These receptors have different affinities towards OT, with K_i(OTR) lower than Ki(V1AR) (Åkerlund et al. 1999). Both GPCRs are reported to activate the $G_{q/11}$ protein as their main signaling pathway. Additionally, OTR initiates G_i protein activation, lowering cAMP production in the cell as well as increasing ERK phosphorylation. In fact, the latter is reported to be partly PTX-sensitive and partly PLC inhibitor-sensitive, meaning both activated G proteins lead to ERK phosphorylation with different biological functions depending on the cell type (Ohmichi et al. 1995; Strakova et al. 1998). Similarly, Calcium mobilization/PLC activation following OT stimulation has been shown to also be a mixed result of the βγ-subunits of G_i and $G_{q/11}$ due to decreased, but not fully abolished signal after PTX-treatment (Molnár and Hertelendy 1990; Phaneuf et al. 1993). For V1AR, ERK phosphorylation following an OT challenge was reported in CHO cells (Tahara et al. 1999).
For the OTR, cAMP accumulation assay (AlphaScreen™) was performed with cells pretreated with forskolin (50 µM) to activate the adenylate cyclase, so a decrease in intracellular cAMP concentrations would imply G_i protein activation. The stimulation with OT lead to significant decrease of 66.55 ± 11.12% in OTR-overexpressing cells in comparison with mock transfected cells. OT-T3 was not able to decrease cAMP content in OTR expressing cells (**Figure 14A**) indicating that OT-T3 is unable to activate G_i mediated inhibition of AC.
Calcium mobilization/PLC-activation was measured using the NFAT-luciferase reporter assay, whereas ERK phosphorylation was assessed by a SRE-luciferase based reporter. To gain more information about the G protein involvement in the PLC activation and ERK phosphorylation signals we obtained, cells expressing OTR were additionally pretreated with PTX, a G_i inhibitor. For OTR, calcium mobilization was identical for both agonists with an EC_{50} of 3.77 ± 0.18 nM for OT and 2.87 ± 0.21 nM for OT-T3. Treatment with PTX significantly reduced signal to around 60% of non-treated cells for both agonists in a similar fashion. However, there was no shift in EC_{50} compared to non-treated cells with 4.18 ± 0.2 nM for OT + PTX and 2.76 ± 0.23 nM for OT-T3 + PTX (**Figure 14B**).
ERK phosphorylation by activated OTR was induced by stimulation with OT (EC_{50} 2.11 ± 0.32 nM). Pretreatment with PTX resulted in the abolishment of the G_i effect with an EC_{50} of 0.8 ± 0.43 nM and a significantly reduced signal to around 47% of OT stimulation. Challenge with OT-T3 mimics this attenuated effect of PTX treatment with an EC_{50} of 1.23 ± 0.30 nM. No additional decrease of signal was obtained for OT-T3 stimulation when pretreated with PTX (EC_{50} of 1.19 ± 0.37 nM) (**Figure 14C**), indicating that ERK phosphorylation resulting from OT-T3 challenge is solely due to $G_{q/11}$ activation, but not G_i.

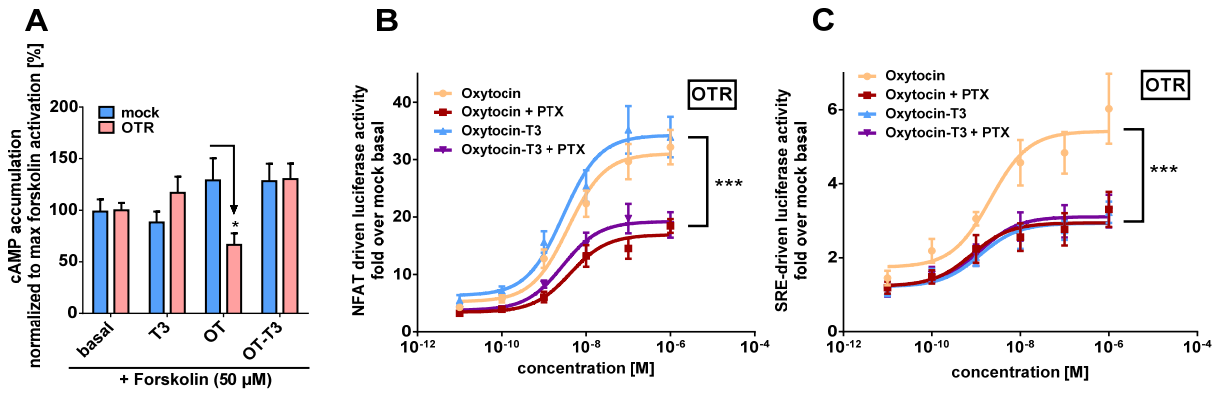

Figure 14: Functional studies on the OTR showed biased agonism of OT-T3.
For Gi activation in (A), COS7 cells were transfected with OTR (red) or empty vector (mock, blue) and stimulated with either T3, OT, OT-T3 (each 1 µM) or not (basal) while treated with forskolin (50 µM) to activate adenylate cyclase. Data represent four independent experiments, each performed in triplicates. For statistical analysis, a two-way ANOVA with multiple comparison was performed, comparing different transfections. Values represent mean ± SEM. Statistical significance is indicated by *p≤0.05.
Calcium mobilization was assessed by a NFAT-reporter in (B) and ERK phosphorylation by a SRE-reporter in (C). HEK293 cells were stimulated with OT (yellow) or OT-T3 (blue) (each 1 µM) and pretreated with PTX (red and violet) or not. Data represent five to nine independent experiments, each performed in triplicates. For statistical analysis, a two-way ANOVA with multiple comparison was performed, comparing different treatments. Values represent mean ± SEM. Statistical significance is indicated by *** p≤0.001

Determination of calcium mobilization and ERK phosphorylation at the V1AR was executed in the identical fashion to OTR. The NFAT-reporter showed identical signaling properties for both agonists on the receptor with an EC_{50} of 6.83 ± 0.31 nM for OT and 6.64 ± 0.30 nM for OT-T3 (**Figure 15A**). ERK phosphorylation on the other hand was significantly increased for the highest OT-T3 concentration of 10^{-6} M and a shifted EC_{50} from 12.69 ± 0.48 nM for OT to 66.4 ± 0.31 nM for OT-T3 (**Figure 15B**).

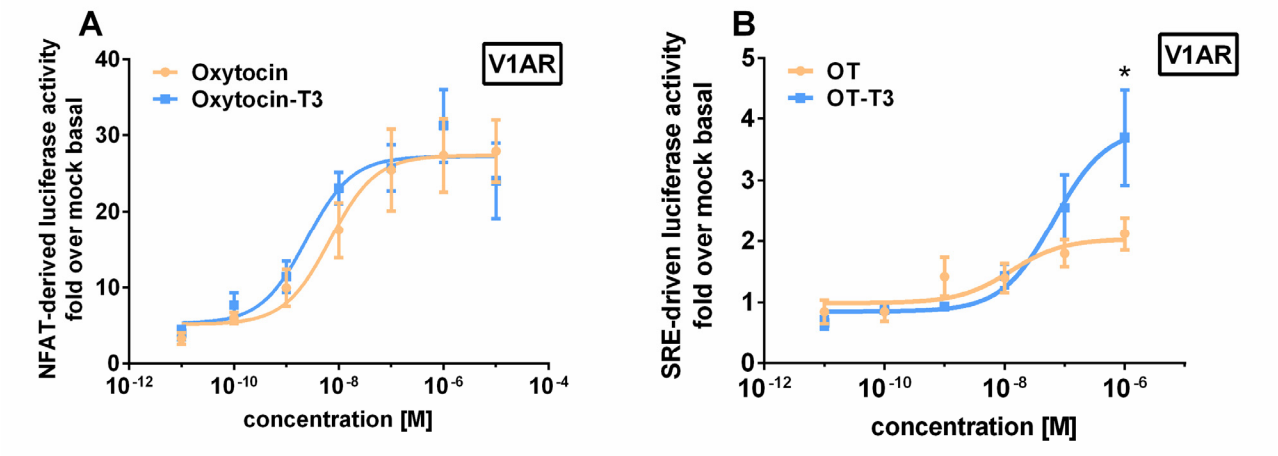

Figure 15: Functional characterization on the V1AR showed biased agonism of OT-T3.
HEK293 cells overexpressing V1AR were stimulated with either oxytocin or OT-T3 (each 1 µM) and calcium mobilization (A) and ERK phosphorylation (B) were measured using a reporter gene assay. Data represent three independent experiments, each performed in triplicates. Values represent mean ± SEM. For statistical analysis, a two-way ANOVA with multiple comparison was performed, comparing different treatments, Statistical significance is indicated by *p≤0.05

4.1.4 Internalization of OTR and V1AR

Both target receptors were investigated for β-arrestin2 recruitment after OT-T3 stimulation using BRET again as an indication for receptor internalization. For establishment of the optimal BRET pairing, preliminary experiments were performed and the combination with the highest ligand-promoted NET BRET was chosen for concentration-dependent experiments. The BRET pairs chosen was ARRB2-NL and OTR-/V1AR-HT with a NET BRET of 5.13 ± 0.63 mBU for OTR and 5.18 ± 0.10 mBU (**Figure S 2C-F**). In these assays, OTR showed identical agonist-promoted BRET signals for both OT and OT-T3 (EC_{50} 6.71 ± 0.15 nM for OT and 5.47 ± 0.11 nM for OT-T3) (**Figure 16A**). Surprisingly, at the V1AR the signal from OT-T3 was reduced to around 9% of maximal OT recruitment of β-arrestin2 (EC_{50} 5.27 ± 0.14 nM for OT). For OT-T3 EC_{50} calculation with appropriate accuracy is not possible. (**Figure 16B**).

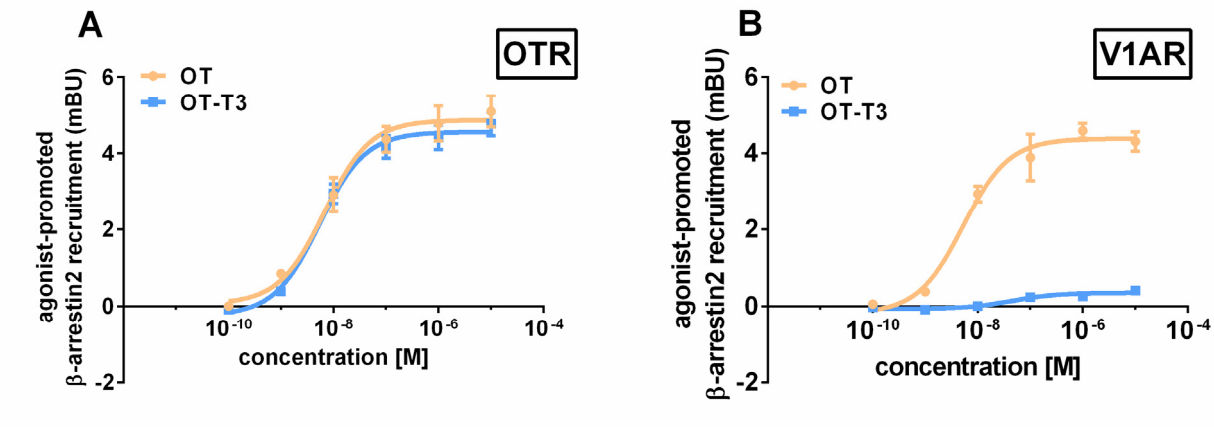

Figure 16: Stimulation of OTR and V1AR with OT and OT-T3 resulted in different β-arrestin2 recruitment pattern. HEK293 cells overexpressing β-arrestin-NanoLuc fusion protein and OTR (A) or V1AR (B) C-terminally tagged with the protein tag HaloTag, binding to the energy acceptor, were stimulated with either OT (yellow) or OT-T3 (blue) in different concentrations and BRET ratios were measured after 5 min of stimulation. Data represent three independent experiments, each performed in triplicates. Values represent mean ± SEM.

4.1.5 Summary of the functional characterization

In summary, two different T3-conjugates have been investigated. They were characterized by their ability to initiate receptor activation and internalization, as well as TH-dependent signaling in presence of the receptor as proof for the “Trojan Horse”-like mechanism. The first investigated conjugate GLP1-T3 was used to establish a toolbox for the investigation of future conjugates. It was able to activate receptor signaling, especially the main G_s-pathway, and internalization, by the means of β-arrestin2 recruitment and decrease of receptor on the cell surface after stimulation in the identical manner to the unconjugated GLP1.
The second conjugate OT-T3, which was designed for correction of symptoms of MCT8-deficiency, has been tested using the toolbox established with GLP1-T3. In comparison to the unconjugated peptide, at the OTR OT-T3 was able to activate the main pathway $G_{q/11}$, but not the G_i-pathway. This led to a reduced phosphorylated ERK signal comparable to the PTX-treated receptor, confirming the missing G_i coupling. The

main signaling pathway, $G_{q/11}$ at the V1AR was not attenuated and ERK phosphorylation was even significantly increased compared to unconjugated peptide in the highest concentration. Nevertheless, this receptor did not show β-arrestin2 recruitment when stimulated with OT-T3 in comparison to a specific interaction after stimulation with unconjugated OT.
The functional characterization of the conjugates resulted in the findings summarized in **Table 24** for GLP1-T3 and **Table 25** for OT-T3. A check mark indicates the activation of the respective signaling pathway or the internalization process, whereas a cross illustrate a missing signal for the respective assay. Arrows indicate the decrease (downwards) or increase (upwards) of signal in comparison to the unconjugated ligand.

Table 24: Summary of the results for the functional characterization of GLP1-T3 at the GLP1R

Ligand	Signaling cascades			Internalization		
	G_s	PLC activation	ERK phos-phorylation	Visually (SNAP-tag)	β-arrestin2 recruitment	Decrease of cell surface expression
GLP1	✓	✗	✗	✓	✓	✓
GLP1-T3	✓	✗	✗	✓	✓	✓

Table 25: Summary of the results for the functional characterization of OT-T3 at the OTR and V1AR

Ligand	Target receptor	Signaling cascades			Internalization
		G_i	PLC activation	ERK phos-phorylation	β-arrestin2 recruitment
OT	OTR	✓	✓	✓	✓
	V1AR	-	✓	✓	✓
OT-T3	OTR	✗	✓	↓	✓
	V1AR	-	✓	↑	✗

4.2 Determination of TH-dependent signaling of the conjugates

In addition to activation of signaling and internalization of the target receptors, it was determined whether the conjugates were able to activate canonical and non-canonical signaling of thyroid hormones. For the canonical signaling, it is known that T3 binds to the TRα1 and relocate into the nucleus. Here, the complex binds to thyroid hormone receptor response elements (TRE), which starts the expression of target genes. To investigate whether T3 is released by the conjugate after internalization and trigger TH-dependent signaling – in other words, whether the “Trojan Horse”-like mechanism works – we used a reporter gene that expressed firefly luciferase under the control of a TRE of four direct repeat (DR4) (Hofmann, Schomburg, and Kohrle 2009). In the following part, results from these reporter gene assays are presented.

4.2.1 Finding a suitable inhibitor for TH transport in HEK293 cells

Membrane transporters are essential for cell regulations and therefore ubiquitous in all cell types (Giacomini et al. 2010). Especially since THs are important for cell function, various sets of transporters, able to transport THs, are endogenously expressed in different cell types (Friesema et al. 2005). In a functional assay for TH-dependent signaling, cell lines like HEK293 cells always show high responses to T3 stimulation.
For most of the membrane transporter, that are able to transport THs, one or more blocker has been found to inhibit the influx and efflux of extracellular T3 or T4. In order to find a suitable TH transporter blocker, we used published inhibitors for all known TH transporters. The drug probenecid is used to increase uric acid excretion in the kidney by inhibition of OATPs (Ho et al. 2000). It is also known to block OATPs in the rat choroid plexus (Pritchard et al. 1999) and therefore is known to block OATP1C1. The tricyclic anti-depressant desipramine (DMI) is known for the inhibition of LATs and MCT8 and MCT10 (Taylor and Ritchie 2007; Roth, Kinne, and Schweizer 2010). LAT1 and 2 are reported to be blocked by 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) (Christensen 1990). The TH derivate 3-iodothyronamin (3-T_1AM) has been shown to block T4 and/or T3 transport of OATP1A2, OATP1C1 and MCT8 (Ianculescu et al. 2010), whereas Bromosulphtalein (BSP) is a known blocker for MCT8 (Friesema et al. 2003; Jayarama-Naidu et al. 2015). Silychristin (SY), a flavonolignan derived from the milk thistle, is a very recent addition to the specific MCT8 blockers (Johannes, Jayarama-Naidu, et al. 2016).
As a preliminary experiment, these blockers were tested in for canonical TH signaling in a reporter gene assay (**Figure 17A**). Cells were co-stimulated with T3 and the different blockers with ten to fifty-fold lower concentrations than previously published, since incubation times were increased from a couple of minutes to an hour (Roth, Kinne, and Schweizer 2010; Zhang et al. 2010; Taylor and Ritchie 2007).
Only one blocker was able to inhibit TH signaling in HEK293 cells, the most promiscuous DMI. It could significantly decrease the cells response to T3 compared to the untreated cells from 10.92 ± 1.01 to 2.62 ± 0.55 fold. Visual control of the cells through the microscope during the assay presented the question of whether the decrease in signaling is due to cytotoxicity of the compound rather than inhibition of transport. Therefore, a cell viability test was performed (**Figure 17B**). In concentrations higher than 50 µM, cell viability was diminished. To find a concentration that is not yet toxic, but sufficiently inhibit TH-dependent signaling, both datasets are depicted together in **Figure 17C**. For ascending concentrations of DMI both cell response and viability decreased in a similar fashion.

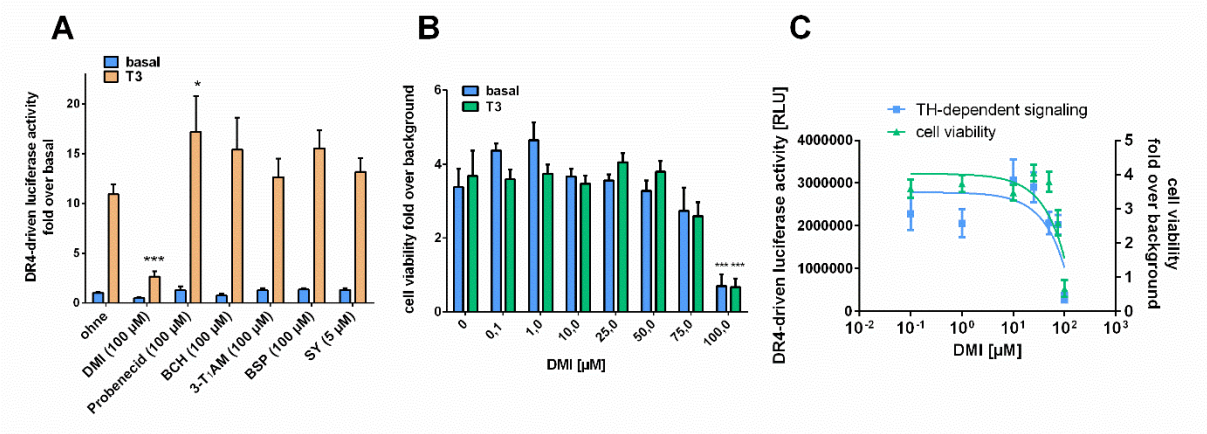

Figure 17: Preliminary assays to determine a suitable blocker for endogenous expressed TH transporter. A: Luciferase based reporter gene studies to compare the different inhibitors that were published as TH transporter blocker. B: Viability test to ensure cell viability at high concentrations of DMI. C: Combined depiction of canonical TH-dependent signaling and cell viability after incubation with varying concentration of DMI. Data represent three to four independent experiments, each performed in triplicates. For statistical analysis, a two-way ANOVA was performed, comparing different treatments with DMI. Values represent mean ± SEM. Statistical significance is indicated by *p≤0.05, ***p≤0.001
DMI: desipramine; BCH: 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid; 3-T_1AM: 3-iodothyronamine; BSP: bromosulphtalein; SY: silychristin

4.2.2 Determination of TH-dependent signaling after challenge with GLP1-T3

In order to ascertain whether the conjugate can activate canonical and non-canonical pathways of TH action, both signaling cascades were investigated by the aforementioned reporter gene assays. The “Trojan Horse”-like mechanism should only work in the presence of the GLP1R and cells that do not express the receptor should not experience T3-dependent signaling after stimulation with GLP1-T3.
Cells were stimulated with GLP1-T3 or T3 in presence or absence of GLP1R for various time periods and concentrations. A significant increase of T3-dependent signaling in the presence of GLP1R was determined after GLP1-T3 challenge for more than 60 min, in comparison to cells that do not express GLP1R. More specifically, signaling increased from 1.527 ± 0.233 x10^6 RLU in the basal state to 11.251 ± 1.360 x10^6 RLU after 6h of incubation with GLP1-T3 (**Figure 18A**). This effect has been shown to be concentration-dependent, though significance between transfections was only present for the highest concentration of 1 µM GLP1-T3 raising from 0.817 ± 0.118 x10^6 RLU to 3.344 ± 0.348 x10^6 RLU (**Figure 18B**, significance indicated with *). Additionally, the mock control showed a significant increase of signaling for this concentration as well (1.922 ± 0.255 x10^6 RLU, significance indicated with #). The stimulation with T3 resulted in an immediate high response irrespective of the expression of GLP1R for different stimulation periods (**Figure 18C**). Furthermore, it rose concentration-dependently for both transfections (**Figure 18E**). Stimulation with GLP1 did not have any effect on T3-dependent signaling, neither time- (**Figure 18D**) nor concentration-dependent (**Figure 18F**).

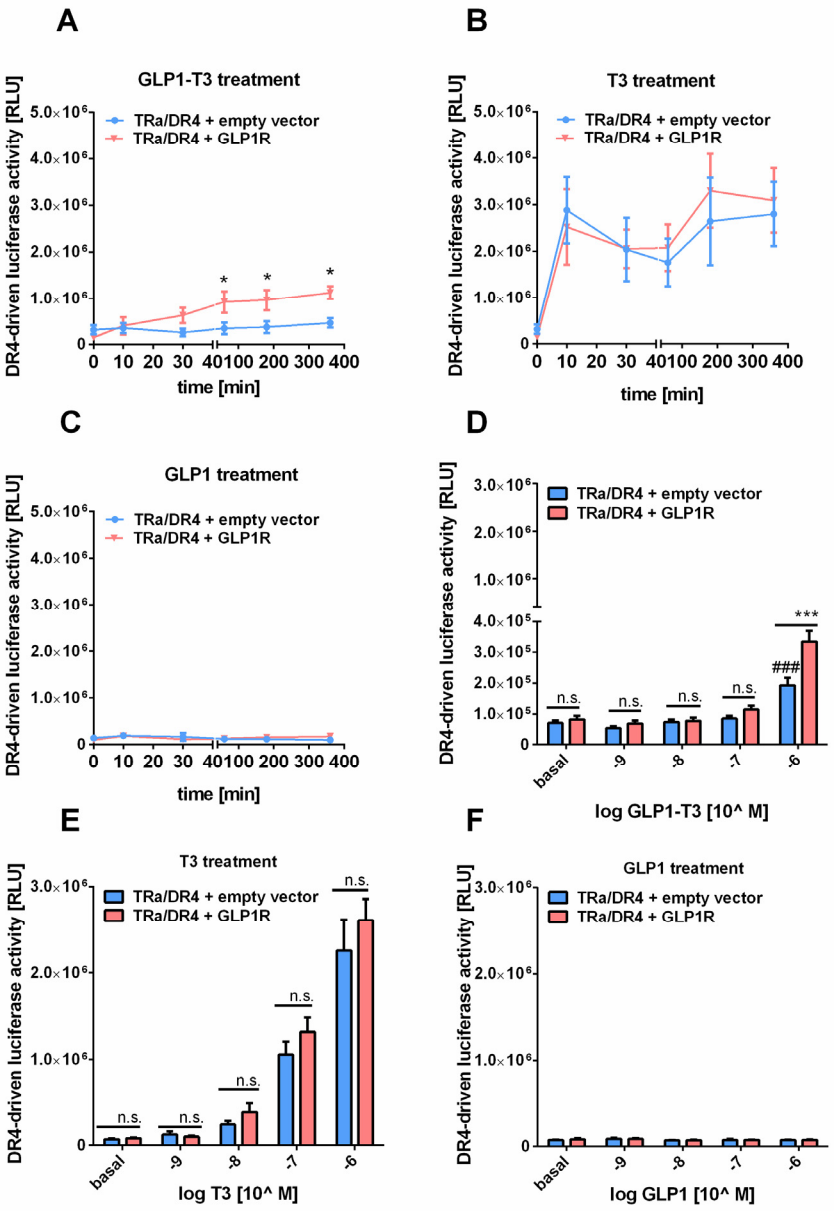

Figure 18: Luciferase based reporter gene studies of nuclear response to TH (canonical pathway) in a time- and concentration-dependent manner. Cells have been co-transfected with the luciferase reporter gene DR4, the thyroid hormone receptor α (TRa) and either GLP1R (red) or empty vector (blue). Cells were stimulated with GLP1-T3 (1 µM) in (A), T3 (B) or GLP1 (C) for various periods (10 min, 30 min, 1h, 3h, 6h) or in different concentrations of GLP1-T3 (D), T3 (E) or GLP1 (F) for one hour. Data represent three independent experiments, each performed in triplicates. For statistical analysis, a two-way ANOVA with multiple comparison was performed, comparing different transfections (*) or different concentrations (#). Values represent mean ± SEM. Statistical significance is indicated by *p≤0.05, **p≤0.01, */###p≤0.001**

In addition, we measured non-canonical signaling of rTRα1 that results in a rapid phosphoinositide 3-kinase activation and mitogen-activated ERK1/2 phosphorylation and used MAPK activation as read-out (Makino et al. 2008; Kalyanaraman et al. 2014). Stimulation with GLP1-T3 resulted a significant increase in T3-dependent signaling in the presence of GLP1R in comparison to the absence of GLP1R (mock transfection with empty vector). More specifically, it increased from 0.3548 ± 0.4468 x10^6 RLU in the basal state to 4.947 ± 0.641 x10^6 RLU after 6h of stimulation (**Figure 19A**). As a control, cells were also treated with T3, which lead to an immediate response with no difference in T3-dependent signaling for cells expressing either GLP1R or empty vector (**Figure 19B**). Additionally, cells treated with GLP1 showed no response in T3-dependent signaling (**Figure 19C**).

Since we were not able to find a transporter blocker that would inhibit endogenous TH transporter in our cell system without also having inhibitory effects on cell viability, no

blocking experiments were performed, which would confirm the entrance of T3 through the receptor.

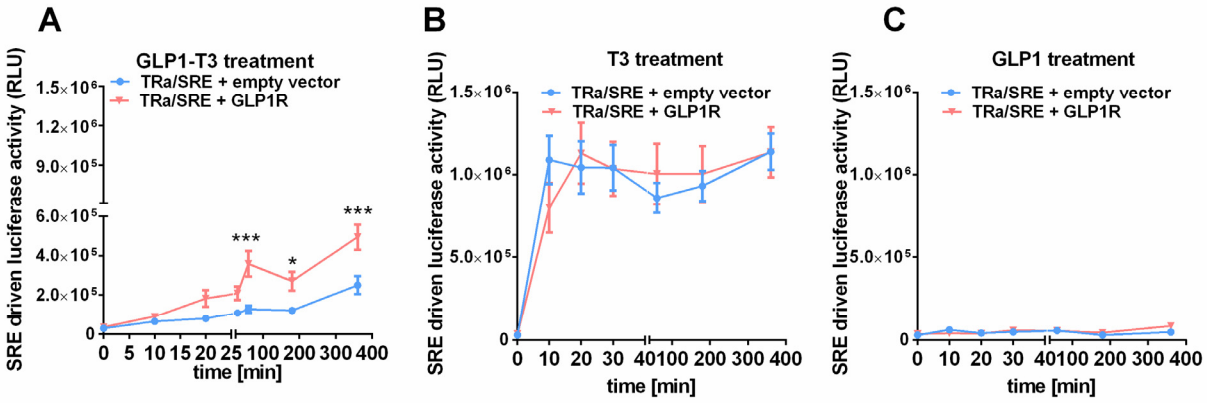

Figure 19: Luciferase based reporter gene studies of non-canonical response to TH in a time - dependent manner. Cells have been co-transfected with the luciferase reporter gene DR4, the thyroid hormone receptor α (TRa) and either GLP1R (red) or empty vector (blue). Cells were stimulated with GLP1-T3 (1 µM) in (A), T3 (B) or GLP1 (C) for various periods (10 min, 30 min, 1h, 3h, 6h). Data represent three independent experiments, each performed in triplicates. For statistical analysis, a two-way ANOVA with multiple comparison was performed, comparing different transfections. Values represent mean ± SEM. Statistical significance is indicated by *p≤0.05, **p≤0.01

4.2.3 Determination of TH-dependent signaling after challenge with OT-T3

Similar to the investigation of GLP1-T3, T3-dependent signaling after stimulation with OT-T3 was investigated in the absence and presence of both target receptors, OTR and V1AR. Again, HEK293 cells were used and a time-dependent assay was employed. The treatment with OT-T3 of OTR expressing cells did not result in a significant increase in canonical signaling compared to mock expressing cells (**Figure 20A**). Similarly, no significant change was measured for V1AR expressing cells, although it showed a tendency to higher values, which were not significant in the statistical analysis (two-way ANOVA, comparing the transfections for every time point) (**Figure 20B**). As a positive control for this assay, cells were also treated with T3 alone. For both receptor transfections, stimulation with T3 resulted in similar values than treatment with OT-T3 with no significant difference for OTR expressing cells in comparison with mock cells (**Figure 20C**) and also again not for V1AR expressing cells, but with a higher tendency (**Figure 20D**). As a negative control, stimulation with OT alone showed overall low values for both receptors (**Figure 20E and F**).Conspicuously, especially when compared to the results from GLP1-T3, values of cells treated with OT-T3 were in the same range with cells treated with T3 for all transfections (around 4 x 10^6 to 1 x10^7 RLU). Since these results raised the question of whether or not the conjugate is stable enough in cell culture conditions, concentration-dependency and non-canonical signaling of OT-T3 was not investigated.

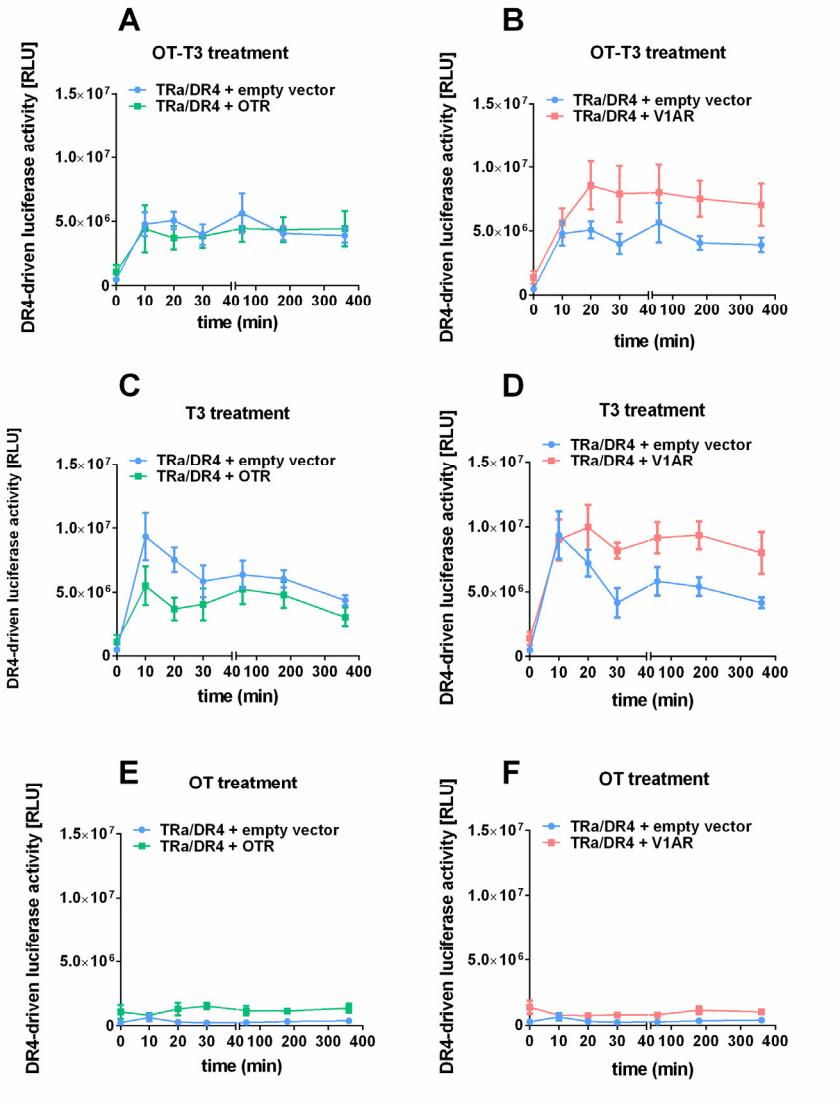

Figure 20: Luciferase based reporter gene studies of canonical response to TH in a time - dependent manner. Cells have been co-transfected with the luciferase reporter gene DR4, the thyroid hormone receptor α (TRa) and either OTR (green), V1AR (red) or empty vector (blue). Cells expressing OTR were stimulated with OT-T3 (1 µM) in (A) or T3 (C). Stimulation of cells expressing V1AR were treated the same with OT-T3 in (B) and T3 in (D). All stimulation took place for various periods (10 min, 20 min, 30 min, 1h, 3h, 6h). Data represent three independent experiments, each performed in triplicates. For statistical analysis, a two-way ANOVA with multiple comparison was performed, comparing different transfections. Values represent mean ± SEM.

4.2.4 Summary of the TH signaling assay results

Additionally, I was able to show increased canonical and non-canonical TH signaling in the presence of GLP1R in comparison to mock transfected cells. These results are depicted in **Figure 21.**

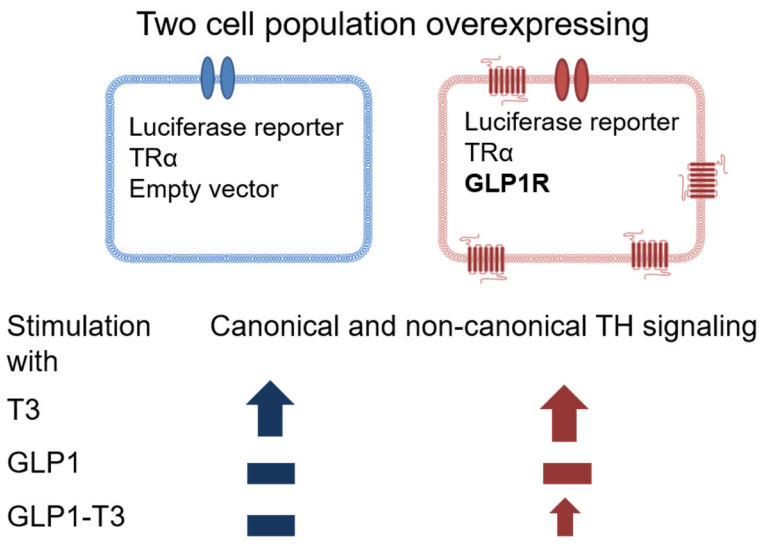

Figure 21: Graphical depiction of the results for canonical and non-canonical TH signaling assays of GLP1-T3. Cells were transfected with a luciferase-based reporter, the TRα and either an empty vector as a mock control or the GLP1R. Cells were then challenged with either T3 as a positive control, GLP1 as a negative control or GLP1-T3, the conjugate between the two compounds. Significant (though not as high as the positive control) increase of both canonical and non-canonical signaling for the conjugate was measured only in the presence of the receptor GLP1R, whereas the mock control did not show any significantly increased values

When investigating canonical TH-dependent signaling, it was revealed that OT-T3 activated the pathway in the same manner as a stimulation with T3 alone and no difference between cells that express either one of the target receptors and mock transfected cells was present. A possible reason is discussed in chapter 5. The results are summarized in **Figure 22**.

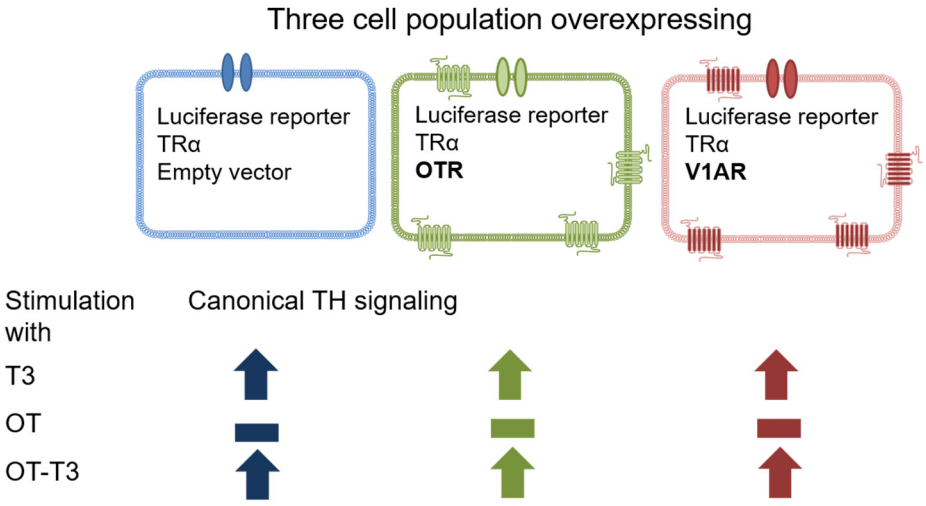

Figure 22: Graphical depiction of the results for canonical TH signaling of OT-T3. Cells were transfected with a luciferase-based reporter, the TRα and either an empty vector as a mock control or the target vectors OTR or V1AR. Cells were then challenged with either T3 as a positive control, OT as a negative control or OT-T3, the conjugate between the two compounds. For all cell populations, OT-T3 increased the TH-dependent signaling in the same range with no significant difference between mock expressing and target receptor expressing cells.

5 Discussion

In the course of this project two T3-conjugated peptides were characterized and validated in terms of receptor activation and internalization, as well as TH-dependent signaling activation. These investigations highlight whether or not the conjugates are working in the intended way, via the “Trojan-Horse”-like approach, as well as identifying possible adverse effects even before *in vivo* investigations. The main aim is to find a perfect receptor-ligand pair that could be used for MCT8-deficiency treatment with minimal side effects. **Figure 23** depicts an overview of general considerations that were made about the perfect receptor-ligand pair as well as the aspects that were investigated in the course of this project.

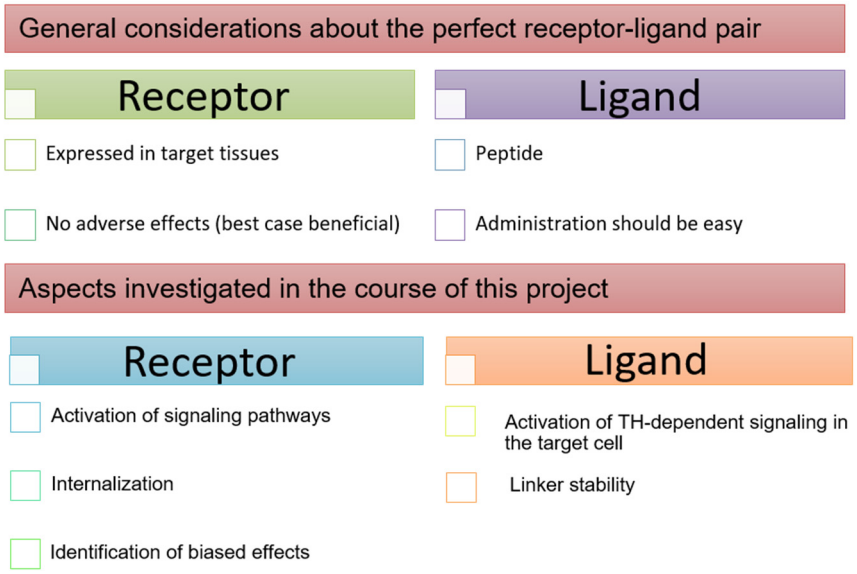

Figure 23: Graphical overview of the considerations regarding the perfect receptor-ligand pair as well as an outline of the aspects investigated in the course of this project.

In the following part, the results from this investigation will be discussed. First, the results of the functional characterization at their respective target GPCRs will be examined. Additionally, it focusses on the internalization of the receptor-ligand complex that follows activation. The second part discusses the activation of TH-dependent signaling which hints to the liberation of T3 after internalization and can be regarded as a proof that the “Trojan Horse”-like mechanism is at play.

5.1 Functional characterization of the conjugates at their respective target GPCR

The “Trojan Horse”-like mechanism utilizes the internalization mechanism of GPCRs. This process is part of the desensitization mechanism of receptors that prevents overstimulation. Hence, a receptor stimulation precedes the endocytosis of the whole receptor-ligand complex. Afterwards T3 should be released within the cytoplasm and

would be able to initiate TH-dependent signaling within the cell. A characterization of the conjugates must therefore happen with a view to functionality on their respective receptors by their peptide part, and to the activation of TH signaling by their T3 part. First, the functional studies were performed including activation of signaling cascades and internalization.

5.1.1 GLP1-T3 activates GLP1R

For the functional characterization of GLP1-T3, the unconjugated GLP1 was used as a positive control. The activation by GLP1-T3 should mimic the activation of the endogenous ligand GLP1. Three signaling cascades have been reported for GLP1R: cAMP as the main pathway, IP3/PLC-activation and MAPK pathway ERK1/2, all of which have been investigated to identify possible adverse effects of the conjugate.

5.1.1.1 G_s pathway at the GLP1R

In the main pathway, the G_s coupled cascade (Baggio and Drucker 2007), GLP1-T3 and unconjugated GLP1 show a similar cAMP concentration that was not significantly different from one another. Therefore, the conjugation of T3 to GLP1 does not influence the pathway that is most important for the receptor activation and a major adverse effect on the receptor can be excluded.

5.1.1.2 $G_{q/11}$/PLC-activation pathway at the GLP1R

There are hints from the literature that GLP1R is also able to activate $G_{q/11}$/PLC activation pathway (Montrose-Rafizadeh et al. 1999; Thompson and Kanamarlapudi 2015), although it is unclear, whether this effect is due to overexpression in *in vitro* assays and/or activation of PLC and plasma membrane calcium channels by the cAMP pathway (Holz, Leech, and Habener 1995). Nevertheless, a basal activity was not reported in any study and therefore, might be a pleiotropic effect of the HEK cells used for this project. Neither GLP1 nor GLP1-T3 were able to increase this basal activity upon stimulation. This leads to the conclusion that the addition of T3 to GLP1 does not lead to diverse activation of the G_q-associated PLC activation pathway and adverse effects can also be excluded for this signaling cascade.

5.1.1.3 MAPK pathway activation at the GLP1R

Additionally, ERK1/2 phosphorylation was tested, since it was also reported for the rat GLP1R in CHO cells and suspected to be caused by the βγ-subunits of G_s (Montrose-Rafizadeh et al. 1999). In the course of this project and with the human GLP1R, we were not able to recreate these findings, since the reporter gene assay for ERK1/2 phosphorylation did not report any activation. One explanation could be the endogenous expression of GIPR (Atwood et al. 2011), another incretin receptor that is reported to impair ERK phosphorylation and calcium release of GLP1R and decrease to 25% of the maximal response when challenged with GLP1 (Roed et al. 2015).

In conclusion, all three assays showed no significant difference between GLP1-T3 and the uncoupled GLP1. From this, it can be concluded, that the hybridization with T3 did not affect the functional properties of GLP1 at its target receptor.

5.1.2 GLP1-T3 initiates GLP1R internalization

Internalization of the receptor after the stimulation with the conjugate is the crucial step in the “Trojan Horse”-like mechanism. Therefore, investigations were performed using several methods.

1. The SNAP-tag staining was used to visualize the internalization. When stained with a non-permeable dye (SNAP Surface 488) and challenged with GLP1 or GLP1-T3, the receptor was clearly internalized for both ligands after 30 min of stimulation. This seems to be a reasonable time frame and similar results have been published already where the SNAP-tag technology was also used in a similar fashion (Roed et al. 2014).
2. This was also confirmed by an internalization assay that utilized a split luciferase (HiBiT), where the surface lost more than 80% of receptor expression in a concentration-dependent manner for both GLP1 and GLP1-T3 with very similar IC_{50} for the two ligands. The cell surface expression of GLP1R was measured after 30 min, verifying the results from the SNAP-tag staining.
3. Recruitment of β-arrestin precedes the internalization process and NanoBRET™ assay (in this case β-arrestin2) was used for these investigations. Similar results for GLP1 have been published with an EC_{50} of around 10^{-8} M (Hager et al. 2016). GLP1-T3 showed a slightly lower potency to recruit β-arrestin2, but was still able to interact with the protein in a concentration-dependent manner.

These results allow the conclusion that the conjugate is able to activate both signaling cascades and the internalization process in a similar fashion to the unconjugated peptide. The conjugate does not exhibit biased signaling on the receptor, a fact that should exclude unexpected adverse effects *in vivo* on the cellular level.

5.1.3 Oxytocin-T3 exhibits biased signaling at both target receptors

Similar functional characterization was performed with the two target receptors for OT-T3, OTR and V1AR. The results show a differential picture of activation by OT-T3. The pathways tested for OTR are G_i, $G_{q/11}$/PLC-activation and ERK1/2 phosphorylation, all pathways have been published for the receptor.

5.1.3.1 OTR signaling

5.1.3.1.1.1 G_i signaling at the OTR

G_i coupling has been known for a long time and confirmed in many publications, and seems to be most important for uterine contractions (Busnelli et al. 2012; Strakova and Soloff 1997). The conjugate was not able to activate G_i mediated AC inhibition upon stimulation. This showed an attenuation of OT function at the OTR when hybridized with T3.

5.1.3.1.1.2 $G_{q/11}$/PLC-activation at the OTR

PLC- activation, mostly described as the main pathway for OTR, has been reported to be partly activated by $G_{q/11}$ and partly by the βγ-subunits of G_i (Phaneuf et al. 1993; Molnár and Hertelendy 1990). Pertussis toxin (PTX), a G_i inhibitor was used to confirm the results from the cAMP assay. PTX is an ADP-ribosylating toxin that adds a ribosyl group to a cysteine residue at the α-subunit of G_i. This results in an uncoupling of the G protein with its receptor and therefore the α-subunit does not dissociate from its βγ-subunit (Locht, Coutte, and Mielcarek 2011). The main pathway of OTR, PLC-activation was not attenuated by the hybridization with T3 in comparison to OT alone. This was surprising, since signaling was PTX-sensitive for both peptides, which clearly suggests that part of the activation is still due to activation of the βγ-subunits of G_i even for OT-T3. It is published that the subunits of G_i do not necessarily dissociate upon activation (Frank et al. 2005; Bünemann, Frank, and Lohse 2003). It could be hypothesized that OT-T3 binding to OTR rearranged the receptor in a way such that it still couples to G_i without activation of the α-subunit but βγ-subunits, which would be fully inhibited by PTX-pretreatment.

5.1.3.1.1.3 MAPK pathway at the OTR

To confirm this, MAPK pathway was investigated. For the OTR, it has been published to be partly activated by PLC activation and partly due to activation of G_i. However, it is not entirely clear whether the G_i part of ERK phosphorylation is due to the βγ-subunits or the activity of the α-subunit and could be a pleiotropic effect, since different sensitivities of MAPK pathway for OTR have been published in different cell lines (Zhong, Yang, and Sanborn 2003; Strakova et al. 1998). PTX-pretreatment lead to a significant decrease of signals, indicating that G_i protein activation is partly responsible for ERK phosphorylation in the used cell system. The signals were significantly dampened when cells were challenged with OT-T3 in comparison to unconjugated OT. The curve resulting from OT-T3 stimulation mimics the curve of PTX-treated cells, leading to the conclusion that the activation of ERK phosphorylation by OT-T3 is produced by the activation of $G_{q/11}$ alone, but not G_i.

Together with the results from the AlphaScreen™ and NFAT assay, these findings suggest that OT-T3 is not able to activate the G_i protein fully, but only its βγ-subunits. In contrast to PTX, the interaction between receptor and G protein still takes place, when stimulated with the conjugate, but the conformational change of the receptor does not activate the α-subunit of G_i and therefore, no inhibition of AC and a decrease in ERK phosphorylation was measured. **Figure 24** depicts an overview of the biased signaling at the OTR for a better understanding.

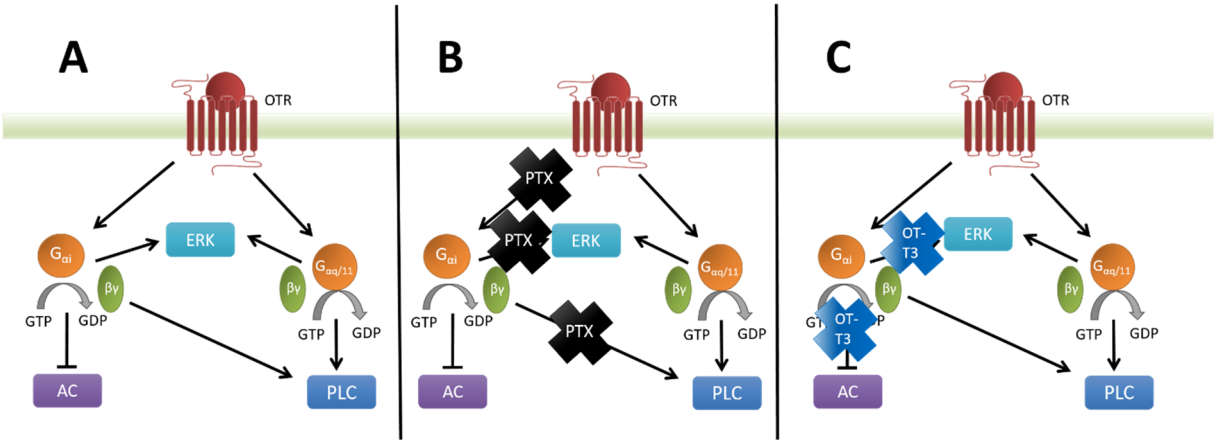

Figure 24: Depiction of the signaling pathways at the OTR and the biased signaling caused by PTX and OT-T3.
A: OTR is known to couple to two different G proteins, G_i and $G_{q/11}$. Upon activation, the trimeric G proteins dissociate in their α-subunit, which has a GTPase function, and their βγ-subunits. The $G\alpha_i$ inhibits AC, whereas $G\alpha_{q/11}$ activates PLC. Additionally, it is known to start MAPK pathway, here depicted as ERK. For this particular GPCR, both, PLC activation and ERK phosphorylation have been published to be caused by activation of both G proteins.
B: Pertussis toxin (PTX) inhibits the coupling of G_i to the receptor and therefore blocks the dissociation of this G protein entirely. This leads to a decrease in PLC activity and ERK phosphorylation.
C: OT-T3 was able to activate the $G_{q/11}$ protein to a full extend, but lacked a certain part of the G_i protein pathway, such as AC inhibition and ERK phosphorylation. Since PLC activity was identical to OT stimulation alone and could be decreased by PTX treatment, the βγ-subunits are assumed to be active, whereas $G\alpha_i$ activity was missing.

In brief, OT-T3 exhibits biased signaling on the OTR with attenuated activation of the G_i pathway with abolished AC inhibition and reduced MAPK activation, but persistent PLC activation. These results hint to possible adverse effects on a cellular level.

5.1.3.2 V1AR signaling

For the second receptor, V1AR two pathways have been investigated: $G_{q/11}$/PLC-activation and ERK phosphorylation.

5.1.3.2.1.1 $G_{q/11}$/PLC-activation at the V1AR

The main pathway, the PLC-activation, has been published for V1AR for both endogenous ligands, AVP and OT (Thibonnier et al. 1994). In comparison to OTR, the maximal response of V1AR was lower, verifying the lower affinity of V1AR to OT (Åkerlund et al. 1999). The activation of this pathway was identical for both peptides,

which leads to the conclusion that the conjugate activates the receptor in the same manner as the unconjugated OT.

5.1.3.2.1.2MAPK pathway at the V1AR

Activation of MAPK pathway at the V1AR has been published for both natural agonists, AVP and, with a slightly lower affinity, OT (Tahara et al. 1999). For the performed assay in the course of this project, signals were significantly increased with high concentrations of OT-T3. This could result from the instability of the conjugate and the resulting activation of non-canonical TH signaling. This has been explained in the introduction (1.1.6.4) and demonstrated to be activated (results part, 0). The reporter used for these assays is the same as for non-canonical TH signaling, but it misses the co-expression of TRα. Still, high concentrations of free T3 might be enough to activate the reporter by endogenous TRs and could be the reason for the significant increase in ERK phosphorylation. This would explain that the effect is most prominent for the highest concentration of conjugate.

In conclusion, both target receptors exhibit biased signaling for OT-T3. Although these effects have to be kept in mind for potential adverse effects of the conjugate, the internalization process is most important for the “Trojan Horse”-like approach. Depending on the signaling effects of the receptors in the target cells, it could even be beneficial to decrease signaling, but still activate the endocytosis. Therefore, the internalization process of the two target receptors was investigated next.

5.1.4 Oxytocin-T3 initiates internalization of OTR, but not V1AR

Afterwards, the recruitment of β-arrestin2 was tested for both target receptors as an indicator for internalization. β-arrestins have been shown to be important mediators of internalization independently of G protein activation (Grundmann et al. 2018), and therefore the recruitment needed to be tested separately.

5.1.4.1 β-arrestin2 recruitment at the OTR

The recruitment of β-arrestins by OTR have been shown to be important for endocytosis of the receptor (Oakley et al. 2001). Our assays showed the identical response to both conjugated and unconjugated peptide in a concentration-dependent manner. The receptor seems to be internalized upon the stimulation with the conjugate, which is an important step in the “Trojan Horse”-like mechanism.

5.1.4.2 β-arrestin2 recruitment at the V1AR

As with most Class A GPCRs, V1AR is also known to be internalized by β-arrestins (Terrillon, Barberis, and Bouvier 2004). Surprisingly, the receptor was not able to recruit β-arrestin2 when challenged with OT-T3, but did so after OT stimulation. This seems to be a specific effect for OT-T3 at the V1AR, since the assay for OTR executed in the same manner showed an identical recruitment for both tested agonists. Therefore, it is unlikely that the instability of the conjugate and free T3 had any influence on the assay, although the effect of stimulation with T3 alone has not been

controlled for. This means that in contrast to OTR, V1AR is not internalized upon stimulation with OT-T3.

From these results, it can be concluded that OTR internalizes when stimulated with OT-T3, whereas V1AR does not. Unfortunately, V1AR was the target receptor that was most attractive for the treatment of MCT8-deficient patients due to the high expression in motoneurons (Caldwell et al. 2008), a potential target for a treatment.
Combining these results with the signaling assays, it is clear that the OT-T3 conjugate does not meet optimal requirements for the “Trojan Horse”-like approach as it was determined for GLP1-T3. Final validation however was made by investigating the canonical TH-dependent signaling in the absence and presence of both target receptors.

5.2 TH-dependent signaling of the conjugates

Stimulation of cells with T3 leads to the regulation of gene expression within every cell. This is mediated by transmembrane transporters that are able to transport TH over the membrane. Within the cells, T3 binds to TRs that function as transcription factors and regulate the expression of target genes by binding to TRE in the promotor region (Lazar, Berrodin, and Harding 1991). This has been defined as the canonical pathway of TH and can be used by a reporter gene assay that utilizes a TRE in control of a luciferase gene. The expression of luciferase is proportional to the TH-dependent signaling that is activated by free T3 within the cell (Hofmann, Schomburg, and Kohrle 2009).
After the functional characterization of the peptide part of the conjugate has been performed, TH-dependent signaling was investigated as a proof that after internalization of the receptor-conjugate complex, T3 is liberated and can act in a similar manner to T3 that would be transported via the transmembrane transporter. First, preliminary experiments were performed to find a suitable TH transporter blocker for the used cell system. This could then be used to exclude that free T3 is being transported by TH transporters and is proof of a functioning “Trojan Horse”-like mechanism. Every cells type is expressing its unique set of TH transporters. To find a suitable blocker, all published TH transporter blockers were tested.
Afterwards, activation of TH-dependent signaling of both conjugates were tested in absence and presence of their target receptors. The “Trojan Horse”-like mechanism would be proven by an increase of TH signaling only in the presence, but not in the absence of their respective receptors.

5.2.1 No tested blocker could inhibit endogenous TH transporters in HEK293 cells

We tested published TH transporter blockers in previously reported concentrations or lower, but for longer incubation periods. Only DMI was able to decrease T3 signaling by a significant amount, but when cell viability was tested, it was discovered that DMI might not block the transport of T3 into the cell, but is in fact a toxic compound. Hence,

it probably decreased the TH signaling by lowering the amount of live cells when incubated with high concentrations or for long periods. To find a concentration that might be able to block TH transport in non-toxic concentrations, cells were incubated with varying concentrations of DMI and both TH-dependent signaling and cell viability were determined. Unfortunately, no concentration was found that would still be able to block TH transport, but would not influence cell viability. It has to be concluded, that the inhibition of TH transport is due to toxicity of the compound rather than a true blockage of the endogenous transporter. Later, a study was published that confirmed toxicity of DMI for concentrations higher than 500 µM, five times the highest amount that was used for the experiments in this project (Dong and Wade 2017). It has to be noted that the incubation time in the published study was 10 min, whereas the incubation time for the experiments in this project was 1 h. For time-dependent experiments, incubation periods up to 6 h hours were planned.

From those preliminary experiments it can only be concluded that DMI is potentially toxic for living cells even in low concentrations when incubated for long periods.

5.2.2 GLP1-T3 activates TH-dependent signaling in the presence of GLP1R

After it was confirmed that GLP1-T3 activates GLP1R and its internalization, TH-dependent signaling was investigated. It was hypothesized that signaling should only occur in the presence of the respective receptor through the “Trojan Horse”-like mechanism when cells are challenged with the conjugate. Two different pathways have been investigated, the canonical pathway utilizing a TRE-controlled luciferase reporter and the non-canonical pathway which uses a SRE-controlled luciferase reporter. Both are co-expressed with the rTRα, a TH receptor that is necessary to activate the reporter.

5.2.2.1 GLP1-T3 activates the canonical pathway

Indeed, GLP1-T3 was able to activate canonical TH-dependent signaling pathway in the presence of the receptor. The signals were significantly increased in comparison to the cells where the receptor was absent. This seems to be time- and concentration-dependent. In comparison to the positive control, the stimulation with T3 alone, where we could see an immediate increase to maximal signals, the values for GLP1-T3 were more than 50% lower and the effect was slower with significant results after 60 min of stimulation. This is a strong indicator that the “Trojan Horse”-like mechanism is at work with GLP1R being internalized and recycled back to the membrane or degraded rather than a transporter that permanently transports TH over the plasma membrane.
Regarding the concentration-ascending experiments, it has to be noted that signaling also increased in mock controlled cells for the highest concentration compared to the basal state. This is most likely due to endogenous expressed GPCRs of the used cell system. It has been published that HEK293 cells express GIPR (Atwood et al. 2011), another incretin receptor that is able to bind to GLP1 with very low affinity (Roed et al. 2015). The interactions with possible endogenously expressed GLP1R or other unknown receptors cannot be excluded.

5.2.2.2 GLP1-T3 activates the non-canonical pathway

A similar experimental set-up was used to measure the non-canonical pathway. Here, the reporter gene was controlled by a SRE. This response element is identical to the one that was used for ERK phosphorylation assays, since the non-canonical pathway is known to activate PI3K as well as MAPK pathways (Makino et al. 2008; Kalyanaraman et al. 2014). The difference to the experiments performed for the activation of MAPK at the GLP1R is the co-expression of transfected rTRα1, which mediates the phosphorylation of ERK. Results from the functional characterization at the receptor show that the abundance of endogenously expressed TRs is not enough to activate the reporter (chapter 4.1.2). GLP1-T3 was able to activate this pathway in a time-dependent manner in the presence of GLP1R that was significantly higher than in mock transfected cells. The curve progression is very similar to the canonical pathway with a significant increase after 60 min that was slowly increasing, but did not reach signals similar to T3 challenge. Again, this indicates that the increase is due to the "Trojan Horse"-like mechanism rather than instability of the conjugate.
Unfortunately, since we were not be able to find a suitable TH transporter blocker, we could not confirm our findings with the inhibition of endogenously expressed transporters. Nevertheless, the significant increase of signaling in comparison to mock controlled cells is evidence that GLP1-T3 is entering the cells via the "Trojan Horse"-like mechanism. Since we have not been able to find a suitable blocker for these experiments that could block endogenous TH transporters in the used cell system, further experiments have not been performed.

In conclusion, GLP1-T3 was able to activate both canonical and non-canonical TH-dependent signaling, probably by internalization of the receptor-conjugate complex and subsequent liberation of T3 within the cell. The fact that challenge with GLP1-T3 only increased signals in cells that were expressing GLP1R is a strong indicator that this conjugate is working in the intended way and would be suitable for *in vivo* studies. The performed assays for this conjugate helped building a toolbox that can be used for future peptide candidates.

5.2.3 Oxytocin-T3 does not display increased TH-dependent signaling in the presence of both target receptors

The second conjugate investigated, OT-T3 already displayed biased signaling on both target receptors with one of them potentially failing internalization after stimulation. Both of the receptors were tested in a time-dependent experiment for TH-dependent canonical signaling. There was no significant difference between GPCR expressing and mock expressing cells. Additionally, the values were in the same range as the T3-stimulated positive controls indicating that the conjugate is not stable in cell culture conditions and T3 was released prematurely outside of the cells before OT-T3 even binds to the receptor. Later, our cooperation partners, who synthesized the peptide-T3 hybrid, confirmed the instability of the compound when incubated with serum. The biased signaling that the conjugate still exhibits is most likely due to the residual linker on the peptide. This confirms that even slight modifications on small peptides can cause a biased response on the receptor.

These findings lead to the conclusion that OT-T3 is not stable enough to be used as a “Trojan Horse”. Our cooperation partners are currently working on designing a new conjugate that is more stable.

5.3 Summary of all findings and conclusions

5.3.1 Summary for GLP1-T3

For the first conjugate, GLP1-T3, that was used to establish a toolbox for investigations of peptide-hormone conjugates in general and functioned as a proof-of-principle compound, we found that it was able to activate its target receptor GLP1R in the identical manner as the unconjugated GLP1. In addition, it can initiate the internalization process of GLP1R, which is crucial for the “Trojan Horse”-like mechanism. The hybridization with T3 seems to have no attenuating effect on receptor binding and the desensitization mechanism. GLP1R is a class B GPCR that binds ligands with its long N-terminal domain rather than a binding pocket in the transmembrane domain (Hoare 2005). This might be one reason the receptor activation is less sensitive to ligand modifications. Additionally, its size of around 30 amino acids (Baggio and Drucker 2007) makes it a good candidate for hybridization with several target side chains that are probably not involved in receptor binding. In this project, it was used to establish the toolbox for validation of future conjugates and originally was intended as a treatment of metabolic syndromes. Therefore, a similar activation and internalization of GLP1R by GLP1-T3 is beneficial for the treatment, since both GLP1 and T3 have metabolic effects in their target tissues. In the case of a conjugate that is intended as a treatment for AHDS, possible side effects by the activation of receptor pathways have to be considered, though the internalization of the receptor is a necessity for the “Trojan Horse”-like mechanism to work.
This mechanism was proven by canonical and non-canonical TH-signaling that was significantly increased in the presence, but not in the absence of GLP1R. This confirms that liberation of T3 after internalization is working rather than a premature release outside of the cell. Since we could not find a suitable TH transport blocker, the results could not be confirmed by inhibition of endogenous TH transporter. All results for GLP1-T3 are depicted in a graphical abstract (**Figure 25**).

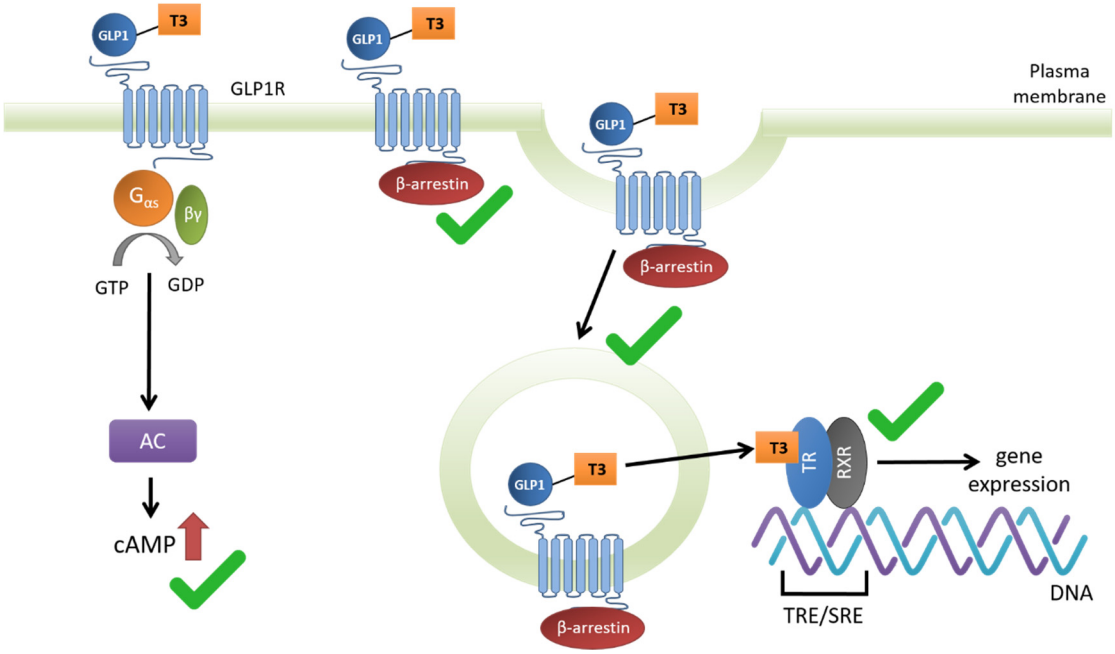

Figure 25: Graphical abstract for the first investigated conjugate GLP1-T3. The ligand was able to activate the main pathway for the respective GPCR, the GLP1R in the identical manner to the unconjugated GLP1. It has also been shown that the conjugate initiated the recruitment of β-arrstin2 to the receptor and decreased the amount of surface-localized GLP1R, suggesting receptor internalization.
In presence of the GLP1R, the conjugate was able to activate both canonical and non-canonical signaling pathways of TH, suggesting T3 entered the cell through the "Trojan Horse"-like mechanism.

5.3.2 Summary for OT-T3

The second conjugate, OT-T3 that was thought to be a candidate to treat MCT8-deficieny was investigated with the established toolbox. In contrast to GLP1, oxytocin is a small nonapeptide that binds to class A GPCRs that have small binding pockets (Barberis, Mouillac, and Durroux 1998).
Any kind of addition to or modification of the peptide can lead to attenuated binding at the receptor and therefore altered signaling. This has been already shown for carbetocin (de-amino-1-monocarba-(2-O-methyltyrosine)-oxytocin), an oxytocin analog that has been deaminated at the N-terminus and the disulphide bridge between cysteine 1 and 6 has been replaced with CH_2-S. This peptide is no longer able to activate G_i coupling and β-arrestin1/2 recruitment (Passoni et al. 2016).
This illustrates that, especially for small peptides and Class A GPCRs, nearly every residue seems to be crucial for ligand binding and receptor conformational changes that result in altered signaling. As stated above, depending on the cell response, altered receptor signaling could be beneficial to minimize adverse effects. On the other hand, receptor desensitization could also have adverse effects on the cell, but it is necessary for the intake of T3 over the "Trojan Horse"-like mechanism. Therefore, a receptor should be chosen that is quickly recycled to the cell surface.
TH-dependent signaling assays revealed instability of the compound which lead to the increase of signal independently of receptor expression, concluding that no "Trojan-Horse"-like mechanism is at work. Therefore, no further experiments were conducted.

All results for OT-T3 are depicted in a graphical abstract (**Figure 26**). Interesting to note however, is the fact that even though the conjugate is most likely unstable and T3 is released prematurely, the signaling properties on both tested target receptors are different from unconjugated OT. It might be concluded that the linker is still attached to OT and seems to impede interaction with the receptor binding pocket.

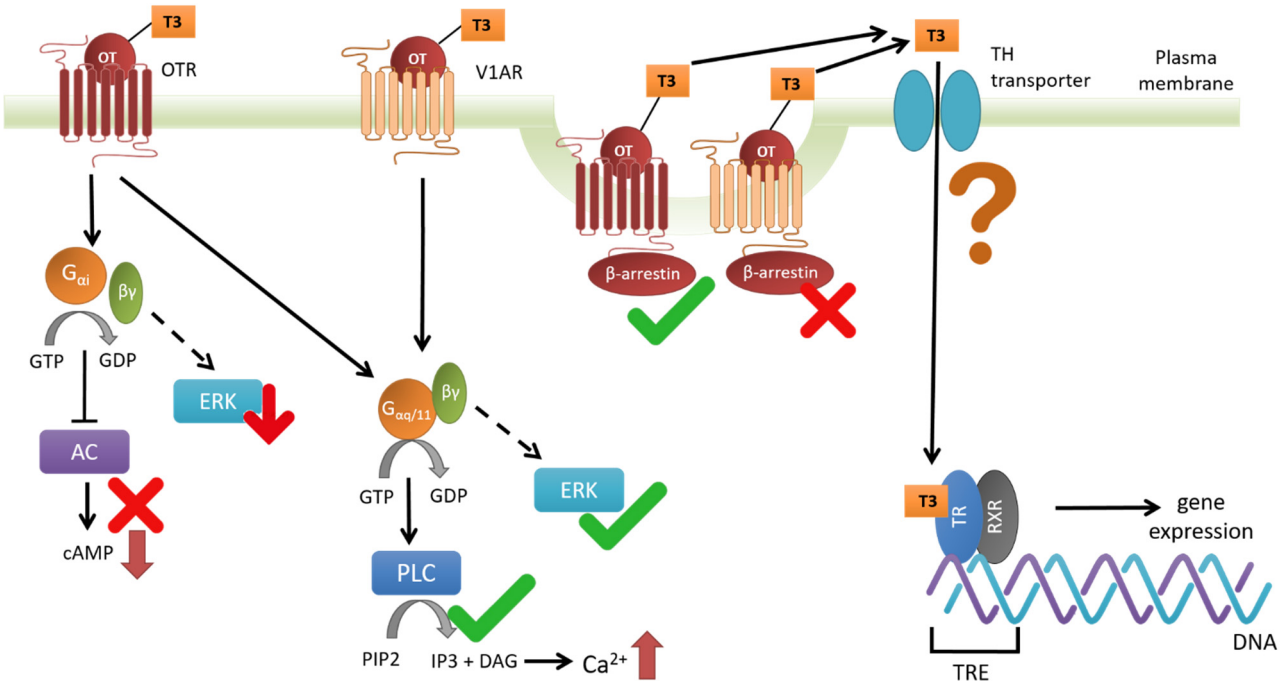

Figure 26: Graphical abstract for the second investigated conjugate OT-T3. The ligand was able to activate the main pathway for the two target GPCRs, OTR and V1AR. For other pathways, biased signaling was identified. OT-T3 was not able to activate G_i-mediated AC inhibition and showed a significantly increased ERK phosphorylation in comparison to the unconjugated OT. It was also able to initiate recruitment of β-arrstin2 to OTR, but not to V1AR, suggesting no internalization at the V1AR.
The conjugate showed stability issues, suggesting that high TH-dependent signaling measured in this project were due to free T3 that has already been cleaved outside of the cell.

5.4 Suitability of small peptides as backbone for "Trojan-Horse"-conjugates

In order to discuss the suitability of the two different peptides used in the course of this project, the different classes of their respective GPCRs and their binding pockets have to be reviewed shortly. The main difference between class A and class B is the long extracellular N-terminal domain of class B GPCRs. For most receptors, the peptide agonists bind to this long extracellular part and the N-terminal region then activates the receptor by interaction with the helical transmembrane domains (Lee, Booe, and Pioszak 2015). Most of the known class A GPCR on the other hand have shorter extracellular domains and agonists bind directly into their binding pocket. Therefore, ligands that activate the receptor at their orthosteric binding site are relatively small.
As the results from this project show, the choice of peptide as the backbone of "Trojan Horse"-conjugates can have a profound effect on signaling behavior exhibited by their

respective receptor. The main difference between GLP1 and OT is their size (GLP1: 30 amino acids, ~ 3500 Da; OT: 8 amino acids, ~1000 Da) and the GPCR class of their target receptors. As a class B receptor, GLP1R binds its ligands in two steps. First, the N-terminal long extracellular part of GLP1R interacts with the C-terminal part of GLP1, then the N-terminus of the ligands is associated with the core of the receptor in the transmembrane domain (Underwood et al. 2010; Runge et al. 2008). Combining this knowledge with the overall size of the peptide, GLP1 displays plenty of options to attach a small hormone, such as estrogen (as was done in (Finan et al. 2012)) or T3 without hindrance of receptor binding.

OTR and V1AR, on the other hand, have small binding pockets within their transmembrane domain. Due to their overall homology to the many existing class A receptors, the residues that are important for specific interaction with the ligands are oriented towards the binding pocket. These have been shown to be conserved over many species in TMH6, whereas residues in TMH7 are more variable to determine the selectivity of the receptor towards its ligands (Koehbach et al. 2013). For OT it has been found that residues in the extracellular N-terminal part of OTR close to the TMH1, as well as ECL1 and 2, are essential for high-affinity binding of agonists, but not for antagonists (Gimpl et al. 2008). Intense research on these receptors show that plenty of contact points between peptide and receptor are necessary for binding specificity and signaling activation. Associated with the small size of the ligand, it is easy to imagine that almost every residue of this peptide has a role in receptor binding. Furthermore, the fact that the closely related peptide AVP, which differs in only two amino acid positions (Ganten et al. 1986), has a vastly different affinity to V1AR than OT (Åkerlund et al. 1999), makes clear that the family of oxytocin and vasopressin receptors and peptides are a fine-tuned network. Therefore, the attachment or modification on these peptides poses a difficult task.

Before synthesis, a structural homology model was used to identify possible modification sites of OT (**Figure 27**). In this model, the C-terminal part pointed to the extracellular site and was therefore chosen as the hybridization site for T3. In detail, it was position 8 (Leu) that was linked to the TH. Since the synthesis of the conjugate was performed by our cooperation partners (Indiana University, Bloomington), the actual linker chemistry is not known.

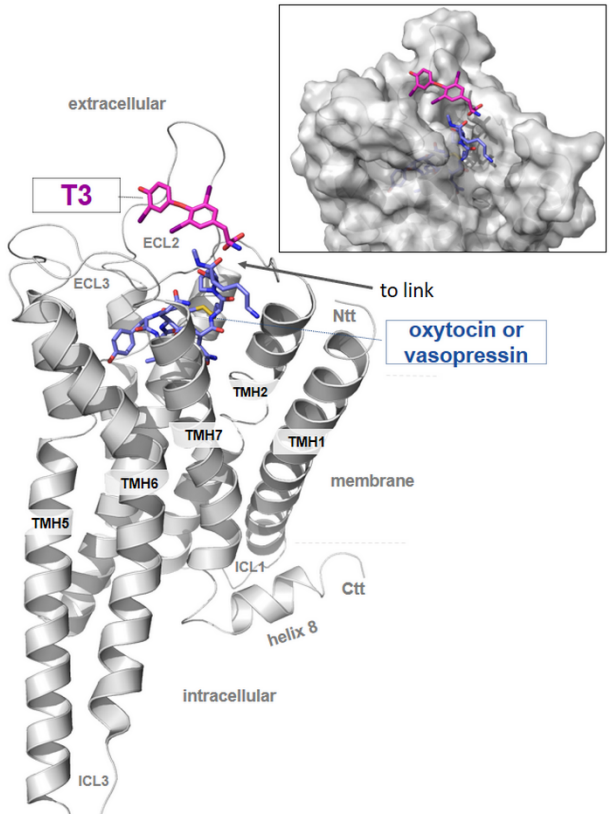

Figure 27: Structural homology model of OTR (backbone ribbon presentation) based on a recently described protocol for modeling of the oxytocin/vasopressin receptor group (Busnelli et al. 2013). OT (blue sticks) is docked at the endogenous orthosteric binding site in the active conformation. The inset shows the surface representation receptor/ligand complex and visualizes that specifically the C-terminal ligand part should be used to modify the ligand, because it points towards the extracellular site. T3 (magenta) is added to the C-terminal part of OT as the suggested hybridization site.
This image by Gunnar Kleinau was used as an indicator as to where T3 should be conjugated to OT.

Recently, Busnelli et. al. published a study with bivalent OTR agonists that are targeted towards OTR dimers. Here, OT was deaminated at position 1 (Cys) and alkane linkers of various length were added at the leucine residue of position 8. They then characterized these agonists and found that a linker containing more than eight alkane groups was able to maintain receptor function (Busnelli et al. 2016). This confirms that the hybridization site for OT-T3 was a good choice, but the chemical composition needs to be improved. This is especially complicated, since the linker should also be able to release T3 within the cell, but not before entering it.
In conclusion, hybridization of larger peptides such as glucagon or GLP1 with small hormones is possible without losing receptor functions. With smaller peptides, modification should be made with caution. The here investigated OT-T3 is not advised to be tested *in vivo*.

5.5 Suitability of OT-T3 for treatment of MCT8-deficiency

The aim of the study was to find a perfect ligand-receptor pair that would be suitable for treatment of MCT8-defciciency. For that, an already available peptide-T3 conjugate, GLP1-T3, was used to establish a toolbox of *in vitro* assays. This conjugate was not intended for MCT8-deficiency treatment, but as a proof-of-principle compound. The candidate that was thought to be suitable for the intended purpose was OT-T3. Irrespective of the fact that the investigated OT-T3 conjugate is not stable enough in cultured conditions and the size of OT makes the hybridization of T3 difficult without losing receptor functions, its suitability for the intended purpose as a treatment for AHDS has to be discussed. Initially, it was thought to be a perfect candidate due to its known positively associated functions in social behavior and bonding. Additionally, its target receptors are highly expressed in the brain overall, especially in the areas that would benefit from increased T3 levels in MCT8-deficient patients, such as areas that are associated with locomotor defects.

However, whether or not OT even crosses the BBB is still up for debate (Leng and Ludwig 2016). The BBB is comprised of epithelia cells, glia cells, and extracellular matrix that separates the peripheral circulation from the CNS. Its permeability is regulated by tight junctions between the epithelium (Lawther, Kumar, and Krovvidi 2011). Several studies claim an intranasal (IN) administration of OT as a way to target the brain directly and bypass the BBB. This is supposed to be facilitated by two routes: the olfactory pathway over olfactory nerve in the nasal mucosa, which directly extend to the cranial cavity (Bahadur and Pathak 2012) and the trigeminal nerve, a cranial nerve which projects from the nasal passages to the brain stem. OT is thought to be internalized into these neurons, transported along the axon followed by exocytosis. Although this method of administration supposedly reaches the brain, most of the IN administered OT will leak into the circulation through the nasal mucosa (Lochhead and Thorne 2012). Another, more reasonable, but debated explanation is the uptake of IN administered drugs into the perivascular space, a lymphatic system of the brain, dubbed the glymphatic system. Whether this system actually exists or is just an artifact in MRI scans is highly controversial (Engelhardt, Vajkoczy, and Weller 2017; Naganawa et al. 2017). From here it can be absorbed by the CSF and would be distributed throughout the whole brain through the perivascular space to the neurons (Lochhead et al. 2015). Since most studies are performed in rodents, they should be regarded with caution. Diffusion and distribution of drugs throughout the brain regions might be faster than in humans due to the considerably smaller size of rodent brains.

After IN administration, OT concentrations are measured in the cerebrospinal-fluid (CSF), a fluid that surrounds the brain and the spinal cord (Davson 1967), in saliva and/or blood. Since a lot of the studies are performed in rodents and in most of them, overall OT concentrations have been determined, several of these investigations are subject to strong criticism. Many suggest that administration of OT to the periphery (for example intravenous=IV) would lead to a release of central OT (Ermisch et al. 1985) and critics argue that this would be also the case for IN administered OT that distributes in the periphery in high concentrations (Leng and Ludwig 2016). A very recent study suggested that this is not the case. Lee and colleagues administered d5-deuterated OT IV and IN to non-human primates (rhesus macaques). Afterwards they measured this labeled peptide in peripheral blood and CSF samples with a novel highly sensitive and specific quantitative mass spectrometric assay, but could not find increased endogenous, non-labeled OT concentrations. Nevertheless, they were also not able to find an advantage of IN versus IV administrations, since there were no increased labeled OT concentrations in the CSF compared to IV administration (Lee et al. 2017). Critics also point out that in most studies measured OT concentrations in the CSF are a small fraction of the originally administered OT and therefore are due to spillage of high concentrations in the circulation and accumulation in the subarachnoid space. This could have adverse effect on organs in the periphery, such as the gastro-intestinal tract and reproductive system (Leng and Ludwig 2016).

Another noticeable fact is the inconsistent methodology used in the published articles. Numerous studies use vastly different methods of measurement of plasma OT. Some studies used ELISA (enzyme-linked immunosorbent assays) (Feldman, Gordon, and Zagoory-Sharon 2011), radioimmunoassays (Szeto et al. 2011; Feldman, Gordon, and Zagoory-Sharon 2011) or LC/MS (Liquid chromatography/mass spectrometry) (Zhang

et al. 2011) of unextracted or extracted plasma (Christensen et al. 2014), making it difficult to compare values from different reports.
In clinical psychology OT is studied for its behavior effects. Patients with schizophrenia or on the autism spectrum disorders are mostly subjected to IN administration of high doses of OT and many studies report reduction of symptoms. However, many of these studies have only a small number of test subjects, short treatment periods and investigate social cognition using tests that are standardized, but can be hard to interpret (Feifel et al. 2010; Pedersen et al. 2011).
In summary, many questions surrounding OT administration are still open. In case there will be a new and stable OT-T3 conjugate, *in vivo* studies in rodents would be necessary in addition to the here presented *in vitro* studies. Presuming the conjugate was able to surpass the BBB after IN administration, T3 would be released in the olfactory bulb, the trigeminal nerve, and/or the glymphatic system and could be transported into other brain regions.
The expression of MCT8 within the human brain is still mostly unknown, since the majority of studies have been performed in mice. As mentioned in the introduction, mct8-KO does not lead to the severe phenotype in mice. Another TH transporter, most likely oatp1c1, is expressed on the murine BBB which can compensate for the decreased influx of T3 into the brain (Mayerl et al. 2014). Fluorescence-based immunostaining of the human brain performed in our institute suggest that MCT8 is mainly expressed in both apical and basolateral membrane of epithelia cells of the human BBB, but not in neurons or the supporting glia cells (unpublished data, Nina-Maria Wilpert). This contrasts with previously published findings in human neurons of several brain areas using chromogenic immunohistochemistry staining (Chan et al. 2014; Alkemade et al. 2011; Wirth, Roth, Blechschmidt, Hölter, Becker, Racz, Zimmer, Klopstock, Gailus-Durner, Fuchs, et al. 2009). Other studies support our findings (Vatine et al. 2017; Roberts et al. 2008), leading to believe that once the BBB was circumvented by IN administration, TH transport into neurons with OT-T3 would be at least possible. This is supported by the fact that OTR is found in an early olfactory neuronal cell line (Gravati et al. 2010) and in the olfactory bulb (Stoop 2012) as well as trigeminal neurons (Tzabazis et al. 2015) of rat brains.

5.6 Prospects of AHDS treatment

Due to the controversy around OT administration, however, other ways for the treatment of MCT8-deficiency to bypass the BBB should be considered. IN administration seems to be very dependent on the properties of the compound used, including but not limited to lipophilicity, acid dissociation constant, and charge density (Chou and Donovan 1998; Kao et al. 2000). A recent publication shows the IN administration of labeled full-length immunoglobulin G, a rather large peptide, into the nervous system of rats, highlighting the brain areas that were reached by the compound, including olfactory bulb, brainstem and part of the frontal cortex (Kumar et al. 2018). Other substances that have been postulated to be suitable for IN administration are dopamine (Dahlin et al. 2000), insulin (Kern et al. 1999) and insulin-like growth factor (Thorne et al. 2004). IN administration of TH has not been tested yet and could be of interest, but needs intensive investigations. Many compounds have

been capsulated with nanoparticles such as methoxy poly(ethylene glycol)-poly(lactic acid) (MPEG-PLA) that improves the passage of the nasal mucosae due to its hydrophobic core, the PLA-part, and its hydrophilic shell, the PEG-part (Zhang et al. 2006). This could be a promising alternative way to deliver TH into the brain.

The last question remaining is whether major improvement of AHDS by any treatment is even possible, since it is a genetic disorder with developmental impairments. Here, the only similar disease that comes to mind is CH, which can be caused genetically, but involves global hypothyroidism. Major improvements in growth and development of CH patients have been observed by treatment with T4 from early childhood (Krude, Kühnen, and Biebermann 2015). As described in the introduction, one of the main differences of AHDS in comparison to CH is the missing supply of maternal THs even during the first trimester of gestation, a crucial phase of neuronal development (de Escobar et al. 2008), due to the inactive transporter on the BBB. Therefore, treatment within pregnancy would be most desirable, although it is rarely tested for an uncommon genetic disorder during pregnancy and most cases are diagnosed postnatally. This is only the case in families with known cases of AHDS. Nevertheless, treatment with OT-T3 during pregnancy would not be advised anyway, due to the labor-inducing properties of OT (Dawood et al. 1978) and the high concentrations of oxytocinase, the enzyme that degrades oxytocin during pregnancy to prevent premature labor (Fekete 1932).

Treatment would most likely start after birth and diagnosis, but how promising the therapy would be is unknown. TH derivates have been used to treat AHDS patients, but were not able to improve the neurological phenotype (Verge et al. 2012). In a mouse study, pregnant mice carrying mct8-deficient and wild type embryos were treated with L-T4 or DITPA. After birth, TH-regulated gene expression was assessed by qPCR to show that the derivate was able to cross the placenta in a similar fashion to L-T4 (Ferrara et al. 2014). This study has to be interpreted with caution, since the mct8-KO mouse model has its limitations as stated above (introduction, chapter 1.2.3). Especially the missing neurological problems due to an additional TH transporter on the BBB of rodents, make this model not ideal for treatment testing. It has also been shown that DITPA is not able to cross a human BBB-model derived from iPSCs (Vatine et al. 2017) and most likely does not so *in vivo*, which explains the results of the human trails.

The main histological characteristics resulting from this are structural immaturity and deficient myelination of CNS-neurons (López-Espíndola et al. 2014), a shared phenotype with untreated CH (Berbel et al. 1994; Koromilas et al. 2010). Myelination has been shown to continue postnatally to the age of four (Bernal 2007). Moreover, TH treatment of an adult mouse model for multiple sclerosis, a demyelinating disease, resulted in regeneration of myelin and differentiation of oligodendrocyte progenitor cells (Harsan et al. 2008). Additionally, case reports of CH show improvements of myelination through magnetic resonance spectroscopy (Jagannathan et al. 1998), leading to the belief that postnatal treatment in the first years of life might also improve the conditions of MCT8-deficient patients. Nevertheless, due to the aforementioned differences between these conditions, it is still unclear at what point in time and to what extent some manifestations are reversible, if at all. Therefore, more research in this field is clearly necessary.

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7 Supplements

7.1 Cloning primers

Table S 1 List of all cloning primers used for this project

Gene	Recipient plasmid	Primer	
		F/R	Sequence
rARRB2	pFC32K	F	AACTGCGATCGCCATGGGGGAGAAACCCGGGACC
		R	ACGCGTTTAAACGCAGAGTTGATCATCATAGTCGTCATCCTTCATCC
GLP1R	pcDps	F	CTGAATTCGCCACCATGGCCGGCGCCCCCGGCCCGCTGC
		R	CCACTAGTTCAGCTGCAGGAATTTTGGCAGGTGGC
	$pSNAP_f$	F	GTCAGCTAGCATGGCCGGCGCCCCC (signal peptide)
		R	GTCAGAATCCGGGGCCGGCCCTGCC (signal peptide)
		F	AAGACTCGAGCGCCCCCAGGGTGCCACT (mature peptide)
		R	TAGATTAATTAATCAGCTGCAGGAGGCCTGGCA (mature peptide)
	pFC14A	F	GTCGGCGATCGCCATGGCCGGCGCCCCCGGCCC
		R	TGTTGTTTAAACGCTGCAGGAGGCCTGGCAAGTGGCTGT
	pBiT3.1-secN	F	TATATCTAGACGCCCCCAGGGTGCCACTGTGTCC
		R	TCGAAAGCTTTCAGCTGCAGGAGGCCTGGCAAGTGGC
OTR	pcDps	F	ATATAGAATTCGCCACCATGGAGGGCGCGCTCGCAG
		R	CTGCACTAGTTCACGCCGTGGATGGCTGGGAGCA
	PFC14A	F	GACCGCGATCGCCATGGAGGGCGCGCTCGCAGCCAA
		R	CGCGGTTTAAACCGCCGTGGATGGCTGGGAGCA
V1AR	pcDps	F	ATATGAATTCGCCACCATGCGTCTCTCCGCC
		R	GAGCACTAGTTCAAGTTGAAACAGGAATGAATTTGATGGACTTGGA
	PFC14A	F	GACCGCGATCGCCATGCGTCTCTCCGCCGGTCCC
		R	TGTCGTTTAAACAGTTGAAACAGGAATGAATTTGATGGACTTGGA

7.2 Genes of interest (cDNA)

All cloned constructs were sequenced using the Sanger-method and the sequence were aligned with the known cDNA from databases such as NCBI (National Center for Biotechnology Information) or Ensembl gene browser using the BLAST (basic local alignment search tool).

ARRB2 (*rattus norvegicus*) NM_012911, 1232 bp, 410 aa

ATGGGTGAAAAACCCGGGACCAGGGTCTTCAAGAAGTCGAGCCCTAACTGCAAGCTCACCGTGT
ACTTGGGCAAGCGTGACTTTGTGGATCACTTGGACAAAGTGGATCCTGTCGATGGTGTGGTGCTT
GTGGATCCTGACTACTTGAAGGACCGGAAAGTGTTTGTGACCCTCACCTGTGCCTTCCGCTATGG
CCGAGAAGACCTGGATGTACTGGGCCTGTCTTTCCGCAAAGATCTGTTCATCGCCACCTACCAG
GCCTTCCCCCCCATGCCCAACCCACCTCGGCCCCCCACCCGCCTACAGGACCGACTGCTGAAG
AAGTTGGGCCAGCATGCCCACCCCTTTTTTTTCACAATACCCCAGAATTTGCCTTGCTCCGTCACA
CTGCAGCCAGGACCGGAGGACACAGGGAAGGCCTGTGGAGTAGACTTTGAGATTCGAGCCTTCT
GTGCCAAATCTATAGAAGAAAAAAGCCACAAAAGGAACTCCGTGCGGCTTATCATCAGAAAGGTA
CAGTTTGCTCCTGAGACACCCGGCCCCCAGCCATCAGCTGAAACCACACGCCACTTCCTCATGT
CTGACCGGAGGTCCCTGCACCTAGAGGCTTCCCTGGACAAAGAGCTGTACTACCATGGGGAACC
CCTCAATGTCAACGTCCACGTCACCAACAATTCTGCCAAGACCGTCAAGAAGATCAGAGTGTCTG
TGAGACAGTATGCCGACATTTGCCTCTTCAGCACCGCGCAGTACAAGTGTCCTGTGGCTCAGCTT
GAACAAGATGACCAGGTGTCTCCCAGTTCCACATTCTGCAAGGTGTACACCATAACCCCGCTGCT
CAGTGACAACCGAGAGAAGCGTGGCCTTGCCCTTGATGGGCAACTCAAGCACGAAGACACCAAC
CTGGCTTCCAGCACCATTGTGAAGGAGGGAGCCAACAAGGAGGTGCTGGGAATCCTAGTATCCT
ACAGGGTCAAGGTGAAGCTGGTGGTGTCTCGAGGCGGGGATGTCTCCGTGGAGCTACCTTTCGT
CCTAATGCACCCCAAGCCCCACGACCACATCACCCTTCCCCGACCCCAGTCAGCCCCCCGGGAA
ATAGACATCCCTGTGGATACCAACCTCATTGAATTCGATACCAACTATGCCACAGACGACGACAT
CGTGTTTGAGGACTTTGCGAGGCTTCGGCTGAAGGGGATGAAGGATGACGACTGTGATGACCAG
TTCTGCTAG

GLP1R (*homo sapiens*) NM_002062.3, 1391 bp, 463 aa

ATGGCCGGCGCCCCCGGCCCGCTGCGCCTTGCGCTGCTGCTGCTCGGGATGGTGGGCAGGGC
CGGCCCCCGCCCCCAGGGTGCCACTGTGTCCCTCTGGGAGACGGTGCAGAAATGGCGAGAATA
CCGACGCCAGTGCCAGCGCTCCCTGACTGAGGATCCACCTCCTGCCACAGACTTGTTCTGCAAC
CGGACCTTCGATGAATACGCCTGCTGGCCAGATGGGGAGCCAGGCTCGTTCGTGAATGTCAGCT
GCCCCTGGTACCTGCCCTGGGCCAGCAGTGTGCCGCAGGGCCACGTGTACCGGTTCTGCACAG
CTGAAGGCCTCTGGCTGCAGAAGGACAACTCCAGCCTGCCCTGGAGGGACTTGTCGGAGTGCG
AGGAGTCCAAGCGAGGGGAAAGAAGCTCCCCGGAGGAGCAGCTCCTGTTCCTCTACATCATCTA
CACGGTGGGCTACGCACTCTCCTTCTCTGCTCTGGTTATCGCCTCTGCGATCCTCCTCGGCTTCA
GACACCTGCACTGCACCAGGAACTACATCCACCTGAACCTGTTTGCATCCTTCATCCTGCGAGCA
TTGTCCGTCTTCATCAAGGACGCAGCCCTGAAGTGGATGTATAGCACAGCCGCCCAGCAGCACC
AGTGGGATGGGCTCCTCTCCTACCAGGACTCTCTGAGCTGCCGCCTGGTGTTTCTGCTCATGCA
GTACTGTGTGGCGGCCAATTACTACTGGCTCTTGGTGGAGGGCGTGTACCTGTACACACTGCTG
GCCTTCTCGGTCTTATCTGAGCAATGGATCTTCAGGCTCTACGTGAGCATAGGCTGGGGTGTTCC
CCTGCTGTTTGTTGTCCCCTGGGGCATTGTCAAGTACCTCTATGAGGACGAGGGCTGCTGGACC
AGGAACTCCAACATGAACTACTGGCTCATTATCCGGCTGCCCATTCTCTTTGCCATTGGGGTGAA
CTTCCTCATCTTTGTTCGGGTCATCTGCATCGTGGTATCCAAACTGAAGGCCAATCTCATGTGCAA
GACAGACATCAAATGCAGACTTGCCAAGTCCACGCTGACACTCATCCCCCTGCTGGGGACTCAT
GAGGTCATCTTTGCCTTTGTGATGGACGAGCACGCCCGGGGGACCCTGCGCTTCATCAAGCTGT
TTACAGAGCTCTCCTTCACCTCCTTCCAGGGGCTGATGGTGGCCATATTATACTGCTTTGTCAACA
ATGAGGTCCAGCTGGAATTTCGGAAGAGCTGGGAGCGCTGGCGGCTTGAGCACTTGCACATCCA
GAGGGACAGCAGCATGAAGCCCCTCAAGTGTCCCACCAGCAGCCTGAGCAGTGGAGCCACGGC
GGGCAGCAGCATGTACACAGCCACTTGCCAGGCCTCCTGCAGCTGA

OTR (*homo sapiens*) NM_000916.3, 1169 bp, 389 aa

ATGGAGGGCGCGCTCGCAGCCAACTGGAGCGCCGAGGCAGCCAACGCCAGCGCCGCGCCGCC
GGGGGCCGAGGGCAACCGCACCGCCGGACCCCCGCGGCGCAACGAGGCCCTGGCGCGCGTG
GAGGTGGCGGTGCTGTGTCTCATCCTGCTCCTGGCGCTGAGCGGGAACGCGTGTGTGCTGCTG
GCGCTGCGCACCACACGCCAGAAGCACTCGCGCCTCTTCTTCTTCATGAAGCACCTAAGCATCG
CCGACCTGGTGGTGGCAGTGTTTCAGGTGCTGCCGCAGTTGCTGTGGGACATCACCTTCCGCTT
CTACGGGCCCGACCTGCTGTGCCGCCTGGTCAAGTACTTGCAGGTGGTGGGCATGTTCGCCTCC
ACCTACCTGCTGCTGCTCATGTCCCTGGACCGCTGCCTGGCCATCTGCCAGCCGCTGCGCTCGC
TGCGCCGCCGCACCGACCGCCTGGCAGTGCTCGCCACGTGGCTCGGCTGCCTGGTGGCCAGC
GCGCCGCAGGTGCACATCTTCTCTCTGCGCGAGGTGGCTGACGGCGTCTTCGACTGCTGGGCC
GTCTTCATCCAGCCCTGGGGACCCAAGGCCTACATCACATGGATCACGCTAGCTGTCTACATCGT
GCCGGTCATCGTGCTCGCTGCCTGCTACGGCCTTATCAGCTTCAAGATCTGGCAGAACTTGCGG
CTCAAGACCGCTGCAGCGGCGGCGGCCGAGGCGCCAGAGGGCGCGGCGGCTGGCGATGGGG
GGCGCGTGGCCCTGGCGCGTGTCAGCAGCGTCAAGCTCATCTCCAAGGCCAAGATCCGCACGG
TCAAGATGACTTTCATCATCGTGCTGGCCTTCATCGTGTGCTGGACGCCTTTCTTCTTCGTGCAG
ATGTGGAGCGTCTGGGATGCCAACGCGCCCAAGGAAGCCTCGGCCTTCATCATCGTCATGCTCC
TGGCCAGCCTCAACAGCTGCTGCAACCCCTGGATCTACATGCTGTTCACGGGCCACCTCTTCCA
CGAACTCGTGCAGCGCTTCCTGTGCTGCTCCGCCAGCTACCTGAAGGGCAGACGCCTGGGAGA
GACGAGTGCCAGCAAAAAGAGCAACTCGTCCTCCTTTGTCCTGAGCCATCGCAGCTCCAGCCAG
AGGAGCTGCTCCCAGCCATCCACGGCGTGA

V1AR (*homo sapiens*) NM_000706.4, 1265 bp, 418 aa

ATGCGTCTCTCCGCCGGTCCCGACGCGGGGCCCTCGGGCAACTCCAGCCCATGGTGGCCTCTG
GCCACCGGCGCTGGCAACACAAGCCGGGAGGCCGAAGCCCTCGGGGAGGGCAACGGCCCACC
GAGGGACGTGCGCAACGAGGAGCTGGCCAAACTGGAGATCGCCGTGCTGGCGGTGACTTTCGC
GGTGGCCGTGCTGGGCAACAGCAGCGTACTGCTGGCTCTGCACCGGACGCCGCGCAAGACGTC
CCGCATGCACCTCTTCATCCGACACCTCAGCCTGGCCGACCTGGCCGTGGCATTCTTCCAGGTG
CTGCCGCAAATGTGCTGGGACATCACCTACCGCTTCCGCGGCCCCGACTGGCTGTGCCGCGTG
GTGAAGCACCTGCAGGTGTTCGGCATGTTTGCGTCGGCCTACATGCTGGTAGTCATGACAGCCG
ACCGCTACATCGCGGTGTGCCACCCGCTCAAGACTCTGCAACAGCCCGCGCGCCGCTCGCGCC
TCATGATCGCGGCCGCCTGGGTGCTGAGCTTCGTGCTGAGCACGCCGCAGTACTTCGTCTTCTC
CATGATCGAGGTGAACAATGTCACCAAGGCCCGCGACTGCTGGGCCACCTTCATCCAGCCCTGG
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GTACCTGCTACGGCTTCATCTGCTACAACATCTGGTGCAACGTCCGCGGGAAGACGGCGTCGCG
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TGTCAGCAGCGTGAAGTCCATTTCCCGGGCCAAGATCCGCACGGTGAAGATGACTTTTGTGATC
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CATGTCCGTCTGGACCGAATCGGAAAACCCTACCATCACCATCACTGCATTACTGGGTTCCTTGA
ATAGCTGCTGTAATCCCTGGATATACATGTTTTTTAGTGGCCATCTCCTTCAAGACTGTGTTCAAA
GCTTCCCATGCTGCCAAAACATGAAGGAAAAATTCAACAAAGAAGATACTGACAGTATGAGCAGA
AGACAGACTTTTTATTCTAACAATCGAAGCCCAACAAACAGTACGGGTATGTGGAAGGACTCGCC
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7.3 Plasmid maps

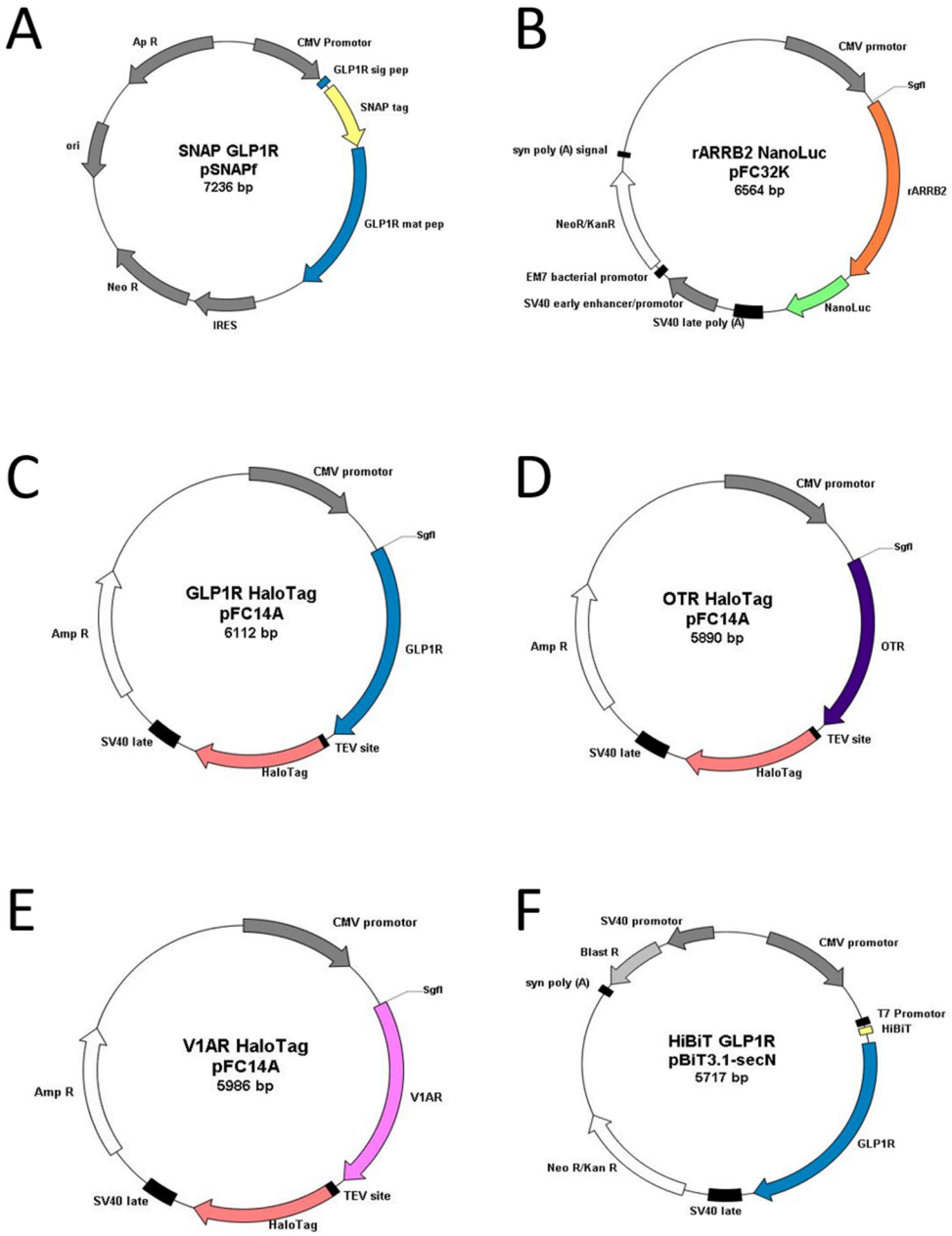

Figure S 1: Overview of all expression vectors that result in a fusion protein. A: SNAP-GLP1R includes the signal peptide in front of the SNAP-tag. The mature peptide follows the tag. B: rARRB2 NanoLuc (C-terminally tagged) was already cloned during my diploma thesis and used for the BRET recruitment assays. C: GLP1 Halotag® (C-terminally tagged), D: V1AR HaloTag® (C-terminally tagged) and E: OTR HaloTag® (C-terminally tagged) were also used for the BRET assays. F: HiBiT-GLP1 was cloned into the pBiT3.1-secN and a 24 aa long linker was kept between the tag and the cDNA of the receptor.

7.4 Establishment of the β-arrestin2 recruitment BRET assay

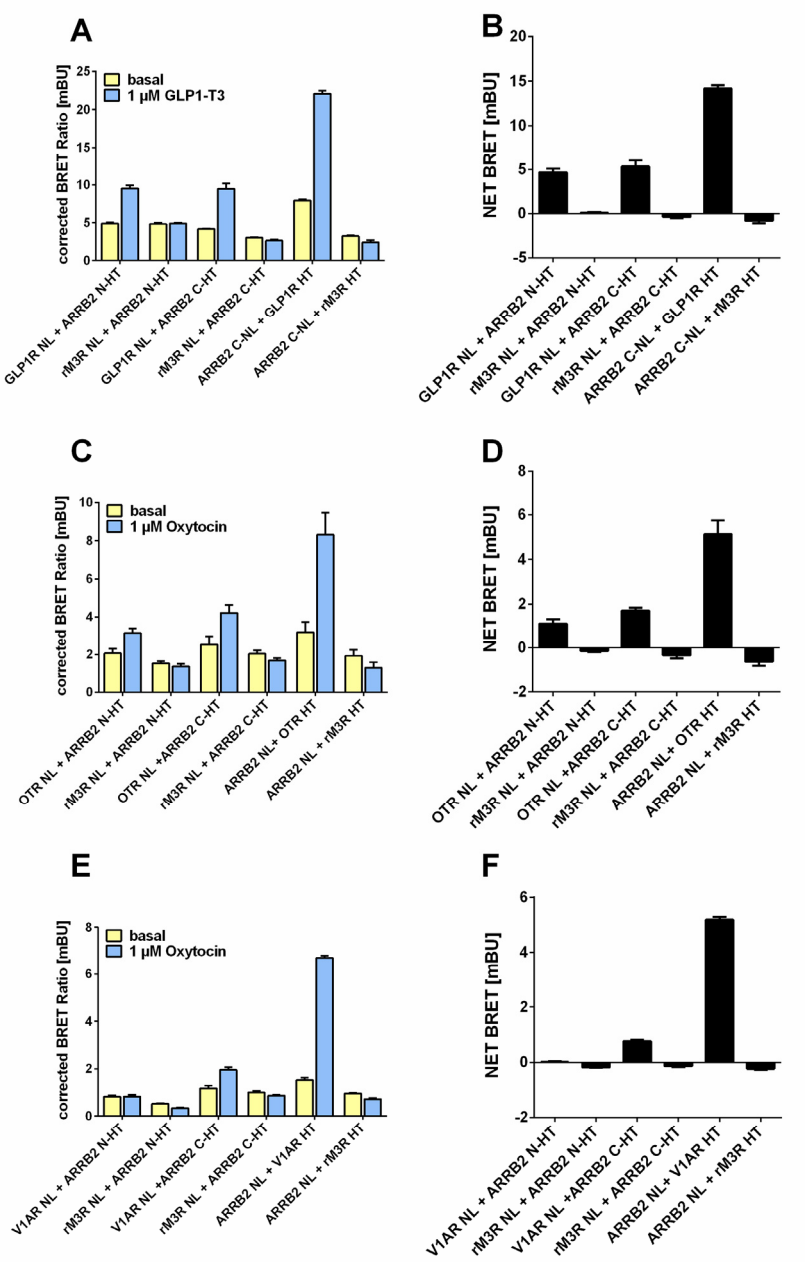

Figure S 2: Establishment for the β-arrestin2 recruitment assay.
For every tested GPCR, the optimal BRET pairing was investigated by preliminary experiments. Here, all available BRET pairings were tested to determine the pairing with the highest ligand promoted BRET change (NET BRET). For all tested receptors, the combination of ARRB2 NL and receptor-HT was showing the highest NET in comparison to the other combinations. The values ranged from 15 mBU for GLP1R (A and B), to 5 mBU for OTR (C and D) and V1AR (E and F). These combinations were then used for concentration-response assays to compare the β-arrestin2 recruitment between peptide and conjugate. Data represent one experiment for GLP1R, two independent experiments for V1AR and three independent experiment, performed in triplicates. Value represent mean ± SEM. NL: NanoLuc, energy donor; HT: HaloTag®, epitope tag that binds the energy acceptor, N-terminal indicated with N-HT and C-terminal indicated with C-HT; mBU: milliBRETUnits

8 Abbreviations

aa	Amino acid
AC	adenylate cyclase
ADHS	Attention-deficit hyperactivity disorder
AHDS	Allan-Herndon-Dudley syndrome
ANOVA	analysis of variance
ASRT	endosomal sorting actin-sorting nexin 27 (SNX)-retromer tubule
ATP	adenosine triphosphate
AVP	arginine vasopressin
BAT	brown adipose tissue
BBB	blood-brain-barrier
BCH	2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid
BLAST	basic local alignment search tool
bp	base pair
BRET	bioluminescence resonance energy transfer
BSA	bovine serum albumin
CALB	calbindin-D28k
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CH	congenital hypothyroidism
CIP	calf intestine phosphatase
CNS	central nervous system
CO_2	carbon dioxide
COS7	*Cercopithecus aethiops* cell line 7
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9
CRYM	µ-crystallin
CSF	Cerebrospinal-fluid
Da	Dalton
DAG	diacylglycerol
DAPI	4′,6-diamidino-2-phenylindole
ddNTP	dideoxynucleotide
DE	Deutschland, Germany
DIO	deiodinase
DIT	diiodothyrosine
DITPA	3,5-diiodothyropropionic acid
DKO	double knock out
DMEM	dulbecco's MEM
DMI	desipramine hydrochloride
DMSO	dimethylsufoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
DR	dopamine receptor
DR4	direct repeat separated by four nucleotides

EC_{50}	Half maximal effective concentration
ECL	extracellular loops
EDTA	Disodium ethylenediaminetetraacetate dihydrate
ELISA	Enzyme-linked immunosorbent assay
ER	estrogen receptor
ER	endoplasmic reticulum
ERK1/2	extracellular signal-regulated kinase 1/2
FBS	fetal bovine serum
FOXE1	forkhead-box protein E1
GAPDH	Glycerinaldehyd-3-phosphat-Dehydrogenase
GCGR	glucagon receptor
GDP	guanine diphosphate
GH	growth hormone
GLP1	Glucagon-like peptide 1
GLP1R	Glucagon-like peptide 1 receptor
GNAS	guanine nucleotide binding protein α subunit
GPCR	G protein coupled receptor
GRK	GPCR kinase
GTP	guanine triphosphate
h	hours
HEK293	human embryonal kidney cell line 293
HPLC	high pressure liquid chromatography
HPT	hypothalamus-pituitary-thyroid
HT	HaloTag®
iBMEC	induced brain microvascular endothelia cell
IBMX	3-Isobutyl-1-methylaxanthine
IC_{50}	Half maximal inhibitory concentration
ICL	intracellular loops
IN	intranasal
IP_3	inositol triphosphate
iPSC	induced pluripotent stem cell
IV	intravenous
KO	knock out
LAT	L-type amino acid transporters
LB	lysogeny broth
LC/MS	Liquid chromatography/mass spectrometry
LDL	low-density lipoprotein
LHR	luteinizing hormone receptor
M	molar
MAPK	mitogen-activated protein kinase
mBU	milliBRET units
MCS	mutiple cloning site
MCT	monocarboxylate transporter
MEM	minimal essential media
MFS	major facilitator superfamily
min	minute
MIT	monoiodotyrosine

MPEG-PLA	methoxy poly(ethylene glycol)-poly(lactic acid)
NCBI	National Center for Biotechnology Information
NEA	non-essential amino acids
NEFL	light neurofilament subunit
NFAT	nuclear factor of activated T-cells
NIS	sodium-iodine-symporter
NKX2.1	NK homeobox 1
NL	NanoLuc
nm	nanometer
OATP	organic anion transporter polypeptide
OT	oxytocin
OTR	oxytocin receptor
PAX8	paired-box-protein 8
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDE	phosphodiesterase
PEG	Poly ethylene glycol
PFA	paraformaldehyde
PI3K	phosphatidylinositol 3-kinase
PIP_2	phosphatidylinositol-4,5 bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA	Poly lactic acid
PLC	protein lipase C
PTHR	parathyroid receptor
PTX	pertussis toxin
PVALB	Ca^{2+}-binding proteins parvalbumin
PVN	paraventricular nucleus
RhoGEF	Rho guanine-nucleotide exchange factors
RLU	relative light unit
RNA	ribonucleic acid
RT	reverse transcriptase
rT3	reverse triiodothyronine
RXR	retinoid X receptor
SLC	solute carrier family
SNX	sorting actin-sorting nexin 27
SON	supraoptic nucleus
SRE	serum response element
STAT	signal transducer and activator of transcription
T2	diiodothyronine
T3	triiodothyronine
T4	thyroxine, 3,3',5,5'-Tetraiod-L-thyronin
TGB	thyroxine-binding globulin
TH	Thyroid hormone
THOX	thyroid oxidase
TMH	transmembrane helices

TPO	thyroperoxidase
TR	thyroid hormone receptor
TRE	thyroid hormone response elements
TRH	thyrotropin-releasing hormone
TRHR	thyrotropin-releasing hormone receptor
Triac	3,5,3'-triiodothyroacetic acid
TSH	thyroid-stimulating hormone
UCP1	uncoupling protein 1
USA	United States of America
V1AR	vasopressin 1A receptor
V1BR	vasopressin 1B receptor
V2R	vasopressin 2 receptor
WAT	white adipose tissue
XPCT	X-linked PEST-containing transporter
β_2AR	β_2 adrenergic receptor
µl	Micro liter
µM	Micro molar

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