Characterization of the neurokinin3- and histamine2-receptors in brown and white adipose tissue

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List of abbreviations

A1AR	a1 adenosine receptor
BA	brown adipocytes
BAT	brown adipose tissue
BMI	body mass index
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CNS	central nervous system
DAG	1,2-diacylglycerine
DNA	deoxyribonucleic acid
Ednra	ET receptor type A
ET-1	endothelin-1
FABP4	fatty acid binding protein 4
FFA	free fatty acids
FR	FR900359
GPCRs	G-protein coupled receptors
HSL	hormone sensitive lipase
H1/2/3/4-receptor	histamine receptor 1/ 2/ 3/ 4
IP3	inositol triphosphate 3
KO	knockout
NE	norepinephrine

NK1/ 3-receptor	neurokinin1-/ 3-receptor
NKB	neurokinin b
NST	non-shivering thermogenesis
OSA	obstructive sleep apnea
PBS	phosphate buffered saline
PNS	peripheral nervous system
PPAR-γ	peroxisome proliferator-activated receptor gamma
P/S	penicillin/ streptomycin
scWAT	subcutaneous white adipose tissue
SNS	sympathetic nervous system
T3	triiodothyronine
TAC3	tachykinin3
TACR3	tachykinin3- receptor
UCP-1	uncoupling protein 1
WA	white adipocyte
WAT	white adipose tissue
WATi	inguinal white adipose tissue

1. Introduction

1.1 Obesity

1.1.1 Definition of obesity

Obesity is an excessive accumulation of fat within the human body, which can be classified by different systems.

A common way to do so is to use the body mass index (BMI) which is recommended by the World Health Organization (WHO).

$$\text{Body mass index} = \text{Weight [kg]} / \text{Height*Height [m}^2\text{]}$$

Tab. 1: Classification of BMI grades

Definition		**BMI**
Underweight		< 18.50
Normal range		18.50-24.99
Overweight		25.00-29.99
Obese	Class 1	30.00-34.99
	Class 2	35.00-39.99
	Class 3	> 40.00

According to the WHO, the BMI is the most useful population-level measure of overweight and obesity as it is the same for both sexes and for all ages of adults (WHO 2017). Nevertheless, the index has its weaknesses as it does not take different body types nor muscle mass into account.

To achieve a greater accuracy at infancy, a method is used which focuses on standard deviations.

Tab. 2: Standard deviations as a means to classify obesity

Children under age five:

Overweight	> 2 standard deviations above median
Obese	> 3 standard deviations above median

Children aged between 5-19 years:

Overweight	> 1 standard deviations above median
Obese	> 2 standard deviations above median

(WHO 2017)

The waist circumference risk threshold integrates the different risks associated with different fat depots, depending on the distribution within the human body and takes the ethnical background into account. This method matches the absolute waist circumference with the ethnical background and discriminates that the same waist circumference value can mean that someone is considered to be of normal weight or overweight depending on the given ethnical background. Intra-abdominal visceral fat is associated with a higher risk for cardiovascular diseases, metabolic syndrome, type 2 diabetes and overall mortality than subcutaneous fat (Després and Lemieux 2006; Lee et al. 2017). This threshold can be used to discriminate elevated cardiovascular risks.

Tab.3: Classification of obesity by the specific waist circumference threshold

	White women	AA women	White men	AA men
Normal weight	72.1 cm	76.2 cm	82.1 cm	78.3 cm
Overweight	86.6 cm	85.3 cm	94.5 cm	91.8 cm
Obese I	96.8 cm	96.6 cm	107.0 cm	104.3 cm
Obese II	110.6 cm	109.9 cm	120.1 cm	118.8 cm

Obese I: BMI >30 kg/m^2

Obese II: BMI >35 kg/m^2

AA: African American (Staiano et al. 2013)

1.1.2 Epidemiology of obesity

Presently, more people worldwide suffer from obesity than being underweight and overweight and obesity cause more deaths than underweight.

Since the 1980s the incidence of obesity has doubled and in total numbers more than 1.9 billion adults were overweight with a portion of over 600 million of them being obese (WHO 2017).

In 2013, the prevalence of adults showing a BMI greater than 25 was about 36,9 % for males and 38,0 % for females. The developed countries are still on the forefront of obesity with adults being highly likely to suffer from it. Comparing both sexes in developed countries, obesity and overweight are more prevalent in men than in women with approximated numbers of 58,0 % to 47,0 % (Ng et al. 2014).

In concordance with developed countries, obesity has also substantially grown in developing countries while illustrating a different sex pattern as women have a higher percentage of overweight or obese individuals than men. The data show figures of an estimated 33,0 % for women in comparison with 29,5 % for men (Ng et al. 2014).

Furthermore, the prevalence of obesity and overweight has significantly risen in infancy and adolescent age likewise. Since 1980, the numbers have gradually increased from 16,9 % for boys and 16,2 % for girls to 23,8 % and 22,6 %, respectively (Ng et al. 2014).

1.1.3 Pathophysiology of obesity

The probability of suffering from obesity is linked to various factors.

A chronic imbalance between calorie consumption and expenditure leads to an increased fat mass within the human body (Muller and Geisler 2017). Unlimited amount of food and a sedentary lifestyle contribute to this mismatch (Jebb and Moore 1999). Energy expenditure defines the calories dissipated by the human body daily. A variety of different organs contribute to whole energy expenditure and each one of them has a very specific metabolic rate. There are four main systems which dictate whole energy expenditure: the nervous system, the metabolic system, the musculoskeletal system and adipose tissue mass. The metabolic system compromises different organs like the respiratory, the circulatory, the urinary and the digestive organs (Heymsfield et al. 2018).

There are strong genetical influences which also define body weight. It could be illustrated that adopted children have a BMI in conformity with their biological parents but not with their adoptive ones. Especially monozygotic children being raised in different families showed great similarity regarding their BMI (Stunkard et al. 1986).

Furthermore, maternal obesity and weight gain during pregnancy is associated with higher risks for perinatal obesity (Starling et al. 2015). In conclusion, genetical and behavioral effects contribute to familial predisposition which is an important predictor for child obesity and its conduction to adulthood (Nielsen et al. 2015).

Besides genetical aspects and a chronic energy imbalance, the choice and composition of food plays a major role in the formation of obesity.

The energy density and subsequently the glycemic load vary comparing different kinds of food. High density food which is commonly seen in processed and industrialized goods causes elevated levels of insulin. High levels of insulin disturb the breakdown of fatty acids and lead to fat storage instead. Therefore eating habits, especially regarding high density food are a major contributor to the development of obesity (Camacho and Ruppel 2017).

1.1.4 Obesity associated diseases

There are various negative health consequences associated with obesity. Obesity is considered to have caused 3,4 million deaths worldwide in 2010. Moreover, it was estimated that a total of 3.9% years of life are lost due to obesity and 3,8 % of disability adjusted life years (DALYs) gained worldwide (Ng et al. 2014).

Obesity affects multiple organ systems within the human body. It increases the risk for cardiovascular disease, hypertension, type 2 diabetes mellitus (type 2 dm) and stroke as well as the overall mortality (Williams et al. 2015).

Cancer entities which are strongly linked to the prevalence of obesity, are bladder, colorectal, pancreatic and prostatic cancer. Moreover, they have been analyzed to have a worse outcome in comparison to their outcome in non-obese patients (Garg et al. 2014).

Obstructive sleep apnea (OSA) is a major driving force for exacerbation of type 2 diabetes, hypertension, obesity and atherosclerosis (Dharia and Brown 2017). Alarmingly, obesity has proven to be the strongest risk factor for the development of the OSA, so that the bidirectional relationship between OSA and obesity can be regarded as a vicious circle (Hamilton and Joosten 2017).

Furthermore, obesity is a major risk factor for osteoarthritis due to chronic enhanced pressure on joints which are exposed to weight-bearing. Interestingly, increased local inflammation caused by obesity adds to the etiological factors also in joints not prone to carrying weight (Bliddal et al. 2014; Sun et al. 2017).

In addition, there is a significant elevation of risk to suffer from autoimmune diseases like systemic lupus erythematosus comparing obese and normal weight women (Tedeschi et al. 2017).

Obesity further negatively alters the male spermatogenesis and sperm function causing abnormal genetic modifications which can eventually lead to male infertility (Liu and Ding 2017).

1.2 Adipose tissues

The human body is composed of different adipose tissues which will be presented and illustrated in the following sections.

1.2.1 Brown adipose tissue

Brown adipose tissue (BAT) is a unique tissue within the human body as it is able to uncouple the respiratory chain and create heat without shivering. This process is known as non-shivering thermogenesis (NST) and is based on the uncoupling protein one (UCP-1) which belongs to a family of mitochondrial carriers located at the inner membrane of the mitochondrion. UCP-1 mediates a proton-leak disrupting the formation of adenosine triphosphate and thus creating heat (Klingenspor et al. 2008).

BAT can be found in the interscapular area as well as in the mediastinum, renal and pararenal regions and in para-aortic and neck fat depots (Heaton 1972). Brown adipocytes contain many mitochondria and small lipid droplets which enhances their efficiency in creating heat. In addition, they are richly vascularized with up to five capillaries surrounding one brown adipocyte which in synergy with their high mitochondria content promotes their brownish appearance (Robidoux et al. 2004). BAT mass and activity greatly depends on age and sex as women and younger people have a higher BAT mass and an elevated activity of BAT (Lee et al. 2013, Pfannenberg et al. 2010).

BAT has long been regarded to be extinct in adulthood due to its gradual decline, especially in the more superficial areas like the interscapular region (Heaton 1972). Therefore, it was concluded that the physiological role was limited to non-shivering thermogenesis in children. In 2009, however, it could be shown in PET/ computer tomography studies (see figure 1) that there is a cold-induced uptake of radioactively

labeled glucose [18-FDG] in brown adipose tissue in human adults (Saito et al. 2009, van Marken Lichtenbelt et al. 2009; Virtanen et al. 2009)

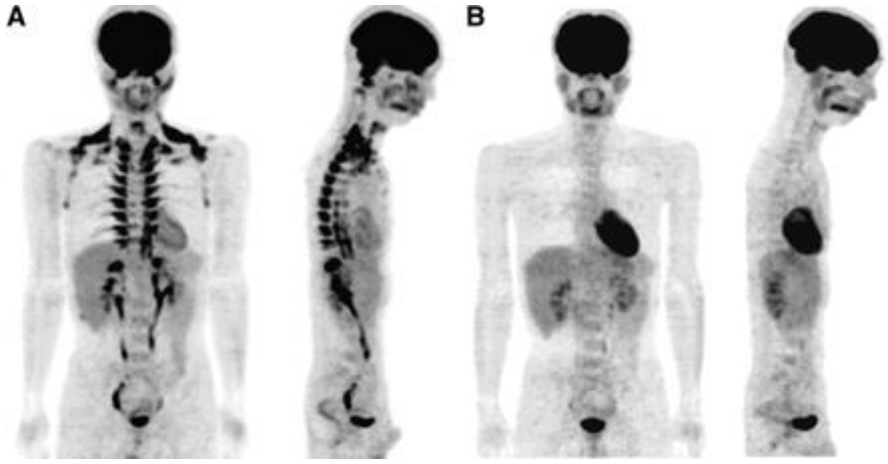

Fig. 1: Whole body FDG-PET of a 25 year old male subject, the image is reproduced from (Saito et al. 2009). [A] A male subject was stimulated to cold at 19 °C while only wearing light clothes. Additionally, the legs were intermittently rested on an ice block. ^{18}F-FDG was injected intravenously after 1 hour of cold stimulation and PET/ CT-scans were performed after 2 hours. The dark areas indicate uptake of radioactively labeled glucose [18-FDG] in brown adipose tissue in cervical, mediastinal, supraclavicular, paravertebral, and supra-/perirenal regions [B] The same patient underwent equal PET/CT-examinations 2 weeks later, but he was exposed to 27 °C with standard clothing and legs were not rested on an ice block. Note the diminished number of dark areas corresponding with a smaller glucose uptake in BAT in comparison to A.

The contribution of BAT to daily energy expenditure is significant as a mere 50 g of fully stimulated BAT could account for up to 20 % of daily energy consumption in human adults (Klingenspor et al. 2012). It has been estimated that activated BAT could salvage as much as an equivalent of 4.1 kg of pure white adipose tissue in the course of a year (Virtanen et al. 2009). In mice exposed to cold, BAT consumed up to 50 % of dietary lipids and glucose which highlights the potential BAT could exert in the prevention of the metabolic disease (Bartelt et al. 2011; Bartelt and Heeren 2012).

The sympathetic nervous system (SNS) plays a key role in the activation of BAT and ß3-adrenoceptor is the most abundantly expressed adrenoceptor in BA (Cannon and Nedergaard 2004). Activation of the G_s-coupled ß3-adrenoceptor leads to an intracellular increase in cyclic adenosine monophosphate (cAMP) and subsequently the activation of the proteinkinase A (PKA). Next, the proteinkinase A phosphorylates the hormone-

sensitive lipase (HSL) which liberates free fatty acids (FFA) from triglycerides. FFA act as a strong activator of the UCP-1 protein. Furthermore, PKA phosphorylates various cytosolic and nuclear proteins- e.g. the transcription factor CREB which promotes the transcription of various genes including UCP-1 (Cannon and Nedergaard 2004). Prolonged exposure to cold stimulates an elevated transcription rate of thermogenic genes in addition to the activation and proliferation of adipose precursor cells (Bronnikov et al. 1992; Cao et al. 2004). Conversely, it was shown that lipolysis is not essential for NST in mice (Shin et al. 2017) and cold-induced thermogenesis depends on adipose triglyceride lipase mediated lipolysis in cardiac muscle but not in BAT (Schreiber et al. 2017).

1.2.2 White adipose tissue

White adipose tissue (WAT) is functionally and morphologically distinct to its counterpart- the brown adipose tissue.

White adipocytes (WA) are composed of a single fat droplet which contains large amounts of triglycerides in the cytoplasm of the cell and WAT is the principal site for energy storage in humans. In case of energy demand, the energy can be liberated by lipolysis and ß-oxidation. WAT is classified by localization as either subcutaneous or visceral (Guerre-Millo 2002).

In recent years, the research focusing on WAT has brought a clearer understanding of additional functions as its role is not exclusively limited to energy storage. In fact, WAT secretes a variety of hormones such as leptin, adiponectin, multiple cytokines and steroid hormones so that it can be regarded as a major endocrine organ (Guerre-Millo 2002). In obesity, this function of WAT is severely dysbalanced and contributes to chronic low grade inflammation levels which in turn lead to metabolic dysfunction and obesity linked disorders (Ouchi et al. 2011). High levels of leptin are found in obese patients and correlate with the BMI and total body fat. Elevated leptin serum levels are associated with type 2 diabetes, atherosclerosis and further obesity-associated conditions and leptin may play a crucial role in the formation and progression of breast cancer. Alarmingly, it could be demonstrated that leptin promotes the growth of breast cancer cells in vitro. Contrary to leptin, circulating adiponectin secreted by WAT correlates negatively with WAT mass

and has opposing effects to leptin. High levels of adiponectin are associated with beneficial health conditions like diminished triglyceride levels and enhanced insulin sensitivity as well as a lower probability to develop type 2 diabetes. Interestingly, adiponectin inhibits breast cancer cell proliferation in vitro by blocking the normal response upon growth factor stimulation and by fostering apoptosis rate (Cleary et al. 2010; Li et al. 2009).

The immune system is closely linked to the state of homeostasis and obesity severely unhinges that susceptible equilibrium. The molecular mechanisms underlying this relationship are complex. Obesity causes an elevated expression of pro-inflammatory mediators in adipose tissue and stimulates immune cells resident in adipose tissue to convert to a pro-inflammatory phenotype (Hotamisligil 2017). TNF-α is a driving force in inflammatory and autoimmune diseases and plays a major role in obesity linked insulin resistance (Ouchi et al. 2011).

1.2.3 Beige adipocytes

Beige adipocytes share many features with classical brown adipocytes like a multilocular appearance, an overlapping expression of thermogenic and adipogenic genes e.g., UCP-1 as well as a similar content of mitochondria but reside within WAT (Rosen and Spiegelman 2014).

Beige and brown adipocytes distinguish themselves through differing expression levels of marker genes and a different cellular origin (Hepler et al. 2017, Waldén et al. 2012). The term browning refers to the appearance of UCP-1 positive beige adipocytes in WAT which can be induced by various stimuli (Rosen and Spiegelman 2014). Treatment with hormones like triiodothyronine (T3), atrial and ventricular natriuretic peptides, the skeletal muscle hormone irisin and the phosphodiesterase-inhibitor sildenafil as well as the pharmacological stimulation of the adenosine A2A-receptor are potent inducers of beige cell formation (Gnad et a. 2014; Mitschke et al. 2013; Rosen and Spiegelman 2014). Moreover, cold response is a strong physiological activator of browning (Chechi et al. 2013).

Depending on the specific localization of fat depots, the abundance of beige cells differ significantly as inguinal and retroperitoneal fat exhibit the highest numbers and perigonadal fat the lowest (Rosen and Spiegelman 2014). Additionally, various white fat depots display a significantly different capacity for browning. Subcutaneous and inguinal fat depots are more prone for browning than epididymal fat depots (Seale et al. 2011). Interestingly, the PRDM16 protein is essential for browning of white fat as ablation of the PRDM16 protein leads to a significant decline in thermogenic gene expression and O_2-consumption in white fat depots whereas brown adipocyte function is unaltered by the ablation (Cohen et al. 2014).

1.2.4 GPCR signaling in adipose tissue

G-protein-coupled receptors (GPCRs) represent one of the largest groups of membrane bound receptors. In addition to significant regulation in various physiological processes, they exhibit an important role in modulating adipose tissue (Wess 1997).

Each GPCR couples to a family of heterotrimeric G-proteins which function as intracellular transducers and interact with a different signaling pathway. The heterotrimeric G-proteins are composed of a Gα, Gβ and Gγ- subunit and activation induces the dissociation of the Gα-subunit from the Gβγ- complex which allows the binding of further signaling molecules (Neves et al. 2002). The Gαs-, the Gαi and the Gαq-subtypes are some of the most well characterized Gα-proteins. They exert their effects through different signaling pathways (Mitchell 2013). Stimulation of the Gαs and Gαi- subunit elevates or diminishes intracellular cAMP-levels, respectively. The Gαq-protein activates the phospholipase C which hydrolyzes phosphatidylinositol phosphate to generate inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Neves et al. 2002). IP_3 and DAG confer and amplify the Gαq-mediated signal. IP_3 activates its IP_3-receptor on the endoplasmic reticulum which causes an influx of Ca^{2+} to the cytoplasm. DAG in turn activates the proteinkinase C. Stimulation of the proteinkinase C and the Ca^{2+}-influx propagate further cellular events (Mizuno and Itoh 2009).

Well studied Gαs-coupled receptors in BA are the ß$_3$-adrenergic receptor and the adenosine A2A receptor (Cannon and Nedergaard 2004, Gnad et al. 2014). As previously illustrated, activation of the ß$_3$-adrenergic receptor leads to the activation of BA as well as

to an elevated transcription rate of thermogenic genes like UCP-1 (Cannon and Nedergaard 2004). Moreover, stimulation of the A2A-receptor induces an increase in energy expenditure as well as a rise of beige adipocytes in WAT. Hence, Gαs-signaling is a key regulator of adipose tissue activity and is mandatory for an unimpaired adipogenesis. This is highlighted by the fact that adipose-specific Gαs deficient mice developed almost no adipose tissue (Chen et al. 2010).

Gαi signaling inhibits the adenylate cyclase and therefore lowers cAMP levels. Overexpression of the A1 adenosine receptor (A1AR) which couples to the Gαi molecule in mice leads to fewer FFA levels and resistance to high fat diet induced insulin sensitivity impairment (Dong et al. 2001). Furthermore, activation of the A1AR increases lipogenesis, lipid accumulation and blocks lipolysis (Eisenstein and Ravid 2014).

In contrast to Gαs signaling, constitutive Gαq signaling is a negative regulator of adipose tissue differentiation. One of the best studied Gαq-coupled receptors in BAT is the endothelin receptor type A (Ednra) (Klepac et al. 2016). Activation of the Ednra with the endogenous ligand ET-1 inhibits the differentiation of BA which is illustrated by a diminished expression of adipogenic and thermogenic markers like PPAR-γ and UCP-1. Accordingly, inhibition of Gαq signaling elevates differentiation of murine brown adipocytes underlined by increased UCP-1 expression and adipogenic markers like aP2 and PPAR-γ (Klepac et al. 2016). Furthermore, constitutive Gαq- signaling *in vivo* in transgenic mice induces WAT-like appearance of BAT. Interestingly, the expression level of Gαq in human WAT displays an inverse UCP-1 correlation. Thus, inhibition of the Gαq signaling pathway could be a potential pharmacological approach to combat obesity (Klepac et al. 2016). Although the Gαq signaling pathway greatly influences the differentiation of BA, only a few receptors causing this negative regulation in BA have been identified. Strikingly, the influence of the Gαq-signaling pathway does not only play a significant role in the regulation of BA differentiation but interaction with the Gαq-signaling pathway in hepatocytes can improve glucose tolerance and diminish HbA1c levels (Zhou et al. 2017).

1.3 Overview of therapeutic treatment options

There are a variety of different approaches to combat obesity that have been focusing on conservative life style interventions. Despite this efforts to combat obesity weight loss is hard to accomplish and even harder to maintain (MacLean et al. 2015). The following sections will give an overview of established strategies for weight management with the associated benefits and side effects.

Conservative therapy

A comprehensive approach towards tackling obesity is essential to achieve a long-term weight management control. Thus, it is necessary to provide the patient with a diversified plan to combat obesity. Weight loss goals should be reasonable and achievable because success encourages the adherence to the therapy. Otherwise, the probability of not following the diet guidelines and falling back into old behavior patterns will be greater.

Weight loss is based on restricting calorie intake in combination with physical exercise and behavioral changes. Exercising alone is not an effective means as weight loss will only be around 0.1 kg per week. Nevertheless, exercising is essential to accomplish weight maintenance (Fock and Khoo 2013).

There are different kinds of weight loss diets which are distinguished by the composition of the macronutrients and the magnitude of the caloric restriction. They can be classified in low calorie/ low fat/ low carbohydrate regimens with a targeted calorie deficit of around 500 kcal/ day. In addition, a very low calorie diet exists with an intake of around 200-800 kcal/ day; but medical supervision is advised due to the potential major side effects (Fock and Khoo 2013). Despite the diversity of diets, the adherence to the dietary regimen plays a more significant role than the composition of the diet itself (Sacks et al. 2009).

A moderate loss of 5-10 % of body weight is regarded as a success as it is sufficient to diminish the risk to development type 2 diabetes and further health impairments associated with obesity (Montesi et al. 2016). In general, a mean weight reduction of 5-8.5 kg is obtained in the first 6 months after lifestyle interventions. After 48 months, an average loss of 3-6 kg can be observed (Franz et al. 2007).

Conservative therapy alone does not provide long lasting effects in a majority of cases. Diet induced weight loss is hard to maintain. An average of 1-2 kg per year is regained after dieting, with numbers greater initially (MacLean et al. 2015). The phenomena of cycles of losing weight and consequently regaining the same amount of weight or even more is the so called “yoyo-effect” (Chilloux and Dumas 2017). People often repeatedly undergo those cycles of dieting and regaining weight without achieving long term weight control. The reasons underlying this crux are diverse as social, behavioral and physiological factors play a fundamental role (MacLean et al. 2015). In addition, whole body energy expenditure is reduced during low-caloric diets (Müller et al. 2016). A study reported numbers as low as only 3 % of people who were able to maintain their weight loss after 4-5 years after their initial diet program (Kramer et al. 1989). Therefore, it is necessary to take surgical and pharmacological interventions into consideration when conservative management of obesity fails (Montesi et al. 2016).

Surgical therapy

Surgical interventions (i.e., bariatric surgery) must be carefully evaluated with regards to their benefits, but also to intra- and postoperative complications. In general, they should be exclusively performed after failure of conservative therapy.

Patients with a BMI > 35 kg/m^2 plus at least one comorbidity associated with obesity or patients with a BMI > 40 kg/m^2 are considered for surgical therapy. There are different approaches but all surgical operations share a common feature in modifying the stomach.

The laparoscopic gastric bypass surgery is the gold standard. It is a combination of a restrictive and malabsorptive operation. Further interventions are the sleeve gastrectomy and the laparoscopic insertion of a modifiable gastric band. In addition to weight loss, in a majority of cases obesity accompanying diseases like type 2 diabetes can be diminished (Williams 2012).

Although significant weight loss can be achieved, surgical interventions are not applicable for every patient. Diseases like COPD and heart failure can inhibit the possibility of surgery as patients cannot be administered general anesthesia without potential risk of life. Furthermore, every operation bears the risk of intra- and postoperative complications like bleeding, insufficiency of anastomoses and death.

Pharmacological therapy

The pharmacological therapy can be a valuable method to foster weight loss as an adjunct to conservative therapy. It can enhance the adherence to dietary and behavioral changes.

Pharmacological therapy is applicable for patients with a BMI > 27 kg/ m^2 (Apovian et al. 2015).

The German society for obesity (DAG) recommends orlistat/ Xenical® as the sole drug for the treatment of obesity. Orlistat works as a pancreatic and gastric lipase inhibitor and reduces the uptake of dietary fatty acids. It is approved for long-term weight management and moderate weight loss can be obtained (Apovian et al. 2015). However, meta-analyses show only minor improvements after intake of orlistat as compared to placebo-groups with regard to weight loss. Overall, orlistat performs inferiorly than other options like surgical interventions or comprehensive conservative approaches. Furthermore, there are some adverse side effects like flatulence, diarrhea, fecal urgency and incontinence which can greatly reduce the adherence to the regular intake of that drug. Importantly, some more severe adverse effects like acute liver failure have been linked to the intake of orlistat but no clear causality could be established so far (Halpern and Halpern 2015).

In contrast to Germany and the European Union (EU), the American Society for Obesity recommends a variety of drugs which exert their effects through different pharmacological mechanisms. Sympathomimetic drugs like phentermine/ Adipex-P® and diethylpropion/ Tenuate® increase the release of noradrenaline and moderate weight loss can be accomplished (Apovian et al. 2015). In combination with topiramate/ Qsymia® which modulates the GABA-receptor, phentermine can achieve greater weight loss and can be used for chronic treatment. Cautious usage of sympathomimetic drugs is advised though, due to their contraindications in case of hypertension and heart diseases (Apovian et al. 2015). The admission for some of the sympathomimetic drugs like phentermine has been withdrawn in the EU due to an unfavorable benefit to side effect ratio (Deutsche Adipositas Gesellschaft (DAG) e.V. 2014, Kang and Park 2012). Although a few sympathomimetic drugs are still admitted in the EU, the use is not recommended because of significant side effects like psychological dependency and central and cardiovascular adverse effects.

Their general application time is limited to brief periods ranging from four to up to 12 weeks (Apovian et al. 2015).

New pharmaceuticals admitted in the EU in 2015 are the combination of bupropion and naltrexone/ Mysimba® and the human incretin derivate liraglutide/ Saxenda® or Durolane®. Bupropion is a dopamine and norepinephrine reuptake inhibitor and naltrexone is an opioid receptor antagonist. Their combined effect on weight loss is not fully understood (Kang and Park 2012). Nonetheless, bupropion/ naltrexone as well as liraglutide induce moderate weight loss and long-term safety data are available. Besides holding various downsides, liraglutide must be injected in addition to being cost inefficient (Apovian et al. 2015).

Experimental trials tested Mirabegron/ Betmiga®, a $ß_3$-agonist, which is commonly used for treatment of the overactive bladder, as a potential stimulator of BAT activity. A significant rise of the resting metabolic rate could be obtained, but a four times higher dose than the currently approved one was selected (Cypess et al. 2015). Although the dose was well tolerated, the broad administration of a sympathomimetic drug should be handled carefully due to the aforementioned side effects.

In summary, a majority of the drugs approved to this day solely facilitate light to moderate weight loss, have side effects or a specific group of patients are excluded from admission. In addition, their applications often do not have lasting results as rebound effects can be observed in a lot of cases after withdrawal of usage. To combat obesity, long-term safety and efficacy has to be achieved and therefore, the new approval of drugs is handled in a strict manner (Kang and Park 2012). Thus, the development of safe and efficient novel pharmaceuticals is of great significance.

1.4 Histamine and neurokinin receptors

Preliminary data (see figure 4 and 21) which were obtained at the institute of pharmacology and toxicology suggested a significant regulation of the neurokinin 3- and histamine 2-receptor expression during differentiation of brown adipocytes. To this date, there was little information about their role in differentiation and function of BA and WA and investigation of both receptors was an appealing research topic. Thus, the following

sections contain detailed information about the receptors and their respective receptor families which were specifically looked at in this doctoral thesis.

1.4.1 Neurokinin receptor family

The neurokinin 3 receptor (NK3) is a 7-transmembrane domain receptor which belongs to the family of neurokinin receptors. So far there are three known human neurokinin receptors (Severini et al. 2002).

The endogenous ligands are a group of evolutionary highly conserved peptides which are called neurokinin b (NKB), substance p and neurokinin a. Neurokinin b (synonym: tachykinin) shows the highest affinity to the neurokinin 3 receptor. Nevertheless, all endogenous ligands can bind to all neurokinin receptors with varying affinities. Interaction between the neurokinin receptors and their inherent ligands leads to the activation of the Gαq-signaling pathway and the subsequent rise of inositol trisphosphate (IP3) and diacylglycerol (DAG) (Khawaja and Rogers 1996).

The neurokinin receptor family is widely distributed both centrally and peripherally. In the central nervous system (CNS), the neurokinin 3 receptor can be found in the cerebral cortex, the nucleus tractus solitarii and the dorsal root of the spinal cord. Substance p is the predominant ligand of the CNS where it modulates the central control of respiration (Khawaja and Rogers 1996).

Within the peripheral areas of the body, neurokinins play a role in pro-inflammatory responses like cigarette smoke-induced plasma exsudation or bronchoconstriction (Khawaja and Rogers 1996).

Mutation of *TAC3* and the *TACR3*, the corresponding gene names for the NKB and the NK3- receptor, respectively, lead to a rare genetic condition, the normosmic idiopathic hypogonadotrophic hypogonadism. Thus, it became apparent that neurokinin b signaling plays a major role in the onset of puberty (Topaloglu et al. 2010).

The neurokinin receptor family also has clinical implications as aprepitant/ Emend® and fosaprepitant/ Ivemend®, two neurokinin 1 (NK1) receptor antagonists, are in use for treatment of chemotherapy-induced nausea and vomiting. Aprepitant/ Emend® can be applied orally, whereas fosaprepitant/ Ivemend ® is the prodrug of aprepitant/ Emend® and

can only be administered intravenously. Due to the efficacy and safety of the NK1-receptor antagonists, they were added to the clinical practice guidelines in the therapy of chemotherapy-induced nausea and vomiting alongside the tested combination of the 5-hydroxytryptamine-receptor antagonist ondansetron/ Zofran® with dexamethasone/ Fortecortin® (Aapro et al. 2015).

So far, little is known about the physiological role of neurokinins and their corresponding receptors in adipose tissue.

1.4.2 Histamine receptor family

The histamine receptor family is a group of G-protein coupled, 7- transmembrane domain receptors composed of four receptors (H1-H4 receptor). The endogenous ligand histamine can be found throughout the body where it acts as a neurotransmitter and tissue hormone. It plays a central role in regulating allergic reactions and in the modulation of the immune system (O'Mahony et al. 2011). These four receptors exhibit different expression patterns as well as distinctive functions (Nieto-Alamilla et al. 2016). Recently, it was demonstrated that all four receptor subtypes are expressed in BAT as well as in subcutaneous and gonodal WAT (Zhao et al. 2019).

The H1- receptor can be found in a wide diversity of cells like hepatocytes, immune cells, vascular smooth muscle cells and neurons (O'Mahony et al. 2011). The major signaling pathway of the H1-receptor is the interaction with the Gαq/11-protein (Panula et al. 2015). Upon extracellular stimulation, the H1-receptor exerts the classical immediate hypersensitivity response which results in multiple pathological processes throughout the body like allergic rhinitis, asthma and severe anaphylaxis. The H1-receptor modulates many functions in the CNS like food and water intake or sleep regulation (O'Mahony et al. 2011). Moreover, H1-signaling protects against the advancement of atherosclerotic plaques in an *in vivo* model prone to atherosclerotic formation due to hyperlipidemia (Yamada et al. 2016). Regarding metabolism, H1-receptor knockout (KO) mice display increased visceral adiposity and a mildly elevated insulin resistance (Wang et al. 2010). Overall, the H1-receptor has a significant role in the appearance of metabolic syndrome-associated diseases (Yamada et al. 2016).

The H2-receptor which this study primarily focuses on is expressed on a broad range of cells like gastric parietal cells, cardiac cells and neurons. It is in charge of important immunoregulatory modifications like mast cell degranulation and T-cell proliferation (O'Mahony et al. 2011). Interestingly, histamine released by mast cells promotes beiging of subcutaneous WAT with increased expression of UCP-1 mRNA and it plays a pivotal role in the upregulation of UCP-1 mRNA expression during winter time (Finlin et al. 2017). Like the H1-receptor, the H2-receptor is responsible in the regulation of metabolic syndrome-associated diseases (Wang et al. 2010; Yamada et al. 2016). H2-receptor KO mice fed with a cholic acid (CA) diet showed a severe non-alcoholic fatty liver disease (NAFDL) phenotype as well as enhanced insulin resistance (Yamada et al. 2016). Furthermore, ranitidine/ Zantac®, a selective antagonist of the H_2-receptor, is in clinical use for stress ulcer prophylaxis in critically ill patients as it inhibits the gastric acid secretion (Alhazzani et al. 2013). In the majority of cells, the H2-receptor couples to the Gαs-protein. Nevertheless, it has been demonstrated that the H2-receptor also interacts with the Gαq-protein (Kühn et a. 1996, Panula et al. 2015). Our laboratory conducted a profile screen of 347 GPCRs expressed in brown preadipocytes and mature brown adipocytes. The H2-receptor was significantly regulated throughout differentiation in this screen and was labeled to be Gαq-coupled in brown adipocytes according to the IUPHAR database (http://www.guidetopharmacology.org/) (Klepac et al. 2016; Sharman et al. 2013).

The structurally similar H3- and H4- receptors are the most recently discovered receptor subtypes and activate the Gαi/o-protein (Nieto-Alamilla et al. 2016). The H3-receptor distinguishes itself as it is almost exclusively expressed in the nervous system whereas the H-receptor can be mainly found on immune cells (Nieto-Alamilla et al. 2016). The H3-receptor alters the transmission of a variety of neural transmitters on presynaptic and postsynaptic levels and H3-receptor deficient mice suffer from severe neuroinflammatory diseases (Nieto-Alamilla et al. 2016; O'Mahony et al. 2011). The H4- receptor has an important role similar to the aforementioned H1- and H2- receptors in the immune response of diseases like asthma and colitis and H4-receptor- antagonists are potential therapeutics for the treatment of atopic dermatitis and psoriasis (O'Mahony et al. 2011, Schaper-Gerhardt et al. 2018). In subcutaneous white adipose tissue (scWAT), stimulation of the H4- receptor triggers browning of WAT and leads to accelerated

metabolic rates. Conversely, H4-receptor knockdown ablates cold-induced browning of scWAT and diminishes lipolysis effects triggered by cold (Zhao et al. 2019).

However, to this date there is little knowledge about the physiological role of the H1-H3-receptor in brown and white adipocyte differentiation and regulation.

1.5 Adipogenic and thermogenic differentiation markers

Differentiation markers are important proteins which are regulated during differentiation of cells and help to determine the specific cell subtype or maturity of cells. In the following section, the adipogenic and thermogenic differentiation markers which were chosen to be specifically examined in the consecutive experiments will be briefly presented.

1.5.1 aP2

aP2 or FABP4 is a cytosolic chaperone protein of the fatty acid binding protein (FABP) family displaying various intracellular and extracellular functions. aP2 is capable of binding to unsaturated and saturated fatty acids, thus regulating lipid trafficking within the cell. In addition to transportation functions, aP2 plays a more complex role in metabolic and inflammatory processes. For instance, aP2 regulates macrophage redox signaling and the activation of the inflammasome in macrophages and is a key player in the linkage of obesity and inflammation in general (Steen et al. 2017).

It is significantly upregulated during differentiation of preadipocytes into adipocytes and is one of the most abundantly expressed proteins in mature adipocytes (Spiegelman and Green 1980).

FABP4-deficient mice showed greater resistance to the formation of atherosclerotic plaques and affliction to metabolic syndrome (Makowski and Hotamisligil 2005). For a long time, research was solely focused on its intracellular functions, but it became apparent that FABP4 additionally undergoes regulated secretion. Elevated plasma levels of aP2 positively correlate with the prevalence of obesity in patients as well as with features of the metabolic syndrome like insulin resistance (Hotamisligil and Bernlohr 2015).

1.5.2 PPAR-gamma

The peroxisome proliferator-activated receptor-γ (PPAR-γ) belongs to a nuclear hormone receptor family which consists of three divergent receptors - PPAR-α, PPAR-γ, PPAR-δ. All receptors show different compound binding sites as well as different expression levels depending on the tissue (Tontonoz et al. 1995).

Through differential promotor usage and alternative splicing two isoforms of the PPAR-γ originate - PPAR-γ1 and PPAR-γ2. PPAR-γ1 mRNA is abundant in large intestine and adipose tissue and further accounts for up to 85% of PPAR-γ mRNA overall, thus making it the predominant PPAR-γ isoform (Fajas et al. 1997).

In interaction with C/EBPα, PPAR-γ functions as the master regulator of adipogenesis in white and brown adipocytes (Spiegelman and Flier 1996). Various downstream targets of PPAR-γ are thermogenic genes like UCP-1 (Tai et al. 1996). Established agonists of PPAR-γ are a diverse group of compounds like eicosanoids, fatty acids and thiazolidinediones which were in use for the treatment of type 2 diabetes, but were withdrawn due to substantial adverse side effects (Kliewer et al. 1997). However, recent studies with genetically modified mice have highlighted a potential comeback for the application of PPAR-γ agonists. Deacetylation of the PPAR-γ protein protects mice from the adverse effects of thiazolidinedione treatment and makes them unsusceptible to obesity. Thus, modification of the PPAR-γ protein and the development of novel PPAR-γ agonists could aid the treatment of type 2 diabetes and obesity (Kraakman et al. 2018).

1.5.3 UCP-1

The UCP-1 is a unique protein of 32 kDA in the mitochondria of brown adipocytes (Nicholls et al. 1978). It is able to short-circuit the electric gradient of the respiratory chain and thereby produce heat.

The UCP-1, or thermogenin as it has been originally referred to, resides within the inner membrane of the mitochondria of BA (Nicholls et al. 1978). Upon activation, the UCP-1 causes a proton influx from the membrane space to the mitochondria matrix. This dissipates the electric gradient which is otherwise used to synthesize ATP in form of heat

(Porter 2006). The activity of UCP-1 is regulated by the presence of purine nucleotides and FFA. As previously illustrated, the FFA are liberated by the HSL from triglycerides. The HSL in turn is regulated by NE via downstream targets, which highlights once again the importance of SNS on the function of BA (Cannon and Nedergaard 2004). Purine nucleotides inhibit the function of UCP-1, but mitochondria of BA are very sensitive to FFA, so that physiological increases of FFA levels can overcome the inhibition of the purine nucleotides and activate UCP-1 (Porter 2017). Although other forms of the protein like UCP-2 or UCP-3 exist, UCP-1 is the only physiologically relevant thermogenic protein within BA (Matthias et al. 2000). The ability to create heat contributes to the maintenance of the core temperature in humans and mammals and it could have been of great significance for the evolutionary success of mammals (Cannon and Nedergaard 2004). Recently, it was established that UCP-1 function is qualitatively similar in human and mouse cells which has been previously unclear (Porter et al. 2016).

1.6 Positive controls for enhanced and diminished Gαq-signaling

Endothelin-1 (ET-1) and the depsipeptide FR900359 (FR) were used in the experiments as positive controls for enhanced and diminished Gαq-signaling, respectively.

ET-1 is a potent agonist of the Ednra-receptor (ETA)- a Gαq-coupled receptor- which is highly expressed in BA (Klepac et al. 2016). Stimulation of ETA leads to a decrease of differentiation markers like aP2, PPAR-γ and UCP-1 in BA. Additionally, it has been shown that enhanced Gαq-signaling diminishes stimulated browning of WA (Klepac et al. 2016). Thus, ET-1 was used as a positive control for the activation of Gαq-signaling in subsequent experiments.

Furthermore, the depsipeptide FR900359 (FR) was utilized as a selective inhibitor of the Gαq/11/14 signaling pathway in the experiments (Schrage et al. 2015). It has been shown that the application of FR enhances the differentiation of murine BA and elevates the expression levels of adipogenic and thermogenic markers like aP2 and UCP-1 in BA and WA (Klepac et al. 2016). FR was therefore used as a positive control for blocked Gαq signaling.

1.7 Aim of this project

Recently, our laboratory showed that Gαq-signaling is a negative regulator of adipose tissue differentiation and that blockage of Gαq-signaling leads to an increased differentiation of murine brown adipocytes (Klepac et al. 2016). However, there are only a few receptors identified signaling via Gαq in BAT (Klepac et al. 2016).

Hence, it is of great interest to examine whether other receptors are involved in the control of BA differentiation via the Gαq-signaling pathway. Strikingly, the NK3-receptor and Hrh2 are described to be Gαq-coupled (Khawaja and Rogers 1996, http://www.guidetopharmacology.org/) and are significantly up- or downregulated during differentiation .

Not much is known about their physiological roles in BAT or WAT. Therefore, I will address the following question, which might help to understand the role the H2- and NK3-receptor play in the formation and preservation of obesity:

- Does pharmacological stimulation of the H2- or NK3-receptor lead to receptor regulated changes in differentiation of BA and WA?
- Do dietary alterations have an impact on the expression of both receptors in different murine adipose tissues?
- Can pharmacological targeting of the aforementioned receptors alter activation of BA?
- Do BA secrete histamine or tachykinin to stimulate themselves in an autocrine or paracrine fashion?
- Could stimulation or antagonism of the Hrh2 and NK3-receptor have potential clinical implications in the treatment of obesity?

To address those questions, pharmacological and molecular biological experiments were conducted.

2. Materials and methods

2.1 Cell culture

Tab 4: Material and equipment for cell culture experiments

Product	Manufacturer
Amthamine (Cat. No. 0668)	Tocris
Biofuge Primo	Heraeus
Bovine Serum Albumin (Cat. No. A7030)	Sigma-Aldrich
Collagenase Type II	Worthington
Conical tubes 15 and 50ml (Cat. No 62.554.502, 62.547.254)	Sarstedt
Countess automated cell counter (Cat. No. C10227)	Invitrogen
Cryogenic vials (Cat. No. 72.379.992)	Sarstedt
Dexamethasone (Cat. No. D4902)	Sigma-Aldrich
Dimethyl sulfoxide (DMSO) (Cat. No. A994)	Roth
DMEM, high glucose, GlutaMAX (TM), +/- pyruvate (Cat. No.31966, 61965)	Gibco
Endothelin-1 (Cat. No. 1160)	Tocris
Fetal bovine serum (Cat. No. S0015)	Biochrom
FR900359 (FR)	provided by the Institute of Pharmaceutical Biology, University of Bonn, D-53115 Bonn, Germany
HERAcel® 150 – Incubator	Heraeus
Herasafe®- Lamina air flow	Heraeus

Histamine (Cat. No. 3545)	Tocris
Insulin (Cat. No. I9278)	Sigma-Aldrich
Leica DMIL- Microscope	Leica Microsystems GmbH
Penicillin/ streptomycin (P/S) (Cat. No A2213)	Merck
Ranitidine (Cat. No. 1967)	Tocris
Rosiglitazone (Cat. No. R2408)	Sigma-Aldrich
SB223412 (Cat. No. 4672)	Tocris
Senktide (Cat. No. 1068)	Tocris
T175 tissue culture flasks (Cat. No. 83.3912.002)	Sarstedt
Trypan Blue Stain (Cat. No. 15250)	Gibco
Trypsin- EDTA (0.05 %), phenol red-Trypsin (Cat. No. 25300054)	Gibco
Varioklav 135 T	Faust
3,3',5-Triiodo-L-thryonine sodium salt (Cat. No T6397)	Sigma-Aldrich
3-Isobutyl-1-methylxanthine (IBMX) (Cat. No. I5879)	Sigma-Aldrich
6-well TC plates (Cat. No.83.3920)	Sarstedt
6-well TPP plates (Cat. No. 92406)	TPP Techno Plastic Products AG
10 cm^2 TC- dishes (Cat. No. 83.3902)	Sarstedt
12-well TC plates (Cat. No. 662160)	Greiner
12-well TPP plates (Cat. No. 92412)	TPP Techno Plastic Products AG
30 µM and 100 µm nylon meshes (Cat. No. NY3002500, NY1H00010)	Millipore

2.1.1 Cell culture of brown adipocytes

Tab.5: BA-isolation buffer

$CaCl_2$	1.3 mM
Glucose	5 mM
HEPES	100 mM
KCL	5 mM
NaCl	123 mM
BSA	1.5 %
Collagenase II	2 mg/ ml

Tab. 6: BA-culture medium

DMEM (high glucose, GlutaMAX^{TM})	Cat. No. 61965
FBS	10 %
HEPES	10 mM
Insulin	4 nM
P/S	1 %
Sodium-Ascorbat	25 µg/ ml
T3	4 nM

<u>Isolation protocol of BAT-dervied preadipocytes</u>

Neonatal C57BL/6 mice were used to obtain BAT-derived preadipocytes for cell culture experiments with brown adipocytes. First, the interscapular BAT was excised from the mice and the tissue was dissected with a scissor and put in 3 mL of BA-isolation buffer (table 5). The BA-isolation buffer was prepared in advance. The reagents were dissolved in H_2O and adjusted to pH=7.4. The solution was sterilely filtered, BSA and Collagenase

were added freshly before usage and the solution was sterilely filtered a second time (Scheibler 2017).

After placement in the BA-isolation buffer, the tissue was disintegrated at 37 °C and it was thoroughly shaken for 30 minutes. A 100 µM nylon mesh was used to filter remaining pieces and the maintained suspension was stored on ice for 30 minutes so that the suspension would separate in different phases. Next, the middle phase was extracted and filtered with a 30 µM nylon mesh. Centrifugation for 10 minutes at 700 g formed a pellet at the bottom of the falcon and superfluous solution was removed. Then, the pellet was resuspended in 2 mL of BA-culture medium (table 6). The cells were seeded in a 6 well TC plate and immortalized after 24 hours with 200 ng of Simian Virus 40 (SV40) large T-antigen under the presence of a phosphoglycerate kinase promoter (Scheibler 2017).

Tab. 7: BA-Growth medium

DMEM (high glucose, GlutaMAX^{TM})	Cat. No. 61965
FBS	10 %
P/S	1 %

Cultivation protocol of BAT-derived preadipocytes

The cells were grown in BA- growth medium (table 7) and the medium was regularly changed. At ~90 % confluence, the cells were washed two times with sterile PBS and detached with Trypsin for 3 minutes at 37 °C. Subsequently, the cell suspension was collected and put in a falcon. Next, the falcon was centrifuged at 1000 rpm for 5 minutes so that a pellet would form and the superfluous solution was removed. The cells were resuspended in BA-growth medium (table 7). Cells were split and seeded in 10 cm^2 TC dishes in BA-growth medium and the aforementioned steps were carried out again until passage 5. For storage, the obtained cells were put in BA-growth medium supplemented with 10 % DMSO and put in cryogenic vials. They were kept at -80 °C for the first 24 hours and at -196 °C for long-term storage (Scheibler 2017).

Tab. 8: BA-Differentiation medium

DMEM (high glucose, GlutaMAX™)	Cat. No. 61965
FBS	10 %
Insulin	1 nM
P/S	1 %
T3	20 nM

Tab. 9: BA-Induction medium

DMEM (high glucose, GlutaMAX™)	Cat. No. 61965
FBS	10 %
Insulin	1 nM
P/S	1 %
T3	20 nM
IMB	0.5 mM
Dexamethasone	1 µM

Differentiation and treatment protocol of BAT-derived preadipocytes

Preadipocytes in passage 6 were used for cell culture experiments. First, the Trypan Blue Stain and the Countess Automated Cell counter were used to count the number of preadipocytes. Depending on whether 6-well or 12-well plates were utilized, 150000 or 80000 cells were seeded per well on TC plates in BA-growth medium (table 7, day -4). The cells were incubated at 5 % CO_2 and 37 °C for two days until day -2 or ~70-80 % confluence. The growth medium was replenished with BA-differentiation medium (table 9) on day -2.

After incubation for two more days, BA were stimulated using the BA- induction medium (table 10, day 0). Subsequently, the cells were cultivated in BA-differentiation medium (day 2) and the medium was changed every second day (day 2, 4, 6) (figure 1).

To observe the effects of a chronic treatment on the differentiation of BA, the sustained treatments were performed from day 0 to day 7 (figure 1). The substances were given in concordance with the change of BA- differentiation medium (day 2, 4, 6). The mature BA were analyzed on day 7. This treatment scheme applies for RNA, protein, and Oil-Red-O-Stain experiments.

The following compounds were used for chronic treatment of BA:

- Amthamine
- ET-1
- FR
- Histamine
- Ranitidine
- SB223412
- Senktide

Differentiation and treatment scheme:

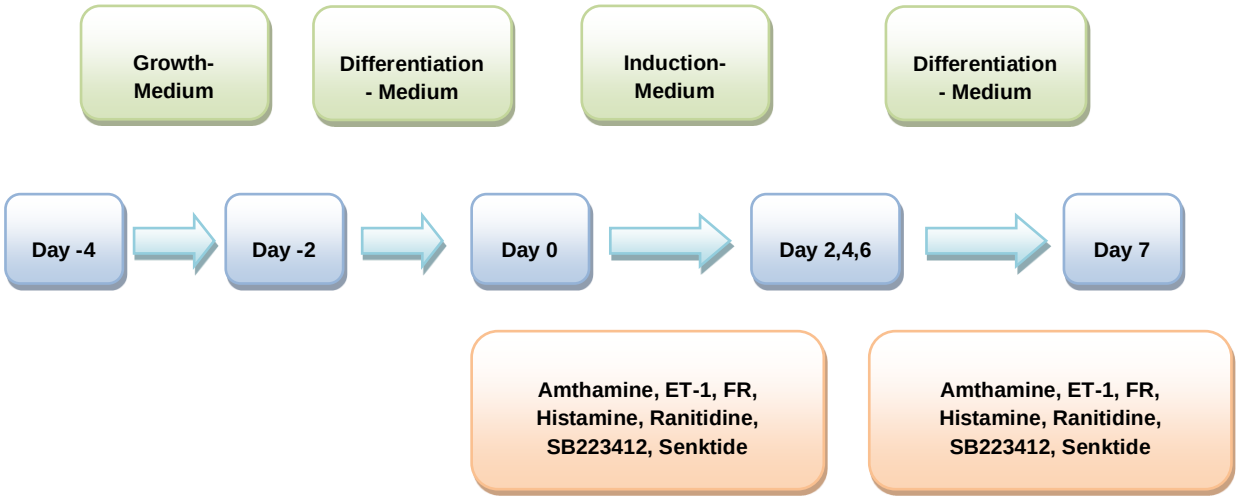

Fig. 2: Differentiation and treatment scheme of BAT-derived preadipocytes

2.1.2 Cell culture of white adipocytes

Tab. 10: WA- Isolation buffer

DMEM (high glucose, GlutaMAX™)	Cat. No. 31966
BSA	0.5 %
Collagenase Type II	1.5 mg/ ml

Tab. 11: WA- Growth medium

DMEM (high glucose, GlutaMAX™)	Cat. No. 31966
FBS	10 %
P/S	1 %

Isolation protocol of WATi-derived preadipocytes

For white adipocytes cell culture experiments, 8-10 weeks old C57BL/6 mice were used to isolate WATi-derived preadipocytes. First, inguinal WAT (WATi) depots were isolated and the tissue was washed in cold PBS (table 8). Next, the tissue was shredded and WA-isolation buffer (table 10) was added at a ratio of 7ml medium per 1 g of tissue. For the WA-isolation buffer, DMEM (high glucose, GlutaMAX™) was utilized and BSA and collagenase were added freshly before usage. Subsequently, the shredded tissues underwent incubation at 37 °C and frequent shaking for 30 minutes. 7 ml of WA-growth medium stopped ongoing collagenase activity. In consecutive steps, cells were centrifuged at 1000 rpm for 10 minutes and the supernatant was removed. 2ml of WA-growth medium was used to dissipate the pellet and the solution was filtered through a 100 µM nylon mesh. The collected cells were then seeded in a T175 culture flask, washed with PBS after 24 hours and the WA-growth medium was replaced every 24 hours until confluence was achieved (Scheibler 2017).

Upon reaching confluence, cells were washed with PBS and detached using trypsin for 5 minutes at 37 °C. The addition of WA-growth medium stopped trypsin activity. Next, cells

were centrifuged for 10 minutes at 1000 rpm. Superfluous solution was removed and the pellet was resolved in WA-growth medium containing 10 % DMSO. The solution separated in different phases and the middle phase was extracted. Subsequently, the cell solution was transferred to cryogenic vials, stored at -80°C and relocated to -200 °C after 24 hours for long-term storage (Scheibler 2017).

Tab. 12: WA-Induction medium

DMEM (high glucose, GlutaMAX™)	Cat. No. 31966
D-biotin	1 mM
Dexamethasone	0.25 mM
FBS	5 %
IMBX	0.5 mM
Insulin	0.172 mM
L-ascorbate	50 mg/ ml
P/S	1 %
Pantothenate	17 mM
Rosiglitazone	1 µM
T3	1 nM

Tab. 13: WA-Maintenance medium

DMEM (high glucose, GlutaMAX™)	Cat. No. 31966
D-biotin	1 mM
FBS	5 %
Insulin	0.172 mM
L-ascorbate	50 mg/ml
P/S	1 %
Pantothenate	17 mM
T3	1 nM

Differentiation and treatment protocol of WATi-derived preadipocytes

The Trypan Blue Stain and the Countess Automated Cell Counter were used to count preadipocytes. The cells were seeded at numbers of 80000 cells per well in a 12 well TPP plate and 150000 cells per well in a 6 well TPP plate. The cells were seeded on day -6 and grown in WA-growth medium (table 13) until they reached confluence. The WA-growth medium was replenished every other day (day -2, -4) and on day 0 cells were stimulated by WA-induction medium (table 14). WA-maintenance medium was replaced from day 2 on every 48 hours (figure 2). The sustained treatment was performed from day 0 till day 7 and was applied in concordance with the change of WA-maintenance medium (day 2,4,6) (figure 2). The mature WA were analyzed on day 7. This treatment routine applies for the RNA experiments.

The following compounds were used for chronic treatment of WA:

- Amthamine
- ET-1
- FR
- Histamine
- Ranitidine
- SB223412
- Senktide

Differentiation and treatment scheme:

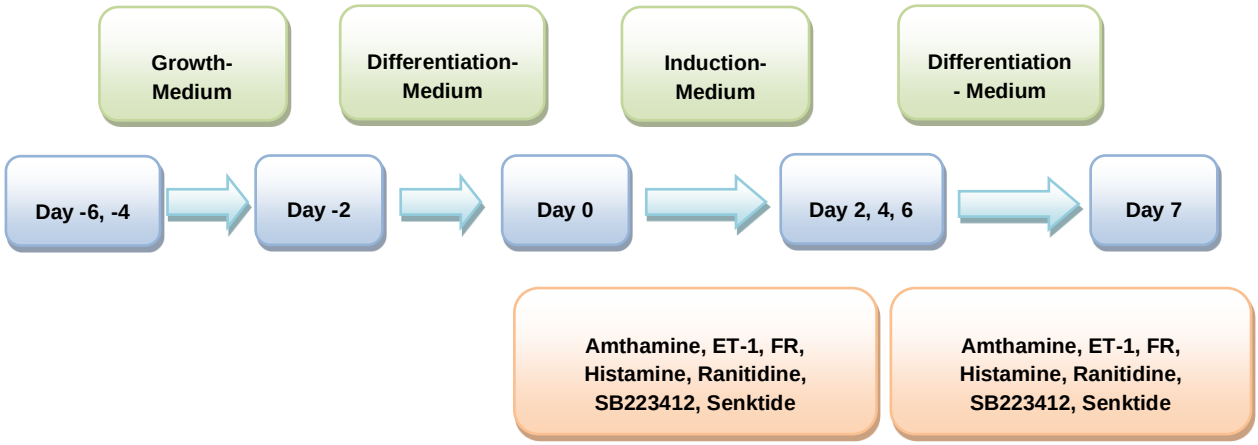

Fig. 3: Differentiation and treatment scheme of WATi-derived preadipocytes

2.2 RNA-methods

Tab. 14: Material and equipment for RNA-methods

Product	Manufacturer
Casting platforms	EmbiTech
Centrifuge (Cat. No. 5415R)	Eppendorf
Diethyl pyrocarbonate- DEPC (Cat. No. K028.1)	Roth
Electrophoresis camber	Peqlab
Ethidium bromide solution 10 mg/ml (Cat. No. 2218.1)	Roth
GelDoc®XR – UV light transilluminator	BioRad
InnuSOLV RNA Reagent (Cat. No. 845-Sb-2090100)	Analytik Jena AG
LightCycler 480 SYBR Green I Master (Cat. No. 04707516001)	Roche
HT7900- real-time PCR machine	Applied Biosystems
Nanodrop200 Spectrophotometer	ThermoScientific
QuantityOne® Software	BioRad
SYBR-Green PCR master mix (Cat. No 4309155)	Applied Biosystems
Thermocycler BiometraTone	Analytik Jena AG
Thermomixer comfort (Cat. No. 2050-120-04)	Eppendorf
Transcriptor First Strand Synthesis Kit (Cat. No. 4896886)	Roche
1kb ladder (Cat. No. 10787018)	Life Technologies

Isolation protocol of RNA and synthesis of cDNA

innuSOLV RNA reagent was used to isolate RNA from cells by lysis. After transfer to reaction tubes the cell solution was purified from remaining fat and protein particles by the addition of chloroform and subsequent centrifugation for 10 minutes at 4 °C and 13000 rpm. The upper phase was carefully extracted and relocated to new reaction tubes. After the addition of isopropanol, RNA precipitated and was compressed to a pellet by centrifugation for 10 minutes at 4 °C and 13000 rpm. Then, the supernatant was removed so that the remaining pellet could be washed with Ethanol. This step was repeated twice. The pellet was air dried for approximately 30 minutes until the superfluous water evaporated and resolved in DEPC-H_2O. RNA concentrations were measured using the Nanodrop200 Spectrophotometer (Scheibler 2017). Next, the Transcriptor First Strand cDNA Synthesis Kit was used for transcription of 1000ng of RNA to cDNA. Synthesis of cDNA was conducted by following manufacturer's instructions. The obtained cDNA was diluted to a final concentration of 2.5 ng/ µl.

The following program was conducted:

Tab. 15: cDNA synthesis cycle

Steps	Time [min]	Temperature [°C]
1	10 min	25 °C
2	30 min	55 °C
3	5 min	85 °C

Quantitative realtime-polymerase chain reaction (qRT-PCR)

To perform the qRT-PCR experiments, 5 µL of SYBR Green PCR mastermix and the corresponding primer pairs (table 19) were added to 12.5 ng of cDNA. Amplification was conducted using a light cycler with the specific amplification cycle listed below. After cDNA amplification, the melting curves were analyzed to assess primer pair quality. Therefore, the analysis of melting curves program was run after the qRT-PCR amplification cycle.

Tab. 16: qRT-PCR amplification cycle

Step	Time [s]	Temperature [°C]	Cycles
1	30 s	25°C	
2	600 s	95 °C	
3	10 s	95°C	40
4	15 s	72°C	40
5	90 s	72°C	40
6	10 s	82°C	40

Tab. 17: Analysis of melting curves

Step	Time [s]	Temperature [°C]
1	15 s	95 °C
2	60 s	60 °C
3	15 s	95 °C

Tab. 18: Primer List

Name	Forward	Reverse
fabp4 (ap2)	5'-TGA AAA AAG TGG GAG TGG GCT TTG-'3	5'-CAC CAC CAG CTT GTC ACC ATC TCG T -3'
cidea	5'-ATT TAA GAG ACG CGG CTT TGG GAC A-'3	5'-TTT GGT TGC TTG CAG ACT GGG ACA T-3'

hprt	5'-ACA TTG TGG CCC TCT GTG TGC TCA-3'	5'-TCA TGA CAT CTC GAG CAA GTC TTT-3'
hrh2	5'-CCC TCA TCT TCA TCA CTG TTG CTG GC-'3	5'-TGT GTT CTC TGA TGG TGG CTG CCT-T-3'
plin1	5‘-CTC TGG GAA GCA TCG AGA AGG TGG T-3‘	5‘-CCT TCA GGG CAT CGG ATA GGG ACA T-3‘
pgc-1α	5’-GCA CAC ACC GCA ATT CTC CTT TGT A-3’	5’- ACG CTG TCC CAT GAG GTA TTG ACC A-3’
pparγ	5’-TCC GTA GAA GCC GTG CAA GAG ATC A-3’	5’-CAG CAC GTT GTC TTG GAT GTC CTC G-3’
tacr3	5'-CGA TAG AGG GAA TCT GAG CGC TGC-3'	5'-CGA TGC CAA TCT TAG TGG CTG TGG CA-3'
ucp-1	5'-TAA GCC GGC TGA GAT CTT GT-3’	5'-GGC CTC TAC GAC TCA GTC CA-3'

PLIN-1 and *tacr3* are the corresponding gene names for perilipin and the NK3-receptor, respectively. *HPRT* served as internal reference control and housekeeping gene in all mRNA experiments. Relative fold changes were calculated by the $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$- method (Livak and Schmittgen 2001).

2.3 Protein methods

Tab. 19: Material and equipment for protein methods

Product	Manufacturer
BioPhotometer D30	Eppendorf
Bovine serum albumin (Cat. No. A7030)	Sigma-Aldrich
Cell scraper (Cat. No. 2015217)	Labomedic
Centrifuge 5430R	Eppendorf
Complete EDTA- free protease inhibitor cocktail (Cat. No. 04693116001)	Roche
Coomassie brilliant blue G-250 (Cat. No. 1.15444.0025)	Merk
Enhanced chemiluminescence (ECL) Western Blotting detection reagent (Cat. No. RPN2106)	Amersham Biosciences
Mini-Protean Tetra cell electrophoresis system	BioRad
Minispin centrifuge (Cat. No. 727706)	Sigma-Aldrich
Nitrocellulose membrane, AmershamProtan 0.45NC (Cat. No. 10600002)	GE Healthcare Life Sciences
PageRulerPrestained Protein Ladder (Cat. No. 26616)	ThermoScientific
Power supply, Consort EV202 (Cat. No Z654418)	Sigma-Aldrich
Roller Mixer SRT6	Stuart
Thermomixer comfort (Cat. No. 205012004)	Eppendorf

Tab. 20: Antibodies

Anti-goat (Cat. No. 705-035-147)	Dianova
Anti- mouse (Cat. No. 115-035-146)	Dianova
Anti- rabbit (Cat. No. 7074)	Cell Signaling
aP2 (Cat. No. sc-18661)	Santa Cruz Biotechnology
PPAR-γ (Cat. No. sc-7273)	Santa Cruz Biotechnology
UCP-1 (Cat. No. sc-7273)	Sigma-Aldrich
Tubulin (Cat. No. MS-719-PO)	Dianova

Protein isolation

Tab. 21: RIPA- buffer (lysis buffer)

Stock solution	
Desoxy-cholic acid Na	0.5 %
NaCl	150 mM
NP -40	1.0 %
SDS	0.1 %
TrisHCl	50 mM (pH 7.5)
Chemicals added before usage	
Complete EDTA-free protease inhibitor	40 µl/ ml
Na_3VO_4 1 mM	10 µl/ ml
NaF 10 mM	50 µl/ ml

In consecutive steps, cells were washed with cold PBS (table 8) and cold RIPA-buffer (table 21) was added to the wells for lysis. Next, cells were scratched off the wells with a cell scraper and transferred with the RIPA-buffer into reaction tubes. After incubation for 30 minutes on ice, the samples were centrifuged for ten minutes at 13000 rpm and 4 °C. The clear middle phase was then extracted, and the protein lysate was transferred to new reaction tubes. Quantification of protein concentration was conducted via the Bradford quantification method explained in the following section.

Bradford quantification method:

Tab. 22: Coomassie solution

Ethanol	5 %
Phosphoric Acid	8.5 %
H_2O	86.49 %
Coomassie brilliant blue G-250	0.01 %

The coomassie solution and 0.15 M NaCl were mixed and added to the isolated protein lysate. After incubation of the solution for one minute at room temperature, a BioPhotometer was used to measure the adsorbance of the solution at 595 nM. The protein concentration was calculated by the adsorbance of the solution.

SDS-polyacrylamid electrophoresis

Tab. 23: Laemmli solution 3×

Bromphenol blue	0.015 %
Glycerol	20 %
H_2O	
SDS	17 %
TrisHCl (pH= 6.8)	125 mM

Tab. 24: Resolving gel

Resolving gel	10 %	15 %
Acrylamide	3.3 ml	5.0 mL
APS 20 %	50 µL	50 µL
H_2O	4.0 ml	5 mL
TEMED	4 µL	4 µL
Tris 1.5 M (pH= 8.8)	2.5 mL	2.5 mL

Tab. 25: Stacking gel

Stacking gel	
Acrylamide	830 µL
APS 20 %	25 µL
H_2O	3.4 mL
TEMED	5 µL
Tris 1.5 M (pH= 8.8)	630 µL

Tab. 26: Electrophoresis buffer 10x

Glycine	2 M
H_2O	
SDS	0.1 %
Tris	250 mM
pH= 8.3	

First, SDS-PAGE gels were prepared and were composed of a stacking and resolving gel component. Resolving gels were made according to the molecular mass of the respective protein, whereas the stacking gels were prepared in the same manner for each SDS-PAGE gel. The recipes were designed for 10 ml of resolving and 5 ml of stacking gel. For greater amounts, the specific proportions were multiplied according to the desired amount.

Then, a specific amount of protein according to the results of the Bradford quantification method was mixed with the Laemmli solution (table 25). The Laemmli solution was kept at -20 °C for long-term storage and 10 % ß-mercaptoethanol was added freshly to the

solution before usage. In consecutive steps, the samples were centrifuged for 30 sec at 13000 rpm and incubated for 5 minutes at 98 °C. Next, the samples were loaded into the prepared SDS-PAGE gels and the electrophoresis was performed at 100 V in room temperature warm electrophoresis buffer which was diluted at a ratio of 1:10 before usage.

Western blot and detection

Tab. 27: TBS ×10

H_2O	
NaCl	1.4 M
SDS	0.1 %
Tris	100 mM
pH= 8.0	

The TBS x10 solution was prepared according to the above-mentioned recipe (table 25). For the TBST solution, TBSx10 was diluted to a ratio of 1:10 with H_2O before usage and after the addition of 0.1 % Tween-20, the solution was protected from light and stored at room temperature. For the blocking solution, 5 % milk powder was resolved in TBST and kept at 4 °C for long-term storage.

After performing the SDS-PAGE electrophoresis, the transfer of the proteins to a nitrocellulose membrane was conducted. The western blotting was carried out at 300 mA for around 105 minutes in cold transfer buffer. The transfer buffer was composed of 10 % electrophoresis buffer 10x, 70 % H_2O and 20 % methanol. In consecutive steps, the membranes were washed two times in TBST and blocked by using the aforementioned blocking solution for 60 minutes under constant motion. After washing the membranes 3 more times for 5 minutes each in TBST, the primary antibody was added to incubate the membranes overnight at 4 °C. Following this incubation, TBST was used to wash the membranes 3 times for 5 minutes each. Then, the secondary antibody was given to the membranes in 1 % milk solution for 60 minutes at room temperature while the membranes

were constantly shaken. Subsequently, the membranes were washed again 3 times for 5 minutes in TBST. Following the addition of ECL detection reagent for 1 minute, proteins levels were detected in the ImageQuant LAS 4000 mini. Quantification of the density of the respective protein was conducted by using Image J software. Tubulin served as an internal control to quantify relative fold changes.

Tab. 28: List of primary antibodies used

Primary antibodies	% resolving gel	Concentration
aP2	15 %	1:1000
PPAR-γ	10 %	1:500
Tubulin		1:1000
UCP-1	10 %	1:500

The primary antibodies were dissolved in 5 % BSA solution diluted in TBST.

Tab. 29: List of secondary antibodies used

Secondary antibodies	Protein	Concentration
Anti-goat	aP2	1 µL/ 5 mL
Anti-mouse	Tubulin, PPAR-γ	0,5 µL/ 5 mL
Anti-rabbit	UCP-1	1 µL/ 5 mL

The secondary antibodies were dissolved in 1 % milk solution and were conjugated with a horseradish peroxidase.

2.5 Acute lipolysis *in vitro*

Tab. 30: Material and equipment for acute lipolysis *in vitro* experiments

Product	Manufacturer
Bovine serum albumin (Cat. No. A7030)	Sigma-Aldrich
DMEM, high glucose, HEPES, no phenol red (Cat. No 21063)	Gibco
Free glycerol reagent (Cat. No. F6428)	Sigma-Aldrich
Glycerol standard (Cat. No. G7793)	Sigma-Aldrich
HERAcell®150- Incubator	Heraeus
Norepinephrine (Cat. No. A9512)	Sigma-Aldrich
Sunrise Adsorbance Reader	Tecan
96-well plates (Cat. No. 83.3924)	Sarstedt
Amthamine, histamine, ranitidine, SB223412, senktide	As listed above

To study the effect of a substance on the activity of an adipocyte, the lipolytic activity can be analyzed. The relative glycerol release of mature BA in cell culture medium is a common benchmark for lipolysis and thus activity of the cells (Balkow et al. 2015). NE is applied as a positive control because it activates lipolysis via the physiological ß$_3$-cAMP-PKA-HSL- pathway (Cannon and Nedergaard 2004). The lipolysis experiments were conducted with mature BA (d+7) which did not receive any treatment during differentiation and were differentiated according to the previously mentioned protocol. Upon reaching maturity, the BA were stimulated acutely for two hours with the respective stimulant and the relative glycerol release was measured.

The following compounds were used for treatment:

- Amthamine
- Histamine
- Ranitidine
- SB223412
- Senktide

For the preparation of the lipolysis medium, DMEM (high glucose, HEPES) was supplemented with 2 % BSA. Next, mature BA were washed three times with lipolysis medium and each well was filled with 300 µL lipolysis medium. The specific compounds (amthamine, histamine, NE [1 µM], ranitidine, SB223412, senktide) were then added to the cells and cells were incubated at 37 °C for two hours. 40 µl of lipolysis medium was taken from each well and mixed with 60 µl of the Free Glycerol Reagent in a 96-well plate, incubated for further five minutes at 37 °C and the adsorbance was then measured in a Sunrise Adsorbance Reader at 540 nM. The provided operator manual was followed to calculate the standard concentration.

2.6 Oil-Red-O-Staining

Tab. 31: Material and equipment for Oil-Red-O-Staining experiments

Product	Manufacturer
Isopropanolol 99 % (Cat. No. 278475)	Sigma-Aldrich
Oil-Red-O (Cat. No. 09755-25G)	Sigma-Aldrich
Syringe filter 0.22 µM (Cat. No. 514-0061)	VWR

Oil-Red-O is a fat-soluble diazol dye which specifically stains triglycerides and cholesteryl esters, but not biological membranes. The intensity of staining is proportional to the differentiation state of adipocytes. Therefore, Oil-Red-O-Staining is an elegant method to

visualize whether pharmacological treatment leads to altered storage of triglycerides (Ramírez-Zacarías et al. 1992).

The Red-O-Stock solution was prepared in advance to the experiments. 500ml Oil-Red-O and 100ml Isopropanol (99 %) were mixed and stirred overnight. Before performing the experiments, the prepared Red-O-stock solution was mixed with H_2O in a 3:2 ratio. The solution was filtered two times to ensure no solid particles remained. A 4 % paraformaldehyde solution diluted in PBS was prepared and kept at 4 °C for long-term storage.

Mature brown adipocytes (d+7) which underwent chronic treatment with a ligand of the H2- and NK3-receptor throughout differentiation were used to perform these experiments. The cells were washed in 4 °C cold PBS twice and fixed for 15 minutes in the 4 % paraformaldehyde solution. After carefully washing the cells two times in 4 °C cold PBS once again, 1 ml of the prepared Red-O-ready to use solution was added to each well. After incubation for four hours at room temperature, the wells were carefully rinsed two times with water to remove superfluous solution.

2.6 cAMP, DAG, histamine and tachykinin ELISA

Tab. 32: Material and equipment for intracellular cAMP, DAG, histamine and tachykinin ELISA

Product	Manufacturer
Amthamine (Cat. No. 0668)	Tocris
Biofuge Primo- centrifuge	Heraeus
cAMP Elisa Kit (Cat. No. ADI-900-066)	Enzo
DAG Assay Kit (Cat. No. MET-5028)	Cell Biolabs INC.
Histamine Enzyme Immunoassay Kit (Cat. No. A05890)	Bertin Pharma
Mouse Tachykinin ELISA Kit (Cat. No. LS-F16411)	LifeSpan Biosciences
Norepinephrine (Cat. No. A9512)	Sigma-Aldrich
Senktide (Cat. No. 1068)	Tocris
Sunrise Adsorbance Reader	Tecan

Measurement of intracellular cAMP concentrations

BA precursor cells were differentiated according to the previously mentioned protocol (differentiation and treatment protocol of BAT-derived preadipocytes). Mature cells were acutely stimulated with NE [1 µM], senktide [100 nM], amthamine [1000 nM] and washed with ice-cold PBS. Controls and samples were lysed with 0.1 M HCL 0, 15 and 30 minutes after stimulation, respectively. Next, samples were analyzed with the cAMP ELISA Kit (Enzo, Cat. No. ADI-900-066) following the manufacturer's instructions. Measurement was performed using a Sunrise Adsorbance Reader (Tecan) at 405nm.

Measurement of intracellular diacylglycerol (DAG) concentrations

BA precursor cells were differentiated according to the previously mentioned protocol (differentiation and treatment protocol of BAT-derived preadipocytes). Mature cells were stimulated 16 hours prior to performing the experiments with the following substances: NE

[1 µM], senktide [100 nM], SB223412 [100 nM], neurokinin B [10-1000 nM], histamine [100 nM], amthamine [100 nM] and ET-1 [10 nM]. Subsequently, samples were prepared and analyzed with the DAG Assay Kit (Cell Biolabs Inc., Cat. No. MET-5028) in accordance with the manufacturer's manual. Measurement was performed using a Sunrise Adsorbance Reader (Tecan) for excitation in the 530-560 nm range and for emission in the 585-595 nm range.

Measurement of histamine and tachykinin in cell culture medium

BA precursor cells were differentiated according to the previously mentioned protocol (differentiation and treatment protocol of BAT-derived preadipocytes). Cells were treated with NE [1 µM]. NE was added acutely on mature BA 16 hours prior to the collection of the supernatant. On day -2 and day 7 of the differentiation protocol, the supernatant of each well was taken. NE was used in these experiments as it positively influences the differentiation of BA. NE is a key stimulator of BA activation and can significantly upregulate UCP-1 expression via interaction with the β_3-adrenoreceptor for example (Cannon and Nedergaard 2004).

Measurement of histamine in cell culture medium

In consecutive steps, samples were analyzed with the Histamine Enzyme Immunoassay kit (Bertin Pharma, Cat. No A05890) following the manufacturer's instructions and measured using a Sunrise Adsorbance Reader (Tecan) at 405 nm.

Measurement of tachykinin in cell culture medium

The samples were centrifuged for 20 minutes at 1000 ×g to remove particulates. After performing the centrifugation, samples were analyzed with the Mouse Tachykinin ELISA Kit (LifeSpan Biosciences) following the manufacturer's instruction. Upon finishing the assay procedure, samples were measured with a Sunrise Adsorbance Reader (Tecan) at 450nm.

2.7 Normal diet and high fat diet mice

Tab. 33: Material and equipment for normal diet and high fat diet mice experiments

Product	Manufacturer
Seven-week-old, male C57BL/6J mice	
Green Line IVC cage	Tecniplast
EF D124505B* mod LS	Ssniff
EF acc. D12492 (II) mod. *	Ssniff

Diet and housing

Seven-week-old, male C57BL/6J mice were purchased and housed in a Tecniplast Green Line IVC cage at 23 °C with a 12 hours day and night circle. First, they were rested for one week in order for them to acclimate. Subsequently, they were either fed a normal chow diet (Ssniff EF D124505B* mod LS) or a high fat diet (Ssniff EF acc. D12492 (II) mod. *) without restriction to water or food. The high fat diet's calories consisted of 60 % fat. The diet was carried out for 12 weeks. After week 20, different adipose tissues (BATi, WATi, WATg) were dissected and purified from the mice and mRNA from these tissues were isolated following the aforementioned protocol. The isolation was carried out to perform RT-PCR experiments with this mRNA to study the effect of the different diets on the receptor expression in the aforementioned adipose tissues.

The experiments were granted by the State Office for Nature, Environment and Consumer protection of North Rhine-Westphalia.

2.8 Statistical analysis

The presented data were analyzed by a two-sided t-test or the One-Way ANOVA test with a post hoc Newman-Keuls multiple comparison test using GraphPad Prism 5 and 6 software, if not stated otherwise. The tachykinin and histamine Elisa were calculated by a four-parameter logistic function using the following software myassays.com. The data is shown as mean plus standard error of the mean. Furthermore, each n represents an individual experiment.

3. Results

3.1 Neurokinin3-receptor- regulation and coupling

Brown adipocytes

Preliminary screening data (experiments performed by Dr. Katarina Klepac, data not shown) suggested the NK3-receptor and H2-receptor to be Gαq GPCRs which were highly upregulated in the case of H2-receptor and downregulated in the case of the NK3-receptor in BA differentiation.

The expression levels of the NK3-receptor mRNA in preadipocytes and mature BA were further analyzed (experiments performed by Dr. Thorsten Gnad, figure 4).

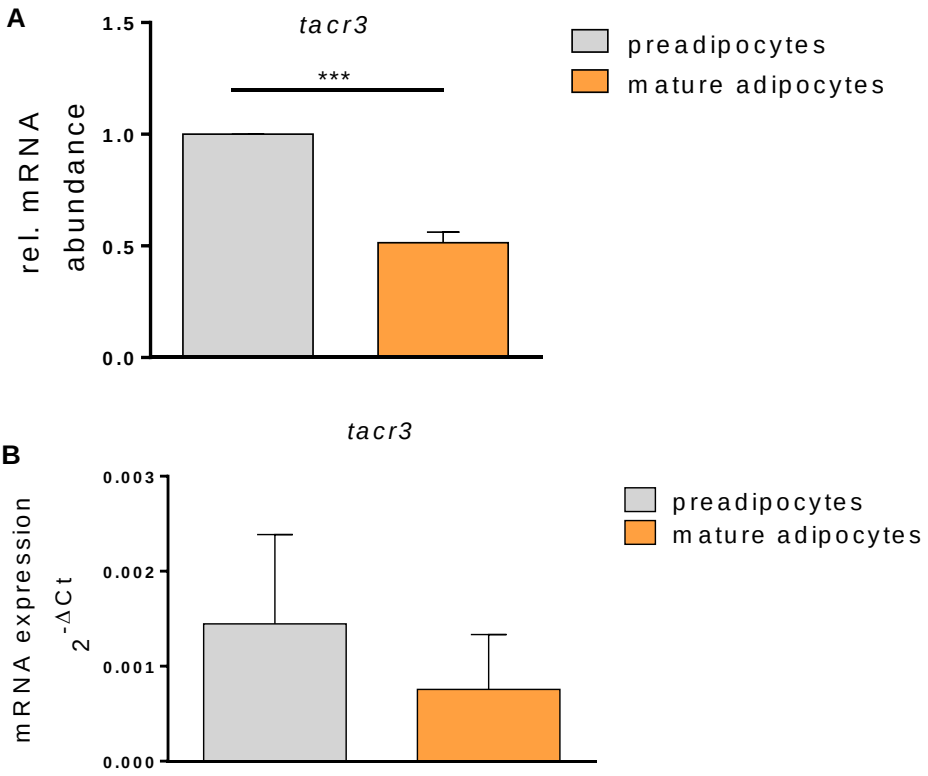

Fig. 4: Expression of NK3-receptor mRNA in preadipocytes and mature BA. (A) Expression levels were normalized to preadipocytes mRNA, (B) mRNA expression was computed by the $2^{-\Delta CT}$-method, *hprt* served as housekeeping gene, mean with s.e.m, n=4, t-test, *** $p < 0.001$, *tacr3* is the corresponding gene name of the NK3-receptor

Interestingly, the NK3-receptor mRNA (figure 4, A) was significantly downregulated by 49 % during the course of differentiation.

White adipocytes

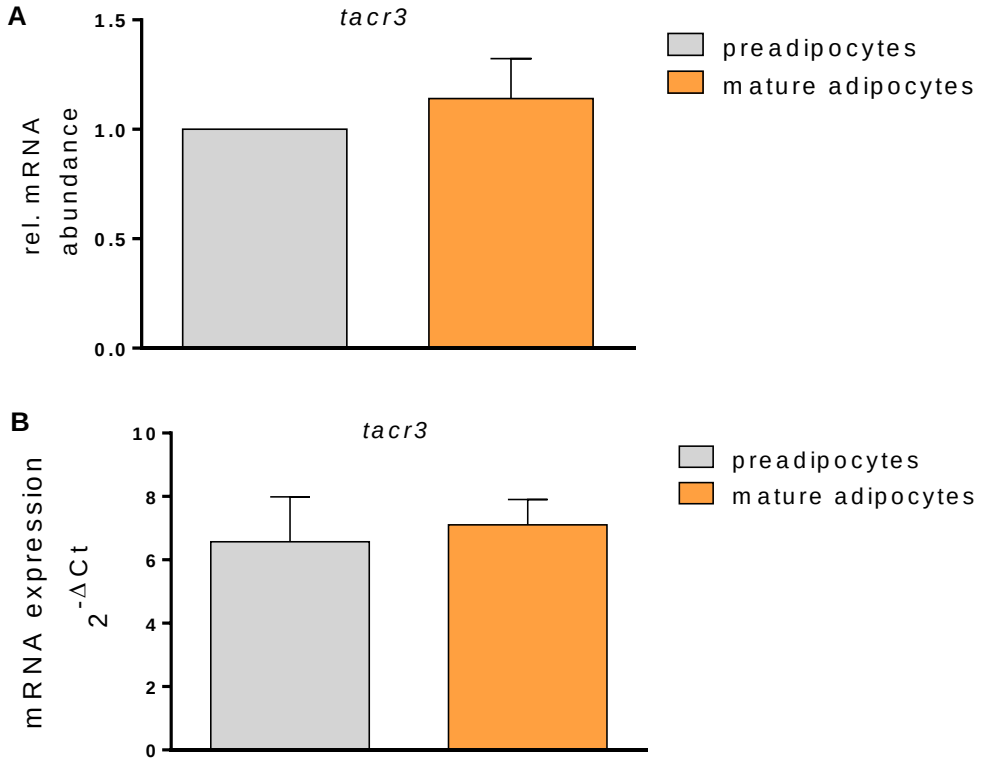

Fig. 5: Expression of NK3-receptor mRNA in preadipocytes and mature WA. (A) Expression levels were normalized to preadipocytes mRNA expression, (B) mRNA expression was computed by the $2^{-\Delta CT}$-method, *hprt* served as housekeeping gene, mean with s.e.m, n=3, t-test, *tacr3* is the corresponding gene name of the NK3-receptor

Preadipocytes and mature WA both express the NK3-receptor (figure 5). In contrast to the downregulation of the NK3-receptor mRNA expression in BA (figure 4), there is no significant change in the NK3-receptor mRNA expression in WA during differentiation.

There is not much knowledge about whether the NK3-receptor leads to alterations in differentiation and functional aspects of BA and WA. Therefore, it was of great interest to further study its physiological role in BA and WA.

NK3-receptor: intracellular DAG assay

As previously mentioned, interaction between the neurokinin receptors and their endogenous ligands leads to the activation of the Gαq pathway. Through the subsequent stimulation of the phospholipase C and further intermediate steps, an increase of inositol

trisphosphate- (IP3) and diacylglycerol (DAG) levels occurs (Khawaja and Rogers 1996). Thus, to confirm the Gαq-coupling of the NK3-receptor in brown adipocytes, a DAG assay (figure 6) was conducted to confirm that stimulation of the NK3-receptor leads to an intracellular rise of the Gαq second messenger DAG.

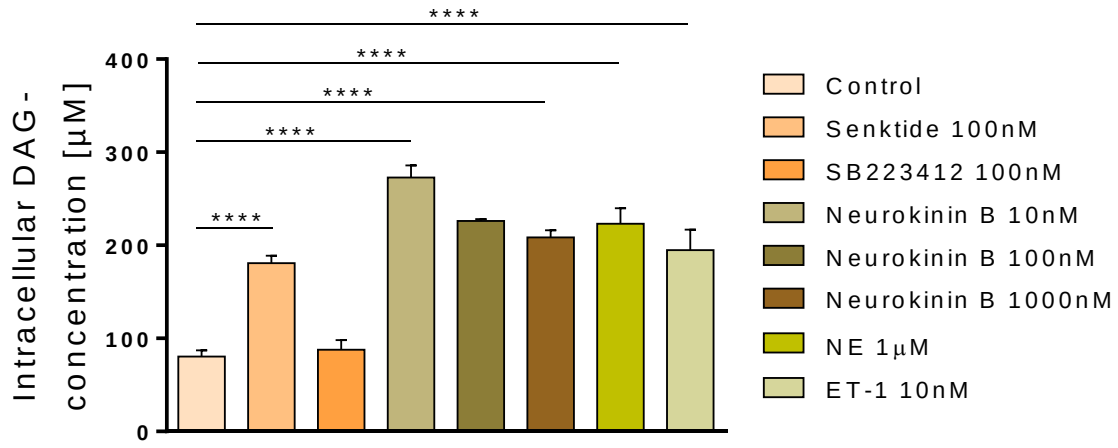

Fig. 6: Release of intracellular DAG [µM] in mature BA upon stimulation of the NK3-receptor. Control (Ctrl.) was not treated. Other cells were acutely stimulated with senktide [100 nM] - agonist to the NK3-receptor, SB223412 [100 nM] – antagonist to the NK3-receptor, neurokinin B [10, 100, 1000 nM]- endogenous ligand of the NK3-receptor, NE [1 µM], and ET-1 [10 nM] – agonist to the EDNRA- for 16 hours, n=3 except neurokinin B [100 nM] n=2, error bars, s.e.m., ANOVA-test, **** p< 0.0001

As expected, stimulation of the NK3-receptor (figure 6) with the agonist senktide [100 nM] as well as with the endogenous ligand neurokinin b in different concentrations led to a significant rise of intracellular DAG. Interestingly, neurokinin B [10-1000 nM] caused a stronger response than senktide [100 nM]. Furthermore, there was no significant difference between stimulation with neurokinin B 10 nM and 1000 nM. This demonstrates a very sensitive response already at small amounts [10 nM] of neurokinin B. SB223412 [100 nM]- antagonist to the NK3-receptor- did not alter the intracellular DAG levels basally. ET-1 [10 nM] as a potent agonist of the Gαq-coupled Ednra-receptor (ETA) also significantly elevated the intracellular DAG levels. NE [1 µM], likewise, caused a strong response presumably via Gαq-coupled alpha adrenergic receptors in brown adipocytes (Kikuchi-Utsumi et al. 1997; Wu et al. 1992).

Overall, the expression of the NK3-receptor mRNA is significantly downregulated throughout differentiation in BA (figure 4), whereas no significant change is observable in WA (figure 5). Stimulation of the NK3-receptor leads to an increase of intracellular DAG (figure 6), whereas antagonism does not alter it.

3.2 Chronic effects of senktide on BA and WA differentiation

Next, the effect of chronic pharmacological stimulation of NK3 on BA and WA differentiation and function was analyzed using the specific NK3- receptor agonist senktide (Endo et al. 2015). Senktide is a synthetic peptide highly selective to the NK3-receptor (Stoessl et al. 1988).

Brown adipocytes

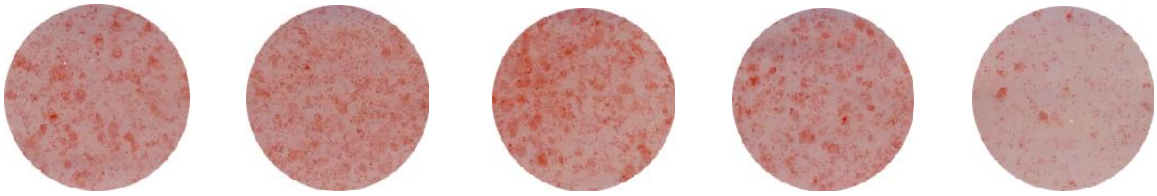

Fig. 7: Representative Oil-Red-O-Stain of BA [senktide].
Control was untreated. Other cells were chronically treated with senktide [1,10, 100 nM] and ET-1 [10 nM] as positive control, n=3

Stimulation of the NK3-receptor with senktide [1 nM, 10 nM, 100 nM] resulted in no major reduction of Oil-Red-O-Stain (figure 7).

Hereafter, the results of chronic stimulation with senktide [1 nM, 10n M, 100 nM] on BA differentiation are demonstrated.

Treatment with senktide did not change the expression of aP2 on mRNA and protein levels (figure 8).

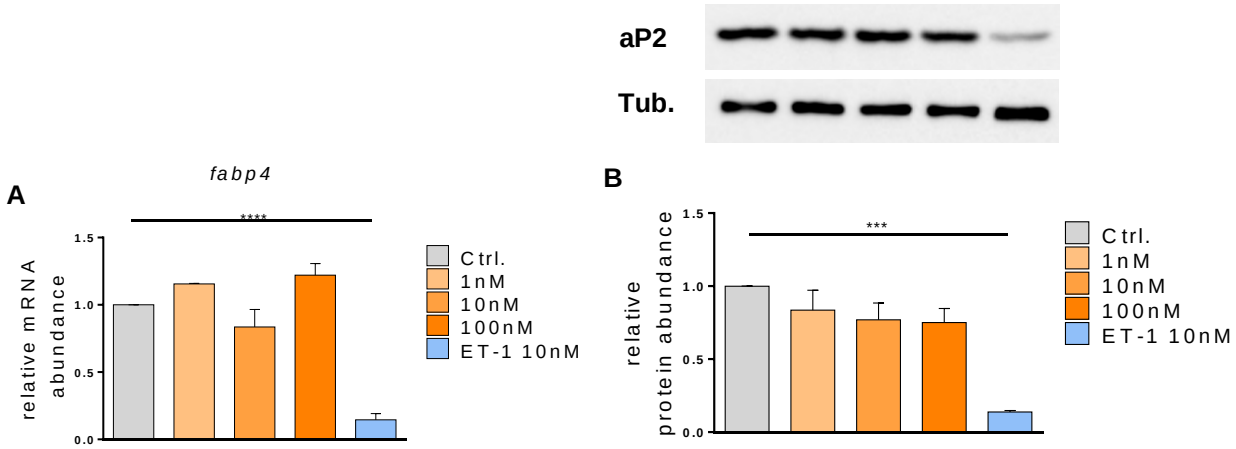

Fig. 8: Expression of aP2 on mRNA-level (A) and protein level (B) in mature BA [senktide]. Control (Ctrl.) was not treated. Other cells were treated with senktide [1 nM, 10 nM, 100 nM] or ET-1 [10 nM] during differentiation, expression levels were normalized to the control, *fabp4* is the corresponding gene name for aP2 (A), mean with s.e.m, (A) and (B): n=3, ANOVA-test, *** p< 0.001, **** p< 0.0001

Unexpectedly, NK3-receptor stimulation with senktide [100 nM] increased the expression of PPAR-γ mRNA by 31 % (figure 9) and PPAR-γ protein levels were likewise elevated by 33 % by treatment with senktide [10 nM], but not at higher nanomolar concentrations.

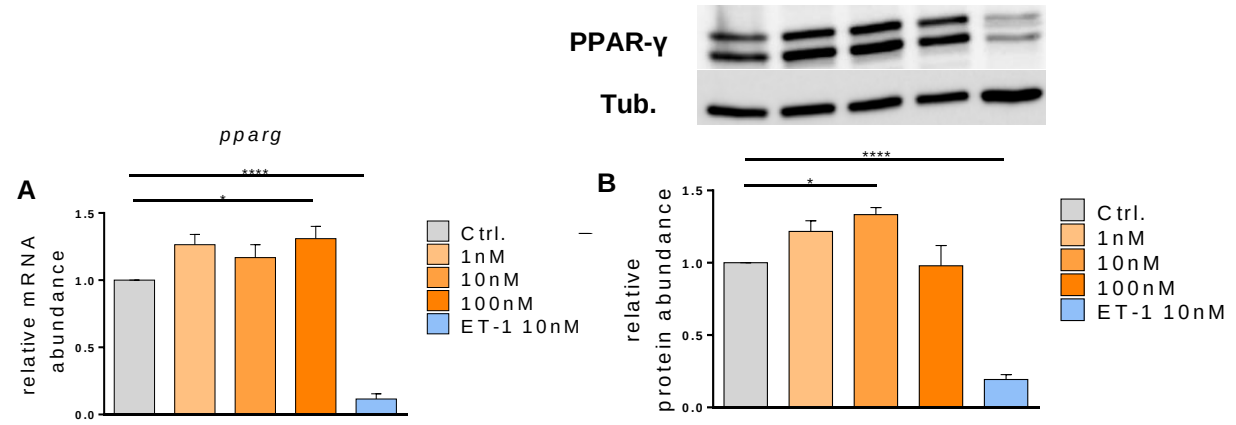

Fig. 9: Expression of PPAR-γ on mRNA-level (A) and protein level (B) in mature BA [senktide]. Control (Ctrl.) was not treated. Other cells were treated with senktide [1 nM, 10 nM, 100 nM] or ET-1 [10 nM] during differentiation, expression levels were normalized to the control, *pparg* is the corresponding gene name for PPAR-ɣ, mean with s.e.m, (A) and (B): n=3, ANOVA-test,* p< 0.05, **** p< 0.0001

Pharmacological stimulation of BA with sustained senktide treatment did not alter UCP-1-expression neither on mRNA nor on protein levels (figure 10). ET-1 [10 nM] significantly decreased the expression of aP2, PPAR-γ and UCP-1 with regard to mRNA and protein levels.

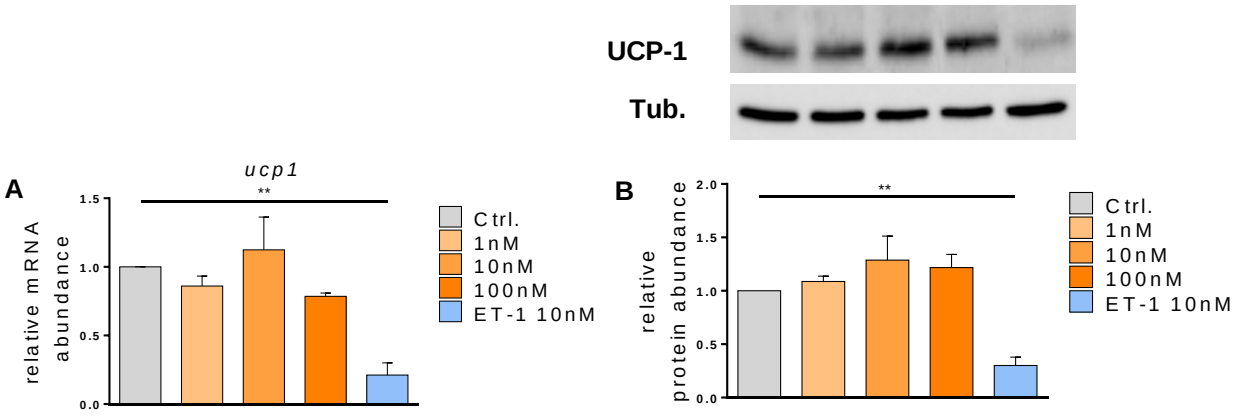

Fig. 10: Expression of UCP-1 on mRNA-level (A) and protein level (B) in mature BA [senktide]. Control (Ctrl.) was not treated. Other cells were treated with senktide [1 nM, 10 nM, 100 nM] or ET-1 [10 nM] during differentiation, expression levels were normalized to the control, *ucp1* is the corresponding gene name for UCP-1 (A), mean with s.e.m, (A) and (B): n=3, ANOVA-test, ** p< 0.01

Summarizing, chronic treatment with senktide induced a non-significant down-regulation of the protein levels of the adipogenic marker aP2 (figure 8) and a significant up-regulation of PPAR-γ protein at low concentrations (figure 9). However, no significant changes in the level of the thermogenic marker UCP-1 were observed (figure 10) in BA.

To examine more intensively whether the augmentation of PPAR-γ levels affects further downstream targets multiple thermogenic and adipogenic markers which are regulated by PPAR-γ were screened (Arimura et al. 2004; Lasar et. al 2018; Lee et. al 2014; Savage 2005; Viswakarma et al. 2007).

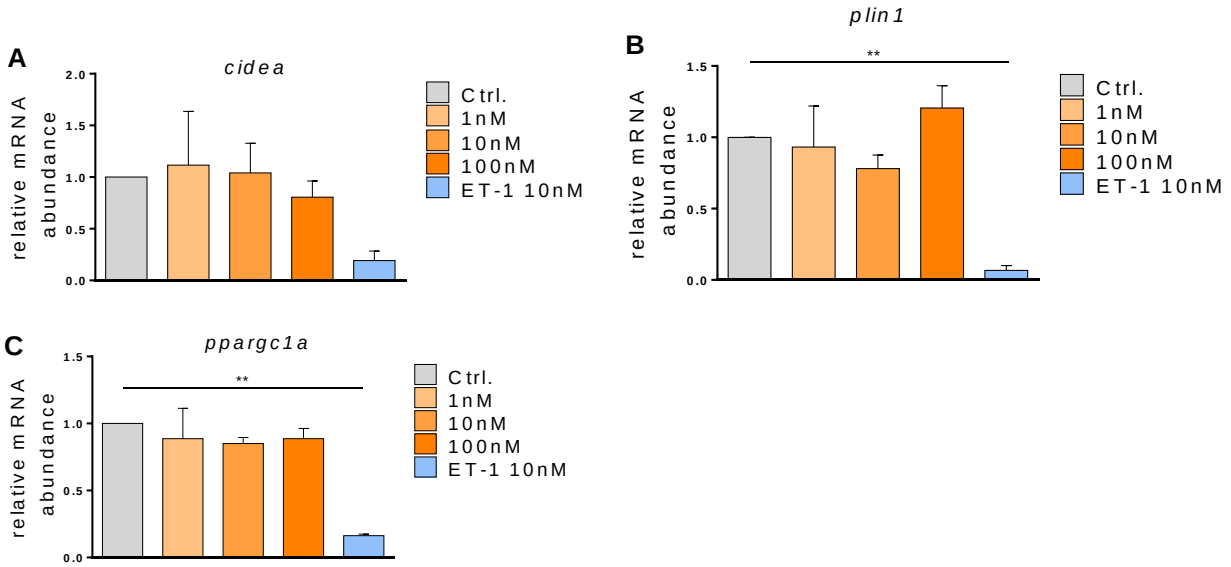

Fig. 11: Expression of CIDE-A (A), Perilipin (B) and PGC1-α (C) on mRNA-level in mature BA [senktide]. Control (Ctrl.) was not treated. Other cells were treated with senktide [1 nM, 10 nM, 100 nM] or ET-1 [10 nm] during differentiation, *cidea/ plin1/ ppargc1a* are the corresponding gene names for CIDE-A (A), Perilipin (B) and PGC1-α (C), expression levels were normalized to the control, mean with s.e.m, (A), (B), (C): n=3, ANOVA-test, ** $p < 0.01$

Treatment with senktide [1, 10, 100 nM] (figure 11) did not lead to a significant change in the levels of CIDE-A, perilipin or PGC1-α, while ET-1 [10 nM] strongly decreased the expression of CIDE-A (A), Perilipin (B) and PGC1-α (C).

The increase of PPAR-γ by the treatment with senktide was surprising as a downregulation of the expression was expected due to the suspected Gαq-coupling. Thus, an intracellular cAMP-ELISA was conducted to analyze whether the NK3-receptor might also couple to the Gαs-protein in BAT. As previously illustrated, activation of the Gαs-coupled receptor results in a rise of intracellular cAMP, and norepinephrine as an agonist of the highly expressed Gαs-coupled ß$_3$-adrenergic receptor in BAT can be used as a positive control (Cannon and Nedergaard 2004).

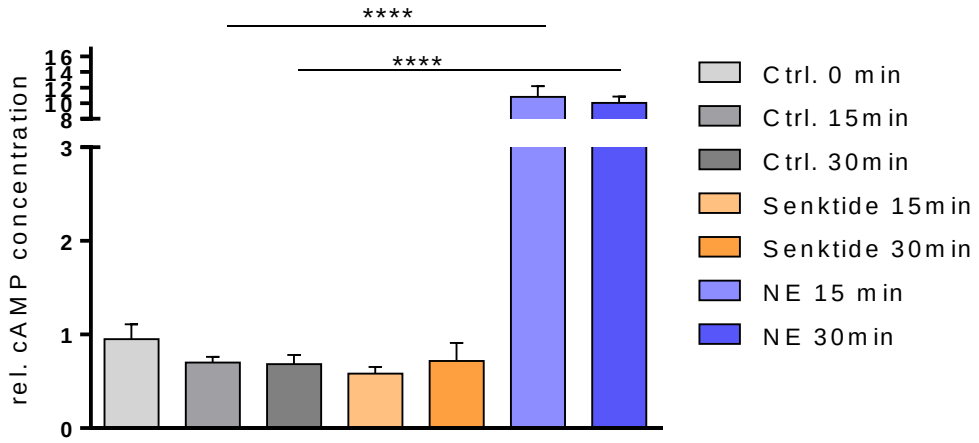

Fig. 12: Intracellular cAMP-levels in mature BA after 0-, 15- or 30-min stimulation with senktide. Control (Ctrl.) was not treated. Other cells were treated with senktide [100 nM] or NE [1 µM], mean with s.e.m, n=6, ANOVA-test, **** $p < 0.0001$

Senktide [100 nM] did not alter the intracellular cAMP-concentration after 15 and 30 minutes in comparison with NE [1 µM] which significantly rose cAMP-levels 10.82-fold and 10.05-fold after 15 and 30 minutes (figure 12).

Besides cAMP, lipolysis was analyzed as readout for BA activation.

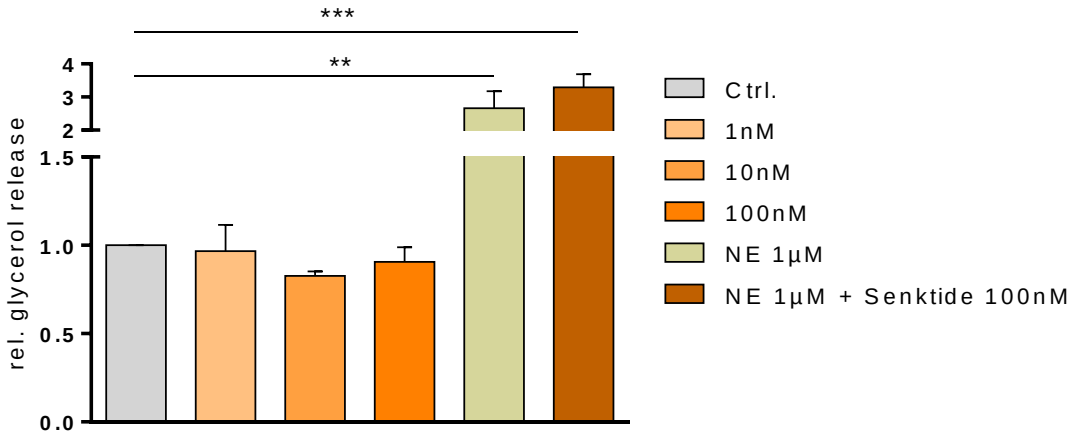

Fig. 13: Relative glycerol release in mature BA after stimulation with senktide. Control (Ctrl.) was not treated. Other cells were treated with senktide [100 nM], NE [1 µM] and NE [1 µM] + senktide [100 nM], mean with s.e.m; senktide [1nM, 10 nM, 100 nM] n=3, NE [1 µM]+ senktide [100 nM] n=4 , ANOVA-test, ** $p < 0.01$, *** $p < 0.001$

Senktide [1, 10, 100 nM] did not significantly affect the glycerol release of the cell (figure 13). Treatment with NE [1 µM] increased the glycerol release by 2.66-fold, whereas

senktide in co-stimulation with NE [1 µM] did not affect the glycerol liberation significantly in comparison with NE [1 µM] alone.

In conclusion, application of senktide did not raise intracellular cAMP-levels (figure 12) and did not change the relative glycerol release in mature BA (figure 13). The surprising upregulation of PPAR-ɣ by the treatment of senktide cannot be explained by these results.

White adipocytes

Hereafter, the chronic stimulation of the NK3-receptor with senktide was analyzed in WA.

As previously illustrated, WA display the capacity for browning and it was of great interest whether browning and thereby an increase in UCP-1 expression can be induced by treatment with senktide. WA do not express UCP-1 commonly as they require certain browning triggers to elevate the expression of genes related to thermogenesis (Merlin et al. 2018).

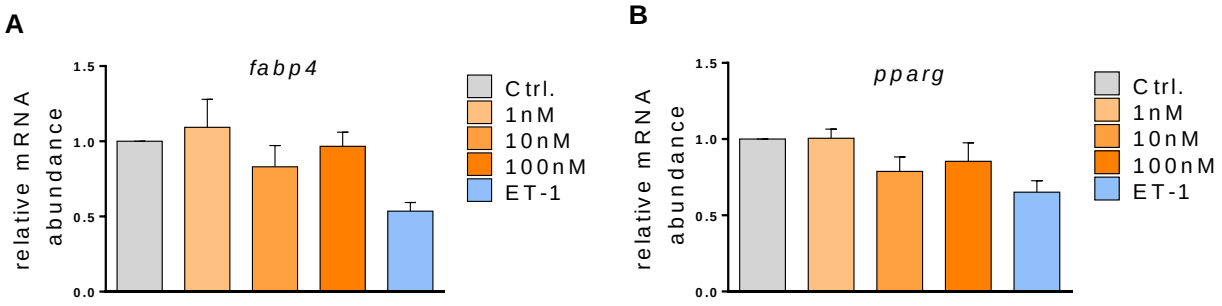

Fig. 14: Expression of aP2 (A) and PPAR-γ (B) on mRNA-level in mature WA [senktide]. Control (Ctrl.) was not treated. Other cells were treated with senktide [1, 10, 100 nM] or ET-1 [10 nM] during differentiation, *fabp4* and *pparg* are the corresponding gene names for aP2 (A) and PPAR-ɣ (B), expression levels were normalized to the control, mean with s.e.m; (A) n=4, (B) n=3, ANOVA-test, * $p < 0.05$

Senktide did not change the expression of aP2 or PPAR-γ in mature WA (figure 14) in contrast to elevation of PPAR-γ mRNA levels in BA (figure 9). Furthermore, neither senktide nor ET-1 triggered expression of UCP-1 in these WA and UCP-1 was therefore not detectable (data not shown). ET-1 [10 nM] significantly diminished the expression of aP2 by 47 % as well as of PPAR-γ by 35 %, albeit non-significantly.

Overall, chronic application of senktide did not have a significant effect on the differentiation of WA and did not induce the expression of UCP-1 in WA (figure 14).

3.3 Chronic effects of SB223412 on BA and WA differentiation

SB223412 is a non peptide antagonist of the NK3-receptor (Sarau et al. 1997) and the effects of the treatment with SB223412 on BA and WA differentiation and function were analyzed in subsequent experiments.

Brown adipocytes

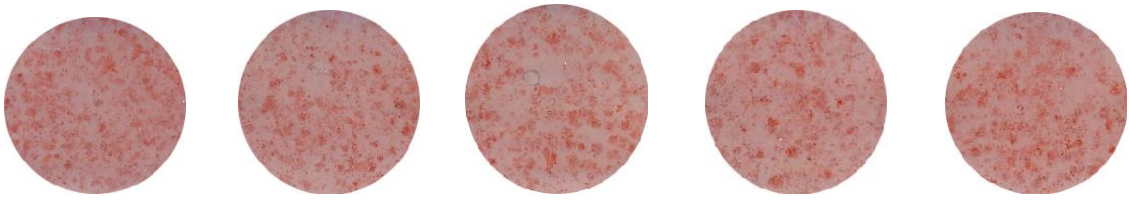

Fig. 15: Representative Oil-Red-O-Stain of BA [SB223412].
Control was untreated. Other cells were chronically treated with SB223412 [10, 100, 1000 nM] and FR [1 µM] as positive control, n=3

As depicted in the representative Oil-Red-O-Stain in figure 15, treatment with SB223412 did not influence the accumulation of triglycerides in comparison to the control. In addition, FR [1 µM] did not have an observable effect.

In the following section, the chronic effects of SB223412 on BA differentiation are shown.

SB223412 caused an increase in aP2 expression on mRNA and protein level at high concentrations (figure 16), albeit non-significantly. FR [1 µM] raised the expression of aP2 by 75 % (A) and by 46 % (B), respectively.

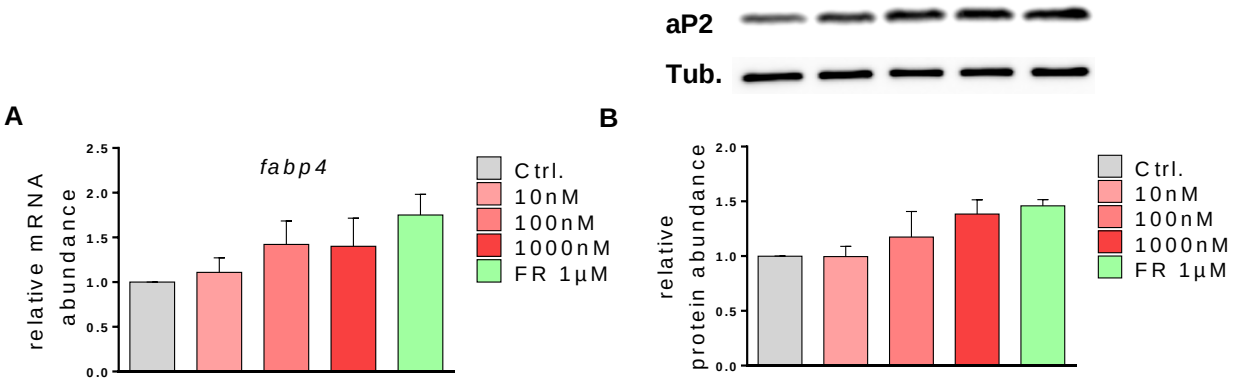

Fig. 16: Expression of aP2 on mRNA-level (A) and protein level (B) in mature BA [SB223412]. Control (Ctrl.) was not treated. Other cells were treated with SB223412 [10 nM, 100 nM, 1000 nM] or FR [1 µM] during differentiation, expression levels were normalized to the control, *fabp4* is the corresponding gene name for aP2 (A), mean with s.e.m, (A) and (B): n=3, ANOVA-test, * $p < 0.05$

Figure 17 displays the expression of PPAR-γ upon stimulation with SB223412. SB223412 [1000 nM] non-significantly increased the expression of PPAR-γ on mRNA level by 51 % but no clear effect can be obverseved on protein levels. FR [1 µM] significantly upregulated the expression of PPAR-γ mRNA by 93 % and non-significantly on protein level (figure 18).

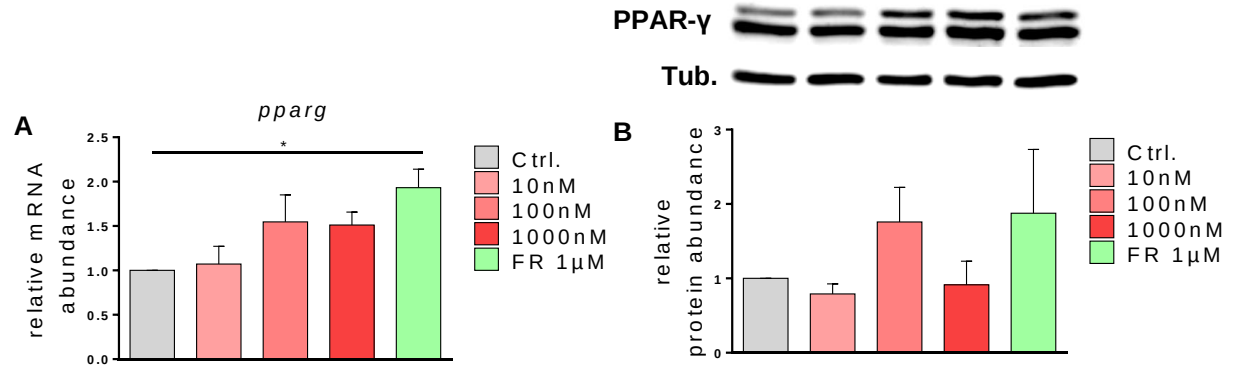

Fig. 17: Expression of PPAR-γ on mRNA-level (A) and protein level (B) in mature BA [SB223412]. Control (Ctrl.) was not treated. Other cells were treated with SB223412 [10 nM, 100 nM, 1000 nM] or FR [1 µM] during differentiation, expression levels were normalized to the control, *pparg* is the corresponding gene name for PPAR-ɣ (A), mean with s.e.m, (A) and (B): n=3, ANOVA-test, * $p < 0.05$

SB223412 did not have a clear and observable effect on the expression of UCP-1 on mRNA and protein levels (figure 18) while FR [1 µM] increased the expression of UCP-1 on mRNA and protein levels, albeit non-significantly.

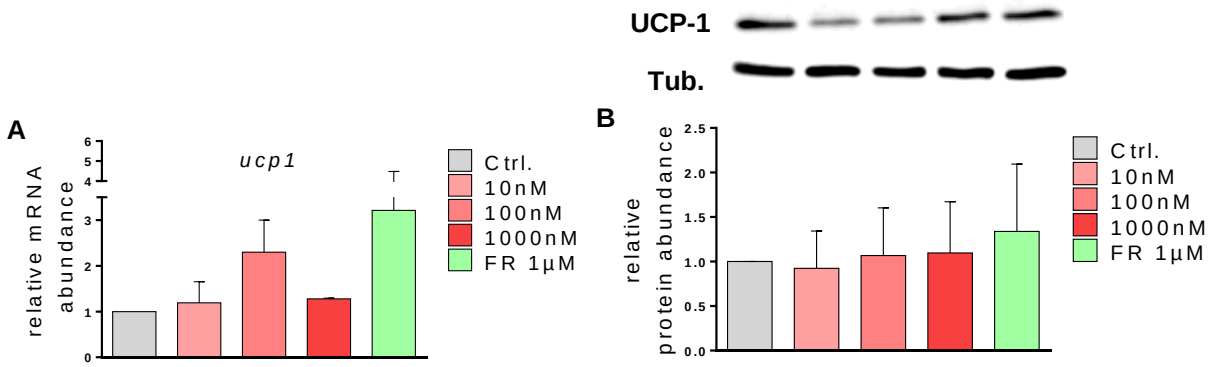

Fig. 18: Expression of UCP-1 on mRNA-level (A) and protein level (B) in mature BA [SB223412]. Control (Ctrl.) was not treated, other cells were treated SB223412 [10 nM, 100 nM, 1000 nM] or FR [1 µM] during differentiation, expression levels were normalized to the control, *ucp1* is the corresponding gene name for UCP-1 (A), mean with s.e.m, (A) and (B): n=3, ANOVA-test, *** $p < 0.001$

Summarizing, chronic treatment with SB223412 had no clear effect on differentiation of BA measured by the adipogenic differentiation marker aP2 (figure 16) and PPAR-γ (figure 17) as well as the thermogenic differentiation marker UCP1 (figure 18).

Next, the effect of SB223412 on the glycerol release of BA as functional parameter of BA activation was scrutinized.

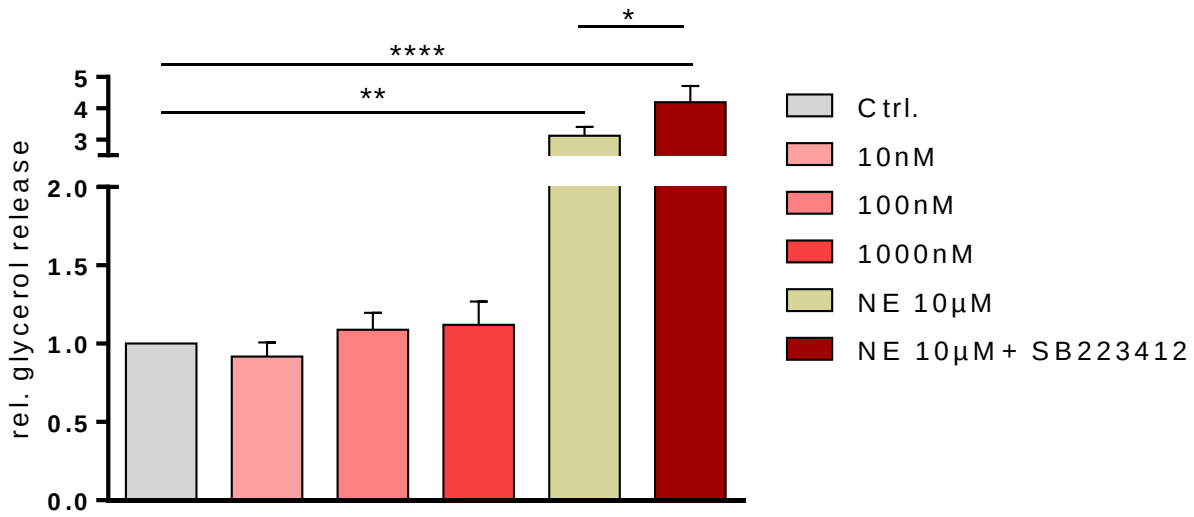

Fig. 19: Relative glycerol release in mature BA after stimulation with SB223412. Control (Ctrl.) was not treated. Other cells were treated with SB223412 [10 nM, 100 nM, 1000 nM], NE [1 µM] and NE [1 µM] + SB223412 [1000 nM], mean with s.e.m; SB223412 [10 nM, 100 nM, 1000 nM] n=4; NE [1 µM] and NE [1 µM] + SB223412 [1000 nM], n=7, ANOVA-test, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$

Figure 19 shows the relative glycerol liberation as a benchmark of lipolytic activity in mature BA after stimulation with SB223412. The glycerol release was unaltered by treatment with SB223412 whereas NE [1 µM] significantly increased the glycerol release. Interestingly, SB223412 [1000 nM] in addition with NE [1 µM] boosted the relative glycerol release in comparison with the sole activation through NE [1 µM] by about 34 %.

White adipocytes

Potential implications of the chronic treatment with SB223412 on differentiation markers of WA were analyzed in subsequent experiments.

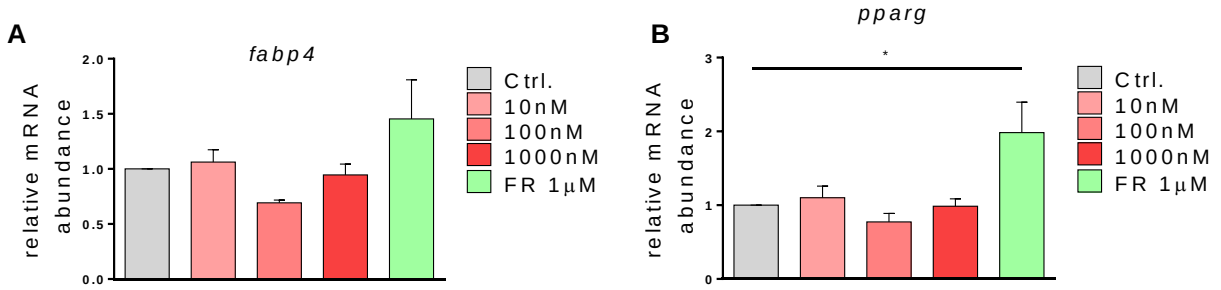

Fig. 20: Expression of aP2 (A) and PPAR-γ (B) on mRNA-level in mature WA [SB223412]. Control (Ctrl.) was not treated. Other cells were treated with SB223412 [10 nM, 100 nM, 1000 nM] or FR [1 µM] during differentiation, expression levels were normalized to the control, *fabp4* and *pparg* are the corresponding gene names for aP2 (A) and PPAR-ɣ (B), mean with s.e.m, (A) and (B): n=3, ANOVA-test, * $p < 0.05$

SB223412 did not change expression levels of aP2 and PPAR-ɣ mRNA (figure 20), albeit a non-significant decline of aP2 mRNA expression upon stimulation with SB223412 [100 nM]. Moreover, SB223412 did not induce the expression of UCP-1 in WA and UCP-1 was therefore not detectable (data not shown). aP2 mRNA and PPAR-ɣ mRNA expression was elevated by FR [1µM].

Summarizing, chronic treatment with SB223412 did not alter the expression of aP2 and PPAR-ɣ mRNA in WA and did not induce the expression of UCP-1 in WA.

3.4 Histamine receptor regulation and coupling

Brown adipocytes

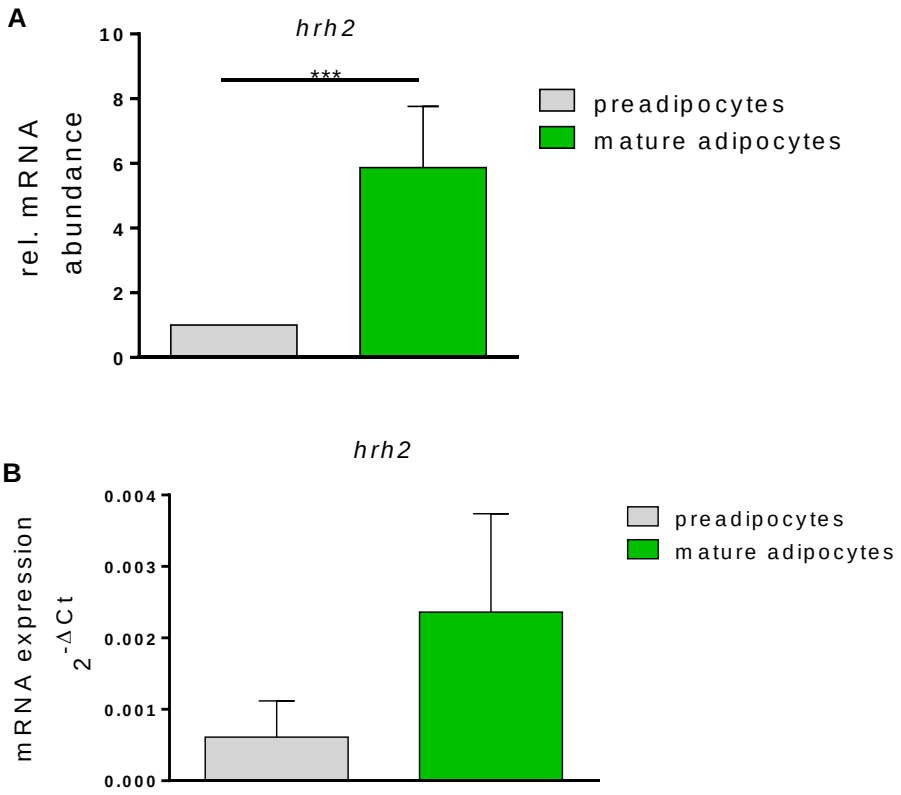

Fig. 21: Expression of H2-receptor on mRNA-level in preadipocytes and mature BA. (A) Expression levels were normalized to preadipocyte mRNA abundance, (B) mRNA expression was computed by the $2^{-\Delta CT}$-method, *hprt* served as housekeeping gene, mean with s.e.m, n=4, t-test, *** $p < 0.001$

Previous preliminary data obtained (experiments performed by Dr. Thorsten Gnad) at the institute of pharmacology and toxicology revealed a significant 5.87-fold upregulation of another putative Gαq-coupled GPCR H2-receptor (Figure 21, A).

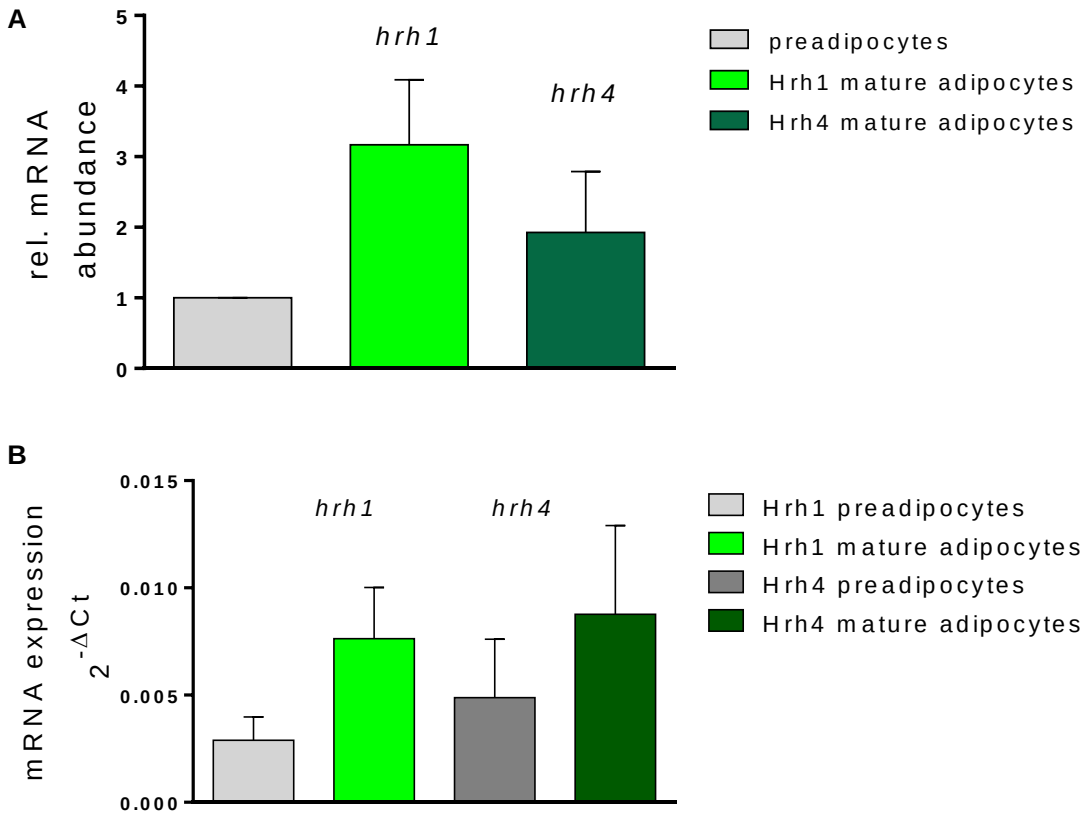

Fig. 22: Expression of H1- and H4-receptors on mRNA-level in preadipocytes and mature BA. (A) Expression levels were normalized preadipocyte mRNA abundance, (B) mRNA expression was computed by the $2^{-\Delta CT}$-method, *hprt* served as housekeeping gene, mean with s.e.m, n=3, t-test, * $p < 0.05$

The H1- and H4-receptors are likewise expressed in BA and are upregulated during differentiation (figure 22), albeit not significantly. The H1-receptor couples to the Gαq/11-protein (Panula et al. 2015) whereas the major signaling pathway of the H4-receptor is the Gαi/o-protein (Sahlholm et al. 2008). In contrast to the study of Zhao et al. (Zhao et al. 2019), there was no reliable H3-receptor mRNA expression detectable in preadipocytes nor in mature brown adipocytes (data not shown).

White adipocytes

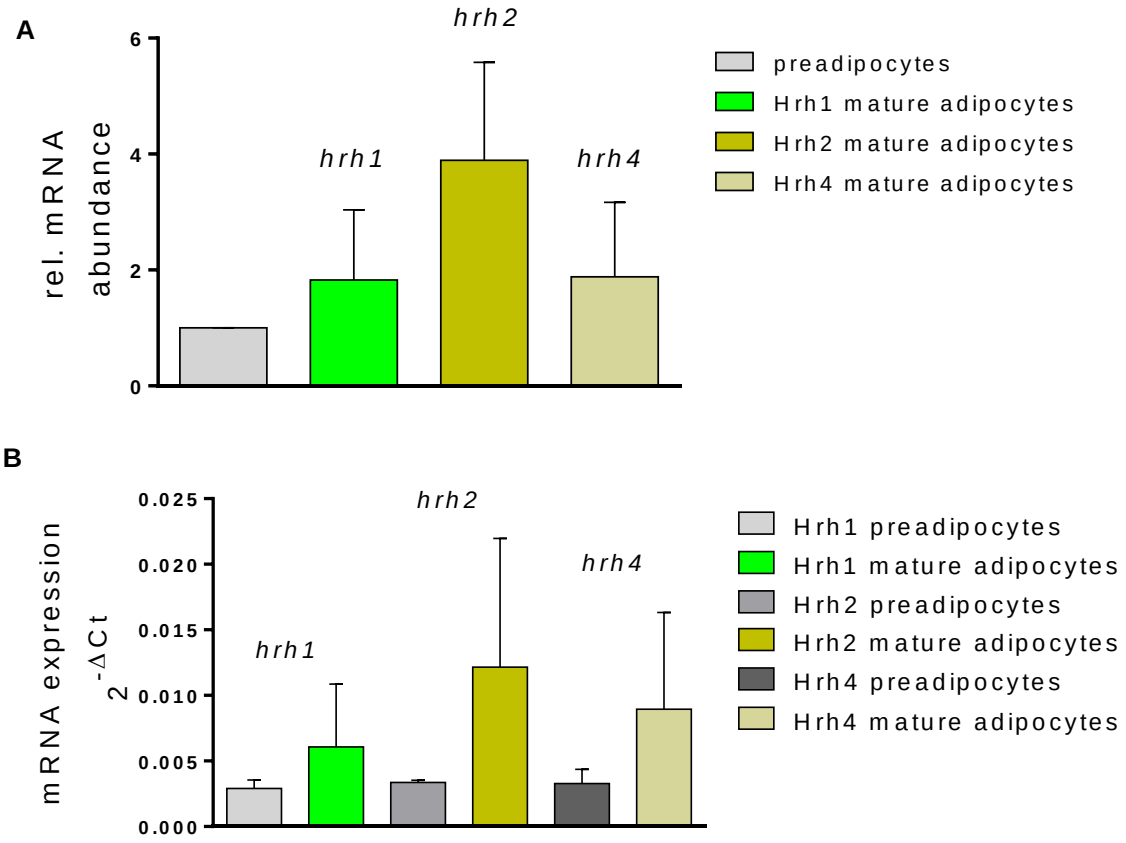

Fig. 23: Expression of H1-, H2 and H4-receptors on mRNA-level in preadipocytes and mature WA**.** (A) Expression levels were normalized to preadipocyte mRNA abundance, (B) mRNA expression was computed by the $2^{-\Delta CT}$-method, *hprt* served as housekeeping gene, mean with s.e.m, n=3, ANOVA-test, * $p < 0.05$

Preadipocytes and mature WA express H1-, H2 and the H4-receptor mRNA (figure 23) and the H1-, H2- and H4-receptor mRNA is upregulated throughout differentiation, although these effects do not reach statistical significance. The H2-receptor mRNA expression is amplified by 3.89-fold which is in similarity to the upregulation of the Hrh2 in BA (figure 21). In WA, there was likewise no effective H3-receptor expression measurable (data not shown).

In conclusion, BA and WA express the H1-, H2- and H4-receptors (figure 21-23) and the H2-receptor (figure 21) is significantly upregulated during differentiation in BA. The other receptors illustrate a non-significant tendency to be upregulated. Contradictory to previous

findings (Zhao et al. 2019) there was no reliable H3-receptor mRNA expression detectable neither in BA nor in WA (data not shown).

G-protein coupling of the histamine h2 receptor

As previously illustrated the H2-receptor is expressed in a broad range of different tissues where it couples to the Gαs-protein in the majority of cases (Panula et al. 2015; Kühn et al. 1996). According to the IUPHAR database (http://www.guidetopharmacology.org/) the H2-receptor was determined to be Gαq-coupled in BA. Hence, a DAG assay was performed (figure 24), a second messenger of the Gαq-coupling signaling pathway, to confirm the Gαq-coupling in BA. To verify the IUPHAR database search results, intracellular cAMP-levels of BA were measured using ELISA after specific stimulation of the H2-receptor with amthamine, a highly selective H2-receptor agonist (Saligrama et al. 2014).

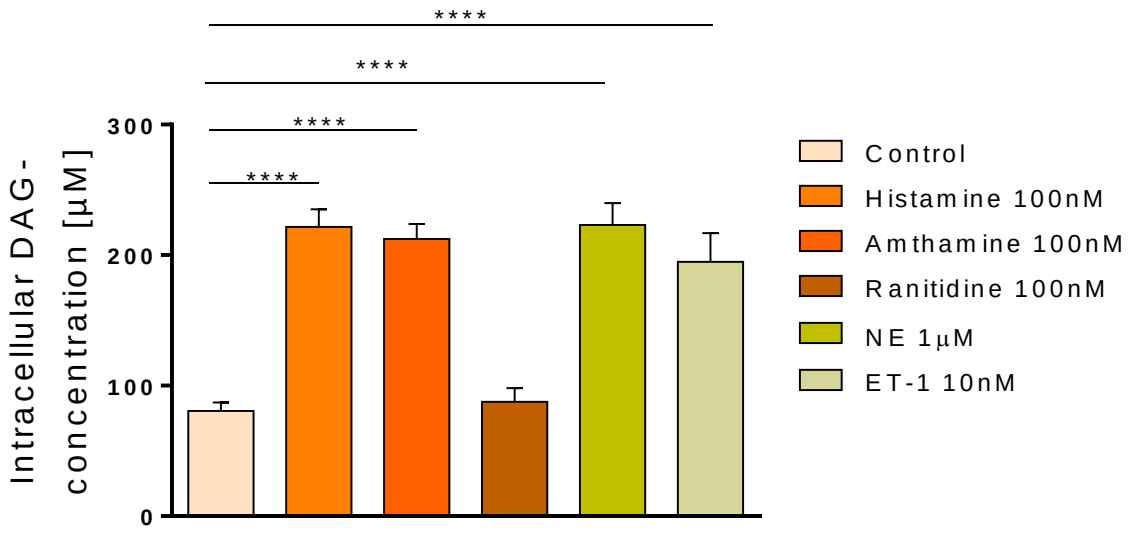

Fig. 24: Release of intracellular DAG [µM] in mature BA upon stimulation of the H2-receptor. Control (Ctrl.) was not treated. Other cells were acutely stimulated with histamine – [100 nM] - endogenous ligand of the H1-H4-receptors, amthamine [100 nM] – agonist of the H2-receptor, ranitidine [10 nM, 100 nM, 1000 nM] – antagonist of the H2-receptor, NE [1 µM] and ET-1 – agonist to the ETA-receptor [10 nM] for 16 hours, n=3, error bars, s.e.m., ANOVA-test, **** p< 0.0001

Acute stimulation of the H2-receptor with histamine [100 nM], endogenous ligand of the H1-H4-receptors, and amthamine [100 nM], agonist of the H2-receptor, significantly rose the intracellular DAG levels. Ranitidine [100 nM], antagonist of the H2-receptor, was used as negative control and did not alter the second messenger. ET-1 [10 nM] caused a strong

response with a 1.94-fold increase. NE [1 µM], likewise, elevated the intracellular DAG levels which could presumably be explained by the fact that Gαq-coupled alpha-adrenergic receptors are expressed in brown adipocytes (Kikuchi-Utsumi et al. 1997; Wu et al. 1992).

Next, the cAMP-Elisa is shown.

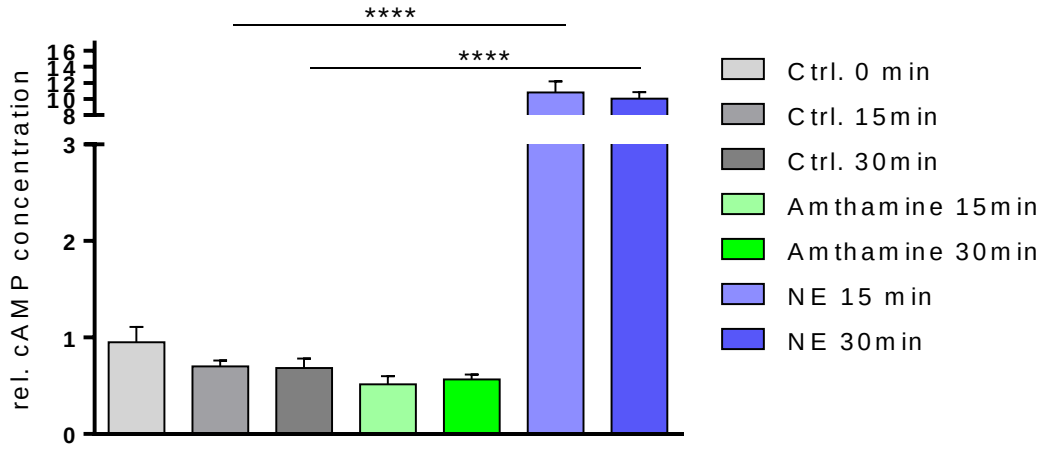

Fig. 25: Intracellular cAMP-levels in mature BA after 0-, 15- or 30-min stimulation with amthamine. Control (Ctrl.) was not treated, other cells were treated with amthamine [1000 nM] or NE [1 µM], mean with s.e.m, n=6, ANOVA-test, **** $p < 0.0001$

Amthamine had no effects on intracellular cAMP-levels (figure 25) as measured 15 and 30 minutes after treatment in comparison to the controls whereas NE [1 µM] significantly raised intracellular cAMP- levels 10.82-fold and 10.05-fold after 15 and 30 minutes, respectively.

In conclusion, stimulation of the H2-receptor significantly increased intracellular DAG levels whereas cAMP was unaltered by H2-receptor stimulation. Hence, the H2-receptor most likely couples to Gαq proteins and not to the Gαs-signaling pathway in BA.

3.5 Chronic effects of histamine on BA and WA differentiation

Histamine is a biogenic amine best known for regulating immune responses (O'Mahony et al. 2011) and the endogenous ligand of the H1-, the H2-, H3- and H4-receptors (O'Mahony et al. 2011). Until today, not much is known about the role of histamine signaling in brown adipocytes. Hence, initial experiments were performed to investigate the effect of histamine on BA differentiation.

Brown adipocytes

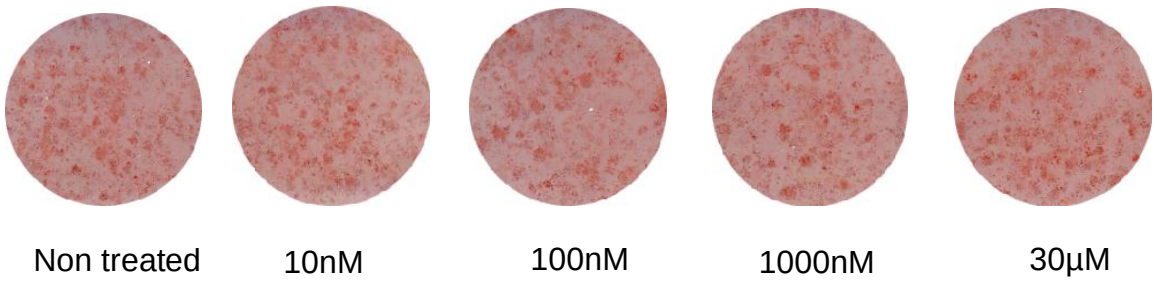

Fig. 26: Representative Oil-Red- O-Stain of BA [histamine].
Control was untreated. Other cells were chronically treated with histamine [10 nM, 100 nM, 1000 nM, 30 µM], n=3

Chronic histamine treatment at different concentrations did not have an noticable influence on the accumulation of triglycerides which is a hallmark of BA differentiation (figure 26).

Moreover, protein expression analysis of the adipogenic markers aP2 and PPARγ showed no effect of chronic histamine stimultion of BA (figure 27).

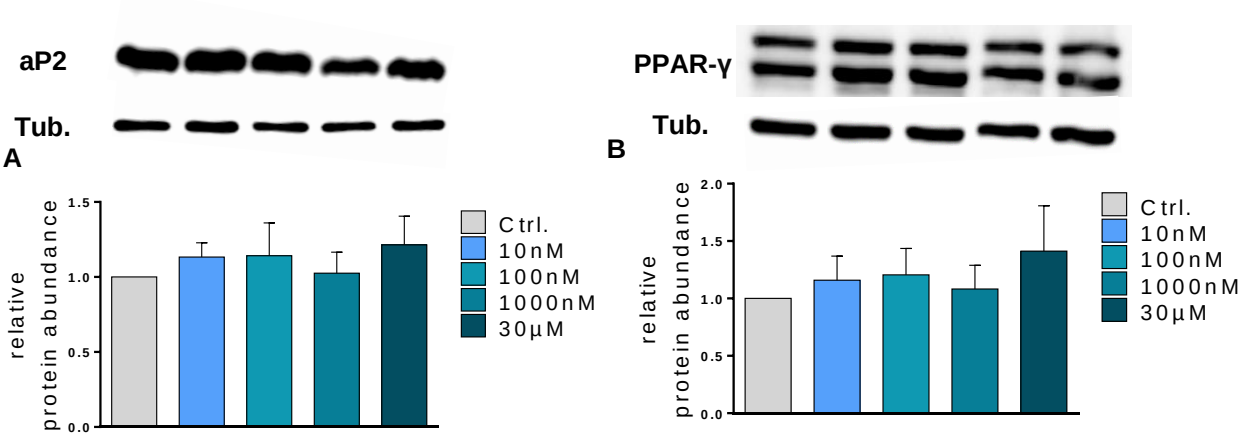

Fig. 27: Expression of aP2 (A) and PPAR-γ (B) on protein level in mature BA [histamine]. Control (Ctrl.) was not treated. Other cells were treated with histamine [10 nM, 100 nM, 1000 nM, 30 µM] during differentiation, expression levels were normalized to the control, mean with s.e.m, (A) n=4, (B) n=5, ANOVA -test, * $p < 0.05$

Analysis of the most important thermogenic marker protein UCP-1 revealed increased protein levels after chronic histamine treatment with 10 nM and 100 nM, albeit not significantly (figure 28) whereas higher concentrations seemed to have no effect.

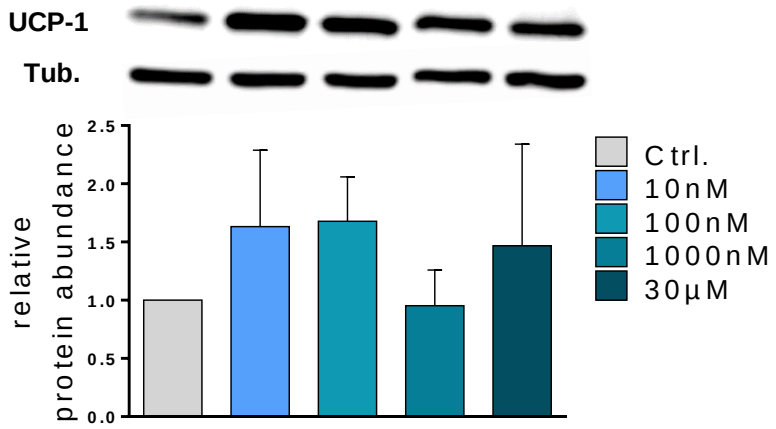

Fig. 28: Expression of UCP-1 on protein level in mature BA [histamine].
Control (Ctrl.) was not treated. Other cells were treated with histamine [10 nM, 100 nM, 1000 nM, 30 µM] during differentiation, expression levels were normalized to the control, mean with s.e.m, n=3, ANOVA-test, * $p < 0.05$

Summarizing, histamine had no significant effect on the protein expression of adipogenic and thermogenic marker genes (figure 27-28).

To analyze possible functional effects of histamine on BA, lipolysis was measured after acute treatment with histamine (figure 29). Low concentration of histamine elevated glycerol release of BA, albeit not significantly, whereas higher concentrations showed no effect.

Co-stimulation of BA with NE [1 µM] and histamine [10 nM, 30 µM] had no additive effect in comparison to NE treatment alone.

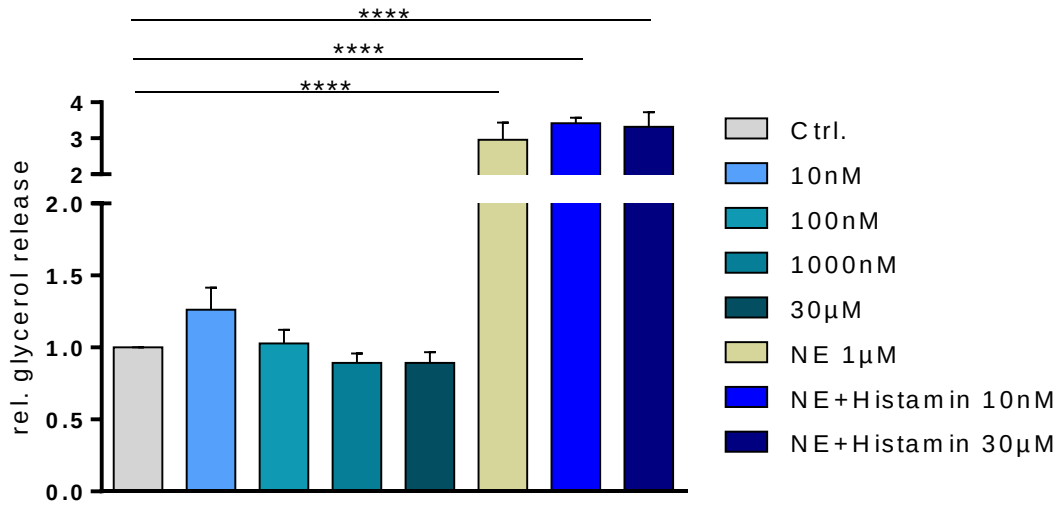

Fig. 29: Relative glycerol release in mature BA after stimulation with histamine. Control (Ctrl.) was not treated. Other cells were treated with histamine [10 nM, 100 nM, 1000 nM, 30 µM], NE [1 µM] n=4; NE [1µM] + histamine [10 nM] n=3, NE [1 µM] + histamine [30 µM] n=4, mean with s.e.m; n=4, ANOVA-test, **** $p < 0.0001$

Hence, acute treatment of BA with histamine does not influence brown fat cell function.

White adipocytes

Recent reports have described a role of histamine receptors in browning – the appearance of thermogenic, UCP-1-positive brown-like adipocytes – within WAT (Finlin et al. 2017; Zhao et al. 2019). To verify these findings and to analyze whether treatment with histamine alters the differentiation of WA, the subsequent experiments were performed.

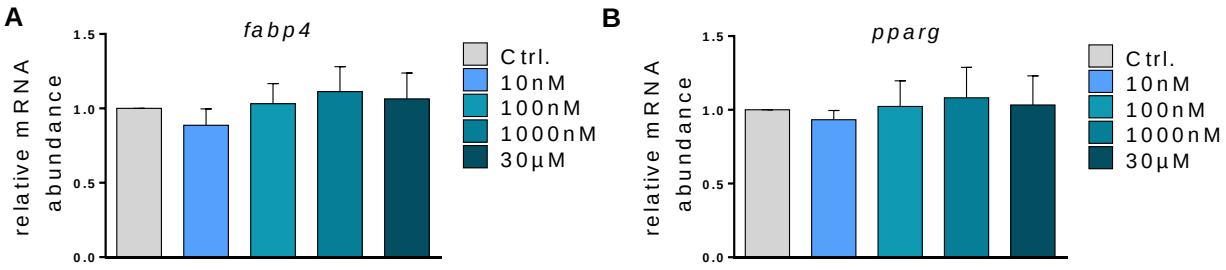

Fig. 30: Expression of aP2 (A) and PPAR-γ (B) on mRNA-level in mature WA [histamine]. Control (Ctrl.) was not treated. Other cells were treated with histamine [10 nM, 100 nM, 1000 nM, 30 µM] during differentiation, expression levels were normalized to the control, *fabp4* and *pparg* are the corresponding gene names for aP2 (A) and PPAR-ɣ (B), mean with s.e.m, (A) and (B): n=4, ANOVA-test, * $p < 0.05$

Chronic treatment of WA with histamine did not change mRNA expression levels of aP2 and PPAR-ɣ (figure 30). In contrast to the findings of the aforementioned publications, no

significant rise in UCP-1 mRNA expression was detectable. WA do not express UCP-1 basally. Hence, no mRNA expression could be detected at all in treated as well as untreated WA (data not shown)

In conclusion, chronic treatment with histamine did not have a significant effect on the expression of adipogenic marker genes aP2 and PPAR-γ. Moreover, histamine did not induce *in vitro* browning of WA (figure 30).

3.6 Chronic effects of amthamine on BA and WA differentiation

Given the presence of several histamine receptors in BA and WA, pharmacological tools were used next to specificly target the H2-receptor. Amthamine is a highly selective H2-receptor agonist (Saligrama et al. 2014) and the following experiments were performed to analyze the effects of amthamine on BA and WA differentiation and function.

<u>Brown adipocytes</u>

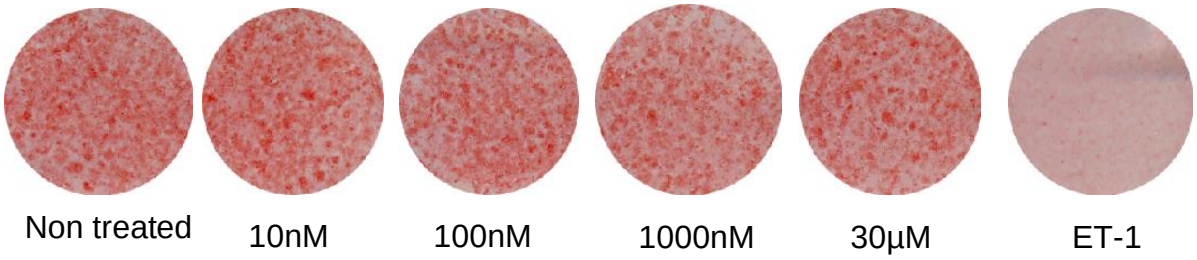

Fig. 31: Representative Oil-Red- O-Stain of BA [amthamine].
Control was untreated. Other cells were chronically treated with amthamine [10 nM, 100 nM, 1000 nM, 30 µM], ET-1 [10 nM] was used as positive control, n=3

Chronic treatment with amthamine at different concentrations did not have an observable effect on BA triglycerides accumulation (figure 31). After treatment with ET-1 [10 nM], a visually significant lower amount of triglycerides was aggregated in the cells.

Figure 32 displays the expression of aP2 on protein levels after chronic stimulation with amthamine. Protein levels were unchanged by amthamine. A decrease of aP2 expression was achieved on protein levels through treatment with ET-1 [10 nM], albeit non-significantly.

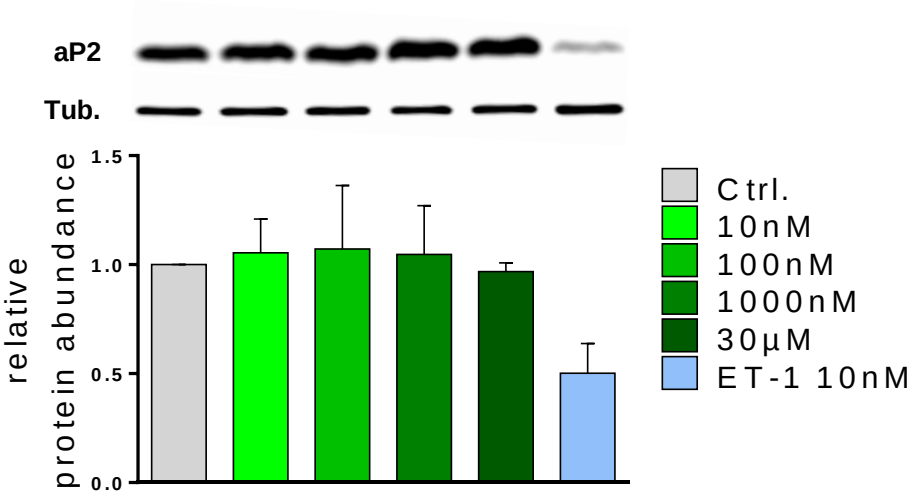

Fig. 32: Expression of aP2 on protein level in mature BA [amthamine]. Control (Ctrl.) was not treated. Other cells were treated with amthamine [10 nM, 100 nM, 1000 nM, 30 µM] or ET-1 [10 nM] during differentiation, expression levels were normalized to the control, mean with s.e.m, n=3, ANOVA-test, * $p < 0.05$

Similar results were obtained after analysis of PPAR-γ levels (figure 33): chronic treatment with amthamine did not induce a significant change in the expression of PPAR-γ in any tested condition. ET-1 [10 nM] diminished the protein levels of PPAR-γ by 56 %, albeit non-significantly.

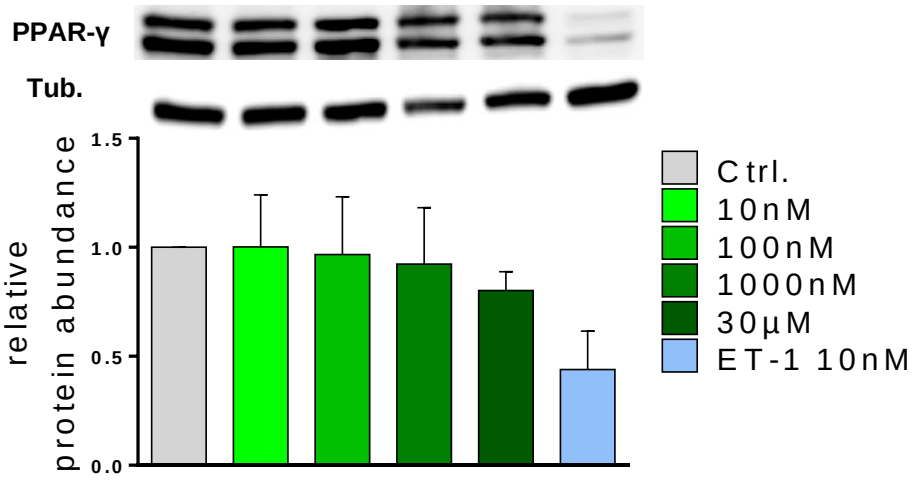

Fig. 33: Expression of PPAR-γ on protein level in mature BA [amthamine]. Control (Ctrl.) was not treated. Other cells were treated with amthamine [10 nM, 100 nM, 1000 nM, 30 µM] or ET-1 [10 nM] during differentiation, expression levels were normalized to the control, mean with s.e.m, n=3, ANOVA-test, * $p < 0.05$

Regarding the expression of UCP-1, protein levels were not significantly changed in comparison to control after treatment with amthamine. ET-1 [10 nM] induced a non-significant 57 % decrease in the expression of UCP-1 protein (figure 34).

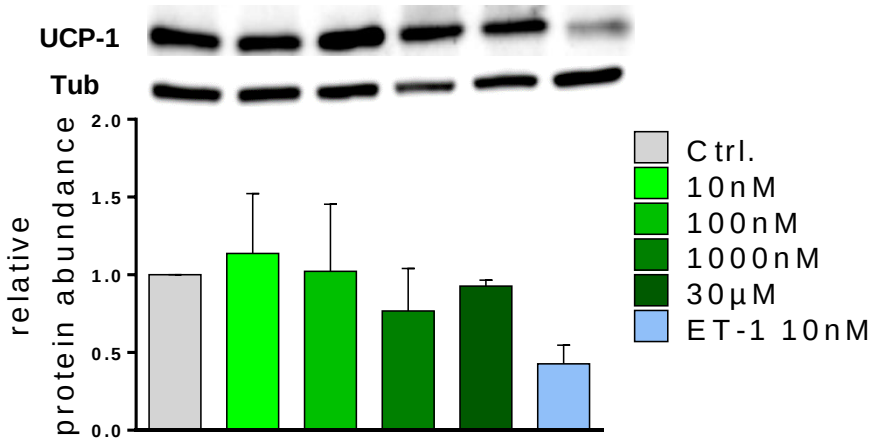

Fig. 34: Expression of UCP-1 on protein level in mature BA [amthamine].
Control (Ctrl.) is not treated. Other cells were treated with amthamine [10 nM, 100 nM, 1000 nM, 30 µM] or ET-1 [10 nM] during differentiation, expression levels were normalized to the control, mean with s.e.m, n=3, ANOVA-test, * $p < 0.05$

In conclusion, chronic treatment with different concentrations of amthamine did not affect differentiation of BA (figure 32-34).

Furthermore, I assessed whether acute stimulation with amthamine modulates BA function by measuring lipolysis (figure 35).

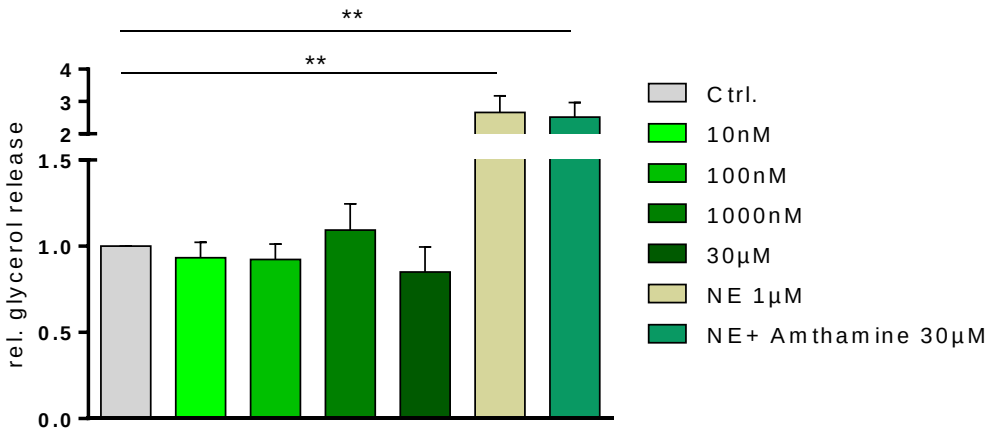

Fig. 35: Relative glycerol release in mature BA after stimulation with amthamine. Control (Ctrl.) was not treated. Other cells were treated with amthamine [10 nM, 100 nM,1000 nM, 30 µM], NE [1 µM] and NE [1 µM] + amthamine [30 µM], mean with s.e.m; n=3, ANOVA-test, ** $p < 0.01$

While NE [1 µM] significantly increased glycerol liberation, amthamine by itself as well as in combination with NE [1 µM] showed no effect (figure 35).

Summarizing, acute application of amthamine did not alter lipolytic activity of mature BA (figure 35).

White adipocytes

As described before, adipogenic and thermogenic markers were analyzed to investigate the effect of amthamine on WA differentiation.

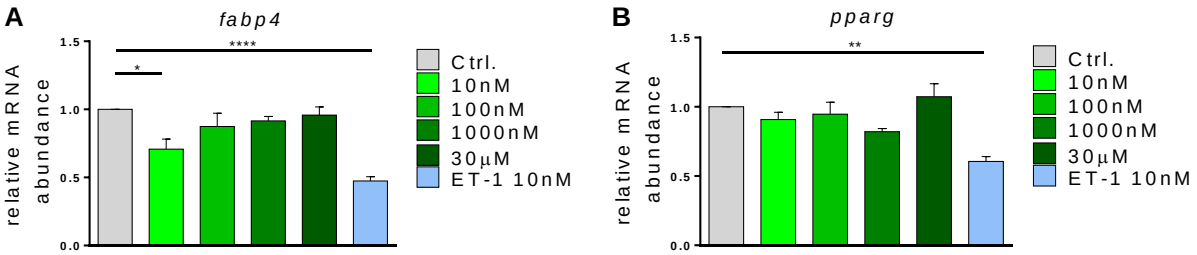

Fig. 36: Expression of aP2 (A) and PPAR-γ (B) on mRNA-level in mature WA [amthamine]. Control (Ctrl.) was not treated. Other cells were treated with amthamine [10 nM, 100 nM, 1000 nM, 30 µM] or ET-1 [10 nM] during differentiation, expression levels were normalized to the control, *fabp4* and *pparg* are the corresponding gene names for aP2 and PPAR-γ mean with s.e.m; (A) and (B): n=4, amthamine [1000 nM] (A) and (B) n=3, ANOVA-test, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$

Interestingly, amthamine at low nanomolar concentrations [10 nM] caused a significant downregulation of aP2 mRNA by 29 % (figure 36) whereas higher nanomolar concentrations did not have an observable effect. Chronic H2-receptor stimulation with amthamine did not alter PPAR-ɣ mRNA (figure 36) expression and did not induce UCP-1 expression (data not shown). ET-1 [10 nM] significantly decreased the expression of aP2 and PPAR-γ mRNA by 53 % and 39 %.

3.7 Chronic effects of ranitidine on BA and WA differentiation

Ranitidine is a selective H2-receptor-antagonist and is in clinical use to prevent gastric stress ulcer disease (Alhazzani et al. 2013). The effect of ranitidine on differentiation and function of BA and WA were analyzed in subsequent experiments.

Brown adipocytes

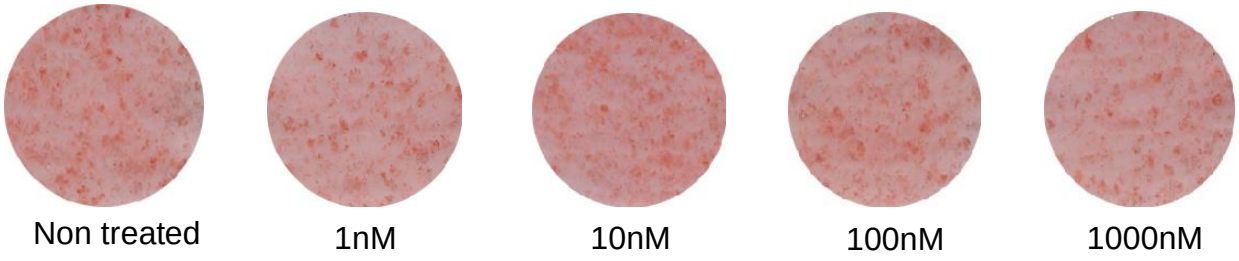

Fig. 37: Representative Oil-Red-O-Stain of BA [ranitidine].
Control was untreated. Other cells were chronically treated with ranitidine [1, 10, 100, 1000 nM], n=3

Initially, lipid droplet accumulation was analyzed after chronic treatment with ranitidine at different concentrations. No effect of ranitidine could be observed (figure 37).

On molecular level, ranitidine upregulated aP2 protein expression at all tested conditions, albeit non significantly (figure 38). FR - an inhibitor of Gαq protein signaling and known enhancer of BA differentiation (Klepac et al., 2016) – non-significantly raised the expression of aP2 on protein level by 84 %.

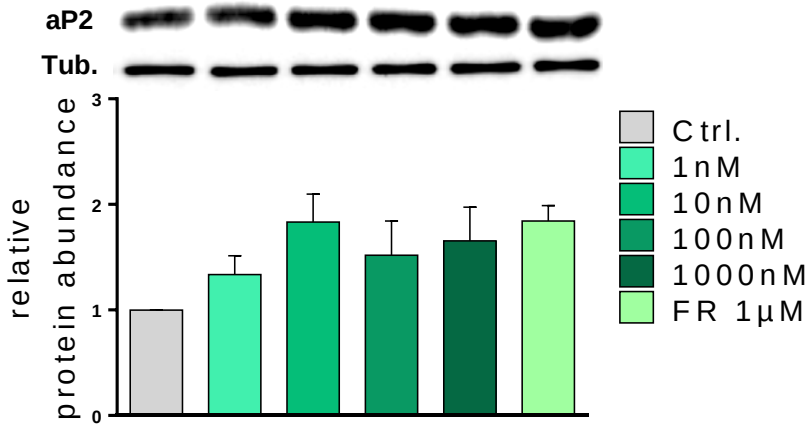

Fig. 38: Expression of aP2 on protein level in mature BA [ranitidine].
Control (Ctrl.) was not treated. Other cells were treated ranitidine [1 nM, 10 nM, 100 nM, 1000 nM] or FR [1 µM] during differentiation, expression levels were normalized to the control, mean with s.e.m, n=4, ANOVA-test, * $p < 0.05$

Ranitidine had no statistically significant effect on PPAR-ɣ protein levels, while FR [1 µM] significantly raised PPAR-ɣ protein expression by 101 % (figure 39).

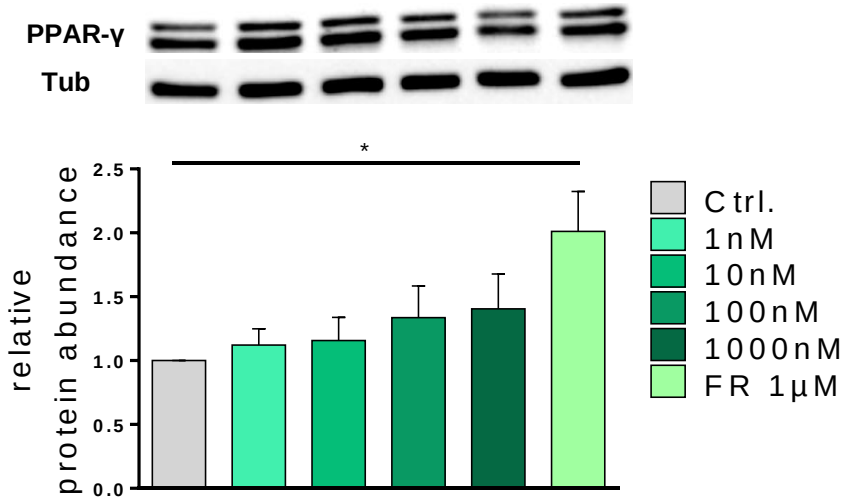

Fig. 39: Expression of PPAR-γ on protein level in mature BA [ranitidine].
Control (Ctrl.) was not treated. Other cells were treated with ranitidine [1 nM, 10 nM, 100 nM, 1000 nM] or FR [1 µM] during differentiation, expression levels were normalized to the control, mean with s.e.m, n=5, ANOVA-test, * $p < 0.05$

Moreover, UCP-1 protein expression was unchanged by ranitidine independent of concentration (figure 40), while FR [1 µM] caused a significant upregulation of 87 % on UCP-1 protein expression levels.

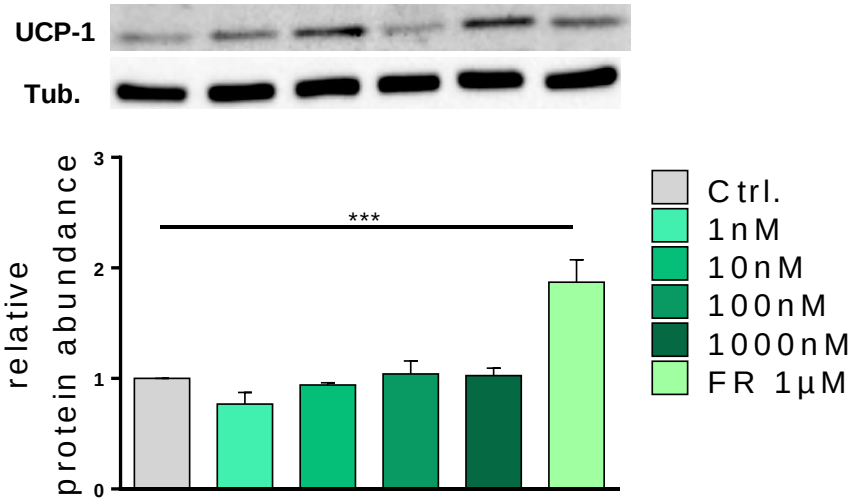

Fig. 40: Expression of UCP-1 on protein level in mature BA [ranitidine]. Control (Ctrl.) was not treated. Other cells were treated with ranitidine [1 nM, 10 nM, 100 nM, 1000 nM] or FR [1 µM] during differentiation, expression levels were normalized to the control, mean with s.e.m, n=3, ANOVA-test, *** $p < 0.001$

In conclusion, chronic treatment with ranitidine in different concentrations non-significantly enhanced the expression of aP2 (figure 38) while expression of PPAR-γ (figure 39) and UCP-1 (figure 40) was unaffected.

Next, the effect of ranitidine on BA function was analyzed. No effect of ranitidine on glyceroly release was detected, independent of concentration (figure 41), while NE [1 µM] induced a 3.4-fold increase of lipolysis in comparison with the control. Treatment of BA with ranitidine together with NE had no additive effect on lipolysis.

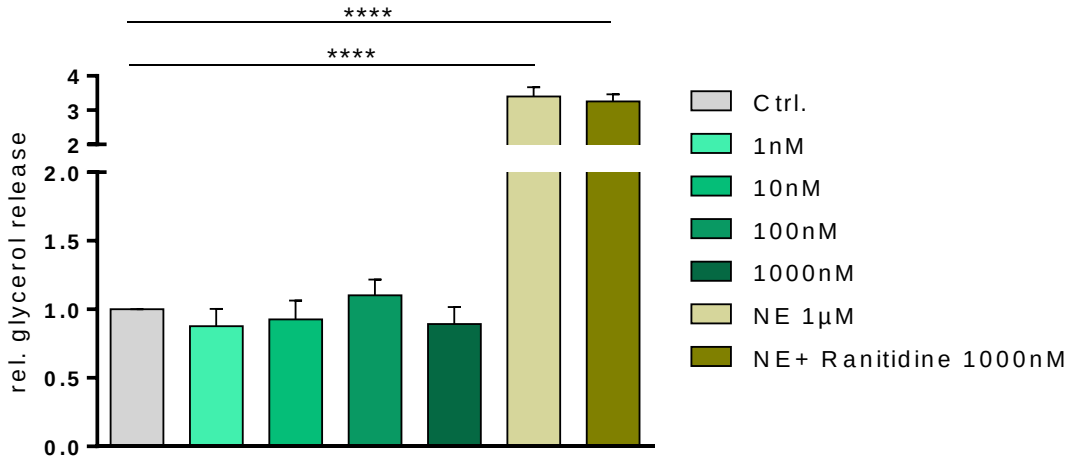

Fig. 41: Relative glycerol release in mature BA after stimulation with ranitidine. Control (Ctrl.) was not treated. Other cells were treated with ranitidine [1 nM, 10 nM, 100 nM, 1000 nM], NE [1 µM] and NE [1 µM] + ranitidine [1000 nM], mean with s.e.m; n=3, ANOVA-test, **** $p < 0.0001$

White adipocytes

So far, nothing has been described about the effect ranitidine on the differentiation of WA. Thus, the following experiments were performed.

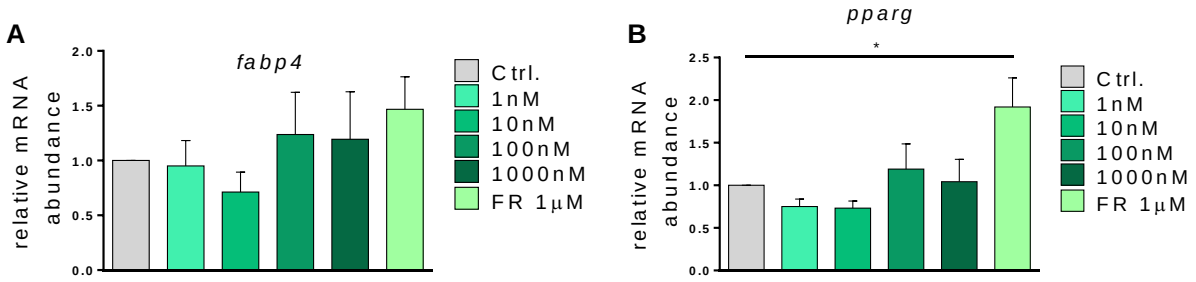

Fig. 42: Expression of aP2 (A) and PPAR-γ (B) on mRNA-level in mature WA [ranitidine]. Control (Ctrl.) was not treated. Other cells were treated with ranitidine [1 nM, 10 nM, 100 nM, 1000 nM] or FR [1 µM], expression levels were normalized to the control, *fabp4* and *pparg* are the corresponding gene names for aP2 (A) and PPAR-ɣ (B), mean with s.e.m; (A) and (B): n=3, ANOVA-test, * $p < 0.05$

In WA, treatment with ranitidine had no clear effect on the expression of aP2 and PPAR-γ mRNA (figure 42) while FR [1 µM] upregulated PPAR- γ mRNA by 91 %. Ranitidine did not induce the expression of UCP-1 mRNA in WA (data not shown).

Summarizing, chronic stimulation of WA with ranitidine did not affect the adipogenic differentiation measured by aP2 and PPAR-γ expression and did not induce the expression of the thermogenic protein UCP-1.

3.8 Histamine and tachykinin release in brown adipocytes

In recent years, the understanding of BAT as a secretory organ has become more evident. BAT-derived adipokines play a major role in the communication between different cell types within BAT and can enhance and suppress activity of BA (Scheideler et al. 2017, Villarroya et al. 2017). So far there is no knowledge whether BA release tachykinin and histamine in an autocrine or paracrine fashion. For a comprehensive understanding of BAT's secretory function, it is of great interest to analyze whether BA release those signaling molecules.

Thus, I conducted a tachykinin-Elisa comparing brown preadipocytes and mature BA treated with and without NE [1 µM] (figure 43).

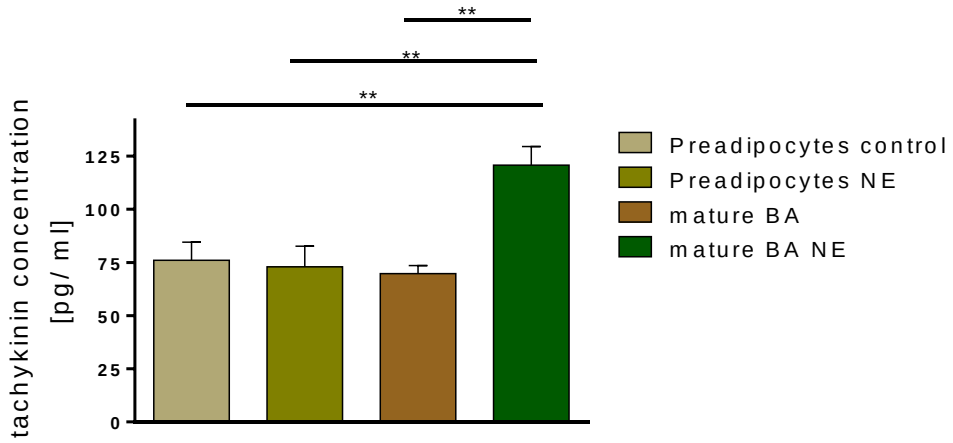

Fig. 43: Release of tachykinin [pg/ ml] to the cell culture medium in premature and mature BA. Control (Ctrl.) was not treated. Other cells were acutely stimulated with NE [1 µM] for 16 hours, supernatant was collected on day -2 (preadipocytes) and day 7 (mature BA), n=3, error bars, s.e.m., ANOVA-test, ** $p < 0.01$

Interstingly, brown preadipocytes as well as mature BA secreted tachykinin *in vitro* (figure 43). Although the expression of the NK3-receptor declines throughout differentiation (see figure 4), the amount of tachykinin liberated by the cells to the cell culture medium is stable between preadipocytes and mature BA. While NE treatment of predipocytes had no effect on tachykinin release, sympathetic stimulation of mature BA significantly elevated tachykinin levels (figure 43)

Hence, treatment of mature BA with NE, the physiological activator of BAT, results in elevated tachykinin release.

Next, I carried out a histamine ELISA (figure 44) to scrutinize whether adipocytes secrete histamine and to examine the difference in the secretion upon acute stimulation with NE [1 µM].

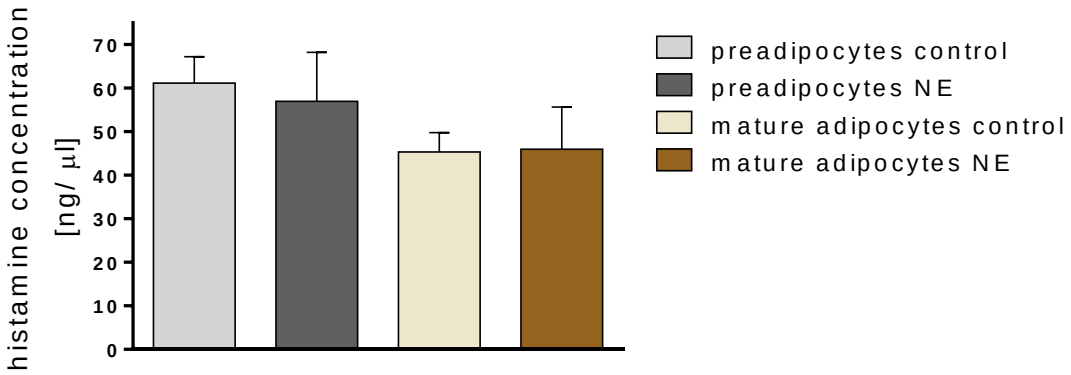

Fig. 44: Release of histamine [ng/ µl] to the cell culture medium in premature and mature BA. Control was not treated. Other cells were acutely stimulated with NE [1 µM] for 16 hours, supernatant was collected on day -2 (preadipocytes) and day 7 (mature BA) n=3, error bars, s.e.m., ANOVA-test, * p< 0.05

Preadipocytes as well as mature adipocytes secreted histamine in vitro (figure 44) and the amount of histamine released to the cell culture medium declined non-significantly throughout differentiation. In contrast to tachykinin (figure 43), treatment of brown preadipocytes as well as mature BA with NE had no additive effect on histamine release (figure 44).

Summarizing, I could show that BA secrete tachykinin and histamine in vitro (figure 43, 44). Stimulation with NE significantly elevates the secretion of tachykinin in mature BA whereas histamine secretion is unaltered by NE. Together, these data indicate that tachykinin as well as histamine could signal in auto/ paracrine manner in BAT.

3.9 Influence of the feeding regimen on NK3- and H2-receptor expression in murine adipose tissue

To further improve our understanding of the NK3- and H2-receptors in the development of obesity, I investigated the expression levels of both receptors in BAT and WAT of lean and obese mice.

While robust expression of both receptors could be detected in BAT of 20-week-old mice fed a normal (ND) or high fat diet (HFD) for 12 weeks, no effect of the feeding regimen could be detected (figure 45).

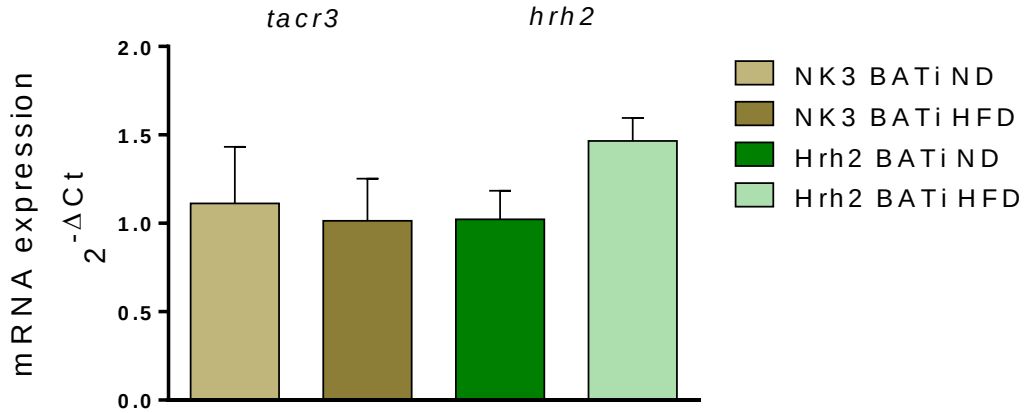

Fig. 45: Expression of NK3- and H2-receptor mRNA in murine BATi.
Eight week male C57BL/6J mice were either fed a normal (ND) or high fat diet (HFD) containing 60 % fat for 12 weeks, mean with s.e.m, NK3 BATi ND n=3, NK3 BATi HFD n=4, Hrh2 BATi ND n=3, Hrh2 BATi ND n=5, *hprt* served as housekeeping gene, mRNA expression was computed by the $2^{-\Delta CT}$-method, t-test, NK3/ Hrh2 BATi ND and HFD were tested against each other, * $p < 0.05$, *tacr3* and *hrh2* are the corresponding gene names for the NK3- and H2-receptor

In contrast, analysis of inguinal (subcutaneous) WAT (WATi), which has a high capacity for browning (Sidossis et al. 2015; Barbatelli et al. 2010), revealed a downregulation of both receptors upon HFD feeding of mice (figure 46), albeit not significantly.

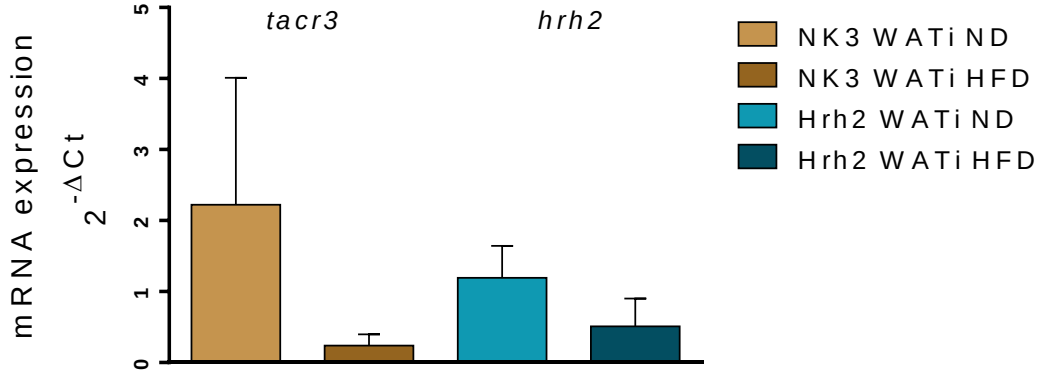

Fig. 46: Expression of NK3- and H2-receptor mRNA in murine WATi.
Eight week male C57BL/6J mice were either fed a normal (ND) or high fat diet (HFD) containing 60 % fat for 12 weeks, mean with s.e.m, NK3 WATi ND n=3, NK3 WATi HFD n=3, Hrh2 WATi ND n=4, Hrh2 WATi HFD n=3, *hprt* served as housekeeping gene, mRNA expression was computed by the $2^{-\Delta CT}$-method, t-test, NK3/ Hrh2 WATi ND and HFD were tested against each other, * $p < 0,05$, *tacr3* and *hrh2* are the corresponding gene names for the NK3- and H2-receptor

In WATi, HFD feeding of mice induced diminished NK3-receptor mRNA expression and elevated H2-receptor mRNA expression compared to mice fed a normal diet (figure 47). These effects were not statistically significant, though.

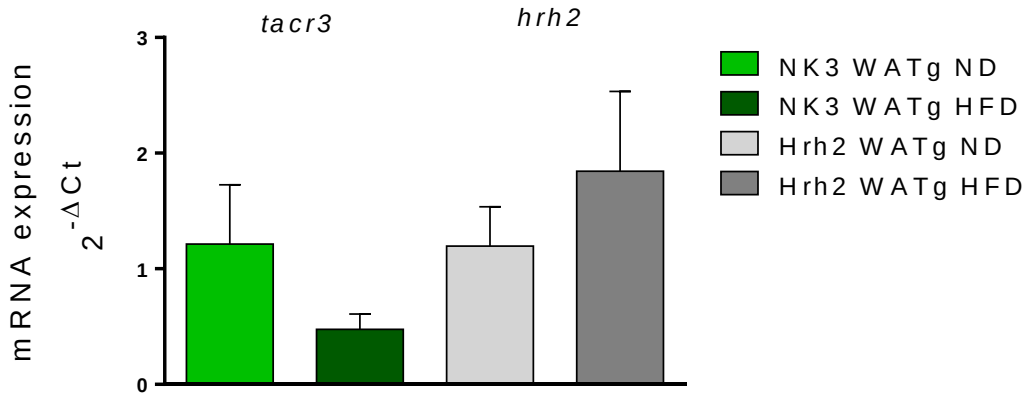

Fig. 47: Expression of NK3- and H2-receptor mRNA in murine WATg.
Eight week male C57BL/6J mice were either fed a normal (ND) or high fat diet (HFD) containing 60 % fat for 12 weeks, mean with s.e.m, NK3 WATg ND n=3, NK3 WATg HFD n=3, Hrh2 WATg ND n=4, Hrh2 WATg HFD n=3, *hprt* served as housekeeping gene, mRNA expression was computed by the $2^{-\Delta CT}$-method, t-test, NK3/ Hrh2 WATg ND and HFD were tested against each other, * p< 0.05, *tacr3* and *hrh2* are the corresponding gene names for the NK3- and H2-receptor

Overall, it was demonstrated that the H2- and NK3-receptor are expressed in all major fat depots (figure 45-47) and that feeding a high fat diet might downregulate the expression of both receptors in inguinal WAT. In gonodal WAT, HFD feeding of mice might provoke a downregulation of the NK3-receptor and an upregulation of the H2-receptor.

4. Discussion

The Gαq-signaling pathway plays a major role in the differentiation of BA as constitutive Gαq-signaling is a negative regulator of brown adipocyte differentiation (Klepac et al. 2016). Thus, inhibition of Gαq-signaling is an intriguing target to elevate brown adipocyte differentiation and thereby whole-body energy expenditure. Strikingly, the expression levels of the Gαq-coupled NK3-receptor (figure 4) and H2-receptor mRNA (figure 21) are significantly regulated during the differentiation of BA. There is little knowledge about their physiological role in brown and white adipose tissue. Therefore, pharmacological studies were performed to investigate the NK3- and H2- receptor in BA and WA and to evaluate their effects on the differentiation and functions of the cells.

4.1 Neurokinin3- receptor in BA and WA

NK3-receptor expression could be demonstrated in preadipocytes and mature adipocytes in BA and WA (figure 4 and 5). In BA, the NK3-receptor mRNA was significantly downregulated by 49 % during differentiation whereas in WA there was no significant regulation of receptor mRNA expression.

4.1.1 Gαq-signaling of the NK3- receptor in BA

I hypothesized that the NK3-receptor is Gαq-coupled in BA as it has been demonstrated that the NK3-receptor is Gαq-coupled in other tissues (Khawaja and Rogers 1996). Pharmacological stimulation with senktide and the endogenous ligand neurokinin b at low nanomolar concentrations led to increased amount of intracellular DAG (figure 6). DAG is a well-established second messenger of the Gαq-signaling pathway (Neves et al. 2002). This data confirms the previous findings that the NK3-receptor is at least partially coupled to the Gαq-signaling pathway in BA.

4.1.2 Chronic effects of senktide on BA and WA differentiation

Senktide is a potent agonist (Endo et al. 2015, Stoessl et al. 1988) of the NK3-receptor and upon chronic treatment with senktide, a decline of differentiation markers was expected.

Brown adipocytes

Senktide had no clear and obvious effect on lipid accumulation within BA (figure 7). Interestingly, low concentrations of senktide [10 nM] significantly increased the expression of PPAR- γ on protein levels (figure 9) but not at higher nanomolar concentrations. No significant alterations of aP2 and UCP-1 expression (figure 8 and 10) was observed upon sustained BA stimulation with senktide. The significant upregulation of PPAR-ɣ was surprising as a downregulation was priorly expected. Thus, I assumed that the NK3-receptor might be interacting with an additional signaling pathway. Interestingly, coupling of one receptor is not strictly limited to one G-protein as it was already demonstrated some time ago that a single receptor can couple to multiple G protein in the same cell line (Liebmann and Böhmer 2000). Interaction between the NK3-receptor with the Gαs-signaling pathway could provide an adequate explanation for the upregulation of PPAR-γ protein. As previously illustrated, Gαs-coupled receptors are key regulators of adipose tissue differentiation as they for instance enhance BA differentiation (Cannon and Nedergaard 2004, Gnad et al. 2014). Stimulation of those Gαs-coupled receptors leads to increased level of the second messenger cAMP (Neves et al. 2002). Hence, coupling of the NK3-receptor with the Gαs-signaling pathway could provide an adequate explanation for the upregulation of PPAR-γ protein.

To test this hypothesis, I performed a cAMP-ELISA (figure 12).

After stimulation with senktide there was no intracellular rise of cAMP (figure 12) detectable, though. Therefore, interference of the NK3-receptor with Gαs-signaling pathway seems unlikely. More testing is warranted to unveil how these effects occur as there are a plethora of further G proteins involved in cell signaling (Syrovatkina et al. 2016). Interestingly, the $G_{\beta\gamma}$-subunit as well as the G_{11}-molecule and the $G_{12/13}$-molecule are ubiquitously expressed within cells, however their influence on differentiation and function of BA is still not very well examined. The aforementioned signaling pathways play a decisive role in general cell metabolism like regulation of adenylyl cyclases and regulation of the Erk1/2 MAP kinase signaling pathway (Birnbaumer 2007). It is conceivable that the interplay between the NK3-receptor with a further G protein regulated signaling pathway besides the established and confirmed Gαq-signaling pathway is responsible for the upregulation of the PPAR-ɣ protein after chronic treatment of BA with

senktide. To prove that concept, further experiments are necessary to determine the effect of those additional signaling pathways on the differentiation of BA.

To investigate the effects of the upregulation of PPAR-γ after chronic treatment with senktide on the differentiation of BA more precisely, CIDE-A, Perilipin and PGC1-α (figure 11) as downstream targets of PPAR-γ were examined (Arimura et al. 2004; Lasar et. al 2018; Lee et. al 2014; Savage 2005; Viswakarma et al. 2007). However, there was no significant regulation of the aforementioned markers upon treatment of BA with senktide (figure 11). Hence, these data fail to support the findings of the upregulation of the PPAR-γ protein. PPAR-γ, though, influences a broad range of additional genes (Fang et al. 2016) so that further genes which are regulated by PPAR- γ should be looked at.

Neither acute stimulation of BA with senktide nor co-stimulation with NE showed significant effects (figure 13) on the glycerol release which is a common benchmark for lipolysis. The data indicate that senktide is not capable of rapidly changing lipolysis in BA and is not able to booster the positive effects of NE on lipolysis.

In conclusion, senktide caused a significant upregulation of PPAR-ɣ on protein levels which was surprising and cannot be fully explained by these results. The overall effect of senktide on BA differentiation, however, was modest and senktide had no effect on the function of BA. It would be interesting to observe the effects of a complete receptor knockdown model or the overexpression of the NK3-receptor in an *in vivo* model on adipose tissue.

<u>White adipocytes</u>

I could show that WA express the NK3-receptor (figure 5) and that there is no significant regulation throughout differentiation.

No significant effects on the expression of aP2 and PPAR-ɣ were observable after chronic treatment with senktide in WA and no induction of UCP-1 was noted (figure 14). UCP-1 is not commonly synthesized in WA and its expression relies on so called browning agents (Merlin et al. 2018). Thus, it is unlikely that stimulation of the NK3-receptor with senktide alters the adipogenic differentiation of WA and is capable of enrolling a thermogenic gene expression pattern.

However, further testing is warranted and *in vitro* browning of WA with stimulation of the NK3-receptor is an intriguing experiment to be conducted in the future.

4.1.3 Chronic effects of SB223412 on BA and WA differentiation

SB223412 is an antagonist of the NK3-receptor (Sarau et al. 1997) and treatment with SB223412 was expected to induce an upregulation of differentiation markers in BA and WA due to its antagonism to the Gαq-coupled NK3- receptor (Khawaja and Rogers 1996, Klepac et al. 2016).

Brown adipocytes

SB223412 had no observable effect on the lipid accumulation visualized by the Oil-Red-O-Stain (figure 15). SB223412 in higher nanomolar concentrations, however, non-significantly elevated the expression of aP2 protein (figure 16) whereas PPAR-ɣ and UCP-1 protein expression (figure 17-18) was not changed. This influence was in line with the prior expected effects as an upregulation of differentiation markers was hypothesized. Low receptor protein abundance of the NK3-receptor could be accountable for the minimal changes observed after chronic administration of SB223412 and senktide on BA differentiation.

While acute administration of SB223412 alone did not alter the relative glycerol release (figure 19), co-stimulation with NE, elevated the activating effects of NE stimulation by 34 % in comparison to NE alone. Hence, NK3-antagonism together with NE co-stimulation could positively regulate lipolysis in BA. However, the underlying mechanism is unclear. Regulation of lipolysis has thus far been linked to the Gαs-signaling pathway via the cAMP, PKA and HSL- axis in BA (Cannon and Nedergaard 2004) and previous studies (Klepac et al. 2016) as well as the collected data in this thesis did not suggest a role of the Gαq-signaling pathway in modulation of lipolysis. However, conflicting data have been published with one study (Schilperoort et. al 2018) proposing a role of the Gαq-signaling pathway and its stimulation in promoting fat oxidation in BA. Concluding, SB223412 in co-stimulation with NE might regulate lipolysis in BA by blocking the Gαq-signaling pathway and thereby alleviating the detrimental effects of enhanced Gαq-signaling but further testing is warranted to mechanistically elucidate this effect.

Summarizing, SB223412 non-significantly upregulated aP2 protein expression at high nanomolar concentrations but the overall effect of SB223412 on the differentiation of BA was modest. SB223412 in co-stimulation with NE boosted the relative glycerol release whereas SB223412 alone was not capable of changing the state of lipolysis. Overall, SB223412 seems no intriguing drug for the treatment of obesity.

White adipocytes

In WA, there were no significant alterations in aP2 and PPAR-γ expression (figure 20). Furthermore, treatment of WA with SB223412 could not induce the production of the UCP-1 and as stated beforehand WA rely on so called browning agents for the synthesis of UCP-1 (Merlin et al. 2018). Hence, the data indicate that treatment of WA with SB223412 cannot initiate a thermogenic gene expression profile and that collectively with the data obtained after stimulation of WA with senktide (see 4.1.2) the NK3-receptor does not regulate WA differentiation. A potential reason could be low NK3-receptor levels in WA. Nonetheless, it would be interesting to observe the effects of *in vitro* browning of WA with blockage of the NK3-receptor.

4.2 Histamine receptors in BA and WA

Recent findings demonstrated the expression of all four histamine receptor subtypes (H1-H4-receptors) in BAT, gonadal and subcutaneous WAT (Zhao et al. 2019). The results (figure 21-23) were partially consistent with previous results as the H1-, H2- and H4-receptors were expressed in premature and mature brown and white adipocytes. Moreover, they illustrated a non-significant tendency to be upregulated through differentiation except for the H2-receptor in BA which showed a significant 5.87-fold increase in relative receptor abundance. However, contradictory to research of Zhao (Zhao et al. 2019), I could not show an effective expression of the H3-receptor in BA nor in WA (data not shown).

4.2.1 Gαq-signaling of the H2-receptor in BA

As previously illustrated, the H2-receptor can be found in a broad range of tissues, where it couples to the $G\alpha_s$-signaling pathway in a majority of cases. However, according to the IUPHAR database (http://www.guidetopharmacology.org/) the H2-receptor was classified

to be Gαq-coupled in BA. Hence, I wanted to prove to which signaling pathway the H2-receptor couples in BA by conducting a DAG- and a cAMP-Elisa (figure 24, 25) as established second messengers of the Gαq- and Gαs-signaling pathway, respectively (Neves et al. 2002). Stimulation with histamine and amthamine led to a significant increase in intracellular DAG (figure 24) whereas acute stimulation of the BA with amthamine did not change intracellular cAMP formation (figure 25). These data indicate that the H2-receptor couples to the Gαq-signaling pathway in BA and does not interact with the Gαs-signaling pathway. In line with this conclusion are the observed effects of treatment with amthamine and ranitidine on the differentiation of BA which are in conformity with the anticipated effects of enhanced Gαq-signaling (Klepac et al. 2016).

4.2.2 Chronic effects of histamine on BA and WA differentiation

Histamine is the endogenous ligand of the four histamine receptors H1-H4-receptors (O'Mahony et al. 2011) and pharmacological experiments with BA and WA were carried out to observe the effects of histamine on BA and WA differentiation.

Brown adipocytes

Chronic treatment with histamine had no comprehensive and significant effect on BA differentiation measured by aP2, PPAR-ɣ and UCP-1 protein expression (figure 27-28) albeit a non-significant increase of UCP-1 protein expression in low concentrations. Various reasons could be accountable for that which are described below. Brown adipocytes express the range of different histamine receptors and the histamine receptors do not only differ in the effects they exert on the various tissues but they couple likewise to different signaling pathways (Karlstedt et al. 2003). The H1-receptor is described to couple to the Gαq/11-protein (Panula et al. 2015). According to the IUPHAR database (http://www.guidetopharmacology.org/), the H4-receptor couples to the Gαi/o-signaling pathway and the H2-receptor to the Gαq-signaling pathway. Therefore, the possible effects of treatment with histamine could have nullified due to the variety of signaling pathways the different histamine receptors interact with. Furthermore, the expression of the histamine receptor mRNA might be regulated so that the abundance of the receptor protein is not sufficient for a greater influence on the differentiation of BA (Schaefke et al.

2018). Last but not least, histamine might not be involved in BA differentiation at all. Hence, no effect of chronic treatment of BA with histamine was observable.

With regard to the function of BA, acute stimulation of BA with histamine and histamine in co-stimulation with NE did not significantly affect the glycerol release in comparison with NE [1µM] (figure 29). The relative glycerol release is a hallmark of lipolysis and hence a functional parameter of BA. Therefore, I can conclude that histamine is not able to acutely change the state of lipolysis in BA.

In conclusion, histamine had no greater influence on BA differentiation and function and does not constitute intriguing potential as pharmacological therapy for obesity.

White adipocytes

It has been previously demonstrated that mast cells are important regulators of WA browning as histamine released by mast cells can initiate a significant upregulation of UCP-1 mRNA expression in WA (Finlin et al. 2017, Zhang et al. 2019). Moreover, aP2 expression is significantly regulated by histamine in WA (Zeng et al. 2007). In this study, however, chronic treatment with histamine did not influence the expression of either marker in WA (figure 30) and failed to support the previous established findings. PPAR-ɣ was likewise unaltered by treatment with histamine (figure 30).

There might be multiple underlying reasons why no significant upregulation of aP2 and UCP-1 mRNA expression was observed. The extended treatment with histamine over a course of 7 days can neutralize the effects observable if acute treatment is applied. Zeng et al. could show that treatment with histamine [20/ 30 µM] induces a maximum increase in aP2 and UCP-2 expression in WA after 6 hours (Zeng et al. 2007). In this study, experiments on WA were performed 24 hours after the last stimulation with histamine so that the maximum effects observable in the mentioned paper are not within the time frame of our experiments.

Moreover, Finlin et al. used a conditioned medium of cold shocked mast cells to treat their WA in order to examine the alterations of treatment with histamine on UCP-1 mRNA expression (Finlin et al. 2017). Thus, it is unclear which substances are contained in the conditioned medium and whether it is necessary that histamine is given alongside possible

other substances contained in the medium to observe the upraise in UCP-1 mRNA expression.

An interesting experiment to be conducted in the future is *in vitro* browning of WA with stimulation of WA by histamine.

4.2.4 Chronic effects of amthamine on BA and WA differentiation

Amthamine is a potent agonist of the H2-receptor (Saligrama et al. 2014). As the H2-receptor was priorly described to be Gαq-coupled, a decline in differentiation was expected upon chronic treatment with amthamine.

Brown adipocytes

In brown adipocytes, chronic treatment with different concentrations of amthamine had no obvious effect on BA triglyceride accumulation (figure 31) nor on protein expression of aP2, PPAR-γ and UCP-1 (figure 32-34). Hence, these data indicate that amthamine and H2-receptor stimulation does not regulate BA differentiation. Low H2-receptor expression levels in BA could be accountable for these results.

Furthermore, neither amthamine alone nor in co-stimulation with NE had a significant effect on the release of glycerol (figure 35). Hence, I can conclude that amthamine does not significantly change the state of lipolysis in BA.

In summary, amthamine did not change the state of differentiation of BA measured by aP2, PPAR-ɣ and UCP-1 protein expression. In addition, amthamine was not capable of acutely altering the state of lipolysis in BA. Therefore, amthamine constitutes no promising target in regulating BA differentiation and function.

White adipocytes

In WA, chronic treatment with amthamine [10 nM] induced a modest decline in the aP2 mRNA expression in WA whereas higher nanomolar concentrations did not regulate aP2 expression (figure 36). Furthermore, amthamine failed to induce UCP1-expression. The downregulation of aP2 was consistent with the prior expectations and these findings propose that H2-receptor stimulation at low nanomolar concentration might weaken adipogenic differentiation of WA. Moreover, the results suggest that amthamine fails to

initiate a thermogenic gene expression profile in WA. Minor H2-receptor protein expression could be a potential reason for the solely moderate effects observed after chronic stimulation of WA with amthamine.

To conclude, amthamine displayed the prior expected effects that treatment with amthamine at low nanomolar concentrations induces a decline in differentiation markers. Overall, the effects were only moderate though and restricted to the differentiation marker aP2.

Further experiments to be planned in the future are *in vitro* browning of WA with stimulation of WA by a H2-receptor agonist.

4.2.5 Chronic effects of ranitidine on BA and WA differentiation

Ranitidine is an antagonist of the H2-receptor (Alhazzani et al. 2013) and an increase of differentiation markers was priorly anticipated upon treatment of BA and WA with ranitidine.

Brown adipocytes

Ranitidine had no observable effect on the accumulation of triglycerides in BA (figure 37). The aP2- protein expression (figure 38) was non-significantly upregulated upon chronic treatment with ranitidine at low nanomolar concentration. These results are in line with the predictions which were made beforehand and might further support the proposed Gαq-coupling of the H2-receptor in BA. Furthermore, there was a non-significant tendency for an increase in the expression of PPAR-ɣ protein (figure 39). The expression of UCP-1 was unchanged upon treatment with ranitidine (figure 40).

There was no substantial effect of ranitidine and ranitidine in co-stimulation with NE on the glycerol release of BA (figure 41). Thus, ranitidine is not able to acutely change the state of lipolysis in BA and does not foster the positive effects of stimulation with NE.

Overall, ranitidine showed non-significant effects on the regulation of aP2 and PPAR-ɣ and no effect on the function of BA.

White adipocytes

Ranitidine non-significantly upregulated aP2 expression at high nanomolar concentrations in WA, whereas PPAR-ɣ expression was unchanged and no induction of UCP-1 expression (figure 42) was noted by ranitidine. Hence, ranitidine is unlikely to initiate a thermogenic gene expression profile in WA.

In conclusion, ranitidine showed the priorly anticipated effects and non-significantly upregulated aP2 expression in BA and WA. Overall, the influence of ranitidine on the differentiation and function of BA and WA was only modest though and ranitidine is unlikely to play a decisive role as treatment for obesity.

Nonetheless, *in vitro* browning of WA under blockage of the H2-receptor has not been conducted so far to our knowledge, constitutes interesting potential and should therefore be performed in the future.

4.3. Histamine and tachykinin secretion in BA

4.3.1 Tachykinin release in BA

As soon as we could show that BA effectively express and regulate the NK3-receptor, the question arose whether they also secrete tachykinin themselves. This was due to the rising understanding that BAT has far more capabilities beyond the sole dissipation of energy in form of heat. It became more apparent in recent years that BAT is an active secretory organ and secretes a variety of different molecules to modulate BAT activity (Scheideler et al. 2017, Villarroya et al. 2017). I could show that preadipocytes as well as mature brown adipocytes release tachykinin and that the amount secreted is stable during differentiation (figure 43). This contrasts with the downregulation of the NK3-receptor in mature BA. Interestingly, acute stimulation with NE caused a significant uprise in the levels of tachykinin. Tachykinin seems to play a role in intercell signaling and in the acute response of BA upon activation through sympathetic stimulation. Hence, tachykinin might be involved in autocrine and paracrine stimulation of mature BA or the modulation of nearby non-BA cells upon stimulation through the SNS as BAT is densely innervated and the SNS is a key regulator of BA differentiation (Hull 1966, Cannon and Nedergaard 2004)

Substance P for instance belongs to the same endogenous peptide group as tachykinin and is known to interact with peripheral nerves by modifying ion channels and excitability of sensory neurons in various pain pathways (Moraes et al. 2014). It would be very appealing to conduct further research on the influence of tachykinin on peripheral nerves and the interplay between the SNS and BAT. This could be done by carrying out denervation studies.

4.3.2 Histamine release in BA

To date, it was unclear whether BA liberate histamine themselves. The conducted histamine ELISA revealed that BA effectively secrete histamine and that the amount of the released histamine non-significantly declines throughout differentiation (figure 44). Additionally, it could be demonstrated that acute stimulation with NE [1 µM] does not affect the amount of the released histamine in comparison with untreated mature adipocytes.

This data argues for a role of histamine in autocrine/ paracrine signaling in BAT. As previously established, histamine plays a key role in immunomodulatory functions as well as angiogenesis (Laakkonen et al. 2017; O'Mahony et al. 2011). As histamine does not seem to be involved in BA differentiation, further research is warranted to examine the effects of histamine signaling in BAT. The interplay between histamine and angiogenesis as well as potential histamine mediated immune modulations in BAT should be investigated. This could be conducted for example by a receptor knockout model or overexpression of the H2-receptor in a cell or in an *in vivo* model.

4.4 Influence of the feeding regimen on NK3- and H2-receptor expression in murine adipose tissues

I investigated the expression of the NK3- and H2-receptors in different adipose tissues in obese and lean mice to check whether dietary alterations impact the expression of those receptors. The goal was to improve our understanding of the role of the NK3 and H2-receptors in the development of obesity.

I could show that either receptor is robustly expressed in BATi, WATi and WATg (figure 45-47) and that the choice of diet has no significant influence on the expression of those receptors albeit a non-significant downregulation of both receptors in mature WATi upon

HFD feeding of mice and a divergent downregulation of the NK3-receptor and upregulation of the H2-receptor in WATg (figure 46, 47).

Hence, these data suggest there is no need for an up-or downregulation of the NK3- or H2-receptors in murine adipose tissue reacting on excessive amounts of fatty acids. This means that the receptors are either sufficiently expressed for the response of the mice to a high fat diet or more likely that they do not play a substantial role in this response. This is underlined by the previous results that no stimulation of either receptor showed major and comprehensive effects on the differentiation of BA and WA.

I heavily focused on the role of the NK3- and H2-receptors in adipogenesis in BA and WA in this study. However, the findings that the diet does not change the expression also assists the hypothesis that the NK3- and H2-receptors might have different functions in BA and WA. Within the peripheral areas of the body, neurokinins play a role in pro-inflammatory responses like cigarette smoke induced plasma exsudation or bronchoconstriction (Khawaja and Rogers 1996). The H2-receptor is in charge of important immunoregulatory modifications like mast cell degranulation and T-cell proliferation (O'Mahony et al. 2011).

Therefore, it is plausible that in BA and WA the NK3- and H2-receptors might also play a role in the inflammatory response of those cells. This needs further experiments like gene expression analysis of pro- and anti-inflammatory cytokines to substantiate this hypothesis.

4.5 Regulation of lipolysis by the Gαq-signaling pathway

In contrast to the upregulation or downregulation of differentiation markers upon chronic treatment, in the majority of experiments there was no significant alteration on the glycerol release after acute NK3- and H2-receptors stimulation and blockage with the tested ligands in BA (figure 13, 19, 29, 35, 41), albeit a significant increase in the relative glycerol release of BA upon co-stimulation with SB223412 and NE (figure 19).

These data might suggest that the NK3- and H2-receptors or stimulation of the Gαq-signaling pathway in general without NE do not interact with lipolysis at all. Lipolysis is already heavily regulated by NE via the physiological $ß_3$-cAMP-PKA-HSL- pathway

(Cannon and Nedergaard 2004). Therefore, the data could indicate that stimulation or blockage of Gαq-coupled receptors does not influence lipolysis in BA without co-stimulation with NE. Conduction of further experiments is warranted to confirm or dismiss the previous proposed hypothesis.

4.6 Missing effects of FR and ET-1 on BA and WA differentiation

Recently, our laboratory demonstrated that Gαq-signaling is a major signaling pathway to influence the differentiation of BA and WA. My colleague K. Klepac used FR [1 µM] and ET-1 [0.3 nM] in her studies to show the effects of diminished or enhanced Gαq-signaling in BA, respectively (Klepac et al. 2016).

Hence, I used FR [1 µM] and a slightly changed ET-1 [10 nM] concentration in my experiments as positive controls.

In contrast to the findings of Klepac et al., FR [1 µM] and ET-1 [10 nM] did not always alter the expression markers significantly in this study. Different reasons could be accountable for that phenomenon. The brown and white adipocytes I used in this study were isolated from different mice as the ones Klepac et al. used in their experiments. Therefore, the expression and activity of the Ednra-receptor may differ significantly. Moreover, the expression of the signaling molecules and the activity of the Gαq/11/14 signaling pathway could be diminished in comparison to the cells Klepac et. al. used in their study. This could explain why there was no significant up- or downregulation of differentiation markers in all cases when FR [1 µM] or ET-1 [10 nM] was applied. To address that problem in the future, a dose response curve could be performed with ET-1 and FR which could show how the isolated BA and WA specifically react upon treatment with the aforementioned compounds.

5. Summary

In recent years, obesity reached pandemic dimensions and the means to have lasting success in fighting obesity are alarmingly limited. For a long time, it was believed that brown adipose tissue only exists in newborns and gradually declines throughout youth but it could be demonstrated that metabolic active brown adipose tissue exists in adults likewise. Brown adipose tissue features a unique protein- UCP-1, which contributes to whole body energy expenditure by uncoupling the respiratory chain and burning energy in form of heat. That recent discovery gave rise to a promising pharmacological approach to combat the pandemic obesity by activating brown adipose tissue or enhancing brown adipose tissue mass and thus burn excess energy. An intriguing target is the Gαq-signaling pathway as it plays a decisive role in the differentiation of brown adipose tissue. Constitutive activation of the Gαq-signaling pathway leads to a decline in differentiation of brown adipocytes. The inhibition of Gαq-signaling could increase adipose tissue differentiation and thereby elevate whole body energy expenditure. Strikingly, the expression levels of the Gαq-coupled NK3- and H2-receptor mRNA are significantly regulated during differentiation. Therefore, the question arose whether activation and inhibition of the aforementioned receptors change the state of differentiation and alter the functions of brown and white adipocytes. I could show that NK3- and H2-receptor are expressed in brown and white adipose tissue and that they are likely Gαq-coupled in brown adipocytes. Sustained stimulation of brown and white adipocytes with aforementioned receptor compounds showed some significant effects on the differentiation of brown and white adipocytes but overall, the data do not suggest a significant role of either receptor in regulation of brown and white adipocyte differentiation. Therefore, it is unlikely that the tested NK3- and H2-receptor ligands will play a role in the treatment of obesity in the future. I could prove that brown adipocytes indeed secrete histamine and tachykinin and that differentiation affects the amount secreted or how the cells respond to stimulation with NE. In addition, the data indicates that acute interaction with the Gαq-signaling pathway does not change the function of brown adipocytes measured by the state of lipolysis. The data proved that stimulation of neither receptor is able to enroll a thermogenic gene expression pattern in white adipocytes. Moreover,

dietary interventions showed no significant effect on the expression of either receptor in an in-vivo model. However, these results were not always in line with the prior anticipated effects and further analysis is necessary on how these results occur.

6. List of figures

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8. References

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