Characterization of A2 adenosine receptors in brown adipose tissue

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List of abbreviations

AC	adenylyl cyclase
ADA	adenosine deaminase
ADK	adenosine kinase
Ado	adenosine
AdoR	adenosine receptor
ADP	adenosine diphosphate
AK	adenosine kinase
AMP	adenosine monophosphate
ANOVA	analysis of variance
aP2	adipocyte Protein 2
APS	ammonium persulfate
ATP	adenosine triphosphate
BA	brown adipocytes
BAT	brown adipose tissue
BAT-MSC	brown adipose tissue derived mesenchymal stem cells
BMI	body mass index
BSA	bovine serum albumin
Ca	calcium
cAMP	cyclic adenosine-3‘,5‘-monophosphate
CD39	ectonucleoside triphosphate diphosphohydrolase-1, cluster of differentiation 39
CD73	ecto-5’-nucleotidase, cluster of differentiation 73
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine-3‘,5‘-monophosphate
Cl	chloride
CMV	cytomegalovirus
CRE	cAMP response element
CREB	cAMP response element binding protein
CT	computed tomography scan
ctrl	control

cyto 5’NT	cytosolic-5‘-nucleotidase
d	day
Dexa	dexamethasone
DEPC	diethyl pyrocarbonate
DM	differentiation medium
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleoside triphosphate
dsDNA	double-stranded deoxyribonucleic acid
EC_{50}	half maximal effective concentration
ECL	enhanced chemiluminescence
ELISA	enzyme-linked immunosorbent assay
Epac	Rap1 guanine-nucleotide-exchange factor
FBS	foetal bovine serum
FFA	free fatty acids
GAPDH	glyceraldehyde 3 phosphate dehydrogenase
H	hydrogen
HCl	hydrochloric acid
Hcy	homocysteine
H_2O	dihydrogen monoxide, water
HRP	horseradish peroxidase
HSL	hormone-sensitive lipase
IBMX	3-isobutyl-1-methylxanthine
IM	induction medium
Ino	inosine
K	potassium
K_M	Michaelis constant
LB	lysogeny broth
LTR	long terminal repeats
LV	lentivirus
MAP	mitogen-activated protein

Mg	magnesium
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cell
Myf5	myoblast-specific myogenic factor 5
N	nitrogen
n	number
Na	sodium
NE	norepinephrine
NEB	New England Biolabs
NP-40	nonoxynol-40
O	oxygen
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PDE	phosphodiesterase
PET	positron emission tomography
PFA	paraformaldehyde
PGC-1α	peroxisome proliferator-activated receptor γ-coactivator-1α
PI3	phosphoinositide 3
PKA	cAMP-dependent protein kinase, protein kinase A
PLC	phospholipase C
PPARγ	peroxisome proliferator-activated receptor γ
PRDM16	PR domain containing 16
P/S	penicillin/streptomycin
qPCR	quantitative polymerase chain reaction
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
rpm	rotations per minute
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
S	sulfur
SAH	S-adenosyl homocysteine
SDS	sodium dodecyl cyclase

SEM	standard error of the mean
SV 40	simian virus 40
T3	triiodothyronine
TBS	Tris-buffered saline
TBST	Tris-buffered saline supplemented with Tween-20
TEMED	N,N,N’,N’-tetramethylethylenediamine
TG	triglyceride
UCP-1	uncoupling protein-1
UV	ultraviolet
WAT	white adipose tissue
WHO	world health organization
WPRE	Woodchuck hepatitis virus posttranscriptional response element
wt	wild type

1. Introduction

1.1 Overweight and obesity

An overweight adult is defined by having a body mass index (BMI) between 25 kg/m^2 and 30 kg/m^2 whereas an obese subject has a BMI greater than 30 kg/m^2. Especially in high income countries, a big part of the population is overweight or obese, but it has recently become a global health care problem, being dramatically on the rise in low- and middle-income countries.

1.1.1 Current data

According to the latest estimates of the World Health Organisation (WHO), there are more than 1.9 billion adult people worldwide being overweight and about 600 million being obese in 2014 (Figure 1). Taken together, that is more than half of the world's adult population. In Germany, the prevalence for having a BMI equal to or greater than 25 kg/m^2 was 54.8 % in 2014. The numbers are increasing every year and by now there are more deaths worldwide linked to overweight and obesity than to underweight and/or starvation (Speakman & O'Rahilly, 2012). Mainly responsible for the development of overweight and obesity is an increase of energy-dense food consumption concomitant with a decrease in physical activity. Only few cases are caused primarily by diseases (e.g. hypothyroidism) or the use of drugs (e.g. antidepressants).

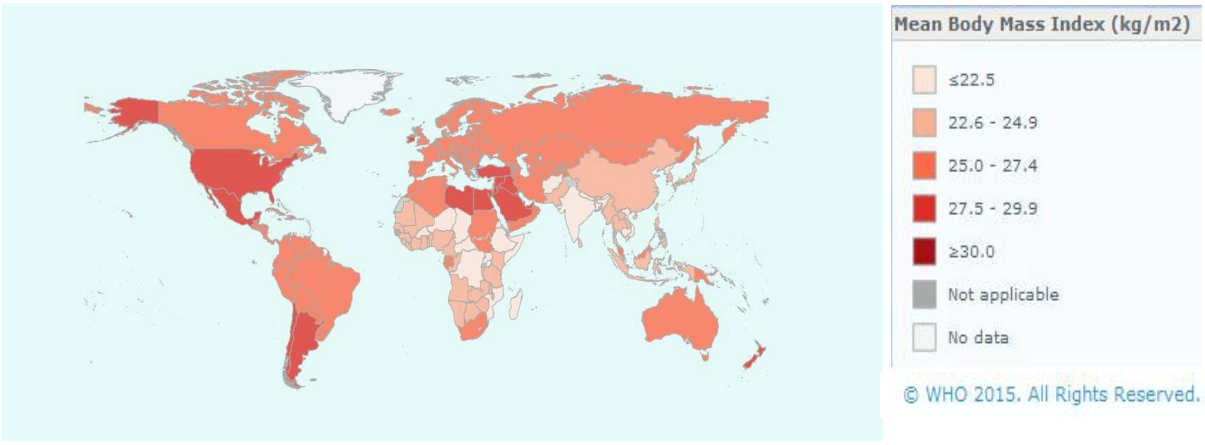


Fig. 1: Mean Body Mass Index (BMI) by country.
Age standardized estimate of the mean BMI in kg/m^2 by country for adults (age ≥ 18) in 2014. (Image: WHO, 2015)

1.1.2 Health consequences

Overweight and obesity are risk factors for many diseases and associated with a reduced life expectancy (WHO, 2015; Figure 2).

A raised BMI is especially linked with cardiovascular diseases, like arterial hypertension and heart diseases, with type 2 diabetes mellitus, musculoskeletal disorders and certain types of cancer (Schmidt, 2015; Valerio, et al., 2014). Obesity increases the risk of ischemic strokes in young adults (Mitchell, et al., 2015) and of later-life disability (Wong, et al., 2015).

An effective weight control would not only promote longevity of the population, but also decrease the medical expenditure of the health care systems (Nagai, et al., 2012; Walls, Backholer, Proietto, & McNeil, 2012).

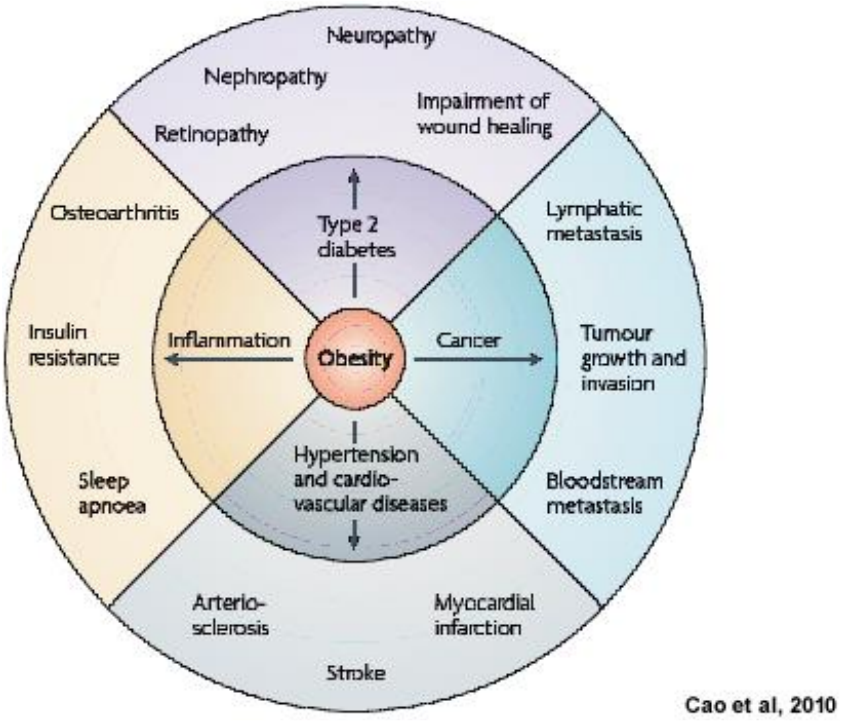


Fig. 2: Health consequences of obesity.
Obesity is a major risk factor for type 2 diabetes mellitus, for hypertension and cardiovascular diseases, for certain types of cancer, like the mamma carcinoma and the colorectal carcinoma and for an increased inflammation. This may lead to different consequences like neuropathy, sleep apnoea, stroke and myocardial infarction and reduces life expectancy (Image: Cao et al, 2010)

1.1.3 Prevention and fighting of overweight and obesity

Overweight and obesity, as well as associated diseases can be prevented by a limitation of the food and energy intake and an increase of the physical activity. However, simply reducing energy intake through diets is usually not successful in the longterm. Large parts of the population do not have sufficient daily physical activity (WHO) and it is questionable how many obese subjects are willing and able to significantly increase exercise/activity. Hence, pharmacological approaches to combat obesity are urgently needed. There are only a few drugs approved to fight distinct forms of obesity by reducing the appetite or decreasing the fat absorption. Three pharmaceuticals are approved in Germany: Orlistat (Xenical®), liraglutide (Saxenda®) and naltrexone/bupropion (Contrave®). The one mostly used in Germany is orlistat, an inhibitor of gastric and pancreatic lipases that is able to reduce the intestinal fat absorption by up to 30 % (Drent, et al., 1995). Several studies showed a significant loss of weight in obese patients, compared to a control group treated with placebos, but frequent side-effects included diarrhoea and even anal incontinency (Chanoine, Hampl, Jensen, Boldrin, & Hauptman, 2005; Davidson, et al., 1999; Drent, et al., 1995; Krempf, et al., 2003). Liraglutide is a glucagon-like peptide-1 (GLP-1) receptor agonist, originally approved for the treatment of type 2 diabetes, which was shown to reduce body weight in obese, non-diabetic patients as well (Pi-Sunyer, et al., 2015). This effect is primarily mediated by reduced appetite and energy intake (van Can, et al., 2014), the main adverse events include nausea and diarrhea (Pi-Sunyer, et al., 2015). Latest studies show promising effects of liraglutide on the maintenance of diet-induced weight lost in obese patients (Lundgren, et al., 2021). The combination of naltrexone, an opioide receptor antagonist and bupropion, a dopamine and norepinephrine reuptake inhibitor, was found to produce weight-loss in obese adults (Wadden, et al., 2011), but the rare serious adverse effects include suicidal thoughts and seizure (Christou & Kiortsis, 2015). If those methods alone are not effective enough, special obesity surgery can be performed to lower the weight by reducing the stomach volume or the bowel length. The operations used most frequently during the past ten years in Germany are the Roux-en-Y gastric bypass and the sleeve gastrectomy (Stroh, 2016). However, these invasive methods are linked with a lot of risks and only applicable for mortally obese subjects, but an appropriate pharmacological alternative is yet to be found.

1.2 Brown adipose tissue

Obesity is characterized by excessive abundance of white adipose tissue (WAT), which is the majority of adipose tissue in the human body. Its main function is to store energy in the form of fat (Karastergiou & Mohamed-Ali, 2010).

Brown adipose tissue (BAT) on the other hand has opposite functions *in vivo* and consumes energy by producing heat through non-shivering thermogenesis (Cannon & Nedergaard, 2004). Because of this ability, it plays an important role in newborns, who have to adapt to a sudden temperature change from the intrauterine to the external environment in combination with a high surface area-to-volume ratio (Silva, 2006). It was believed for many years that BAT athrophies quickly postnatally in humans, but in 2009 a series of studies showed that adult humans also possess metabolically active BAT (Cypess, et al., 2009; van Marken Lichtenbelt, et al., 2009; Virtanen, et al., 2009). Hence, BAT has become an interesting target of pharmacological research.

In humans, small amounts of BAT are found in the neck, in the supraclavicular and axillary regions, in the paravertebral and perirenal regions and around the major vessels (Virtanen, et al., 2009). It can also be found in WAT, what suggests that both adipose tissues might be interspersed in some depots (Clarke, Brglevska, Lau, Ramdave, & Hicks, 2007). Interestingly, studies showed that BAT can be activated by cold and that obese subjects have significantly decreased BAT activity (van Marken Lichtenbelt, et al., 2009).

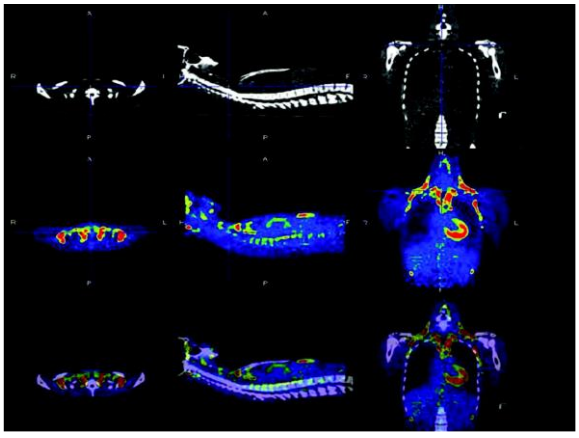


Fig. 3: Metabolically active BAT in human adults.
Combined computer tomographic (CT) and positron emission tomographic (PET; with ^{18}F-flourodeoxyglucose) images of the human thorax. Transverse (left), sagittal (middle) and coronal (right) slices. Activated brown adipose tissue appears red and green. (©Image: Dr. Wouter van Marken Lichtenbelt, University of Maastricht, Maastricht, the Netherlands)

Nevertheless, an age-related decline in BAT activity could still be shown (Cypess, et al., 2009; Ouellet, et al., 2011).

1.2.1 Differentiation of brown fat cells

There have been two different types of brown fat cells identified so far: the “classical” brown adipocytes and brown-like fat cells, referred to as beige or brite (brown in white) cells, which can be induced by cold exposure and other stimuli (Enerback, 2010; Frontini & Cinti, 2010).

The classical brown fat occurs in typical locations, like the supraclavicular and the perirenal region and it mostly consists of a high number of brown adipocytes with a quite similar appearance (Cohen & Spiegelman, 2015). These cells arise from a lineage that expresses the myoblast-specific myogenic factor 5 (Myf5) gene, shared with skeletal muscle cells and independent from white adipocytes (Seale, et al., 2008).

The beige cells on the other hand occur in white fat depots, especially in the subcutaneous adipose tissue (Wu, et al., 2013) and come from the same Myf5 negative lineage the white adipocytes come from (Seale, et al., 2008; Wu, et al., 2013).

Several transcription factors have been identified to be involved in the development of brown fat cells. While the nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ) is necessary for white and brown adipocytes (Tontonoz, Hu, & Spiegelman, 1994), especially the PPARγ coactivator-1α (PGC-1α) and the transcriptional regulator PR domain containing 16 (PRDM16) seem to play a major role in the differentiation of brown fat cells (Seale, et al., 2008; Uldry, et al., 2006).

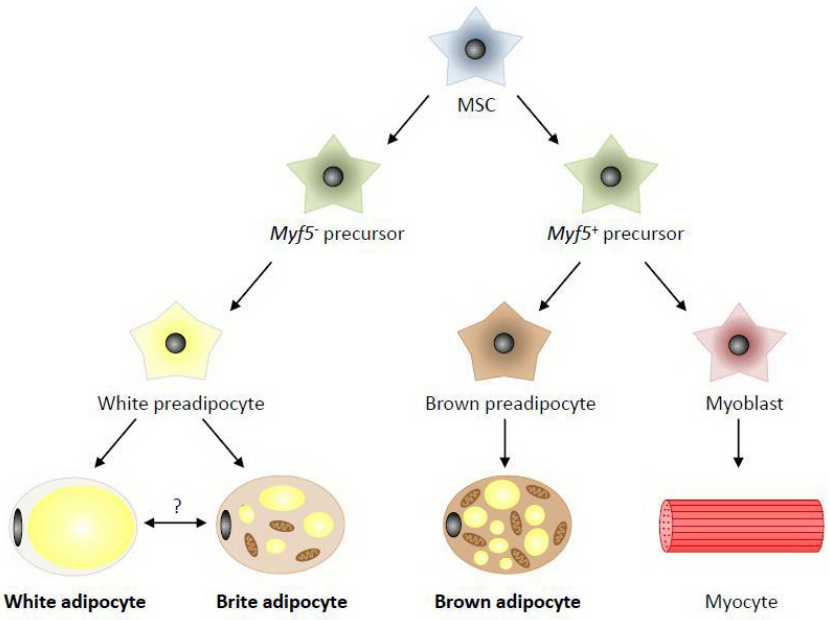


Fig. 4: Differentiation of brown and brown-like (brite) adipocytes.
Mesenchymal stem cells (MSC) can develop into different adipocyte precursor cells. The further differentiation is controlled by transcription factors like PPARγ, PGC-1α and PRDM16. (Image: Siersbaek, et al., 2012)

1.2.2 Brown adipose tissue function

Being stimulated by cold exposure or high energy intake, the sympathetic nervous system activates BAT via the release of catecholamines and the activation of β-adrenergic receptors on the brown fat cells (Cannon & Nedergaard, 2004). The receptors are coupled to stimulating G-proteins (G_s-proteins) and activate the adenylate cyclase to elevate the intracellular amount of cAMP after stimulation. This leads to an activation of different signalling cascades, e.g. proteinkinase A (PKA) signalling which increase lipolysis of fat and thereby the amount of free fatty acids in brown adipocytes. Mainly responsible for the ability of brown adipose tissue to produce heat by non-shivering thermogenesis is the unique brown fat protein uncoupling-protein-1 (UCP-1) (Nedergaard, et al., 2001). The expression and function of UCP-1 is upregulated by the presence of free fatty acids and inhibited by adenosine triphosphate (ATP). The protein is located in the inner membrane of the mitochondria and uncouples the proton gradient, built by the respiratory chain, from the ATP synthesis by allowing the protons to re-enter the mitochondrial matrix, which generates heat (Nedergaard, et al., 2001). Therefore the

brown adipocytes are rich of mitochondria, packed with well-developed christae and possess multilocular lipid droplets (Cannon & Nedergaard, 2004).

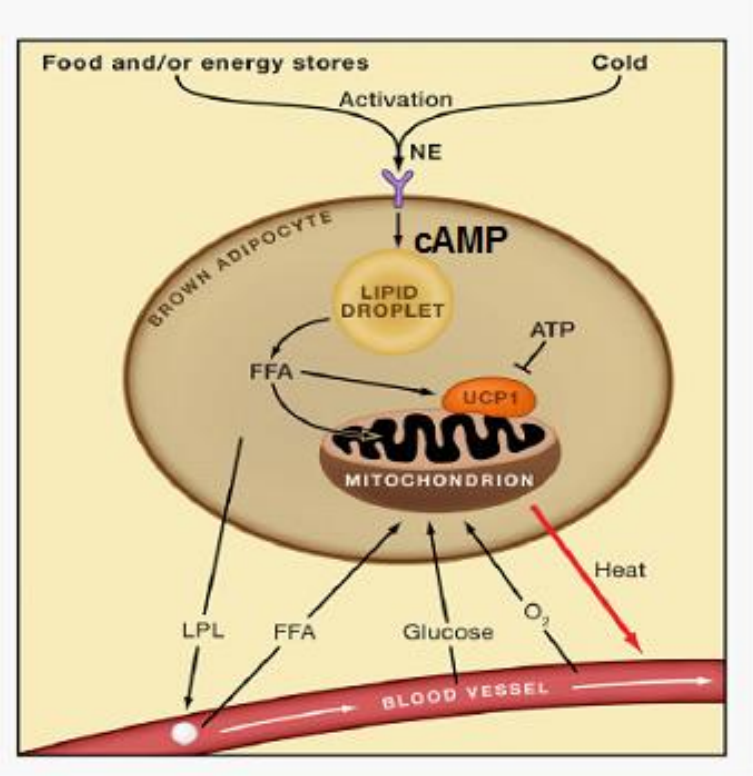


Fig. 5: Brown adipose tissue function.
BAT is highly innervated by the sympathetic nervous system. Catecholamins like norepinephrine (NE) are released after activation and increase the intracellular cAMP and the lipolysis in the brown adipocytes via G_s-coupled receptors. Free fatty acids (FFA) and glucose are metabolized by the cells, but the synthesis of adenosinetriphosphat (ATP) is uncoupled from the respiratory chain in the mitochondria by uncoupling-protein-1 (UCP-1) and heat is generated. (Image: Cannon & Nedergaard, 2004)

Because an activation of BAT for an anti-obesity therapy with cold exposure or β-adrenergic agonists is clinically not practicable, as it is either not well-tolerated (cold) or has to many side-effects (e.g. blood pressure), alternative strategies must be explored.

1.3 Adenosine

Adenosine is a naturally occurring endogenous purine nucleoside composed of the nitrogenous base adenine attached to a cyclic ribose sugar molecule (ribofuranose) moiety (Eltzschig, 2009).

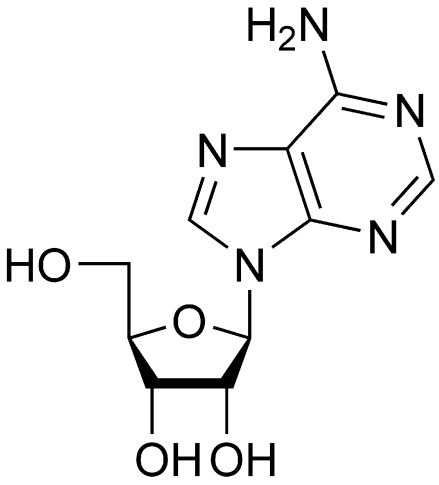


Fig. 6: Adenosine structure.
Schematic illustration of the nucleoside adenosine. The base adenine is linked to a cyclic sugar molecule of ribose. H = hydrogen, N = nitrogen, O = oxygen.

1.3.1 Adenosine metabolism

There are different ways for adenosine to be formed (Figure 7). The nucleoside can be synthezised de novo during the energy-consuming purine biosynthesis (Layland, Carrick, Lee, Oldroyd, & Berry, 2014) or by hydrolysis of *S*-adenosyl homocysteine (SAH) (Deussen, Lloyd, & Schrader, 1989), but it is mainly generated by the breakdown of intra- or extracellular adenine nucleotides like ATP (Zimmermann, 2000).

Extracellular ATP is rapidly hydrolyzed to adenosine by two ecto-enzymes working in concert. The nucleoside triphosphate diphosphohydrolase CD39 splits off the first two phosphate groups (Robson, et al., 2005) and the ecto-5'-nucleotidase CD73 hydrolyses AMP to adenosine (Picher, Burch, Hirsh, Spychala, & Boucher, 2003). Intracellular, the most important enzyme for the formation of adenosine is the AMP-selective cytosolic 5'-nucleotidase, which hydrolyzes AMP into adenosine and inorganic phosphate (Darvish, Pomerantz, Zografides, & Metting, 1996). The amount of intra- and extracellular

adenosine is adjusted by efficient equilibrative transporters (Parkinson, Xiong, & Zamzow, 2005).

The two enzymes mainly responsible for catabolising adenosine are adenosine kinase (ADK), which catalyzes the phosphorylation of adenosine to produce AMP (Drabikowska, Halec, & Shugar, 1985) and adenosine deaminase (ADA), which catalyzes the deamination of adenosine to inosine (Ford, et al., 2000). The capacity and the K_m of ADA are much higher than the ones of ADK, so ADK is thought to be the principal enzyme responsible for regulating the level of adenosine under physiological conditions (Pak, Haas, Decking, & Schrader, 1994).

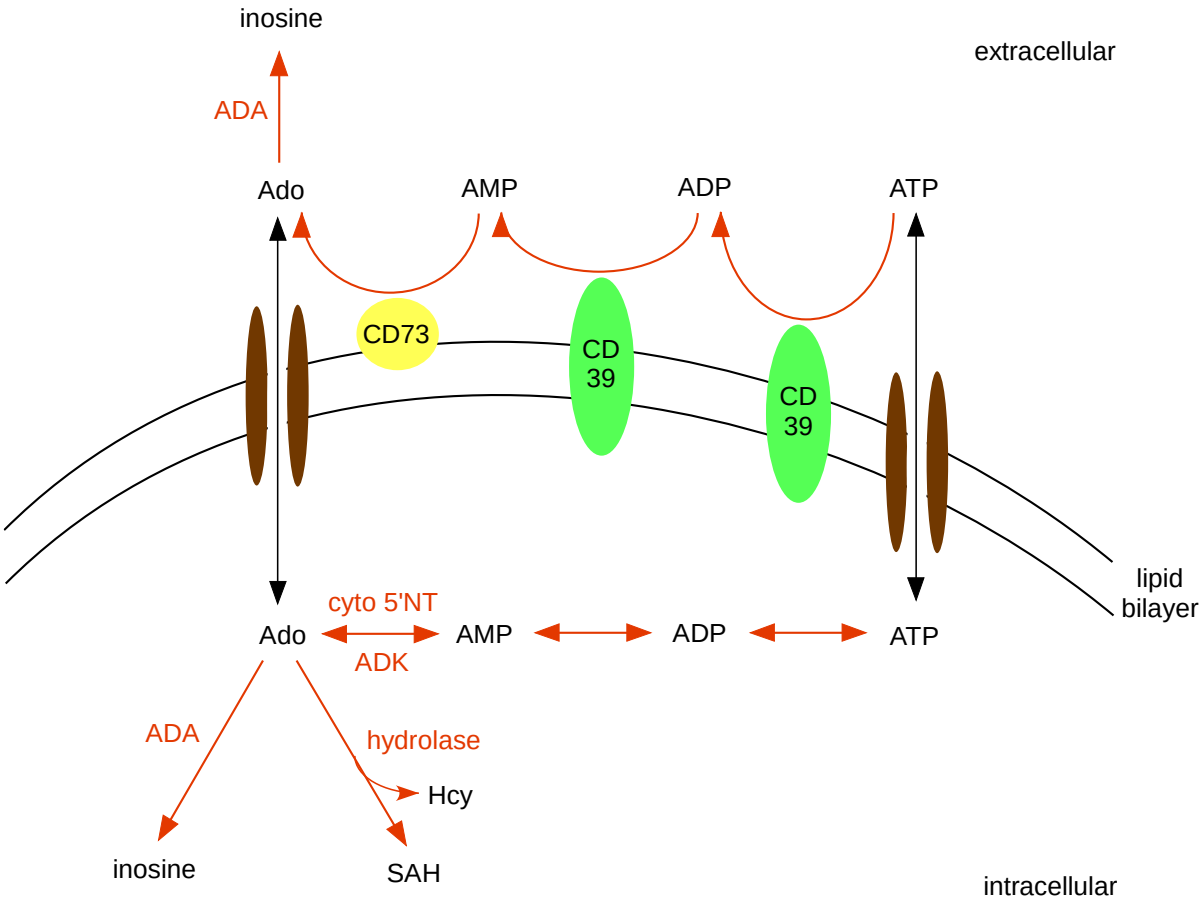


Fig. 7: Adenosine metabolism.
Schematic illustration of adenosine metabolism intra- and extracellular. Ado = adenosine, AMP = adenosine monophosphate, ADP = adenosine diphosphate, ATP = adenosine triphosphate, SAH = S-adenosyl homocysteine, Hcy = homocysteine, ADK = adenosine kinase, ADA = adenosine deaminase, cyto 5'NT = cytosolic 5' nucleotidase.

1.3.2 Adenosine receptors

Four different adenosine receptor subtypes have been identified up to now, the A1 receptor, the A2A and A2B receptors and the A3 receptor, which are ubiquitously expressed (Fredholm, 1995; Palmer & Stiles, 1995). The receptors are all coupled to heterotrimeric GTP-binding and GTP-hydrolyzing proteins (G-proteins) and their proposed molecular structure is typical for G-protein-coupled receptors, with 7 transmembrane domains connected by three intra- and three extracellular loops and flanked by an extracellular N-terminus and an intracellular C-terminus (Linden, 1991; Meng, et al., 1994; Pierce, et al., 1992; Salvatore, et al., 1993). The A1, the A2A and the A3 receptors can be activated by adenosine concentrations between 0.01 µM and 1 µM which are present under basal conditions, while the A2B receptors require a higher concentration that exceeds 10 µM to be significantly activated (Fredholm, Irenius, Kull, & Schulte, 2001).

The A1 and A3 adenosine receptors are coupled to a G-protein with a G_i α-subunit (Figure 8) that inhibits the activity of the adenylyl cyclase and thereby reduces the amount of intracellular cAMP and the activity of PKA (Dunwiddie & Fredholm, 1989; Gessi, et al., 2008; Salvatore, et al., 1993). The A1 adenosine receptor has been shown to reduce activity of the hormone-sensitive lipase (HSL) and the adipose triglyceride lipase in adipose tissue via this pathway (Dhalla, et al., 2009). Both receptors are also linked to various kinase pathways like phosphoinositide 3 (PI3) kinase and mitogen-activated protein (MAP) kinases (Jacobson & Gao, 2006; Zhong, et al., 2003). Activation of the A1 receptor can directly activate K^+ channels (Linden, 1991) and the A3 receptor can stimulate phospholipase C (PLC) via a G_q-protein (Gessi, et al., 2008).

The A2A and the A2B adenosine receptors are coupled to G_s-proteins (Figure 8), which stimulate the adenylyl cyclase. The enzyme catalyzes the conversion of ATP to 3',5'-cyclic AMP (cAMP), the amount of cAMP increases and PKA is activated (Feoktistov & Biaggioni, 1997; Fredholm, Chern, Franco, & Sitkovsky, 2007). PKA activates hormone-sensitive lipase (HSL) in adipose tissue by phosphorylation, which in turn hydrolyzes triglycerides to free fatty acids and glycerol. PKA also activates the transcription factor cAMP response element-binding protein (CREB) by phosphorylation, which can in turn mediate gene expression (Nemeth, et al., 2003). The A2A receptor mediated increase in cAMP also directly activates the Rap1 guanine-nucleotide-exchange factor Epac and the

associated signalling pathway (Fredholm, et al., 2007). A2B receptor stimulation can in addition trigger the activation of PLC via a G_q-protein (Ryzhov, Goldstein, Biaggioni, & Feoktistov, 2006).

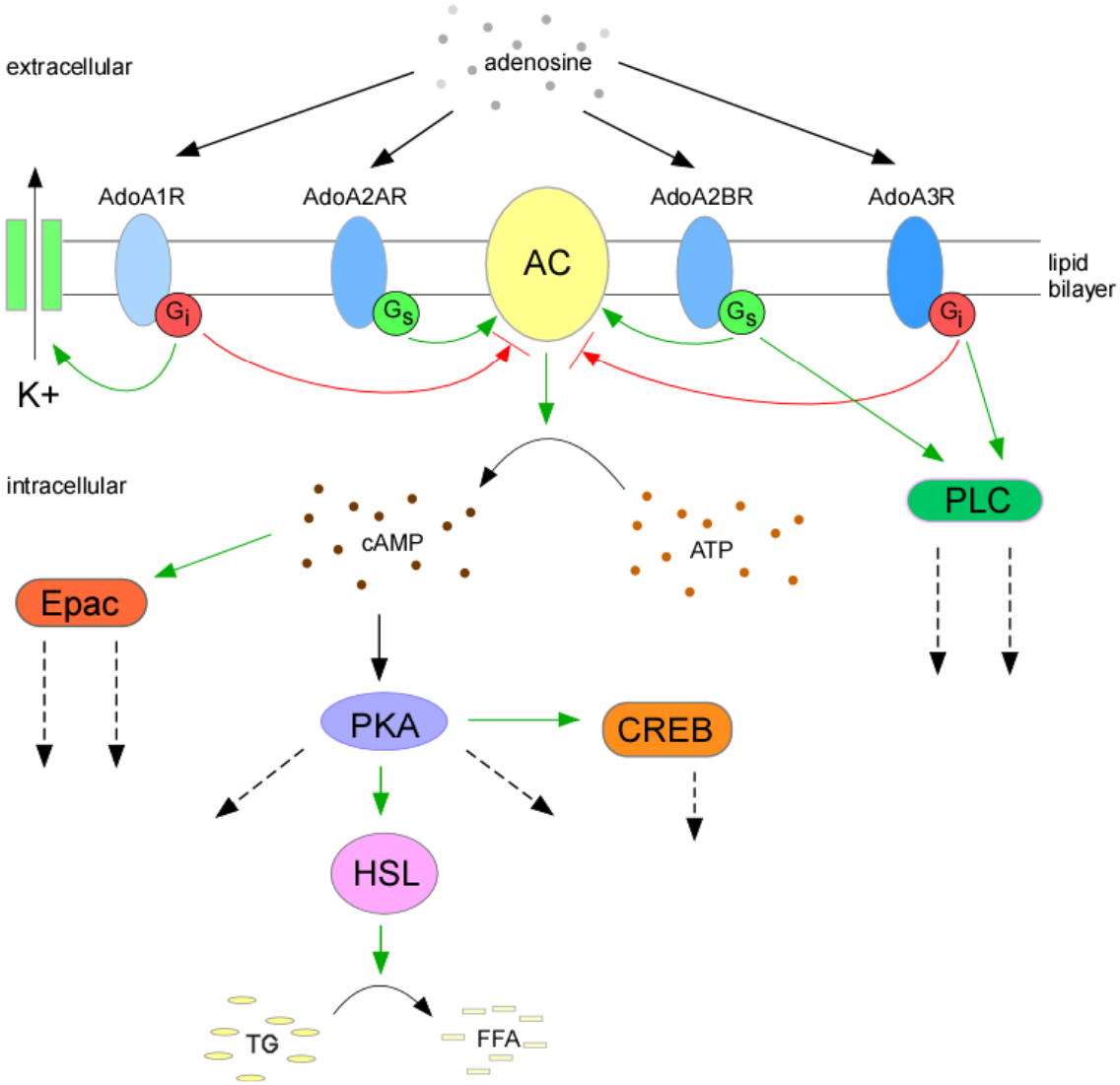


Fig. 8: Adenosine signalling pathways.
Adenosine is generated extracellular or is transported from intra- to extracellular and can bind to and activate one of the four G-protein coupled receptors. The signalling mainly occurs by the adenylyl cyclase (AC) – cAMP – protein kinase A (PKA) pathway. PKA activates hormone-sensitive lipase (HSL) in adipose tissue by phosphorylation, which in turn hydrolyzes triglycerides (TG) to free fatty acids (FFA) and glycerol. The G_i-coupled A1 and A3 receptors inhibit the pathway, while the G_s-coupled A2A and A2B receptors stimulate it. Increasing levels of cAMP directly activate Rap1 guanine-nucleotide-exchange factor Epac. In addition, the A1 receptor can directly activate K^+ channels and the A2B and A3 receptors are able to stimulate phospholipase c (PLC) via G_q-proteins. CREB = cAMP response element-binding protein.

1.3.3 Adenosine function

Adenosine receptors occur in most of human tissues and adenosine has effects across many different organ systems, some of them essential for the human physiology. Some research groups suggest that the nucleoside is one of the factors that adjust metabolism to the needs of the organism (Fredholm, Johansson, & Wang, 2011).

It has been long known that adenosine has the ability to increase the blood flow in most vascular beds including the coronary circulation (Berne, 1963), mainly by the activation of A2A and A2B adenosine receptors (Kilpatrick, Narayan, Mentzer, & Lasley, 2002). An exception are the afferent arterioles of the kidney where adenosine leads to a vasoconstriction (Hansen & Schnermann, 2003), mediated by the activation of A1 receptors (Brown, et al., 2001).

Because of its ability to decelerate the atrioventricular conductor or to block it for a few seconds (Urthaler & James, 1972), adenosine is used as an antiarrhythmic medication to treat a number of supraventricular tachycardia (Eltzschig, 2009).

Adenosine also has some major effects on the central nervous system. It is able to inhibit the release of excitatory neurotransmitters and to decrease the rates of firing of nerves by activation of A1 receptors (Fredholm, Chen, Masino, & Vaugeois, 2005) and seems to play an important role in the regulation of sleep and wakefulness (Palchykova, et al., 2010), acting most likely via A2A adenosine receptors (Scammell, et al., 2001).

1.3.4 The effects of adenosine on adipose tissue

All four adenosine receptor subtypes are expressed in white adipose tissue (Johansson, Yang, Lindgren, & Fredholm, 2007) and like described above (1.3.2), adenosine acting via the abundant A1 receptors is an important inhibitor of lipolysis in WAT (Johansson, et al., 2008). This insulin-like effect was discovered quite early (Dole, 1962) and the major physiological role of A1 receptors in regulating lipolysis is well accepted.

But less is known about the role of adenosine for brown adipose tissue function. Early studies have shown that adenosine has an effect on brown adipocytes that is similar to the findings in WAT, inhibiting lipolysis in cells from hamsters and rats and reducing the sensitivity to catecholamines (Schimmel & McCarthy, 1984; Szillat & Bukowiecki, 1983; Woodward & Saggerson, 1986). However, it still has to be investigated which receptors are expressed on brown adipocytes and which one mediates the effects of adenosine on

BAT, especially regarding the latest findings about metabolic active BAT in human adults (see 1.2).

1.4 Aim of the MD thesis

The role of adenosine in regulating white adipose tissue function has been studied before (Dhalla, et al., 2009). However, the effects of adenosine on brown adipocytes remain a subject of interest, especially the role of A2A and A2B adenosine receptors in BAT function.

The purpose of the MD thesis is to characterize the role of A2 adenosine receptors in regulating brown adipose tissue function by using special agonists and antagonists and overexpression of the receptors on murine brown adipocytes.

This thesis therefore focuses on the following questions:

1) Which adenosine receptors are expressed in murine brown adipocytes?
2) Which effects do special adenosine receptor agonist and antagonists have on adipogenesis of brown adipocytes and which receptor subtype mediates these effects?
3) Do specific adenosine receptor agonists and antagonists have an effect on the expression of UCP-1 and which receptor subtype mediates this effect?
4) Does adenosine affect murine brown adipocyte lipolysis?
5) Which intracellular signalling pathways mediate adenosine function in murine BAT?
6) Is the effect of adenosine on BAT function additive to the effect of norepinephrine?

2. Material and Methods

2.1 General

All material used in this study, if not further specified were bought from the following companies: Calbiochem (Darmstadt), Carl Roth GmbH (Karlsruhe), Merck (Darmstadt), Sigma-Aldrich (München) and VWR (Darmstadt). Water used in this study was purified and distilled by an EASYpure UV/UF system (WeteA, Wilhelm Werner GmbH, Leverkusen). If not stated otherwise, all commercial kits and enzymes were used according to the manufacturer's recommendations.

2.2 Cell culture methods

2.2.1 Material and equipment

Material:

Cryogenic vials, Sarstedt (Cat. No. 72.379.992)

Collagenase II, Worthington, UK (Cat. No. CLS2)

Dexamethasone, Sigma-Aldrich (Cat. No. D-4902)

Dish (100 mm), Sarstedt (Cat. No. 83.1802.001)

Dulbecco's modified Eagle's medium (DMEM):

Glutamax I + 4500mg/l Glucose, - Pyruvate; Gibco, Karlsruhe (Cat. No. 61965059)

Dulbecco's modified Eagle's medium (DMEM):

Glutamax I + 4500mg/l Glucose, + Pyruvate; Gibco, Karlsruhe (Cat. No. 61965059)

Falcon tube (15 ml, 50 ml), Sarstedt (Cat. No. 62.554.001, 62.548.004)

Flask (175 cm^2), Sarstedt (Cat. No. 83.1812.002)

Foetal bovine serum (FBS), Biochrom AG, Berlin (Cat. No. S0115)

Insulin, Sigma-Aldrich (Cat. No. I-9278)

Isobuthylmethylxanthine (IBMX), Sigma-Aldrich (Cat. No. I-5879)

Nylon meshes, Millipore, Schwalbach

Penicillin / Streptomycin (P/S), Biochrom AG, Berlin (Cat. No. A2213)

Pipettes (5 ml, 10 ml, 25 ml), Sarstedt (Cat. No. 86.1253.001, 86.1254.001, 86.1685.001)

Triiodthyronine-Na (T3), Sigma-Aldrich (Cat. No. T-6397)

Trypsin-ethylen diaminetetraacetic acid (EDTA) 0,05 %, Invitrogen, Berlin (Cat. No. 25300-096)
Well plate (6-, 12-well), Sarstedt (Cat. No. 86.1836, 83.3921)
Well plate (24-, 96-well), Sarstedt (Cat. No. 83.1839, 83.3924)

Equipment:
Centrifuge, Biofuge Primo, Heraeus, Hanau
Incubator, HeraCell 150, Heraeus
Laminar air flow, HeraSafe, Heraeus
Microscope, DMIL, Leica
Neubauer counting chamber, Labomedic, Gießen

2.2.2 Isolation and culture of primary BAT-MSCs

The interscapular brown fat pads of new born mice were used to isolate the BAT-MSCs (Haas et al., 2009). They were dissected and afterwards incubated in a collagenase digestion buffer at 37° C for 30 minutes with vigorous shaking. The remaining tissue residues were filtered by a 100 µm nylon mesh and the mixture subsequently placed on ice for 30 min, before another step of filtration through a 30 µm nylon mesh. Then it was centrifuged at 700x g for 10 minutes and the pellet was resuspended in dissection medium. The including cells were counted with a Neubauer counting chamber and seeded on 6-well plates. They were grown at 37° C and 5 % CO_2.

Collagenase digestion buffer

NaCl	123 mM
KCl	5 mM
$CaCl_2$	1.3 mM
Glucose	5 mM
HEPES	100 mM

dissolved in H_2O, adjusted to pH 7.4, sterile filtered and stored at 4° C
the following substances were added freshly before use:

BSA	1.5 %
Collagenase II	0.2 %

sterile filtered and stored at 4° C

Dissection medium

DMEM Glutamax I + 4500 mg/l Glucose, - Pyruvate	
FBS	10 %
P/S	1 %
Insulin	4 nM
T3	4 nM
HEPES	10 mM
Sodium ascorbate	25 µg/ml

2.2.3 Immortalization of primary BAT-MSCs

24 h after isolation, the primary BAT-MSCs were infected with a lentiviral vector containing the Simian Virus 40 (SV 40) large T-antigen for immortalization (day 1). Afterwards they were expanded in growth medium at 37° C and 5 % CO_2. The cells were used for experiments up to passage 5 (p1 – p5).

Growth medium

DMEM Glutamax I +4500 mg/l Glucose, - Pyruvate	
FBS	10 %
P/S	1 %

2.2.4 Cell culture and storing of primary BAT-MSCs

The immortalized primary BAT-MSCs were cultured in growth medium at 37° C and 5 % CO_2, the medium being changed every 24 hours. By the time the culture was confluent, the cells were washed with warm PBS (37° C), treated with Trypsin for 5 min at 37° C in order to detach them and then resuspended in growth medium. Now the cells could be seeded for further experiments or stored for a longer period of time. Therefore the cell suspension was centrifuged for 5 min at 160 x g and the generated cell pellet resuspended in freezing medium and transferred to cryogenic vials. In order to reduce the temperature slowly, the vials were put on ice for 15 min, put in a freezer at -80° C for 24 hours and finally stored in liquid nitrogen (-196° C).

Freezing medium

Growth medium	
DMSO	10 %

In order to use the cryo-preserved cells for further experiments, they were thawed rapidly in a water bath at 37° C and then mixed with preheated growth medium. After being centrifuged for 5 min at 160 x g, the medium was discarded to remove the DMSO and the cell pellet was resuspended in fresh growth medium and seeded on culture plates.

2.2.5 Adipogenic differentiation of immortalized BAT-MSCs

Immortalized BAT-MSCs are *in-vitro* differentiated into brown adipocytes due to an eleven day protocol established in our lab (Haas et al., 2009). Approximately 150.000 cells per well (6-well) or 120.000 cells per well (12-well) were seeded and treated with 2 ml of growth medium per well for 48 hours (day -4). Then the medium is changed to differentiation medium for another 48 hours (day -2), so the MSC are predisposed to preadipocytes, before the adipogenesis of the confluent cultures is induced by treating them with induction medium (day 0). After 2 days the induction phase is over and the medium is changed to differentiation medium again (day 2) which is renewed every second day until the cells were considered brown adipocytes on day 7.

Differentiation medium (DM)

DMEM Glutamax I + 4500 mg/l Glucose, - Pyruvate	
FBS	10 %
P/S	1 %
Insulin	20 nM
T3	1 nM

Induction medium (IM)

Differentiation medium	
Dexamethasone	1 µM
IBMX	0.5 mM

2.2.6 Infection of cells with lentiviral vectors

The cells were seeded in 6-wells or 12-wells like described before (2.2.5). When they adhered to the plate after a few hours, the medium was discarded and the cells were treated with 800 µl of fresh growth medium per well, containing 30 ng (6-well) or 20 ng (12-well) of the lentiviral vector. After being incubated for 24 hours at 37° C, 1 ml of additional growth medium was given to each well and the plates were incubated for 24 hours at 37° C again. Finally the medium was discarded, 2 ml of the differentiation medium were added and the normal adipogenic differentiation of the cells proceeded.

2.3 Biochemical methods

2.3.1 Material and equipment

Material:

Acrylamide, Rotiophorese Gel 30 (37.5:1), Carl Roth GmbH (Cat. No. 3029.1)

BSA (essential fatty acid free), Sigma-Aldrich (Cat. No. A-7030)

Cell scraper, Labomedic, Gießen (Cat. No. 2015217)

Coomassie dye, Coomassie brilliant blue, Merck (Cat. No. 1.15444.0025)

Enhanced chemiluminescence solution (ECL) reagent, Amersham Biosciences, Buckinghamshire, UK (Cat. No. 1059250/243)

L-(-)-Norepinephrine bitartate salt monohydrate (NE), Sigma-Aldrich (Cat. No. A-9512)

Oil RedO, Sigma-Aldrich (Cat. No. O-0625)

Protease inhibitor cocktail, Complete EDTA-free, Roche, Mannheim (Cat. No. 11873580)

Equipment:

Centrifuge, 5415R, Eppendorf, Hamburg

Centrifuge, Biofuge Primo, Heraeus, Hanau

Centrifuge, Sigma 8k with 12510-H rotor, Sartorius, Göttingen

Electrophoresis / Blotting system, Mini Trans Blot System, BioRad, München

Microplate absorbance reader, Sunrise Basic, Tecan, Austria

Photometer, Biophotometer, Eppendorf, Hamburg

Thermomixer, 5350, Eppendorf, Hamburg

Western blot analysis software, Quantity One, BioRad

Primary antibodies:
Adenosine A1-R, 1:500, Santa Cruz Biotechnology, Santa Cruz, USA (Cat. No. Sc-28995)
Adenosine A2A-R, 1:500, Santa Cruz Biotechnology, Santa Cruz, USA (Cat. No. Sc-32261)
Adenosine A2B-R, 1:500, Santa Cruz Biotechnology, Santa Cruz, USA (Cat. No. Sc-28996)
aP2, 1:1000, Santa Cruz Biotechnology, Santa Cruz, USA (Cat. No. Sc-1473)
C/EBPa, 1:1000, Santa Cruz Biotechnology, Santa Cruz, USA (Cat. No. Sc-365318)
GAPDH, 1:1000, Epitomics, USA (Cat. No. 2251-S)
PPARy, 1:1000, Santa Cruz Biotechnology, Santa Cruz, USA (Cat. No. Sc-7273)
Tubulin, 1:1000, Dianova, Hamburg (Cat. No. DLN-09992)
UCP-1, 1:500, Santa Cruz Biotechnology, Santa Cruz, USA (Cat. No. Sc-6529)

Secondary antibodies:
Goat-HRP, 1:5000, Chemicon (Cat. No. AP309P)
Mouse-HRP, 1:10000, Dianova, Hamburg (Cat. No. 115-035-146)
Rabbit-HRP, 1:5000, Cell-Signaling, Danvers, USA (Cat. No. 7074)

2.3.2 Oil Red O Staining of differentiated adipocytes

The Oil Red O Staining is a method to visualize lipid droplets in tissues or cells and is used to visualize the grade of differentiation of mature adipocytes (Haas et al., 2009).
It was performed on day 7 of the 11-day differentiation protocol (2.2.5). The medium was discarded and the cells were washed with PBS. In order to fix them to the plates they were treated with 1 ml per well of 4 % PFA for 15 min at room temperature. The PFA is discarded and the fixed cells were washed with PBS, before the Oil Red O working solution was applied for 2-4 hours at room temperature. Finally the coloured cells were washed with H_2O.

Oil Red O stock solution (5 mg/ml)

Isopropyl alcohol (99 %)

Oil Red O 0.5 %

Dissolved with a magnetic stir bar overnight and stored at RT

Oil Red O working solution

H_2O

Oil Red O stock solution 60 %

Mixed and filtered twice through a paper filter

2.3.3 Preparation of total protein lysates

In order to isolate the proteins from adherent cells, the medium was discarded and the cells were washed with ice-cold PBS. Then 200 µl of the ice-cold lysis buffer (RIPA) were added to each well and the plates were put on ice. A scraper was used free the cells from the plates and the lysates were transferred to Eppendorf tubes. The samples were put in an ultrasonic bath for 1 min and incubated on ice for about 5 min, before being centrifugated at 14,000 rpm and 4° C for 10 min. The pure phase between the fatty layer and the pellet was transferred in new Eppendorf tubes and the protein concentration determined via Bradford Protein Assay (2.3.4). Each sample should contain the same protein concentration. The adjusted samples were mixed with 3-fold concentrated Laemmli (Laemmli, 1970) and incubated at 95° C for 5 minutes (37° C for 15 min, if the samples are G-protein-coupled-membrane-proteins) to give negative charge to all proteins and to reduce disulfide bonds. Afterwards they could be used for SDS-PAGE (2.3.5) or they were shock frozen in liquid nitrogen and stored at -80° C.

Lysis buffer (RIPA)

Tris HCl	10 mM / pH 7.4
NaCl	150 mM
NP-40	1 %
Desoxy-cholic acid-Na	1 %
Sodium dodecyl sulphate (SDS)	0.1 %

sterile filtered and stored at 4° C

before use the following substances were added	
Complete EDTA-free	40µl/ml
NaF	10 mM
Na_3VO_4	1 mM

Laemmli buffer (3x)

Tris HCl	125 mM / pH 6.8
Glycerol	20 %
SDS	17 %
Bromphenol blue	0.015 %
Dissolved in H_2O and stored at -20° C	
Before use the following substance was added	
ß-mercaptoethanol	5 %

2.3.4 Quantification of protein concentration with the Bradford protein assay

The Bradford assay is a colorimetic procedure for measuring the total protein amount in a sample, using the absorbance shift of Coomassie brilliant blue G-250 that is caused by an interaction with certain amino acid residues (Bradford, 1976). The free dye displays an absorbance maximum at 470 nm, while the protein-bound anionic form has its maximum at 595 nm. 2 µl of each protein lysate sample were mixed with 98 µl of 0.15 M NaCl and incubated with 1 ml of Coomassie solution for 2 min. The absorbance was measured at 595 nm and a range of different BSA standard dilutions used to determine the protein concentration.

Coomassie solution

Coomassie brilliant blue g-250	0.01 %
Ethanol (95 %)	5 %
Phosphoric acid (85 %)	8.5 %
dissolved in H_2O and stored at 4° C	

2.3.5 One dimensional SDS-polyacrylamide-gelelectrophoresis (SDS-PAGE)

The SDS-PAGE is a widely used method to separate proteins according to their electrophoretic mobility, determined by their length, conformation and charge. The denatured Laemmli protein samples (2.3.3) were loaded on a stacking gel that was poured on top of a differentially buffered separating gel. During the discontinuous gel electrophoresis the proteins concentrate at the stacking / separating gel interface, before getting separated according to their molecular size in the separating gel under denaturating conditions. The gelelectrophoresis was performed in SDS-PAGE running buffer at 100 V, 300 mA and RT, using a Mini Trans Blot system (BioRad).

Stacking gel (5 ml)	**5 %**
H_2O	3.4 ml
Rotiophorese Gel 30 % (acrylamide mix)	0.83 ml
Tris-HCl 1 M (pH 6.8)	0.63 ml
Ammonium persulfat (APS) 20 %	0.025 ml
N,N,N',N'-tetramethylethylenediamine (TEMED)	0.005 ml

Separating gel (10 ml)	**8 %**	**10 %**	**12 %**	**15 %**
H_2O	4.6 ml	4 ml	3.3 ml	2.3 ml
Rotiophorese Gel 30 %	2.7 ml	3.3 ml	4 ml	5 ml
Tris HCl 1.5 M (pH 8.8)	2.5 ml	2.5 ml	2.5 ml	2.5 ml
APS 20 %	0.05 ml	0.05 ml	0.05 ml	0.05 ml
TEMED	0.004 ml	0.004 ml	0.004 ml	0.004 ml

10 x SDS-PAGE running buffer	
Tris	25 nM
Glycine	2 M
SDS	0.1 %
dissolved in H_2O and stored at RT	

2.3.6 Western Blotting and immunodetection

Western Blotting is used to detect specific proteins with the compatible antibodies. The stacking gel of the SDS-PAGE was removed and the separating gel placed in transfer buffer. In order to transfer the proteins electrically from the polyacrylamide gel onto a nitrocellulose membrane, both were arranged in a transfer construction containing transfer buffer. Dependent on the protein size to be transferred, time and current were chosen. Small proteins were blotted at 230 mA for 50 min, bigger ones at 300 mA for 70 min.

Afterwards the membrane was briefly washed in Tris-buffered saline (TBS) mixed with Tween-20 (TBS-T) and incubated for 1 hour in blocking buffer at RT on an orbital shaker in order to block unspecific binding sites. The membrane was washed 3 times with TBS-T again, before being incubated with the primary antibody diluted in blocking buffer and NaN_3 overnight at 4° C. After 3 more washes with TBS-T the next morning, the membrane was incubated with the appropriated horseradish peroxidise (HRP)-coupled secondary antibody for 1 hour at RT. Subsequently the membrane was washed with TBS-T 3 times and finally subjected to freshly prepared enhanced chemiluminescent (ECL) in order to detect the wanted protein using ImageQuant software.

To be able to quantify the amount of expression of the wanted protein, the membrane was stripped afterwards for 30 min and the immunodetection procedure was repeated with tubulin. It is a protein that is expressed in most cells and functions as a loading control.

Transfer buffer

10x SDS PAGE running buffer	10 %
Methanol	20 %

dissolved in H_2O and stored at RT

10 x TBS

Tris	100 mM
NaCl	1.4 M

dissolved in H_2O, adjusted to pH 8.0 with HCl and stored at RT

TBS-T (0.1 %)

10 x TBS	10 %
Tween-20	0.1 %

dissolved in H_2O and stored at RT, protected from the light

Blocking buffer I

Skimmed milk powder	5 %

dissolved in TBS-T and stored at 4° C

Blocking buffer II

BSA	5 %

dissolved in TBS-T and stored at 4° C

2.3.7 Measurement of lipolytic activity in mature brown adipocytes

Lipolysis is a main aspect of BAT function and is therefore measured in order to investigate the activity of brown adipocytes. Triglycerides are hydrolyzed enzymatical into free fatty acids and glycerol during lipolysis, so the release of free glycerol by the cells is proportional to their lipolytic activity. The release can be measured by coupled enzyme reactions.

First the 6- or 12-well plates with the differentiated adipocytes were washed with the lipolysis medium 3 times to get rid of the free fatty acids, then 800 µl of the medium were added to each well. The stimulating substances, like norepinephrine or adenosine, were added and the cells were incubated at 37° C and 5 % CO_2 for 2 hours. In order to determine the amount of free glycerol, 40 µl of the medium from each well were transferred to a 96-well and mixed with 60 µl of the free glycerol reagent (Sigma), before being incubated at 37° C for 10 min. Finally the absorbance was measured at 540 nm in a plate reader. 40 µl of the pure medium mixed with 60 µl of the free glycerol reagent were used as a blank and 5 µl of the standard solution (Sigma) mixed with 95 µl of the free glycerol reagent as a standard. The standard was used to calculate the glycerol concentration in the samples and the results were normalized to the protein concentration in the cells, which was determined afterwards using protein lysates and Bradford assay.

Lipolysis medium

DMEM (no phenol red, 4.5 g/l D-glucose)	
BSA (essential fatty acid free)	2 %

2.3.8 Quantitative determination of cAMP in culture supernatants

A direct cAMP enzyme-linked immunosorbent assay (ELISA) kit (#ADI-901-066) was used to measure the amount of cAMP poured by mature brown adipocytes after being stimulated with specific substances.

The cells were treated with 2 ml of the cAMP assay medium each well, the substances were added and the plates were incubated at 37° C for 15 min. The medium was discarded afterwards and the cells were subjected to 600 µl of HCl (0.1 M) for 10 min at RT in order to lyse the cells and inactivate the phosphodiesterases (PDE). The mixture of cell components and HCl was transferred to a 1.5 ml Eppendorf tube and 30 µl of the acetylating reagent were added to each sample before they were centrifuged at a minimum of 600 x g for 10 min. The supernatants were used as samples for the ELISA. Therefore 100 µl of each supernatant were mixed with 50 µl of the neutralizing reagent on a 96-well plate and incubated with the first antibody on a plate shaker at RT for 2 hours. After being emptied and washed with the washing buffer, 200 µl of the substrate solution were added to each well and the plate was incubated at RT for 1 hour. Finally the enzyme reaction was stopped by adding 50 µl of the stop solution to each well and the absorbance was measured at 405 nm against a blank and different standard solutions. The cAMP concentration was calculated from standard and normalized to the protein concentration of the samples, which was determined afterwards using the Bradford assay.

cAMP assay medium

DMEM #61695	
FBS	5 %
P/S	1 %
IBMX	0.5 mM

2.4 Molecular biological methods

2.4.1 Material and equipment

Material:

Dish, 100 mm, Sarstedt (Cat. No. 83.1802.001)

Ethidium bromide, Carl Roth GmbH (Cat. No. 2218.1)

LightCycler® SYBR Green I Master, Roche, Mannheim (Cat. No. 4887352)

NucleoBond® PC 500 EF Kit, MACHEREY-Nagel, Düren (Cat. No. 740550)

Platinum® Taq Polymerase High Fidelity, Invitrogen, Karlsruhe (Cat. No. 11304-011)

QIAquick gel extraction kit, QUIAGEN, Hilden (Cat. No. 28704)

Restriction enzymes, New England Biolabs, Schwalbach

T4 DNA Ligase, Invitrogen, Karlsruhe (Cat. No. 11304-011)

TaqCORE Kit, Qbiogen, Montreal, Kanada (Cat. No. EPTQK 109)

Transcriptor First Strand Synthesis Kit, Roche, Mannheim (Cat. No. 4896866)

Equipment:

Autoclave, Varioclave 135 T, Faust, Meckenheim

Electrophoresis chamber, Run One™, Peqlab, Erlangen

Incubator, Certomat IS, Sartorius, Göttingen

LightCycler® 480, Roche

Microwave, Severin, Sundern

Real-time PCR machine, Mx 3000P Multiplex, Stratagene, Agilent Technology

Quantitative PCR system, Santa Clara, USA

Thermocycler, T1, Biometra, Göttingen

Thermomixer 5350, Eppendorf, Hamburg

Ultracentrifuge, Optima L-100 XP, Beckman & Coulter, USA

UV light transilluminator, GelDoc® XR, BioRad, München

2.4.2 Transformation of competent bacteria

The competent Escherichia coli (E. coli) bacteria were defrosted on ice and mixed with the Plasmid DNA in an Eppendorf tube. After being incubated on ice for 10 min, a heat shock procedure was performed. The mixtures were put in a water bath at 42° C for 42

sec in order to get the bacterial membranes permeable for the DNA. Afterwards the tubes were put on ice for 2 min and 1 ml of LB+ medium is added to each of them. The samples were incubated in a shaker at 37° C and 225 rpm for 1 h in order to get the bacteria proliferated and centrifuged at 2000 rpm for 10 min after that. Finally the old medium was discarded, the bacteria pellet was resuspended in fresh LB+ medium and plated on LB+ plates with ampicilline overnight at 37° C.

LB+ medium

NaCl	0.5 %
Peptone	1 %
Yeast extract	0.5 %
Glucose	0.1 %

dissolved in H_2O, adjusted to pH 7.5, autoclaved and stored at 4° C

LB+ plates

LB+ medium

Agar-agar	1.5 %

autoclaved, poured into 100 mm Petri dishes and stored at 4° C

Additives

Ampicilline	50 µg/ml

2.4.3 Preparation of plasmid DNA from bacterial cultures – Mini Preparation

A glass tube with 4 ml of LB+ medium and 4 µl of ampicilline was prepared for every bacteria colony that had grown after the transformation (2.4.2). The colonies were transferred to the tubes with pipette tips and cultured overnight in a shaker at 225 rpm and 37° C. A protocol from Sambrook and Russel for alkaline lysis was used afterwards in order to check the transformed plasmid DNA. It allows a rapid isolation of plasmid DNA with a sufficient degree of purity for further restriction check cuts and sequencing.

2.4.4 Preparation of plasmid DNA from bacterial cultures – Maxi Preparation

The method was used to get a maximal output of plasmid DNA. The samples were sequenced after Mini preparation and the cultures where the right ones belong to were distributed to 2 flasks containing 300 ml of LB+ medium and ampicilline. The bacteria were cultured overnight in a shaker at 225 rpm and 37° C. The next day, the flasks were centrifuged at 5000 rpm and 4° C for 15 min and the medium was discarded. The plasmid DNA was isolated endotoxin-free according to the manufacturer's instructions of the NucleoBond® PC 500 EF Kit.

2.4.5 Enzymatic manipulation of DNA

Restriction enzymes were used to cut the DNA at specific sites. There are 3 different kinds of restriction enzymes which differ in the location they cleave the DNA. The type I and type III restriction enzymes cut the DNA randomly far from respectively outside their recognition sites, while the type II restriction enzymes cleave the DNA within or close to the binding motif. This is why this type is mostly used as a molecular tool.

All restriction enzymes used in this study were purchased from New England Biolabs (NEB, Schwalbach). The digestion was performed according to the manufacturer's instructions, using the restriction buffers supplied with the enzymes by NEB. In general the following conditions were used:

DNA digestion	
DNA	1-4 µg
Buffer (10 x)	10 %
BSA (10 x)	10 %
Restriction enzymes	5 – 20 U
filled up to 30 µl with H_2O and incubated for 1 h at 37° C	

In order to create a phosphodiester bond between DNA fragments, the DNA ligation was performed according to the following protocol.

DNA ligation

DNA backbone (vector)	100 ng
DNA insert	10 - 80 ng
Ligation buffer 10x (Invitrogen)	10 %
T4 DNA ligase (Invitrogen)	5 %

filled up to 10 µl with H_2O and incubated overnight at 16° C

The ligation mixes were used for the transformation of the competent E. coli bacteria (2.4.2).

2.4.6 Agarose gel electrophoresis

Agarose gel electrophoresis was performed in order to separate, identify and purify DNA fragments. Therefore the agarose gel was prepared by adding the agarose powder (0.7 – 2 %) to 1 x TBE buffer and boiling it in the microwave until the agarose was dissolved. Afterwards ethidium bromide (800 ng/ml) was added, the comb was placed in the casting platform and the solution was poured into the platform. The gel was allowed to solidify at RT and then transferred into an electrophoresis chamber filled with 1 x TBE buffer. Each sample was mixed with 6 x loading buffer before being loaded on the agarose gel. 10 µl of the DNA ladder was used as a marker and H_2O as a negative control. Finally electrophoresis was performed at 100 V and RT. The DNA bands were visualized by ultraviolet (UV) light at 366 nm, using the UV light transilluminator and QuantityOne® Software.

TBE buffer 10 x

Tris-HCl	0.9 M
Boric acid	0.9 M
EDTA (pH 8.0)	20 mM

dissolved in H_2O and stored at RT

Loading buffer 6 x

TBE buffer 10 x	60 %
Ficoll type 400	18 %

EDTA (pH 8.0)	0.12 mM
Bromphenol blue	0.15 %
Xylencyanol FF	0.15 %
dissolved in H_2O and stored at -20° C	

Additives

Ethidium bromide	10 mg/ml

The right DNA bands were identified, cut out of the agarose gel and transferred to 1.5 ml Eppendorf tubes. The extraction of the DNA was performed with the QIAquick gel extraction kit (QUIAGEN) according to the manufacturer's instructions.

2.4.7 Isolation of RNA from mature brown adipocytes

In order to isolate the RNA from mature brown adipocytes, the cells were washed with ice-cold PBS, before 1 ml of Trizol was added to each well. The cells were scaped of with a pipette and the mix of cells and Trizol was transferred to a 1.5 ml Eppendorf tube. Next, 200 µl of chloroform ($HCCl_3$) were added to each sample, they were shaken for about 15 s and then incubated for 5 min at RT. The tubes were centrifuged at 13.000 rpm and 4° C for 10 min and the pure upper phase was transferred to a new 1.5 ml Eppendorf tube afterwards. 500 µl of isopropanol (99 %) were added to each of these tubes and they were shaken again. After being centrifuged at 13.000 rpm and 4° C for 10 min, the supernatant of the samples was discarded and 1 ml of ethanol (75 %) was added to each. The mixtures were vortexed and then centrifuged at 13.000 rpm and 4° C for 5 min. The supernatants were carefully discarded afterwards and the pellets were dried at RT for about 20 min. Finally the appropriate amount of DEPC-H_2O was added to each tube and the RNA pellets were solved by shaking the samples at 550 rpm and 55° C for about 10 min.

The RNA concentration of the samples was measured using NanoDrop® and they were used for further experiments or frozen at -80° C.

2.4.8 Polymerase chain reaction (PCR)

The PCR is a widely used enzymatic method in molecular biology to amplify and quantify DNA or certain sequences of the DNA (Saiki et al., 1988). Essential components of the PCR are the DNA template, the two primers, a heat-stable DNA polymerase (*Taq* polymerase) and the source for the newly built DNA, the dNTPs. The method is based on thermal cycling, normally it is a three steps procedure: the mixture is heated to 95° C what leads to a denaturation of the DNA, then the temperature is lowered to the annealing temperature of the primers (52 – 65° C) and finally raised to 72° C for the DNA synthesis of the *Taq* polymerase.

All PCR reactions were performed using the TaqCORE kit (Qbiogen).

2.4.9 Reverse transcriptase PCR (RT-PCR)

The RT-PCR is performed in order to produce complementary DNA (cDNA) out of isolated RNA samples. The Transcriptor First Strand Synthesis Kit from Roche and diverse hexamer primers were used according to the manufacturer's instructions.

RT-PCR reaction mix

mRNA	500 ng
diluted with DEPC-H_2O in a total volume of 11 µl	
Buffer	4 µl
dNTPs	2 µl
Random Hexamer Primer	2 µl
Inhibitor	0.5 µl
Reverse transcriptase	0.5 µl

RT-PCR program

First round *1	**Second round *35**	**Third round *1**
95° C for 5'	95° C for 30''	72° C for 5'
	52° C for 30''	4° C for unlimited period of
	72° C for 45''	time

Each sample was diluted with 180 µl of DEPC-H_2O for the quantification of the cDNA by qPCR (2.4.10) afterwards.

2.4.10 Quantitative real time PCR (qPCR)

The qPCR is a method used for the amplification and simultaneous quantification of cDNA. The SYBR Green dye produces a fluorescent signal by interacting with double-stranded DNA (dsDNA), whose intensity is proportional to the amount of dsDNA in the sample. It increases with every step of the amplification and can be measured in real time by the Roche LightCycler® 480.

qPCR reaction mix	
Light Cycler SYBR Green I Master 2x	5 µl
cDNA (1:10)	5 µl
Primer forward (5 pmol/µl)	0.5 µl
Primer reverse (5 pmol/µl)	0.5 µl

2.4.11 Touchdown PCR

The touchdown PCR is used in order to amplify a specific region of the DNA and to reduce the non-specific background. Therefore an annealing temperature a few degrees above the optimum of the primers was chosen in the beginning, so that only the specific primers bind to the DNA. The annealing temperature was gradually lowered every step what led to a higher rate of amplification of the replicated specific regions of the DNA from the former cycles.

Touchdown PCR reaction mix	
Buffer (+$MgCl_2$) 10x	5 µl
$MgSO_4$ (50 mM)	2 µl
DNA template	1 µl
dNTPs	1 µl
Primer forward (1:10)	1 µl
Primer reverse (1:10)	1 µl
Platinum Taq polymerase	0.2 µl

diluted with DEPC-H_2O to a total volume of 50 µl

Touchdown PCR program

First round *3	**Second round *3**	**Third round *3**
95° C for 1’	95° C for 30’’	95° C for 30’’
95° C for 30’’	55° C for 30’’	54° C for 30’’
57° C for 30’’	72° C for 3’	72° C for 3’
72° C for 3’		
Fourth round *3	**Fifth round *30**	**Sixth round**
95° C for 30’’	95° C for 30’’	72° C for 10’
53° C for 30’’	52° C for 30’’	4° C for unlimited time
72° C for 3’	72° C for 3’	period

2.5 Statistical analysis

All values are presented as means ± standard error of the mean (SEM). Statistical differences were determined using the unpaired Student’s *t* test. P-value less than 0.05 indicates a significant result (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Comparisons among several groups were performed by analysis of variance (ANOVA) followed by Student-Newman-Keul post-hoc test.

3. Results

3.1 Adenosine receptor expression in brown adipocytes

First, the expression of the different adenosine receptors in brown adipocytes and brown adipose tissue (BAT) was investigated. As mentioned before, there are four known subtypes of adenosine receptors, the A1 receptor, the A2A and A2B receptors and the A3 receptor. Qualitative (Figure 9 A) and quantitative PCR (Figure 9 B and 9 C) was used to evaluate the mRNA expression level of these subtypes during differentiation of brown adipocytes.

All four adenosine receptor subtypes are expressed in preadipocytes as well as in brown adipocytes (Figure 9 A), the A2B receptor being the most abundant (Figure 9 B). The amounts of receptor mRNA were shown in relation to the A1 receptor, which is known to play an important role in white adipocytes. Next, the amount of receptor mRNA of each receptor subtype in mesenchymal stem cells (MSC) was compared to the amount in differentiated brown adipocytes. It is shown that the A1 as well as the A2A receptor are significantly upregulated during differentiation, while the expression level of the A2B receptor is not changed (Figure 9 C).

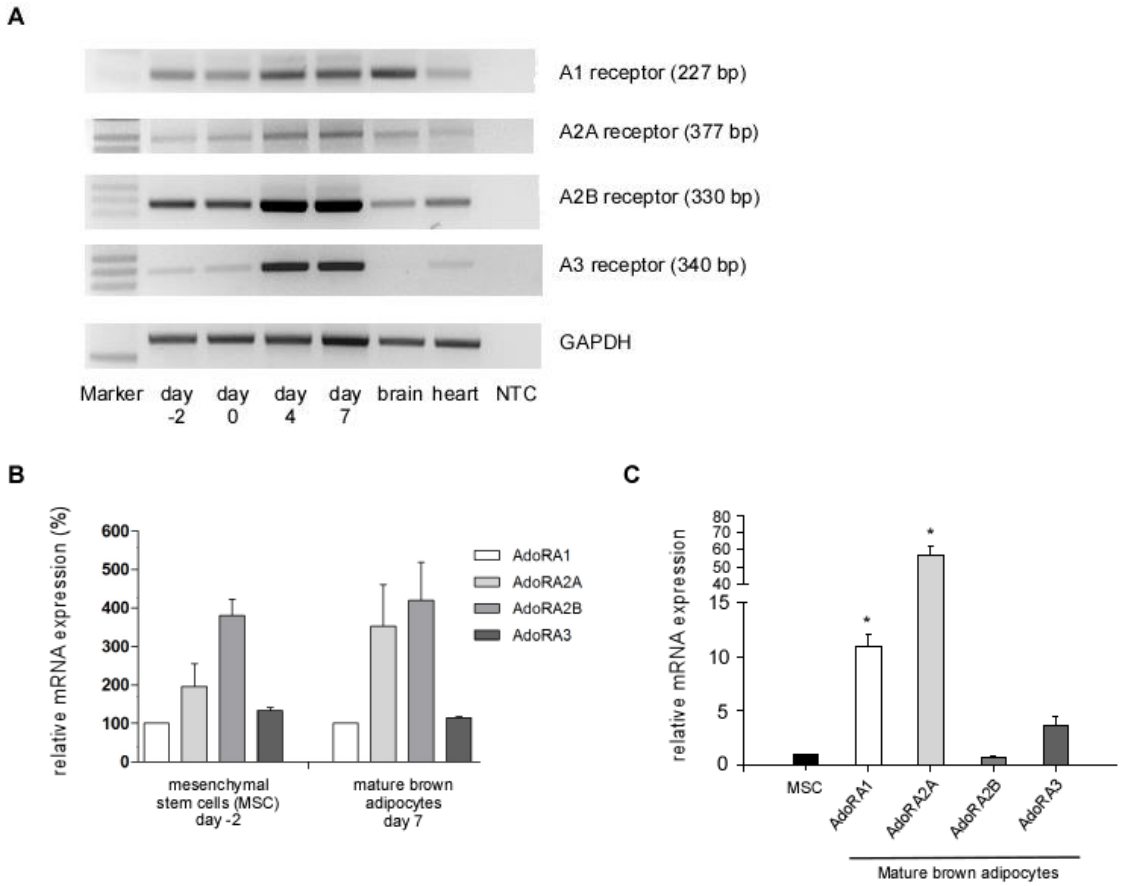


Fig. 9: Expression of adenosine receptors in murine brown adipocytes.
(A) RT-PCR analysis of all four adenosine receptors in brown adipocytes on four different days of the eleven day differentiation protocol and in the brain and heart of mice. Water instead of RNA was used as a negative control (NTC) and GAPDH as an internal control. (B) The relative expression of the adenosine receptor mRNA in comparison to the A1 receptor on day -2 and day 7. (C) The relative adenosine receptor mRNA expression level in mature brown adipocytes in comparison to the level on day -2. *$p < 0,05$. Data are given as mean + s.e.m. (N=3, n=3) and analyzed using two-tailed Student's t test (B and C).

Parts of the data of Figure 9 B and 9 C were preliminary work of Dr. Thorsten Gnad (N=1, n=1).

3.2 Influence of pharmacological stimulation of adenosine receptors on brown adipocyte differentiation

Previous studies in our laboratory showed that cyclic guanosine monophosphate (cGMP) is a strong inducer of brown fat differentiation (Haas, et al., 2009). Also, previous studies using the osteoblastic cell line 7F2 showed that increased A1 receptor expression induces adipocyte differentiation, whereas the over-expression of A2B receptors inhibits

adipogenesis and stimulates an osteoblastic phenotype (Gharibi, Abraham, Ham, & Evans, 2011). The same group discovered that A1 and A2A receptors significantly increased during the differentiation of mesenchymal stem cells (MSC) to white adipocytes. While A1 receptors mainly stimulated lipogenic activity of adipocytes, the A2A receptor activation boosted adipocytic differentiation and lipid accumulation (Gharibi, Abraham, Ham, & Evans, 2012).

Therefore, this study aimed to investigate the effect of specific adenosine receptor antagonists and agonists, i.e. modulation of intracellular cyclic adenosine monophosphate (cAMP) on brown cell differentiation.

To elevate cAMP concentration, brown adipocytes were treated with the specific A1 (PSB36) and A3 (MRS1523) antagonists and the A2A (CGS21680) and A2B (Bay 60-6583) agonists during standard differentiation protocol. Oil Red O staining was used to visualize lipid droplet formation. Compared to control, no significant changes between treatments were observed (Figure 10 A).

For further investigation, the levels of important adipogenic marker proteins like CCAAT-enhancer-binding-protein α (C/EBP α), peroxisome-proliferator-activated receptor γ (PPARγ) and adipocyte protein 2 (aP2) were measured by Western blotting.

Only chronic treatment with the A3 receptor antagonist MRS increased the expression level of C/EBP α and aP2 significantly compared to control brown adipocytes. The A1 receptor antagonist and both A2 agonists had no significant effect on the protein levels of marker genes (Figure 10 B-D)

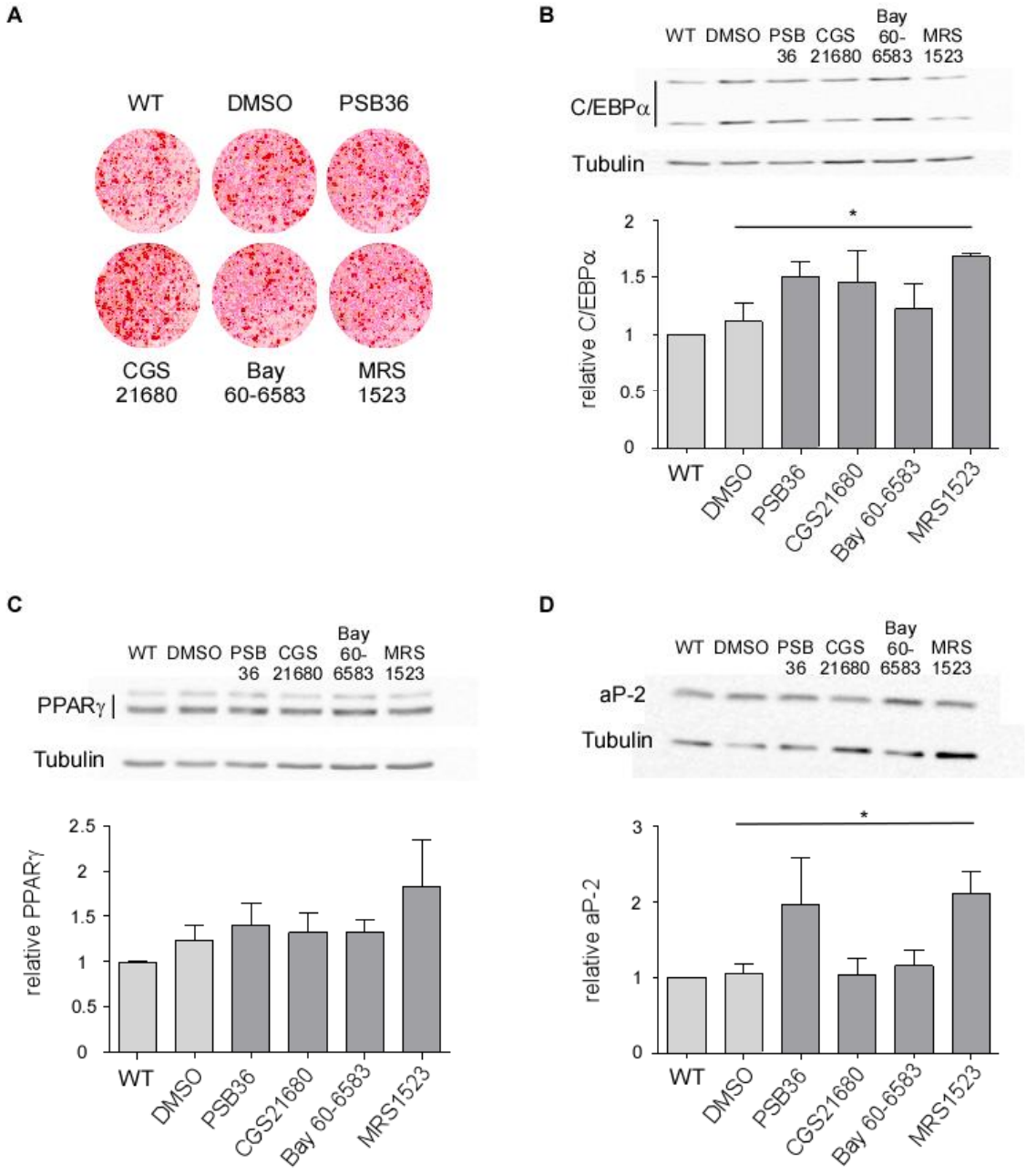


Fig. 10: Effect of adenosine receptor agonists and antagonists on adipogenesis in brown adipocytes.
(A) Oil Red O stain of mature brown adipocytes on day 7. Treatment with DMSO, 30 nM of the A1 receptor antagonist PSB36, 150 nM of the A2A receptor agonist CGS21680, 300 nM of the A2B receptor agonist Bay 60-6583 or 30 nM of the A3 receptor antagonist MRS1523 from day -2 till day 7 as indicated. Western Blot analysis and the corresponding densitometric analysis of CCAAT-enhancer-binding protein α (C/EBPα) (B), peroxisome-proliferator-activated receptor γ (PPARγ) (C) and adipocyte protein 2 (aP-2) (D). Tubulin was used as an internal control. Treatment with DMSO, 30 nM of PSB36, 150 nM of CGS21680, 300 nM of Bay 60-6583 or 30 nM of MRS1523 from day -2 till day 7 as indicated. *p < 0,05. Data are shown as mean + s.e.m. (N=3, n=3) and analyzed using two-tailed Student's t test (B-D).

3.3 Effect of pharmacological stimulation of adenosine receptors on thermogenic capacity of brown adipocytes

Brown adipose tissue (BAT) consumes energy through non-shivering thermogenesis by uncoupling the ATP-synthesis in the mitochondria and producing heat instead of ATP (Cannon & Nedergaard, 2004). Responsible for this process is the unique brown fat protein Uncoupling-Protein-1 (UCP-1). Hence, we investigated whether chronic treatment of brown adipocytes during differentiation with the A1 and A3 adenosine receptor antagonists and the A2 agonists effects the expression of UCP-1 and thereby the thermogenic capacity of these cells. Cells were chronically treated with the respective substances during differentiation and the expression level of UCP-1 was measured afterwards via Western blotting.

Only the A1 adenosine receptor antagonist PSB36 showed a tendency to increase the level of UCP-1 in brown adipocytes, however, this effect was not significant (*p-value* = *0,2233*). Inhibition or activation of the other adenosine receptors did not change the UCP-1 levels of brown adipocytes.

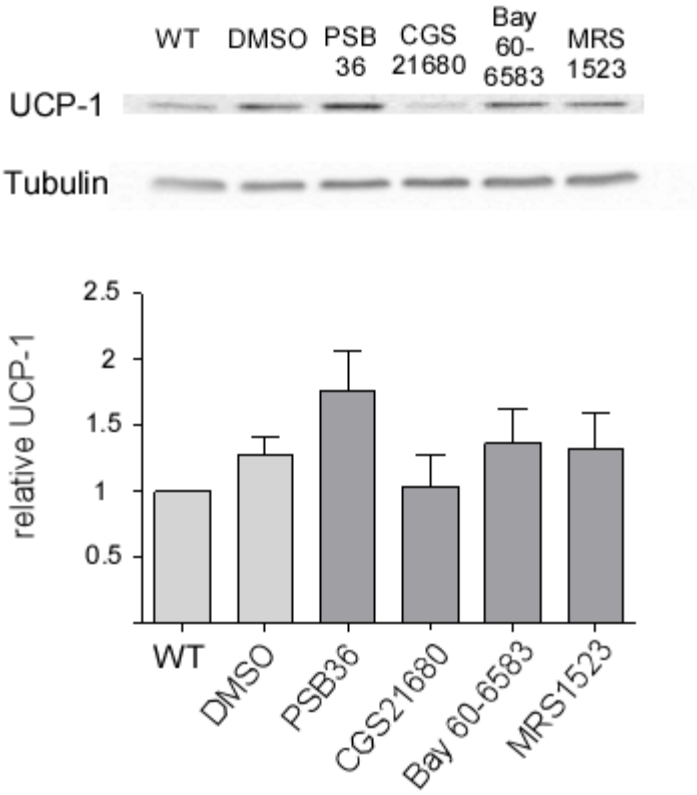


Fig. 11: Chronic treatment with the specific adenosine receptor agonists and antagonists does not effect UCP-1 protein levels.
Western Blot analysis and the corresponding densitometric analysis of uncoupling protein 1 (UCP-1). Tubulin was used as an internal control. Treatment with DMSO, 30 nM of the A1 receptor antagonist PSB36, 150 nM of the A2A receptor agonist CGS21680, 300 nM of the A2B receptor agonist Bay 60-6583 or 30 nM of the A3 receptor antagonist MRS1523 from day -2 till day 7 as indicated. *p < 0,05. Data are shown as mean + s.e.m. (N=3, n=3) and analyzed using two-tailed Student's t test.

3.4 Stimulation of the A2 receptors causes an activation of brown adipocyte function

The activation of brown adipocytes by the sympathetic nervous system leads to an increased amount of intracellular cAMP by stimulating the adenylyl cyclase via beta-receptors (Cannon & Nedergaard, 2004). High levels of cAMP activate the proteinkinase A (PKA). PKA activates hormone-sensitive lipase (HSL) which hydrolyzes triglycerides to free fatty acids and glycerol. The free fatty acids bind to UCP-1 and modulate it and thereby activate brown adopcyte function (Nedergaard, et al., 2001).

To investigate the role of adenosine and the different adenosine receptors in the activation of brown adipocyte function, intracellular levels of cAMP were analyzed with ELISA.

3.4.1 Adenosine and specific A2A and A2B receptor agonists increase intracellular cAMP in brown adipocytes

The stimulation of beta-adrenergic receptors with NE leads to an increasing level of intracellular cAMP and to an activation of brown adipocyte function (Cannon & Nedergaard, 2004). In contrast to beta-receptors, A1 and the A3 adenosine receptors are Gi-coupled and their stimulation decreases the amount of intracellular cAMP. Vice versa, inhibition of A1 and A3 might increase cAMP levels. A2A and A2B receptors are Gs-coupled and therefore increase cAMP upon stimulation.

In order to investigate the role of the different adenosine receptors in activation of brown adipocyte function, cells were treated with the specific A1 antagonist PSB36, the A2A agonist CGS21680, the A2B agonist Bay 60-6583 and the A3 antagonist MRS1523 and the intracellular cAMP level was measured by ELISA.

Adenosine as well as A2A and A2B receptor stimulation increased the level of intracellular cAMP in brown adipocytes significantly. Interestingly, adenosine and A2 stimulation reached 57 % (adenosine), 62 % (A2A) and 58 % (A2B), respectively, of the maximal response provoked by norepinephrine. Blockade of A1 and A3 receptors elevated intracellular cAMP levels, albeit not significantly (Figure 12).

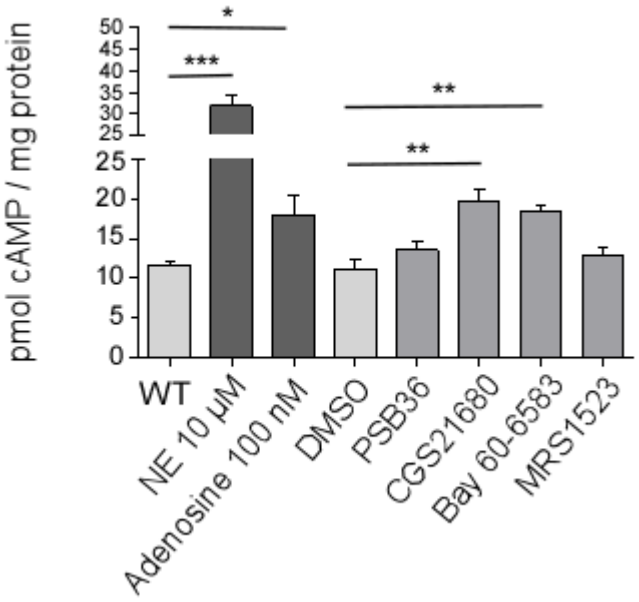


Fig. 12: Adenosine and specific A2A and A2B agonists increase intracellular cAMP in brown adipocytes.
Quantification of intracellular cAMP in mature brown adipocytes. Norepinephrine (NE) was used as a positive control. Treatment of cells on day 7 with 10 µM of NE, 100 nM of adenosine, DMSO, 30 nM of the A1 receptor antagonist PSB36, 150 nM of the A2A receptor agonist CGS21680, 300 nM of the A2B receptor agonist Bay 60-6583 or 30 nM of the A3 receptor antagonist MRS1523 for 15 min as indicated. *p < 0,05, **p < 0,01, ***p < 0,001. Data are shown as mean + s.e.m. (N=3, n=3-5) and analyzed using ANOVA with Newman-Keuls post hoc test.

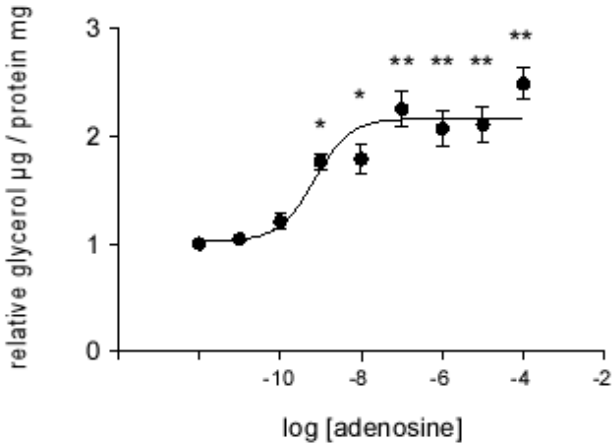
3.4.2 Adenosine elevates lipolysis in brown adipocytes and has an additive effect to norepinephrine

Lipolysis, i.e. the hydrolysis of fat into glycerol and free fatty acids is a hallmark of brown adipocyte activity. Here, we measured lipolysis/glycerol release by the cells after short-term treatment with adenosine and the specific agonists and antagonist of the receptors.
It is known that brown adipocytes are activated by the sympathetic nervous system via norepinephrine (NE) (Cannon & Nedergaard, 2004), resulting in elevated lipolysis. Adenosine has previously been described to be inhibitory on adipocyte lipolysis or oxygen consumption in hamster and rat (Schimmel & McCarthy, 1984; Szillat & Bukowiecki, 1983; Woodward & Saggerson, 1986). This study wanted to investigate the role of adenosine on murine brown fat cell activation. The release of free glycerol from the cells after an acute stimulation with adenosine was measured using a lipolysis assay and normalized to the protein concentration of the samples.

Adenosine elevated lipolysis of murine brown adipocytes significantly (2.5-fold) and in a dose-dependent manner. Half maximal stimulation was already reached at low nanomolar concentrations (EC_{50} = 0.7 nm), indicating a high sensitivity of these cells for adenosine (Figure 13 A).

It is well established that there is a co-transmission of ATP together with NE after stimulation of nerve terminals (von Kugelgen & Starke, 1991). ATP can be rapidly degraded to adenosine via ectonucleotidases (Picher, et al., 2003; Robson, et al., 2005). Hence, adenosine might have an additive effect to NE-mediated activation of brown adipocytes. Mature brown adipocytes were pre-treated with a low concentration of NE followed by increasing concentrations of adenosine and cellular lipolysis was measured. Adenosine increased the release of free glycerol in a dose-dependent manner while stimulating the cells with NE shortly before (Figure 13 B), possibly indicating a physiological co-stimulation of BAT by the sympathetic nervous system with NE and adenosine. Vice versa, the combination of adenosine with NE increased the maximum glycerol release provoked by adenosine alone by 24 per cent (Figure 13B).

A



B

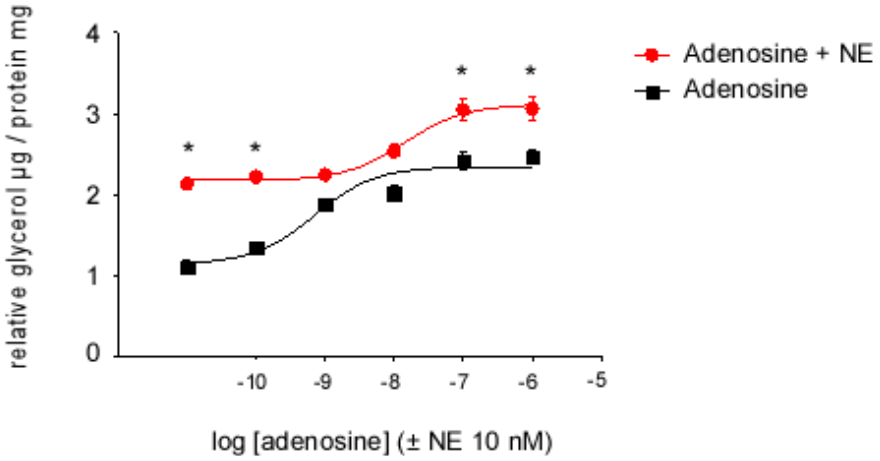


Fig. 13: Adenosine elevates lipolysis in brown adipocytes and has an additive effect to norepinephrine.
Analysis of the free glycerol release of mature brown adipocytes after stimulation with different concentrations of adenosine (A) and adenosine ± norepinephrine (NE) (B). Treatment of cells on day 7 with an increasing adenosine concentration from 10^{-10} M to 10^{-4} M (A) and an increasing adenosine concentration from 10^{-10} M to 10^{-6} M ± 10 nM of NE (B) for 2 hours as indicated. *p < 0,05, **p < 0,01. Data are shown as mean ± s.e.m. (N=5, n=10) (A) and (N=4, n=6) (B) and analyzed using two-paired Student's t test.

Parts of the data of Figure 13 A were preliminary work of Dr. Thorsten Gnad (N=1, n=1).

3.4.3 Specific A2A and A2B adenosine receptor agonists elevate lipolysis

To further investigate the role of the different adenosine receptors, the release of free glycerol by brown adipocytes was measured after treatment with the specific antagonists and agonists described in *3.4.2*.

Stimulation of cells with A2A or A2B agonists resulted in significantly elevated lipolysis compared to control. A2A receptor stimulation increased brown adipocyte activation 2.3-fold, whereas the A2B receptor agonist provoked a 1.9-fold increase compared to control. Both A2 receptor activations had similar effects compared to adenosine. Interestingly, A1 receptor inhibition increased the glycerol release significantly, indicating a basal activity of this receptor and/or existence of extracellular adenosine. A3 receptor blockade did not show any significant effect (Figure 14).

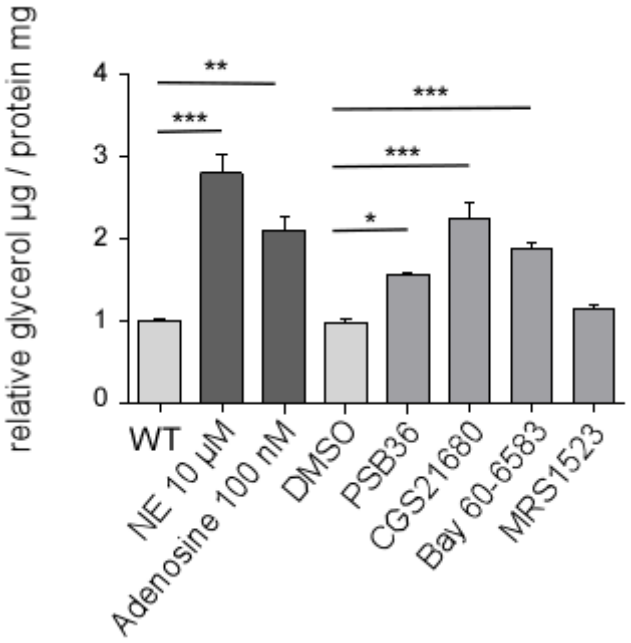


Fig. 14: Specific A2A and A2B adenosine receptor agonists elevate lipolysis.
Analysis of the free glycerol release of mature brown adipocytes. Norepinephrine (NE) was used as a positive control. Treatment of cells on day 7 with 10 µM of NE, 100 nM of adenosine, DMSO, 30 nM of PSB36, 150 nM of CGS21680, 300 nM of Bay 60-6583 or 30 nM of MRS1523 for 2 hours as indicated. *p < 0,05, **p < 0,01, ***p < 0,001. Data are shown as mean + s.e.m. (N=4, n=4) and analyzed using two-paired Student's t test.

This data shows that brown adipocytes may be activated by specific A2A and A2B receptor agonists and thereby suggests that these receptors might play an important role in the activation of brown adipocyte function.

3.5 Overexpression of the A2A and the A2B adenosine receptors effects adipogenesis in brown adipocytes

To further investigate the role of the A2A and the A2B adenosine receptors in brown adipocytes, a genetic approach using lentiviral overexpression of the receptors was used. Brown adipocytes were infected with full-length A2A- or A2B-receptor coding virus constructs (Figure 15 A) and cells were differentiated. To ensure that the virus infection itself has no effect on the cell differentiation, control lentiviral vector (rll156) was used. First, the efficacy of virus-mediated overexpression was controlled by analysing A2A and A2B receptor mRNA expression using qPCR. The amount of A2A receptor mRNA was increased by 19-fold, whereas A2B expression was elevated 71-fold compared to control virus-infected cells (Figure 15 B-C)

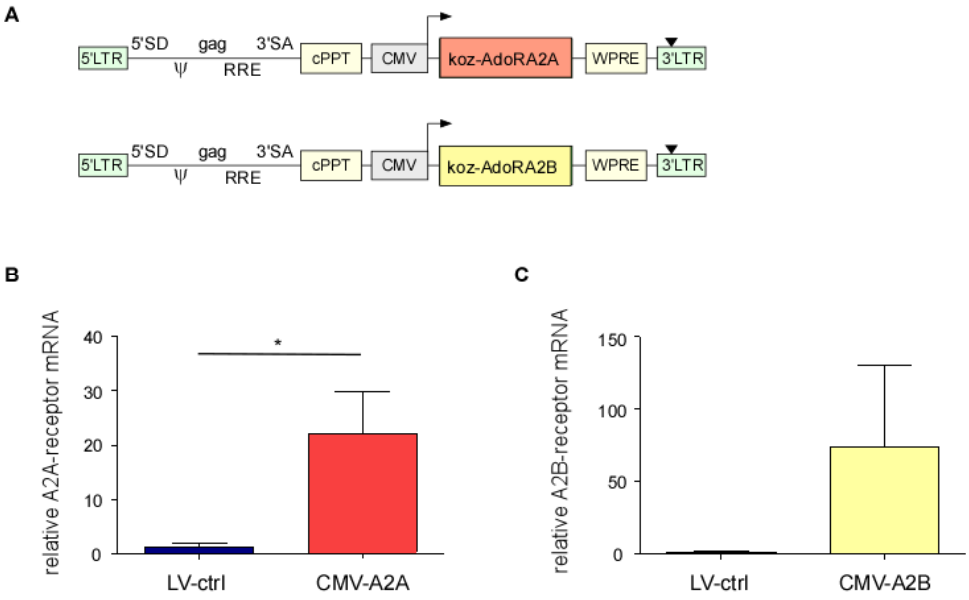


Fig. 15: Successful overexpression of the A2A and the A2B adenosine receptor.
(A) The lentiviral constructs used for the infection of the brown adipocytes. Cells were infected with approximately 30 ng (6-well) or 20 ng (12-well) of virus on day -3 of the eleven day differentiation protocol as indicated. QPCR analysis of the receptor mRNA of the A2A (B) and the A2B receptor (C) in mature brown adipocytes in comparison to cells infected only with the control virus. *p < 0,05. Data are shown as mean + s.e.m. (N=3, n=3) and analyzed using two-tailed Student's t test (B and C).

3.5.1 Overexpression of the A2A receptor increases the number of lipid droplets in brown adipocytes and the expression of adipogenic marker proteins

Next, effects of the A2 adenosine receptor overexpression on the differentiation of brown adipocytes were investigated. Therefore, mature cells were stained with Oil-Red-O solution on the last day of differentiation. Uninfected brown adipocytes were treated with cGMP during differentiation as a positive control. The overexpression of the A2A receptor alone increased the number of Oil Red O-stained lipid droplets in comparison to cells infected with the control virus. Surprisingly, additional chronic treatment with the A2A receptor agonist CGS21680 showed no further effect, but reduced the amount of lipids to the level of the uninfected cells. The overexpression of the A2B receptor and its combination with the A2B receptor agonist Bay 60-6583 showed no effect (Figure 16).

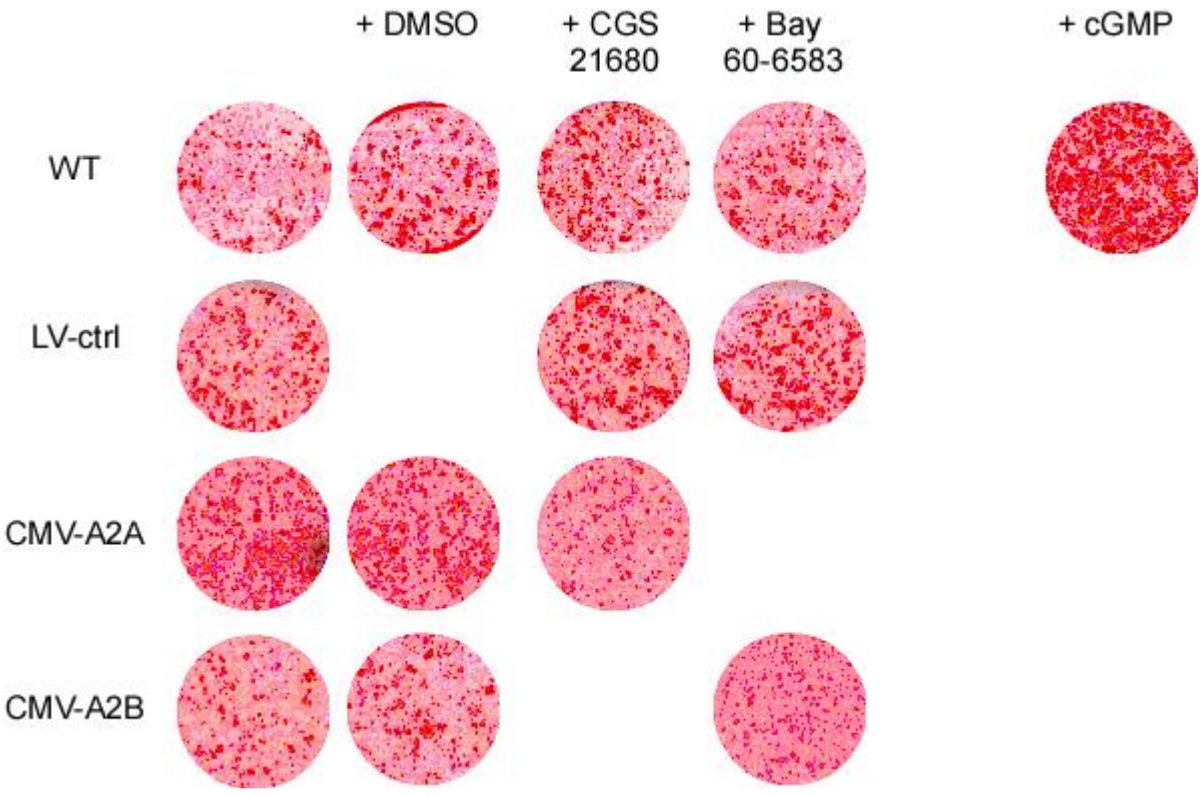


Fig. 16: Overexpression of the A2A receptor increases the number of lipid droplets in brown adipocytes.
Oil Red O stain of mature brown adipocytes on day 7. Infection of the cells with 30 ng of virus on day -3 of the eleven day differentiation protocol. Treatment with DMSO, 150 nM of CGS21680, 300 nM of Bay 60-6583 or cGMP from day -2 till day 7 as indicated.

In order to investigate the effect of the A2A and A2B adenosine receptor overexpression on the adipogenesis of brown adipocytes on molecular basis, the levels of the adipogenic marker proteins C/EBPα, PPARγ and aP-2 were determined via Western Blotting.

The expression levels of C/EBPα and PPARγ were increased by overexpression of the receptors in comparison to cells infected with the control virus. The amount of C/EBPα was significantly elevated by 2.2-fold by overexpression of the A2A adenosine receptor. The overexpression of the A2B receptor elevated the amount of the protein by 1.9-fold, however, the change was not significant (Figure 17 A). The level of PPARγ did also not change significantly, but was increased 2.06-fold in the cells with the A2A receptor over-expressed and by 2.19-fold in the cells with the A2B receptor being overexpressed (Figure 17 B). Interestingly, the combination of the overexpression and the stimulation with the specific agonist for both A2 adenosine receptors had no further positive effect on the amount of all three proteins, but decreased the expression level of aP-2 significantly. These cells only reached 36.29 (A2A) respectively 38.99 (A2B) per cent of the aP-2 expression level of the cells infected by the control virus (Figure 17 C).

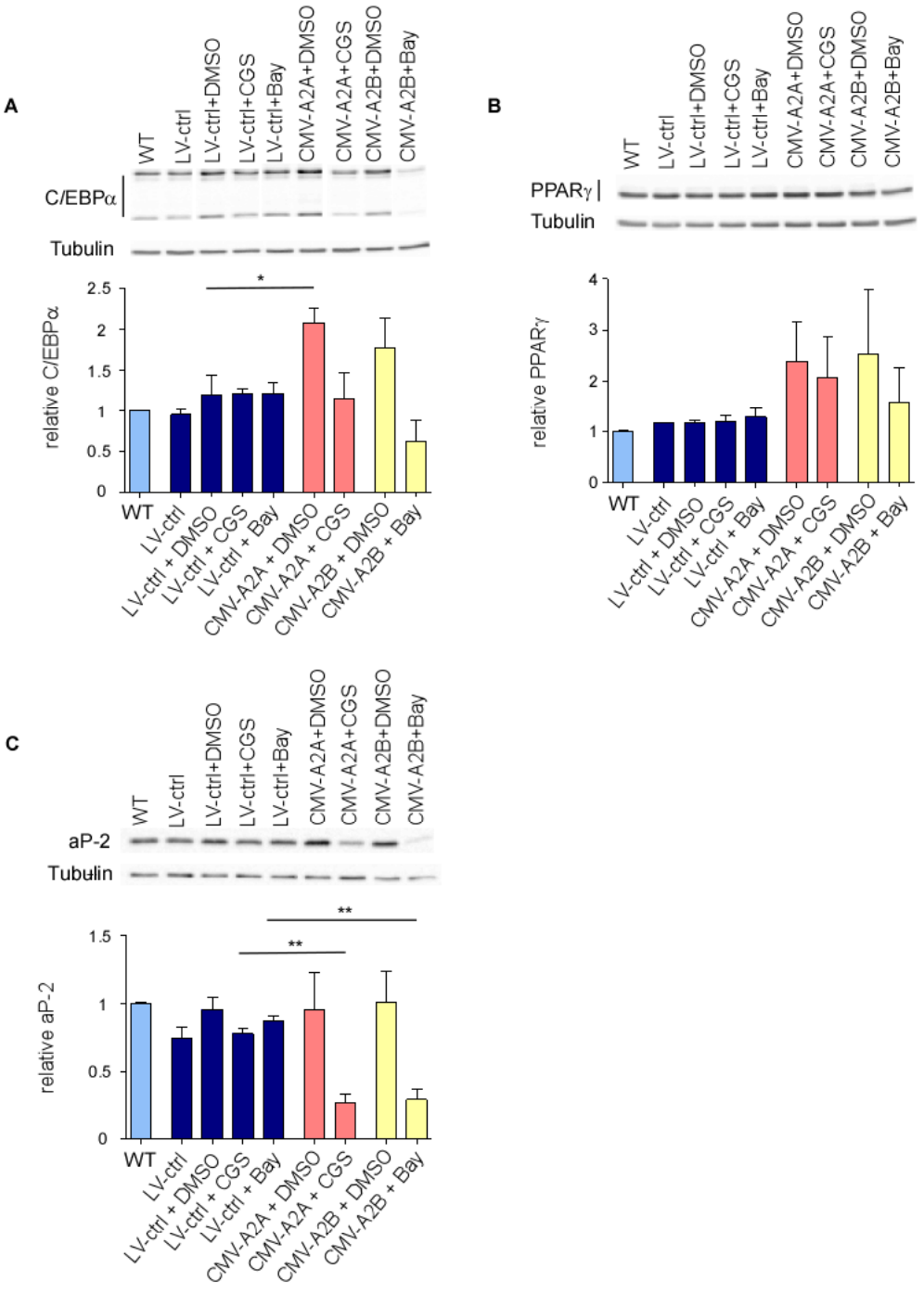


Fig. 17: Overexpression of A2 adenosine receptors increases the expression of adipogenic marker proteins.
Western Blot analysis and the corresponding densitometric analysis of CCAAT-enhancer-binding protein α (C/EBPα) (A), peroxisome-proliferator-activated receptor γ (PPARγ) (B) and adipocyte protein 2 (aP-2) (C). Tubulin was used as an internal control. Infection of the cells with 30 ng of virus on day -3 of the eleven day differentiation protocol. Treatment with DMSO, 150 nM of CGS21680 or 300 nM of Bay 60-6583 from day -2 till day 7 as indicated. *p < 0,05, **p < 0,01. Data are shown as mean + s.e.m. (N=3, n=3) and analyzed using two-paired Student's t test.

3.6 Overexpression of the A2A adenosine receptor influences the expression level of UCP-1

Besides the adipogenic pathway, thermogenesis in mature brown adipocytes was also investigated after overexpression of the A2A and A2B adenosine receptors. Therefore, the expression level of UCP-1 in cells infected with rll-156, CMV-A2A and CMV-A2B with and without additional stimulation by agonists was analyzed via Western blotting.

Surprisingly, only the increased abundance of the A2A adenosine receptor enhanced the expression level of UCP-1 in brown adipocytes, while the combination of overexpression and chronic treatment with the A2A receptor agonist CGS21680 showed no increase of the thermogenic protein in comparison to cells infected with the control virus. Similar results were obtained when overexpressing A2B and stimulating with A2B receptor agonist Bay 60-6583. The overexpression of the A2A receptor alone increased the expression of UCP-1 compared to the cells infected by the control virus by 3.34-fold, while the overexpression of the A2B receptor elevated UCP-1 significantly by 1.35-fold (Figure 18).

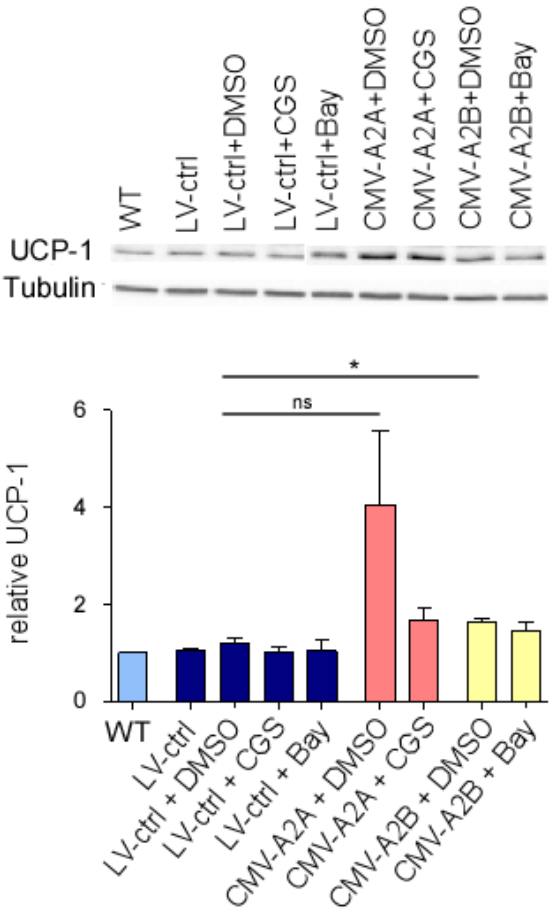


Fig. 18: Overexpression of the A2A adenosine receptor influences the expression level of UCP-1.
Western Blot analysis and the corresponding densitometric analysis of Uncoupling Protein 1 (UCP-1). Tubulin was used as an internal control. Infection of the cells with 30 ng of virus on day -3 of the eleven day differentiation protocol. Treatment with DMSO, 150 nM of CGS21680 or 300 nM of Bay 60-6583 from day -2 till day 7 as indicated. *p < 0,05. Data are shown as mean + s.e.m. (N=3, n=3) and analyzed using two-paired Student's t test.

3.7 Overexpression of the A2 adenosine receptors stimulates brown adipocyte function

It was already shown that a stimulation of mature brown adipocytes with adenosine or the A2A and A2B adenosine receptor agonists CGS21680 and Bay 60-6583 activated cell function by increasing the intracellular cAMP and elevating lipolysis. In order to study whether the overexpression of the A2 adenosine receptors has an effect on the lipolysis in brown adipocytes, cells were infected with the control lentivirus rll-156, the CMV-A2A and the CMV-A2B and differentiated according to the normal protocol. The mature cells were treated with adenosine or the specific A2 receptor agonists for 2 hours and the release of free glycerol was measured.

Interestingly, the overexpression of the receptors alone significantly increased the lipolysis in the brown adipocytes in comparison to the cells infected with the control virus by 1.78-fold (A2A) and 2.19-fold (A2B), again indicating extracellular adenosine acting on cellular A2 adenosine receptors. Brown adipocytes overexpressing the A2A or the A2B receptor in combination with stimulation with adenosine or the specific receptor agonists also showed a significantly higher release of free glycerol in comparison to the stimulated cells infected with rll-156. Being stimulated with adenosine, the cells with the A2 receptors over-expressed increased the lipolysis in comparison to the cells infected by the control virus by 1.51-fold (A2A) and 1.58-fold (A2B). Being stimulated with the specific receptor agonists, these cells released 56.42 (A2A) respectively 31.60 (A2B) per cent more of free glycerol compared to the control cells stimulated with the same substances (Figure 19).

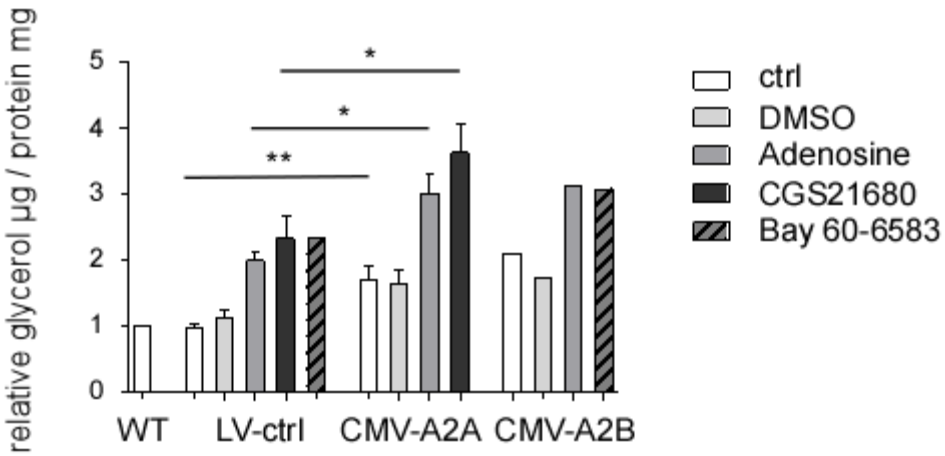


Fig. 19: Overexpression of the A2 adenosine receptors stimulates brown adipocyte function.
Analysis of the free glycerol release of mature brown adipocytes. Infection of the cells with 30 ng of virus on day -3 of the eleven day differentiation protocol. Treatment of cells on day 7 with DMSO, 100 nM of adenosine, 150 nM of CGS21680 or 300 nM of Bay 60-6583 for 2 hours as indicated. *p < 0,05, **p < 0,01. Data are shown as mean + s.e.m. (N=2-4, n=2-6) and analyzed using two-paired Student's t test.

These data confirmed the possibility of an activation of brown adipocyte function by a stimulation of the A2 adenosine receptors and that increasing receptor expression alone was capable of increasing brown adipocyte function.

4. Discussion

4.1 Adenosine effects on brown adipose tissue function

Brown adipose tissue has become an attractive target of pharmaceutical research since it was known that it is present and metabolically active in adult humans (Cypess, et al., 2009; van Marken Lichtenbelt, et al., 2009; Virtanen, et al., 2009). An activation of BAT as a potential therapeutic target may represent a novel strategy in the treatment of obesity (Ravussin & Galgani, 2011), that has become a global health care problem, according to WHO information. The activation of BAT by the sympathetic nervous system is well described. The stimulation of β-adrenergic receptors, coupled to G_s-proteins, activates the cAMP / PKA pathway (Cannon & Nedergaard, 2004). PKA phosphorylates HSL and thereby increases lipolysis, the release of free fatty acids in BAT (Shih & Taberner, 1995), which activate UCP-1 (Nedergaard, et al., 2001). It also positively regulates the expression of UCP-1 by activation of the transcription factor CREB , which binds to the promoter of the UCP-1 gene (Cao, et al., 2004; Thonberg, et al., 2002).

However, a BAT-centered therapy based on β-adrenergic agonists would be clinically not feasible, because of the massive side-effects like cardio-vascular stimulation. Cypess et al. showed 2015 that the β3-adrenergic-receptor agonist mirabegron, which is in the clinic to treat over-active bladder, is able to activate human BAT in young and healthy male subjects, but also significantly increased the heart beat and the systolic blood pressure (Cypess, et al., 2015). Obesity is often associated with high blood pressure (Berchtold, Sims, Horton, & Berger, 1983), so the cardiovascular side effects might be even extensive for the patients who need to be treated. As long as there is no way found to minimize these side effects, alternative strategies must be explored.

Adenosine is a co-transmitter in the sympathetic nerve system and is known to be released in BAT during stimulation of sympathetic nerves (Gourine, et al., 2009). Like the β-adrenergic receptors, adenosine receptors are coupled to G-proteins and mainly act via the cAMP / PKA pathway. While the A1 and the A3 adenosine receptors are coupled to G_i-proteins and inhibit the pathway (Linden, 1991; Salvatore, et al., 1993), stimulation of the A2A and the A2B adenosine receptors activates the pathway via G_s-proteins (Meng, et al., 1994; Pierce, et al., 1992).

Adenosine is also able to increase active CREB, for example in macrophages via the A2A receptor (Nemeth, et al., 2003), but on the other hand it is known to inhibit lipolysis in white adipose tissue via the A1 receptor by reducing the activity of HSL (Dhalla, et al., 2009). Previous studies show similar inhibitory effects of the purinergic transmitter on BAT from hamster or rat (Schimmel & McCarthy, 1984; Woodward & Saggerson, 1986). Nevertheless, the underlying signalling pathways and the involved adenosine receptors still remain unclear.

Here, it is shown that all four adenosine receptor subtypes are expressed in murine BAT, the A2A and A2B receptors being the most abundant. Moreover, this study demonstrates that adenosine surprisingly activates murine brown adipocytes at low nanomolar concentrations, the effect being additive to a stimulation with norepinephrine. Using specific agonists and applying an over-expression model in murine brown fat cells, it was shown that adenosine, acting on A2A and A2B receptors, enhances lipolysis. This is most likely attributed to the cAMP / PKA pathway, the second messenger being increased after stimulation of the A2 adenosine receptors. Furthermore, a chronic stimulation of the A2A adenosine receptor led to an enhanced expression of UCP-1, suggesting an important role of the receptor for the thermogenesis of murine brown adipocytes.

4.2 Adenosine – a co-activator of murine BAT acting via the cAMP / PKA pathway

Previous studies reported an inhibiting effect of adenosine on lipolysis in brown adipocytes (BA) from hamsters and rats and a decrease of the sensitivity to catecholamines (Schimmel & McCarthy, 1984; Szillat & Bukowiecki, 1983; Woodward & Saggerson, 1986). In contrast, this study reveals an activating effect of adenosine on murine brown adipocytes *in vitro* at low nanomolar concentrations. By measuring the release of free glycerol after an acute stimulation, it could be shown that adenosine increases lipolysis twofold compared to control. Importantly, adenosine had an additive effect in combination with norepinephrine reaching a threefold increase of glycerol release. Indeed, BAT is known to be activated by the sympathetic nervous system (Cannon & Nedergaard, 2004) and adenosine is known to act as co-transmitter of this system (Gourine, et al., 2009). Regarding that, the possibility of adenosine being a physiological co-activator of BAT seems quite probable. Besides, the contrary results of

the early studies mentioned above can at least partially be explained by a different expression profile of adenosine receptors in the used cells. The brown adipocytes of hamsters for example have the same amount of A1 and A2A receptors while A2B or A3 receptors could not be detected (Gnad, et al., 2014).

An important question addressed in this present thesis was which intracellular signalling pathway mediates adenosine function in BAT. It is well known that adenosine mainly acts via the adenylyl cyclase / cAMP / PKA pathway, inhibiting it by activation of the A1 or A3 receptor (Dunwiddie & Fredholm, 1989; Salvatore, et al., 1993) or stimulating it by activation of the A2A or A2B receptor (Meng, et al., 1994; Pierce, et al., 1992). Given these findings, this study investigated the effect of adenosine on the amount of intracellular cAMP in murine brown adipocytes by measuring it with ELISA after an acute stimulation of BA with the substances. It could be demonstrated that adenosine nearly doubles the amount of cAMP in brown fat cells compared to control, while norepinephrine treatment even resulted in a threefold increase. Thus, adenosine acts via the adenylyl cyclase / cAMP / PKA pathway to increase lipolysis and activate BAT function (Figure 20). These effects most probably are mediated by phosphorylation and activation of HSL by PKA, like it has been shown in BAT after stimulation of β3-receptors (Shih & Taberner, 1995). HSL hydrolyzes triglycerides to free fatty acids (Vaughan, Berger, & Steinberg, 1964). The lipolysis is also essential for activation of thermogenesis, because the unique brown fat protein UCP-1 is strongly activated by free fatty acids (Hagen, Zhang, Vianna, & Lowell, 2000).

The question remains, how adenosine is released in BAT. Two possible mechanisms include the breakdown of ATP released from sympathetic nerves and the autocrine or paracrine release from brown adipocytes. Some findings of this study suggest an important role of the second pathway. The blocking of the inhibitory A1 receptors alone, as well as the over-expression of the A2-receptors, without any further treatment of the cells with Adenosine or one of the A2-receptor agonists, increased lipolysis significantly, which implicates the receptors being activated and therefore the existence of extracellular adenosine. The release by brown adipocytes seems to be the most probable explanation, especially given the fact that white adipocytes already have been proven to release adenosine (Kather, 1990).

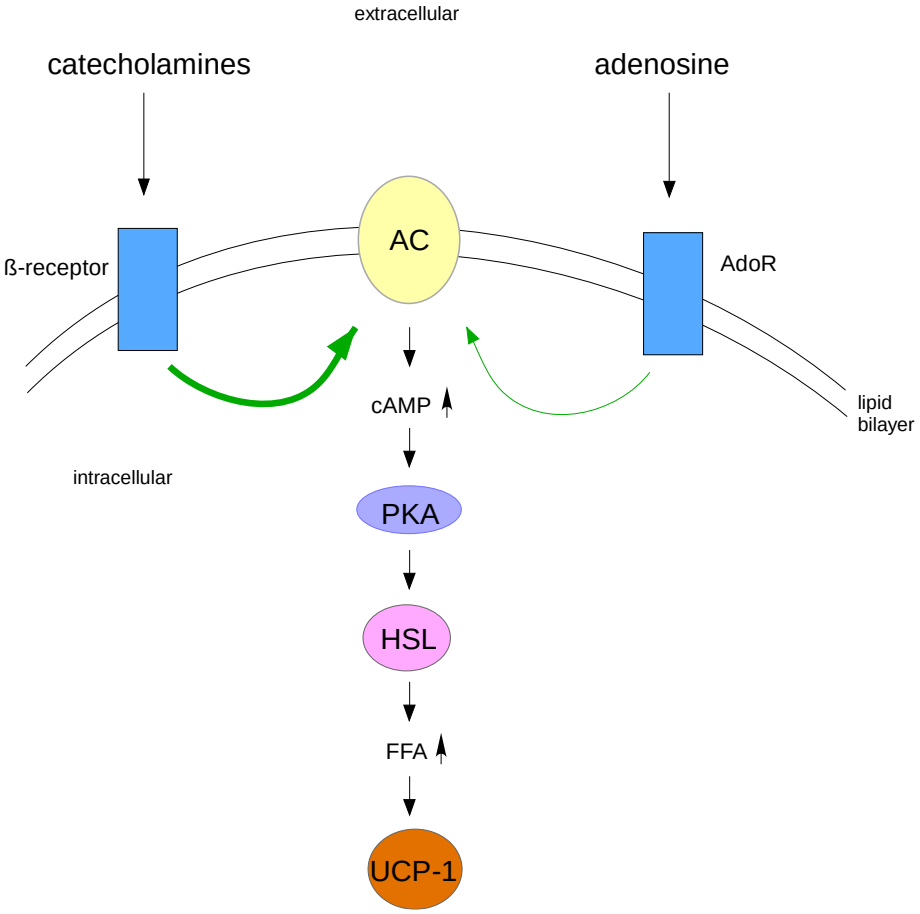


Fig. 20: Activation of BAT function by catecholamines and adenosine.
Catecholamines and adenosine activate BAT function by stimulation of the adenylyl cyclase (AC) / cAMP / proteinkinase A (PKA) pathway, which activates HSL and thereby increases lipolysis and free fatty acids (FFA). The thermogenic protein uncoupling protein 1 (UCP-1) is strongly activated by FFA.

4.3 Adenosine effects on BAT are mediated by A2 receptors

Previous studies showed that A2B adenosine receptor stimulation is beneficial for insuline signalling in WAT (Eisenstein, et al., 2014; Johnston-Cox, et al., 2012). On the other hand Johansson and co-workers demonstrated the critically important role of A1 receptors for the inhibitory effect of adenosine on white adipose tissue by using the A1 receptor knockout mouse (Johansson, et al., 2008). Assuming a similar role of the A1 receptor for brown adipose tissue, the expression level of all adenosine receptor subtypes was investigated in this study using quantitative PCR and compared to the expression of the A1 receptor. Surprisingly, the amount of A2A and A2B receptor mRNA in murine mature brown adipocytes was three- or fourfold higher than the amount of A1

receptor mRNA, respectively, suggesting an superior role of these G_s-coupled receptors in BAT.

Importantly, these findings might explain adenosine-mediated increase of cAMP and the stimulation of lipolysis in murine brown adipocytes. The A1 and the A3 adenosine receptors are coupled to G_i-proteins and known to inhibit the cAMP-pathway and lipolysis (Dunwiddie & Fredholm, 1989; Johansson, et al., 2008) while the A2 receptors are coupled to G_s-proteins and known to stimulate it (Feoktistov & Biaggioni, 1997; Fredholm, et al., 2007), so the effect of adenosine on murine BAT is most likely mediated via the A2A and / or A2B receptor.

Additionally, specific agonists for the A2 receptors were used. Sole stimulation of either the A2A or the A2B receptor elevated the amount of intracellular cAMP and the release of free glycerol to a similar extend as adenosine. Finally a lentiviral mediated over-expression of A2A or A2B receptor in murine brown adipocytes was applied to further investigate the role of the receptors in activation of BAT function. It could be shown that cells overexpressing either receptor had further elevated cAMP levels and showed increased lipolysis in response to adenosine.

Taken together my findings could confirm that adenosine effects on murine BAT function are based on the stimulation of A2A and A2B receptors.

4.4 Role of A2A receptors for thermogenesis in BAT

It was already pointed out that stimulation of the A2A adenosine receptor increases lipolysis and thereby the amount of free fatty acids in brown adipocytes, leading to an activation of UCP-1.

In addition to cAMP levels and lipolysis, I analyzed the expression level of UCP-1 in BA with the receptor being over-expressed. Western Blot analysis revealed a fourfold increase on the amount of UCP-1 in comparison to untreated cells and cells treated with a control virus. The results were not significant, nevertheless they may show an important role of the A2A receptor for thermogenesis in BAT.

This study shows that an activation of the A2A receptor elevates the intracellular level of cAMP and PKA is known to be a major mediator of cAMP function (Su, et al., 1995). Previous studies demonstrated that PKA phosphorylates and activates the transcription factor CREB in BAT (Thonberg, et al., 2002), which binds to cAMP-response elements

(CREs) on the UCP-1 promoter and activates its expression (Cao, et al., 2004). This could represent a possible pathway for adenosine acting via the A2A receptor (Figure 21), especially because adenosine is known to activate CREB in macrophages (Nemeth, et al., 2003). Therefore, UCP-1 upregulation after adenosine stimulation could be beneficial for BA capacity to burn energy after an initial activation leading to lipolysis and the release of free fatty acids.

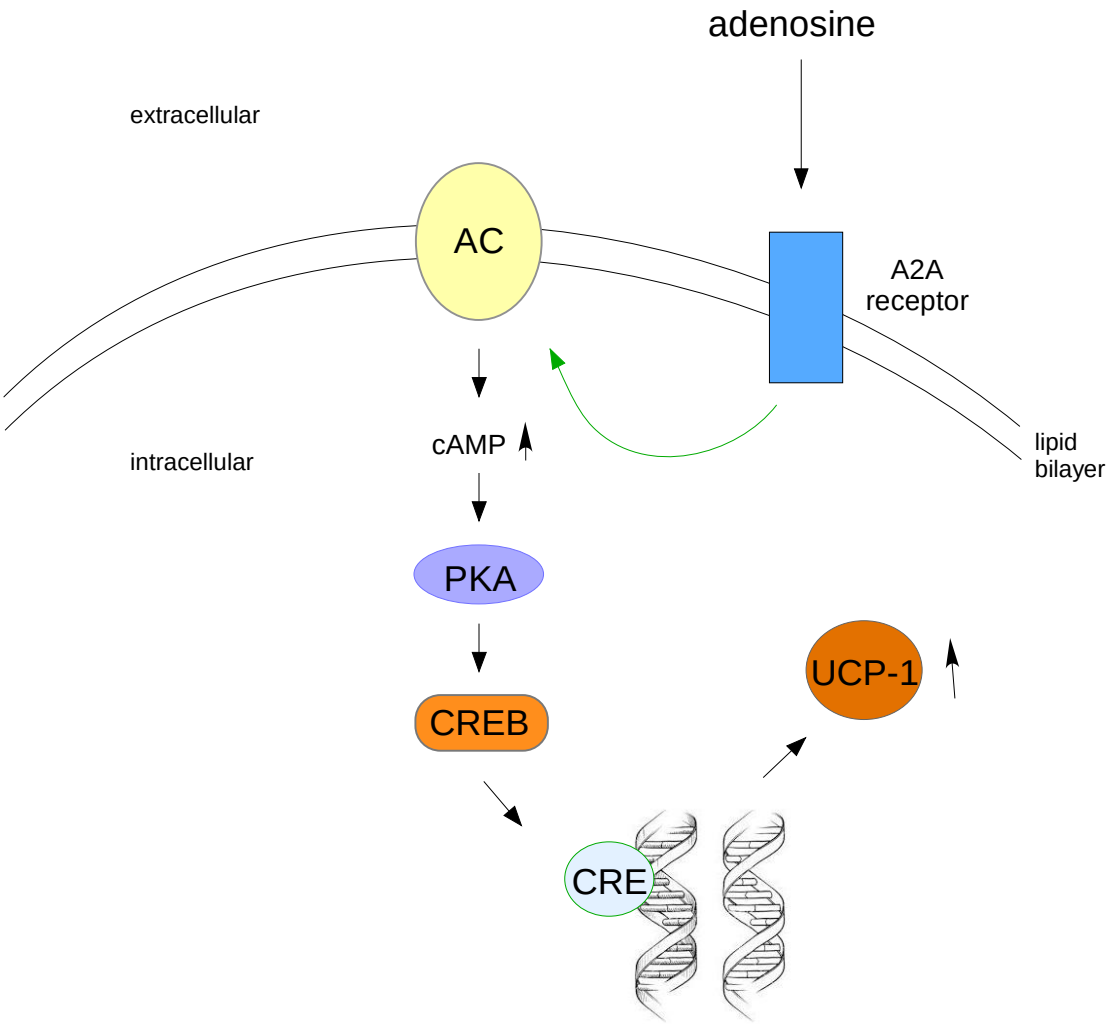


Fig. 21: Adenosine may stimulate the expression of UCP-1 via A2A receptors.
The activated A2A adenosine receptor stimulates adenyly cyclase and thereby increases the amount of intracellular cyclic adenosine monophosphate (cAMP). Protein kinase A (PKA), as a major mediator of cAMP function, activates cAMP response element-binding protein (CREB), which binds to cAMP-response elements on the uncoupling protein 1 (UCP-1) promoter and activates its expression.

4.5 The A2 adenosine receptors – Novel pharmacological targets in the therapy of obesity?

This study suggests an important role of the A2A and the A2B adenosine receptor for the activation of brown adipocyte function. Especially the A2A receptor may become an important pharmacological target in the therapy of obesity as it not only activates the fat cells function but also elevates the level of UCP-1 and thereby promotes thermogenesis. However, the data was gained with studies on murine brown adipocytes *in-vitro.* The effect of adenosine on human BAT remains unclear, especially because previous studies demonstrated a different effect of the purinergic transmitter in other species, like hamsters and rats (Schimmel & McCarthy, 1984; Woodward & Saggerson, 1986). If there were shown similar effects on human brown adipocytes *in-vitro*, *in-vivo* studies could be performed, for example with A2A adenosine receptor knock-out mice. These studies might answer other open questions. Are the A2 adenosine receptors essential for an activation of BAT? Is an overactive BAT able to influence body weight or are there counterregulatory mechanisms? How is WAT influenced by an stimulation of A2 adenosine receptors? Initial data here show the A2A adenosine receptors as predominant in human BAT/BA, too (Gnad, et al., 2014). Latest studies even show that human BAT is activated by adenosine and stimulation of the A2A receptor (Lahesmaa, et al., 2018) and that a stimulation of adenosine receptors in BAT could be of physiological relevance (Ruan, et al., 2018).

The use of adenosine as a drug for a long-term stimulation is not practicable, because of the short half-life in vivo (Moser, Schrader, & Deussen, 1989). The side-effects also remain a problem that needs to be solved. As adenosine receptors are not only expressed on fat cells, but on many important tissues of the human body, side effects of a therapy with adenosine or a special A2 receptor agonist would most likely occur. Adenosine has been used as a drug to treat supraventricular tachycardia for a long time (Eltzschig, 2009), because of its ability to decelerate the atrioventricular conductor (Urthaler & James, 1972) and the specified side effects cover for example pain in the thoracal region, a decrease of the heart rate, ventricular and supraventricular extrasystoles, a block of the atroventricular conductor, ventricular tachycardia, atrial fibrillation, dyspnoea, headache, vertigo and a decrease of the blood pressure (Arzneimittel pocket 2015). A specific stimulation of either the A2A or the A2B receptor

would probably reduce the side effects, but still the outcome of a treatment with special agonists is not predictable. The A2A receptor is for example involved in the regulation of sleep and wakefulness (Scammell, et al., 2001) and the A2B receptor mediates the release of interleukin-4 by human mast cells (Ryzhov, et al., 2006). Both receptors also play a role in regulating vascular tone (Kilpatrick, et al., 2002), so a treatment with specific agonists would most likely effect the blood pressure. A dual specific agonist for the A2A and the A2B receptor might be a solution, because of the high expression of these receptors on BAT. The dose could be reduced and the side effects minimized. Nevertheless, it is a promising approach and worth further investigation.

5. Summary

Pandemic obesity and its associated diseases like type 2 diabetes, coronary heart failures, stroke and cancer has become one of the leading worldwide health problems. Obesity is characterized for the excessive abundance of white adipose tissue (WAT), which main function is to store energy in the form of fat. Brown adipose tissue (BAT) on the other hand consumes energy through non-shivering thermogenesis. Since it was shown that adult humans possess metabolic active BAT in 2009, pharmacological activation of brown fat cells promises to be a new strategy in order to treat obesity. BAT function is stimulated by neurons of the sympathetic nervous system. ATP is a co-transmitter released together with NE from sympathetic nerves, adenosine is a breakdown product of ATP.

In my thesis, I was not only able to show that adenosine stimulates BA function, but also that lipolysis is increased by adenosine while stimulating the cells with NE shortly before. This could indicate a possible co-stimulation of BAT with NE and adenosine by the sympathetic nervous system and open up new pharmacological approaches for the activation of BAT. First, however, the question arises through which of the adenosine receptors this effect is mediated. I could show that all adenosine receptor subtypes are expressed in BA, the A2B receptor being the most abundant. The A2A receptor is strongly upregulated during differentiation. A2A and A2B receptor agonists and antagonists do not have a significant effect on the differentiation of brown adipocytes. However, stimulation of A2A and A2B using specific agonists activates BA with significant increase of intracellular cAMP and elevated lipolysis. Over-expression of the A2A receptor, without direct stimulation with an agonist, lead to an increased differentiation of brown adipocytes. I could also show a stimulating effect on BA function by the over-expression of the two receptors.

This data suggests an important role of the A2A and A2B receptor for the activation of BA function. Especially the A2A receptor may become an important pharmacological target in the therapy of obesity as it has been shown to be highly abundant in human BAT. As adenosine receptors are not only expressed on fat cells, but many important tissues of the human body, potential side effects of a therapy with adenosine or specific adenosine receptor agonists remain a problem to be solved.

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